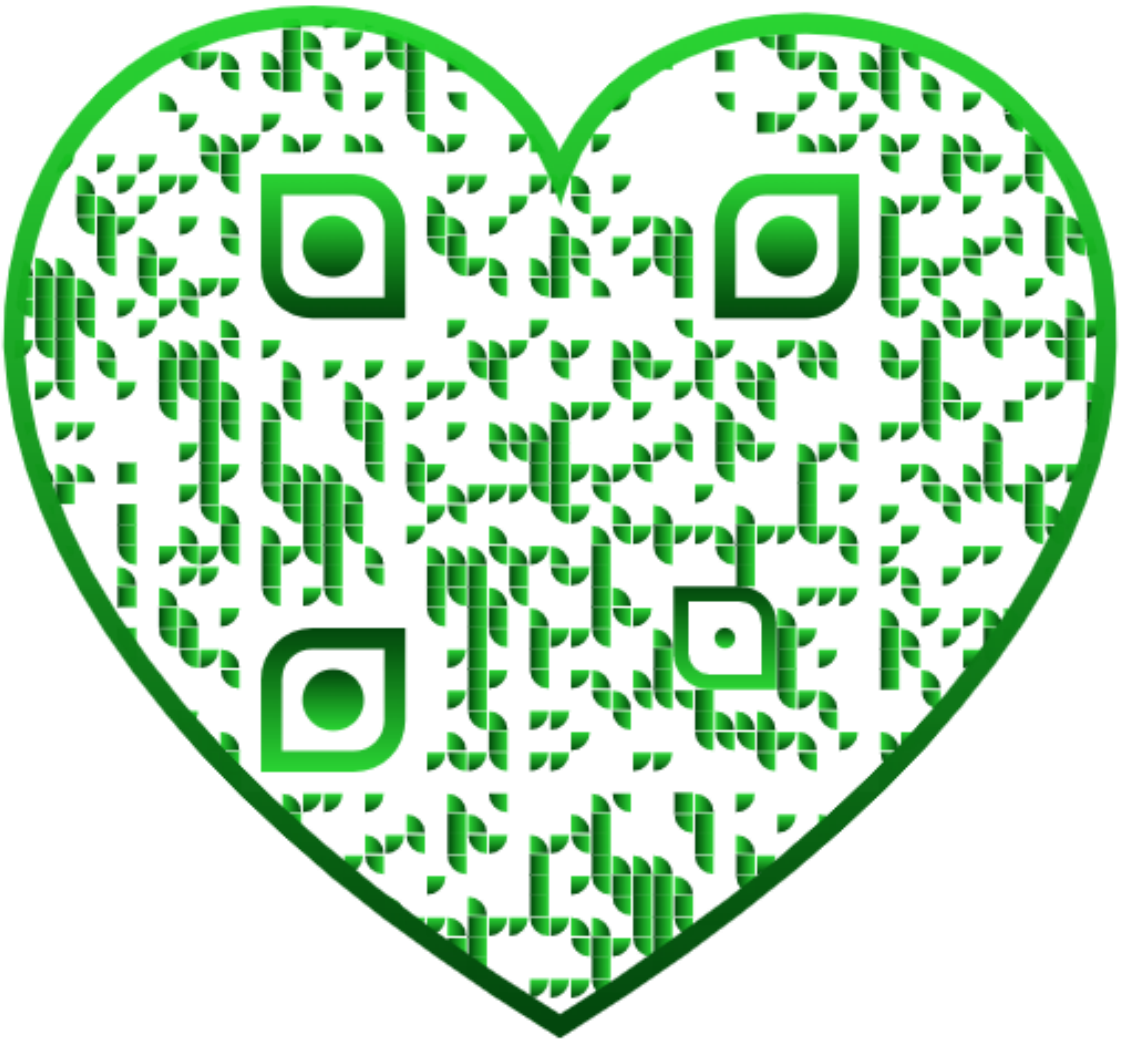
9TH EDITION

Casarett & Doull's TOXICOLOGY

THE BASIC SCIENCE OF POISONS

CURTIS D. KLAASSEN

Mc Graw Hill Education



Casarett and Doull's
TOXICOLOGY
The Basic Science of Poisons

> "What is there that is not poison?
> All things are poison and nothing (is)
> without poison. Solely the dose
> determines that a thing is not a poison."

Paracelsus (1493–1541)

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Casarett and Doull's
TOXICOLOGY

The Basic Science of Poisons

Ninth Edition

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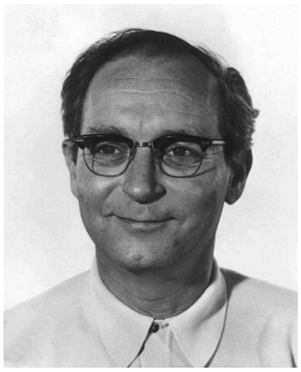
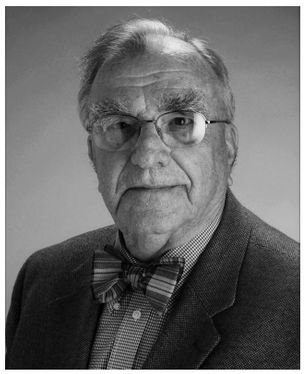


Louis James Casarett

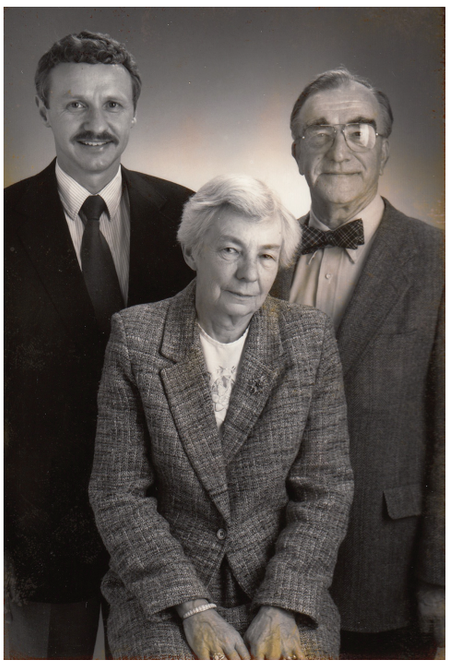


John Doull

Fifty years ago when I started lecturing graduate students there was no comprehensive toxicology textbook, and thus one often needed many hours in the library reading the literature to prepare for a lecture. Thus, I was thrilled when Lou Casarett and John Doull decided to edit a textbook in toxicology because it would enable me to give much better lectures with much less preparation time. The textbook provided a review of the literature on each topic in toxicology written by an expert in the area.

The origin of this textbook started at NIH Toxicology Study Sections meetings in the late 1960s and early 1970s. All members of the Study Sections agreed there was a growing need for a textbook in toxicology, in fact many members of those Study Sections became authors of various chapters in the book.

At the time, Lou Casarett was a professor at the University of Hawaii and John Doull was a professor at the University of Kansas. As a result, Lou spent time in Kansas City with John selecting authors of the book, whereas John and his family spent a summer in Hawaii in finalizing the organization of the book and writing chapters for the first edition. Unfortunately, shortly thereafter and before the first edition was published, Lou died of brain cancer.



Klaassen, Amdur, Doull

The first edition was entitled *Toxicology: The Basic Science of Poisons* and was published in 1975. John Doull asked Mary Amdur, a friend of Lou Casarett, and myself, a younger toxicologist at the University of Kansas, to help him edit the second edition of the textbook. Mary suggested that the names of the two first editors be added to the title of the textbook, and thus the second and all subsequent editions have been entitled *Casarett and Doull's Toxicology: The Basic Science of Poisons.* The second, third, and fourth edition were edited by Doull, Amdur, and Klaassen. Mary Amdur died in 1998 and John Doull in 2017.

This ninth edition is dedicated not only to Lou Casarett, John Doull, and Mary Amdur, but all authors who have contributed to the nine editions of this book. These authors have summarized the knowledge in their area of expertise to help faculty prepare lectures as well as to help students learn the discipline. To emphasize the importance that previous authors have had on the education of toxicologists over the decades, their names are acknowledged in the chapter they previously authored.

Lou Cantilena, MD, PhD, author of the "Clinical Toxicology" chapter of this book and previous editions, was killed, along with his daughter, in an airplane accident in December 2017. Lou was piloting his daughter home for the Christmas holiday from Kansas City, where she was finishing her MD and PhD studies at the University of Kansas. Professionally, Dr. Cantilena will be remembered for his contributions to the Poison Control Centers and for treating poisoned patients, educating physicians for the military, doing clinical trials in order to discover more effective and less addicting treatments for pain, and consulting with the Food and Drug Administration on the management of drug-induced *torsades de pointes*. Lou's positive attitude, enthusiasm, smile, sincerity, and devotion to his family are hallmarks of his legacy.

Curtis D. Klaassen, PhD, DABT, ATS, FAASLD

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Preface

The ninth edition of *Casarett and Doull's Toxicology: The Basic Science of Poisons*, as in previous editions, is meant primarily as a text for, or an adjunct to, graduate courses in toxicology. Because the eight previous editions have been widely used in courses in environmental health and related areas, an attempt has been made to maintain those characteristics that will again provide information on the many facets of toxicology, especially the principles, concepts, and modes of thoughts that are the foundation of the discipline. Mechanisms of toxicity are emphasized. Research toxicologists will find this book an excellent reference source to find updated material in areas of their special or peripheral interests.

The overall framework of the ninth edition is similar to that of the previous editions. The seven units are General Principles of Toxicology (Unit I), Disposition of Toxicants (Unit II), Non-Organ-Directed Toxicity (Unit III), Target Organ Toxicity (Unit IV), Toxic Agents (Unit V), Environmental Toxicology (Unit VI), and Applications of Toxicology (Unit VII).

This edition reflects the progress made in toxicology during the last few years. The examples are the importance of apoptosis, autophagy, cytokines, growth factors, oncogenes, cell cycling, receptors, gene regulation, protective mechanisms, repair mechanisms, transcription factors, signaling pathways, transgenic mice, knock-out mice, humanized mice, polymorphisms, microarray technology, second-generation sequencing, genomics, proteomics, epigenetics, exposome, microbiota, read across, adverse outcome pathways, high-content screening, computational toxicology, innovative test methods, organ-on-a-chip, etc. in understanding the mechanisms of toxicity and the regulation of chemicals. This edition is markedly updated from the previous edition; over one-third of the chapters in this ninth edition are authored by scientists that have not been previously involved with the textbook. References in this edition include not only traditional journal and review articles, but Internet sites too.

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Preface to the First Edition

This volume has been designed primarily as a textbook for, or adjunct to, courses in toxicology. However, it should also be of interest to those not directly involved in toxicologic education. For example, the research scientist in toxicology will find sections containing current reports on the status of circumscribed areas of special interest. Those concerned with community health, agriculture, food technology, pharmacy, veterinary medicine, and related disciplines will discover the contents to be most useful as a source of concepts and modes of thought that are applicable to other types of investigative and applied sciences. For those further removed from the field of toxicology or for those who have not entered a specific field of endeavor, this book attempts to present a selectively representative view of the many facets of the subject.

Toxicology: The Basic Science of Poisons has been organized to facilitate its use by these different types of users. The first section (Unit I) describes the elements of method and approach that identify toxicology. It includes those principles most frequently invoked in a full understanding of toxicologic events, such as dose–response, and is primarily mechanistically oriented. Mechanisms are also stressed in the subsequent sections of the book, particularly when these are well identified and extend across classic forms of chemicals and systems. However, the major focus in the second section (Unit II) is on the systemic site of action of toxins. The intent therein is to provide answers to two questions: What kinds of injury are produced in specific organs or systems by toxic agents? What are the agents that produce these effects? A more conventional approach to toxicology has been utilized in the third section (Unit III), in which the toxic agents are grouped by chemical or use characteristics. In the final section (Unit IV) an attempt has been made to illustrate the ramifications of toxicology into all areas of the health sciences and even beyond. This unit is intended to provide perspective for the nontoxicologist in the application of the results of toxicologic studies and a better understanding of the activities of those engaged in the various aspects of the discipline of toxicology.

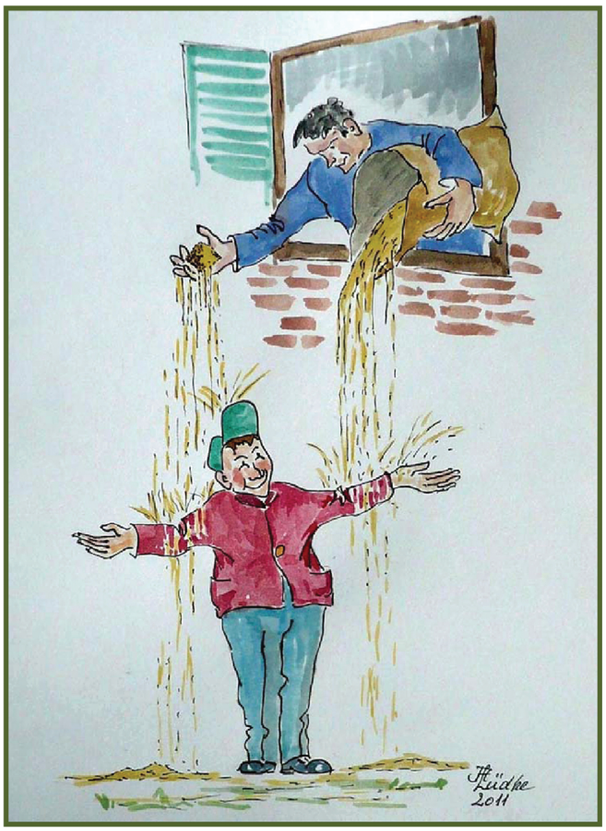
It will be obvious to the reader that the contents of this book represent a compromise between the basic, fundamental, mechanistic approach to toxicology and the desire to give a view of the broad horizons presented by the subject. While it is certain that the editors' selectivity might have been more severe, it is equally certain that it could have been less so, and we hope that the balance struck will prove to be appropriate for both toxicologic training and the scientific interest of our colleague.

L.J.C.
J.D.

Although the philosophy and design of this book evolved over a long period of friendship and mutual respect between the editors, the effort needed to convert ideas into reality was undertaken primarily by Louis J. Casarett. Thus, his death at a time when completion of the manuscript was in sight was particularly tragic. With the help and encouragement of his wife, Margaret G. Casarett, and the other contributors, we have finished Lou's task. This volume is a fitting embodiment of Louis J. Casarett's dedication to toxicology and to toxicologic education.

J.D.

Dose and Dose-Rate matter



Unit I

General Principles of Toxicology

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chapter 1

The Evolving Journey of Toxicology: A Historical Glimpse

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ABOUT TOXICOLOGY

Humans are smart but vulnerable. We need to be prepared for countless unforeseen events that could compromise our health and well-being. Toxicology arose as a way to understand, prevent, mitigate, and treat the potentially harmful consequences of many of the substances we are exposed to.

According to the Society of Toxicology (SOT) (http://www.toxicology.org/about/vp/vision.asp):

Toxicology is the study of the adverse effects of chemical, physical, or biological agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects.

The National Library of Medicine's (NLM) *Collection Development Manual* elaborates by noting:

Toxicology studies the agents responsible for adverse effects, the mechanisms involved, the damage that may ensue, testing methodologies to determine the extent of damage, and ways to avoid or repair it. Toxicology is traditionally associated with chemical exposures, such as the effects of drugs, industrial chemicals, pesticides, food additives, household products, and personal care items. Toxinology, a sub discipline of toxicology, studies biological exposures, such as insect stings, poisonous mushrooms and plants, venomous snakes and aquatic life. The third category of toxicology is concerned with physical hazards, such as radiation and noise.

One of the key points to understand, as noted above, is that although toxicology in the popular mind is confined to chemicals and, probably, in practice most of the research and concern occur in this realm, other agents such as radiation and substances derived from biological organisms are equally relevant to the field.

The word *toxicology* is derived from the Latinized form of the Greek word *toxicon*, meaning "arrow poison." *Poison*, as a noun, dates back to the Old French *poison* or *puison*, meaning, originally, a drink, especially a medical drink, but later signifying more of a magical potion or poisonous drink. Another point of terminology concerns the commonly misused term *toxin*. Despite past and informal uses of the term, it formally should be used to refer to toxic substances produced biologically. Thus, technically, chemicals such as formaldehyde or asbestos, say, would *not* be considered toxins. There are any number of other terms which could be used to delineate the broader category of substances which are toxic, regardless of origin. Examples are *toxicant*, *toxic agent*, and *toxic substance*. *Xenobiotics* is a term referring to substances, whether toxic or not, foreign to a given organism.

Finally, in this brief lesson on toxicology nomenclature, one needs to clarify the use of the words *poisonous* and *venomous* when used as animal adjectives. Though often used interchangeably, they are, in fact, rather distinct. A venom requires a delivery mechanism. Thus, because a snake, for example, injects its venom (or toxin) into its victim, it is considered a *venomous* animal. Instead, a toxic mushroom must be ingested to make its effect felt. Thus, it should instead be deemed *poisonous*.

Toxicology is largely concerned with the interaction of toxicants and biological organisms. While toxicodynamics investigates the effect of the toxicant on the organism, toxicokinetics looks at how the organism affects the toxicant (e.g., absorption, biotransformation, distribution, and elimination). Mechanisms of toxicity at cellular and biochemical levels play a key role in determining why an agent has the effects it does. Toxic responses may be directed to particular organs or systems, for example, kidney, liver, and nervous system. Another way to consider effects is as clastogenic or mutagenic, resulting in carcinogenic or teratogenic effects. Often the focus of research is on a particular chemical or class of chemicals,

such as pesticides, metals, or solvents. Environmental contamination and toxicology are tightly bound fields of study, and toxicology has much to contribute to an understanding of air, water, and soil pollution. Establishing the safety of drugs relies upon toxicology as does ensuring the safety of our water and food supply. Envenomations, whether by snakes, spiders, scorpions, aquatic life, or other creatures, as well as poisoning by plants and fungi are also within toxicology's scope.

Toxicology today is a highly interdisciplinary science that borrows from and intersects with other sciences such as chemistry, biology, pharmacology, medicine, physiology, biochemistry, molecular biology, pathology, and environmental science. Increasingly, it is also appropriating the tools of the computational sciences as one way to improve the precision of safety assessment, screen large numbers of chemicals efficiently, cut costs, and reduce animal use. Toxicology can be parsed into branches in a variety of ways. One such set of groupings follows:

Descriptive Toxicology: The emphasis is on the testing of toxicants, typically on animals. It focuses on the dose–response relationship and extrapolation to humans.

Mechanistic Toxicology: Looks at how the agent induces its biochemical or physiological effect on the organism, that is, modes of action. *Biochemical and Molecular Toxicology* is a synonym for this branch.

Clinical Toxicology: This branch's focus is on the effects of drugs and other chemicals on humans, particularly, but also on other animals. Its work is often involved with drug overdoses and other poisonings, and determining the substance involved and its amount in the body. Sometimes used synonymously with *Medical Toxicology* although technically, in terms of profession, a medical toxicologist tends to have an MD while a clinical toxicologist has a PharmD. A veterinarian who specializes in toxicology, typically, has a DVM.

Forensic Toxicology: Concerned with the cause of death from toxic agents, often in instances of drug abuse or misuse. With a focus on homicides and suicides, this branch of toxicology goes hand-in-hand with the work of the police and medical examiners.

Environmental Toxicology: Investigates the effects of toxicant exposures on the general environment and living organisms therein. Thus, pollution of air, water, and soil, and effects on plants and wildlife would fall within this branch. Ecotoxicology, a more specialized area, is devoted to the effects of toxic chemicals on populations, communities, and terrestrial, freshwater, and marine ecosystems. Environmental toxicologists can further define their work in even more specialized terms, for example, aquatic toxicology.

Occupational Toxicology: Deals with the study of chemical and other agents in the workplace, worker exposures, safety and health, and standard setting. Industrial Hygiene covers a very similar terrain.

Regulatory Toxicology: Focuses on ways in which humans and the environment can be protected from toxic effects, through regulations and standard setting. Considers scientific decision-making within a societal and legal framework. Relies heavily upon risk assessment.

Toxicogenomics: Concerned with the compilation and synthesis of information regarding gene and protein expression in order to understand molecular mechanisms involved in toxicity. Toxicogenomics calls upon proteomics, metabolomics, and transcriptomics to identify biomarkers that predict toxicity and genetic susceptibility to harmful substances. Environmental pollutants, pharmaceuticals, and other potentially toxic substances are all within the scope of toxicogenomics research.

Computational Toxicology: Deals with the use of modern computational approaches and information technologies to elucidate mechanisms of toxicity. May also be referred to as toxicoinformatics.

Virtually every branch of toxicology listed overlaps with at least one other. Other ways to parse the discipline are by agents under consideration, such as venoms, pesticides, metals, solvents, drugs, and radiation. One can also look, instead, at target biological systems which the agent may affect, for example, liver, kidney, skin, and heart. As for toxins, they can be categorized by their biological origin, such as insect-, plant-, reptile-, or marine-derived toxins. Some toxicologists spend their careers focused very tightly on a subject, while others graze across many research fields.

ABOUT HISTORY

History is *about* the past; it is not the past. The past is passive, objective, all encompassing. History is active, subjective, and selective. The further back in time that we look, the more problematic it is for us to reach, in the present, conclusions about what happened in the past. Examples, particularly from ancient eras, described below, will show how tales accepted without question are currently being re-examined and revised, and remind us that history is also relative.

Science begins with observation. In the distant past, our observational skills did not extend beyond our senses. We put our senses, to good use, nevertheless, in assessing toxicity and safety even in prehistorical times (i.e., before the written record). Our hominin ancestors used trial and error extensively to explore their environment. In terms of toxicology, they would make careful note of which substances, particularly potential food sources, were safe and which were hazardous. Although it might very well be after the damage was done, they and their tribe and descendants would quickly learn to differentiate between the safe and toxic. Toxic substances, of course, were to be avoided, although it soon became clear that they could be used against enemies.

There are numerous ways to approach the history of toxicology because there are many histories, such as those of the branches outlined in the previous section. Complicating the presentation of a uniform history is the fact that these individual histories overlap. Given the space limitations of this chapter, we will focus on chemicals and proceed chronologically, taking occasional detours as necessary.

TOXICOLOGY IN ANTIQUITY

Ancient China

Shen Nong, the legendary founder of Chinese Herbal Medicine, also known as the farmer god (for he also taught his people how to farm), and said to live circa 2800 BC, saved his subjects from the worry of trying different potential food plants to decide whether they were poisonous. He was said to have tasted hundreds of herbs daily to differentiate the poisonous from the medicinal or just plain edible. Although the toxins he encountered made him sick frequently, he somehow survived them. He is also considered the author of perhaps the world's first pharmacological compendium, *Divine Farmer's Classic of Materia Medica*. His text, a compilation of oral traditions, was compiled in the 3rd century AD. Legend also has it that Shen Nong discovered tea when, sitting under a Camellia tree, dried leaves fell into the water he was boiling to drink (Wilkinson, 2007; Yang, 1998).

Du (毒) is the standard word for poison or toxicity in Chinese. It was understood by the ancient Chinese that drugs (herbals in this instance) were potentially toxic and dose played a role. Aconite, derived from the plant wolfsbane and possessing extreme potential toxicity, was widely used medicinally in small doses in China over 2000 years ago. It was usually applied externally, often processed in some way or mixed with other drugs, to treat various wounds, but was also ingested as a tonic to restore qi (the vital energy defined by Chinese medicine) and extend life. At the same time, sources from that era show that unadulterated aconite in larger doses was often used to murder (Liu, 2014). Today we know that the alkaloids in aconite have a narrow therapeutic index and their use is not generally recommended. Interestingly, it took several thousand years for the role of dose in toxicity to be firmly articulated in the West by Paracelsus, who is discussed later in this chapter.

The ancient Chinese poison, Gu, is one of many potions residing in that blurry historical space between fact and legend. Presumably, a variety of venomous creatures such as snakes, lizards, scorpions, and insects were confined in a container and left to devour each other until only one was left. This survivor thus concentrated in its body the toxins of all its former cell mates and the venom extracted from it was believed to be superbly potent.

Ancient India

Ancient India was no stranger to the knowledge and uses of poisons. Poisoned weapons of various sorts were well known. A Sanskrit verse reads, "Jalam visravayet sarmavamavisravyam ca dusayet," or "Waters of wells were to be mixed with poison and thus polluted" (Khajja et al., 2011). Sushruta was an Indian surgeon. Volume 5 of his medical and surgical compendium, *Suśrutasaṃhitā*, a foundational work in Ayurveda (traditional Indian medicine), contains several chapters related to poisons and poisoning, including descriptions of vegetable and mineral poisons (Sthavara) and animal poisons (Jangama), as well as advice on medical treatment of snake bites and insect bites (Wisdom Library, n.d.). *Agada Tantra*, one of the eight clinical specialties of Ayurvedic medicine, is specifically associated with toxicology (Manohar, 2014; Wujastyk, 2003).

India also has a long tradition of tales about the so-called "venomous virgin" (visakanya), first mentioned in the *Suśrutasaṃhitā*. This maiden, sometimes referred to as the "poison damsel," would, as a young girl, be fed "tolerably minute, but gradually increasing, amounts of poison or snake venom, and that by the time she was an attractive young woman, the level of toxin in her body would be so high that she could be sent to an enemy king as a gift. Upon kissing her, making love to her, or even just sharing glass of wine with her, he would instantly fall dead" (Slouber, 2015). The Rig Veda itself, one of the four texts sacred to Hinduism, includes hymns related to poisons (Wikisource, n.d.-a).

Ancient Egypt

Ancient Egypt was for nearly 30 centuries one of the world's preeminent civilizations and has left us a legacy of unrivalled art, architecture, and religious traditions. Animals played an important role in its belief systems. Egyptian gods and goddesses often took on a hybrid human–animal physical form.

Venomous snakes and insects were well known and the focus of toxicology as it existed in ancient Egypt. One of the major documents examining snakebite, and surviving in most of its entirety to our time are the Brooklyn Papyri (held by the Brooklyn Museum), 525–600 BC (Sanchez and Harer, 2014). Its two sections describe individual snakes and treatment for snakebites, respectively. Paragraph 15 of the Papyri, for example, describes the snake known by the Egyptians as Apophis which, mythologically, personified evil. Scholars believe this may be the Boomslang (*Dyspholidus typhus*) in the Colubridae family. Symptoms and signs of snake envenomation are presented in the Papyri. The treatments offered could be general, for any snakebite, or specific. Bites by snakes known to be lethal generally received no treatment. Therapeutic measures, overall, were largely symptomatic. One treatment that comes up with frequency is the use of Allii Cepae, the onion, used in various preparations depending on the bite. Often this was used in conjunction with induced vomiting to rid the body of the poison:

Paragraph 41: Very good remedies to be made for those suffering from all snake bites: Onion, ground finely in beer. Eat and spit out for one day. (then follows an incantation)
Paragraph 42: As for the onion, it should be in the hand of the priest of Serqet, wherever he is. It is that which kills the venom of every snake, male or female. If one grinds it in water and one smears a man with it, the snake will not bite him. If one grinds it in beer and sprinkles it all over the house one day in the new year, no serpent male or female will penetrate therein. (Nunn, 1996)

Toxicity is addressed to a lesser extent in other important papyri such as the Berlin, Edwin Smith, and Ebers papyri.

Cleopatra VII, born in 69 BC, is one of the most fascinating personalities to flourish in Egypt when Greece and Rome held sway. During her reign as Pharaoh, Egypt was a Hellenistic (i.e., Greek) province, part of the Ptolemaic dynasty, established after the death of Alexander the Great. After Cleopatra's death, Egypt was annexed by Rome. And while her romantic exploits with Julius Caesar and Mark Anthony have been grist for generations of writers and artists, it is her death that holds toxicological interest for us. After the Battle of Actium (on Greece's west coast), which ended in defeat for the Egyptians, and learning that Marc Anthony killed himself by a self-inflicted sword wound, Cleopatra decided to follow suit. It is said that she had her servants bring her a basket of figs, in which one or more asps (Egyptian cobra) were hidden, and holding one to her breast, she succumbed to its venomous bite. A recent analysis questions the feasibility of a maid capable of carrying a basket of one or more Royal Cobras (9.8–13 ft in length, and weighing some 13 lbs) camouflaged by figs (Tsoucalas and Sgantzos, 2014). Other evidence on the time frame of her dying support this doubt. It has now been suggested that a more likely scenario was that she was murdered, perhaps with a poisonous draught by Octavian, the victor in their battle. He may have then spread the rumor of her suicide to avoid turmoil in the streets (against him) by the subjects who adored her.

Pontus, Mithridates, and Theriacas

The kingdom of Pontus in northeastern Turkey played an interesting role in the history of poisons and antidotes. Mithridates VI, its ruler beginning in 120 BC, was a fierce adversary of Rome, engaging it in battle three times. Ultimately, he succumbed to defeat by Pompey in the third war and committed suicide. Even as a boy, Mithridates experimented with poisons and antidotes, even on himself. Son of a father who was murdered with poison and a mother who would have poisoned him in order to ascend to the throne, he went into hiding for a period of years. He returned to capture his rightful position by likewise using poison, probably arsenic. With a background like that, one could hardly consider it paranoia that he feared assassination by poison and took precautions to avoid it (Mayor, 2010).

His approach was to ingest small doses of toxicants to become immune to them. His lifelong pursuit was to create a universal

antidote, which came to be known as a theriac, his particular one called a Mithridatium, by creating a concoction of tiny amounts of deadly poisons and antidotes. Not as far-fetched as it seems, recent science reveals that exposure over thousands of years to arsenic among certain Andean highland populations may have resulted in a level of resistance in their modern-day descendants (Schlebusch et al., 2013).

There have been many speculations about what the ingredients of the Mithridatium were, but we do not know for certain, and may never know. Returning to Mithridates' defeat by Pompey, legend holds that the ignominy of it led him to want to end his life. He retreated, with a poison, to the highest tower of his castle with his daughters. His daughters insisted that they be administered the poison first. After they died, he drank the balance. He weakened, but did not die, and his disorientation prevented him from stabbing himself with his own sword as he attempted. Instead, at least in one version of his actual death, he appealed to his bodyguard, Bituitus, to impale him with a sword.

Ancient Greece

Nicander of Colophon (fl 130 BC), a Greek poet and physician, is the author of two of the oldest extant works on poisons—*Theriaka* and *Alexipharmaka*—both written in hexameter verse (Gow and Scholfield, 2014; Touwaide, 2014b). The *Theriaka* concerns venomous animals. As such they have a delivery system through which injection of their venom can be harmful to humans and other organisms. A large portion of this volume is devoted to snakes. Among other information, he describes 15 snakes, including several cobras, and the symptoms in humans associated with envenomation, followed by discussion of remedies. Additional narrative is devoted to spiders, scorpions, insects, lizards, and fish. His *Alexipharmaka*, a briefer poem, deals with 21 poisons from the vegetable, mineral, and animal kingdoms. Among them are aconite, white lead, and hemlock. As in his companion work, Nicander describes the poison, its symptoms, and antidotes.

The Greek philosopher, Socrates (469–399 BC), whose wisdom was kept alive through the ages via his disciple, Plato, became an iconic figure in the history of toxicology through his death. Convicted of corrupting the youth of Athens and disrespecting the gods, he was sentenced to death. The received knowledge of the ages, historiographically transmitted, is that his execution was to be carried out in suicidal fashion, with Socrates condemned to drink an extract of hemlock, a poisonous plant (*Conium maculatum*) well known to the ancients. Recently, scientific evidence has called this into question largely because the account provided in Plato's *Phaedo* describes a clinical disorder not caused by hemlock poisoning (Dayan, 2009), although the debate has yet to be resolved and some sources point to a possible mixture of hemlock and opium (Arihan et al., 2014).

Alexander the Great (born 356 BC) plays a role in the history of toxicology in Greece in that the cause of his death is an unsolved mystery as well (Mayor, 2014). He is said to have drunk vast quantities of wine at a banquet in Babylon, after which he suffered severe abdominal pain. Over days, things went from bad to worse and he developed partial paralysis finally dying two weeks later. Rumors of poisoning began circulating in no time. He had enough enemies. Some even thought that Aristotle, his former tutor, poisoned him. Some of his friends guessed that he succumbed to a legendary poison taken from the waterfall of the Styx River, not only the mythological entrance to Hades, but an actual place in the north central Peloponnese. Ancient writers have considered the river poisoned. Though possibilities abound and speculation is widespread, the true cause of Alexander's death has never been confirmed.

Recent discoveries suggest that even the Oracle at Delphi, perhaps the most important and sacred shrine in ancient Greece, is, in a curious fashion, toxicologically significant. Associated with the Greek god Apollo, people would pilgrimage to Delphi with questions usually about what events would occur in the future. They would address their questions to the Pythia, a role filled by various women at different time. Plutarch, the celebrated Greek biographer and essayist, served as one of the priests at the temple of Apollo at Delphi. He noted that *pneuma* (a kind of gas or vapor) was emitted in the *adyton*, a small inner sanctum type area (de Boer, 2014). The Pythia would sit on a tripod-shaped chair, given a chance to inhale the *pneuma*, and go into a trance, after which a priest would address to her the questions asked by the petitioners. Similar accounts appear in ancient texts by others including Plato. Modern-day research attempted to assess the likelihood of an actual gas affecting the mental states of these priestesses. A 2002 paper bringing together the skills of a geologist, archaeologist, and clinical toxicologist reviewed the various research studies, concluding that "the probable cause of the trancelike state used by the Pythia at the oracle of Delphi during her mantic sessions was produced under the influence of inhaling ethylene gas or a mixture of ethylene and ethane from a naturally occurring vent of geological origin" (Spiller et al., 2002).

Toxicology is also heir to a rich mythological tradition. After Hercules, for example, killed the nine-headed sea monster known as the Hydra, as part of his second labor, he cut it open and dipped his arrows in its venom, providing him with what may have been the first biological weapon for use in future battles. Achilles, one of the prominent heroes in Homer's *Iliad* was a victim of just such a poison. Immersed as an infant in the river Styx by his mother to make him immortal, she failed to realize that in holding him by the heel, that very part of the body would make him susceptible to future danger. And so, it was that in the final battle of the Trojan War, he was killed by a poisoned arrow shot into this heel. These are but two examples of how poisons were incorporated into myth and legend in ancient Greece and elsewhere.

Ancient Rome

The Romans of antiquity were also knowledgeable in the principles and practice of toxicology. Interestingly, the Latin word *venenum* can mean either poison or remedy, and one would typically modify the term according to the usage intended (i.e., *bonum venenum* or *malum venenum*).

Dioscorides (born 40 AD), a native of Anazardus, Cilicia, Asia Minor, was a physician who traveled through the Roman Empire with Emperor Nero's army. He would collect samples of local medicinal herbs as he encountered them. The information he gleaned became material for his encyclopedic *De materia medica*, compiled in the 1st century AD, and relied upon for centuries as the most extensive and reliable herbal available. In it he classified poisons as animal, plant, or mineral (Timbrell, 2005). More specifically, *De Venenis* and *De venenosis animalibus*, ascribed to Dioscorides but probably not written by him, covered poisons in general and animal venoms, respectively, and were very influential works in toxicology down through the ages (Touwaide, 2014a).

Galen, another Roman Empire era physician, born (129 AD) in Pergamon, had a monumental impact on the understanding and practice of medicine. He became court physician to Marcus Aurelius. He was a firm subscriber to the theory of the humors (blood, yellow bile, black bile, and phlegm), the origins of which may go back to ancient Egypt but which were first articulated about medicine by Hippocrates. Galen formulated his own Galeni Theriaca and claimed it improved upon the one concocted by Mithridates (Karaberopoulos et al., 2012). He wrote about assorted theriac

compounds in his books *De Antidotis* I and II and *De Theriaca ad Pisonem*. Indeed, he tested them by bringing roosters into contact with snakes.

Poisoning, especially among the ruling classes, was frequently practiced, typically (but not exclusively) by women upon their husbands or other inconvenient relatives. If they did not have the skills to do the deed themselves, they sought professional poisoners, usually women as well. One of the most notorious of the lot was Locusta. As the story is told, she was summoned by Agrippina, the wife of Emperor Claudius, to kill him so that Agrippina's son, Nero, from a previous marriage would become the new Roman emperor. Locusta supplied Agrippina with a batch either of poisoned or poisonous mushrooms. Though taken quite ill, the mushrooms did not kill Claudius outright. Quick thinking (though history is not quite clear by whom) led Agrippina to convince Claudius to let her run a feather down his throat to expel the poison. The feather itself, though, was coated with a lethal dose of poison which killed Claudius and thus Nero assumed the throne. Though Locusta was imprisoned, it was not long before Nero had her released and, in fact, employed her to poison Britannicus, a son of Claudius from a previous marriage and thus a threat to the new emperor. Nero ultimately pardoned Locusta for all past crimes and she was allowed to establish a school to train others in her art.

The legal framework of toxicology is sometimes dated back to the age of the Roman military and political leader Sulla. Under the *lex Cornelia de sicariis et veneficis* (81 BC), punishment was imposed for anyone who prepared, sold, bought, kept, or administered a noxious poison (venenum malum) (Hobenreich and Rizzelli, 2014).

A theory proposed in 1983 by Jerome Nriagu popularized the idea that the metal lead was responsible for the fall of the Roman Empire. It has been stated that the ruling classes, in particular, were exposed to lead contamination in water supplies, cooking, and the production of wine, ultimately decreasing their fertility and reproductive capacity. More recent archaeological investigations have found that although clinical lead poisoning probably did occur, the mean skeletal lead content of populations at the time was less than half that of present-day Europeans in the same regions. The assertion that lead was the primary culprit in Rome's decline and fall has been largely refuted (Cilliers and Retief, 2014a, 2014b).

Lead has continued to plague mankind, in occupational and other exposures, through the ages. Interestingly, in 1921 a global treaty the White Lead (Painting) Convention was adopted. It was meant to largely prohibit the use of white lead as a pigment in paint. With no thanks to the Lead Industries Association, this was never ratified by the United States (Hernberg, 2000). Herbert Needleman, a physician, was instrumental in helping us understand how lead affects children, particularly with his 1979 study in the *New England Journal of Medicine* noting deficits in children with high dental lead levels (Rosner and Markowitz, 2005). Still a concern in inner cities, lead periodically makes the headlines, as in the case of its seepage into the drinking water of Flint, Michigan, in 2016.

THE MIDDLE AGES AND RENAISSANCE

As we transition from antiquity to the Middle Ages at about 400 AD, toxicology continues to have a presence in European society vis-à-vis both poisoning as a means of dispatching enemies but increasingly in trying to establish its scientific foundation. Some of the well-accepted tenets of the toxicology of this time such as the hypothesis that the saliva of rabid dogs was a poison on a par with snake venom would see revision, but the scientific method was at least beginning to take hold.

The Venetian Council of Ten was a governing body in Venice from around 1310 until 1797. They were known for conducting secret tribunals whereby figures perceived as a threat to the state were ordered executed. Many of these executions were carried out by poisoning. There were several attempts on the life of Francesco Sforza of Milan, while Mehmed II, Sultan of the Ottoman Empire, was allegedly ordered to be poisoned by the Council (Jutte, 2015).

Poisoners continued to find steady employment but some reputations, as will be seen in the following paragraphs, were ill-deserved. Poisoning as an assassination method was widespread during the 14th to 16th centuries in Europe. Letters to Grand Duke Cosimo I de'Medici affirm as much. Animal venoms, phytotoxins, and mineral poisons were all employed. Cosimo himself was suspected of poisoning and was in possession of a poison recipe among his confidential documents and his library contained several books in which poisons were discussed. He was also involved in a plot to assassinate Piero Strozzi, part of a rival banking empire, by poisoning his wine. Poisoning was clearly a family affair with the Medicis, and Cosimo's sons Ferdinando and Francesco were equally complicit in it. Despite persistent rumors that Francesco and his wife, Bianca, were poisoned with arsenic by the former's brother, Ferdinando, the official cause of death was listed as malaria. Although recent forensic examinations still do not entirely agree, it now appears most likely that malaria was indeed the culprit (Fornaciari and Bianucci, 2010). Many legends surround Catherine de'Medici who moved to France to marry the future King Henry II. Despite multiple purported victims, there is no definitive evidence that she poisoned anyone. Developing and testing antidotes was also part of the Medicis' stock-in-trade (Pratte et al., 2014; Barker, 2017).

Another powerful and infamous Italian family, originally from Spain, and on whom were pinned numerous heinous crimes, poisoning among them, were the Borgias. There were claims, for example, that Cesare murdered a servant who was a lover of his sister, Lucretia, in front of their father Pope Alexander. Cesare was also said to have poisoned Cardinal Juan Borgia. The reputation of Lucretia herself was stained with allegations, by enemies of the Borgias, that she was a poisoner. Documents uncovered recently in the Vatican archives refute these and other claims concerning the Borgias and it is now thought that, though saints by no means, their undeserved reputation for extensive poisonings and murders stems from rumors spread and repeated by their enemies (Dal Bello, 2012; Cobb, 2017).

In 17th century France, during the reign of Louis XIV there had been a series of poisonings which have not, at least to date, been subject to any of the above revisionism. It became known as *L'affaire des poisons* (the Affair of the Poisons) and originated with the trial of Madame de Brinvilliers, convicted of poisoning her father and two brothers and attempting to poison other family members. Prior to her execution she implicated, without specifically naming them, many others, who were subsequently prosecuted and sentenced to death. One of the most notorious was the celebrated Catherine Deshayes, also known as La Voisin, an acknowledged sorceress, who did a very good business in poisons, abortions, and black masses. La Voisin was finally burned at the stake in 1680 for her crimes (Duramy, 2012; Somerset, 2014).

Giulia Tofana was yet one more notorious 17th century Italian poisoner, thoroughly skilled at her trade. It is thought that two women in Palermo, Francesca la Sarda and Teofania di Adamo, jointly concocted and marked a poison known as "Acqua Tufania" for which they were executed. Some of their associates fled to Rome and, under the leadership of Giulia Tofana, possibly Teofania's daughter, they carried on the business, even after the death of Giulia.

The poison became known as Aqua Tofana. Arsenic was likely a primary ingredient. It was sold throughout Italy to domestically unsatisfied women seeking freedom from their husbands. Aqua Tofana became an almost generic term for particularly potent poisons and the term has appeared in various sources, including medical textbooks, for some two centuries. Although originally producing violent symptoms, it ultimately became associated with a class of toxicants known as "slow poisons," which rather than existing in fact may have simply been a speculative class of agents designed to fuel the imaginations of the easily swayed (Dash, 2015).

As already mentioned, the Middle Ages and Renaissance were times not only of commonplace poisonings, particularly among the aristocracy and ruling classes, but of an increasingly sophisticated understanding of toxicology. Moses Maimonides, the great Jewish philosopher, theologian, and scientist, wrote his *Treatise on Poisons and their Antidotes*, originally in Arabic, in 1198. Part I was concerned with bites from snakes and rabid dogs (toxicology, remember, was still in its formative stage), and stings of scorpions and insects. Part II dealt with poisons in food and minerals, as well as remedies. He made a distinction between "hot" and "cold" poisons which, it has been claimed, may be equivalent to modern-day hemolysins and neurotoxins. Maimonides also emphasized preventive measures (Rosner, 1968; Furst, 2001; Maimonides, 2009).

The study of toxicants was so widespread in Persian and Arabic countries during the Middle Ages that the era has come to be known as the golden age of medieval toxicology. Among prominent toxicologists who wrote noteworthy treatises on the subject were Jābir (Jaber) ibn Hayyān (721–815 AD), Ibn Maāsawyah (Yuhanna ibn Masawyah, Abu Zakariya, 777–857 AD), and Ibn Waḥshīyah al-Nabti (9–10th century AD). Known by his Latin name of Avicenna in the West, Abū ʻAlīAal-Ḥusayn ibn Abd Allāh ibn Sīnā was perhaps the most noteworthy physician/scientist/philosopher of the Islamic world. His celebrated "Canon of Medicine" remained the most popular medical textbook for some six centuries (Nasser et al., 2009). Covering a broad range of topics, it includes detailed descriptions of venoms and other poisons, such as opioids and oleander, as well as instructions related to antidotes (Ardestani et al., 2017). He even explored the effect of alcohol on opium poisoning:

Patients may have concurrent alcohol poisoning. It can have a synergistic effect with opium poisoning and decrease its lethal dose. On the other hand, alcohol may serve as an opium antidote. This effect depends on the amount of ingested alcohol.

Many of his observations have been confirmed by current medical knowledge (Heydari et al., 2013).

On a very practical level, as was seen even in the Roman era, it became clear to ordinary people, especially those whose work entailed significant exposure to certain natural materials such as minerals, that their very occupations could be harmful. Georgius Agricola (1494–1555) born in the kingdom of Saxony, currently part of Germany, studied many subjects and completed his medical education in Padua. He has come to be known as "the father of mineralogy" largely as a result of his best known monograph, *De Re Metallica*, published in 1556.

Inevitably we reach the point where we address the incalculable contributions of the unorthodox medical revolutionary, Theophrastus von Hohenheim, called Paracelsus (1493/94–1541). Born in Einsiedeln, a municipality now in modern-day Switzerland, he was a wanderer and iconoclast, and strongly tied to the alchemical tradition. He theorized that there were four pillars of medicine: natural philosophy, astronomy, alchemy, and medical virtue. He went his own way and was not highly regarded by the medical establishment or local government officials. Indeed, as a lecturer at the University of Basel (as well as the city's municipal physician until being forced to flee), he burned the standard medical textbooks of the day, such as those of Avicenna and Galen (Borzelleca, 1999). History, though, has vindicated many of his teachings. In addition to his medical works, he was a keen observer and investigator of toxic effects of various agents and wrote a treatise about their effects upon miners. He concludes this work with a discussion of metallic mercury and criticizes its use at the time as therapy for people afflicted with syphilis (Gantenbein, 2017).

The most famous toxicological adage associated with Paracelsus is "The dose makes the poison," which is a distillation of what he wrote in his *Seven Defenses*, designed to defend his controversial teachings in the face of his adversaries:

Wenn jhr jedes Gifft recht wolt außlegen/ Was ist das nit Gifft ist? alle ding sind Gifft/ vnd nichts ohn Gifft/ allein die Dosis macht/ dz ein ding kein Gifft ist.
When you want to correctly evaluate a poison, what is there that is not poison? All things are poison and nothing is without poison; only the dose determines that something is not a poison.

This was surely known in various and sundry ways, certainly by experience, long before the time of Paracelsus, but never had it been so well articulated. We may, today, look upon the latter portion of this statement as an oversimplification. After all, what about factors other than dose which influence toxicity—gender, age, pre-existing conditions, genetics, the microbiome, etc.? This is all well and good, and it is not unusual for quite valid eureka moments to be refined over time, but for a concise encapsulation of one of the key components of what and when something is a poison, and which continues to serve as a bedrock of toxicology, Paracelsus deserves the laurel crown and the oft-cited appellation, "Father of toxicology." An understanding of the dose–response relationship is no less significant to our understanding of toxicology today than it was 500 years ago.

It is tempting to declare Paracelsus' legacy as ironclad. However, proponents of a theory originating in the 19th century known as hormesis are today suggesting that substances known to be toxic at elevated doses may actually have a beneficial effect at very low doses. Non-monotonic dose–response (NMDR) curves graphically describe hormesis. Hormesis remains a controversial theory among toxicologists.

Paracelsus was but one example of the tenuous link between alchemy and toxicology. The alchemist Jan Baptist Van Helmont, though once a disciple of Paracelsus, ultimately went his own way. Van Helmont did acknowledge that almost everything in nature is possessed of some secret poison but that somehow it overlay a core of goodness. He referred to the bible and medical alchemical theories to support his views and reveal ways to remove the poison (Hedeson, 2017).

Other key figures were Pietro d'Abano who compiled a treatise devoted to poisons and their remedies, *De venenis*, which sought to return to the pure Greek roots of toxicology; the Paduan physician Girolamo Cardano who offered a careful analysis on the relationship between poison and putrefaction; Gerolamo Mercuriale who focused on reconciling ancient and contemporary definitions of poison; and Andrea Bacci who argued against a universal definition of poison and also said that its unusual powers made it similar to other natural substances such as the magnet (Gibbs, 2017; see http://fredgibbs.net/posts/universals-and-particulars-of-poison).

Interest has always been keen on both preventing and treating poisoning. Various products of biological origin, typically solid and hard, were said to serve in this capacity. They include stones, shark teeth, bezoars, and horns, sometimes embellished and worn as jewelry, and used in table settings or even in some instances found

in graves. A bezoar stone is an indigestible mass found in the gastrointestinal system, especially the stomach. Etymologically, the word derives from the Farsi words, *bāk* (purification) and *zahr* (poison) and, indeed, the stones were described in ancient Arabic medical literature since the 8th century and used as antidotes by Persian, Arab, and Jewish physicians. Belief in bezoars made its way to Europe and is mentioned in Johannes de Cuba's *Hortus Sanitatis* in 1485 and Pietro d'Abano described their use in 1565 (Barroso, 2014, 2017).

Fossil shark teeth (Glossopetrae), as well, have found application as prophylactics, detectors, and neutralizers of poisons. In medieval times, it was said that such teeth mounted in silver announced poisons by "sweating" or changing color. Their ability to detect poison and protect humans from poisoning is cited in Lapidaries such as those of Marbode (11th century), Sloane (16th century), and Jean de Mandeville. Miocence specimens of *Otodus megalodon* from Malta were said to be the most efficacious of the shark's teeth. Due to a 16th century shortage of bezoar stones, a substitute that came to be known as Goa Stones was formulated. In addition to various precious stones, coral, ambergris, and musk, they often contained pulverized fossil shark teeth. Often gold-plated, they could be housed in containers of elaborate silver or gold. Scrapings from these stones mixed in wine, beer, or other beverages could purportedly ward off the effects of any poisons (Duffin, 2017).

Alicorn, that is, the horn of the mythical unicorn, was thought to have medicinal and poison detecting qualities. By the end of the 14th century, the idea became established that it too like shark teeth could detect poison by perspiring in the presence of adulterated food and drink. One of the earliest medieval sources about the medicinal power of unicorns (though the horn per se is not mentioned) is the *Physica b*y Hildegard of Bingen (1098–1179) (Lavers, 2017). James Primrose noted: "It can scarce be said, whether to the Bezaar stone, or to the Unicornes horn the common people attributes greater vertues, for those are thought to be the prime Antidotes of all" (Primrose, 1651). Narwhal teeth or the horns of many another animal were likely passed off as unicorn horns. In 1389, John of Herse made a pilgrimage to Jerusalem and observed, "Near the field Helyon in the Holy Land is the river Mara whose bitter water Moses struck with his staff and made sweet so that the children of Israel could drink thereof. Even now evil and unclean beasts poison it after the going down of the sun; but in the morning the unicorn comes from the sea and dips its horn into the stream and thereby expels the poison so that the other animals can drink of it during the day. The fact which I describe I have seen with my own eyes" (Unitarian Review, 1879). There was not, though, universal acceptance of the anti-toxic legitimacy of unicorn products (including powder). Two respected French authorities, Ambroise Paré (1510–1590), court physician to four French kings, and the pharmacist Laurent Catelan (1568–1590) from Montpellier, had differing views on alicorn, with the former a detractor of its efficacy and the latter a proponent (Gerritsen, 2007). Eventually, as with much else, the antidotal property of unicorn horns was consigned to legend.

18TH AND 19TH CENTURIES

Hermetical traditions such as alchemy did not suddenly disappear come 1700. Isaac Newton himself was a passionate alchemist, as was Robert Boyle, often considered the father of modern chemistry. That said, the scientific method gained increasing prominence in the 18th and 19th centuries as a way of understanding our universe, and toxicology benefited from this more sophisticated and methodical approach. A number of scientists made important contributions to toxicology during this time.

Richard Mead (1673–1754) is the author of the first book in English devoted solely to poisons, *A Mechanical Account of Poisons in Several Essays*. He described the signs and symptoms of snake envenomation, performed chemical tests on venom, and experimented on snakes (to study their venom delivery system) and other animals (Seifert, 2011).

Bernardino Ramazzini, born in Carpi, Italy, and educated at the University of Parma, was a physician whose seminal achievements have earned him the moniker Father of Occupational Medicine (Pope, 2004). While the connection between workers' illnesses and their workplace environment, including materials to which they are exposed, had been noted by the ancients, Ramazzini's analysis of this linkage raised the issue to an entirely new level. The first edition of his most famous book, *De Morbis Artificum Diatriba* (*A Treatise on the Diseases of Workers*), published in 1700, is the first comprehensive and systematic work on occupational diseases (Felton, 1997). It outlined the health hazards of chemicals and other substances, including repetitive motions, encountered by workers in over 50 occupations. Among Ramazzini's many enlightening observations, and one in which he quotes Hippocrates, is the following:

"When you come to a patient's house, you should ask him what sort of pains he has, what caused them, how many days he has been ill, whether the bowels are working and what sort of food he eats." So says Hippocrates in his work Affections. I may venture to add one more question: what occupation does he follow?

The spirit of Ramazzini lives on in the Collegium Ramazzini (CollegiumRazazzini), an independent, international academy founded in 1982 by Irving J. Selikoff and others, to advance the study of occupational and environmental health issues. It holds conferences, symposia, and training courses, and publishes statements and research papers.

Another key figure in occupational toxicology is Percivall Pott (1714–1788), born in London. In 1774 he published an essay, *Chirurgical Observations Relative to the Cataract, the Polypus of the Nose, the Cancer of the Scrotum*. In this he made the link between the profession of chimney sweeps (regarding soot lodging in the folds of scrotal skin) and scrotal cancer (Brown and Thornton, 1957). This was the first occupational link to cancer and Pott's investigations contributed to the science of epidemiology. It wasn't until the 1920s that benzo[a]pyrene was identified as the actual chemical responsible (Dronsfield, 2006).

There were many scientists spanning the 18th and 19th centuries who played significant roles in making toxicology the discipline that it is. The ability to synthesize new chemicals and the added ability to detect their presence, especially in small amounts, marked the beginning of the modern era of toxicology. For centuries, poisonings were confirmed only by confession or eye witness accounts. Making the leap from merely suspecting adulteration or poisoning to irrefutable proof was a major milestone for toxicology. Four scientists who made remarkable advances in the area of chemical detection were Karl Wilhelm Scheele, Christian Friedrich Samuel Hahnemman, Johann Daniel Metzger, and Valentine Rose. Scheele discovered oxygen before Joseph Priestley, although he published his results later. He is also credited with the discovery of hydrofluoric, hydrocyanic, and arsenic acids, and devised methods for detecting arsenic in body fluids and corpses. Hahnemman discovered a test for arsenic oxide. Rose and Metzger discovered the first methods for detecting elemental arsenic and arsenic oxides in fluids and tissues (Farrell, 1994). In 1836, the English chemist

James Marsh developed what came to be known as the Marsh test, a groundbreaking method for detecting arsenic.

The medical celebrity Mathieu Joseph Bonaventure Orfila (1787–1853) is often claimed by Spain (where he was born and studied) and France (where he continued his studies, worked, and died) (Bertomeu-Sanchez and Nieto-Galan, 2006; Bertomeu-Sanchez, 2009). While very influential in applying the concepts of chemistry to medicine, it was in toxicology that he excelled and for which he is best known. He became Dean of the Paris Medical Faculty and was a founding member of the Academy of Medicine. At a time when animal experimentation was somewhat less frowned upon, he experimented widely with dogs, varying the amount of poison (such as arsenic) administered and the route of administration, and tested antidotes and treatments. He authored *Traite des poisons*, one of the most popular textbooks of the first half of the 19th century (Orfila, 1814–1815). He subsequently extracted the sections on antidotes and treatments and published them in a compact free-standing volume designed not only for physicians but also for lay audiences that may not have access to medical care but need to know what to do in the event of a poisoning emergency.

Orfila was called to act as a medical expert in various criminal cases. He is best known for a case involving Marie Lafarge, charged with poisoning her husband. Eyewitnesses had seen her buying arsenic (used to exterminate rats) and stirring a white powder into her husband's food. Upon his exhumation, no evidence of arsenic was found using the newly improved test for arsenic devised by James Marsh, although doubts remained whether the physicians were performing the test properly. Orfila was summoned and found definite traces of arsenic in the body, and demonstrated that it did not come from the surrounding soil. Marie Lafarge was found guilty of murder and received a death sentence, later commuted to life in prison. The case cemented Orfila's reputation as the greatest toxicologist of the day.

And yes, indeed, not only Paracelsus, but also Orfila has been called "Father of Toxicology," but of course representing a different era, and for different reasons. "Father of Forensic Toxicology," or "Father of Modern Toxicology," might be more precise. Let's hope that all these "Father of Toxicology" claims don't result in any paternity suits.

In France, Francois Magendie (1783–1855) was best known for his pioneering contributions in neuroscience and neurosurgery, and experimental physiology. His studies on the effects of drugs on different parts of the body though led to the introduction of compounds such as strychnine and morphine into medical practice (Tubbs et al., 2008). His research into the mechanisms of toxicity of these and other alkaloids furthered the science of toxicology.

Claude Bernard (1813–1878), Magendie's most celebrated pupil, made several physiological discoveries including the role of the pancreas in digestion, the regulation of the blood supply by vasomotor nerves, and the glycogenic function of the liver. His work also led to an understanding of the self-regulating process of living organisms we now refer to as homeostasis. He won acclaim for his book *Introduction à l'Etude de la Médecine Expérimentale* (*An Introduction to the Study of Experimental Medicine*), a classic in the field. He stressed the importance of starting with a hypothesis and having results which are reproducible, thereby furthering the paradigm of the modern scientific method. In the realm of toxicology, Bernard demonstrated that the mechanism of action of curare resulted from its interference in the conduction of nerve impulses from the motor nerve to skeletal muscle. The sensory nerves were left intact. In addition to curare, he studied the toxicological properties of other neuroactive compounds such as opium, atropine, strychnine, and nicotine (Bernard, 1857; Conti, 2002). He was also the first to describe the hypoxic effects of carbon monoxide. Bernard was attuned to how the perturbation of biological systems by toxic agents can be of value to basic science. He stated:

Poisons can be employed as means for the destruction of life or as agents for the treatment of the sick but in addition there is a third of particular interest to the physiologist. For him the poison becomes an instrument which dissociates and analyses the most delicate phenomena of living structures and by attending carefully to their mechanism in causing death he can learn indirectly much about the physiological processes of life …

While Orfila, as we have seen, also experimented on dogs, and was one of many scientists, including Magendie, to subscribe to animal experimentation, Bernard established it as part of the scientific method. He stated:

Experiments on animals are entirely conclusive for the toxicology and hygiene of man. The effects of these substances are the same on man as on animals, save for differences in degree.

Bernard, though an acknowledged seminal figure in experimental medicine, was criticized over his vivisection experiments on unanesthetized animals. The debate over the moral ramifications of animal experimentation gained steam during his lifetime. Interestingly, his wife was appalled by this part of his work. She left him, took their daughters, and with them became ardent antivivisectionists (Cavan, n.d.).

Greatly influenced by Orfila, Robert Christison (1797–1882), a Scottish physician, was interested in underpinning medical jurisprudence, especially toxicology, with a scientific foundation. Early on, he investigated the detection and treatment of oxalic acid poisoning and followed this up with investigations on arsenic, lead, opium, and hemlock. His celebrated book, *Treatise on Poisons,* first published in 1829, went through four editions. In addition to his work on poisons, he made important contributions in nephrology (Wikisource, n.d.-b).

Substance abuse, dependence, and addiction have plagued people throughout all time. Published in 1821, Thomas De Quincey's penetrating *Confessions of an English Opium Eater* is an autobiographical account of his opium (more properly laudanum, for he took his opium with alcohol) addiction. His book covers both *The Pleasures of Opium* and *The Pains of Opium*. This may have been the first look at drug addiction but was followed by countless others, fact and fiction, in numerous artistic genres, literary, visual, and even musical: to name a few (some made into movies) Aldous Huxley (*The Doors of Perception*), Hunter S. Thompson (*Fear and Loathing in Las Vegas*), William S. Burroughs (*Naked Lunch* and *Junky*), and Irvine Welsh (*Trainspotting*). Billy Wilder's film, *The Lost Weekend* (1945), featuring Ray Milland, is a classic about alcoholism and Frank Sinatra stars as a heroin addict in *The Man with the Golden Arm* (1955).

THE MODERN ERA

Radiation

The late 19th century is about the time when an understanding of radiation and its potentially hazardous effects began to surface. As is the case with chemicals and biological agents, radiation can be and has been of enormous benefit to society in general and has resulted in countless positive health outcomes via diagnosis and therapy. Nonetheless, precautions are necessary because radiation hazards can be devastating. In 1895, Wilhelm Röntgen discovered x-rays, electromagnetic energy waves with wavelengths

some 1000 times shorter than those of light. He also learned that x-rays could penetrate human flesh. In 1896, Nikola Tesla intentionally exposed his fingers to x-rays and reported burns. In that same year Henri Becquerel discovered that uranium salts naturally emitted similar rays. Marie Curie, a student of Becquerel, named the phenomenon "radioactivity." She went on to discover thorium, polonium, and radium, and received the Nobel Prize twice (once with her husband and Becquerel in physics and later in chemistry). Tragically, her death was attributed to aplastic anemia, likely contracted from her extensive work with radioactive materials (Jorgensen, 2016).

Soon after radium's discovery, it was manufactured synthetically and was believed to have almost magical healing properties. It appeared in food products such as bread, chocolate, toys (because of its luminescence), toothpaste, cosmetics, suppositories, and products to treat impotence. One of the first revelations about the true potency of radioactivity and the scope of its potential danger concerned the unfortunate girls who became radium watch dial painters in the early 1900s. These "radium girls" were hired by the U.S. Radium Corporation to apply radium paint to watch and clock faces so they would glow in the dark. They were instructed to use their lips to shape the brushes to a fine point. By 1927, over 50 women died due to radium paint poisoning, and many of the survivors suffered significant health problems (Mullner, 1999).

The detonation of the world's first atomic bomb in 1945, the Trinity Test, an outgrowth of the Manhattan Project, took place in the New Mexico desert where the nuclear age literally burst upon the scene. There were no doubts, at this point, about the damage such a bomb could inflict and did. On August 6, 1945, while World War II was raging, an American B-20 aircraft dropped an atomic bomb over the city of Hiroshima, killing nearly 100,000 people on impact and decimating virtually the entire city. Maybe half of that number of people were killed when a second atomic bomb was dropped on Nagasaki. Tens of thousands of people in both cities would later die of radiation exposure or otherwise suffer devastating injuries (Blow, 2015). The Treaty on the Non-Proliferation of Nuclear Weapons (NPT), which entered into force in 1970 and was extended indefinitely in 1995, seeks to "prevent the spread of nuclear weapons and weapons technology, to promote cooperation in the peaceful uses of nuclear energy and to further the goal of achieving nuclear disarmament and general and complete disarmament" (UNODA, n.d.).

Although nuclear weapons were developed and used to intentionally wreak destruction and havoc, nuclear power plants are designed to harness the force of the atom for peaceful purposes, that is, to generate energy. However, things do not always go as planned. In 1979, the Three Mile Island plant in Pennsylvania suffered a malfunction that led cooling water to escape from the reactor, and the nuclear fuel rods suffered a partial meltdown. Thankfully, there were no detectable health effects in the population at large. In contrast, the people in the area of the Ukraine where the Chernobyl plant was located experienced a dramatic meltdown in 1986 and were not so fortunate. There was no containment structure and a plume of radioactive material was sent skyward. An estimated 30 people died from radiation poisoning over a period of weeks and several thousand more were put at risk for cancer. In 2011, a massive earthquake and tsunami disabled the power supply and cooling of three Fukushima Daiichi reactors in Japan. All three cores melted within days. No deaths from radiation sickness was reported but over 100,000 people were evacuated from their homes (NPR, n.d.; World Nuclear Association, 2017).

Food and Drugs

The science of qualitative and quantitative chemical detection was applied most effectively to the detection of chemicals in body fluids, drugs, and food. In modern society, we have grown so accustomed to regulations that ensure high standards of purity for most commercial products that it is difficult to remember a time when there were no such protections in place. The realization that there was indeed a need for them evolved gradually. Events leading up to the passage of the Pure Food and Drug Act of 1906 are a good place to start since much of what we consider the modern era of toxicology occurred in and around early efforts to regulate the commerce of food and drugs.

Toxicology has developed and continues, to some extent, to develop as a reactive (rather than proactive) field. Thus, chemical laws and regulations often are enacted in reaction to major or widespread exposure incidents. An early demonstration of this phenomenon is in the efforts to ensure the safety of certain substances to which virtually everyone was exposed, that is, food and drugs. As early as 1848, chemical analyses of agricultural products were carried out in the U.S. Patent office under the Department of the Interior by Lewis Caleb Beck, an American physician and chemist who researched the adulterants in many drugs commonly prescribed by physicians of the time (Kinch, 2016). In 1846 he published *Adulterations of Various Substances Used in Medicine and the Arts with Means of Detecting Them: Intended as a Manual for the Physician, the Apothecary, and the Artisan*. His publication helped promote the Drug Importation Act of 1848. At the time, there were six major ports of entry within the United States, namely New York, Boston, Baltimore, Philadelphia, New Orleans, and Charleston, where pharmaceuticals entered the American market. The 1848 law required the U.S. Customs Service to inspect and stop any adulterated drugs from entering the U.S. market. Inspectors were typically experienced physicians and pharmacists who could more easily detect a counterfeit substance. They were also armed with the added ability to conduct qualitative tests, such as those detailed in Beck's publication, to determine if a drug was adulterated.

The Department of Agriculture, which would eventually give rise to the Food and Drug Administration (FDA), was established under Abraham Lincoln in 1862. The Division of Chemistry rested within this department and employed a single chemist Charles Mayer Wetherill. In 1883, Harvey W. Wiley, who was to play a highly influential role in safeguarding the country's food and drugs, took over as the Division's fourth chemist. The Division of Chemistry became the Bureau of Chemistry in 1901 and in 1902 Wiley was granted $5000 to administer what came to be called the "Poison Squad" experiments. These experiments involved asking healthy volunteers to consume measured amounts of preservatives routinely added to food items to determine whether they were safe for human consumption. The experiments were carried out in a controlled setting with meals prepared by a designated cook and chemist William R. Carter (Pray, 2003). Although cringeworthy by today's ethical standards, some of the chemicals fed to these young men were borax, benzoic acids, and formaldehyde. While many still question the validity of these sensational experiments, the publicity helped to enlighten consumers about the potential dangers of adulterated foods and the importance of accurate labeling.

Wiley was not alone in his pursuit to rid the market of impure foods and drugs. Journalists as well took up the cause of exposing quack medicines and adulterated food staples thereby fueling Wiley's efforts. The so-called muckraking journalists of the early 20th century exposed hundreds of patent medicines as misleading, harmful, and sometimes deadly. One example (of many) was the

case of acetanilide, a nonsteroidal anti-inflammatory drug used to treat pain and reduce fever, but highly toxic. In 1905, Samuel Hopkins Adams published, in *Collier's Weekly*, "The Great American Fraud," a sensational article exposing the hoax of patent medicines (Adams, 1905). Upton Sinclair's 1906 book, *The Jungle*, detailed unsanitary conditions of workers in the meat packing industry. "The Jungle" was published as a serial in 1905 and then as a book in 1906. Despite the many efforts to pass legislation to ensure food and drug safety prior to 1906, nothing seemed to get through both the House and Senate and, unfortunately, many bills languished for years. Wiley worked tirelessly to institute food and drug legislation throughout his tenure at the FDA (1883–1912) and during this time over 100 food and drug bills were introduced in Congress with nearly all failing to gain any traction.

The Pure Food and Drugs Act and the Meat Inspection Act were passed on the very same day in 1906 by the then president Theodore Roosevelt. The former law became known as the "Wiley Bill" due to Harvey Wiley's efforts. The Bureau of Chemistry was reorganized in 1927 into the Food, Drug, and Insecticide Administration, later renamed the Food and Drug Administration and ultimately moved out of the Department of Agriculture entirely and into what is now the Department of Health and Human Services.

To backtrack a bit in time, England's attention to the adulteration of food and drugs actually preceded that of the United States by a half century. Friedrich Accum, Wiley's counterpart in the United Kingdom, published a book in the 1820s titled *A Treatise on the Adulterations of Food, and Culinary Poisons* with the subtitle *There Is Death in the Pot*. Accum wrote about hundreds of poisonous additives commonly used in food products to either sweeten, color, or bulk up foods. He also pointed a finger at the perpetrator, giving the names and addresses of the offending manufacturers, which was unprecedented at the time (Accum, 1820; Oser, 1987). Accum became extremely unpopular among wealthy shop owners and he eventually left the country. Friedrich Accum and, later, Thomas Wakley and Arthur Hill Hassall were the figures most responsible for the campaign to prevent food adulteration which eventually resulted in food and drug legislation in the United Kingdom (Oser, 1987).

The 1906 Pure Food and Drug Act in the United States did not have the broad impact that was intended. Wiley and other supporters were hopeful that the law would have far reaching implications and broadly protect the food supply. However, as written, its main purpose was to ban foreign and interstate traffic of adulterated, falsely advertised, or mislabeled food and drug products. It empowered the U.S. Bureau of Chemistry to inspect products and refer offenders to prosecutors, but gave no prosecutorial power to the agency itself. For example, during the Jamaican Ginger poisonings detailed in the next paragraph, the FDA was not involved in the investigation or prosecution of the crime until well after the case was resolved by a judge. The law required that the active ingredients be placed on the label of a drug's packaging and that drugs could not fall below purity levels established by the United States Pharmacopeia (USP) or the National Formulary. The USP and National Formulary guidelines were established some years earlier by a group of physicians and pharmacists, and served as a foundation for the Pure Food and Drugs Act. Although the law was popular, it was virtually impossible to enforce. The 1906 law prevented the manufacture, sale, or transportation of adulterated, misbranded, poisonous, or deleterious foods, drugs, medicines, and liquors. The new law led to the establishment of government-run analytical laboratories, and the conditional removal of certain ingredients such as ethanol, herbal mixtures, and coloring agents in most but not all cases. Many sections of the Act were overturned by the then Associate Justice Oliver Wendell Holmes and the U.S. Supreme Court in 1911. Wiley left the Bureau of Chemistry in 1912. The 1906 Act was not perfect, but it was a perfect jump start to the subsequent food and drug reform laws in the United States.

Prohibition in the United States ran from 1920 to 1933. During this time, there were very few legal means for obtaining alcohol. One of the few remaining options for alcohol consumption was via a doctor's prescription which would allow one to procure whiskey or rum from a pharmacist. Meanwhile, it was legal to purchase over-the-counter patent medicines or elixirs containing alcohol. Some disreputable drug companies began increasing the alcoholic content of their medicines or inventing new ones composed almost entirely of alcohol. One infamous concoction was Jamaica Ginger, which contained between 70% and 80% alcohol by weight. The U.S. Treasury Department required changes to the ingredients of Jamaica Ginger to discourage its abuse. The minimum requirement of ginger solids per cubic centimeter of alcohol resulted in a bitter concoction that was not palatable. Inspectors would often boil down the liquid and weigh the solids to ensure that the concoction was formulated appropriately. Two bootleggers (Harry Gross and his brother-in-law Max Reisman) developed an alternative recipe that could pass the inspection and taste well enough to sell by adding tri-ortho-cresyl phosphate (TOCP) to the mixture. In early 1930 reports began to pour in detailing strange paralysis of the legs, arms, and wrists with little to no recovery in large numbers of people throughout the midwest. By 1931 the disease, which had come to be known colloquially as Ginger Jake paralysis, had reached epidemic proportions affecting an estimated 10,000 people across the country from New York to California. Doctors eventually traced the illness back to the Jamaica Ginger elixir, but since the typical ingredients (as listed in the U.S. Pharmacopeia) were not known to cause disease they immediately suspected a contaminant was responsible. The matter was taken up by the Public Health Service's National Institutes of Health (NIH), which was newly formed from the Hygienic Laboratory in 1930. It was there that the adulteration with tri-ortho-cresyl phosphate was discovered. There were over 35,000 members of the United Victims of Ginger Paralysis Association (Morgan and Penovich, 1978). The Ginger Jake episode and other cases of false therapeutic claims made it clear that change needed to come to the 1906 law, and change it did, propelled by the sulfanilamide poisonings of 1937–1978.

Sulfa drugs were a 20th century miracle for the treatment of bacterial and fungal infections. The first sulfa drug, Protonsil, showed no effect in vitro with bacterial assays but was extremely effective in vivo. It was later discovered that Protonsil is metabolized to sulfanilamide in vivo and the science of the bioactivation of drugs was revealed. The discovery of sulfanilamide was heralded as a major event in combating bacterial diseases. However, for a drug to be effective there needed to be an equally effective delivery system. Sulfanilamide is highly insoluble in an aqueous solution. Originally prepared as an elixir in ethanol, chemists discovered that the drug was more soluble in diethylene glycol. Therefore, the latter solvent replaced it, and a sweet syrup was added to make it more palatable to children. The new preparation was labeled an "elixir." Many patients, most of whom were children, died of acute kidney failure resulting from metabolism of the glycol to oxalic acid and glycolic acid. The drug and its metabolites crystallized in the kidney tubules, leading to renal failure (Wax, 1994). This tragedy led to the passage of the 1938 Food, Drug, and Cosmetic (FD&C) Act, also known as the Copeland Bill, named for Senator Royal S. Copeland. It contained provisions for both misbranding and adulteration. A cosmetic was deemed to be adulterated if it "contains any poisonous or deleterious substance that may render it injurious to users

under customary conditions of use." The misbranding provisions prohibited labeling that is "false or misleading in any particular." The law also required that a package's ingredients and their amounts, as well as the name and address of the manufacturer, packer, or distributor, be clearly displayed on the label. To enforce the statute, the FDA was given search, seizure, and prosecutorial powers.

The sulfanilamide disaster played a critical role in the development of toxicology and inspired the research of Eugene Maximillian Geiling in the Pharmacology Department of the University of Chicago that elucidated the mechanism of toxicity of the sulfanilamide elixir (diethylene glycol). These studies began at the heart of the investigations in the late 1930s (Geiling et al., 1938). Studies of the glycols were simultaneously carried out at the FDA by a group led by Arnold Lehman, another legendary modern toxicologist.

Frances Oldham Kelsey was a research assistant in Geiling's lab at the University of Chicago during the sulfanilamide investigations and was responsible for conducting the animal toxicity testing with sulfanilamide. She earned a PhD from the University of Chicago in 1938 and graduated from Chicago's medical school in 1950. She started working at the FDA in 1960 where she was tasked with reviewing new drug applications for U.S. approval. Among her first assignments was a new drug thalidomide (Kevadon), an anti-nausea medication, also used to alleviate morning sickness in pregnant women, recently licensed by the William S. Merrell drug company based in Cincinnati, Ohio. The company had already distributed the drug to over 1200 U.S. doctors with the expectation that it would be approved quickly. Drugs could go on the market 60 days after the manufacturer filed an application with the FDA. It was often the practice of pharmaceutical companies to supply doctors with the new drugs and they were encouraged to test them on patients. Kelsey held up the application and asked Merrell for more information regarding its safety. By 1961 it became clear that thalidomide posed a serious safety risk. Infant deaths and deformities were occurring at an alarming rate across Europe and the German manufacturer began pulling the drug from the market in late 1961. By 1962 the application for approval in the United States was withdrawn completely. Though never licensed in the United States, physicians distributed the drug as samples to patients. The government estimated that more than 2 million tablets were distributed to around 20,000 patients in the United States and by late 1962 there were at least 17 babies with thalidomide-related defects. Worldwide, there were more than 10,000 babies born with thalidomide-related defects and countless pregnancies that ended in miscarriage (the exact number is unknown). The tragedy could have been far worse in the United States if not for the efforts of Frances Kelsey. The thalidomide tragedy led to the 1962 Kefauver-Harris Amendments to the FDA signed by the then President John F. Kennedy. With these amendments, the FDA was given the authority to require proof of efficacy (rather than just safety) before a new drug could gain approval. The amendments created the groundwork for the multi-phased approval process involving clinical trials, which is still very much in use today. Interestingly, under strict controls, in recent years thalidomide has been reintroduced as a treatment for certain symptoms of leprosy.

Even with the current laws in place, occasionally a drug must be highly regulated, recalled, or removed from the open market for reasons such as toxicity, impurities, lack of efficacy, or abuse potential. Clinical trials are conducted on populations significantly smaller than those eventually using the drug. Side effects not detected prior to approval often become apparent in the larger population. All other factors being equal, many effects are harder to detect in a small sample size. Increasing the sample size enhances the statistical power of a test which is the situation after approval when the drug is taken by many more people. Although drugs are often voluntarily removed from the market, there are cases where the FDA orders a drug to be recalled or removed. Mylotarg (gemtuzumab ozogamicin), for example, was approved under an accelerated approval process in 2000 for the treatment of acute myelogenous leukemia. In 2010 the drug was voluntarily withdrawn from the market by its manufacturer Pfizer. A phase 3 comparative controlled clinical trial demonstrated an increase in mortality. Additionally, the drug was not considered to be more effective over conventional cancer therapies available at the time. Vioxx (rofecoxib) was one of the largest worldwide (by Merck) recalls ever. This nonsteroidal, anti-inflammatory medication for arthritis was responsible for perhaps over 27,000 heart attacks and cardiac deaths. These effects did not emerge in the original clinical trials but subsequent trials confirmed the danger.

From around 1938 to 1971, millions of pregnant women were prescribed diethylstilbestrol (DES) as a hormone-replacement therapy and to prevent miscarriages and premature births. Research during the 1950s showed it was not effective. Before long it was discovered that DES caused a rare vaginal cancer (clear cell adenocarcinoma) in girls and young women who had been exposed to DES in the womb (Herbst et al., 1971). It was recalled from the market in 1971.

In some cases, a drug may be removed from the market temporarily to protect consumers. In 1982, there were several deaths eventually linked to Tylenol brand acetaminophen capsules. The capsules were laced with potassium cyanide (Wolnik et al., 1984). Several copycat crimes followed this incident; most notably, the conviction of Stella Nickell in 1987. Stella Nickell laced Excedrin capsules with cyanide, killing both her husband and a woman who purchased the tampered product. Crimes such as these made clear the need for tamper-evident packaging and led to the passage of the Federal Anti-Tampering Act of 1983. Tamper-evident packaging created visual evidence for the consumer that a product was opened or damaged prior to purchase. The new packaging didn't provide 100% protection against tampering but made it much more difficult to tamper.

The FDA is routinely scrutinized by Congress, the public, drug companies, and consumer advocacy groups. Amendments and other changes are issued as the need arises according to the changing landscape of drug use, discovery, and development.

Among the latest of these changes is the process by which the FDA plans to review applications for new drugs in the future. The FDA implemented an initiative to harmonize the review and approval process for new drugs with the SEND initiative in 2016. SEND stands for the Standard for Exchange of Nonclinical Data and is an implementation of the Clinical Data Interchange Standards Consortium (CDISC) Standard Data Tabulation Model (SDTM) for nonclinical studies. The primary purpose of SEND is to present nonclinical data consistently regardless of the source of the data.

Pesticides Research and Chemical Warfare: A Surprising Alliance

Naturally derived pesticides have been used to protect crops for thousands of years. The first recorded use of insecticides took place some 4500 years ago with the Sumerians who dusted elemental sulfur on their crops. Three thousand two hundred years ago, the Chinese used mercury and arsenic compounds to control body lice (Unsworth, 2010). Synthetic pesticide development and use is a product of the 20th century. The histories of synthetic pesticide use and chemical warfare agents go at least partially hand in hand. Their research and development was widespread throughout the

United States and Europe during the early 20th century. Many of the chemical warfare agents manufactured during World War I and II were discovered while conducting pesticide research. The chemicals under investigation were typically noxious chlorine derivatives and were discovered to be mildly to extremely toxic to humans. Not surprisingly, the peacetime attention to pesticide research was diverted to weaponizing many of these fortuitous discoveries during wartime. The effort behind the wartime manufacture of these agents was immense and after the war there was a surplus of what may arguably be considered the deadliest chemicals ever invented. The post-war effort was primarily geared toward disposal of these agents, although many were merely transferred and stockpiled in various countries outside of Germany. From 1946 through 1948 large amounts of various chemical weapons confiscated during World War II were dumped into the Baltic Sea after the war in a military campaign known as "Operation Davy Jones' Locker" (Kaffka, 1995). These materials continue to contaminate the waters and poison fishermen and wildlife as they are slowly released from their containers. The containers were not suitable for long-term storage and degraded over time.

Germany was responsible for much of the large-scale production of pesticides and warfare gases used in the early to mid-1900s. Fritz Haber, a German scientist, sought a way to capture nitrogen in the air for use in large-scale fertilizer production. His success, with further contributions from Carl Bosch, at nitrogen fixation (the Haber-Bosch process), garnered him the Nobel Prize in 1918. The Haber-Bosch process was instrumental in the manufacture of nitrogen-based explosives for the German Army during World War I (Hager, 2009). Some argue that the Germans would have run out poisonous gases if not for Fritz Haber and Carl Bosch. Bosch also researched the weaponization of toxic substances such as chlorine, phosgene, and mustard gas, leading to the largest deployment of chemical weapons in modern history. During World War I, the Germans launched a chemical attack using chlorine gas in Ypres, Belgium in 1915. Phosgene, which is now used in the manufacture of pesticides and plastics, was employed extensively by the Germans during World War I and accounted for nearly 85% of all gas-related fatalities during that war (Marrs et al., 2007). Tabun was the first nerve agent to be synthesized in 1937 by the IG Farben scientist Gerhard Schrader during his research to discover new organophosphate insecticides. The human toxicity of tabun was realized by accident during its development in 1935. Tabun causes acetylcholinesterase inhibition in the peripheral and central nervous systems. The symptoms that result include trembling, convulsions, and respiratory paralysis. During World War II, tabun was manufactured as a part of the Grün 3 program in Brzeg Dolny, Poland in 1942. The plant was seized by the Soviet Army and moved to Russia. The production and stockpiling of chemical warfare agents continued throughout World War II. In the 1930s Willy Lange (a German biochemist) and Gerhard Schrader also discovered organophosphate cholinesterase inhibitors including sarin, soman, cyclosarin, and other less potent organophosphate insecticides. This class of chemicals was destined to become a driving force in the study of neurophysiology and toxicology for several decades (Sneader, 2006).

The United States embarked upon an active research program to study the effects of exposure to these nerve agents and to develop a means of defense. Much of this early research occurred at the University of Chicago. The growth of toxicology in academia grew out of these studies of organophosphate pesticides. Eugene Geiling and Kenneth Dubois at the University of Chicago in the 1940s were instrumental in these early studies. Their dedication to fostering the education of so many other scientists in the field of toxicology was pivotal to the development of toxicology programs around the country.

It should also be noted that, pesticides aside, the spread of toxicology through academic development of eager scientists was accomplished decades earlier through the work of Oswald Schmiedeberg (1838–1921) and Louis Lewin (1850–1929) at the University of Strasbourg and Berlin in Germany, respectively. Schmiedeberg trained approximately 120 students in toxicology and Lewin, who trained under Matthias Eugen Oscar Liebreich at the Pharmacological Institute of Berlin (1881), studied the chronic toxicity of narcotics and other alkaloids. Lewin also published much of the early research on the toxicity of methanol, glycerol, acrolein, and chloroform (Lewin, 1920, 1929). Lewin wrote in his book *Gifte und Vergiftungen* (1929) of the causal connection between dental amalgam fillings and illness. One of his famous patients was the well-known chemistry professor Alfred Stock (1876–1946), who suffered from mercury poisoning due to chronic exposure to mercury vapors which was common among chemists at the time. Lewin informed Stock of the toxicity of mercury exposure from dental amalgams. In 1926 in an article in *Zeitschrift für Angewandte Chemie* (*Journal of Applied Chemistry*), Stock sided with the claim that mercury released from amalgam fillings caused poisoning and demanded that the use of mercury for this purpose be stopped.

Though Schmiedeberg and Lewin had a 60- to 70-year head start, the efforts at the University of Chicago were no less significant. Geiling and Dubois's toxicology lab there investigated the effects of chemical warfare agents synthesized by chemists working at the National Defense Research Council's Office of Scientific Research and Development. This organization (NDRC/OSRD) was founded in 1940 by Franklin Delano Roosevelt to manage and conduct scientific research related to the problems underlying the development, production, and use of devices and materials used for warfare. The university had a very large smokestack on the grounds making it an ideal place on campus to study the effects of poisonous gases. The toxicology lab at the University of Chicago was active for about 30 years, dissolving in the late 1960s (Doull, 2001). The lab produced many scientists who became leaders in the field of toxicology. These scientists went to other academic institutions, government agencies, and industrial laboratories and were instrumental in establishing many toxicology laboratories and programs throughout the United States, consequently spreading their knowledge and influence and lending credibility to the discipline. Members of Geiling and DuBois's group were the leaders in organophosphate toxicology. DuBois's colleagues, principally Sheldon Murphy, continued to be in the forefront of this special area of study for many years. Geiling and Dubois wrote the first undergraduate toxicology text, *Textbook of Toxicology*, in 1959 (DuBois and Geiling, 1959). In 1975, Louis Casarett and John Doull (1923–2017) followed with what has become the most widely accepted toxicology text in academic programs—*Toxicology: The Basic Science of Poisons*, currently in its ninth edition and edited by Curtis D. Klaassen of the University of Kansas. John Doull was a revered scientist and mentor who remained active in toxicology through most of the first quarter of the 21st century.

The importance of the early research on the organophosphates has taken on special meaning in the years since 1960. Organophosphate insecticides are typically short-lived and do not persist in the environment or bioaccumulate up the food chain. For this reason, many were used as a replacement for DDT and other persistent organochlorine insecticides. Today, a third generation of insecticides, mainly pyrethroids, has replaced many of the organophosphates formerly used.

DDT was recognized as an insecticide by Paul Hermann Müller in 1939, a discovery which won him the Nobel Prize in Physiology in 1972. DDT was extremely effective in preventing the spread of

malaria in developing countries. It was the chemical of choice for controlling insect populations in the United States as well. Not long after its introduction, it was discovered to have detrimental effects on wildlife, particularly on certain species of birds. The chemical caused fragility in eggshells and thus many birds didn't reproduce effectively and populations diminished over time. The work and research of Rachel Carson brought this to the attention of environmental scientists and to the public when she published her findings in the book *Silent Spring* in 1962 (Carson, 1962). Although she encountered considerable opposition from those in the research community, her conclusions could not be refuted. The subsequent 1972 ban of DDT was a milestone in toxicological history for several reasons. For one, it was the first ban of a chemical based upon its effects on wildlife. Further, the grassroots effort surrounding the desire to ban DDT joined other environmental advocacy movements, which together were instrumental in influencing the government to create the Environmental Protection Agency (EPA) in 1970. Although DDT can still be legally manufactured in the United States, its use is prohibited. However, it is still used on some continents such as South America and South Africa and has effectively decreased the incidence of certain diseases such as malaria, typhus, and bubonic plague.

Another popular and critically important book which has had a strong influence on toxicology as it relates to pesticides and other chemicals (such as DES discussed above) is Theo Colburn's *Our Stolen Future* published in 1996 with a foreword by the then Vice President Al Gore. It brought the concept of endocrine disruption, while not a new one, to the public and scientific forefront. The book argued that endocrine active (or estrogen mimicking) compounds may be eliciting effects at doses considerably lower than toxicities caused by other mechanisms, and that reproductive and developmental risks can be significant. U.S. federal agencies have since funded a multitude of research projects related to endocrine disruption.

The Poison Control Center Movement and High Profile Poisonings

Pesticides were not the only class of chemicals synthesized and used in great quantities in the mid-20th century. Many other potentially harmful industrial chemicals flooded the market. Poisoning in the home increased principally in young children with the advent and widespread use of laundry and cleaning agents. In the 1950s the American Academy of Pediatrics formed a special committee to consider the problem of poisoning in children. Louis Gdalman had already begun the first poison information services, however modest, in the 1930s at Saint Luke's Hospital in Chicago. He kept a detailed card index of the most common poisons along with their treatments and antidotes and maintained and answered a call line or hotline for emergencies as well. By 1953 the Chicago Area Poisoning Control Program was established with Saint Luke's at its epicenter in Chicago and Louis Gdalman as its head. Today this poison control center is known as the Chicago and Northeastern Illinois Regional Poison Control Center. Poison control centers were founded in Boston and New York soon after the one in Chicago, and by 1955 there were a total of 17 centers. There were 661 poison control centers in 1978 at the height of the poison control center movement. The number of active centers since then has dramatically decreased due, largely, to federal and state funding cuts (Institute of Medicine Committee on Poison Prevention and Control, 2004). There were approximately 54 active poison control centers nationwide in 2014 and they provided telephone guidance for nearly 2.2 million human poison exposures. Most poison control centers are also able to handle queries related to companion animals, and the ASPCA (American Society for the Prevention of Cruelty to Animals), founded in 1866, manages its own specialized Animal Poison Control Center, established in 1996. It operates 24 hours a day, 365 days a year. The invention of child-safety-lock packaging reduced many accidental pediatric ingestions and the iconic figure Mr. Yuk taught children to identify hazardous substances in the home. Ways to protect consumers from chemical products in the home was one issue that was fervently addressed with numerous campaigns geared toward education and prevention.

Poisonings, even apart from their irregular use in chemical warfare or inadvertent household exposures, have continued unabated from ancient times forward. Several of these have been of high profile personalities and some have been suicides. In 1978, Jim Jones, founder of the Peoples Temple, led over 900 of his followers, one third of them children, to their deaths, by ordering them to drink a cyanide-laced punch drink in Jonestown, Guyana. An umbrella outfitted with a firing mechanism was used to administer the poison ricin to the leg of Georgi Markov, a Bulgarian dissident and writer. He died several days later. Nazi leaders such as Hitler, Himmler, and Goering committed suicide with cyanide and were responsible for the poisoning deaths of millions of Jews during World War II. In 1995, five plastic bags of liquid sarin were punctured with metal-tipped umbrellas in Tokyo subway cars during rush hour releasing the deadly nerve gas. The Aum Shinrikyo religious cult, led by Shoko Asahara, was found responsible. In 2004, prior to his election as President of Ukraine, Viktor Yuschchenko was poisoned with dioxin, resulting in severe facial disfigurement due to chloracne. In 2006, Alexander Litvinenko, a former officer of the Russian state security organization FSB was poisoned by, and died from, radioactive polonium-210. In February 2017, Kim Jong Nam, the half-brother of North Korean Leader Kim Jong Un, was assassinated at a Malaysian airport when two women rubbed his face with the lethal nerve agent VX. It is clear that poisons continue to be a weapon of choice in politics and in society (Famous Deaths).

Mass Environmental Exposures, the U.S. EPA, and Environmental Legislation

Years prior to the advent of a full-fledged grassroots environmental movement, a variety of events made clear the fragility of our environment. The Donora Smog, for example, was a historic air inversion in Pennsylvania that killed 20 people and sickened 7000 more in 1948. In 1952, during the so-called Great Smog of London, over 5 days, more than normal coal emissions mixed with fog in a temperature inversion resulted in thousands of deaths and tens of thousands of hospitalizations. Recent research has determined that Londoners were breathing in the fog equivalent of acid rain. Sulfate was a big contributor to the fog, and sulfuric acid particles were formed from sulfur dioxide released by coal-burning for residential use and power plants (Wang et al., 2016). It was the Great Smog that led to passage of the 1956 Clean Air Act in the United Kingdom. In Cleveland, the Cuyahoga River is remembered as the body of water polluted from decades of industrial waste which infamously caught fire in 1969 (and, in fact, on earlier occasions as well). One seminal event, a harbinger of environmental awareness, was the celebration of the first Earth Day on April 22, 1970, an ongoing annual event.

More direct cause–effect incidents involving chemicals also began coming to light. Most companies didn't originally have built-in strategies for removing the waste they created. A common modus operandi was to create landfills for dumping the chemical byproducts that accumulated because of the manufacturing process.

The increase in the manufacture of chemicals translated to both an increase in direct human exposure via ingestion of products kept in the home, and an increase in indirect human exposure via leaching of dumped chemicals into the ground water, air, and food supply. One of the earliest and most pivotal demonstrations of the issue of chemical dumping in the United States that is, and one that would influence the course of toxicology, was the environmental disaster that came to be known as Love Canal. Epidemiological and other scientific studies confirmed the tragedy.

Love Canal in Niagara Falls, New York was used as a dump site by the Hooker Chemical Company for over a decade. In the 1970s, long after it was capped and an entire community built on top of it, weather patterns forced chemical waste into the groundwater and at surface. The entire area was found to be contaminated with a variety of toxic chemicals, which led to a cluster of illnesses among the residents living in the area. The activism around the contamination and subsequent cleanup led to legislation that would ensure that other chemically contaminated sites would receive government funding for cleanup and move families to prevent further exposure. This law, the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), commonly known as Superfund, was enacted by Congress on December 11, 1980. Administered by the EPA, it authorizes the cleanup of uncontrolled or abandoned hazardous-waste sites as well as accidents, spills, and other emergency releases of pollutants and contaminants into the environment.

Love Canal paved the way for other communities and sites to be cleaned up as well. Contaminated communities were found, for example, in Times Beach, Missouri where dioxin was discovered, and in Woburn, Massachusetts, where the primary contaminant of concern was trichloroethylene. Hexavalent chromium was discovered in Hinkley, California, and asbestos contamination in Libby, Montana. The EPA was given the responsibility to develop risk assessment methodology to determine health risks from exposure to effluents and to attempt to remediate these sites. Exposure to chemicals from these waste sites tend to be highly variable and unpredictable because they typically involve exposure to a mixture of chemicals. The effort behind the Love Canal incident led to broad-based support for research into the mechanisms of action of individual chemicals and complex mixtures.

Regrettable as it is that the consequences of toxic environmental exposure fall upon anyone, it is even more unfortunate that the burden is often borne by communities otherwise disadvantaged or in the minority, be it as a result, for example, of poverty, race, or education. Environmental justice, which advocates for the fair treatment of people of all persuasions with regard to the development, implementation, and enforcement of environmental laws, regulations, and policies, is but one example. The National Environmental Justice Advisory Council (NEJAC), a federal advisory committee to the EPA, was established in 1993.

Superfund was amended as a virtually direct result of the release of methyl isocyanate from a Union Carbide insecticide plant in Bhopal, India, in 1984. With an immediate death toll of some 4000, a final death toll of many thousands more, and even more victims who suffered and are still suffering lingering effects, Bhopal disaster remains probably the worst industrial accident in history. An important law authorized by Title III of the 1986 Superfund Amendments and Reauthorization Act (SARA) is the Emergency Planning and Community Right-to-Know Act (EPCRA). It requires public records of chemicals managed at facilities, and provides the EPA with the authority to work with states and localities to prevent accidents and develop emergency plans in case of dangerous releases of chemicals ("EPA Superfund"). The EPA's Toxics Release Inventory (TRI), a publicly accessible online database, is an outgrowth of EPCRA. It lists, among other annually collected data, the numbers of pounds of certain potentially hazardous chemicals released to the environment.

Outside of this fairly straight line to regulations protecting the American public from chemical releases, there was no shortage of other disasters throughout history. For decades in the early part of the 20th century, one of Japan's Chisso Corporation plants began releasing methylmercury in industrial wastewater to Minamata Bay. It bioaccumulated in the aquatic life in the Bay and was eaten by the local populace, as well as animals. With the situation not discovered until 1956, it took a severe toll on the population. Over 2000 victims suffered from severe nervous system symptoms, and many of those died (Hachiya, 2006). Referencing this disaster as well as many other health concerns of the chemical, the 2013 Minamata Convention on Mercury is a global treaty to protect human health and the environment from the adverse effects of the chemical and its compounds ("Mercury Convention"). Itai-itai, another disease outbreak in Japan, was caused by cadmium poisoning, resulting from the release of large quantities of this chemical into the Jinzū River from mining operations. Weak and brittle bones are among the main effects. Again, it took decades for this to come to light and investigations were not undertaken in earnest by the Toyama Prefecture until 1961. In Italy, the 1976 Seveso disaster was the result of an industrial accident. Named after the Italian town of Seveso, it resulted in the exposure of thousands of people to dioxin. Chloracne was among the main sequelae and there was an excess risk of lymphatic and hematopoietic tissue neoplasms in the most exposed zones (Pesatori et al., 2009). Man-made as well as naturally occurring environmental accidents involving chemicals have occurred throughout the world. Some of these exposures were avoidable and some were not. When natural phenomenon leads to chemical exposures we are often left without a clear understanding of the cause. On August 15, 1984, Lake Monoun in West Province, Cameroon exploded in a limnic eruption, in which dissolved carbon dioxide suddenly erupted from deep lake waters, forming a gas cloud with suffocating potential. The gas killed 37 people. At that time, such eruptions involving volcanic lakes were unknown. It was still unclear how to deal with this type of disaster 2 years later when on August 21, 1986 a similar and even more deadly eruption occurred at Lake Nyos, about 100 km (62 mi) NNW. The Lake Nyos eruption killed approximately 1746 people and more than 3000 livestock. Lake Monoun, Lake Nyos, and Lake Kivu are the only known volcanic lakes in the world to have high concentrations of gas dissolved deep below the surface (Kling et al., 2005). The buildup of these gases can result in a limnic eruption. Currently efforts are underway to understand these volcanic lakes and devise ways to safely degas them without harming humans or surrounding plant and animal life. With each new environmental mishap or disaster, we are reminded of the fragility of human life and the ecosystem. We can learn by understanding how and why these exposures occur and either prevent or prepare for the next incident.

The Toxic Substances Control Act (TSCA) is a U.S. law passed by the United States Congress in 1976 and administered by the United States Environmental Protection Agency. The law regulates the introduction of new or already existing chemicals. When the TSCA was put into place, all existing chemicals were considered to be safe for use. There were, however, some 62,000 chemicals that were never tested by the EPA because they were "grandfathered" in and statutorily not considered an "unreasonable" risk. The TSCA did not require any toxicity testing before submitting a Pre-Manufacturing Notice (PMN). No safety information was required to be included in the PMN. The EPA had to rely on

computer modeling to determine whether the new chemical "could" present an unreasonable risk. The 2013 reform to the law aimed to fix the key flaws in TSCA's safety standard. These flaws led to the EPA's inability to ban asbestos. The new changes allowed the EPA to order testing without first having to show potential risk, and making more information about chemicals available to states, health professionals, and the public by limiting trade-secret allowances. The Frank R. Lautenberg Chemical Safety for the 21st Century Act revised the standard TSCA used to determine whether regulatory control of a certain chemical is warranted. If a chemical presents an unreasonable risk of injury to health or the environment, TSCA requires the EPA to initiate rulemaking to reduce risks to a reasonable level. The bill requires that standard to be based on exposure to a chemical under its conditions of use.

A sampling of other significant environmental legislation administered by the EPA includes the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (1947), which regulates pesticides, the Clean Air Act (1970), the Clean Water Act (1972), the Safe Drinking Water Act (1974), and the Resource Conservation and Recovery Act (RCRA) (1976), giving the agency the authority to control hazardous waste from "cradle to grave," all strengthened in various ways with amendments since their initial implementation.

The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is a European Union regulation dated December 18, 2006. REACH requires all companies manufacturing or importing chemical substances into the European Union in quantities of one ton or more per year to register these substances with the European Chemicals Agency (ECHA) in Helsinki, Finland. Since REACH applies to some substances that are contained in products ("articles" in REACH terminology), any company importing goods into Europe could also be affected.

Occupational Safety and Health and Industrial Toxicology

As we have seen, concerns about occupational safety date back to antiquity. Modern industrial toxicology in the United States, though, was born out of the Bureau of Labor Statistics (BLS) in the early 1900s with the first report on industrial hygiene commissioned in 1903 by Carroll D. Wright, the Bureau's first commissioner. Charles P. Neill, Commissioner of the Bureau when the 1906 Meat Inspection Act and Food and Drug Act were passed, was responsible for inspecting the meat packing factories just prior to the passage of these laws. Neill was an advocate for industrial health and safety issues and made them a priority for the Bureau. After the Federal Employees' Compensation Act of 1908, Neill began to put even greater emphasis on industrial hygiene and safety. He was aware of and impressed by Alice Hamilton, Chief Medical Examiner for the Illinois State Commission on Occupational Diseases, and convinced her to work for the BLS. She was given the official title of "Special Investigator of Industrial Diseases" and one of her first assignments was to investigate companies in the United States that manufactured white lead. She discovered 358 cases of lead poisoning between 1910 and 1911, 16 of which were fatal. She also discovered incidents of lead poisoning in the pottery making industry and in the painter's trade. Alice Hamilton was a modern-day pioneer of occupational safety comparable to Ramazzini in his day. She traveled the country documenting the diseases associated with various occupations, and while not officially employed by the government during her early excursions, her research and reports were respected and procured by the government. She conducted surveys throughout the United States and abroad. She read extensively on worker protection. While the subject was documented and recognized as an official branch of medicine in Europe, workplace-related diseases and preventive measures received scant attention in the United States. Government officials at that time were unconcerned, and assured her that the working conditions in the United States were better than those in other countries so there was no need for industrial safety. However, a survey conducted by the BLS under Carroll Wright proved otherwise. Investigations of the match-making industry revealed hundreds of workers suffering from "phossy jaw" after being exposed to phosphorous dust particles. The disease caused painful swelling of the gums and jaw. Abscesses in the jaw that resulted in partial or total removal of the jawbone were common. Often the condition led to organ failure and death. Phossy Jaw was well known in both Europe and the United States at the time. European industrial medicine experts warned of this occupational disease in their countries, but the United States did little to protect or warn its workers. Hamilton was determined to document and make public information about these diseases so that something could be done to protect workers. During her surveys, she gained entry into countless factories, becoming well informed of the manufacturing processes for various trades. She was the leading authority on lead poisoning and reported on the high mortality rates of workers in the lead industries. Her reports undoubtedly invigorated the laws that were passed, as many were a direct response to tragedies or diseases she uncovered in workplaces across the country. The seed for occupational safety was planted by a small group of concerned officials, but it was cultivated by Hamilton who became the eyes and ears of the movement (Hamilton, 1943).

The BLS was not the only organization to address occupational health and safety concerns. The Bureau of Mines, for example, was created in 1910, within the Department of the Interior, and health and safety were within its purview. The National Safety Council was established in 1911. An Office of Industrial Hygiene and Sanitation was established within the Public Health Service in 1914. The *Journal of Industrial Hygiene* started publication in 1918. By the late 1940s many of the country's largest companies (such as Dow, Du Pont, and Union Carbide) began to establish internal toxicology laboratories to help ensure worker safety. Frank A. Patty who served as Director of Industrial Hygiene for General Motors authored *Industrial Hygiene and Toxicology* in 1948. His book, now known as *Patty's Industrial Hygiene and Toxicology* has gone through many editions and is a standard of the field. The Occupational Safety and Health Administration (OSHA) was founded in 1971 with the first guidelines for standards of safety following in 1972. Hamilton published *Industrial Hygiene* in 1925, the first American textbook in the field. She published *Industrial Toxicology* in 1934.

Harriet Hardy was a physician from Massachusetts. She became interested in industrial toxicology and began working for the Massachusetts Division of Occupational Medicine. She began studying the diseases of workers in the fluorescent bulb industries in Ipswich, Lynn and Salem, Massachusetts around 1945 (Castleman, 1994). She discovered that many of the workers contracted berylliosis. Berylliosis is caused by the inhalation of dust or fumes containing beryllium. The disease presents itself with coughing, weight loss, shortness of breath, and scarring of the lungs. While beryllium was a main area of study for Dr. Hardy throughout her career, she also studied anthrax and mercury poisoning. Alice Hamilton invited Hardy to update *Industrial Toxicology*. *Hamilton and Hardy's Industrial Toxicology*, now in its 6th edition, is another classic of the field. The OSHA passed in 1970 by President Nixon also created the National Institute for Occupational Safety and Health (NIOSH). Although OSHA is the regulatory agency that establishes limits to

chemical exposures in the workplace and investigates workplace hazards, NIOSH conducts research to help to reduce workplace illnesses and accidents.

MISCELLANEOUS ORGANIZATIONS

While organizations are discussed throughout this chapter in the context of the particular subject under consideration, we gather together here several additional professional societies, governmental organizations, and other bodies that been influential in toxicology.

The discipline and profession of toxicology has grown enormously in the past century. There are numerous scholarly societies, professional and government organizations, conferences, textbooks, and educational programs, all dedicated to toxicology. The Society of Toxicology (SOT) was founded in 1961 by Fredrick Coulston, William Deichmann, Kenneth DuBois, Victor Drill, Harry Hayes, Harold Hodge, Paul Larson, Arnold Lehman, and C. Boyd Shaffer (Hays, 1986). There were 108 charter members who joined SOT the first year. There are now thousands of members of SOT in addition to members of affiliate organizations who attend the annual conference. To name just a few other groups devoted to more specialized areas of toxicology, consider the International Society of Toxinology (est. 1962), the American Academy of Clinical Toxicology (est. 1968), the Society of Forensic Toxicology (est. 1970), the Society of Toxicologic Pathology (est. 1971), the Society of Environmental Toxicology and Chemistry (est. 1979), the American College of Toxicology (est. 1979), the Society of Risk Analysis (est. 1980), and the International Society for the Study of Xenobiotics (est. 1981).

The FDA continues to play a primary role in toxicology research and regulations. The National Center for Toxicological Research (NCTR), founded in 1971 to support FDA's ability to make science-based decisions, is considered its research arm. The National Institute of Environmental Health Sciences (NIEHS) is a branch of the National Institutes of Health (NIH) and was founded in 1969 to study the effects of the environment on human disease. NIEHS is home to the National Toxicology Program, an interagency program of the Department of Health and Human Service (DHHS), and dedicated to testing and evaluating substances in the environment. Other U.S. government agencies, although their mission does not focus on toxicology, are in fact involved in it to one extent or another. Examples are the Department of Transportation, which regulates hazardous materials, and the Department of Housing and Urban Development, which considers toxic chemicals in dwellings. The Consumer Product Safety Commission is a regulatory agency charged with protecting the public from unreasonable risks of injury or death associated with the use of consumer products, including household chemicals. Certainly, state and local jurisdictions also must deal with issues relating to toxicology and environmental health.

The Gordon Research Conferences "provide an international forum for the presentation and discussion of frontier research in the biological, chemical, and physical sciences, and their related technologies" (Gordon Research Conferences, n.d.). They have played a key role in the history of toxicology and in furthering its research. A series of conferences were held on toxicology and safety evaluation, beginning with one chaired by Bernard Oser in 1956. Indeed, it was at the conference in 1961 at Kimball Union Academy in Meriden, New Hampshire that the first organizational meeting for SOT was held. There continue to be several Gordon Research Conferences of toxicological relevance each year.

There are also several organizations that offer certification in toxicology. The American Board of Toxicology is considered the "gold standard" in the field for many and its diplomats receive the "DABT" designation upon successful completion of the exam. The Academy of Toxicological Sciences, the American Board of Medical Toxicology, the American Board of Forensic Toxicology, and the American Board of Veterinary Toxicology are also among the main certifying organizations within the various toxicology disciplines.

The Federation of European Toxicologists and European Societies of Toxicology, or EUROTOX, fosters the science and teaching of toxicology throughout Europe. On an even broader global level, the International Union of Toxicology, founded in 1980, has as its members toxicology societies from around the world, and hosts the triennial International Congress of Toxicology (ICT) in addition to the Congress of Toxicology in Developing Countries (CTDC).

INTERNATIONAL ENVIRONMENTAL CONVENTIONS AND OTHER GLOBAL EFFORTS

Given that toxic agents do not respect national borders, it is important to seek, where possible, international agreements on controlling them. Globally, there are treaties that have had, and continue to have, a strong component related to chemicals management. Multilateral Environmental Agreements (MEAs) is the broad term used to encompass such agreements, some of which are directly relevant to toxicology in terms of managing potentially hazardous chemicals. While such international conventions were signed even in the early years of the 20th century, most of them are an outgrowth of several United Nations conferences, that is, the UN Conference on the Human Environment (Stockholm, 1972), the UN Conference on Environment and Development, aka the Earth Summit (Rio de Janeiro, 1992), and the UN World Summit on Sustainable Development (Johannesburg, 2002). The big three MEAs particularly relevant to chemicals management are the Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and their Disposal (adopted 1989; entered into force 1992), the Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade (adopted 1998; entered into force 2004), and the Stockholm Convention on Persistent Organic Pollutants (adopted 2001; entered into force 2004).

Complementing the treaties above is the Strategic Approach to International Chemicals Management (SAICM), a non-binding policy framework to promote chemical safety around the world. Its overall objective is "the achievement of the sound management of chemicals throughout their life cycle so that by the year 2020, chemicals are produced and used in ways that minimize significant adverse impacts on the environment and human health." SAICM's objectives are grouped into five themes: risk reduction; knowledge and information; governance; capacity-building and technical cooperation; and illegal international traffic. SAICM's final decision-making meeting before the 2020 goal, the fourth session of the International Conference on Chemicals Management, was held in Geneva in 2015.

ANIMAL ALTERNATIVES, RISK ASSESSMENT, AND GREEN CHEMISTRY

While we have already seen how poisoning incidents and environmental accidents can spur legislation and consequently influence and advance the course of toxicological science, another issue has

played a major role as well. The practice of using animals in scientific experiments with the ultimate aim of advancing biomedical research and safeguarding human health has had a long and checkered history. Greeks such as Aristotle and Erasistratus performed experiments on living animals as early as the 4th century BC (Hajar, 2011). Though animal experimentation was generally well intentioned and has resulted in significant breakthroughs in improving health, there have always been ethical concerns and continued questions about relevance and cost. Over the years, more and more, the public advocated for, and toxicologists employed, alternative means to assess the toxicity and safety of toxicants. The need for less expensive, and more efficient and germane, means of testing were spurs to the search for animal alternatives, in support of the significant argument of compassion.

W.M.S. Russell and R.L. Burch first proposed the concept of the Three Rs, standing for Replacement, Reduction, and Refinement, in 1959 (Russell and Burch, 1959). These ethical principles are widely adhered to throughout the world as a way to significantly limit the number of animals used in scientific experimentation. The term *alternatives*, as an approximate synonym for the Three Rs, was coined by the distinguished physiologist David Smyth in 1978 (Smyth, 1978). The U.S. Animal Welfare Act, signed into law in 1966, regulates the treatment of animals in research and exhibition, and was a good first step in addressing the issues of animal use but it wasn't until the technology advanced sufficiently that true inroads were made into alternatives to animal testing, which in turn were an impetus to strengthen the framework of risk assessment.

The EPA defines human health risk assessment as "the process to estimate the nature and probability of adverse health effects in humans who may be exposed to chemicals in contaminated environmental media, now or in the future." In 1983, the National Research Council (NRC), in its publication, *Risk Assessment in the Federal Government* (also known as the Red Book), set forth a critical paradigm for assessing risks consisting of four steps: (1) hazard identification, (2) dose–response assessment, (3) exposure assessment, and (4) risk characterization. They also drew a distinction between this scientifically grounded process and the process of risk management, which ideally relies upon it, but brings into play economic, legal, social, technological, and political factors, as well as public values (National Research Council, 1983). A complementary NRC publication in 2009 focused on improving both the technical analysis supporting risk assessment and the utility of risk assessment, that is, making it more relevant to and useful for risk-management decisions and offered several recommendations (National Research Council, 2009). An NRC companion volume of another sort urged strengthening activities in exposure science (National Research Council, 2012). Given that exposure assessment is a critical step in the risk assessment process, it has been surprisingly underemphasized as a scientific companion to toxicology. The committee preparing this report "envisions a shift toward a toxicologic assessment program that has an interface with exposure science and is influenced by and responsive to human and environmental exposure data." Most recently, in 2017, the National Academies issued the report, "Using 21st Century Science to Improve Risk-Related Evaluations" (https://www.nap.edu/catalog/24635/using-21st-century-science-to-improve-risk-related-evaluations). It considers advances in molecular and cellular biology, omics technologies, analytical methods, bioinformatics, and computations tools, looking as well at exposure science, and makes recommendations for integrating these new scientific approaches into risk-based evaluations.

The Precautionary Principle is a relatively recent means of integrating ethical and common sense concerns into the risk assessment process. The 1998 Wingspread Statement on the Precautionary Principle summarizes it as follows:

When an activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not fully established scientifically. In this context the proponent of an activity, rather than the public, should bear the burden of proof.

While not new conceptually (e.g., better safe than sorry), formalizing it has helped incorporate the principle into various policies and laws. Although not scientifically grounded, it invokes common sense for many people, scientists included. The Stockholm Treaty on Persistent Organic Pollutants, for example, invokes the Precautionary Principle to govern genetically modified organisms and some toxic chemicals (http://sehn.org/wingspread-conference-on-the-precautionary-principle/).

Tied in with both modern approaches to non-animal testing and bringing risk assessment into the 21st century is the concept of "Green Chemistry." The term was coined in 1991 by Paul Anastas, at the time an EPA staff chemist, who also developed and launched the EPA's Green Chemistry Program (Anastas, 2009). It was after publication of his 1998 groundbreaking book *Green Chemistry: Theory and Practice* that this approach to creating safer chemical products was better appreciated by toxicologists (Anastas and Warner, 2000). Sometimes called "sustainable chemistry," its focus is on the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances and applies across the life cycle of such products. Sustainability itself has become one of the early 21st century's buzzwords. There is no universal definition of sustainable development, but one of the most widely quoted and earliest comes from the 1987 Brundtland Commission Report, also known as *Our Common Future*: "Development that meets the needs of the present without compromising the ability of future generations to meet their own needs" (Brundtland Commission, 1987). This very broad definition easily encompasses green chemistry and the direction toxicology should be taking in the future.

INFORMATION RESOURCES

Often overlooked in discussions, historical or otherwise, of any science, is its informatics framework. The National Library of Medicine (NLM), currently a component of the National Institutes of Health (NIH), began life in 1836 in the office of the U.S. Army Surgeon General. The Index Catalogue to its collection of monographs and periodicals was launched in 1880 and Index Medicus, the first comprehensive index of journal articles, in 1879. Toxicology papers have been in scope since those early years. The Toxicology Information Program was established at NLM in 1967 at the behest, a year earlier, of the President's (i.e., Lyndon Johnson) Science Advisory Committee. The Committee concluded that "there exists an urgent need for a much more coordinated and more complete computer based file of toxicological information than any currently available and, further, that access to this file must be more generally available to all those legitimately needing such information" (PSAC, 1966). The program was renamed the Toxicology and Environmental Health Information Program (TEHIP) in 1994.

The TOXLINE database, intended to be a comprehensive bibliographic resource for scientific literature on toxicology, started operation in 1972 and ultimately was incorporated into the larger TOXNET system ("Toxnet"). Today, TEHIP offers an extensive array of free online databases including TOXLINE, the Hazardous Substances Data Bank (HSDB), the chemical dictionary ChemIDplus, the Household Products Database, Haz-Map, an

occupational health and toxicology database, EPA's Toxics Release Inventory, and many more.

The EPA's Aggregated Computational Toxicology Online Resource (ACToR), with data on over 500,000 chemicals, is another major portal as is the OECD's eChemPortal, initiated in 2004 in response to the request by the World Summit on Sustainable Development to improve the availability of hazard data on chemicals. Finally, the European Chemicals Agency (ECHA) offers a unique source of information on well over 100,000 chemicals (https://echa.europa.eu/information-on-chemicals).

WHERE ARE WE HEADED?

Looking to the future, toxicology, no differently than other sciences, will continue to rely heavily upon the knowledge gained from basic research. New techniques and technologies have only improved the field of toxicology. The sequencing of the human and other genomes has markedly affected all biological sciences. Toxicology is no exception. Today new animal models, especially zebrafish, *Caenorhabditis elegans*, and *Drosophila melanogaster* (all of which have orthologs of human genes), are widely used in toxicology. The understanding of epigenetics is opening novel approaches to the fetal origin of adult diseases including cancers, diabetes, and neurodegenerative diseases and disorders. The discovery of micro RNAs and siRNAs has significantly improved our ability to understand the function of certain genes. The 21st century genome editing tool CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) will likely find its way into a variety of toxicological applications.

Small interfering RNA (siRNA) or short interfering RNA is a class of double-stranded RNA molecules ranging from 20 to 25 base pairs in length. Small interfering RNA functions through the RNA interference (RNAi) pathway and works by blocking the expression of specific genes with a complementary nucleotide sequence. The result of this interaction prevents translation of the target mRNA. David Baulcombe and Andrew Hamilton discovered these inhibitory elements in plants, and later the phenomenon was discovered in *C. elegans* by Craig Mello and Andrew Fire (Fire et al., 1998; Hamilton and Baulcombe, 1999). Their discoveries gave rise to a new tool for biomedical research and drug discovery. Significant therapeutic advances have been made in rare genetic diseases, infectious diseases, and other more common illnesses based on the siRNA platform. RNAi-based therapeutics include drugs targeting age-related macular degeneration (AMD), respiratory syncytial virus (RSV), hepatitis C, cancer, and renal failure. The development of these unique and specific therapies and the platform around gene silencing is responsible for the increase in pharmaceutical companies and academic centers devoted to this area of research. The number of new and existing companies and academic programs with RNAi programs is too numerous to list here, but they are the platform from which new advances in research and drug discovery will mature.

Contemporary toxicology is spreading its research tentacles in a variety of directions. The toxicological study of nanomaterials promises to yield significant findings based upon quantum size effects and large surface area to volume ratios. They may pose unique threats to the environment and humans. The recently articulated concept of the exposome, in a sense of the environmental equivalent of the human genome, considers the many complex exposures we are subjected to throughout our lives, including diet, lifestyle, and social influences. Systems biology is increasingly being used to identify biomarkers of toxicant exposure and to understand molecular mechanisms of toxic pathways. Researchers are uncovering the significant role of the microbiome in affecting toxicity. Meanwhile, organs-on-chips are a new technology which may, in the future, revolutionize toxicity testing.

Few disciplines can point to both basic sciences, direct applications, and societal influences at the same time. Toxicology may be unique in this regard. The mechanisms of action of the xenobiotics studied by toxicologists, in the tradition of Claude Bernard, continue to be the tools of modern biology. Adverse outcome pathways (AOPs), today, are being recognized as a new construct for organizing biological information. Data, its generation and application, have always been a critical element in science. Today, big data, open data, and data science are all the rage, even though there seem to be no uniform definitions. In general terms, though, *big data* refers to data sets that are extremely large and require advanced computation to reveal patterns and trends. *Open data* refers to data that can be shared freely by all. One of the primary objectives is to accommodate interoperability to allow different data sets to work in tandem. *Data science* is an even more generic term encompassing big data, open data, and more. The 2017 annual conference of the Society of Toxicology convened an informational session on "Supporting Open Data in Toxicology." Its goals were "to provide basic conceptual frameworks to increase open access to toxicological data, encourage cross-discipline collaboration, link existing toxicological research data with computational toxicology and Tox21, and ensure long-term sustainability for toxicological data resources into the future."

Tox21 (Toxicology in the 21st Century) is a federal collaboration among the EPA and various NIH branches aiming to develop better toxicity assessment methods to quickly and efficiently test whether certain chemical compounds have the potential to disrupt processes in the human body that may lead to negative effects (https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21). Since its inception in 2008, it has focused its chemical screening initiatives on two themes: (1) generating fit-for-purpose cellular models for secondary screening, and (2) developing a high-throughput gene expression core facility. In a related vein, Evidence-based Toxicology took a cue from Evidence-based Medicine to more coherently adapt assessment and validation of toxicological test methods and testing strategies (Hoffmann et al., 2016). The Evidence-based Toxicology Collaboration (EBTC), founded in 2011 at the Johns Hopkins Bloomberg School of Public Health, sees itself as "guided by the themes of transparency, objectivity and consistency."

One of the great challenges remaining is the issue of mixtures. While most research focuses on single chemicals, we are, in fact, exposed to many chemicals at a time and over time. Learning how they interact with each other in causing their effects upon organisms is a critical question. Related to this is the issue of the effects of chemicals or combinations thereof in common household products including furniture, cars, electronics, and baby products.

The history of toxicology is rich with fascinating narratives that span many scientific disciplines. There are few fields which have interacted so widely and intimately with its sister sciences. Toxicologists are shaped in academia where they learn and develop the primary skillset to conduct basic research to understand mechanisms of chemical interaction and biological processes. Graduate and undergraduate programs continue to develop and improve. Toxicology is taught in schools of public health, medical schools, and schools of pharmacy inside and outside of the United States. Toxicologists from academic laboratories continue to seed other academic institutions, government organizations, and private industries, as the guardians of human, animal, and environmental health.

The upward trajectory of toxicology continues unabated. Its scientific foundation is becoming more assured, precise, and relevant.

Challenges will remain and part of the drag on its thrust will be intermittent funding and political constraints but these will not be powerful enough to interfere with long-term progress. Toxicology will continue to build upon its history, and build a trail of new history. A better understanding of toxicant exposures, individual and combined, and their effects upon living organisms will lead to an era when the global environment will be significantly safer and the world's populace healthier. Reaching this goal will take time and labor but it is achievable as we look toward the future and learn from the past.

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REFERENCES

Accum F. *A Treatise on Adulteration of Food, and Culinary Poisons, Exhibiting the Fraudulent Sophistications of Bread, Beer, Wine, Spirituous Liquors, Tea, Oil, Pickles, and Other Articles Employed in Domestic Economy and Methods of Detecting Them*. London: Longman, Hurst, Rees, Orme, and Brown; 1820.

Adams S. *The Great American Fraud*. New York: Collier and Son; 1905.

Anastas P. *Green Chemistry? He Invented the Term*. New York: New York Academy of Sciences; 2009.

Anastas PT, Warner JC. *Green Chemistry: Theory and Practice*. New York: Oxford University Press; 2000.

Ardestani MM, Rahimi R, Esfahani MM, Habbal O, Abdollahi M. The golden age of Medieval Islamic toxicology. In: Wexler P, ed. *History of Toxicology and Environmental Health. Toxicology in the Middle Ages and Renaissance*. Academic Press; 2017:11–30.

Arihan O, Arihan SK, Touwaide A. The case against Socrates and his execution. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014:69–82:chap 8.

Barker S. Poisons and the prince: toxicology and statecraft at the Medici Grand Ducal Court. In: Wexler P, ed. *History of Toxicology and Environmental Health. Toxicology in the Middle Ages and Renaissance*. Academic Press; 2017:71–81.

Barroso MS. Animal stones and the dark age of Bezoars. In: Wexler P, ed. *History of Toxicology and Environmental Health. Toxicology in Middle Ages and Renaissance*. Academic Press; 2017:115–124.

Barroso MS. The Bezoar stone: a princely antidote, the Tavora Sequeir Pinto Collection—Oporto. *Aca Med-Hist Adriat*. 2014;12(1):77–98.

Bernard C. *Leçons sur les Effets des Substances Toxiques et Medicamenteuses*. Paris: Baillière; 1857.

Bertomeu-Sanchez JR. Popularizing controversial science: a popular treatise on poisons by Mateu Orfila (1818). *Med Hist*. 2009;53(3):351–378.

Bertomeu-Sanchez JR, Nieto-Galan A, eds. *Chemistry, Medicine, and Crime (Mateu J.B. Orfila (1787-1853) and His Times*. Sagamore Beach, MA: Science History Publications; 2006.

Blow M. *The Atomic Bomb*. New Word City, Inc.; 2015.

Borzelleca JF. Paracelsus: herald of modern toxicology. *Toxicol Sci*. 1999;53(1):2–4.

Brown JR, Thornton JL. Percivall Pott (1714–1788) and chimney sweepers' cancer of the scrotum. *Br J Ind Med*. 1957;14(1):68–70.

Brundtland Commission. *Our Common Future*. New York: Oxford University Press; 1987.

Carson R. *Silent Spring*. Boston, MA: Houghton Mifflin; 1962.

Castleman B. Remembering Harriet Hardy, M.D. *New Solut*. 1994;4(3):33–34.

Cavan S. Animal experimentation: the tragic legacy of science; n.d. Available at: http://userwww.sfsu.edu/kazbeki/animexper.html. Accessed August 8, 2017.

Cilliers L, Retief F. Lead poisoning and the downfall of Rome: reality or myth? In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014a:118–126:chap 11.

Cilliers L, Retief F. Poisons, poisoners, and poisoning in ancient Rome. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014b:127–137:chap 12.

Cobb C. The case against the Borgias. In: Wexler P, ed. *History of Toxicology and Environmental Health. Toxicology in the Middle Ages and Renaissance*. Academic Press; 2017:53–62.

Conti F. Claude Bernard's Des Fonctions du Cerveau: an ante litteram manifesto of the neurosciences? *Nat Rev Neurosci*. 2002;3(12):979–985.

Dal Bello M. La leggenda nera. I Borgia (a TV series). Città Nuova. 2012.

Dash M. Aqua Tofana: slow poisoning and husband-killing in 17th century Italy. A blast from the past; 2015. Available at: https://mikedashhistory.com/2015/04/06/aqua-tofana-slow-poisoning-and-husband-killing-in-17th-century-italy/.

Dayan AD. What killed Socrates? Toxicological considerations and questions. *Postgrad Med J*. 2009;85:34–37.

de Boer JZ. The oracle at Delphi: the Pythia and the pneuma, intoxicating gas finds, and hypotheses. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014:83–91.

Doull J. Toxicology comes of age. *Annu Rev Pharmacol Toxicol*. 2001:41:1–21.

Dronsfield A. Percival Pott, chimney sweeps and cancer. Education in Chemistry. Royal Society of Chemistry; 2006. Available at: https://eic.rsc.org/feature/percival-pott-chimney-sweeps-and-cancer/2020205.article.

DuBois K, Geiling EMK. *Textbook of Toxicology*. New York: Oxford University Press; 1959.

Duffin CJ. Fossil sharks' teeth as alexipharmics. In: Wexler P, ed. *History of Toxicology and Environmental Health. Toxicology in the Middle Ages and Renaissance*. Academic Press; 2017:125–134.

Duramy BF. Women and poisons in 17th century France. *Chi-Kent L Rev*. 2012;87(2):347–370.

Farrell M. Pioneer forensic toxicologists: Marsh, Orfila and their predecessors. *Criminologist*. 1994;18(1):33–36.

Felton JS. The heritage of Bernardino Ramazzini. *Occup Med (Lond)*. 1997;47(3):167–179.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998;391(6669):806–811.

Fornaciari G, Bianucci R. I e Bianca: Una Scomoda Verita [Francesco I and Bianca: An inconvenient truth]. *Paleopatologia.It*; 2010. Available at: http://www.paleopatologia.it/attivita/pagina.php?recordID=24.

Furst A. Moses Maimonides. *Toxicol Sci*. 2001;59(2):196–197.

Gantenbein UL. Poison and its dose: Paracelsus on toxicology. In: Wexler P, ed. *History of Toxicology and Environmental Health. Toxicology in the Middle Ages and Renaissance*. Academic Press; 2017:1–10.

Geiling EMK, Paul MD, Cannon MD. Pathologic effects of elixir of sulfanilamide (diethylene glycol) poisoning: a clinical and experimental correlation: final report. *JAMA*. 1938;111(10):919–926.

Gerritsen SP. Gewina. 2007;30(1):1–20.

Gibbs F. http://fredgibbs.net/posts/universals-and-particulars-of-poison; 2017. Accessed August 8, 2017.

Gordon Research Conferences. https://www.grc.org; n.d.

Gow ASF, Scholfield AF. *Nicander: The Poems and Poetical Fragments*. London: Cambridge University Press; 2014.

Hachiya N. The history and the present of Minamata disease: entering the second half a century. *Jpn Med Assoc J*. 2006;49(3):112–118.

Hager T. *The Alchemy of Air: A Jewish Genius, a Doomed Tycoon, and the Scientific Discovery That Fed the World but Fueled the Rise of Hitler*. New York: Broadway Books; 2009.

Hajar R. Animal testing and medicine. *Heart Views*. 2011;12(1):42.

Hamilton A. *Exploring the Dangerous Trades*. Boston, MA: Little, Brown; 1943. [Reprinted by Northeastern University Press, Boston, MA; 1985.]

Hamilton AJ, Baulcombe D. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*. 1999;286(5441):950–952.

Hays HW. *Society of Toxicology History, 1961–1986*. Washington, DC: Society of Toxicology; 1986.

Hedeson GD. Jan Baptist Van Helmont and the medical-alchemical perspectives of poison. In: Wexler P, ed. *History of Toxicology and Environmental Health. Toxicology in the Middle Ages and Renaissance*. Academic Press; 2017:91–100.

Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med*. 1971;284:878–881.

Hernberg S. Lead poisoning in a historical perspective. *Am J Ind Med*. 2000;38(3):244–254.

Heydari M, Hashempu MH, Zargaran A. Medicinal aspect of opium as described in *Avicenna's Canon of Medicine*. *Acta Medico-Historica Adriatica*. 2013;11(1):101–112.

Hobenreich E, Rizzelli G. Poisoning in ancient Rome: the legal framework, the nature of poisons, and gender stereotypes. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 2: Toxicology in Antiquity. Academic Press; 2014:42–51:chap 12.

https://echa.europa.eu/information-on-chemicals.

https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21.

https://www.nap.edu/catalog/24635/using-21st-century-science-to-improve-risk-related-evaluations.

http://sehn.org/wingspread-conference-on-the-precautionary-principle/.

http://www.toxicology.org/about/vp/vision.asp.

Hoffmann S, Hartung T, Stephens M. Evidence-based toxicology. *Adv Exp Med Biol*. 2016;856:231–241.

Institute of Medicine Committee on Poison Prevention and Control. Historical context of poison control. In: *Forging a Poison Prevention and Control System*. Washington, DC: National Academies Press, 2004:80–105:chap 4.

Jorgensen TJ. *Strange Glow: The Story of Radiation*. Princeton, NJ: Princeton University Press; 2016.

Jutte D. *The Age of Secrecy: Jews, Christians, and the Economy of Secrets, 1400–1800*. New Haven, CT: Yale University Press; 2015:56–57.

Kaffka A. *Sea-Dumped Chemical Weapons: Aspects, Problems, and Solutions*. Boston, MA: Kluwer Academic; 1995.

Karaberopoulos D, Karamanou M, Androutsos G. The art of medicine: the theriac in antiquity. *Lancet*. 2012;376:1942–1943.

Khajja BS, Sharma M, Singh R, Mathur GK. Forensic study of Indian toxicological plants as botanical weapon (BW): a review. *J Environ Anal Toxicol*. 2011;1(3):112.

Kinch M. *A Prescription for Change: The Looming Crisis in Drug Development*. Chapel Hill, NC: University of North Carolina Press; 2016.

Kling GW, Evans WC, Tanyileke G, Kusakabe M, Ohba T, Yoshida Y, Hell JV. Degassing lakes Nyos and Monoun: defusing certain disaster. *Proc Natl Acad Sci USA*. 2005;102(40):14185–14190.

Lavers D. Origin of myths related to curative, antidotal and other medicinal properties of animal "horns" in the Middle Ages. In: Wexler P, ed. *History of Toxicology and Environmental Health*; *Toxicology in the Middle Ages and Renaissance*. Academic Press; 2017:101–114.

Lewin L. *Die Gifte in der Weltgeschichte: Toxikologische, allgemeinverstandliche Untersuchungen der historischen Quellen*. Berlin: Springer; 1920.

Lewin L. *Gifte und Vergiftungen*. Berlin: Stilke; 1929.

Liu Y. Poisonous medicine in ancient China. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 2: Toxicology in Antiquity. Academic Press; 2014:89–97.

Maimonides M. *On Poisons and the Protection against Lethal Drugs: A Parallel Arabic-English Edition*. Gerrit Bos, ed. McVaugh MR, trans. Provo, UT: Brigham Young University; 2009.

Manohar PR. Toxicity of Ayurveda medicines and safety concerns: the need to revive the branch of toxicology in Ayurveda. *Anc Sci Life*. 2014;34(1):1–2.

Mayor A. *The Poison King: The Life and Legend of Mithradates, Rome's Deadliest Enemy*. Princeton, NJ: Princeton University Press; 2010.

Mayor A. Alexander the Great: a questionable death. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014:52–59.

Marrs TT, Maynard RL, Sidell F. *Chemical Warfare Agents: Toxicology and Treatment*. West Sussex, England: Wiley; 2007.

Morgan JP, Penovich P. Jamaica ginger paralysis. Forty-seven-year follow-up. *Arch Neurol*. 1978;35(8):530–532.

Mullner R. *Deadly Glow: The Radium Dial Worker Tragedy*. Washington, DC: American Public Health Association; 1999.

Nasser M, Tibi A, Savage-Smith E. Ibn Sina's Canon of Medicine: 11the century rules for assessing the effects of drugs. *J R Soc Med*. 2009;102(2):78–80.

National Research Council. *Risk Assessment in the Federal Government: Managing the Process*. Washington, DC: National Academy Press; 1983.

National Research Council. *Science and Decisions: Advancing Risk Assessment*. Washington, DC: National Academies Press; 2009.

National Research Council. *Exposure Science in the 21st Century: A Vision and a Strategy*. Washington, DC: National Academies Press; 2012.

NPR. http://www.npr.org/2011/03/17/134568383/japan-three-mile-island-chernobyl-putting-it-all-in-perspective; n.d.

Nunn JF. *Ancient Egyptian Medicine*. Norman, OK: University of Oklahoma Press; 1996:144.

Orfila MJB. *Traite des Poisons Tires des Regnes Mineral, Vegetal et Animal, ou, Toxicologie Generale Consideree sous les Rapports de la Physiologie, de la Pathologie et de la Medecine Legale*. Paris: Crochard; 1814–1815.

Oser B. Toxicology then and now. *Regul Toxicol Pharmacol*. 1987; 7:427–433.

Pesatori AC, et al. Cancer incidence in the population exposed to dioxin after the "Seveso accident" twenty years of follow-up. *Environ Health*. 2009;8:30.

Pope MH. Bernardino Ramazzini: the father of occupational medicine. *Spine*. 2004;29(20):2335–2338.

Pratte ELH, Bryant S, Handley C, Hall M. Pick your poison. *The Chemist*. 2014;87(2):19–29.

Pray S. *A History of Nonprescription Product Regulation*. New York: Pharmaceutical Product Press; 2003.

PrimroseJ.https://quod.lib.umich.edu/e/eebo2/A91017.0001.001?view=toc.

PSAC (President's Science Advisory Committee). *Handling of Toxicological Information*. Washington, DC: White House; 1966.

Rosner D, Markowitz G. Standing up to the lead industry: an interview with Herbert Needleman. *Public Health Rep*. 2005;120:330–337.

Rosner F. Moses Maimonides' treatise on poisons. *JAMA*. 1968; 205(13):914–916.

Russell WMS, Burch RL. *The Principles of Humane Experimental Technique*. London: Methuen; 1959.

Sanchez GM, Harer WB. Toxicology in ancient Egypt. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014:2–10:chap 1.

Schlebusch CM, et al. Possible positive selection for an arsenic-protective haplotype in humans. *Environ Health Perspect*. 2013;121(1):53–58.

Seifert S. Richard Mead: one for the ages. *Clin Toxicol (Phila)*. 2011;49(1):53–55.

Slouber M. The venomous virgin: fact or fantasy? In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 2: Toxicology in Antiquity. Academic Press; 2015:98–103.

Smyth D. *Alternatives to Animal Experiments*. Scolar Press in Association with The Research Defence Society; 1978.

Sneader W. *Drug Discovery: A History*. Wiley; 2006. Available at: http://onlinelibrary.wiley.com/book/10.1002/0470015535.

Somerset A. *The Affair of the Poisons: Murder, Infanticide, and Satanism at the Court of Louis XIV*. St. Martin's Press; 2014.

Spiller HA, Hale JR, De Boer JZ. The Delphic oracle: a multidisciplinary defense of the gaseous vent theory. *J Toxicol Clin Toxicol*. 2002;40(2):189–196.

The Unitarian Review and Religious Magazine, Boston: Office of the Unitarian Review, 1879;12:130.

Timbrell J. *The Poison Paradox*. Oxford, MI: Oxford University Press; 2005.

Touwaide A. Harmful botanicals. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014a:60–68:chap 7.

Touwaide A. Nicander, Thêriaka, and Alexipharmacka: venoms, poisons, and literature. In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014b:44–51:chap 5.

Tsoucalas G, Sgantzos M. The death of Cleopatra: suicide by snakebite or poisoned by her enemies? In: Wexler P, ed. *History of Toxicology and Environmental Health*; Vol. 1: Toxicology in Antiquity. Academic Press; 2014:12–20:chap. 2.

Tubbs RS, et al. Francois Magendie (1783–1855) and his contributions to the foundations of neuroscience and neurosurgery. *J Neurosurgery*. 2008;108:1038–1042.
UNODA (United Nations Office for Disarmament Affairs). https://www.un.org/disarmament/wmd/nuclear/npt/; n.d.
Unsworth J. History of pesticide use; 2010. http://agrochemicals.iupac.org/index.php?option=com_sobi2&sobi2Task=sobi2Details&catid=3&sobi2Id=31. Accessed August 8, 2017.
Wang G, et al. Persistent sulfate formation from London fog to Chinese haze. *Proc Natl Acad Sci USA*. 2016;113(48):13630–13635.
Wax PM. Elixir sulfanilamide-Massengill revisited. *Vet Hum Toxicol*. 1994;36(6):561–562.
Wikisource. Rig Veda; n.d.-a. Available at: https://en.wikisource.org/wiki/The_Rig_Veda/Mandala_1/Hymn_191. Accessed August 8, 2017.
Wikisource. Christison, Robert (DNBoo). Dictionary of National Biography, 1885–1900, Volume 10; n.d.-b. Available at: https://en.wikisource.org/wiki/Christison,_Robert_(DNB00). Accessed August 8, 2017.
Wilkinson P. *Chinese Myth: A Treasury of Legends, Art, and History (The World of Mythology)*. New York: Routledge; 2016.
Wisdom Library. http://www.wisdomlib.org/hinduism/book/sushruta-samhita-volume-5-kalpasthana/d/doc142971.html; n.d. Accessed August 8, 2017.
Wolnik KA, Fricke FL, Bonnin E, Gaston CM, Satzer RD. The Tylenol tampering incident-tracing the source. *Anal Chem*. 1984;56(3):466a–470a, 474a.
World Nuclear Association. http://www.world-nuclear.org/information-library/safety-and-security/safety-of-plants/fukushima-accident.aspx; 2017.
Wujastyk D. *The Roots of Ayurveda: Selections from Sanskrit Medical Writings*. London: Penguin; 2003:144.
Yang S, ed. *The Divine Farmer's Materia Medica: A Translation of the Shen Nong Ben Cao*. Boulder, CO: Blue Poppy Press; 1998.

SUPPLEMENTAL READING

Adams F, trans. *The Genuine Works of Hippocrates*. Baltimore, MD: Williams & Wilkins; 1939.
Albert A. *Selective Toxicity*. London: Methuen; 1951.
Beeson BB. Orfila—pioneer toxicologist. *Ann Med Hist*. 1930;2:68–70.
Bernard C. Analyse physiologique des proprietes des systemes musculaire et nerveux au moyen du curare. *C R Acad Sci (Paris)*. 1856;43:325–329.
Bryan CP. *The Papyrus Ebers*. London: Geoffrey Bales; 1930.
Clendening L. *Source Book of Medical History*. New York: Dover; 1942.
Gaddum JH. *Pharmacology.* 5th ed. New York: Oxford University Press; 1959.
Garrison FH. *An Introduction to the History of Medicine*. 4th ed. Philadelphia, PA: Saunders; 1929.
Holmstedt B, Liljestrand G. *Readings in Pharmacology*. New York: Raven Press; 1981.
http://www.ranker.com/list/death-by-poison-list-of-famous-poison-victims/notable-famous-deaths.
http://www.theaic.org/pub_thechemist_journals/Vol-87-No-2/Vol-87-No2-Article-4.pdf.
Hutt PB, Hutt PB II. A history of government regulation of adulteration and misbranding of food. *Food Drug Cosmet J*. 1984;39:2–73.
Leung AY. Traditional toxicity documentation of Chinese Materia Medica—an overview. *Toxicol Pathol*. 2006; 34:319–326.
Orfila MJB. *Secours a Donner aux Personnes Empoisonees et Asphyxiees*. Paris: Feugeroy; 1818.
Poland A, Knutson JC. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons, examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol*. 1982;22:517–554.
Ramoutsaki IA, Ramoutsakis YA, Tsikritzis MD, Tsatsakis AM. The roots of toxicology: an etymological approach. *Vet Hum Toxicol*. 2000;42:111.
Unschuld PU. *Medicine in China: A History of Pharmaceutics*. Berkeley, CA: University of California Press; 1986.
US FDA. *Toxicologic Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*. Washington, DC: U.S. Food and Drug Administration, Bureau of Foods; 1982.
Williams RT. *Detoxication Mechanisms*. 2nd ed. New York: Wiley; 1959.

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chapter 2

Principles of Toxicology

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Introduction to Toxicology

Subdisciplines of Toxicology

Spectrum of Undesired Effects
- Allergic Reactions
- Idiosyncratic Reactions
- Immediate versus Delayed Toxicity
- Reversible versus Irreversible Toxic Effects
- Local versus Systemic Toxicity
- Interactions of Chemicals
- Tolerance

Characteristics of Exposure
- Route and Site of Exposure
- Duration and Frequency of Exposure

Dose–Response Relationships
- Individual, or Graded, Dose–Response Relationships
- Quantal Dose–Response Relationships
- Dose Extrapolation Across Species
- Shapes of Dose–Response Curves
- Monotonic Dose–Response Curves
  - *Threshold and Linear, Nonthreshold Models*
- Nonmonotonic Dose–Response Curves
  - *Essential Nutrients*
  - *Hormesis*
  - *Endocrine Active Chemicals*
- Assumptions in Deriving the Dose–Response Relationship
- Evaluating the Dose–Response Relationship
  - *Therapeutic Index*
  - *Margins of Safety and Exposure*
  - *Potency versus Efficacy*

Assessing Toxicological Responses
- Causation in Toxicology
- Mechanisms and Modes of Action
- Adverse Outcome Pathways

Variation in Toxic Responses
- Selective Toxicity
- Species Differences
- Modifying Factors
  - *Genetics*
  - *Age*
  - *Sex*
  - *Circadian Rhythm*
  - *Microbiome*

Toxicity Testing
- Acute Toxicity Testing
- Subacute (Repeated-Dose) Toxicity Testing
- Subchronic Toxicity Testing
- Chronic Toxicity Testing
- Developmental and Reproductive Toxicity
- Mutagenicity
- Carcinogenicity
- Neurotoxicity Assessment
- Immunotoxicity Assessment
- Sensitization
- Eye and Skin Irritation and Corrosion
- Other Toxicity Tests

Systems Toxicology
- Transcriptome
- Epigenome
- Proteome
- Metabonomics/Metabolomics
- Exposome
- High-Content Screening
- Computational Toxicology
- Innovative Testing Models

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References

INTRODUCTION TO TOXICOLOGY

Toxicology is the study of the adverse effects of chemical, biological, or physical agents on living organisms and the environment. These toxic substances include naturally occurring harmful chemicals, or *toxins,* as well as foreign substances called *xenobiotics.* Toxins are poisons that originate from plants and microbial organisms and also include venoms released by animals in order to injure predators. Aflatoxin is an example of a toxin; it is produced and released from the fungus *Aspergillus* that grows on foods such as corn and nuts. Exposure to aflatoxin is associated with an increased risk of liver cancer. By comparison, xenobiotics include a variety of synthetic chemicals with different intended purposes. Pharmaceuticals are xenobiotics developed to treat disease, whereas pesticides are used to deter pests. In addition, a large number of chemicals are used in manufacturing and industrial processes. For example, the chemical "dioxin" (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]) is generated during the production and/or combustion of certain chlorinated organic chemicals. A unique skin toxicity, called chloracne, has been observed in individuals exposed to dioxin. Some toxic substances can be produced by both natural and anthropogenic activities. For example, polyaromatic hydrocarbons are produced by the combustion of organic matter through ordinary processes (e.g., forest fires) and human activities (e.g., combustion of coal for energy production and cigarette smoking). Arsenic, a toxic metalloid, largely appears in groundwater as a natural contaminant, but also enters groundwater from other sources as well. Generally, such toxic chemicals are referred to as toxicants, rather than toxins, because, although they may be naturally produced, they are not produced by biological systems.

Toxic chemicals may also be classified in terms of their physical state (gas, dust, liquid, size; e.g., nanoparticles); their chemical stability or reactivity (explosive, flammable, corrosive); general chemical structure (aromatic amine, halogenated hydrocarbon, etc.); or ability to cause significant toxicity (extremely toxic, very toxic, slightly toxic, etc.). Classification of toxic chemicals on the basis of their biochemical mechanisms of action (e.g., alkylating agent, cholinesterase inhibitor, and endocrine disruptor) is usually more informative than classification by general terms such as irritants and oxidizers. However, more descriptive categories such as air pollutants, occupation-related exposures, and acute and chronic poisons may be useful to associate toxic chemicals that result in similar adverse events or are encountered under particular conditions. There is no single classification that is applicable to the entire spectrum of toxic chemicals. Instead, a combination of classification systems is generally needed to best characterize toxic substances. In this textbook, for example, toxic chemicals are discussed in terms of their target organs (liver, kidney, hematopoietic system, etc.), use (pesticide, solvent, food additive, etc.), source (animal and plant toxins), and adverse effects (cancer, mutation, etc.).

Virtually every known chemical has the potential to produce injury or death if it is present in a sufficient quantity. Paracelsus (1493 to 1541), a Swiss/German/Austrian physician, scientist, and philosopher, phrased this well when he noted, "What is there that is not poison? All things are poison and nothing [is] without poison. Solely the dose determines that a thing is not a poison." This principle is often summarized with the phrase that "the dose makes the poison."

Chemicals differ in their ability to produce serious injury or death. Table 2-1 shows the dose of chemicals needed to produce death in 50% of treated animals (lethal dose 50 [LD_{50}]). Some chemicals produce death in microgram doses and are commonly denoted as extremely poisonous. Other chemicals may be relatively harmless after doses in excess of several grams. It should be noted, however, that measures of acute lethality such as LD_{50} do not accurately reflect the full spectrum of toxic responses, or hazards, associated with exposure to a chemical. For example, some chemicals may have carcinogenic, teratogenic, or neurobehavioral effects at doses that produce no evidence of acute or immediate injury. In addition, there is a growing recognition that a number of factors can account for an individual's susceptibility to a range of responses. These include age, genetics, diet, underlying diseases, and concomitant exposures among many other factors. Finally, it should be recognized that, for a particular chemical, multiple different effects can occur in a given organism, each with its own "dose–response relationship."

Table 2-1

Approximate Acute LD_{50} Values of Some Representative Chemicals

CHEMICAL	LD_{50} (MG/KG)*
Ethyl alcohol	10,000
Glyphosate	5,600
Sodium chloride	4,000
Ferrous sulfate	1,500
Morphine sulfate	900
Phenobarbital sodium	150
Chlorpyrifos	18
Picrotoxin	5
Strychnine sulfate	2
Nicotine	1
VX nerve gas	1
D-Tubocurarine	0.5
Hemicholinium-3	0.2
Tetrodotoxin	0.10
Dioxin (TCDD)	0.001
Botulinum toxin	0.00001

**LD_{50} is the dose (mg/kg body weight) causing death in 50% of exposed animals.*

A *toxicologist* is an individual trained to examine and communicate the nature of a toxicant's properties and identify approaches to prevent or mitigate harm done to human, animal, and environmental health. Toxicological research identifies the cellular, biochemical, and molecular mechanisms of action of toxic chemicals and determines the extent to which these actions cause functional perturbations in critical organ systems. Using these data, a toxicologist then assesses the relationship between toxicant exposure (or dose) to the response (or outcome) and in turn the probability of an adverse event to occur. This determination requires an assessment of *risk* which is the quantitative estimate of the potential effects of a chemical on human and environmental health at particular exposure levels (e.g., pesticide residues in food and chemical contaminants in drinking water). The variety of potential adverse effects and the diversity of chemicals in the environment make toxicology a broad applied science that draws upon multiple disciplines including chemistry, biology, physiology, pathology, pharmacology, molecular biology, physics, statistics, and more. Because of the many facets that require toxicological examination, the field is often divided into subdisciplines that require specialization in particular areas. Our society's dependence on chemicals and the need to assess potential hazards have made toxicologists an increasingly important part of the decision-making processes.

SUBDISCIPLINES OF TOXICOLOGY

The professional activities of toxicologists fall into three main categories: mechanistic, hazard assessment, and regulatory. Although all three categories have distinctive characteristics, each contributes to the others, and all are vitally important to chemical risk assessment (see Chap. 4).

A *mechanistic toxicologist* identifies the cellular, biochemical, and molecular mechanisms by which chemicals exert toxic effects on living organisms (see Chap. 3 for a detailed discussion of mechanisms of toxicity). The results of mechanistic studies have implications in many areas of toxicology. In risk assessment, mechanistic data may be useful in determining whether an adverse outcome (e.g., cancer and birth defects) observed in laboratory animals may occur in humans. For example, the relative toxic potential of most organophosphorus (OP) insecticides in humans, rodents, and insects can be somewhat predicted on the basis of an understanding of common mechanisms (inhibition of acetylcholinesterase) and differences in biotransformation and accumulation of these insecticides among the three species. Similarly, mechanistic data may be very useful in identifying adverse responses in experimental animals that may not be relevant to humans. For example, the propensity of the widely used artificial sweetener saccharin to cause bladder cancer in rats has been demonstrated to be irrelevant to humans at normal dietary intake rates. This is because mechanistic studies have

demonstrated that bladder cancer is induced only under conditions where saccharin is at such a high concentration in the urine that it forms a crystalline precipitate (Cohen, 1998). Dose–response studies suggest that such high concentrations would not be achieved in the human bladder even after extensive dietary consumption.

Mechanistic data are also useful in the design and production of safer alternative chemicals and in therapies for chemical poisoning and treatment of disease. For example, the drug thalidomide was originally marketed in Europe and Australia as a sedative drug for pregnant women. However, it was banned for clinical use in 1962 because of devastating birth defects that occurred if the drug was ingested during a critical period in pregnancy. Mechanistic studies over the past several decades have demonstrated that thalidomide may have a unique molecular mechanism of action that interferes with the expression of certain genes responsible for blood vessel formation (a process called angiogenesis). With an understanding of this mechanism, thalidomide has been "repurposed" as a valuable treatment for certain infectious diseases (e.g., leprosy) and multiple myeloma. This provides an interesting example of how a highly toxic drug with selectivity toward a specific population (pregnant women) can be used relatively safely in other individuals with proper precautions.

In addition to aiding directly in the identification, treatment, and prevention of chemical toxicity, an understanding of the mechanisms of toxicant action contributes to the knowledge of basic physiology, pharmacology, cell biology, and biochemistry. The advent of new technologies in molecular biology and genomics now provides mechanistic toxicologists with the tools to explore exactly how humans may differ from laboratory animals in their response to toxic substances.

A *hazard assessment toxicologist* conducts toxicity testing that provides comprehensive information for the evaluation of a chemical's safety and to meet important regulatory requirements. The appropriate toxicity tests in cell culture systems or experimental animals (as described later in this chapter and other chapters) are designed to yield information to identify potential hazards and associated risks posed to humans and the environment from exposure to specific chemicals. A *hazard* is a chemical or action that causes harm, whereas the *risk* is the likelihood for a hazard to result in harm. Risk is determined by the extent of the exposure to the hazard. The concern may be limited to effects on humans, as in the case of drugs and food additives. Toxicologists in the chemical industry, however, must be concerned not only with the risk posed by a company's chemicals (insecticides, herbicides, solvents, etc.) to humans but also with potential effects on fish, birds, and plants, as well as other factors that might disturb the balance of the ecosystem. Hazard assessment studies in toxicology rely upon mechanistic experimentation to provide important clues to a chemical's mechanism of action and provide valuable information used in the risk assessments performed by regulatory toxicologists.

A *regulatory toxicologist* has the responsibility for deciding, on the basis of data provided by descriptive and mechanistic toxicologists, whether a drug or other chemical poses a sufficiently low risk (or, in the case of drugs, a favorable risk/benefit profile) to be marketed for a stated purpose. The Food and Drug Administration (FDA) is responsible for allowing drugs, cosmetics, and food additives to be sold in the U.S. market according to the Federal Food, Drug and Cosmetic Act (FFDCA) (Table 2-2). The U.S. Environmental Protection Agency (EPA) is responsible for regulating most other chemicals according to a variety of different legislative acts, including the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), the Toxic Substances Control Act (TSCA), the Resource Conservation and Recovery Act (RCRA), the Safe Drinking Water Act, and the Clean Air Act. In 1996, the U.S. Congress passed the Food Quality Protection Act (FQPA) that fundamentally changed the pesticide and food safety laws to consider stricter safety standards particularly for infants and children, who were recognized as more susceptible to health effects of pesticides. In 2016, amendments to the TSCA were included in the Frank R. Lautenberg Chemical Safety for the 21st Century Act. Under this legislation, the U.S. Congress has mandated a requirement for the EPA to evaluate existing chemicals on strict timelines, update risk-based safety standards, and increase the public's access to information regarding chemicals. This legislation also calls for greater utilization of alternative testing methods that reduce the use of animals for experimentation. As a result, *computational toxicologists* are needed to develop and implement computer-based models to predict adverse health effects resulting from the interaction of chemicals with biological organisms. Computational toxicology is important for the interagency Toxicity Testing in the 21st Century (Tox21) initiative between the National Toxicology Program, FDA, National Institute of Environmental Health Sciences, and National Center for Advancing Translational Sciences.

The EPA is also responsible for enforcing the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA, later revised as the Superfund Amendments Reauthorization Act [SARA]), more commonly called the Superfund Act. This regulation provides direction and financial support for the cleanup of waste sites that contain toxic chemicals that may present a risk to human health or the environment. The Occupational Safety and Health Administration (OSHA) of the Department of Labor was established to ensure that safe and healthful conditions exist in the workplace. The National Institute for Occupational Safety and Health (NIOSH) as part of the Centers for Disease Control and Prevention (CDC) in the Department of Health and Human Services (DHHS) is responsible for conducting research and making recommendations for the prevention of work-related injury and illness, making *occupational toxicology* an important component of regulatory toxicology. The Consumer Product Safety Commission (CPSC) is responsible for protecting consumers from hazardous substances used in the household, whereas the Department of Transportation (DOT) certifies that materials shipped in interstate commerce are labeled and packaged in a manner appropriate for the amount of commensurate hazard. The Nuclear Regulatory Commission (NRC), established in 1974, regulates the civilian use of nuclear material to protect public health and safety, and the environment. Regulatory toxicologists are also involved in the establishment of standards for the amounts of chemicals permitted in ambient air, industrial atmospheres, and drinking water, often integrating scientific information from basic descriptive and mechanistic toxicology studies with the principles and approaches used for risk assessment (see Chap. 4).

In addition to the above categories, there are other specialized areas of toxicology such as forensic, clinical, and environmental toxicology. *Forensic toxicology* is the application of analytical chemistry to toxicology. This field covers the medicolegal aspects of the deleterious effects of chemicals on animals and humans. The expertise of forensic toxicologists is used to aid in establishing the cause of death and determining its circumstances in a postmortem investigation (see Chap. 32). *Clinical toxicology* is the realm of medical science concerned with disease caused by or uniquely associated with toxic substances (see Chap. 33). Generally, clinical toxicologists are physicians who receive specialized training in emergency medicine and poison management. Efforts are directed at treating patients poisoned with drugs or other chemicals and at the development of new techniques to treat intoxications. Public information

Table 2-2

Federal Agencies That Regulate Exposure to Toxic Chemicals in Drugs, Cosmetics, Consumer Products, Food, Work, and the Environment

SECTORS	AGENCIES AND COMMITTEES	RESPONSIBILITIES	MAJOR LEGISLATION
Drugs and cosmetics	U.S. Food and Drug Administration (FDA)	Approve investigational new drug (IND) applications to initiate clinical trials; approve pharmaceuticals for marketing under a new drug application (NDA); regulate chemical exposures in skin creams, lotions, powders, and makeup products	1938, Federal Food, Drug and Cosmetic Act (FFDCA)
Consumer products	U.S. Consumer Product Safety Commission (CPSC)	Establish exposure standards for hazardous substances in consumer products including toys, cribs, cleaning products; require warning labels on products that are corrosive, toxic, irritants, or sensitizers	1972, Consumer Product Safety Act Federal Hazardous Substance Act
Food additives	U.S. Food and Drug Administration (FDA)	Approve new direct additives (flavors, texturing agents, preservatives, processing aids, etc.) that can be used in food; determine additives that are generally recognized as safe (GRAS) and do not require FDA approval for use	1938, Federal Food, Drug and Cosmetic Act (FFDCA) 1958, Delaney Clause 1996, Food Quality Protection Act (FQPA)
Environment	U.S. Environmental Protection Agency (EPA)	"Establish exposure standards for pesticides, water, and air pollutants, and hazardous wastes"; prepare voluntary health advisories for drinking water and set ambient air quality standards	1947, Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) 1970, Clean Air Act 1972, Clean Water Act 1976, Resource Conservation and Recovery Act (RCRA) 1976, Toxic Substances Control Act 1980, Comprehensive Environmental Response, Compensation and Liability Act (CERCLA)—Creation of Superfund 1984, Federal Hazardous and Solid Waste Amendments 2016, Frank R. Lautenberg Chemical Safety for the 21st Century Act
Occupational Exposures	U.S. Occupational Safety and Health Administration (OSHA)	Set legal standards for worker exposure including permissible exposure limits (PELs) and short-term exposure limits (STELs)	1970, Occupational Safety and Health Act

about treatment and prevention is often provided through the national network of poison control centers. *Environmental toxicology* focuses on the impact of chemical pollutants in the environment on biological organisms. Although toxicologists concerned with the effects of environmental pollutants on human health fit into this definition, it is most commonly associated with studies of chemicals in nonhuman organisms such as fish, birds, terrestrial animals, and plants. Often, the terminology of *environmental health sciences* is used to describe environmental toxicology that is concerned with human health. *Ecotoxicology* is a specialized area within environmental toxicology that focuses on the impacts of toxic substances on population dynamics in an ecosystem. The transport, fate, and interactions of chemicals in the environment constitute a critical component of both environmental toxicology and ecotoxicology.

Information from the toxicological sciences, gained by experience or research, has a growing influence on our personal lives as well as on human and environmental health across the globe. Complementary fields such as *exposure science* advance toxicology by studying the magnitude and duration of contact between toxic chemicals and biological organisms. Knowledge about the toxicological effects of a compound and the extent of exposure has important implications for drugs, consumer products, waste cleanup, manufacturing processes, regulatory action, civil disputes, and broad policy decisions. The growing influence of toxicology on societal issues carries with it substantial ethical, legal, and societal implications for toxicological research and testing. Recognizing the many critical roles that toxicologists play throughout society, the Society of Toxicology, a professional organization for toxicologists, has developed the Code of Ethics that frames the expected behaviors and attitudes of its members (Table 2-3). This guidance covers a number of important activities including the conduct of scientific inquiries, communication of findings and assessments of risk, protections for animals, patients, and the environment, as well as transparency in conflicts of interest reporting. Adherence to these professional behaviors and ideals is critical for ensuring the public's trust of the data and assessments conducted by toxicologists.

Table 2-3

Toxicology Code of Ethics

- Conduct their work with objectivity and themselves with integrity. Being honest and truthful in reporting and communicating their research.
- Hold as inviolate that credible science is fundamental to all toxicological research and is the basis for communicating results.
- Recognize a duty to communicate information concerning health, safety, and toxicity in a timely and responsible manner, with due regard for the significance and credibility of the available data.
- Give due consideration to the ethical, legal, social, and policy implications of their research and communications.
- Be a thoughtful advocate for human, animal, and environmental health.
- Abstain from professional judgments influenced by undisclosed conflict of interest, disclose any material conflicts of interest, and avoid situations that imply a conflict of interest.
- Observe the spirit, as well as the letter of laws, regulations, and ethical standards with regard to the conduct of human and animal research.
- Practice high standards of environmental and occupational health, and safety for the benefit of themselves, their co-workers, their families, their communities, and society as a whole.
- Provide equal opportunity and equal consideration to all members without regard to sex, gender identity or expression, race, color, national or ethnic origin, religion or religious belief, age, marital status, sexual orientation, disabilities, or veteran status.

Adapted from the 2012 version of the Society of Toxicology (https://www.toxicology.org/about/vp/code-of-ethics.asp).

SPECTRUM OF UNDESIRED EFFECTS

The spectrum of undesired effects elicited by chemicals can be broad. Some effects are harmful, whereas others are not. Prescription drugs produce a number of effects, but typically only one of these actions is intended to be therapeutic; all of the other responses are referred to as *side effects*. However, some of these side effects may be preferred for another therapeutic indication. For example, the drug sildenafil was originally developed to reduce blood pressure in patients. During the clinical studies of sildenafil in hypertensive patients, male subjects reported sustained erections as a side effect. Recognizing the potential to repurpose this investigational medication, the company subsequently gained approval of sildenafil for the treatment of erectile dysfunction under the brand name Viagra® (Boolell et al., 1996). Some side effects of drugs are never desirable and are always harmful to the well-being of animals and humans. These are referred to as the *adverse, deleterious,* or *toxic* effects of the drug.

Allergic Reactions

A chemical allergy is an adverse reaction of the immune system to a chemical in response to a previous exposure to that chemical or to a structurally similar one. The term *hypersensitivity* is most often used to define this allergic response, but *allergic reaction* and *sensitization reaction* may also describe the situation when a prior exposure (sensitization) of the chemical is required to produce its subsequent toxic effect. Once sensitization has occurred, allergic reactions may result from exposure to relatively very low doses of chemicals. For a given individual, allergic reactions can be dose-related. For example, it is well known that the allergic response to poison ivy or poison sumac in sensitized individuals is related to the extent of the skin's exposure to urushiol oils found in the leaves. Some sensitization reactions, such as allergic reactions to nuts, shellfish, and other foods as well as certain antibiotics and xenobiotics, may be severe and occasionally fatal.

Most chemicals and their metabolic products are not sufficiently large enough in size to be recognized by the immune system as a foreign substance. Thus, these chemicals must first interact with a native protein to form an antigen (or immunogen). A molecule that combines with an endogenous protein to elicit an allergic reaction is termed a *hapten.* The hapten–protein complex (antigen) is then capable of stimulating the immune system to form antibodies over a period of 1 or 2 weeks. Upon subsequent exposure to the chemical, the antigen is recognized by the antibody leading to a range of allergic responses of varying severities (from mild to fatal anaphylactic shock). The pattern of allergic response differs across species. In humans, involvement of the skin (e.g., dermatitis, urticaria, and itching) and the eyes (e.g., conjunctivitis) is most common, whereas in guinea pigs, bronchiolar constriction leading to asphyxia is observed. While skin and eye reactions most often occur in humans, chemically induced asthma (characterized by bronchiolar constriction) certainly can be seen. Hypersensitivity reactions are discussed in more detail in Chap. 12.

Idiosyncratic Reactions

Chemical idiosyncrasy refers to the abnormal reactivity of an individual to a chemical based on its genetics or other individual sensitivity factors (Goldstein et al., 1974; Uetrecht, 2007; Roth and Ganey, 2011). Idiosyncratic reactions have historically been defined as responses that are qualitatively similar to that observed in other individuals but occur at low doses due to an individual's extreme sensitivity (Goldstein et al., 1974). More recent definitions describe *idiosyncratic drug reactions* as adverse effects unrelated to the therapeutic action of a drug that are typically not observed in most treated patients (Uetrecht and Naisbitt, 2013). It has been proposed that idiosyncratic drug responses involve a combination of individual differences in the ability to (1) form a reactive intermediate (usually through oxidation to an electrophilic intermediate), (2) detoxify the reactive intermediate (usually through hydrolysis or conjugation), (3) mount an immune response through human leukocyte antigens (HLAs) as well as T cells, and/or (4) cause cell death (Uetrecht, 2007; Pan et al., 2017). The relatively low incidence of these occurrences likely include patient-specific factors that extend beyond genetics, including inflammatory stress, infection, mitochondrial dysfunction, and environmental factors (Roth and Ganey, 2011). Typically, there is a delay between the time drug therapy is initiated and the clinical presentation of symptoms. In some cases, reinitiation of a medication can rapidly precipitate an idiosyncratic reaction. Often, the risk of an idiosyncratic reaction does not increase with higher doses of a drug. Although such reactions are often rare and may occur at an incidence of 1 in 10,000 exposed people or less, they are one of the common reasons why drugs are withdrawn from the market.

Idiosyncratic reactions can occur in any organ system; however, the skin, liver, hematopoietic, and immune systems are the most often affected. Within the skin, idiosyncratic drug reactions can range from mild to life-threatening and include maculopapular rashes, urticaria, drug reactions with eosinophilia

and systemic symptoms (DRESS), toxic epidermal necrolysis, and Stevens–Johnson syndrome. Examples of drugs and chemicals associated with idiosyncratic reactions in the skin include dinitrochlorobenzene (industrial ingredient), piperacillin (antibiotic), abacavir (HIV antiviral drug), allopurinaol (gout drug), nevirapine (HIV antiviral drug), and carbamazepine (antiseizure drug). Liver-related idiosyncratic drug reactions can be hepatocellular or cholestatic in nature and have been reported for halothane (anesthetic), nitrofurantoin (antibiotic), amoxicillin/clavulonate (antibiotic), and flucloxacillin (antibiotic). Hematologic idiosyncratic drug reactions can differentially affect neutrophil, platelet, and red blood cell populations and can be observed with a number of medications including heparin (anticoagulant), alpha-methyldopa (antihypertensive drug), and chloramphenicol (antibiotic).

Genetic variants in HLAs that encode major histocompatibility complex (MHC) proteins in humans have been strongly implicated in hypersensitivity reactions for a number of drugs and chemicals (Pavlos et al., 2012; Pan et al., 2017). MHC Class I proteins, including HLA-A, HLA-B, and HLA-C, present peptides from proteins that break down inside a cell to cytotoxic $CD8^+$ T cells. The genetic variant HLA-B*5701 is an important risk factor for hypersensitivity reactions resulting from exposure to the HIV medication abacavir (skin toxicity) (Mallal et al., 2008) and the antibiotic flucloxacillin (liver toxicity) (Daly et al., 2009). Similarly, the HLA-B*1502 allele is a risk factor for idiosyncratic drug reactions including Stevens–Johnson syndrome associated with the antiseizure drug carbamazepine (Chung et al., 2004; Chen et al., 2011). Other genetic variants, including those in metabolic enzymes such as glutathione *S*-transferase, are thought to contribute to some extent to idiosyncratic drug reactions (Lucena et al., 2008). Because of the life-threatening nature of idiosyncratic drug reactions, it is critical that researchers continue to identify modifying factors that initiate and/or heighten these responses.

Immediate versus Delayed Toxicity

The toxic effects of a chemical can develop rapidly after a single exposure, or may be delayed for some time. *Immediate toxicity* can be observed for hydrogen cyanide or nerve gases such as sarin. Exposure to high doses of these chemicals can have rapid effects on the cardiovascular, respiratory, and neurological systems culminating in death within minutes. Because of their high toxicity and expeditious actions, hydrogen cyanide, sarin, and similar chemicals have been used as chemical warfare agents.

Delayed toxicities of chemicals may take months or years to be recognized. Because of the long latency period, decades after exposure to a carcinogenic chemical will pass before tumors are observed in humans. There are a number of examples of delayed toxicities due to xenobiotic exposure. Women were prescribed diethylstilbestrol (DES) during pregnancy in order to prevent preterm birth. The daughters of these women have a greatly increased risk of developing vaginal cancer, in young adulthood, approximately 20 to 30 years after in utero exposure to DES (Hatch et al., 1998). Also, delayed neurotoxicity is observed after exposure to some organophosphate insecticides that act by covalent modification of an enzyme referred to as *neuropathy target esterase* (NTE), a neuronal protein with serine esterase activity (Johnson, 1969, 1975). Binding of certain organophosphate chemicals to this protein initiates degeneration of long axons in the peripheral and central nervous system (CNS). The most notorious of the compounds that produce this type of neurotoxic effect is the industrial chemical triorthocresylphosphate. The effect is not observed until at least several days after exposure to the toxic compound.

Reversible versus Irreversible Toxic Effects

Some toxic effects of chemicals are *reversible*, whereas others are *irreversible*. The likelihood of a toxic response to be reversed largely depends on the ability of an injured tissue to adapt, repair, and regenerate. Therefore, for tissues such as the liver and gastrointestinal tract that have a high ability to regenerate, many injuries are reversible. Within days of exposure to the hepatotoxicant acetaminophen, the liver mounts an adaptive response that includes the proliferation of remaining centrilobular hepatocytes and the differentiation of oval stem cells. However, there are limits to this repair capacity, and very high doses may overwhelm this adaptive response leading to permanent loss of function and potentially fatal liver failure. Because of the rapid proliferation of crypt epithelial cells, the gastrointestinal tract is highly sensitive to anticancer drug cytotoxicity, but is also able to repair and repopulate intestinal villi with new enterocytes.

By comparison, the CNS has a much more limited ability to divide and replace damaged neurons making damage largely irreversible. This is exemplified by the persistent loss of neurons that utilize the neurotransmitter, dopamine, which results in Parkinson's-like symptoms observed in patients exposed to the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ selectively targets dopaminergic neurons in the substantia nigra pars compacta, which leads to neurodegeneration and clinical signs that range from slowed reaction time and motion (bradykinesia) to cogwheel rigidity (Langston et al., 1983).

Cancers and birth defects caused by chemical exposures, once they occur, are often also considered irreversible toxic effects. For example, exposure to the antiangiogenic drug thalidomide during sensitive windows of fetal development prevents the formation of limbs—an effect that cannot be reversed. As a result, children are born with truncated or missing arms and legs, a condition called *phocomelia.* Therefore, it is important to understand the regenerative and reparative capacity of a target organ in order to counteract a chemical's toxic effects.

Local versus Systemic Toxicity

Toxic responses are also characterized according to the proximity between the site of chemical exposure and the site(s) of molecular action. *Local effects* are those that occur where contact is first made by the toxicant and the biological system. Such effects are produced by the ingestion of toxic substances or the inhalation of irritant materials. For example, chlorine gas reacts with lung tissue at the site of contact, causing damage and swelling of the lungs, with possibly fatal consequences, even though very little of the chemical is absorbed into the bloodstream. By comparison, *systemic effects* require the absorption and distribution of a toxicant from its entry point to a distant site where the deleterious effects are produced. Most substances—except those that are highly reactive, such as strong acids and bases—produce systemic effects. In some cases, both local and systemic effects can be demonstrated for a single chemical. For example, tetraethyl lead produces effects on skin at the site of absorption and then is delivered systemically to cause its typical effects on the CNS and other organs. If the local area of injury is markedly affected by the toxicant, there may also be indirect systemic effects due to secondary events. For example, kidney damage after a severe acid burn is an indirect systemic effect because the toxicant does not reach the kidney.

Most chemicals that produce systemic toxicity do not cause a similar degree of toxicity in all organs; instead, they usually elicit their major toxicity in only one or two organs. These sites are referred to as the *target organs* of toxicity for a particular chemical.

While it may be true in some cases, the target organ of toxicity is not always the site of the highest concentration of the chemical. For example, lead accumulates in bones, but its toxic effects occur in soft tissues, particularly the brain. Instead, the bones act as a reservoir that can leach and release stored lead and cause neurological injury even years after exposure. The insecticide dichlorodiphenyltrichloroethane (DDT) concentrates in adipose tissue due to its lipophilic chemical property, but produces no known toxic effects in that tissue. However, the primary target organs for toxicity of DDT are the brain and female reproductive system. Thus, migrating birds who accumulated DDT in body fat at high levels during the summer months had no adverse effects until toxic concentrations of DDT were released from stored body fat into the blood and then brain and reproductive system during migration in the fall, leading to both acute mortality from neurotoxicity and decreased reproductive success.

Interactions of Chemicals

Throughout the day, an individual may come in contact with many chemicals at any given time (in the workplace, cosmetics, medications, diet, hobbies, etc.). As a result, it is necessary to consider how various chemicals may interact with each other. Interactions may impact a number of physiologic processes including absorption, protein binding, receptor signaling, and the biotransformation and excretion of one or both of the interacting toxicants. As a consequence, the cumulative response(s) of an individual to combinations of toxicants may be increased or decreased.

The study of chemical interactions often provides a better understanding of key mechanisms of toxicity. A number of terms have been used to describe pharmacological and toxicological interactions (Fig. 2-1):

- An *additive* effect occurs when the combined responses of two chemicals is equal to the sum of the responses to each chemical given alone (e.g., 2 + 3 = 5). For example, when two organophosphorous insecticides are given together, inhibition of acetylcholinesterase enzymes (AChE) is usually additive, based on the relative ability of each one to inhibit AChE.
- A *synergistic* effect is observed when the combined responses of two chemicals are much greater than the sum of the response to each chemical when given alone (e.g., 2 + 2 = 20). For example, both carbon tetrachloride and ethanol are hepatotoxic compounds, but together they produce much more liver injury than expected based on the extent of damage at a given dose when administered alone.
- *Potentiation* occurs when one substance does not produce any toxicity on a particular tissue or system but when added to another chemical makes that chemical much more toxic (e.g., 0 + 2 = 10). Isopropanol, for example, is not hepatotoxic on its own, but when it is administered in combination with carbon tetrachloride, the hepatotoxicity of carbon tetrachloride is much greater than when it is given alone.
- *Antagonism* occurs when two chemicals administered together interfere with each other's actions or one interferes with the action of the other (e.g., 4 + 6 = 8; 4 + (−4) = 0; 4 + 0 = 1).

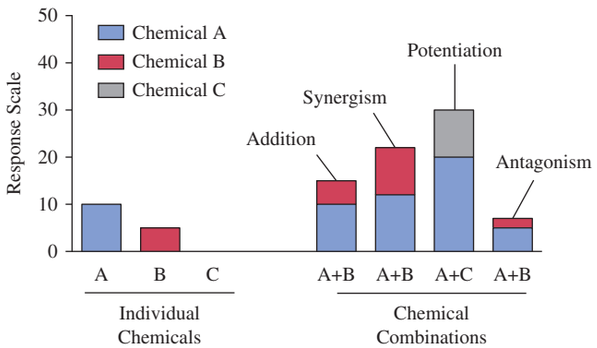

Figure 2-1. *Illustration of responses following various chemical interactions.* Data illustrate a theoretical response to three different chemicals, either alone or in combination. As shown, chemicals A and B have measureable responses when used alone, whereas chemical C does not. Four different examples of chemical interactions, namely additive, synergism, potentiation, and antagonism, are plotted.

Antagonism of the toxic effects of chemicals is often desirable in identifying important mechanisms of toxicity as well as in developing antidotes. There are four major types of antagonism: receptor, chemical, dispositional, and functional (Fig. 2-2).

- *Receptor antagonism* occurs when two chemicals that bind to the same receptor produce less of an effect when given together relative to the addition of their separate effects (e.g., 4 + 6 = 8) or when one chemical antagonizes the effect of the second chemical (e.g., 0 + 4 = 1). Receptor antagonists are often termed *blockers.* This concept is exploited in the clinical treatment of poisonings. For example, the receptor antagonist naloxone treats the respiratory depressive effects of morphine and other morphine-like narcotics by competitively binding to the same receptor. Thus, rapid administration of naloxone can be life-saving for someone who has overdosed on morphine. Another example of receptor antagonism is the use of the antiestrogen drug tamoxifen to lower breast cancer risk among women at high risk for this estrogen-related cancer. As a partial agonist, tamoxifen competitively blocks estradiol from binding to its receptor. Treatment of organophosphorous insecticide poisoning with atropine is another example of an antagonism of toxicity by blocking a physiological "ligand–receptor interaction." In this example, atropine binds to the acetylcholine receptor and thus blocks the excess acetylcholine that accumulates after inhibition of the cholinesterase enzyme by the organophosphorous compound (see Chap. 22).
- *Chemical antagonism or inactivation* is simply a direct chemical reaction between two compounds that produces a less toxic product. For example, 2,3-dimercaptosuccinic acid (DMSA; succimer) chelates or binds to metal ions, such as arsenic, mercury, and lead, leading to decreases in their toxicity. The use of the strongly basic low-molecular-weight protein protamine sulfate to form a stable complex with heparin, which abolishes its anticoagulant activity, is another example. The direct binding or sequestration of the two chemicals prevents the toxicant from interacting with its receptor and in turn reduces downstream signaling and the propensity to cause cellular damage.
- *Dispositional antagonism* occurs when the disposition—that is, the absorption, distribution, biotransformation, or excretion of a chemical—is altered such that the concentration and/or duration of the chemical at the target organ is reduced. Thus, the prevention of absorption of a toxicant by use of activated charcoal, and the increased excretion of a chemical by administration of an osmotic diuretic or alteration of the pH of the urine are examples of dispositional antagonism. If the parent compound is responsible for the toxicity of the chemical (such as the anticoagulant warfarin) and its metabolic breakdown products are less toxic than the parent compound, increasing the compound's biotransformation (metabolism)

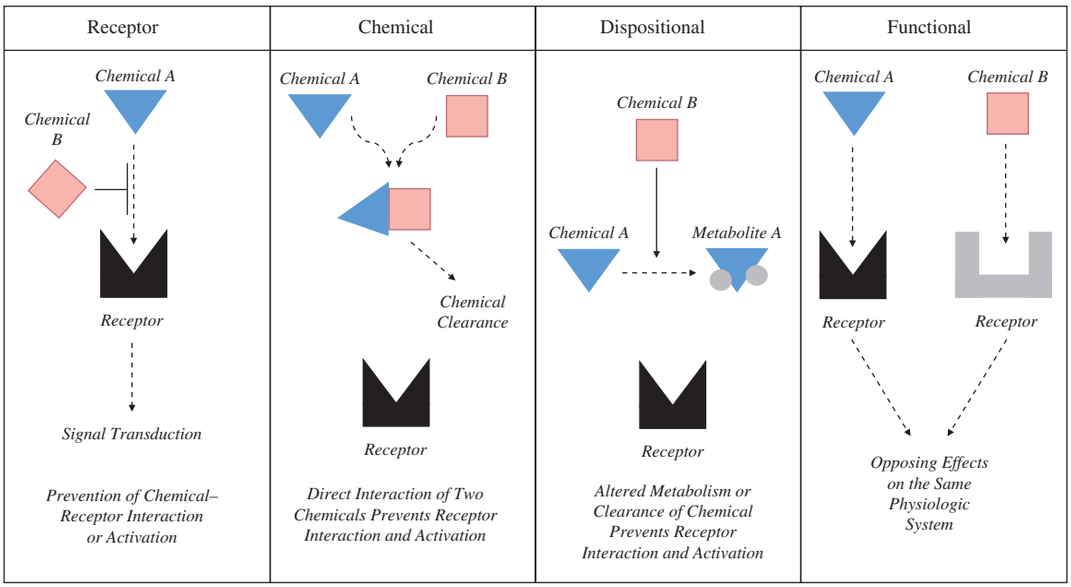

Figure 2-2. *Antagonism can occur by multiple mechanisms.* Four different types of antagonism are shown and include receptor, chemical, dispositional, and function. In each scenario, two chemicals (A [triangle] and B [square]) and their antagonistic relationships are shown.

by administering a drug that increases the activity of the metabolizing enzymes (e.g., a "microsomal enzyme inducer" such as phenobarbital) will decrease its toxicity. However, if the chemical's toxicity is largely due to a metabolic product (as in the case of the organophosphate insecticide parathion), inhibiting its biotransformation by an inhibitor of microsomal enzyme activity (e.g., piperonyl butoxide) will decrease its toxicity.

- *Functional antagonism* occurs when two chemicals counterbalance each other by producing opposing effects on the same physiological function, often through different signaling pathways. For example, blood pressure can markedly fall during severe intoxication with a barbiturate, which can be effectively antagonized by the intravenous administration of a vasopressor such as norepinephrine. In this case, the barbiturate works through $GABA_A$ receptors and norepinephrine activates α-adrenergic receptors to produce opposing effects on vascular tone.

Tolerance

Repeated exposure to a chemical can reduce its pharmacologic and/or toxicologic responses, a process called *tolerance*. *Cross-tolerance* occurs when structurally related chemicals cause diminished responses. Typically, days or weeks of repeated exposure are required for tolerance to occur. Depending upon the chemical and biological effects, there may be multiple mechanisms that cause tolerance:

- *Dispositional tolerance* occurs when the amount of chemical reaching the site of action decreases over time, leading to the reduced responsiveness of the tissue to stimulation. A chemical known to produce dispositional tolerance is phenobarbital. The barbiturate, phenobarbital, produces tolerance to itself by increasing the expression of enzymes in the liver that are responsible for its biotransformation to pharmacologically inactive products, a process known as "biotransformation enzyme induction."
- *Chemical or cellular tolerance* may result from a lower availability of receptors and/or mediators (e.g., neurotransmitters). Drugs of abuse such as morphine interact with the opioid receptor; with repeated exposure to morphine, a protein called beta-arrestin-2 binds to the opioid receptor leading to desensitization and tolerance. Comparatively less is known about the cellular mechanisms responsible for altering the responsiveness of a tissue to a toxic chemical than is known about dispositional tolerance.

CHARACTERISTICS OF EXPOSURE

Toxicity to a biological system requires that sufficient *concentrations* of the "active" form of a chemical accumulate at the site of action for a requisite period of *time*. Many chemicals possess relatively low toxicity in their "native" form but, when acted on by enzymes in the body, are converted to intermediate forms that interfere with normal cellular biochemistry and physiology. Thus, whether a toxic response occurs is dependent on multiple factors: chemical and physical properties of the chemical, the exposure scenario, how the chemical is metabolized by the system, the concentration of the active form at the particular target site(s), and the overall susceptibility of the biological system to injury. In order to characterize fully the potential hazard of a specific chemical, one needs to know not only the type of effect it produces and the dose required to produce that effect, but also the information about the chemical, exposure, and disposition. Two major factors that influence toxicity as it relates to the exposure situation for a specific chemical are the *route of exposure* and the *duration and frequency of exposure*.

Route and Site of Exposure

The major routes for entry of toxic chemicals into the body are the gastrointestinal tract (ingestion), the lungs (inhalation), and the skin (topical, percutaneous, or dermal). Chemicals generally produce the greatest effect and the most rapid response when given directly into the bloodstream (the intravenous route). Chemicals

can also enter the body to varying degrees through other routes. An approximate descending order of effectiveness for the other routes would be inhalation, intraperitoneal, subcutaneous, intramuscular, intradermal, oral, and dermal. The vehicle, or the inert material in which the toxicant is dissolved, and other formulation ingredients can markedly alter chemical absorption after ingestion, inhalation, or topical exposure. In addition, the route of administration can influence the toxicity of chemicals. For example, a chemical that acts on the CNS, but is efficiently detoxified in the liver, would be expected to be less toxic when given orally than when given via inhalation, because the oral route requires that nearly all of the dose pass through the liver before reaching the systemic circulation and then the CNS. Typically, different routes of toxicant entry into the body have been associated with certain types of exposures. Occupational exposure to chemicals most frequently results from breathing contaminated air (inhalation) and/or direct and prolonged contact of the skin with the substance (dermal exposure), whereas accidental and suicidal poisonings occur most frequently by oral ingestion.

A comparison of toxic doses (TDs) of a chemical by different routes of exposure often provides useful information about the extent of absorption into the body. In instances when the TD after oral or dermal administration is similar to the TD after intravenous administration, the assumption is that the chemical is absorbed readily and rapidly. Conversely, in cases where the TD by the dermal route is several orders of magnitude higher than the oral TD, it is likely that the skin provides an effective barrier to absorption of the chemical. Toxic effects by any route of exposure can also be influenced by the concentration of the chemical in its vehicle, the total volume of the vehicle, and the properties of the vehicle to which the biological system is exposed, as well as the rate at which exposure occurs. For more details on the absorption of toxicants, see Chap. 5.

Duration and Frequency of Exposure

The duration and frequency for exposure of experimental animals to chemicals is classified according to four categories: acute, subacute, subchronic, and chronic (Fig. 2-3). *Acute exposure* refers to exposure to a chemical for less than 24 hours. While acute exposure usually refers to a single administration, repeated exposures may be given within a 24-hour period for some slightly toxic or practically nontoxic chemicals. Acute exposure by inhalation refers to continuous exposure for less than 24 hours, most frequently for 4 hours. Repeated exposure is divided into three categories: subacute, subchronic, and chronic. *Subacute exposure* refers to repeated exposure to a chemical for 1 month or less, *subchronic* for 1 to 3 months, and *chronic* for more than 3 months, although usually this refers to studies with at least 1 year of repeated dosing. These three categories of repeated exposure can be by any route, but most often they occur by the oral route.

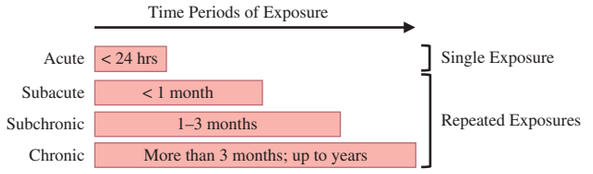

Figure 2-3. *Time periods of chemical exposure.* Single exposures to chemical are observed in acute studies of short duration. Repeated exposures to chemicals can occur over a range of time periods spanning short (subacute), intermediary (subchronic), and long periods (chronic).

In situations of human exposure to chemicals, the frequency and duration of exposure are not well-defined compared to controlled animal studies; nonetheless, many of the same terms are used to describe general exposure situations. Thus, workplace or environmental exposures may be described as *acute* (occurring from a single incident or episode), *subchronic* (occurring repeatedly over several weeks or months), or *chronic* (occurring repeatedly for many months or years). There are multiple examples of acute, subchronic, and chronic toxicities in humans. In 1984, leakage of methyl isothiocyanate from a chemical company led to acute exposure and toxicity in over 200,000 people in India with an estimated 2000 fatalities occurring in the first 24 hours. By comparison, children and workers that come in contact with high levels of the toxic metal lead in their environment or workplace can result in discernable neurological deficits following subchronic exposure for weeks or months. Typically, it takes many years for the chronic toxicities of ethanol (e.g., liver cirrhosis) and cigarette smoking (e.g., chronic obstructive pulmonary disease) to be observed.

For many chemicals, the toxic effects that follow a single exposure are quite different from those produced by repeated exposure. For example, the primary, acute toxic manifestation of benzene is CNS depression, but repeated exposures can result in bone marrow toxicity and an increased risk for leukemia. Acute exposure to chemicals that are rapidly absorbed is likely to produce immediate toxic effects but can also produce delayed toxicity that may or may not be similar to the toxic effects of chronic exposure. Conversely, chronic exposure to a toxic chemical may produce some immediate (acute) effects after each administration in addition to the long-term, low-level, or chronic effects of the toxic substance.

Toxicokinetic studies are performed by sampling the blood or tissue at various times after exposure to determine the concentration of a chemical and better understand the influence of exposures on toxicity endpoints. Concentration-time profiles for a given chemical are influenced by the frequency of exposures. The relationship between elimination rate and frequency of chemical exposure is shown in Fig. 2-4. A chemical that produces severe effects with a single dose may have no effect if the same total dose is given in

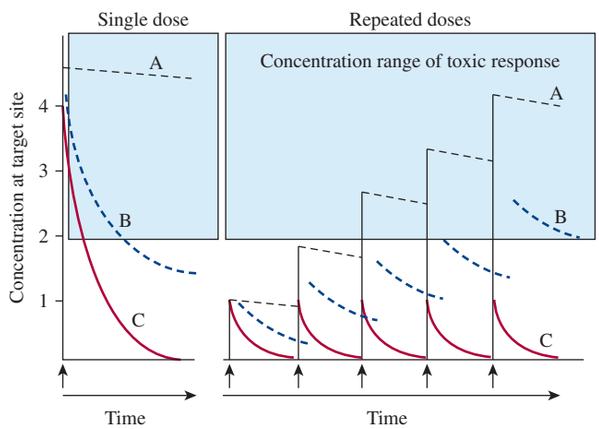

Figure 2-4. *Diagrammatic view of the relationship between dose and concentration at the target site under different conditions of dose frequency and elimination rate.* (Line A) A chemical with very slow elimination (e.g., half-life of 1 year). (Line B) A chemical with a half-life approximately equal to frequency of dosing (e.g., 1 day). (Line C) Rate of elimination faster than the dosing frequency (e.g., 5 hours). Blue-shaded area is representative of the concentration of chemical at the target site necessary to elicit a toxic response.

several intervals. For the chemical depicted by line B in Fig. 2-4, in which the half-life for elimination (time necessary for 50% of the chemical to be removed from the bloodstream) is approximately equal to the dosing frequency, a theoretical toxic concentration (shown conceptually as two concentration units in Fig. 2-4) is not reached until the fourth dose, whereas that concentration is reached with only two doses for chemical A, which has an elimination rate much slower than the dosing interval (time between each repeated dose). Conversely, for chemical C, where the elimination rate is much shorter than the dosing interval, a toxic concentration at the site of toxic effect will never be reached regardless of how many doses are administered. Of course, it is possible that residual cell or tissue damage occurs with each dose even though the chemical itself is not accumulating. The important consideration, then, is whether the interval between doses is sufficient to allow for complete repair of tissue damage. It is evident that with any type of repeated exposure, the production of a toxic effect not only is influenced by the frequency of exposure but may also, in fact, be entirely dependent on the frequency rather than the duration of exposure. Chronic toxic effects may occur, therefore, if the chemical accumulates in the biological system (rate of absorption exceeds the rate of biotransformation and/or excretion), if it produces irreversible toxic effects, or if there is insufficient time for the system to recover from the toxic damage within the exposure frequency interval. For additional discussion of these relationships, see Chaps. 5 and 7.

DOSE–RESPONSE RELATIONSHIPS

The relationship between the "dose" and "response" to a chemical is the foundation of modern toxicology. *Dose–response relationships* are defined as the association between the amount of a toxicant administered and the extent to which changes are observed in a biological system. When describing chemical exposures, it is important to consider both the dose of the chemical administered or measured in the environment (*external dose*) as well as the amount of chemical absorbed and found at the site of biological activity (*internal dose*). There are a number of toxicokinetic factors that will be important in determining the internal dose of most chemicals, which often can vary between tissues. In addition to assessing internal doses, one's ability to quantify a biological response will depend upon defining an endpoint that can be quantified.

The dose of a chemical or toxic agent can be expressed as a mass or concentration. The units of the concentration will depend upon whether the toxic chemical is found in the solid, liquid, or gaseous state. An internal blood concentration of the analgesic acetaminophen in excess of 150 mg/L a few hours after ingestion is considered a toxic exposure. Air pollutant concentrations are often referred to as the amount of the toxic agent in a cubic meter of air (mg/m^3). Another common approach to describing concentrations is based on the units of mass relative to the total number of units of mass. For example, the presence of the lead in drinking water should be less than 15 parts per billion (ppb). Another way to describe this drinking water standard would be 15 μg/L. When considering the administration of chemicals to humans and animals, doses are conventionally expressed as milligrams per kilogram.

Dose–response relationships are routinely divided into two types: (1) the individual dose–response relationship, which describes the response of an *individual* organism to increasing doses of a chemical, often referred to as a "graded" response because the measured effect is continuous over a range of doses, and (2) a quantal dose–response relationship, which characterizes the distribution of individual responses to different doses in a *population* of organisms. For a given chemical, there are likely multiple different molecular, biochemical, and cellular effects (responses), each with its own "dose–response" relationship. These can include changes in receptor binding, enzymatic cofactor availability, cellular energetics, and markers of cellular or organ damage. As a result, the toxic responses of a chemical vary as one goes from lower to higher doses. In turn, there are likely different types of responses across increasing doses (e.g., loss of cellular ATP reserves versus stimulation of cell proliferation) as well as differences in the magnitude or extent of a specific response (e.g., moderate versus marked histopathological changes in a tissue with higher exposure to a chemical). In the instance of population-level "dose–response" characterization, it is important to recognize that the observed response is an integration of the cumulative individual dose–response relationships occurring across multiple molecular and biochemical events in potentially a number of different cell types. As a result, subtle effects that occur at low doses (e.g., changes in gene expression in response to a chemical that activates a nuclear transcription factor, such as dioxins binding to the "aryl hydrocarbon receptor," AhR) may be less evident than responses that appear at higher doses (e.g., cellular necrosis).

Individual, or Graded, Dose–Response Relationships

Individual dose–response relationships are characterized by a continuous scale of doses that lead to an increase in the magnitude of a specific response. The graded dose–response relationship requires the careful selection of a range of doses for evaluation and identification of a specific biochemical process. For example, Fig. 2-5 shows the dose–response relationship between different dietary doses of the organophosphorous insecticide chlorpyrifos and the extent of inhibition of two different enzymes acetylcholinesterase and carboxylesterase in the rat brain and liver, respectively (Lassiter et al., 1999). In the brain, the degree of inhibition of both enzymes is clearly dose-related, although the degree of inhibition per unit dose is different for the two enzymes. From the shapes of these two dose–response curves it is evident that, in the brain, cholinesterase will be inhibited at a chlorpyrifos dose of 3 mg/kg that does not alter the activity of carboxylesterase. At higher doses of chlorpyrifos (5 mg/kg and higher), both enzymes will be inhibited, but the extent of inhibition will be greater for cholinesterase than carboxylesterase. The primary toxicological response that results from chlorpyrifos exposure is directly related to the degree of cholinesterase enzyme inhibition in the brain. Thus, clinical signs and symptoms for chlorpyrifos would follow a dose–response relationship similar to that for the brain cholinesterase enzyme. However, as noted above, for many chemicals, more than one effect may result because of multiple various target sites in different tissues. Thus, the observed response to varying doses of a chemical in the whole organism is often complicated by the fact that most chemicals have multiple sites or mechanisms of toxicity, each with their own "dose–response" relationship and subsequent adverse effect.

Quantal Dose–Response Relationships

In contrast to the "graded" or continuous-scale dose–response relationship that occurs in individuals, the dose–response relationships in a *population* are by definition quantal—or "all or none"—in nature, that is, at any given dose, an individual in the population is classified as either a "responder" or a "nonresponder." Although these distinctions of "quantal population" and "graded individual" dose–response relationships are useful, the two types of responses are conceptually identical. The ordinate in both cases is simply

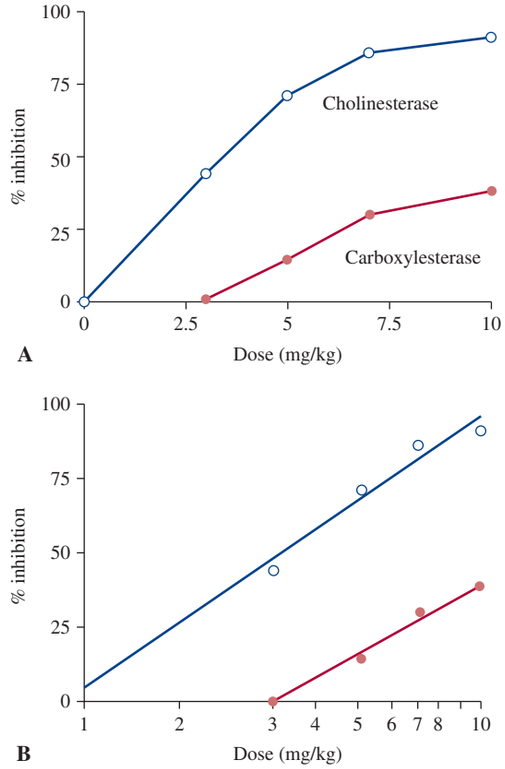

Figure 2-5. *Dose–response relationship between different doses of the organophosphate insecticide chlorpyrifos and esterase enzyme inhibition in the brain.* Open circles and blue lines represent acetylcholinesterase activity and closed circles represent carboxylesterase activity in the brains of pregnant female Long–Evans rats given five daily doses of chlorpyrifos. (**A**) Dose–response curve plotted on an arithmetic scale. (**B**) Same data plotted on a semi-log scale. (Data from Lassiter et al., 1999, with permission.)

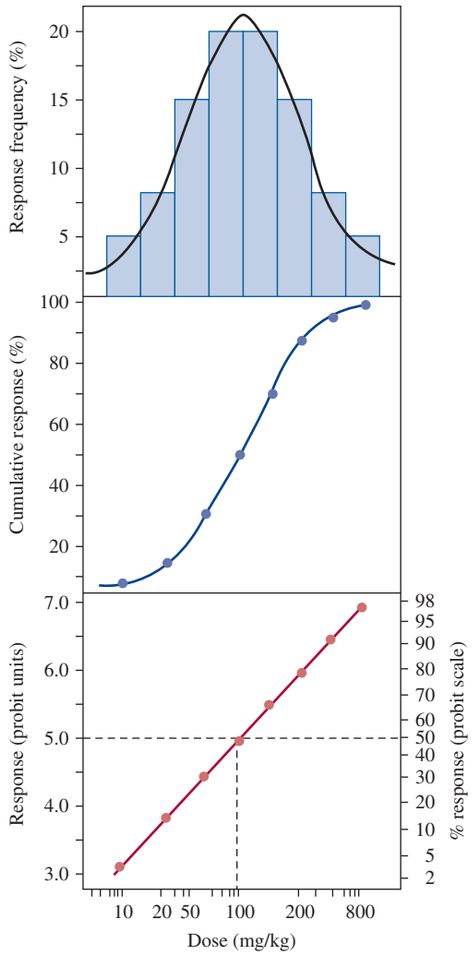

Figure 2-6. *Diagram of quantal dose–response relationship.* The abscissa is the log dose of the chemical. In the top panel the ordinate is response frequency, in the middle panel the ordinate is percent response, and in the bottom panel the response is in probit units (see text).

labeled the *response,* which may be the degree of response in an individual or system or the fraction of a population responding, and the abscissa is the range of administered *doses*.

A widely used approach for estimating the response of a population to a toxic exposure is the "effective dose" (ED). Generally, the midpoint, or 50%, response level for the population is used, giving rise to the "ED_{50}" value. However, any response level, such as an ED_1, ED_{10}, or ED_{30}, could be chosen. Where death is the measured endpoint, the ED_{50} would be referred to as the "lethal dose" LD_{50}. Historically, determination of the LD_{50} was often the first experiment performed with a new chemical. Today, it is widely recognized that the LD_{50} is of little value as a measure of hazard, although it does provide a useful "ball park" indication of the relative hazard of a compound to cause serious, life-threatening poisoning from a single exposure. While death is an obvious quantal endpoint to measure, it should be noted that any quantal response could be used. For example, the LD_{50} of lead or DDT is not a relevant endpoint when characterizing hazards of the chemicals to children or wildlife, respectively. Even continuous variables (e.g., graded data) can be converted to quantal responses. For example, an antihypertensive drug that lowers blood pressure might be evaluated in a population by assigning a "responder" as an individual whose blood pressure was lowered by 10 mm Hg or more. Note that, in this example, an individual who responded to a change in blood pressure of 50 mm Hg would be classified the same as an individual with a change of only 10 mm Hg, yet an individual with a change of 8 mm Hg would be classified as a "nonresponder."

The top panel of Fig. 2-6 shows that quantal dose responses typically exhibit a normal or Gaussian distribution. The frequency histogram in this panel also shows the relationship between dose and effect. The bars represent the percentage of animals that responded at each dose minus the percentage that responded at the immediately lower dose. One can clearly see that only a few animals responded to the lowest dose and the highest dose. Larger numbers of animals responded to doses intermediate between these two extremes, and the maximum frequency of response occurred in the middle portion of the dose range. Thus, we have a bell-shaped curve known as a *normal frequency distribution.* The reason for this normal distribution is that there are differences in the susceptibility of individuals to chemical toxicity; this is known as biological variation. Animals responding at the left end of the curve are referred to as *hypersusceptible,* and those at the right end of the curve are termed *resistant.* If the number of individuals responding at each consecutive dose are added together, a cumulative, quantal dose–response relationship is obtained. When a sufficiently large number of doses are used with a large number of animals per dose, a sigmoid dose–response curve is observed, as depicted in the middle panel of Fig. 2-6. With the lowest dose (6 mg/kg), 1% of the animals responded. A normally distributed sigmoid curve such as this approaches a response of 0% as the dose is decreased and

approaches 100% as the dose is increased, but—theoretically—it never passes through 0% and 100%. However, the minimal ED of any chemical that evokes a stated all-or-none response is referred to as the *threshold dose*.

For a normally distributed population response, the sigmoid curve has a relatively linear portion between 16% and 84%. These values represent the limits of one standard deviation (SD) of the mean (and the median) in a population with truly normal or Gaussian distribution. However, it is usually not practical to describe the dose–response curve from this type of plot because one does not usually have large enough sample sizes to define the sigmoid curve adequately, particularly at low and high doses. In a normally distributed population, the mean ±1 SD represents 68.3% of the population, the mean ±2 SD represents 95.5% of the population, and the mean ±3 SD equals 99.7% of the population. Because quantal dose–response data usually exhibit a Gaussian distribution, one can convert the percent response to units of deviation from the mean or normal equivalent deviations (NEDs). Thus, the NED for a 50% response is 0; the NED of ±1 is equated with an 84.1% response. Traditionally, units of NED are converted by the addition of 5 to the value to avoid negative numbers; these converted units are called *probit units* (Bliss, 1957). The probit (from the contraction of *prob*ability un*it*), then, is an NED plus 5. In this transformation, a 50% response becomes a probit of 5, a ±1 deviation becomes a probit of 6, and a – 1 deviation is a probit of 4.

The data given in the top two panels of Fig. 2-6 are replotted in the bottom panel, with the response plotted in probit units. The data in the middle panel (which was in the form of a sigmoid curve) and the top panel (a bell-shaped curve) form a straight line when transformed into probit units (bottom panel). In essence, what is accomplished in a probit transformation is an adjustment of quantal data from an assumed normal population distribution to a straight line. The ED_{50} is obtained by drawing a horizontal line from the probit unit 5, which is the 50% response point, to the dose–effect line. At the point of intersection, a vertical line is drawn, and this line intersects the abscissa at the ED_{50} point. It is evident from the line that information with respect to the ED for 90% or for 10% of the population also may be derived by a similar procedure. Mathematically, it can be demonstrated that the range of values encompassed by the confidence limits is narrowest at the midpoint of the line (ED_{50}) and widest at both extremes (ED_{10} and ED_{90}) of the dose–response curve (dotted lines in Fig. 2-7). In addition to the ED_{50}, the slope of the dose–response curve can also be obtained. Fig. 2-7 demonstrates the dose–response curves for the response of two compounds. Compound A exhibits a "flat" dose–response curve, showing that a large change in dose is required before a significant change in response will be observed. However, compound B exhibits a "steep" dose–response curve, where a relatively small change in dose will cause a large change in response. It is evident that the ED_{50} for both compounds is the same (8 mg/kg). However, the slopes of the dose–response curves are quite different. At one-half of ED_{50} of the compounds (4 mg/kg), less than 1% of the animals exposed to compound B would respond but 20% of the animals given compound A would respond.

In Figs. 2-6 and 2-7, the doses (mg/kg) have been specified on a logarithmic scale. For normally distributed quantal data, this transformation provides greater linearity in the dose–response relationship. However, this is not always the case. Some radiation effects, for example, give a better probit fit when the dose is expressed arithmetically rather than logarithmically. There are also situations where other functions (e.g., exponentials) of dose provide a better fit to the data than does the log function. Likewise, additional models beyond probits including Hill, logit, and Weibull

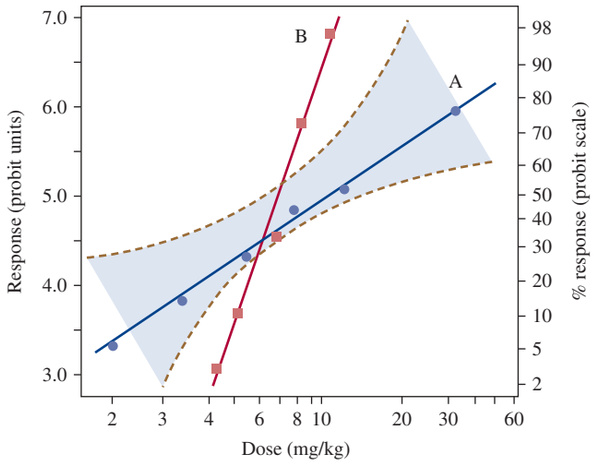

Figure 2-7. *Comparison of dose–response relationship for two different chemicals, plotted on a log dose-probit scale.* Note that the slope of the dose–response is steeper for chemical B than for chemical A. Dotted lines represent the confidence limits for chemical A.

can be used to express dose–response relationships with rigorous mathematical analyses (Demidenko et al., 2017).

Dose Extrapolation Across Species

In the field of toxicology, researchers often work across multiple animal species and in turn there is a need to compare doses from one species to another. This is particularly important in drug development where preclinical data generated in laboratory animals are used to justify and design experiments for clinical testing in humans. While body weight is typically used to calculate a dose of a chemical for an individual organism, it is ineffective to generalize across species. Therefore, the extrapolation of doses across species often requires *allometry*, a field of study that examines relationships between body weight and other biological and physical parameters such as rate of basal metabolism (caloric consumption), heart rate, and blood flow. Allometric studies have revealed that the relationship between body weight and various other physiological parameters can be closely estimated by the following formula: $Y = aW^b$, where Y is the biological parameter of interest (such as metabolic rate) and a and b are constants that relate Y to body weight (Kleiber, 1932). In general, organ sizes between species seem to scale best when b is equal to 1, whereas metabolically derived parameters scale better when b is 0.67 to 0.75 (Sharma and McNeill, 2009).

One simple approach to estimate doses across species is to use body surface area (BSA) rather than body weight for comparison. The relationship between BSA and body weight across most mammalian species is described by the formula $SA = 10.5 \times (\text{body weight [grams]})^{0.67}$ (Chappell, 1992). Empirical comparisons of toxicity data across species confirm that this relationship is appropriate for toxicological scaling. For example, Travis and White (1988) analyzed a number of toxicity testing data sets for 27 different chemotherapeutic drugs for which toxicity data were available in mouse, rat, hamster, dog, monkey, and human (Travis and White, 1988). They found that the exponent of body weight that gave the best correlation with toxicity was 0.73, with 95% confidence bounds of 0.69 to 0.77.

The FDA recommends calculating the *maximum recommended starting dose* for first-in-human clinical trials of an investigational drug using animal data, body surface allometry, and incorporation of a safety factor (CDER, 2005). This requires knowledge of the

no observable adverse effect level (NOAEL) from animal studies. The NOAEL is the highest dose of chemical tested in an animal species that does not result in significant toxicities compared to control animals. Using the NOAEL dose in animals, conversion to a human equivalent dose (HED) using BSA can be made (Table 2-4) (CDER, 2005). For species not listed in the table, the equation HED = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$ should be used. These conversions are largely used for safety testing. The role of using BSA for estimating pharmacologically active doses across species has been examined (Reagan-Shaw et al., 2008); however, it has been recommended that additional factors beyond BSA be incorporated in estimating equivalent doses (Blanchard and Smoliga, 2015).

Not all toxic responses will necessarily scale across species in the same way, nor should allometric scaling be applied to all types of chemicals. For example, acute lethality correlated better across species when body weight, rather than BSA, was used (Rhomberg and Wolff, 1998). Likewise, chemicals that are more amenable to allometric scaling are those that undergo renal excretion (rather than the biliary route), that exhibit minimal hepatic metabolism, that possess limited tissue distribution, and whose molecular targets are similar across species (Sharma and McNeill, 2009).

Computational modeling, particularly physiologically based pharmacokinetic modeling (PBPK), is increasingly being used to scale doses across species. PBPK modeling uses key physiologic parameters and mechanistic data to mathematically describe and predict the disposition of chemicals (Lipscomb et al., 2012) (see Chap. 7). Examples of critical factors used in these assessments include tissue volumes, rates of diffusion, perfusion, transport, membrane permeability, and partition coefficients. Using this information, quantitative relationships that describe a chemical's toxicokinetics across species are derived. In order to apply toxicokinetic data to estimate risks of toxicity, it is also important to consider the toxicodynamic effects of the chemical and whether differences would be anticipated across species. Toxicokinetic–toxicodynamic models can be developed that quantitatively define relationships between exposure and biological responses (Ashauer et al., 2011). This approach has been applied to a variety of quantal and graded dose–response relationships including those assessing growth, development, and reproduction.

Table 2-4

Conversion of Animal Doses to Human Equivalent Doses (HED) for a 60-kg Person Based on Body Surface Area

SPECIES	DIVIDE ANIMAL DOSE (MG/KG) BY
Mouse	12.3
Hamster	7.4
Rat	6.2
Ferret	5.3
Guinea pig	4.6
Rabbit	3.1
Dog	1.8
Monkey	3.1
Marmoset	6.2
Squirrel monkey	5.3
Baboon	1.8
Micro-pig	1.4
Mini-pig	1.1

Shapes of Dose–Response Curves

Toxicology studies are designed to investigate responses across a wide range of doses. Characterization of adverse responses at low doses often depends upon having sensitive measures that detect meaningful, biologic changes. It is often assumed that toxicants will elicit *monotonic* responses where increasing doses will cause a steady unidirectional change in response (e.g., increase or decrease). Monotonic dose–response curves assume shapes that are "uphill" (Fig. 2-8A) or "downhill" (Fig. 2-8B) in nature. Along monotonic dose–response curves, there are dose-dependent transitions that are important in the mechanism(s) of toxicity. A review of 13 toxicants demonstrated a number of saturable and/or inducible kinetic and dynamic processes that underlie these transitions. These include a variety of processes including chemical activation (e.g., butadiene) and detoxification (e.g., methylene chloride), co-substrate depletion (e.g., acetaminophen), receptor interactions (e.g., hydroxyflutamide), repair (e.g., vinyl chloride), and altered homeostasis (e.g., manganese) (Slikker et al., 2004a, 2004b). Typically, different slopes are observed along the dose-dependent transitions when the degree of involvement of these various processes changes.

Dose–response relationships also exist that are *nonmonotonic,* where multiple inflection points exist along the curve that change the shape. The shapes of nonmonotonic dose–response curves are variable and can resemble a biphasic "valley" (U-shaped [Fig. 2-8C] or J-shaped [Fig. 2-8E]) or an "entire hill" (an inverted U-shaped [Fig. 2-8D]). As discussed below, essential nutrients can exhibit U-shaped and inverted U-shaped curves. Similarly, some chemicals are thought to confer benefit to the organism at low exposures (called "hormetic effects" or "hormesis") and be harmful at higher doses. Still other dose–response curves resemble a "roller coaster" with multiple up and down inflection points (Fig. 2-8F). Long-standing regulatory approaches have focused on monotonic dose–response curves to extrapolate the effects anticipated at low doses. However, a review of regulatory framework has begun to examine nonmonotonic dose responses, particularly for endocrine active chemicals (NRC, 2014).

Monotonic Dose–Response Curves

Threshold and Linear, Nonthreshold Models One of the important transitions that occurs in the investigation of dose-dependent responses is found at low doses. Extrapolation of the shape of monotonic dose responses at low doses can be performed by assessing *thresholds* and or using *linear, nonthreshold* extrapolation (Fig. 2-9). A *threshold* is a dose at which toxicity is first observed; at doses below this level, the probability of an individual responding is zero. By comparison, *linear, nonthreshold* models assume there is a direct and proportional relationship between dose and response, even at very low doses. The identification of a threshold in monotonic dose responses depends on the particular response being quantified, the sensitivity of the measurement, and the number of subjects studied. For the individual dose–response relationship, thresholds for many toxic effects certainly exist, although interindividual variability in response and qualitative changes in response pattern with dose make it difficult to establish a true "no effects" threshold in a population of people, for any chemical. The

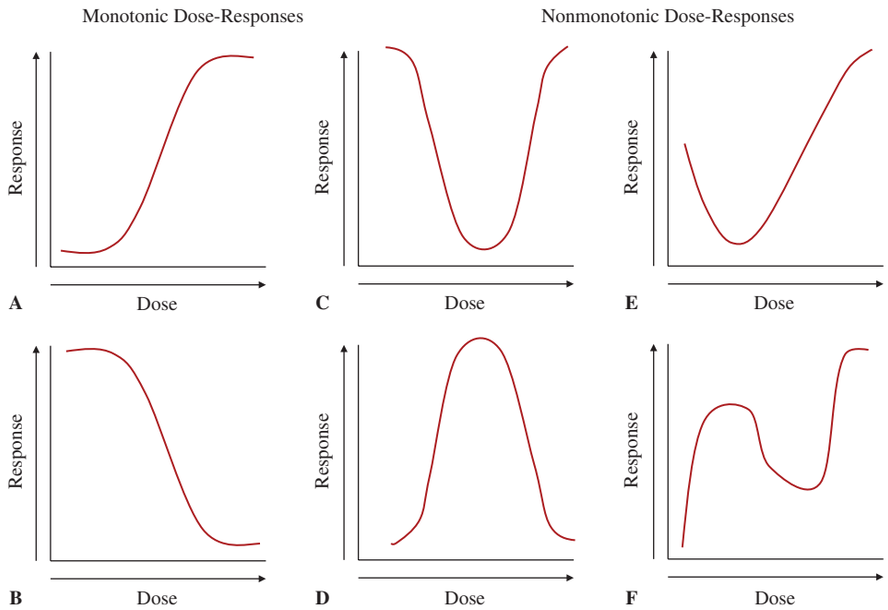

Figure 2-8. *Hypothetical curves for monotonic dose responses and nonmonotonic dose responses.* Traditional monotonic dose responses can exhibit increasing (**A**) and decreasing (**B**) changes in response with higher doses. Nonmonotonic dose responses exhibit a number of shapes including a U-shape (**C**), inverted U-shape (**D**), J-shape (**E**), and variable slope (**F**).

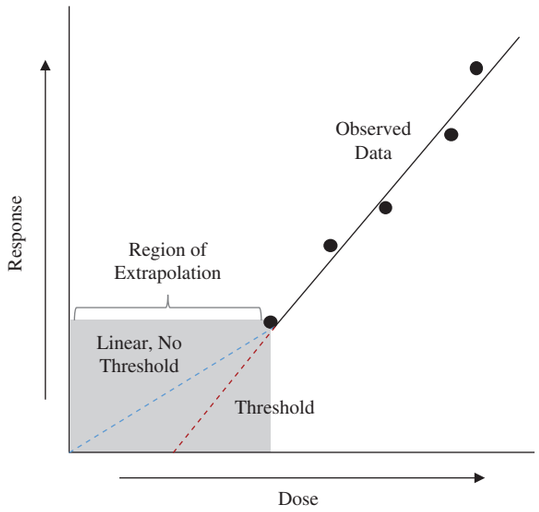

Figure 2-9. *Extrapolation of dose responses at low doses.* Experimental data are shown as individual dots. The gray box represents the region where empiric data are lacking and the shape of the curve needs to be extrapolated. Two approaches, threshold and linear, no threshold are shown. The threshold approach continues the linear regression of the experimental data, maintaining the same slope. The linear, no threshold approach extrapolates the curve using the lowest data point and regresses back to the zero response intercept. This extrapolation is typically performed for genotoxic carcinogens.

biological basis of thresholds for acute and many chronic responses is well-established and frequently can be demonstrated on the basis of mechanistic information, although whether thresholds exist for some types of chronic responses, such as cancer and hormonally mediated effects, remains controversial.

The concept of thresholds has been applied to noncarcinogenic chemicals by employing a qualitative risk characterization approach known as *threshold of toxicological concern* (TTC). TTC suggests that there are levels of exposure for chemicals below which the risk to human health is not appreciable. It was first proposed in the context of food safety (Kroes et al., 2000, 2004) but has been extended to cosmetic ingredients and impurities found in pharmaceutical preparations (Muller et al., 2006; Kroes et al., 2007). TTC integrates available data including exposure, chemical structure, disposition, biotransformation, and toxicity and can be used to prioritize chemicals for further testing.

As noted above, the existence of thresholds for chronic responses is less well-defined, especially in the area of chemical carcinogenesis. A classic example of the difficulty of establishing thresholds experimentally is provided by the "ED_{01}" study, where over 24,000 mice and 81 different treatment groups were used to determine the shape of the dose–response relationship for the prototypical carcinogen 2-acetylaminofluorene (2-AAF) (Fig. 2-10). The study was designed to identify a statistically significant response of 1% (0.01 probability). The mice were exposed to 2-AAF at one of seven different doses in the dose range of 30 to 150 ppm (plus 0 dose control) (Littlefield et al., 1980). Multiple time points for tissue collections were used to determine how quickly tumors developed. Both liver and bladder tumors demonstrated increasing incidence with increasing dose, but the shapes of the two curves were dramatically different. For liver tumors, no clear threshold was evident based on the doses utilized, whereas for bladder tumors an apparent threshold was evident. However, the apparent threshold, or no observable adverse effect level (NOAEL), for bladder cancer was lower at 33 months (45 ppm) than at 24 months (75 ppm) (Fig. 2-10). Of course, the ability to detect a low incidence of tumors depends on the number of animals used in the study. Thus, although a threshold (a dose below which no response occurs) appears evident for bladder tumors, one cannot say for certain that tumors would not occur if more animals had been included in the lower dose groups.

At low doses of carcinogens, the extrapolated shape of the curve for risk assessment varies depending upon the mechanism of

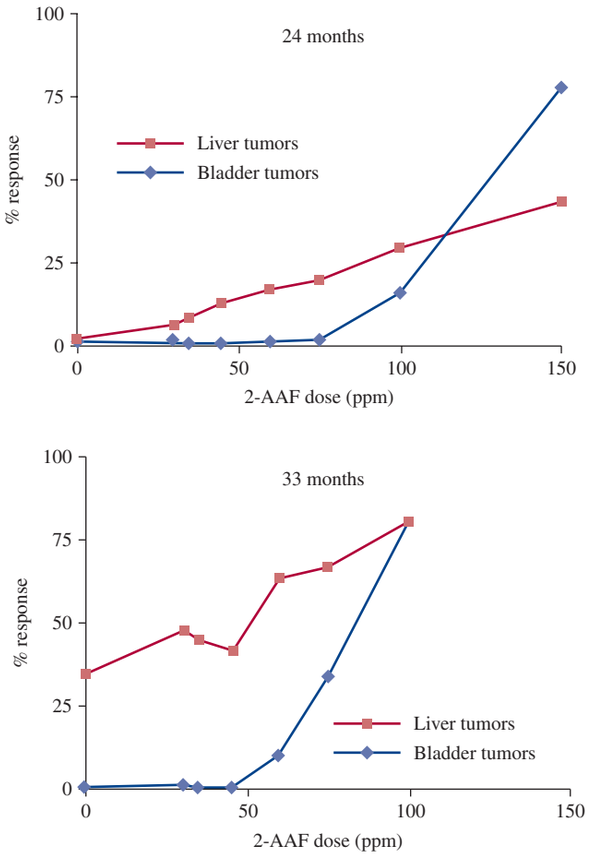

Figure 2-10. *Dose–response relationship for carcinogens—rodents and 2-acetylaminofluorene (2-AAF).*

toxicity. *Non-genotoxic carcinogens*, those that do not interact with DNA (e.g., hypolipidemic drug clofibrate), are thought to exhibit thresholds of dose response, whereas *genotoxic carcinogens* that directly modify and damage DNA (e.g., 2-AAF) have been assumed to increase the risk of cancer even at the lowest possible dose. For ionizing radiation, the upper confidence limit of the lowest dose that causes cancer has been used historically for linear regression to the zero-intercept, an approach called *linear, nonthreshold* risk modeling. Using epidemiologic data from the atomic bomb survivors in the Hiroshima and Nagasaki tumor registries, the 2006 Biological Effects of Ionizing Radiation VII Report recommended the continued use of the linear, nonthreshold dose–response curve for low linear-energy-transfer ionizing radiation (e.g., x-rays and gamma rays) and contended that "it is unlikely that there is a threshold below which cancers are not induced, but at low doses the number of radiation-induced cancers will be small" (NRC, 2006). As a result, the *linear, nonthreshold* dose–response model for ionizing radiation and cancer risk has been adapted by a number of global regulatory agencies and often applied to other genotoxic carcinogens. However, there are remarkable concerns regarding the basis for these recommendations and the continued regulatory use of the linear, nonthreshold model (Calabrese, 2017). This prescribed approach has been criticized for not considering adaptive mechanisms such as DNA repair and clearance of cells with damaged DNA (Tubiana, 2005; Neumaier et al., 2012; Siegel et al., 2017). Recent mechanistic research investigating the relationship between DNA adducts and tumor incidence question the fundamental data used in the linear, nonthreshold risk model, particularly at very low doses of carcinogen exposure.

Emerging data with rainbow trout embryos exposed to a carcinogen has allowed an even more statistically stringent analysis of the shape of the dose–response curve at very low doses. Applying this model to the genotoxic carcinogen dibenzo[*def,p*]chrysene (DBC, also referred to as dibenzo[*a,l*]pyrene), estimates of the shape of the dose–response curve down to a response level of 1 additional tumor in 5000 animals could be obtained, because very large numbers of animals could be exposed. Over 40,000 trout were exposed to different doses ranging over a factor of 500 (DBC, lowest dose 0.45 ppm, highest dose 225 ppm), with over 8000 animals in the control and low-dose groups (Bailey et al., 2009). Because DBC is a potent mutagen, it was assumed that both the rate of DNA adduct formation and the tumor incidence would be linear throughout the dose range. However, there was a clear deviation from linearity at the lower doses, such that the extrapolated dose–response curve crossed the *y*-axis at one cancer in a million exposed animals at a dose 500- to 1500-fold (depending on the statistical model) higher than would have been predicted from the linear extrapolation below the 10% response range (ED_{10}). Remarkably, although the tumor response exhibited a clear "threshold," the formation of DBC–DNA adducts was quite linear through the lowest dose used. While these findings offer novel insight into very low dose–response relationships of early and latent effects, additional debate over the value of the threshold and the linear, nonthreshold approaches for carcinogenesis risk assessment is expected to continue.

Another study that has raised questions about the veracity of the nonthreshold response to chemical carcinogens examined the ability of an "anticarcinogen" called CDDO-Im (closely related to antioxidant chemicals found in broccoli) to prevent DNA damage and subsequent liver cancers in rats treated with the potent mutagen and liver carcinogen, aflatoxin B1 (AFB1) (Johnson et al., 2014). Although the dose of AFB1 given to a group of laboratory rats in this study caused a high level of AFB–DNA adducts to form, and nearly all of the animals (96%) developed liver cancer, pretreatment of a second group of animals given the same dose of AFB1 with CDDO-Im completely prevented the liver tumors, but reduced the DNA damage by only 70%. Thus, while DNA damage from a genotoxic chemical may be "linear at low doses," because of the complex, multistep process of chemical carcinogenesis, coupled with inducible "adaptive response" processes such as DNA repair, actual development of cancers may not follow the linear, low-dose (nonthreshold) response, even for potent mutagenic carcinogens.

Nonmonotonic Dose–Response Curves

Essential Nutrients For natural or endogenous chemicals that are required for normal physiological function and survival (e.g., vitamins and essential trace elements such as chromium, cobalt, zinc, manganese, and selenium), the "graded" dose–response relationship in an individual over the entire dose range can be U-shaped (Fig. 2-11). As essential elements for life, nutrient and vitamin concentrations need to be maintained within a specific range that allows for homeostasis. Doses or concentrations outside of this range can lead to *deficiency* (observed at very low doses when concentrations are below daily minimum requirements) or *toxicity* (observed at high, excessive doses). Often, the biological adverse responses that occur during periods of deficiency or toxicity will differ.

One example that illustrates the importance of proper maintenance of nutrient concentrations and homeostasis is manganese. Manganese is found in all mammalian tissues and is needed for bone formation and the metabolism of macromolecules including

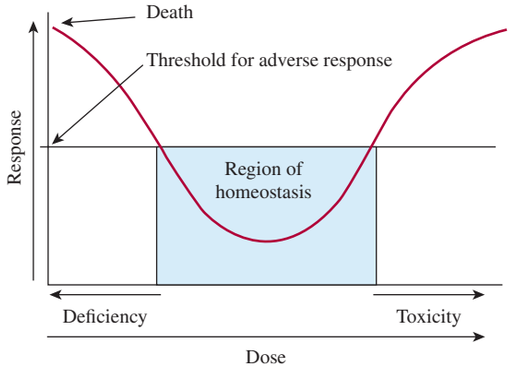

Figure 2-11. *Individual dose–response relationship for an essential substance such as a vitamin or trace element.* It is generally recognized that, for most types of toxic responses, a threshold exists such that at doses below the threshold, no toxicity is evident. For essential substances, doses below the minimum daily requirement, as well as those above the threshold for safety, may be associated with toxic effects. The blue-shaded region represents the "region of homeostasis"—the dose range that results in neither deficiency nor toxicity.

amino acids, lipids, and carbohydrates. Manganese often works as a cofactor for the functioning of enzymes involved in important metabolic processes. The intake of manganese typically occurs through the diet. However, during periods of manganese deficiency, a number of adverse health outcomes have been reported in animals including growth delays, seizures, and impaired fertility. In contrast, excessive exposure to manganese can lead to manganism or neurotoxicity due to the accumulation of manganese in the brain. Similar examples of U-shaped dose–response relationships are observed for other essential elements. High doses of vitamin A can cause liver toxicity and birth defects, high doses of selenium can affect the brain, and high doses of estrogens may increase the risk of breast cancer, even though low amounts of all these substances are essential for life.

Hormesis Evidence suggests that some non-nutritional toxic substances may also impart beneficial or stimulatory effects at low doses but that, at higher doses, produce adverse effects. This concept of "hormesis" has been described for radiation effects (Mitchel, 2006) but may also pertain to other chemicals (Calabrese and Baldwin, 1998; Calabrese and Blain, 2005). For example, chronic alcohol consumption is well recognized to increase the risk of esophageal cancer, liver cancer, and cirrhosis of the liver at relatively high doses, and this response is dose-related. However, there is also substantial clinical and epidemiological evidence that low to moderate consumption of alcohol reduces the incidence of coronary heart disease and stroke (Hanna et al., 1997).

The role of hormesis in chemical carcinogenesis has been evaluated in animals. Altered hepatic foci are preneoplastic lesions that represent precursors to liver tumors in rodents and can be identified by detection of specific markers such as glutathione *S*-transferase. In a rat carcinogenesis bioassay, the contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) had divergent effects on the promotion of altered hepatic foci; at low doses, TCDD reduced the number of foci in the liver, whereas at higher doses TCDD significantly increased the number of foci (and presumably, the risk of hepatocarcinogenesis) (Pitot et al., 1987). Likewise, the exposure of rats to the insecticide 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) similarly decreased the baseline number of hepatic foci at lower doses but enhanced their formation at higher doses (Sukata et al., 2002). In both of these examples, the dose–response curves exhibited a J-shape (Fig. 2-8E). The magnitude of low-dose hormetic effects are often modest (approximately 30% to 60% change in the endpoint) and may not reach statistical significance, particularly when compared to changes observed at high doses. Because of these phenomena, there is great interest in understanding biological responses at low doses of toxicant exposure and the applicability of these findings to human health.

Endocrine Active Chemicals Exogenous chemicals that exert their primary toxic effects via modification of hormonal responses (endocrine disruptors) appear to have effects at relatively low doses that are not seen at higher doses, raising debate about the traditional concept of "dose–response" for endocrine active chemicals. Chemicals that have been shown to interfere with endocrine pathways in vivo and/or in vitro have heterogeneous sources and include industrial byproducts, plasticizers, plastics, pesticides, fungicides, and pharmaceuticals, as well as naturally occurring phytoestrogens. In vitro exposure of normal and cancerous prostate cells to low doses of the plasticizer bisphenol A (0.01 to 100 nM) resulted in biphasic centrosome amplification between two and eightfold with maximal inverted U-shape responses observed at a bisphenol A concentration of 0.1 nM (Tarapore et al., 2014). Nonmonotonic dose–response curves, where the slope of the dose–response curve changes sign (positive or negative) at least one time in the tested range of endocrine active chemicals, have been reported for other chemicals as well (Vandenberg et al., 2012). Diverse mechanisms have been proposed to explain the shape of the nonmonotonic dose–response curves for endocrine active chemicals including dose-dependent cytotoxicity, cell- and tissue-specific receptors and cofactors, receptor selectivity and down-regulation, receptor competition, and endocrine negative feedback loops (Vandenberg et al., 2012).

The EPA has focused its testing efforts on chemicals that interact with and modify the signaling of estrogen, androgen, and thyroid pathways. Specifically, the ability of endocrine active chemicals to modify metabolic, reproductive, and neurological development following early life exposure has garnered the greatest attention for potential human health risk. Studies of endocrine active chemicals require rigorous experimental design and important considerations ensuring a lack of chemical contamination in control groups, inclusion of positive controls, and the use of responsive species for the endpoint(s) in question (Vandenberg et al., 2012). Critiques of the existence of nonmonotonic dose–response curves for endocrine active chemicals have included a lack of consideration of a defined scope, data reproducibility, consistency (including omission of negative studies), and statistical analysis using a weight-of-evidence approach from existing studies (Rhomberg and Goodman, 2012; Lamb et al., 2014). The increasing debate recently led scientists to apply systematic review methodologies to evaluate the low-dose toxicity of endocrine active chemicals (National Academies of Sciences, Engineering, and Medicine, 2017). The recommended definition of *low dose* in the systematic review was defined as the "external or internal exposure that falls within the range estimated to occur in humans" as determined by environmental surveillance and biomonitoring, reliable biomarkers of exposure, and/or computational models (National Academies of Sciences, Engineering, and Medicine, 2017). This would typically mean that the effects occurred at exposures in animals or humans below the NOAEL. The effect of phthalates on male reproductive tract development was used as a case study in the systematic review. From the analysis of available data, the committee concluded that there was a moderate level of evidence that fetal exposure to low doses of di(2-ethylhexyl) phthalate is associated with reduced anogenital distance

in humans. Evaluation of study quality and bias, assessment of the confidence in the body of evidence, and interpretation of data using contemporary statistical approaches will likely be refined as additional classes of endocrine active chemicals are evaluated.

Assumptions in Deriving the Dose–Response Relationship

A number of assumptions need to be considered before traditional dose–response relationships can be used appropriately. The first is that the response is due to the chemical administered. To describe the relationship between a toxic material and an observed effect or response, one must know with reasonable certainty that the relationship is indeed a causal one. For some data, it is not always apparent that the response is a result of chemical exposure. For example, an epidemiological study might result in the discovery of an "association" between a response (e.g., disease) and one or more variables. Frequently, the data are presented similarly to the presentation of "dose response" in pharmacology and toxicology. Use of the dose response in this context is suspect unless other convincing evidence supports a causal connection between the estimated dose and the measured endpoint (response). A major challenge in environmental epidemiology studies where associations between exposure to a toxic substance and the incidence of a disease are made is the presence of "confounders," extraneous variables that influence both exposure and outcome, either positively or negatively. When confounders are not carefully controlled, statistically significant, but spurious, associations may be found. Or, conversely, "real" associations might be missed. Unfortunately, in nearly all retrospective and case–control studies and even in many prospective studies, the dose, duration, frequency, and routes of exposure are seldom quantified, and other potential etiologic factors are frequently present. In its most strict usage, then, the dose–response relationship is based on the knowledge that the effect is a result of a known toxic agent or agents.

A second assumption seems simple and obvious: the magnitude of the response is in fact related to the dose. This assumption relies upon identifying a molecular target with which the chemical interacts to initiate the response and determining that the production of a response and the degree of response are related to the concentration of the chemical at the target site. In turn, the concentration at the site would in fact be related to the dose administered.

The third assumption of the dose–response relationship is that there exists both an appropriate method of quantifying and a precise means of expressing the toxicity. The ideal endpoints are those closely associated with the molecular events resulting from exposure to the toxicant. For a given chemical, there may be multiple dose–response relationships, one for each toxicity endpoint. A hypothetical chemical that produces cancer through genotoxic effects, liver damage through inhibition of a specific enzyme, and CNS effects by reducing neurotransmitter release would likely have three distinct dose–response relationships. Early in the assessment of a chemical's toxicity, little mechanistic information is available; thus, establishing a dose–response relationship based on the molecular mechanism of action is often impossible. In the absence of a mechanistic endpoint of toxicity, one looks to a measure of toxicity that is unequivocal and relevant to the toxic effect. Such measures are often referred to as "effects-related biomarkers." The selection of a toxic endpoint for quantification is not always straightforward. Although many endpoints are quantitative and precise, they are often indirect measures of toxicity. Changes in enzyme levels in blood can be indicative of tissue damage. For example, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are used to detect liver injury. Quantification of serum ALT and AST as effects-related biomarkers is informative because their enzyme activity in the blood is directly related to the extent of damage to liver cells.

Evaluating the Dose–Response Relationship

Therapeutic Index Fig. 2-12 illustrates a hypothetical quantal dose–response curve for a desirable effect of a chemical (effective dose, ED) such as anesthesia, a toxic effect (toxic dose, TD) such as liver injury, and the lethal dose (LD). Each of these responses spans a range of doses and increases at varying rates (or estimated slopes). These curves highlight the importance of the (1) selection of relevant pharmacological/toxicological endpoints and (2) comparisons made between endpoints. The concept of the "therapeutic index" (TI) can be used to illustrate this relationship. Although the TI is directed toward a comparison of the therapeutic ED to the TD of a chemical, it is similarly applicable to considerations of comparative toxicities between chemicals. In its broadest sense, the TI is defined as the ratio of the dose required to cause a toxic effect to the dose needed to produce the desired therapeutic response. Similarly, an index of comparative toxicity is obtained by the ratio of doses of two distinct materials to produce an identical response or the ratio of doses of the same material necessary to yield different toxic effects.

The most commonly used index is the median dose that elicits an effective or toxic response in 50% of a population (ED_{50} or TD_{50}). The TI of a drug is an approximate statement about the relative safety of a drug expressed as the ratio of the adverse endpoint or TD (historically, the LD) to the effective therapeutic dose:

$$TI = \frac{TD_{50}}{ED_{50}}.$$

From Fig. 2-12 one can approximate a TI by using median doses. The larger the ratio, the greater relative safety is assumed. The ED_{50} is approximately 20, and the TD_{50} is about 60; thus, the TI is 3, a number indicating that reasonable care in exposure to the drug is necessary to avoid toxicity. However, the use of the median effective and median toxic doses is not without disadvantages, because median doses do not reflect the slopes of the dose–response curves for therapeutic and toxic effects.

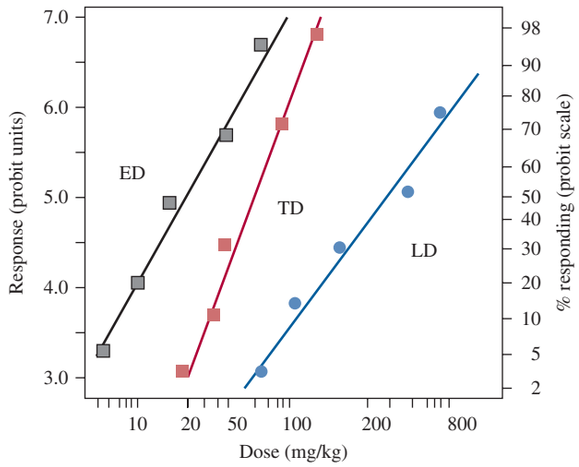

Figure 2-12. *Comparison of effective dose (ED), toxic dose (TD), and lethal dose (LD).* The plot is of log dose versus percentage of population responding in probit units.

Margins of Safety and Exposure One way to overcome the limitation of comparing variable slopes between the effective and toxic dose–response curves is to calculate the ED_{99} for the desired effect (efficacy in 99% of the population) and the TD_1 for the undesired effect (toxicity in 1% of the population), typically after a single administration of a chemical. These conservative parameters are used in the calculation of the *margin of safety* (MOS):

$$\text{MOS} = \frac{\text{TD}_1}{\text{ED}_{99}}.$$

For chemicals with no beneficial or effective dose and/or repeated exposures, the ratio of TD_1 to ED_{99} has little value. Thus, for nondrug chemicals, the term *MOS* is used in risk assessment procedures as an indicator of the magnitude of the difference between an estimated "exposed dose" to a human population and the *no observable adverse effect level* (NOAEL) or other benchmark dose determined in experimental animals.

A measure of the degree of accumulation of a chemical and/or its toxic effects can also be estimated from quantal toxicity data. The *chronicity index* of a chemical is a unitless value obtained by dividing its one-dose TD_{50} by its 90-dose (90-day) TD_{50}, with both expressed in milligrams per kilogram per day. Theoretically, if no cumulative effect occurs over the doses, the chronicity index will be 1. If a compound caused cumulative effects, the chronicity index would be 90.

Frequently, dose–response curves from repeated-dose experimental animal studies (subacute, subchronic, or chronic) are used to estimate the NOAEL, or some other "benchmark" measure of minimal toxic response, such as the dose estimated to produce toxic effects in 10% of the population (TD_{10}) (see also Chap. 4). These estimates of minimal TD, derived from quantal dose–response curves, can be used in risk assessment to derive a *margin of exposure* (MOE) index. This index compares the estimated daily exposure, in milligrams per kilogram per day, that might occur under a given set of circumstances with some estimated value from the quantal dose–response relationship (e.g., NOAEL or TD_{10}). Like the MOS, the MOE is often expressed as a ratio of these two values. Thus, for example, if an estimate of human exposure to a pesticide residue yielded a value of 0.001 mg/kg/day, and a TD_{10} of 1 mg/kg/day was determined for that same pesticide, the MOE would be 1000. This value indicates that the estimate of daily exposure under the described set of conditions is 1/1000 the estimated daily dose that would cause evident toxicity in 10% of exposed animals. (See Chap. 4 for a more complete discussion of benchmark doses, points of departure, NOAELs, and MOE.)

Potency versus Efficacy Additional terminology is often used to describe the dose–response relationship for a particular chemical. *Efficacy* is an assessment of the extent to which a chemical can elicit a response and is often interpreted using the ordinate axis (or *y*-axis). By comparison, the *potency* is determined by the range of doses (on the *x*-axis) over which a chemical produces increasing responses. Fig. 2-13 depicts the dose–response curves for four different chemicals. Chemical A is said to be more potent than chemical B because of their relative positions along the dose axis. Likewise, chemical C is more potent than chemical D. Maximal *efficacy* reflects the magnitude achieved on the response axis (with approximately 80% responding). Chemicals A and B have equal maximal efficacy, whereas the maximal efficacy of C (approximately 30% responding) is less than that of chemical D (approximately 85% responding).

ASSESSING TOXICOLOGICAL RESPONSES

Causation in Toxicology

In recent years, the increasing accessibility of scientific data through electronic means, including social media, has provided consumers with greater information about the safety of chemicals in their diet, environment, household, and consumer products. News headlines may alert the public to potential adverse health effects following exposure to a particular chemical. In each story, some level of uncertainty will exist that questions whether the observed negative outcomes are directly caused by the chemical. Because of this uncertainty, some basic strategies may be used as one attempts to differentiate observed associations between a chemical and an outcome due to causal or noncausal relationships. In 1965, Sir Austin Bradford Hill set forth a series of considerations intended to infer the likelihood that a particular outcome or disease resulted from a specific "exposure" (Hill, 1965). At the time, these considerations were largely aimed at evaluating relationships using epidemiological and occupational data; however, a similar framework can be applied to evaluate experimental and observation data regarding the toxicity of chemicals. They are summarized as follows:

1. *Strength of association.* Hill suggested that strong associations between independent and dependent variables could support a causal relationship. For example, a 10 times increase in death rate due to a specific chemical exposure in a defined population could serve as strong evidence in favor of a potential relationship. However, Hill was careful to not readily dismiss

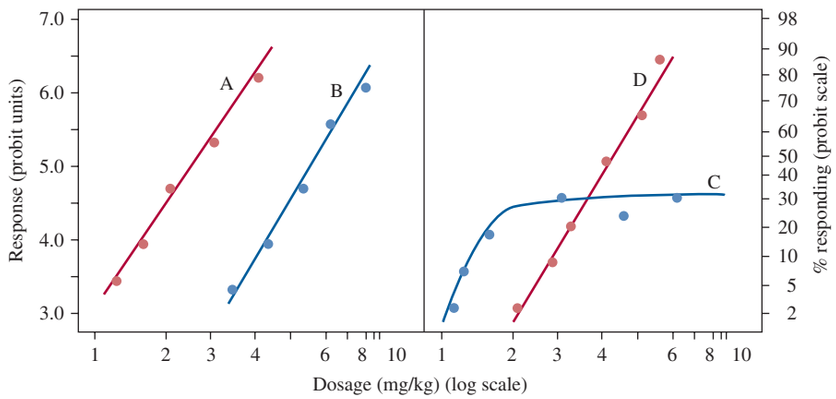

Figure 2-13. *Schematic representation of the difference in the dose–response curves for four chemicals (**A to D**), illustrating the difference between potency and efficacy (see text).*

observations between modest changes in an endpoint and a precipitating event.
2. *Consistency of findings*. Hill recommended the replication of results by different studies, and particularly advocated using different experimental approaches that could reach similar conclusions. With each replication of the findings, the likelihood that a particular error was responsible for the association would lessen.
3. *Specificity of association.* The likelihood of a cause-and-effect relationship is strengthened by how tightly linked an outcome is to exposure to a particular chemical. One limitation of this viewpoint, as Hill noted, is that diseases are often multifactorial in their pathogenesis which may preclude one's ability to establish specificity.
4. *Temporal sequence*. The ability to assign a cause-and-effect relationship requires that the "cause" occur before the effect.
5. *Biological gradient.* Establishment of a clear dose–response relationship, such that greater exposure to a chemical is linked to a more severe outcome, provides further support for causation.
6. *Biological or theoretical plausibility*. The ability to postulate a reasonable mechanism of action can be used to strengthen the likelihood of causation. This does not, however, address the potential that the current state of knowledge is limited.
7. *Coherence with established knowledge*. The association between the cause and effect should reasonably align with the current understanding of disease pathogenesis.

In addition to these seven viewpoints, Hill also suggested that the ability of intervention to limit exposure to the chemical and in turn reduce the likelihood or extent of disease would further support a "cause-and-effect" relationship. Likewise, the ability to draw analogies between related chemicals, due to physicochemical properties or molecular structures (or core features), can lessen the depth of experimental validation needed to assign causation when comparing to a previously characterized toxicant. This approach, often termed *read-across*, has been increasingly implemented since 2009 when the European Union (EU) banned the marketing of cosmetic products tested on animals (Cronin et al., 2013). The read-across computational approach allows scientists to utilize existing and extrapolated data to assess the likelihood that structurally related chemicals share a propensity for toxicity.

Hill cautioned that his viewpoints should serve as a starting point to evaluate the potential for cause-and-effect relationships and did not advocate their use as "hard-and-fast rules of evidence" (Hill, 1965). As Hill noted, "All scientific work is incomplete—whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have or postpone the action that it appears to demand at a given time." Instead, this framework can help establish the "weight of evidence" supporting the likelihood that a chemical exposure causes a particular health outcome. The advancement of molecular toxicology, in the context of the Bradford Hill considerations, has improved the identification of mechanisms of toxicity and proposed surrogate endpoints (such as sensitive biomarkers) that support biological plausibility and delineate temporal relationships between cause and effect (Fedak et al., 2015). Building upon the viewpoints of Hill and others, Guzelian et al. (2005) put forth a systematic, evidence-based approach for defining causation in toxicology. Similar to principles put forth in medicine, evidence-based toxicology is composed of three methodological steps. First, the toxicologist should collect and evaluate the relevant data regarding the chemical and outcome including source, exposure, dose, and diagnosis. Second, the relevant knowledge should be collected in order to frame the question as well as collate and evaluate the relevant literature. Third, the data and knowledge should be integrated in order to formulate a conclusion. This includes evaluation of general causation, dose–response relationships, timing, alternative causes or confounders, rigorous statistical analyses of the results of individual studies and meta-analyses (combined studies), and coherence of the findings.

Experimentation and hazard identification are inherent to identifying cause-and-effect relationships. However, the risk or probability that toxicity will occur depends upon specific exposure scenarios. As a result, dose–response relationships and evaluation of human exposure paradigms are needed to characterize the risk and perform an assessment of the toxicological risk. The marketing of pharmaceuticals requires the systematic testing of numerous endpoints in order to ensure efficacy and safety before new drugs can be approved by the FDA. A series of appropriate animal and human studies need to be completed in order for a pharmaceutical company to apply for a new drug application. By comparison, complete data sets are often not available or required before introducing a new chemical, particularly for industrial chemicals, into commerce. The National Toxicology Program reports that "more than 80,000 chemicals are registered for use in the United States" and estimates an additional 2000 new chemicals are introduced each year (NTP, 2017a). Because few of these industrial chemicals undergo rigorous safety testing, the NTP coordinates safety testing within the U.S. federal government. In the absence of empirical data regarding the safety of new and existing chemicals, the Precautionary Principle was defined at the Wingspread Conference in 1998: "When an activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not fully established scientifically" (Raffensperger and Tickner, 1999; Myers and Raffensberger, 2006). While there have been various regulatory interpretations of the precautionary principle (COEC, 2000; UK-ILGRA, 2007), the intended purpose of the precautionary principle is the protection of the public's health and the environment. As such, the precautionary principle advocates regulatory restrictions that limit human exposure before sound empirical data regarding the safety of a chemical are in place. Following this approach, the burden of proof is placed upon the manufacturer to establish the lowest level of risk before commercial usage. The role of toxicological science as well as ethical considerations in regulatory decision making continues to be a source of considerable debate (Marchant, 2003; Goldstein and Carruth, 2004; Goldstein, 2006). Challenges to the precautionary principle suggest there is a lack of clarity in its application and question how much certainty is needed to guarantee there is little to no risk of harm. Moreover, it has been proposed that precaution is a "built in element" of the traditional risk-assessment paradigm through the use of safety factors and conservative scenarios and represents a more scientific approach to protect human health (Smith et al., 2008). Because of the large role that ethics play in the protection of public health and advocacy of populations (Simon, 2011), continued debate over the decision-making process is anticipated.

Mechanisms and Modes of Action

Fundamental to the field of toxicology is the identification of chemical hazards, determination of how chemicals elicit these adverse events, and assessment of the risk these toxicities bear on humans and/or the environment. *Mechanisms of action* refer to the detailed molecular and biochemical changes that are required for a molecule to cause damage. This is a precise determination of "how"

and the "extent" to which molecular events cause toxicity. Some possible mechanisms of action for carcinogenesis include the ability of the chemical to act as an electrophile, be genotoxic, alter DNA repair, cause epigenetic changes, modulate receptor signaling, and enhance proliferation (Smith et al., 2016). When approaching mechanisms of toxicant action, a number of steps including the delivery of the chemical to the site of action, interaction with key signaling targets, cellular dysfunction, and disrepair will collectively determine the extent and reversibility of toxicity (Fig. 2-14). Various factors such as pharmacokinetics, cell signaling, and maintenance, as well as molecular, cellular, and tissue responses will modulate each of these steps.

At times when these fundamental mechanisms are unknown, toxicologists instead describe the *mode of action* for a toxicant. The mode of action describes the functional or anatomical changes stimulated by a toxicant at a cellular level. The term "mode of action" is more often used in regulatory toxicology and has been described as a "biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data" (Meek et al., 2003). Often, weight of evidence considerations such as those described above by Bradford Hill are used to determine whether there is sufficient verification of how a substance influences key physiologic processes in a way that can be incorporated into risk assessment (Dellarco and Baetcke, 2005). Of particular interest in the determination of a mode of action is the evaluation of biological plausibility and coherence with existing data, as well as temporal and dose–response relationships in evaluating the strength and specificity of the association. This approach has been applied to nuclear transcription factor–mediated liver carcinogenesis in rodents with a focus on three signaling pathways: the aryl hydrocarbon receptor, the constitutive androstane receptor, and the peroxisome proliferator–activated receptor (Andersen et al., 2014; Budinsky et al., 2014; Corton et al., 2014; Elcombe et al., 2014).

In investigating mode of action, a number of considerations are made regarding (1) key events, (2) associative events, and (3) modulating factors (Dellarco and Baetcke, 2005; Andersen et al., 2014). *Key events* are causal precursors that are necessary for the adverse outcome. An example key event might be the formation of a specific DNA adduct. *Associative events* are processes that are not required for the mode of action but can serve as indicators or surrogate markers for the key events. An example of an associative event could be cellular proliferation in a nontarget tissue. Also important are *modulating factors* that can alter the dose–response relationship or likelihood of inducing a key event(s) leading to an adverse outcome. The generation of reactive oxygen species, or oxidative stress, is considered one type of modulating factor. Others include altered cellular communication, activation of certain transcriptional pathways, immune responsiveness, life stage, genetic variation, and others (Simon et al., 2014).

Adverse Outcome Pathways

In recent years, there has been growing interest in developing additional framework beyond the mode-of-action assessment that takes a broader view of common pathways of toxicity and may be applicable for more than just a particular chemical or class of chemicals. Thus, toxicological evaluation has adopted the use of adverse outcome pathways (AOPs) to advance the concept of "toxicity pathways" recommended in the 2007 National Research Council's report on *Toxicity Testing for the 21st Century: A Vision and a Strategy*. AOPs provide a conceptual framework and practical tool to portray and associate sequential biological events at the

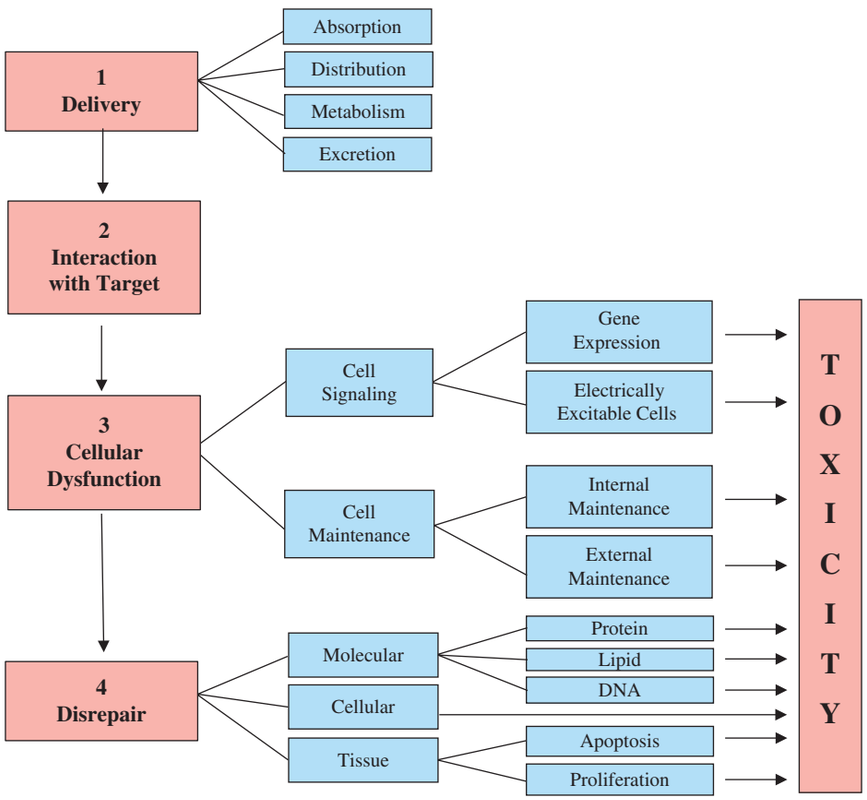

Figure 2-14. *Steps that result in chemical toxicity.*

molecular, cellular, and organ levels following exposure to a chemical that ultimately results in toxicity to an organism (Fig. 2-15) (Ankley et al., 2010; Villeneuve and Garcia-Reyero, 2011). AOPs first emerged in the field of ecotoxicology. AOP modeling utilizes data from computational (quantitative structure activity relationships), in vitro, and in vivo studies and integrates both mode and mechanism of action to describe toxicological processes.

AOPs are composed of the following:

- Molecular Initiating Events
- Key Events and Their Relationships
- Adverse Outcomes

In each AOP, a chemical's structural and physical properties are first evaluated using in silico approaches that may or may not include metabolism of the chemical to other forms. Then, the chemical is assessed for critical molecular initiating events, which include receptor/ligand interactions, macromolecule interactions, protein modifications and alkylation, nuclear receptor activation, and changes in membrane fluidity. With advancing biological complexity, a logical sequence of cellular, organ, and organism responses will be identified—each representing linked key events in the AOP that are required for progression of the toxicity. Responses that include the molecular initiating events and the cellular response are typically classified as the *toxicity pathway.* While the relationships between key events are most often linear, there is the potential for multiple steps to converge or diverge into various key events. The organism responses consist of target organ toxicities, malformations, cancer development, and/or lethality revealed from in vivo testing. Collectively, the molecular initiating events and key events comprise the *mode of action pathway.* While AOPs are similar to modes of action for a chemical, the AOP is anchored to both the molecular initiating event as well as the *adverse outcome,* a specialized key event of regulatory significance, which strengthens the certainty of the overall model. Lastly, the AOP pathway will advance to understanding how toxicant responses will impact an entire population.

Typically, studies performed for AOP pathway development will assess the ability of chemicals to alter normal cellular pathways in appropriate human cells or tissues, particularly during the screening stage. Critical to the refinement and strength of an AOP model is the utilization of complementary approaches central to systems toxicology testing (described later in this chapter). Recently, fundamental principles in developing AOPs have been described (Villeneuve et al., 2014a, 2014b). Most importantly, AOPs are not considered specific to a particular chemical. Likewise, AOPs are modular with key events and key event relationships potentially being used in multiple pathways. Researchers can build AOPs using the Adverse Outcome Pathway Knowledge Base (AOP-KB) from the Organization of Economic Cooperation and Development (OECD), an international organization that develops standardized testing guidelines. Within an AOP, the models can designate whether there are mechanistic data supporting the relationship between key events or acknowledge when a plausible or predictive linkage exists based on limited available or computational data. To strengthen the quality of AOPs and standardize their development, the OECD has released updated guidelines that detail weight-of-evidence evaluation based on the aforementioned Bradford Hill considerations (Becker et al., 2015a, 2017; OECD, 2017). The development of AOPs is an iterative process with continual refinement as new mechanistic data are obtained. The potential utility for AOPs includes prioritization of chemicals for further testing, identification of hazards, classification, and potentially risk assessment, especially for data-rich chemicals such as polychlorinated biphenyls, where the key initiating events in both experimental animals and humans are well-established, and pronounced species differences are known.

VARIATION IN TOXIC RESPONSES

Selective Toxicity

Selective toxicity means that a chemical produces injury to one kind of living matter (such as a cell or organism) without harming another form of life even though the two may exist in intimate contact. They may be related to each other as parasite and host or may be two tissues in one organism. However, by taking advantage of the biological diversity between species, it is possible to develop chemicals that are lethal for an undesired species and harmless for other species. In agriculture, for example, there are fungi, insects, and even competitive plants that injure the crop, and thus selective pesticides are needed. Similarly, animal husbandry and human medicine require pharmaceuticals, such as antibiotics, that are selectively toxic to the infectious microbe but do not produce damage to the host.

Selective toxicity results because the chemical (1) is equally toxic to both organisms but accumulates preferentially in the target or (2) alters a unique cellular or a biochemical feature that is absent or irrelevant in the unaffected species. One source of variation that can lead to selectivity are differences in the absorption, biotransformation, or excretion of the toxicant. The selective toxicity of an insecticide spray may be partly due to a larger surface area per unit weight that causes the insect to absorb a proportionally greater

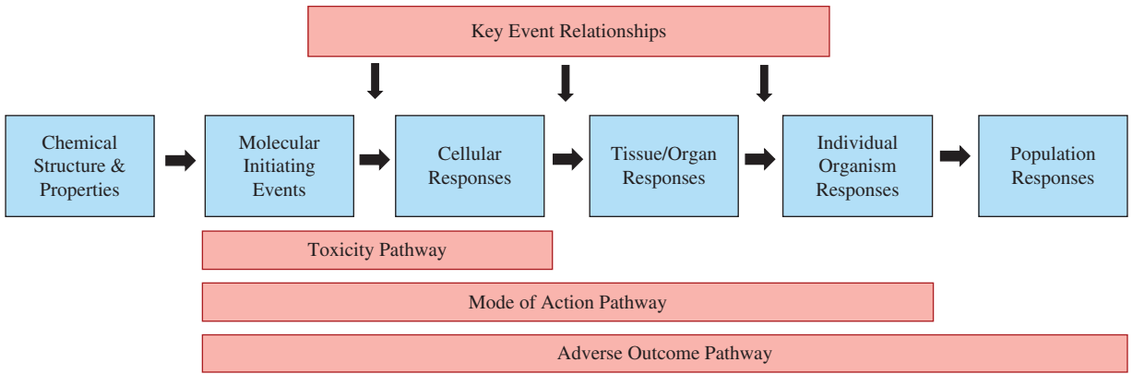

Figure 2-15. *Key parts of adverse outcome pathways (AOPs).*

dose than does the mammal being sprayed. The effectiveness of radioactive iodine in the treatment of hyperthyroidism (as well as its thyroid carcinogenicity) is due to the selective ability of the thyroid gland to accumulate iodine. Another major reason why chemicals are toxic to one, but not to another, type of tissue is that there are differences in metabolism or transport that dictate accumulation of the ultimate toxic compound in various tissues.

Selective toxicity caused by differences in cellular structure is exemplified by comparing plant and animal cells. Plants differ from animals in many ways—for example, absence of a nervous system, an efficient circulatory system, and muscles as well as the presence of a photosynthetic mechanism and cell walls. The fact that bacteria contain cell walls and humans do not has been utilized in developing selective antibiotics, such as penicillin and cephalosporins, that kill bacteria but are relatively nontoxic to mammalian cells. Selective toxicity can also be a result of a difference in biochemistry in the two types of cells. For example, bacteria do not absorb folic acid but synthesize it from *p*-aminobenzoic acid, glutamic acid, and pteridine, whereas mammals cannot synthesize folic acid but have to absorb it from the diet. Thus, sulfonamide drugs are selectively toxic to bacteria because the sulfonamides, which resemble *p*-aminobenzoic acid in both charge and dimension, antagonize the incorporation of *p*-aminobenzoic acid into the folic acid molecule—a reaction that humans do not carry out.

Species Differences

Experimental animals are routinely used in toxicology as surrogates for humans; however, it is important to recognize the quantitative and qualitative differences in response to toxic substances among various species. Even among phylogenetically similar species (e.g., rats, mice, guinea pigs, and hamsters), large differences in response can be observed. For example, the LD_{50} for TCDD differs by more than 1000-fold between guinea pigs and hamsters. Not only the lethal dose for TCDD but also the particular target organs affected vary widely among species.

Species differences in response to carcinogens represent an important issue in regulatory risk assessment. As discussed in Chap. 8, extrapolation of laboratory animal data to infer human cancer risk is a routine component of regulatory decision making. Large interspecies differences in chemical carcinogenicity are not unusual. For example, mice are highly resistant to the hepatocarcinogenic effects of the fungal toxin aflatoxin B_1. Dietary doses as high as 10,000 ppb failed to produce liver cancer in mice, whereas in rats dietary doses as low as 15 ppb produced a significant increase in liver tumors (Wogan et al., 1974). The mechanistic basis for this dramatic difference in response appears to be entirely related to species differences in the expression of a particular form of glutathione *S*-transferase (mGSTA3-3) that has unusually high catalytic activity toward the carcinogenic epoxide of aflatoxin (Eaton and Gallagher, 1994). Mice express this enzyme constitutively, whereas rats normally express a closely related form with much less detoxifying activity toward aflatoxin epoxide. Interestingly, rats do possess the gene for a form of glutathione *S*-transferase with high catalytic activity toward aflatoxin epoxide (rGSTA5-5) that is inducible by certain dietary antioxidants and drugs. Thus, dietary treatment can dramatically change the sensitivity of a species to a carcinogen. Other examples in which large species differences in response to carcinogens have been observed include the development of renal tumors from 2,3,5-trimethylpentane and D-limonene in male rats (Lehman-McKeeman and Caudill, 1992), the production of liver tumors from "peroxisomal proliferators" such as the antilipidemic drug clofibrate and the common solvent trichloroethylene (Roberts, 1999), and the induction of nasal carcinomas in rats after inhalation exposure to formaldehyde (Monticello and Morgan, 1997). Identifying the mechanistic basis for species differences in response to chemicals is an important part of toxicology because only through a thorough understanding of these dissimilarities can the relevance of animal data to human response be verified.

Table 2-5

Examples of Toxic Equivalency Factors for Dioxin-Like Chemicals from the World Health Organization

	CONSENSUS TOXICITY FACTORS		
CHEMICAL	RAT	HUMAN	WHO-TEF
2378-TCDD	1	1	1
12378-PeCDD	0.5	1	1
123478-HxCDD	0.2	0.03	0.1
123678-HxCDD	0.06	0.06	0.1
123789-HxCDD	0.3	0.002	0.1
1234678-HpCDD	0.04	0.2	0.01

Adapted from Larsson et al. (2015).

Where the *mode of action* (MOA) of a toxic chemical is well understood, species differences in response can be estimated with some degree of confidence by comparing the relative potency of a chemical to activate the MOA (e.g., a specific receptor) in tissues from various species. For example, the toxic chemicals referred to as "dioxins," including certain dioxin-like polychlorinated biphenyls (DL-PCBs), produce all or nearly all of their toxic effects by activating an intracellular nuclear transcription factor called the aryl hydrocarbon receptor (AhR). Activation of the AhR is considered to be a "key initiating event" in an *adverse outcome pathway* (AOP) for dioxins and dioxin-like chemicals (Becker et al., 2015b). There are large species differences in susceptibility to the toxic effects of dioxins and especially DL-PCBs. Thus, measuring the ability of the different DL-PCBs to activate the human versus rat AhR has led to the development of "Toxic Equivalency Factors" that provide for a more scientifically accurate way to adjust for differences in species susceptibility to these common environmental pollutants (Table 2-5) (Larsson et al., 2015).

Modifying Factors

Not all humans respond to toxicants in the same manner and to the same degree as each other. Multiple factors modify one's susceptibility to adverse outcomes. Particularly important modifiers include genetic variation among a population, age and life stages, sex and hormonal status, microbiome, and circadian rhythm. Specific examples that illustrate the important role(s) for these factors are provided below and throughout this book. Other influences that can impact the extent of toxicity include the concomitant use of tobacco, alcohol, nutraceutical, pharmaceutical and illicit drugs, exercise, nutrition, and co-exposures in the workplace and at home.

Genetics Hereditary differences in a single gene that occur in more than 1% of the population are referred to as *genetic polymorphisms*. Increasingly, genetic variants are being identified

that impact a variety of target organ toxicities. For example, it is recognized that approximately 50% of the Caucasian population has a gene deletion for the enzyme glutathione *S*-transferase M1. This enzyme has no apparent significant physiological function, and thus homozygotes for the gene deletion (e.g., those who lack both copies of the normal gene) are functionally and physiologically normal. However, epidemiological studies have indicated that cigarette smokers who express two null alleles may be at slightly increased risk of developing lung cancer compared with smokers who have one or both copies of the normal gene (Mohr et al., 2003).

Some individuals are abnormally sensitive to nitrites and certain other chemicals that oxidize the iron in hemoglobin to produce *methemoglobin,* a form that is incapable of carrying oxygen to the tissues. This abnormal phenotype is inherited as an autosomal recessive trait and is characterized by a deficiency in NADH-cytochrome b_5 reductase activity. The genetic basis for this response has been identified as a single nucleotide change in codon 127, which results in replacement of serine with proline (Kobayashi et al., 1990). The consequence of this genetic deficiency is that these individuals may suffer from a serious lack of oxygen delivery to tissues after exposure to doses of methemoglobin-producing chemicals (aniline dyes, the antibiotic trimethoprim, and the anesthetic drug benzocaine) that would be harmless to individuals with normal NADH-cytochrome b_5 reductase activity.

Clinically, pharmacogenetic variants can have a critical influence on a patient's susceptibility to a drug toxicity. These variants may be in the coding or regulatory regions and influence pharmacokinetic/pharmacodynamics responses. The Pharmacogenetics Knowledge Database (PharmGKB) is an online resource that curates the literature on drug-related gene variants for use by scientists and researchers. Because some variants can significantly alter the efficacy and/or toxicity of drugs, the Clinical Pharmacogenetics Implementation Consortium (CPIC) routinely publishes peer-reviewed, evidence-based clinical guidelines that inform clinicians of particular variant alleles that pharmacogenetic testing should be ordered while initiating therapy. Within the CPIC guidelines, important pharmacokinetic- and toxicity-related genes include the (1) phase I metabolizing enzymes cytochrome P450 2C9 (antiseizure drug phenytoin, anticoagulant warfarin), 2C19 (antidepressant citalopram, antiplatelet drug clopidogrel), and 2D6 (analgesic codeine), (2) phase II metabolizing enzymes UDP glucuronysyl transferase 1A1 (UGT 1A1) (antiviral drug atazanavir) and thiopurine *S*-methyltransferase (antineoplastic drug mercaptopurine), and (3) transporters such as the solute carrier SLCO1B1 (hypolipidemic drug simvastatin) (see Chap. 6).

The development of new approaches to identifying associations between diseases or adverse outcomes and common genetic variants (polymorphisms) has changed from a focus on individual candidate genes to "genome-wide association studies" (GWAS) and next-generation sequencing of DNA. GWAS are based on a scan of hundreds of thousands of specific genetic variants (markers called "tag SNP") across the genome of persons affected by a particular disorder or adverse-response phenotype and persons who are not affected, with robust statistical tests to identify associations between a specific genetic marker and the phenotype (e.g., disease state or adverse drug response). Next-generation sequencing allows millions or billions of DNA strands to be sequenced in parallel, often revealing unique and rare variants. These tools require critical bioinformatic and statistical infrastructure for interpretation and have resulted in the discovery of many "gene–environment interactions," including associations between adverse drug responses and particular genetic polymorphisms. Moving from the single, "candidate gene" approach to genome-wide studies has led to the development of the relatively new fields of pharmacogenomics and toxicogenomics. These areas provide an exciting opportunity for mechanistic toxicologists to identify and protect genetically susceptible individuals from harmful environmental exposures, and to customize drug therapies that enhance efficacy and minimize toxicity, based on an individual's genetic makeup.

Age Life stage, and in turn age, is an important factor that can alter susceptibility to toxicity. Metabolic processes that aid in xenobiotic clearance are often altered at juvenile and advancing ages. For example, newborns have relatively low gastric emptying, gastrointestinal motility, and expression of the metabolic enzymes including CYP2D6, CYP2E1, and CYP3A4. Reduced metabolic capacity can decrease the clearance of some chemicals and increase the risk of toxicity. For example, juvenile rodents exhibit a heightened susceptibility to the insecticides parathion and chlorpyrifos as a result of delayed expression of carboxylesterase enzymes that detoxify organophosphate metabolites (Atterberry et al., 1997; Karanth and Pope, 2000). Conversely, the inability of juveniles to bioactivate some chemicals such as acetaminophen to their toxic intermediates appears to protect against liver injury.

Likewise, as humans age to greater than 70 years old, there are a number of pharmacokinetic changes that can impact xenobiotic disposition and toxicity. Reductions in liver, gastrointestinal, and kidney functions as well as lower receptor activity (e.g., beta-adrenergic receptor) along with more prevalent underlying pathologies can heighten the risk of adverse events. Therefore, a number of pharmaceuticals need to be used judiciously in geriatric patients including anticholinergic drugs (e.g., diphenhydramine), anti-inflammatory drugs (e.g., nonsteroidal anti-inflammatory drugs), opioids (e.g., oxycodone), and beta-adrenergic blockers (e.g., metoprolol). In order to prevent medication toxicities, the Beers Criteria have been established, which are recommendations of drugs that should not be used in older patients (American Geriatrics Society Beers Criteria Update Expert, 2015).

Sex Along with genetics and age, sex can be a determinant of xenobiotic disposition and toxicity. One of the notable sex-related differences in humans is the effect of alcohol. Alcohol is absorbed and metabolized differently in females and males. This is in part due to the lower extent of body water in women compared to men of a similar weight. Toxicities such as liver disease and brain damage due to alcohol consumption appear to be more frequent and/or earlier in females compared to males.

A significant amount of toxicology research has compared sexually dimorphic differences between male and female rodents. Male rats metabolize cocaine, diazepam, indinavir, and tolbutamide at a faster rate than female rats (Mugford and Kedderis, 1998). A variety of mechanisms to explain these differences have been shown including growth hormone regulation, sex steroids, and imprinting. The secretion of growth hormone is remarkably different between male and female rodents; females release steady amounts of growth hormone throughout the day, whereas males experience large fluctuations at regular intervals. In the rat liver, *Cyp2c11* (male-specific) and *Cyp2c12* (female-specific) are two sexually dimorphic genes transcriptionally regulated in response to pulsatile or continuous growth hormone secretion (Waxman et al., 1991; Sundseth et al., 1992). Critical transcription factors including the signal transducer and activator of transcription 5b (STAT5b) regulate liver gene expression in a sexually dimorphic manner. In addition to growth hormone–related signaling, sex hormones including estradiol and testosterone influence the expression and

function of drug metabolizing enzymes and transporters, which can alter xenobiotic disposition and toxicity.

Circadian Rhythm Circadian rhythm is a 24-hour cycle that regulates a number of molecular and physiological processes. Within the 24-hour cycle, there are diurnal (light cycle), nocturnal (dark cycle), and crepuscular (transition) periods. The circadian clock consists of a cellular clock with specific genes that oscillate in expression. Timing in the circadian system is affected by a number of factors including light, activity, food consumption, and social cues. While most changes in physiological processes during the 24-hour period are not readily apparent, they can still impact susceptibility to toxicity.

Studies have revealed that the duration of sleep within rats treated with phenobarbital can vary almost twofold depending upon the time frame of administration (Scheving et al., 1993). Similarly, the number of rat lethalities due to amphetamines and cytosine arabinoside is heightened when administered during the dark cycle. The extent of renal injury due to the cancer drug cisplatin is affected by the timing of dosing in mice. Greater elevations in blood urea nitrogen in cisplatin-treated mice were observed when expression of the kidney-specific transporter that mediates its proximal tubular uptake was maximal (Oda et al., 2014).

More recently, a large prospective trial in humans sought to determine whether diurnal variation in the timing of poisonings influenced the likelihood of survival (Carroll et al., 2012). After controlling for a variety of factors including age and sex, a significant association was observed between higher risk of mortality in patients with toxic oleander seed exposures and evening ingestions (5226 patients, $p < 0.001$). A trend for circadian-related mortalities due to organophosphate pesticide poisonings was also observed.

Several epidemiological studies have suggested that disruption of circadian rhythm, such as inconsistent night shift work, which occurs frequently in nurses and airline attendants, may be linked to an increase in risk of breast cancer in women (Liu et al., 2016). This epidemiological association has been supported by laboratory studies demonstrating that hormones such as melatonin are influenced by clock genes, and that perturbations in light cycle change expression of these genes, resulting in changes in cell cycle regulation (Blakeman et al., 2016). However, a recent meta-analysis of three large prospective cohorts of women working night shifts failed to find any association with breast cancer risk (Travis et al., 2016). Thus, while the importance of clock genes and circadian rhythm in modulating biological processes relevant to toxicology are evident, uncertainty remains as to the impact of disruption of circadian rhythm on breast cancer risk in women.

Microbiome Within the body, bacteria outnumber human cells by a ratio of 10:1. Typically, anaerobic and facultative aerobic bacteria comprise the resident microflora of the intestinal tract. The influence of commensal microbes on human health, including toxicologic responses, is garnering greater attention with the advent of highly sensitive methods in metagenomics. The ability to sequence the 16S rRNA subunit allows for comprehensive profiling of bacterial species. For example, exposure of mice to the AhR activator and environmental contaminant, 2,3,7,8-tetrachlorodibenzofuran, can shift the microbiota from enrichment of *Firmicutes* to *Bacteroidetes* species (Zhang et al., 2015). As a consequence of this change, bile acid pathways are disrupted leading to changes in host metabolic homeostasis.

Within the field of toxicology, there are a number of examples illustrating how bacterial-mediated metabolism, including beta-glucuronidase, azo reductase, and cardiac glycoside reductase enzyme activities, can alter the toxicity of xenobiotics (Klaassen and Cui, 2015). The beta-glucuronidase activity of microflora is an important determinant of the gastrointestinal toxicity of a number of drugs. For example, the cancer drug irinotecan and nonsteroidal anti-inflammatory drug diclofenac are excreted into the intestines as glucuronide conjugates. Cleavage of the glucuronic acid moiety by intestinal bacteria generates products that are toxic to enterocytes. In the case of irinotecan, use of a synthetic bacterial beta-glucuronidase enzyme inhibitor in mice blocked the severe, and often life-threatening, diarrhea associated with this cancer drug (Wallace et al., 2010). Similarly, pharmacological inhibition of the beta-glucuronidase enzyme specific to bacteria reduced the mucosal injury associated with diclofenac in mice (LoGuidice et al., 2012; Saitta et al., 2014). Microflora can also alter teratogenicity. The benzodiazepine drug nitrazepam is teratogenic in rats causing cleft palate, limb reductions, and other malformations. Treatment of pregnant rats with antibiotics lowered the bacterial conversation of nitrazepam to 7-aminonitrazepam and prevented perinatal malformations (Takeno and Sakai, 1991). Collectively, these examples show how microbiota can alter xenobiotic metabolism and in turn influence susceptibility to toxicity.

TOXICITY TESTING

Two principal concepts underlie descriptive animal toxicity testing. The first is that the effects produced by a compound in laboratory animals, when properly qualified, are applicable to humans. This premise applies to all of experimental biology and medicine. Most, if not all, known chemical carcinogens in humans are carcinogenic in some species, but not necessarily in all species of laboratory animals. It has become increasingly evident that the converse—that all chemicals identified as carcinogenic in laboratory animals are also carcinogenic in humans—is not true. However, for regulatory and risk assessment purposes, positive carcinogenicity tests in animals are usually interpreted as indicative of potential human carcinogenicity. If a clear understanding of the mechanism of action of the carcinogen indicates that a positive response in animals is not relevant to humans, a positive animal bioassay may be considered irrelevant for human risk assessment (see Chap. 4). This variation in carcinogenic potential appears to be due in many instances to differences in biotransformation of the procarcinogen to the ultimate carcinogen, or differences in target receptor regulation between species.

The second concept is that the exposure of experimental animals to high doses of chemicals is a necessary and valid method of discovering possible hazards in humans. This principle is based on the quantal dose–response concept that the incidence of an effect in a population is greater as the dose or exposure increases. Practical considerations in the design of experimental model systems require that the number of animals used in toxicology experiments always be small compared with the size of human populations at risk. Obtaining statistically valid results from such small groups of animals requires the use of relatively large doses so that the effect will occur frequently enough to be detected. However, the use of high doses can create problems in interpretation if the response(s) obtained at high doses does not occur at low doses. For example, it has been shown that bladder tumors observed in rats fed very high doses of saccharin will not occur at the much lower doses of saccharin encountered in the human diet. At the high concentrations fed to rats, saccharin forms an insoluble precipitate in the bladder that subsequently results in chronic irritation of bladder epithelium, enhanced cell proliferation, and ultimately bladder tumors (Cohen, 1998, 1999). In vitro studies have shown that precipitation

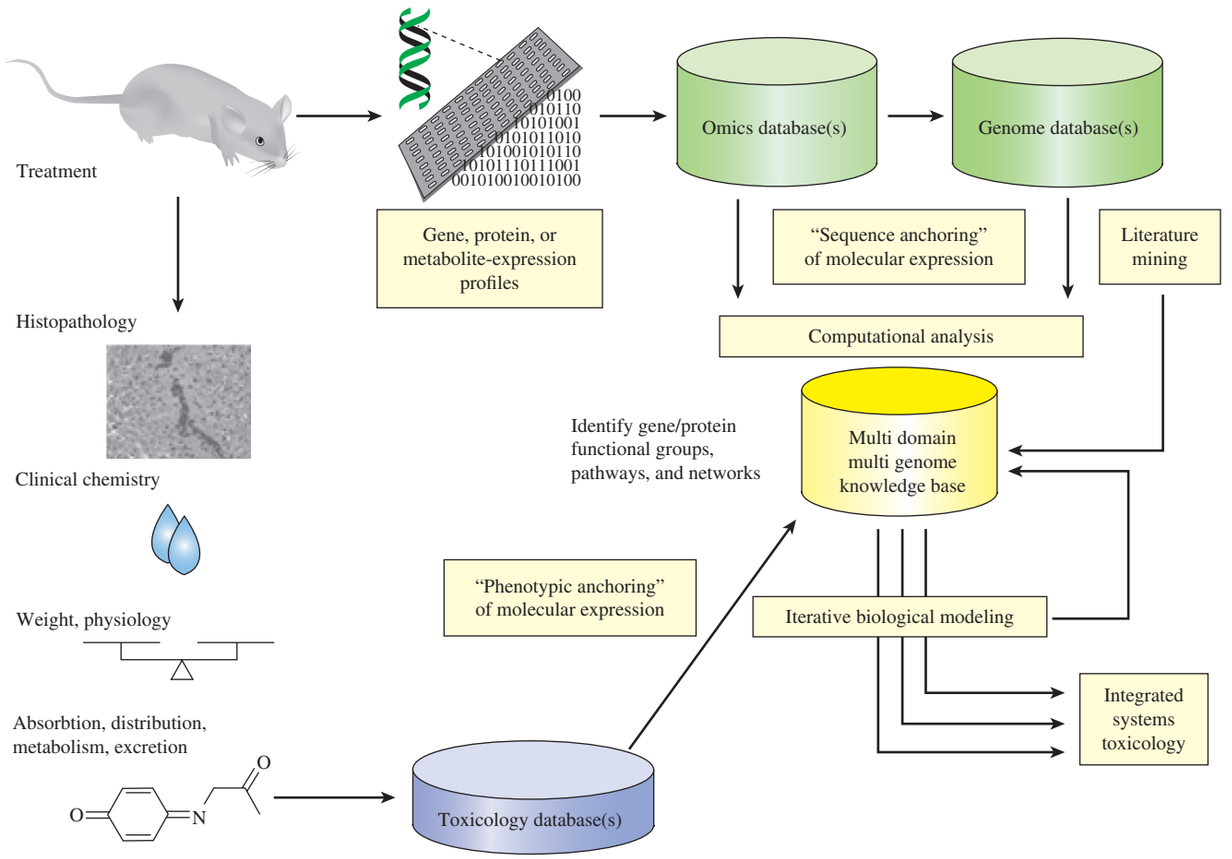

Figure 2-16. *Conceptual approach for incorporating "omics" technologies and resulting large databases into toxicological evaluation.* Data from experiments that evaluate the effects of a chemical on global patterns of gene expression (transcriptomics), protein content (proteomics), and small molecules/metabolites (metabonomics/metabolomics), combined with genomic information from both the test species (e.g., rats and mice) and the target species of interest (e.g., humans), are analyzed by computational tools (bioinformatics) for unique or potentially predictive patterns of toxicity. Essential to the use of omics data for predictive toxicology/safety assessment is the ability to reliably tie observed omics patterns to traditional measures of toxicity, such as histopathology and clinical chemistry (phenotypic anchoring). (From Waters and Fostel, 2004, with permission.)

of saccharin in human urine will not occur at the concentrations that could be obtained from even extraordinary consumption of this artificial sweetener. Even for mutagenic chemicals that form DNA adducts, the response at high doses may not be linear at low doses, as discussed earlier for dibenzo[*def,p*]chrysene. However, for another DNA-reactive carcinogen, aflatoxin B_1, the low-dose data approximated the high-dose response in a linear fashion (Williams, 2012). Examples such as these illustrate the importance of considering the molecular, biochemical, and cellular mechanisms responsible for toxicological responses when extrapolating from high to low dose and across species.

Toxicity tests are not designed to demonstrate that a chemical is safe but rather they serve to identify and characterize the toxic effects a chemical can produce. Although there are no set toxicology tests that have to be performed on every chemical intended for commerce, a tiered approach typical of many hazard assessment programs is illustrated in Fig. 2-16. Depending on the eventual use of the chemical, the toxic effects produced by structural analogs of the chemical, as well as the toxic effects produced by the chemical itself, contribute to the determination of the toxicology tests that should be performed. The FDA, EPA, and OECD have developed good laboratory practice (GLP) standards and other guidances that stipulate how procedures must be performed and documented. These guidelines are expected to be followed when toxicity tests are conducted in support of introducing a chemical to the market.

Although different countries often have different testing requirements for toxicity testing/product safety evaluation, efforts to "harmonize" such testing protocols have resulted in more standardized approaches. The International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use includes regulatory authorities from Europe, Japan, and the United States (primarily the FDA), as well as experts from the pharmaceutical industry in the three regions, who worked together to develop internationally recognized scientific and technical approaches to pharmaceutical product registration. ICH has adopted guidelines for most areas of toxicity testing (Table 2-6). In addition to safety assessment (ICH guidelines designated with an "S"), ICH has also established guidelines on quality (Q), efficacy (E), and multidisciplinary (M) topics. (See http://www.ich.org/products/guidelines.html for a description of current ICH guidelines).

Typically, a tiered approach is used, with testing dependent upon the results of initial studies. A general framework for how new chemicals are evaluated for toxicity is shown in Fig. 2-16. Early studies require careful chemical evaluation of the compound or mixture to assess purity, stability, solubility, and other

Table 2-6

International Conference on Harmonization (ICH) Codification of "Safety" Protocols

Carcinogenicity studies	
S1A	Need for Carcinogenicity Studies of Pharmaceuticals
S1B	Testing for Carcinogenicity of Pharmaceuticals
S1C(R2)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals
Genotoxicity studies	
S2 (R1)	Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use
Toxicokinetics and pharmacokinetics	
S3A	Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies
S3B	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies
Toxicity testing	
S4	Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing)
Reproductive toxicology	
S5(R2)	Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility
Biotechnological products	
S6(R1)	Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals
Pharmacology studies	
S7A	Safety Pharmacology Studies for Human Pharmaceuticals
S7B	The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals
Immunotoxicology studies	
S8	Immunotoxicity Studies for Human Pharmaceuticals
Anticancer pharmaceuticals	
S9	Nonclinical Evaluation for Anticancer Pharmaceuticals
Photosafety studies	
S10	Photosafety Evaluation of Pharmaceuticals
Joint safety/efficacy (multidisciplinary) topic	
M3(R2)	Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals
M7(R1)	Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk

From http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html. Accessed August 8, 2017.

physicochemical factors that could impact the ability of the test compound to be delivered effectively to animals. Once this information is obtained, the chemical structure of the test compound is compared with similar chemicals for which toxicological information is already available. Structure–activity relationships (SAR) may be derived from a review of existing toxicological literature, and can provide additional guidance on the design of acute and repeated-dose experiments, and what specialized tests need to be completed. Once such basic information has been compiled and evaluated, the test compound is then administered to animals in acute and repeated-dose studies.

Because of increased societal pressure to reduce or eliminate the use of animals in toxicity testing, while also ensuring that new chemicals do not pose unreasonable risks to human health or the environment, regulatory agencies have been encouraging new approaches in descriptive toxicity testing that do not rely on laboratory animals. For example, the EU promulgated an important regulatory initiative for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). The implementation of REACH "will have significant impact on applied toxicology and exposure assessment by stimulating innovation in sampling and analysis, toxicology testing, exposure modeling, alternative toxicity testing, and risk assessment practices" (Williams et al., 2009). Alternative, in vitro approaches to toxicity assessment are beginning to transform the way that product safety evaluation is performed.

The recognition that many of the existing chemicals in commercial use today, as well as new chemicals being introduced into commerce, have little toxicological information has prompted calls for new "high-throughput" approaches to toxicity testing. This would allow at least a basic hazard characterization for the thousands of untested chemicals currently in the marketplace, as well as the many new chemicals that are introduced each year. A report from the National Academy of Sciences/National Research Council in 2007 called for a "paradigm shift" in how toxicity testing is done (NAS/NRC, 2007). A key component of this new vision on toxicity testing is the use of an extensive battery of in vitro tests

to evaluate AOPs for chemicals. The prospect is that new technologies in genomics, transcriptomics, proteomics, metabolomics, and bioinformatics can be combined with automated high-throughput technologies to create a tiered structure for toxicity testing. The approach to using biochemical and molecular pathway-based analyses, rather than apical endpoints (e.g., target organ damage, mutagenesis, carcinogenesis, reproductive and developmental effects), to identify potentially problematic chemicals early in their development is particularly attractive from a time frame and economic perspective. However, it is also recognized that validation of screening tests is critically important. In 2000, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established as a component of the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). ICCVAM's mission is to develop and validate alternative testing methodologies that replace, reduce, and refine the use of animals. Validation of in vitro screening tests can reduce the number of animals needed for safety testing and where applicable entirely replace some traditional approaches. Nonetheless, the established in vivo studies described in the following section will continue to serve an important role in hazard evaluation, especially for high-priority chemicals. While guidelines vary between organizations (ICH, NTP, EPA, and OECD), there are conceptual and practical similarities that are commonly employed.

Acute Toxicity Testing

Safety testing is initiated with acute studies that identify target organs and provide an estimate of the intrinsic toxicity for a substance. Typically, these studies are performed in rodents (rats and mice) and aim to approximate the LD (e.g., LD_{50}) of a chemical within 14 days after a single exposure. These data are used to inform the design and dose selection for longer-term (subchronic, chronic) studies.

There are a number of variables that are considered (and often controlled) in the design of acute toxicity tests. When performing acute toxicity tests, there will typically be one control group and four to five treatment groups that each contain three to five animals per group. Both sexes in two species will often undergo this testing protocol. Typically, the route of administration will be the same that is anticipated or intended for human exposure, most often oral. A number of approaches are recommended to determine the doses and dosing: (1) fixed doses (5 to 2000 mg/kg OECD Guideline 420), (2) stepwise method (one group is tested at a time and observations determine the next step (OECD Guideline 423), and (3) up-and-down procedure, where an initial starting dose is estimated from in silico and in vitro testing and then successive doses are selected using half-log increases (OECD Guideline 425). Only a *single* dose of the vehicle and test chemical is administered. Inhalation exposures occur once for a 4-hour period. Assessments include twice-daily examination of animal weight, behavioral modifications, morbidity, and food consumption. After 14 days, all animals undergo a complete necropsy including gross observations, organ weights, and histopathological evaluation. The list of tissues evaluated histologically is extensive with over 40 organs collected and assessed.

The LD_{50} is not a biological constant. Many factors influence toxicity and thus may alter the estimation of the LD_{50} in any particular study. Factors such as animal strain, age, and weight, type of feed, caging, pretrial fasting time, method of administration, volume and type of suspension medium, and duration of observation have all been shown to influence adverse responses to toxic substances. Because of this inherent variability in LD_{50} estimates, it is now recognized that for most purposes it is only necessary to characterize the LD_{50} within an order of magnitude range such as 5 to 50 mg/kg, 50 to 500 mg/kg, and so on.

There is a growing interest to develop in silico and in vitro methods to reduce the number of animals needed for acute systemic toxicity tests. For example, the *Registry of Cytotoxicity* (RC), originally published in German in 1998, was developed by linear regression analysis of the cytotoxic concentration values determined in mammalian cells in culture and the LD_{50} values reported in the literature from various laboratory species. Using this approach, the authors predicted (within a reasonable dose range) the acute oral LD_{50} for 252 of 347 xenobiotics, and the intravenous LD_{50} for rats and/or mice for 117 of 150 xenobiotics (Halle, 2003). Of course, such in vitro approaches do not fully account for dispositional effects that could result in large species differences in acute toxicity, but do provide a rapid first approximation of acute toxicity without the use of experimental animals and suggest a starting dose range for in vivo studies.

In recent years many regulatory agencies have begun to accept alternative in vitro tests for dermal and eye irritation tests that do not use live laboratory animals. Indeed, such in vitro approaches to acute toxicity testing are now being more widely accepted for other types of acute toxicity testing. The National Toxicology Program provides an up-to-date listing of such alternative tests that are accepted by U.S. regulatory agencies (NTP, 2017b).

Subacute (Repeated-Dose) Toxicity Testing

Subacute toxicity tests are performed to obtain information on the toxicity of a chemical after repeated administration and as an aid to establish doses for subchronic studies. A typical protocol is to give three to four different doses of the chemicals to the animals by mixing them in their feed. For rats, 10 animals per sex per dose are often used; for dogs, three doses and three to four animals per sex are used. Clinical chemistry and histopathology are performed after either 14 or 28 days of exposure, as described in the section "Subchronic Toxicity Testing."

Subchronic Toxicity Testing

Subchronic exposure can last for different periods of time, but 90 days (13 weeks) is the most common test duration. The principal goals of the subchronic study are to establish a *no observed adverse effect level* (NOAEL) and to further identify and characterize the specific organ or organs affected by the test compound after repeated administration. One may also obtain a *lowest observed adverse effect level* (LOAEL). The numbers obtained for NOAEL and LOAEL will depend on how closely the doses are spaced and the number of animals examined. Determinations of NOAELs and LOAELs have regulatory implications. For example, the EPA utilizes the NOAEL to calculate the *reference dose* (RfD), which may be used to establish regulatory values for "acceptable" pollutant levels (Barnes and Dourson, 1988) (see Chap. 4). An alternative to the NOAEL approach referred to as the *benchmark dose* uses all the experimental data to fit one or more dose–response curves (Crump, 1984). These curves are then used to estimate a benchmark dose that is defined as "the statistical lower bound on a dose corresponding to a specified level of risk" (Allen et al., 1994a). Often, the benchmark response is defined as a 10% increase in the number of test subjects exhibiting a specific pathological lesion. Advantages of determining a benchmark dose rather than a NOAEL include a lesser dependency upon the experimental doses selected, consideration of the shape of the dose–response curve, and an ability to account for variability and uncertainty within

the data (Davis et al., 2011). Although subchronic studies are frequently the primary or sole source of experimental data used to determine both the NOAEL and the benchmark dose, these concepts can be applied to other types of toxicity testing protocols, such as that for chronic toxicity or developmental toxicity (Allen et al., 1994a, 1994b; Faustman et al., 1994) (see also Chap. 4). If chronic studies have been completed, these data are generally used for NOAEL and LOAEL estimates in preference to data from subchronic studies.

A subchronic study is usually conducted in two species (often rat and dog for FDA, and rat or mouse for EPA) by the route of intended exposure (usually oral). At least three doses are employed (a high dose that produces toxicity but does not cause more than 10% fatalities, a low dose that produces no apparent toxic effects, and an intermediate dose) with 10 to 20 rodents and 4 to 6 dogs of each sex per dose. Each animal should be uniquely identified with permanent markings such as ear tags, tattoos, or electronically coded microchip implants. Only healthy animals should be used, and each animal should be housed individually in an adequately controlled environment. Prior to the initiation of a subchronic (and chronic) safety study, a period of 1 to 2 weeks will be used for pretesting where animals in the control and (anticipated) treated groups are compared for various baseline values, such as urinalysis. When the test compound is administered in the diet over a prolonged period of time (subchronic and chronic studies), the concentration in the diet should be adjusted periodically (weekly for the first 12 to 14 weeks) to maintain a constant intake of material based on food consumption and rate of change in body weight (Wilson et al., 2008). Animals should be observed once or twice daily for signs of toxicity, including changes in body weight, diet consumption, changes in fur color or texture, respiratory or cardiovascular distress, motor and behavioral abnormalities, and palpable masses. All premature deaths should be recorded and necropsied as soon as possible. Severely moribund animals should be terminated immediately to preserve tissues and reduce unnecessary suffering. At the end of the 90-day study, all remaining animals are terminated and blood and tissues collected for further analysis. The gross and microscopic conditions of the organs and tissues (about 15 to 20) and the weight of the major organs (about 12) are recorded and evaluated. Hematology and blood chemistry measurements are usually done before, in the middle of, and at the termination of exposure. Hematology measurements usually include hemoglobin concentration, hematocrit, erythrocyte counts, total and differential leukocyte counts, platelet count, clotting time, and prothrombin time. Clinical chemistry determinations commonly made include glucose, calcium, potassium, urea nitrogen, serum ALT/AST, gamma-glutamyl transpeptidase (GGT), lactic dehydrogenase, alkaline phosphatase, creatinine, bilirubin, triglycerides, cholesterol, albumin, globulin, and total protein. Urinalysis is usually performed in the middle of and at the termination of the testing period and often includes determination of specific gravity or osmolarity, pH, proteins, glucose, ketones, bilirubin, and urobilinogen as well as microscopic examination of formed elements. If humans are likely to have significant exposure to the chemical by dermal contact or inhalation, subchronic dermal and/or inhalation experiments may also be required. Subchronic toxicity studies not only characterize the dose–response relationship of a test substance after repeated administration but also provide data for a more reasonable prediction of appropriate doses for chronic exposure studies.

For chemicals that are to be registered as drugs, acute and subchronic studies (and potentially additional special tests if a chemical has unusual toxic effects or therapeutic purposes) must be completed before the company can file an Investigational New Drug (IND) application with the FDA. If the application is approved, clinical trials can commence. At the time clinical trials are performed, chronic exposure of the animals to the test compound can be carried out in laboratory animals along with additional specialized tests.

Chronic Toxicity Testing

Long-term or chronic exposure studies are performed similarly to subchronic studies except that the period of exposure is longer than 3 months. In rodents, chronic exposures are usually for 6 months to 2 years. Chronic studies in nonrodent species are usually for 1 year but may be longer. The length of exposure is somewhat dependent on the intended period of exposure in humans. For example, for pharmaceuticals, the ICH S4 guidance calls for studies of 6 months in duration in rodents, and 9 months in nonrodents. However, if the chemical is a food additive with the potential for lifetime exposure in humans, a chronic study up to 2 years in duration is likely to be required.

Dose selection is critical in these studies to ensure that premature mortality from chronic toxicity does not limit the number of animals that survive to a normal life expectancy. Most regulatory guidelines require that the highest dose administered be the estimated *maximum tolerable dose* (MTD, also commonly referred to as the "minimally toxic dose"). This is generally derived from subchronic studies, but additional longer studies (e.g., 6 months) may be necessary if delayed effects or extensive cumulative toxicity are indicated in the 90-day subchronic study. The MTD has had various definitions. It has been defined by some regulatory agencies as the dose that suppresses body weight gain slightly (i.e., 10%) in a 90-day subchronic study. However, regulatory agencies may also consider the use of parameters other than weight gain, such as physiological and pharmacokinetic considerations and urinary metabolite profiles, as indicators of an appropriate MTD. Generally, one or two additional doses, usually fractions of the MTD (e.g., one-half and one-quarter MTD), and a control group are tested.

Developmental and Reproductive Toxicity

The effects of chemicals on reproduction and development also need to be determined. *Developmental toxicology* is the study of adverse effects on the developing organism occurring anytime during the life span of the organism that may result from exposure to chemical or physical agents before conception (either parent), during prenatal development, or postnatally until the time of puberty. *Teratology* is the study of defects induced during development between conception and birth (see Chap. 10). *Reproductive toxicology* is the study of the occurrence of adverse effects on the male or female reproductive system that may result from exposure to chemical or physical agents (see Chap. 21).

Several types of animal tests are utilized to examine the potential of a chemical to alter development and reproduction. Six critical periods are delineated during these studies often spanning more than one period: premating to conception, conception to implantation, implantation to closure of the hard palate, hard palate closure to parturition, and birth to weaning and weaning to sexual maturity. General fertility and reproductive performance (*segment I*) tests are usually performed in rats with two or three dose levels (20 rats per sex per dose) of the test chemical (neither produces maternal toxicity) (ICH, 2000). Males are given the chemical 60 days and females 14 days before mating. The dams are given the chemical throughout gestation and lactation. Typical observations made include the percentage of females that become pregnant, the number of stillborn and live offspring, and the weight, growth, survival, and general condition of the offspring during the first 3 weeks of life.

The potential of chemicals to disrupt normal embryonic and/or fetal development (teratogenic effects) is also determined in laboratory animals. Current guidelines for these *segment II* studies call for the use of two species, including one nonrodent species (usually rabbits). Teratogens are most effective when administered during the first trimester, the period of organogenesis. Thus, the animals (usually 12 rabbits and 24 rats or mice per group) are usually exposed to one of three doses during organogenesis (days 7 to 17 in rodents and days 7 to 19 in rabbits), and the fetuses are removed by cesarean section a day before the estimated time of delivery (gestational days 29 for rabbit, 20 for rat, and 18 for mouse). The uterus is excised and weighed and then examined for the number of live, dead, and resorbed fetuses. Live fetuses are weighed; half of each litter is examined for skeletal abnormalities and the remaining half for soft tissue anomalies.

The perinatal and postnatal toxicities of chemicals also are often examined (*segment III*). This test is performed by administering the test compound to rats from the 15th day of gestation throughout delivery and lactation and determining its effect on the birth weight, survival, and growth of the offspring during the first 3 weeks of life.

A multigenerational study may be performed, often in place of segment III studies, to determine the effects of chemicals on the reproductive system. At least three dose levels are given to groups of 25 female and 25 male rats shortly after weaning (30 to 40 days of age). These rats are referred to as the F_0 generation. Dosing continues throughout breeding (about 140 days of age), gestation, and lactation. The offspring (F_1 generation) have thus been exposed to the chemical in utero, via lactation, and in the feed thereafter. When the F_1 generation is about 140 days old, about 25 females and 25 males are bred to produce the F_2 generation, and administration of the chemical is continued. The F_2 generation is thus also exposed to the chemical in utero and via lactation. The percentage of F_0 and F_1 females that get pregnant, the number of pregnancies that progress to full term, the litter size, the number of stillborn, and the number of live births are recorded. Viability counts and pup weights are recorded at birth and at 4, 7, 14, and 21 days of age. The fertility index (percentage of mating resulting in pregnancy), gestation index (percentage of pregnancies resulting in live litters), viability index (percentage of animals that survive 4 days or longer), and lactation index (percentage of animals alive at 4 days that survived the 21-day lactation period) are then calculated. Gross necropsy and histopathology are performed on some of the parents (F_0 and F_1), with the greatest attention being paid to the reproductive organs, and gross necropsy is performed on all weanlings. It should be noted that these studies provide little data regarding developmental neurotoxicity and immunotoxicity, which require more specialized behavioral and functional tests.

The aforementioned testing for reproductive and developmental toxicities is most relevant for pharmaceuticals (OECD, 2002). Additional guidelines for testing nontherapeutic chemicals share some similarities and differences with ICH S5(R2). Most often, developmental tests for industrial chemicals are not segmented and can be folded into repeated-dose toxicity studies (EPA, 2000; OECD, 2015c). In addition to standard developmental toxicity testing, there is interest in utilizing alternate models to screen and prioritize chemicals for animal studies (Knudsen et al., 2011).

Mutagenicity

Mutagenesis is the ability of chemicals to cause changes to DNA that are transmitted during cell division. Mutations can occur in either of the two cell types: germ and somatic cells. *Germline mutations* damage DNA in sperm and ova, which undergo meiotic division and therefore have the potential for transmission of mutations to future generations. If mutations are present at the time of fertilization in either the egg or the sperm, the subsequent combination of genetic material may not be viable. Alternatively, the mutation may not affect early embryogenesis but cause the death of the fetus at a later developmental period. Congenital abnormalities may also result from mutations. *Somatic mutations* refer to nonheritable mutations in all other cell types. Somatic mutations may lead to cell death or transmission of the genetic defect through mitotic division. Mutagenic tests are often used to screen for potential carcinogens.

Numerous in vivo and in vitro procedures have been devised to test chemicals for their ability to cause mutations. Some genetic alterations are visible with the light microscope. In this case, cytogenetic analysis of bone marrow smears is used after the animals have been exposed to the test chemical. Because some mutations are incompatible with normal development, the mutagenic potential of a chemical can also be evaluated by the dominant lethal test. This test is usually performed in rodents. The male is exposed to a single dose of the test compound and then is mated with two untreated females weekly for 8 weeks. The females are killed before term, and the number of live embryos and the number of corpora lutea are determined.

The test for mutagens that has received the widest attention is the *Salmonella*/microsome test developed by Bruce Ames (Ames et al., 1975). This test uses several mutant strains of *Salmonella typhimurium* that lack the enzyme phosphoribosyl ATP synthetase, which is required for histidine synthesis. These strains are unable to grow in a histidine-deficient medium unless a reverse or back mutation to the wild-type form has occurred. Other mutations in these bacteria have been introduced to heighten the sensitivity of the strains to mutagenesis. The two most significant additional mutations enhance penetration of substances into the bacteria and decrease the ability of the bacteria to repair DNA damage. Because many chemicals are not mutagenic or carcinogenic unless they are metabolized to a toxic intermediate, rat liver microsomes containing biotransformation enzymes can be added to the medium containing the mutant strain and the test chemical. The number of reverse mutations is then quantified by the number of bacterial colonies that grow in a histidine-deficient medium.

In addition to the Ames test, there are a number of additional mutagenicity assays routinely used in safety testing. These include in vitro mammalian cytogenetic tests for chromosomal damage (such as metaphase chromosome aberrations) and the mouse lymphoma thymidine kinase (Tk) gene mutation assay. In vivo testing for micronuclei in rodent hematopoetic cells typically accompanies these in vitro assays. DNA strand breakage or covalent binding can also be assessed in rodents. With the advent of techniques that readily allow manipulation of the mouse genome, transgenic animals have been developed to enable in vivo assessment of chemical mutagenicity. For example, two commercially available mouse strains, the "MutaMouse" and "Big Blue," contain the *lac* operon of *Escherichia coli* that has been inserted into genomic DNA using a lambda phage to DNA to produce a recoverable shuttle vector. Stable, homozygous strains of these transgenic animals (both mice and rats have been engineered) are exposed to potential mutagenic chemicals. Transgenic rodent mutation tests allow for relevant metabolism and DNA processes to occur. Following 28 days of repeated dosing, genomic DNA is isolated in multiple organs and evaluated for point mutations. Collectively, these data provide a comprehensive view of the mutagenicity of the test compound.

More recently, ICH has called for consideration of the genotoxic potential of impurities found in small-molecule pharmaceutical

products. The M7(R1) guideline integrates structure–activity relationships and Thresholds of Toxicological Concern–based intake of the impurity following a theoretical lifetime of daily exposure. Following analysis, each impurity is classified to one of five classes based on the potential for mutagenicity and carcinogenicity, each with different risk characterization strategies.

Carcinogenicity

Carcinogenicity studies are both time consuming and expensive, and are usually only performed when there is reason to suspect that a chemical may be carcinogenic, or when there may be widespread, long-term exposures to humans (e.g., widely used food additives, drinking water contaminants, or pharmaceuticals that are likely to be administered repeatedly for long periods of time). Chemicals that test positive in several mutagenicity assays are likely to be carcinogenic, and thus are frequent candidates for oncogenicity bioassay assessment. In the United States, the NTP often evaluates nondrug chemicals for carcinogenic potential. For pharmaceuticals, the FDA may require the manufacturer to conduct carcinogenicity studies as part of the preclinical assessment, depending on the intended use of the drug, and the results of mutagenicity assays and other toxicological data.

Studies to evaluate the carcinogenic (oncogenic) potential of chemicals are usually performed in rats and mice and extend over the average lifetime of the species (18 months to 2 years for mice, 2 to 2.5 years for rats). To ensure that 30 rats per dose survive the 2-year study, 60 rats per group per sex are often started in the study. Both gross and microscopic pathological examinations are made not only on animals that survive the chronic exposure but also on those that die prematurely. The use of the maximum tolerated dose (MTD) in carcinogenicity has been the subject of controversy. The premise that high doses are necessary for testing the carcinogenic potential of chemicals is derived from the statistical and experimental design limitations of chronic bioassays. For nonmutagenic pharmaceuticals, ICH S1C provides the following guidance on dose selection for carcinogenicity studies: "The doses selected for rodent bioassays for non-genotoxic pharmaceuticals should provide an exposure to the chemical, which (1) allow an adequate margin of safety over the human therapeutic exposure, (2) are tolerated without significant chronic physiological dysfunction and are compatible with good survival, (3) are guided by a comprehensive set of animal and human data that focus broadly on the properties of the chemical and the suitability of the animal and (4) permit data interpretation in the context of clinical use."

Another approach for establishing maximum doses to use in chronic animal toxicity testing relies upon basic human pharmacokinetic data that are available (e.g., new pharmaceuticals that have completed phase I clinical trials). For chronic animal studies performed on drugs where single-dose human pharmacokinetic data are available, a daily dose that would provide an area under the curve (AUC) in laboratory animals equivalent to 25 times the AUC in humans given the highest (single) daily dose to be used therapeutically may be used, rather than the MTD.

Most regulatory guidelines require that both benign and malignant tumors be reported. Statistical increases above the control incidence of tumors (either all tumors or specific tumor types) in the treatment groups are considered indicative of a chemical's carcinogenic potential. Thus, the conclusion as to whether a given chronic bioassay is positive or negative for carcinogenic potential of the test substance requires careful consideration of background tumor incidence. Properly designed chronic carcinogenicity studies require that a concurrent control group matched for variables such as age, diet, and housing conditions is used. For some tumor types, the "background" incidence of tumors is surprisingly high. Fig. 2-17 shows the background tumor incidence for various tumors in male and female F344 rats used in 27 NTP 2-year rodent carcinogenicity studies. The data shown represent the percent of animals in control (nonexposed) groups that developed the specified tumor type by the end of the 2-year study. These studies involved more than 1300 rats of each sex. Fig. 2-18 illustrates the aggregated results for tumor development in control (nonexposed) mice from thirty 2-year carcinogenicity studies performed by the NTP. The data represent the results observed in over 1400 male and 1400 female B6C3F1 mice.

There are several key points that can be derived from these summary data:

1. Tumors, both benign and malignant, are not uncommon events in animals even in the absence of exposure to any known carcinogen.
2. There are numerous different tumor types that develop "spontaneously" in both sexes of both rats and mice, but at different rates.
3. Background tumors that are common in one species may be uncommon in another (e.g., testicular interstitial cell adenomas are very common in male rats but rare in male mice; liver adenomas/carcinomas are about 10 times more prevalent in male mice than in male rats).
4. Even within the same species and strain, large gender differences in background tumor incidence are sometimes observed (e.g., adrenal gland pheochromocytomas are about seven times more prevalent in male F344 rats than in female F344 rats; lung and liver tumors are twice as prevalent in male B6C3F1 mice as in female B6C3F1 mice).

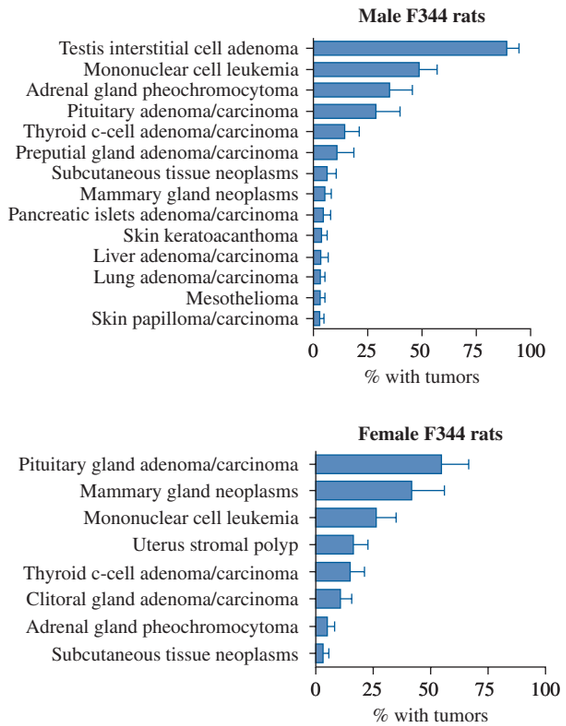

Figure 2-17. *Most frequently occurring tumors in untreated control rats from recent NTP 2-year rodent carcinogenicity studies.* The values shown represent the mean ± standard deviation of the percentage of animals developing the specified tumor type at the end of the 2-year study. The values were obtained from 27 different studies involving a combined total of between 1319 and 1353 animals per tumor type.

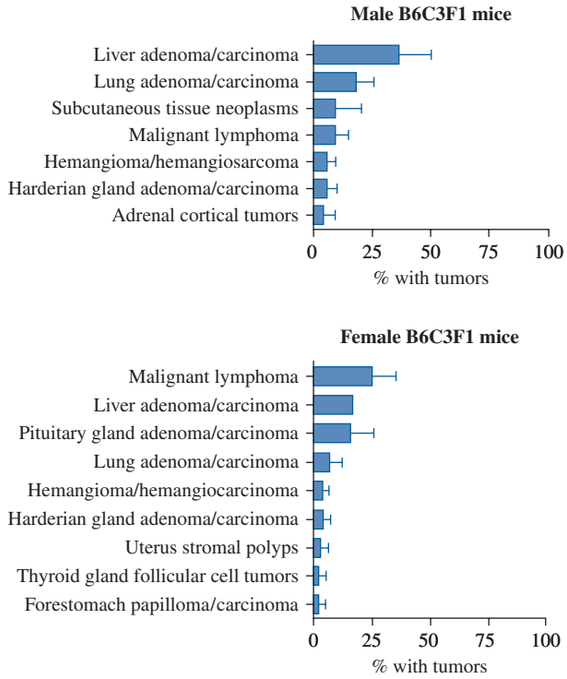

Figure 2-18. *Most frequently occurring tumors in untreated control mice from recent NTP 2-year rodent carcinogenicity studies.* The values shown represent the mean ± standard deviation of the percentage of animals developing the specified tumor type at the end of the 2-year study. The values were obtained from 30 different studies involving a total of between 1447 and 1474 animals per tumor type.

5. Even when the general protocols, diets, environment, strain and source of animals, and other variables are relatively constant, background tumor incidence can vary widely, as shown by the relatively large standard deviations for some tumor types in the NTP bioassay program. For example, the range in liver adenoma/carcinoma incidence in 30 different groups of unexposed (control) male B6C3F1 mice went from a low of 10% to a high of 68%. Pituitary gland adenomas/carcinomas ranged from 12% to 60% and 30% to 76% in unexposed male and female F344 rats, respectively, and from 0% to 36% in unexposed female B6C3F1 mice.

Collectively, these data demonstrate the importance of including concurrent control animals in carcinogenicity studies. Often, comparisons of the concurrent control results to "historic" controls accumulated over many years of study may identify potentially spurious "false-positive" results. The relatively high variability in background tumor incidence among groups of healthy, highly inbred strains of animals maintained in highly controlled environments highlights the dilemma in interpreting the significance of both positive and negative results in regard to the human population, which is genetically diverse, has tremendous variability in diet, nutritional status, and overall health, and lives in an environment full of potentially carcinogenic substances, both natural and human-made.

Neurotoxicity Assessment

Neurotoxicity is defined as the adverse changes in the chemistry, structure, or function of the nervous system following exposure to a chemical or physical agent (examined in Chap. 16). Neurotoxic effects can occur in central or peripheral nervous systems and disrupt sensory, motor, autonomic, or cognitive functions. The developing nervous system is particularly sensitive to chemical exposures (see Chap. 10); therefore, testing on adult and perinatal/juvenile animals is important in assessing the potential risk of neurotoxicology. The *functional observational battery* (FOB) was developed based on early basic screens for behavioral function in mice (Irwin, 1968; Moser, 2000). The FOB used in young adult rats by the EPA evaluates changes in a number of endpoints: autonomic functions (lacrimation, salivation, piloerection, exophthalmos, urination, defecation, and pupil size), abnormal motor movements, reactivity to stimuli, level of activity, gait, forelimb/hindlimb strength, foot splay, and others (OECD, 1997; EPA, 1998c). In addition to the FOB, motor activity and neuropathology are assessed. These studies are performed in at least 10 rats of each sex using at least three dose levels compared to a vehicle control group.

Because of the heightened sensitivity of immature animals to the neurotoxicity of some chemicals, there are a number of methods and procedures for evaluating developmental neurotoxicology (Claudio et al., 2000; Cory-Slechta et al., 2001; Dorman et al., 2001; Garman et al., 2001; Mileson and Ferenc, 2001). Standardized protocols are used to evaluate developmental neurotoxicity in offspring from pregnant and lactating rats exposed perinatally to test chemicals (EPA, 1998d; OECD, 2007). These protocols include tests of neurobehavioral function, such as auditory startle, learning and memory function, changes in motor activity, and neuropathological examination and morphometric analysis across multiple age ranges (pre-weaning, adolescence, and young adults). In 2012, the OECD approved the "Extended One Generation Reproductive Toxicity Study" (OECD, 2012a). A main advantage is that criteria are described that may document that a two-generation study is not needed, thereby reducing substantially the number of animals, and the cost of testing. The document states, "Decisions on whether to assess the second generation and to omit the developmental neurotoxicity cohort and/or developmental immunotoxicity cohort should reflect existing knowledge for the chemical being evaluated, as well as the needs of various regulatory authorities" (OECD, 2012a).

Immunotoxicity Assessment

Under normal conditions, the immune system is responsible for host defense against pathogenic infections and certain cancers. However, xenobiotic exposures can alter immune system development and/or function and lead to immunostimulation (sensitization or autoimmunity) or immunosuppression (see Chap. 12). Our understanding of the biological processes underlying immune system dysfunction remains incomplete. However, advances in molecular biology (including use of transgenic/knockout mice), analytic methods (including RNA sequencing, cell sorting, and multiparameter flow cytometry), animal models (including adoptive transfers in immunocompromised mice and host resistance to viral, bacterial, or tumor cell challenge), and other methods are greatly improving our knowledge.

The development of hypersensitivity can vary among chemicals because of differing intrinsic immune mechanisms. Importantly, traditional dose–response relationships may not necessarily exist. For example, a single or incidental exposure to beryllium, an element used in numerous manufacturing and industrial capacities, has been associated with chronic beryllium disease in some individuals. This disease is a type 4 cell-mediated hypersensitivity reaction that leads to the formation of lung granulomas containing epithelioid cells, macrophages, and T cells. Sensitization to beryllium is more often observed in individuals with a nonsynonymous polymorphism in the *HLA-DPB1* gene that alters antigen recognition (Richeldi et al., 1993). By comparison, autoimmunity occurs when the immune

system attacks normal tissues and can be observed in type II and III hypersensitivity reactions. A wide range of chemicals including therapeutic drugs (methyldopa, isoniazid), metals (mercury), pesticides (hexachlorobenzene), and industrial materials (vinyl chloride) have been associated with autoimmunity. The anesthetic halothane is a well-studied chemical that causes autoimmunity. The trifluoroacetylhalide metabolite of halothane binds to proteins in the liver forming neoantigens that are recognized by the immune system leading to hepatitis.

Immunosuppression is another form of immune system toxicity that can result in a failure to respond to pathogenic infection, a prolonged infection period, or expression of a latent infection or cancer. Therapeutic immunosuppressants are designed and used to prevent organ transplant rejection or suppress inflammation. However, a large number of environmental and industrial chemicals have been associated with immunosuppression, including organochlorine pesticides, lead, and halogenated aromatic hydrocarbons (including TCDD), and exposures that occur during critical stages may present special risks to development.

With the increased development of immunomodulatory biologic drugs, proper safeguards in testing are needed to prevent massive cytokine dysregulation. A pivotal example involved six healthy volunteers who had enrolled in a phase 1 clinical trial in the United Kingdom who developed a severe cytokine response to an anti-CD28 monoclonal antibody leading to systemic organ failures (Suntharalingam et al., 2006). Such cases are stark reminders of the challenges faced in understanding how the immune system is regulated, establishing reliable test systems for identifying such risks prior to human use, and developing safe means for testing these chemicals in humans.

The current practice for evaluating potential toxic effects of novel chemical and therapeutic products on the immune system involves a tiered approach to immunotoxicity screening. The ICH S8 guidance (Table 2-6), which applies to the nonclinical (animal) testing of human pharmaceuticals, is the accepted standard in the United States, the EU, and Japan. Initial data regarding a new drug's ability to alter immune system functioning in an unintended manner can be observed in standard toxicity tests. Evaluation of hematological changes, including differential effects on white blood cells and immunoglobulin changes, increases in infections or tumors, and alterations in lymphoid organ weights or histology, in standard toxicology studies can provide strong evidence of potential effects to the immune system. Immune system dysfunction would signal the need of more detailed evaluations, including the evaluation of functional effects (e.g., T-cell-dependent antibody response or natural killer cell activity), flow cytometric immunophenotyping, or host resistance studies. The NTP uses a tier-based system to screen immunotoxicology endpoints including histopathology and clinical pathology (complete blood count and hematology) for the immune system as well as measures innate immunity (macrophage function and natural killer cell assays), cell-mediated immunity (cytotoxic T lymphocyte activity), and humoral-mediated immunity (antibody production following antigen challenge).

Sensitization

Skin sensitization, also known as allergic contact dermatitis, is the immune-mediated reaction of the skin to a chemical. The appearance of skin sensitization can range in humans from erythema, edema, and vesicles to bullae, whereas in rodents only erythema and edema may be observed. In recent years, there has been great interest in reducing the number of animals needed for testing a chemical's propensity to cause skin sensitization. EPA and OECD guidelines describe protocols for evaluating skin sensitization (OECD, 1992, 2010a, 2010b, 2015d, 2015e, 2016; EPA, 2003). These studies range from the Guinea Pig Maximization test and include the adjuvant and non-adjuvant Buehler test. Guinea pigs (and sometimes mice) are exposed to the test chemical topically or intradermally, followed for 3 to 4 weeks, and evaluated for skin irritation and erythema. More recent tests of sensitization include the use of the local lymph node assay in mice as well a variety of validated in vitro methods such as the reconstructed human epidermis test model, ARE-Nrf2 luciferase test in human keratinocytes, and the human cell line activation test (h-CLAT).

Eye and Skin Irritation and Corrosion

Irritant chemicals cause reversible injury to the eyes or skin, whereas *corrosive* damage is irreversible. Similar to sensitization screening, a variety of new validated methods are available to test a chemical's ability to cause irritation and/or corrosion. EPA protocols utilize albino rabbits for both ocular and dermal testing (EPA, 1998a, 1998b). Eye injury is assessed between 3 and 21 days after application of the chemical to the rabbit's eye and compared to the contralateral untreated eye in the same animal. Typical ocular endpoints include evaluation of the cornea (opacity), iris (congestion and reaction to light), and the conjunctivae (redness and chemosis). Similar testing is recommended by OECD (OECD, 2002). In vitro methods for assessing ocular damage have been validated and recommended for testing purposes (OECD, 2012b, 2013b, 2015f, 2015g). These include use of SIRC (Seruminstitut rabbit cornea) cells and reconstructed human cornea-like epithelium for cell viability/cytotoxicity assays, isolated chicken corneas for fluorescein retention and morphological changes, and Madin–Darby Canine Kidney cells grown on transwell inserts and evaluated for fluorescein leakage across the monolayer.

Dermal toxicity testing is performed by applying patches containing the test chemical to a shaved section of skin for increasing periods of time (minutes to hours). Adjacent regions of skin are used as a control within the same animal. The skin is evaluated for erythema/eschar and edema and reversibility of these findings during a 14-day period. The EPA has also begun to enact alternate testing methods (including the bovine corneal opacity and permeability assay, epiocular assay, cytosensor microphysiometer assay) to assess the ability of pesticides to cause eye irritation. Other chemicals can be tested using these alternate methods on a case-by-case basis. OECD guidelines for dermal irritation and corrosion also include similar protocols for erythema and edema on the skin of albino rabbits (OECD, 2015a), as well as three-dimensional human skin models containing an epidermis and functional stratum corneum (OECD, 2013a, 2015b).

Other Toxicity Tests

Most of the tests described above will be included in a "standard" toxicity testing protocol because they are required by the various regulatory agencies. Additional tests may be required or included in the protocol to provide information relating a special route of exposure, such as inhalation. Inhalation toxicity tests in animals are usually carried out in a dynamic (flowing) chamber rather than in static chambers to avoid particulate settling and exhaled gas complications. Such studies usually require special dispersing and analytic methodologies, depending on whether the chemical to be tested is a gas, vapor, or aerosol. The duration of exposure for inhalation toxicity tests can be acute, subchronic, or chronic, but acute studies are more common with inhalation toxicology. Other special types of animal toxicity tests include toxicokinetics (absorption, distribution, biotransformation, and excretion), the development of

appropriate antidotes and treatment regimens for poisoning, and the establishment of analytic techniques to detect residues of chemicals in tissues and other biological materials.

SYSTEMS TOXICOLOGY

The integration of classical toxicology testing with the "quantitative analysis of large networks of molecular and functional changes occurring across multiple levels of biological organization" has resulted in the field of *systems toxicology* (Sturla et al., 2014). Systems toxicology originated from advancements in high throughput technologies and a need to improve prediction in safety testing. Similar to systems biology (Weston and Hood, 2004), systems toxicology anchors phenotypic data with a battery of molecular functions to understand how a living organism functions or responds to chemicals at the cellular and tissue levels. The molecular functions include *transcriptomics* (complete set of RNA transcripts found in the genome), *epigenomics* (global modifications to genetic material), *proteomics* (profile of proteins present within cells or tissues), *metabonomics/metabolomics* (endogenous and exogenous by-products of cellular metabolism), and *lipidomics* (a subset of the metabolome that is enriched by lipids). Each of these molecular processes is vulnerable to changes by exposure to chemicals. In addition to characterizing molecular and phenotypic changes, systems toxicology also integrates the *exposome,* or the cumulative environmental exposures (including chemicals, diet, lifestyle, etc.) over a lifetime that impacts the development and progression of diseases, into the testing paradigm (Wild, 2005). Together, this multi-faceted approach to toxicology testing enables the identification of precipitating events that subsequently lead to cellular injury or dysfunction.

The ability of toxicologists to identify mechanisms by which chemicals initiate toxicities has been improved by the recent application of high-content screening and novel models. Likewise, the increasing utilization of in vitro testing schemes along with advanced computational approaches has enhanced the predictive screening and prioritization of toxicity testing for chemicals. Multiple in vitro molecular endpoints and in silico models are integrated together to identify common pathways responsible for toxic responses to chemicals. This requires the collection of large data sets (i.e., big data), application of mathematical models, computer simulations, and validation of models with experimental findings (Hartung et al., 2017). The goal of predictive toxicity testing is then to prioritize the testing of a large number of chemicals and in turn reduce the number of animals needed for research and regulatory studies.

Transcriptome

Among the first changes that a cell may exhibit following exposure to a toxic substance is a change in gene expression. The transcriptome (all of the mature mRNA species present in a cell at a given point in time) is dynamic and represents the steady state between the rate of synthesis (transcription) and degradation of mRNAs in a cell. The reverse transcription–polymerase chain reaction (RT-PCR) allows one to quantitatively determine the relative number of mRNA species in a sample for specific genes. With general primers, it is also possible to amplify the entire transcriptome quantitatively to make many complete copies of the transcriptome in a test tube. Thus, large amounts of material for analysis can be obtained from a relatively small number of cells. Using microarray technologies, where tens of thousands of unique oligonucleotides (or cDNAs) are anchored on a solid matrix, toxicologists can quantitatively assess the expression of thousands of unique mRNAs in a single sample, thus capturing an "expression profile" of the entire transcriptome in a single analysis. While microarrays dramatically changed the field of toxicology by permitting large data set comparisons of toxicant responses, this technology has been limited by cross-hybridization concerns and difficulty in quantifying low and high enriched genes.

Next-generation sequencing (NGS) allows use of DNA or RNA samples for whole-genome sequencing, RNA sequencing, targeted sequencing (such as exome sequencing or 16S sequencing), and other approaches. Whole-transcriptome sequencing identifies and quantifies the abundance of all RNA transcripts including coding mRNAs and noncoding RNAs (such as microRNAs). Compared to microarrays, RNA-sequencing has higher resolution and reproducibility and can be used to study alternative splicing events and novel transcripts. Because large data sets are generated with RNA sequencing (over 27,000 genes), bioinformatics is critical for the proper analysis of data. RNA sequencing is gaining greater utilization in toxicology to provide unique profiles of toxicants, such as a cellular response to DNA damage or oxidative stress.

Epigenome

The epigenome includes global changes in the DNA genome that affect gene expression. Multiple epigenetic marks including DNA methylation, histone modifications, and small RNA transcripts contribute to the overall epigenome of a cell or tissue.

Conrad Hal Waddington first postulated in the 1930s that it was not just the genes that shaped development but also the environment that shapes the genes (Holliday, 2006). Epigenetics has been defined in various ways, with perhaps the strictest definition being "a mitotically or meiotically heritable change in gene expression that occurs independently of an alteration in DNA sequence" (Youngson and Whitelaw, 2008). Typically, gene expression is silenced or suppressed, or in some instances activated, by DNA methylation or histone deacetylation—changes that do not alter the nucleotide sequence of the silenced genes. Epigenetic changes can potentially be transgenerational, as suggested in some animal models, which has important implications for toxicological assessment (Rosenfeld, 2010; Skinner, 2011). Given the growing recognition of epigenetics as a means by which environmental factors can alter biological responses, genomic analyses in toxicology may also include techniques to identify toxicant-induced changes in DNA methylation patterns (Watson and Goodman, 2002; LeBaron et al., 2010).

Classical approaches to toxicology have thoroughly documented the potential for a variety of environmental toxicants, such as thalidomide, alcohol, lead, mercury, and PCBs, to cause adverse effects on the developing organism. However, subtle epigenetic changes that are not associated with cytotoxicity or mutations can also result from environmental exposures and thus may have important toxicological implications. Epigenetic changes have been demonstrated to occur from exposure to a variety of environmental hazards, including tobacco smoke, metals, alcohol, phthalates, and bisphenol A (Baccarelli and Bollati, 2009; Bernal and Jirtle, 2010; Perera and Herbstman, 2011; Cheng et al., 2012). Furthermore, epigenetic changes can occur through nutrition, methyl content of diet, intake of folic acid and vitamins, or even social and maternal behavior toward the offspring (Cummings et al., 2010). Epigenetic changes have been causally implicated in cancer, neurodevelopment disorders, autoimmune diseases, diabetes and metabolic disorders, asthma, behavioral disorders, and endocrine disorders (Nystrom and Mutanen, 2009; Attig et al., 2010; Godfrey et al., 2011; Zhang and Ho, 2011). Thus, epigenetic changes induced by xenobiotics, dietary factors, and maternal behavior have implications for safety assessment and risk assessment for xenobiotics (Szyf, 2007; Goodman et al., 2010; LeBaron et al., 2010).

Methylation of DNA is an important determinant of gene expression in cells and tissues, and exogenous chemicals can interfere with transcriptional function by altering DNA methylation (Watson and Goodman, 2002). Although such epigenetic changes do not modify the genomic sequence, they theoretically can result in heritable phenotypic changes. Evidence of multigenerational epigenetic changes from environmental exposures has yet to be revealed in humans (Baccarelli and Bollati, 2009); however, several animal models have demonstrated transgenerational epigenetic changes (Skinner, 2011). Thus, analyses in toxicology may also include techniques to identify toxicant-induced epigenetic changes in DNA and histones and their potential consequences (Watson and Goodman, 2002; Szyf, 2007).

Although changes in gene expression and epigenetic regulation often contribute to, or are reflective of, phenotypic changes that occur in response to a toxic substance, the transcriptome and epigenome are still somewhat far removed from the ultimate biochemical functions that dictate the actual biological function of the cell. Because the functional expression of a gene generally requires the translation of the mRNA to a protein, there is also great interest in looking at the "proteome"—the entire complement of *proteins* that are present in a cell or tissue at a given point in time.

Proteome

The *proteome* represents the complete set of proteins expressed within a cell, organ, or organism. Increases and decreases in proteins may result from changes in transcription or from post-translational modifications that enhance translation or turnover. Enrichment of different proteins as a result of toxicant exposure can be tested using targeted and untargeted approaches. Mass spectrometry is the primary technology used for proteomics because of its high sensitivity and accuracy in identifying proteins, down to the subfemtomolar range. Because of size limitations for accurate mass spectrometry, protein mixtures are usually digested to smaller peptide fragments. The mixture of peptide fragments is resolved into individual components, and the identity of the specific peptides is determined based on high-resolution mass analysis and sequential degradation (sequential loss of single amino acids) of the peptides by various means. The large and complex set of peptide mass fragments is then analyzed using bioinformatics and compared with a large database of mass fragments of known peptides/proteins. Comparative proteomics in an untargeted fashion is enabled by use of isotope tagging for relative and absolute quantification (iTRAQ). Targeted proteomics is performed with selected reaction monitoring (SRM) using predefined sequences.

Mass spectrometry can be used to not only identify and quantify protein enrichment but also assess posttranslational modifications including phosphorylation and ubiquitylation. These alterations to proteins can impact their localization, activity, and/or interaction with other macromolecules. The mycotoxin deoxynivalenol is a contaminant of food and considered an innate immune system toxicant. Exposure of mice to deoxynivalenol resulted in novel phosphorylation of 90 phosphoproteins within the spleen (Pan et al., 2013). Computational analyses characterized the functions of the modified proteins and pointed to potential mechanisms of deoxynivalenol toxicity including changes in the cytoskeleton, apoptosis, and lymphocyte signaling.

Metabonomics/Metabolomics

These two terms are often used interchangeably to describe the analysis of small molecules that serve as substrates, products, and cofactors in the multitude of enzymatic reactions and other metabolic processes that define living cells. Metabonomics has been defined as "the comprehensive and simultaneous systematic profiling of metabolite levels and their systematic and temporal change through such effects on diet, lifestyle, environment, genetic and pharmaceuticals, both beneficial and adverse, in whole organisms" (Lindon et al., 2003, 2006). The term "metabolomics" has been used principally in studies in plants and in vitro or single-cell systems (Fiehn, 2002). Regardless of the specific term used (metabonomics will be used here), the concept of quantitatively analyzing toxicant-induced changes in the "metabolic profile" (the "metabonome") of a cell, tissue, or body fluid can include xenobiotics, their metabolites as well as perturbations in endogenous molecules.

Two approaches for identifying and measuring hundreds, or even thousands, of small molecules in biological samples have emerged—nuclear magnetic resonance (NMR) and/or mass spectrometry. NMR provides structural identification of metabolites; however, it is not as sensitive as mass spectrometry. Similar to transcriptomics and proteomics, metabonomics can be performed in targeted experiments that quantify a predefined set of metabolites or using an untargeted approach. Within the field of metabonomics, there is growing interest in specific types of metabolites such as lipids or the *lipidome.* Lipids not only represent energy sources but also serve as cellular signaling molecules. Use of untargeted lipidomics has revealed dynamic changes in mouse livers exposed to TCDD including repression of de novo fatty acid synthesis and beta-oxidation along with enhanced uptake of lipids (Nault et al., 2017). When integrated with transcriptomic profiling by RNA sequencing, lipidomics provided insight into the mechanisms of TCDD-induced hepatic steatosis.

Exposome

The concept of the exposome was introduced in 2005 by Wild to define a systems approach to advance exposure science in a manner that complements the genome. The *exposome* represents the cumulative environmental exposures (including diet, lifestyle, exercise, inherent metabolic activity, infections, and xenobiotics) across an entire lifespan that affect human health (Wild, 2005; Rappaport and Smith, 2010; Rappaport, 2011; Miller and Jones, 2014). Defining the exposome depends upon highly sensitive analytical methods that can detect changes in biomarkers of exposure, effect, and/or susceptibility that represent the interaction between an exposure and a biological system (Sturla et al., 2014). Biomarkers include exogenous chemicals (or their metabolites), biochemical, physiological, or other alterations in an organism, and intrinsic and acquired factors that will influence susceptibility to toxicity. Two approaches have been developed to characterize the exposome (Lioy and Rappaport, 2011). The "bottom-up" approach uses each category of external exposure (air, water, diet, radiation, lifestyle, etc.) to quantify the cumulative concentrations of contaminants. This is a challenging approach because of the potential to miss sources of exposure. By contrast, the "top-down" approach utilizes untargeted omic methods to quantify exposures or surrogate markers of exposure. The discovery and validation of appropriate biomarkers has enabled the conductance of environment-wide association studies (EWAS) (Patel et al., 2010; Thomas, 2010). In EWAS studies, candidate biomarkers are used to evaluate the causality, prevention, detection, prognosis, and treatment of diseases with regards to the total environment. EWAS studies have been used to study type 2 diabetes and polychlorinated biphenyls (Patel et al., 2010), as well as asthma severity and dust mite exposure (Sordillo et al., 2015).

Building on the adverse outcome pathway (AOP) concept discussed earlier, aggregate exposure pathways (AEPs) are being developed that integrate external, internal, and target site exposures apical to the molecular initiating events (Escher et al., 2017). This approach provides a more comprehensive view of AOPs that consider source and infer critical concentrations that inform subsequent biological effects.

High-Content Screening

High-content screening or cellomics is an automated microscopy analysis that often uses fluorescence to visual cellular or subcellular changes in a high-throughput format (Boutros et al., 2015). Using microtiter plates, toxicologists can use images to visualize temporal and spatial changes in cells, organs, or small organisms (such as zebrafish). Quantitative measurements include morphology, apoptosis, autophagy, cell proliferation, DNA damage, mitochondria functioning, mitosis, oxidative stress, and cytotoxicity to name a few. In fact, multiple phenotypes can be profiled simultaneously (Persson and Hornberg, 2016). One of the earliest examples of the utility of high-content screening for the field of toxicology was a study of chemical-induced liver injury (Xu et al., 2008). Over 300 drugs and chemicals were profiled for their ability to perturb intracellular glutathione, damage mitochondria, and cause oxidative stress in primary human hepatocytes. This screening strategy demonstrated a 50% to 60% true-positive rate for predicting hepatotoxicity potential with a very low rate of false-positives (0% to 5%). Increasingly, high-content screening is being applied to predict not only hepatotoxicity but also cardiotoxicity, neurotoxicity, genotoxicity, and nephrotoxicity.

Computational Toxicology

Hundreds of new chemicals are introduced into our lives each year and add to the tens of thousands of chemicals that we are exposed to on a routine basis. The current paradigm of animal testing precludes the comprehensive safety evaluation of all industrial chemicals. Therefore, *computational toxicology* approaches that integrate biology, chemistry, and computer science are needed to evaluate chemicals for potential adverse liabilities and prioritize them for further testing. This discipline uses models and algorithms based on existing data sets for defined endpoints to predict the likelihood that a new chemical entity will possess the same toxic liabilities. An early example of this approach in drug development is the "Rule of Five" which used simple physicochemical properties such as hydrophobicity, molecular weight, composition, and bonding patterns to approximate a chemical's solubility and permeability after oral administration (Lipinski et al., 2001). Likewise, computational identification of quantitative structural activity relationships (QSARs) has been a long-standing approach to predict target organ toxicities (Weinstein et al., 1985). One of the earliest QSAR toxicology examples is the evaluation of analogs for DDT for structural conformations and orientations in relationship to biological activity (Weinstein et al., 1985). Use of QSAR allows toxicologists to mathematically assess how closely a new chemical aligns to known toxicants and prioritize them for further testing. This screening approach is particularly useful when evaluating thousands of chemicals of unknown toxicity potential. A related approach to QSAR that has been used for years to estimate a chemical's toxicity is *read-across*. The process of read-across is routinely used for hazard identification in risk assessment and can provide qualitative or quantitative information regarding how a particular property or activity will predict a toxicity endpoint from one chemical using data obtained on the same endpoint for a related substance (OECD, 2014).

For the majority of computational toxicology studies, in vivo toxicity data are used as the primary data sets and supplemented with in vitro and in silico studies to perform read-across. When in vitro data are used it is important to consider factors that are absent in cell culture that can influence toxicity findings. These may include a lack of metabolism or parameters such as blood flow rate. Therefore, physiologically based pharmacokinetic (PBPK) modeling is particularly useful when conducting quantitative in vitro to in vivo extrapolation (QIVIVE) (Yoon et al., 2012) (see Chap. 7). Using these approaches, one can integrate dosimetry data, species-specific physiological processes, and physicochemical properties along with in vitro data using appropriate scaling factors.

One feature that is common among all of the various "omics" technologies is the ability to generate very large volumes of data. Both the data management and statistical evaluation of omic studies represent an enormous challenge. The emerging field of "big data" has developed to address these challenges. Since 2004, the National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM), National Institutes of Health (NIH) have hosted PubChem's BioAssay database, which is a public repository for small molecule screening data. As of 2017, there were more than 1.2 million assays and 2.3 million chemical structures in the database (Wang et al., 2017). Automated profiling tools can capitalize on these rich sources of data and be used with QSAR to screen bioassay results from high-throughput screens and apply in vitro–in vivo correlations to identify toxicophores that predict the potential of a chemical to cause injury (Kim et al., 2016).

ToxCast (or Toxicity Forecaster) is a comprehensive computational toxicology initiative by the EPA that integrates computational toxicology and high-content screening to evaluate chemicals for potential toxic effects. In the first phase of ToxCast (2009), over 300 well-studied chemicals such as pesticides were tested in over 500 assays. In the second phase of ToxCast (2013), this testing was expanded to 2000 molecules from a broad range of chemical classes (industrial and consumer product chemicals, food additives, etc.) in even more high-throughput tests (over 700). These data were generated as part of the interagency Toxicity Testing in the 21st Century (Tox21) initiative between the NTP, FDA, National Institute of Environmental Health Sciences, and National Center for Advancing Translational Sciences. This tiered screening approach intends to identify potential chemical liabilities and prioritize chemicals for comprehensive animal testing.

Innovative Testing Models

Novel in vitro approaches for predictive safety testing include organs- and body-on-a-chip and stem cells. Organs-on-a-chip are microfluidic flow-based systems that are engineered with multiple cell types that spatially recapitulate three-dimensional organs. Three-dimensional cultures offer multiple advantages compared to traditional cell culture including enhanced cellular survival, differentiation, cell interactions, and orientation similar to native tissues.

Microfabricated devices allow for cell–cell communication and dynamic mechanical forces such as breathing (lungs), shear (circulation), peristalsis (intestines), and blinking (eyes) that are important for organ functioning. Using a lung-on-a-chip, it was shown that alveolar epithelial cells grown at the air–liquid interface under 10% mechanical strain that mimicked breathing had enhanced nanoparticle translocation and generation of oxidative stress (Huh et al., 2010). As each organ-on-a-chip advances in complexity, there is greater interest in using multiorgan systems (or body-on-a-chip). Connections between individual tissue chips such as the liver allow for xenobiotic biotransformation in hepatocytes and investigation

of metabolites on target organs. Other in vitro models that are gaining increasing utilization are stem cells. Models include induced pluripotent, embryonic, and adult stem cells. Differentiation of stem cells using specific defined media with growth and morphogenic factors have been used for organ testing including liver, brain, kidney, heart, intestine, and skin. Likewise, embryonic stem cells are used for screening developmental toxicants using reporter assays and neurodevelopment markers (Suter-Dick et al., 2015; Pamies and Hartung, 2017). Stem cell–derived testing can include screening methods as well as integration in three-dimensional tissue-like structures.

Innovative animal models that are advancing toxicology research include the use of targeted genetic knockout and knock-in mouse models. Knockout mice are genetically modified mice that have an inactivated gene or cluster of genes, whereas knock-in mice are generated to express a specific gene or modified gene. Generation of knockout and knock-in transgenic mice have been critical in understanding the toxicity of chemicals. For example, use of knockout mice lacking the peroxisome proliferator–activated receptor alpha or knock-in mice expressing the human ortholog has revealed species differences in chemical-induced hepatocellular proliferation (Lee et al., 1995; Cheung et al., 2004).

One limitation of traditional rodent safety studies is the lack of genetic variation in toxicological responses. To recapitulate the genetic variation observed within the human population, a number of genetically diverse mouse strains have been developed. Beginning in 2005, the Collaborative Cross developed a large panel of genetically defined strains that covered about 90% of the genetic diversity within laboratory mice through multiple intercrosses. Using 36 inbred mouse stains and whole-genome association analysis, it was revealed that novel polymorphisms correlated well with acetaminophen-induced liver injury (Harrill et al., 2009). Validation using the orthologous human gene (CD44) in two independent liver injury cohorts confirmed that this genetic factor could influence susceptibility for acetaminophen toxicity. Subsequently, a second mouse genetic line was developed, called the Diversity Outbred population. The Diversity Outbred mice represent independent lineages that recapitulate human interindividual variation in toxicity responses. Linkage analysis of this animal panel identified two sulfotransferases as important genetic determinants of benzene genotoxicity (French et al., 2015). With the increasing demands to identify chemical hazards and determine mechanisms of toxicity, additional innovative models for safety testing are expected in upcoming years.

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REFERENCES

Allen BC, Kavlock RJ, Kimmel CA, Faustman EM. Dose-response assessment for developmental toxicity. II. Comparison of generic benchmark dose estimates with no observed adverse effect levels. *Fundam Appl Toxicol*. 1994a;23:487–495.

Allen BC, Kavlock RJ, Kimmel CA, Faustman EM. Dose-response assessment for developmental toxicity. III. Statistical models. *Fundam Appl Toxicol*. 1994b;23:496–509.

American Geriatrics Society Beers Criteria Update Expert P. American Geriatrics Society 2015 Updated Beers Criteria for Potentially Inappropriate Medication Use in Older Adults. *J Am Geriatr Soc*. 2015;63:2227–2246.

Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat Res*. 1975;31:347–364.

Andersen ME, Preston RJ, Maier A, Willis AM, Patterson J. Dose-response approaches for nuclear receptor-mediated modes of action for liver carcinogenicity: results of a workshop. *Crit Rev Toxicol*. 2014;44:50–63.

Ankley GT, Bennett RS, Erickson RJ, et al. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem*. 2010;29:730–741.

Ashauer R, Agatz A, Albert C, et al. Toxicokinetic-toxicodynamic modeling of quantal and graded sublethal endpoints: a brief discussion of concepts. *Environ Toxicol Chem*. 2011;30:2519–2524.

Atterberry TT, Burnett WT, Chambers JE. Age-related differences in parathion and chlorpyrifos toxicity in male rats: target and nontarget esterase sensitivity and cytochrome P450-mediated metabolism. *Toxicol Appl Pharmacol*. 1997;147:411–418.

Attig L, Gabory A, Junien C. Nutritional developmental epigenomics: immediate and long-lasting effects. *Proc Nutr Soc*. 2010;69:221–231.

Baccarelli A, Bollati V. Epigenetics and environmental chemicals. *Curr Opin Pediatr*. 2009;21:243–251.

Bailey GS, Reddy AP, Pereira CB, et al. Nonlinear cancer response at ultralow dose: a 40800-animal ED(001) tumor and biomarker study. *Chem Res Toxicol*. 2009;22:1264–1276.

Barnes DG, Dourson M. Reference dose (RfD): description and use in health risk assessments. *Regul Toxicol Pharmacol*. 1988;8:471–486.

Becker RA, Ankley GT, Edwards SW, et al. Increasing scientific confidence in adverse outcome pathways: application of tailored Bradford Hill considerations for evaluating weight of evidence. *Regul Toxicol Pharmacol*. 2015a;72:514–537.

Becker RA, Dellarco V, Seed J, et al. Quantitative weight of evidence to assess confidence in potential modes of action. *Regul Toxicol Pharmacol*. 2017;86:205–220.

Becker RA, Patlewicz G, Simon TW, Rowlands JC, Budinsky RA. The adverse outcome pathway for rodent liver tumor promotion by sustained activation of the aryl hydrocarbon receptor. *Regul Toxicol Pharmacol*. 2015b;73:172–190.

Bernal AJ, Jirtle RL. Epigenomic disruption: the effects of early developmental exposures. *Birth Defects Res A Clin Mol Teratol*. 2010;88:938–944.

Blakeman V, Williams JL, Meng QJ, Streuli CH. Circadian clocks and breast cancer. *Breast Cancer Res*. 2016;18:89.

Blanchard OL, Smoliga JM. Translating dosages from animal models to human clinical trials—revisiting body surface area scaling. *FASEB J*. 2015;29:1629–1634.

Bliss CL. Some principles of bioassay. *Am Sci*. 1957;45:449–466.

Boolell M, Allen MJ, Ballard SA, et al. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res*. 1996;8:47–52.

Boutros M, Heigwer F, Laufer C. Microscopy-based high-content screening. *Cell*. 2015;163:1314–1325.

Budinsky RA, Schrenk D, Simon T, et al. Mode of action and dose-response framework analysis for receptor-mediated toxicity: the aryl hydrocarbon receptor as a case study. *Crit Rev Toxicol*. 2014;44:83–119.

Calabrese EJ. Flaws in the LNT single-hit model for cancer risk: an historical assessment. *Environ Res*. 2017;158:773–788.

Calabrese EJ, Baldwin LA. Hormesis as a biological hypothesis. *Environ Health Perspect*. 1998;106(suppl 1):357–362.

Calabrese EJ, Blain R. The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. *Toxicol Appl Pharmacol*. 2005;202:289–301.

Carroll R, Metcalfe C, Gunnell D, Mohamed F, Eddleston M. Diurnal variation in probability of death following self-poisoning in Sri Lanka—evidence for chronotoxicity in humans. *Int J Epidemiol*. 2012;41:1821–1828.

CDER. *Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. Rockville, MD: U.S. Department of Health and Human Services Food and Drug Administration; 2005.

Chappell WR. Scaling toxicity data across species. *Environ Geochem Health*. 1992;14:71–80.

Chen P, Lin JJ, Lu CS, et al. Carbamazepine-induced toxic effects and HLA-B*1502 screening in Taiwan. *N Engl J Med*. 2011;364:1126–1133.
Cheng TF, Choudhuri S, Muldoon-Jacobs K. Epigenetic targets of some toxicologically relevant metals: a review of the literature. *J Appl Toxicol*. 2012;32:643–653.
Cheung C, Akiyama TE, Ward JM, et al. Diminished hepatocellular proliferation in mice humanized for the nuclear receptor peroxisome proliferator-activated receptor alpha. *Cancer Res*. 2004;64:3849–3854.
Chung WH, Hung SI, Hong HS, et al. Medical genetics: a marker for Stevens–Johnson syndrome. *Nature*. 2004;428:486.
Claudio L, Kwa WC, Russell AL, Wallinga D. Testing methods for developmental neurotoxicity of environmental chemicals. *Toxicol Appl Pharmacol*. 2000;164:1–14.
COEC. *Communication from the Commission on the Precautionary Principle*. Brussels: Commission of the European Communities; 2000.
Cohen SM. Cell proliferation and carcinogenesis. *Drug Metab Rev*. 1998;30:339–357.
Cohen SM. Calcium phosphate-containing urinary precipitate in rat urinary bladder carcinogenesis. *IARC Sci Publ*. 1999;147:175–189.
Corton JC, Cunningham ML, Hummer BT, et al. Mode of action framework analysis for receptor-mediated toxicity: the peroxisome proliferator-activated receptor alpha (PPARalpha) as a case study. *Crit Rev Toxicol*. 2014;44:1–49.
Cory-Slechta DA, Crofton KM, Foran JA, et al. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. I: Behavioral effects. *Environ Health Perspect*. 2001;109(suppl 1):79–91.
Cronin M, Madden J, Enoch S, Roberts D. *Chemical Toxicity Prediction: Category Formation and Read-Across*. London: Royal Society of Chemistry; 2013.
Crump KS. An improved procedure for low-dose carcinogenic risk assessment from animal data. *J Environ Pathol Toxicol Oncol*. 1984;5:339–348.
Cummings JA, Clemens LG, Nunez AA. Mother counts: how effects of environmental contaminants on maternal care could affect the offspring and future generations. *Front Neuroendocrinol*. 2010;31:440–451.
Daly AK, Donaldson PT, Bhatnagar P, et al. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet*. 2009;41:816–819.
Davis JA, Gift JS, Zhao QJ. Introduction to benchmark dose methods and U.S. EPA's benchmark dose software (BMDS) version 2.1.1. *Toxicol Appl Pharmacol*. 2011;254:181–191.
Dellarco VL, Baetcke K. A risk assessment perspective: application of mode of action and human relevance frameworks to the analysis of rodent tumor data. *Toxicol Sci*. 2005;86:1–3.
Demidenko E, Glaholt SP, Kyker-Snowman E, Shaw JR, Chen CY. Single toxin dose-response models revisited. *Toxicol Appl Pharmacol*. 2017;314:12–23.
Dorman DC, Allen SL, Byczkowski JZ, et al. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. III: Pharmacokinetic and pharmacodynamic considerations. *Environ Health Perspect*. 2001;109(suppl 1):101–111.
Eaton DL, Gallagher EP. Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol*. 1994;34:135–172.
Elcombe CR, Peffer RC, Wolf DC, et al. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: a case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. *Crit Rev Toxicol*. 2014;44:64–82.
EPA. *Health Effects Test Guidelines OPPTS 870.2400 Acute Eye Irritation*. Washington, DC: U.S. Environmental Protection Agency; 1998a.
EPA. *Health Effects Test Guidelines OPPTS 870.2500 Acute Dermal Irritation*. Washington, DC: U.S. Environmental Protection Agency; 1998b.
EPA. *Health Effects Test Guidelines OPPTS 870.6200 Neurotoxicity Screening Battery*. Washington, DC: U.S. Environmental Protection Agency; 1998c.
EPA. *Health Effects Test Guidelines OPPTS 870.6300 Developmental Neurotoxicity Study*. Washington, DC: U.S. Environmental Protection Agency; 1998d.
EPA. *Health Effects Test Guidelines OPPTS 870.3550 Reproduction/Developmental Toxicity Screening Test*. Washington, DC: U.S. Environmental Protection Agency; 2000.
EPA. *Health Effects Test Guidelines OPPTS 870.2600 Skin Sensitization*. Washington, DC: U.S. Environmental Protection Agency; 2003.
Escher BI, Hackermuller J, Polte T, et al. From the exposome to mechanistic understanding of chemical-induced adverse effects. *Environ Int*. 2017;99:97–106.
Faustman EM, Allen BC, Kavlock RJ, Kimmel CA. Dose-response assessment for developmental toxicity. I. Characterization of database and determination of no observed adverse effect levels. *Fundam Appl Toxicol*. 1994;23:478–486.
Fedak KM, Bernal A, Capshaw ZA, Gross S. Applying the Bradford Hill criteria in the 21st century: how data integration has changed causal inference in molecular epidemiology. *Emerg Themes Epidemiol*. 2015;12:14.
Fiehn O. Metabolomics–the link between genotypes and phenotypes. *Plant Mol Biol*. 2002;48:155–171.
French JE, Gatti DM, Morgan DL, et al. Diversity outbred mice identify population-based exposure thresholds and genetic factors that influence benzene-induced genotoxicity. *Environ Health Perspect*. 2015;123:237–245.
Garman RH, Fix AS, Jortner BS, et al. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. II: Neuropathology. *Environ Health Perspect*. 2001;109(suppl 1):93–100.
Godfrey KM, Inskip HM, Hanson MA. The long-term effects of prenatal development on growth and metabolism. *Semin Reprod Med*. 2011;29:257–265.
Goldstein A, Aronow L, Kalman SM. *Principles of Drug Action*. New York: Wiley; 1974.
Goldstein BD. The precautionary principle: is it a threat to toxicological science? *Int J Toxicol*. 2006;25:3–7.
Goldstein BD, Carruth RS. Implications of the precautionary principle: is it a threat to science? *Int J Occup Med Environ Health*. 2004;17:153–161.
Goodman JI, Augustine KA, Cunnningham ML, et al. What do we need to know prior to thinking about incorporating an epigenetic evaluation into safety assessments? *Toxicol Sci*. 2010;116:375–381.
Guzelian PS, Victoroff MS, Halmes NC, James RC, Guzelian CP. Evidence-based toxicology: a comprehensive framework for causation. *Hum Exp Toxicol*. 2005;24:161–201.
Halle W. The registry of cytotoxicity: toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. *Altern Lab Anim*. 2003;31:89–198.
Hanna EZ, Chou SP, Grant BF. The relationship between drinking and heart disease morbidity in the United States: results from the National Health Interview Survey. *Alcohol Clin Exp Res*. 1997;21:111–118.
Harrill AH, Watkins PB, Su S, et al. Mouse population-guided resequencing reveals that variants in CD44 contribute to acetaminophen-induced liver injury in humans. *Genome Res*. 2009;19:1507–1515.
Hartung T, FitzGerald RE, Jennings P, et al. Systems toxicology: real world applications and opportunities. *Chem Res Toxicol*. 2017;30:870–882.
Hatch EE, Palmer JR, Titus-Ernstoff L, et al. Cancer risk in women exposed to diethylstilbestrol in utero. *JAMA*. 1998;280:630–634.
Hill AB. The environment and disease: association or causation? *Proc R Soc Med*. 1965;58:295–300.
Holliday R. Epigenetics: a historical overview. *Epigenetics*. 2006;1:76–80.
Huh D, Matthews BD, Mammoto A, et al. Reconstituting organ-level lung functions on a chip. *Science*. 2010;328:1662–1668.
ICH. S5(R2): Detection of toxicity to reproduction for medicinal products and toxicity to male fertility. International Conference on Harmonisation; 2000.
Irwin S. Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacologia*. 1968;13:222–257.
Johnson MK. The delayed neurotoxic effect of some organophosphorus compounds. Identification of the phosphorylation site as an esterase. *Biochem J*. 1969;114:711–717.
Johnson MK. Organophosphorus esters causing delayed neurotoxic effects: mechanism of action and structure activity studies. *Arch Toxicol*. 1975;34:259–288.

Johnson NM, Egner PA, Baxter VK, et al. Complete protection against aflatoxin B(1)-induced liver cancer with a triterpenoid: DNA adduct dosimetry, molecular signature, and genotoxicity threshold. *Cancer Prev Res (Phila)*. 2014;7:658–665.

Karanth S, Pope C.Carboxylesterase and A-esterase activities during maturation and aging: relationship to the toxicity of chlorpyrifos and parathion in rats. *Toxicol Sci*. 2000;58:282–289.

Kim MT, Huang R, Sedykh A, et al. Mechanism profiling of hepatotoxicity caused by oxidative stress using antioxidant response element reporter gene assay models and big data. *Environ Health Perspect*. 2016;124:634–641.

Klaassen CD, Cui JY. Review: mechanisms of how the intestinal microbiota alters the effects of drugs and bile acids. *Drug Metab Dispos*. 2015;43:1505–1521.

Kleiber M. Body size and metabolism. *Hilgardia*. 1932;6:315–353.

Knudsen TB, Kavlock RJ, Daston GP, et al. Developmental toxicity testing for safety assessment: new approaches and technologies. *Birth Defects Res B Dev Reprod Toxicol*. 2011;92:413–420.

Kobayashi Y, Fukumaki Y, Yubisui T, Inoue J, Sakaki Y. Serine-proline replacement at residue 127 of NADH-cytochrome b5 reductase causes hereditary methemoglobinemia, generalized type. *Blood*. 1990;75:1408–1413.

Kroes R, Galli C, Munro I, et al. Threshold of toxicological concern for chemical substances present in the diet: a practical tool for assessing the need for toxicity testing. *Food Chem Toxicol*. 2000;38:255–312.

Kroes R, Renwick AG, Cheeseman M, et al. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. *Food Chem Toxicol*. 2004;42:65–83.

Kroes R, Renwick AG, Feron V, et al. Application of the threshold of toxicological concern (TTC) to the safety evaluation of cosmetic ingredients. *Food Chem Toxicol*. 2007;45:2533–2562.

Lamb JC, Boffetta P, Foster WG, et al. Critical comments on the WHO-UNEP State of the Science of Endocrine Disrupting Chemicals—2012. *Regul Toxicol Pharmacol*. 2014;69:22–40.

Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*. 1983;219:979–980.

Larsson M, van den Berg M, Brenerova P, et al. Consensus toxicity factors for polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls combining in silico models and extensive in vitro screening of AhR-mediated effects in human and rodent cells. *Chem Res Toxicol*. 2015;28:641–650.

Lassiter TL, Barone S Jr, Moser VC, Padilla S. Gestational exposure to chlorpyrifos: dose response profiles for cholinesterase and carboxylesterase activity. *Toxicol Sci*. 1999;52:92–100.

LeBaron MJ, Rasoulpour RJ, Klapacz J, et al. Epigenetics and chemical safety assessment. *Mutat Res*. 2010;705:83–95.

Lee SS, Pineau T, Drago J, et al. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol*. 1995;15:3012–3022.

Lehman-McKeeman LD, Caudill D. Biochemical basis for mouse resistance to hyaline droplet nephropathy: lack of relevance of the alpha 2u-globulin protein superfamily in this male rat-specific syndrome. *Toxicol Appl Pharmacol*. 1992;112:214–221.

Lindon JC, Holmes E, Nicholson JK. Metabonomics techniques and applications to pharmaceutical research & development. *Pharm Res*. 2006;23:1075–1088.

Lindon JC, Nicholson JK, Holmes E, et al. Contemporary issues in toxicology, the role of metabonomics in toxicology and its evaluation by the COMET project. *Toxicol Appl Pharmacol*. 2003;187:137–146.

Lioy PJ, Rappaport SM. Exposure science and the exposome: an opportunity for coherence in the environmental health sciences. *Environ Health Perspect*. 2011;119:A466–467.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 2001;46:3–26.

Lipscomb JC, Haddad S, Poet T, Krishnan K. Physiologically-based pharmacokinetic (PBPK) models in toxicity testing and risk assessment. *Adv Exp Med Biol*. 2012;745:76–95.

Littlefield NA, Farmer JH, Gaylor DW, Sheldon WG. Effects of dose and time in a long-term, low-dose carcinogenic study. *J Environ Pathol Toxicol*. 1980;3:17–34.

Liu T, Zhang C, Liu C. The incidence of breast cancer among female flight attendants: an updated meta-analysis. *J Travel Med*. 2016;23(6):pii: taw055.

LoGuidice A, Wallace BD, Bendel L, Redinbo MR, Boelsterli UA. Pharmacologic targeting of bacterial beta-glucuronidase alleviates nonsteroidal anti-inflammatory drug-induced enteropathy in mice. *J Pharmacol Exp Ther*. 2012;341:447–454.

Lucena MI, Andrade RJ, Martinez C, et al. Glutathione *S*-transferase m1 and t1 null genotypes increase susceptibility to idiosyncratic drug-induced liver injury. *Hepatology*. 2008;48:588–596.

Mallal S, Phillips E, Carosi G, et al. HLA-B*5701 screening for hypersensitivity to abacavir. *N Engl J Med*. 2008;358:568–579.

Marchant GE. From general policy to legal rule: aspirations and limitations of the precautionary principle. *Environ Health Perspect*. 2003;111:1799–1803.

Meek ME, Bucher JR, Cohen SM, et al. A framework for human relevance analysis of information on carcinogenic modes of action. *Crit Rev Toxicol*. 2003;33:591–653.

Mileson BE, Ferenc SA. Methods to identify and characterize developmental neurotoxicity for human health risk assessment: overview. *Environ Health Perspect*. 2001;109(suppl 1):77–78.

Miller GW, Jones DP. The nature of nurture: refining the definition of the exposome. *Toxicol Sci*. 2014;137:1–2.

Mitchel RE. Low doses of radiation are protective in vitro and in vivo: evolutionary origins. *Dose Response*. 2006;4:75–90.

Mohr LC, Rodgers JK, Silvestri GA. Glutathione *S*-transferase M1 polymorphism and the risk of lung cancer. *Anticancer Res*. 2003;23:2111–2124.

Monticello TM, Morgan KT. Chemically-induced nasal carcinogenesis and epithelial cell proliferation: a brief review. *Mutat Res*. 1997;380:33–41.

Moser VC. The functional observational battery in adult and developing rats. *Neurotoxicology*. 2000;21:989–996.

Mugford CA, Kedderis GL. Sex-dependent metabolism of xenobiotics. *Drug Metab Rev*. 1998;30:441–498.

Muller L, Mauthe RJ, Riley CM, et al. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. *Regul Toxicol Pharmacol*. 2006;44:198–211.

Myers NJ, Raffensberger C *Precautionary Tools for Reshaping Environmental Policy*. Cambridge, MA: MIT Press; 2006.

NAS/NRC. *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Washington, DC: National Academies Press; 2007.

National Academies of Sciences, Engineering, and Medicine. *Application of Systematic Review Methods in an Overall Strategy for Evaluating Low-Dose Toxicity from Endocrine Active Chemicals*. Washington, DC: National Academies Press; 2017.

Nault R, Fader KA, Lydic TA, Zacharewski TR. Lipidomic evaluation of aryl hydrocarbon receptor-mediated hepatic steatosis in male and female mice elicited by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Chem Res Toxicol*. 2017;30:1060–1075.

Neumaier T, Swenson J, Pham C, et al. Evidence for formation of DNA repair centers and dose-response nonlinearity in human cells. *Proc Natl Acad Sci U S A*. 2012;109:443–448.

NRC. *Health Risks from Exposure to Low Levels of Ionizing Radiation: BEIR VII Phase 2*. Washington, DC: National Academies Press; 2006.

NRC. Review of the Environmental Protection Agency's State-of-the-Science Evaluation of Nonmonotonic Dose-Response Relationships as they Apply to Endocrine Disruptors. Washington, DC: National Academies Press; 2014:1–65.

NTP. Available at: https://ntp.niehs.nih.gov/about/index.html. 2017a.

NTP. Available at: https://ntp.niehs.nih.gov/pubhealth/evalatm/acceptmethods/index.html. 2017b.

Nystrom M, Mutanen M. Diet and epigenetics in colon cancer. *World J Gastroenterol*. 2009;15:257–263.

Oda M, Koyanagi S, Tsurudome Y, Kanemitsu T, Matsunaga N, Ohdo S. Renal circadian clock regulates the dosing-time dependency of cisplatin-induced nephrotoxicity in mice. *Mol Pharmacol*. 2014;85:715–722.

OECD. *Test No. 406: Skin Sensitisation*. Paris: Organisation for Economic Cooperation and Development; 1992.

OECD. *Test No. 424: Neurotoxicity Study in Rodents*. Paris: Organisation for Economic Cooperation and Development; 1997.

OECD. *Test No. 405: Acute Eye Irritation/Corrosion*. Paris: Organisation for Economic Cooperation and Development; 2002.

OECD. *Test No. 426: Developmental Neurotoxicity Study*. Paris: Organisation for Economic Cooperation and Development; 2007.

OECD. *Test No. 429: Skin Sensitisation*. Paris: Organisation for Economic Cooperation and Development; 2010a.

OECD. *Test No. 442A and B: Skin Sensitization*. Paris: Organisation for Economic Cooperation and Development; 2010b.

OECD. *Test No. 443: Extended One Generation Reproductive Study*. Paris: Organisation for Economic Cooperation and Development; 2012a.

OECD. *Test No. 460: Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants*. Paris: Organisation for Economic Cooperation and Development; 2012b.

OECD. *Test No. 431: In Vitro Skin Corrosion: Reconstructed Human Epidermis (RHE) Test Method*. Paris: Organisation for Economic Cooperation and Development; 2013a.

OECD. *Test No. 438: Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*. Paris: Organisation for Economic Cooperation and Development; 2013b.

OECD. *Test No. 194: Guidance on Grouping of Chemicals, 2nd edition*. Paris: Organisation for Economic Cooperation and Development; 2014.

OECD. *Test No. 404: Acute Dermal Irritation/Corrosion*. Paris: Organisation for Economic Cooperation and Development; 2015a.

OECD. *Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*. Paris: Organisation for Economic Cooperation and Development; 2015b.

OECD. *Test No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*. Paris: Organisation for Economic Cooperation and Development; 2015c.

OECD. *Test No. 442C: In Chemico Skin Sensitisation*. Paris: Organisation for Economic Cooperation and Development; 2015d.

OECD. *Test No. 442D: In Vitro Skin Sensitisation*. Paris: Organisation for Economic Cooperation and Development; 2015e.

OECD. *Test No. 491: Short Time Exposure In Vitro Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*. Paris: Organisation for Economic Cooperation and Development; 2015f.

OECD. *Test No. 492: Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*. Paris: Organisation for Economic Cooperation and Development; 2015g.

OECD. *Test No. 442E: In Vitro Skin Sensitisation*. Paris: Organisation for Economic Cooperation and Development; 2016.

OECD. *Test No. 184: Revised Guidance Document on Developing and Assessing Adverse Outcome Pathways*. Paris: Organization for Economic Cooperation and Development; 2017.

Pamies D, Hartung T. 21st Century cell culture for 21st century toxicology. *Chem Res Toxicol*. 2017;30:43–52.

Pan RY, Dao RL, Hung SI, Chung WH. Pharmacogenomic advances in the prediction and prevention of cutaneous idiosyncratic drug reactions. *Clin Pharmacol Ther*. 2017. [Epub ahead of print].

Pan X, Whitten DA, Wu M, et al. Early phosphoproteomic changes in the mouse spleen during deoxynivalenol-induced ribotoxic stress. *Toxicol Sci*. 2013;135:129–143.

Patel CJ, Bhattacharya J, Butte AJ. An environment-wide association study (EWAS) on type 2 diabetes mellitus. *PLoS One*. 2010;5:e10746.

Pavlos R, Mallal S, Phillips E. HLA and pharmacogenetics of drug hypersensitivity. *Pharmacogenomics*. 2012;13:1285–1306.

Perera F, Herbstman J. Prenatal environmental exposures, epigenetics, and disease. *Reprod Toxicol*. 2011;31:363–373.

Persson M, Hornberg JJ. Advances in predictive toxicology for discovery safety through high content screening. *Chem Res Toxicol*. 2016;29:1998–2007.

Pitot HC, Goldsworthy TL, Moran S, et al. A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose-response relationships to altered hepatic foci. *Carcinogenesis*. 1987;8:1491–1499.

Raffensperger C, Tickner J. *Protecting Public Health and the Environment: Implementing the Precautionary Principle*. Washington, DC: Island Press; 1999.

Rappaport SM. Implications of the exposome for exposure science. *J Expo Sci Environ Epidemiol*. 2011;21:5–9.

Rappaport SM, Smith MT. Epidemiology. Environment and disease risks. *Science*. 2010;330:460–461.

Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J*. 2008;22:659–661.

Rhomberg LR, Goodman JE. Low-dose effects and nonmonotonic dose-responses of endocrine disrupting chemicals: has the case been made? *Regul Toxicol Pharmacol*. 2012;64:130–133.

Rhomberg LR, Wolff SK. Empirical scaling of single oral lethal doses across mammalian species based on a large database. *Risk Anal*. 1998;18:741–753.

Richeldi L, Sorrentino R, Saltini C. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. *Science*. 1993;262:242–244.

Roberts RA. Peroxisome proliferators: mechanisms of adverse effects in rodents and molecular basis for species differences. *Arch Toxicol*. 1999;73:413–418.

Rosenfeld CS. Animal models to study environmental epigenetics. *Biol Reprod*. 2010;82:473–488.

Roth RA, Ganey PE. Animal models of idiosyncratic drug-induced liver injury—current status. *Crit Rev Toxicol*. 2011;41:723–739.

Saitta KS, Zhang C, Lee KK, Fujimoto K, Redinbo MR, Boelsterli UA. Bacterial beta-glucuronidase inhibition protects mice against enteropathy induced by indomethacin, ketoprofen or diclofenac: mode of action and pharmacokinetics. *Xenobiotica*. 2014;44:28–35.

Scheving LE, Scheving LA, Feuers RJ, Tsai TH, Cope FO. Chronobiology as it relates to toxicology, pharmacology, and chemotherapy. *Regul Toxicol Pharmacol*. 1993;17:209–218.

Sharma V, McNeill JH. To scale or not to scale: the principles of dose extrapolation. *Br J Pharmacol*. 2009;157:907–921.

Siegel JA, Pennington CW, Sacks B. Subjecting radiologic imaging to the linear no-threshold hypothesis: a non sequitur of non-trivial proportion. *J Nucl Med*. 2017;58:1–6.

Simon T. Just who is at risk? The ethics of environmental regulation. *Hum Exp Toxicol*. 2011;30:795–819.

Simon TW, Simons SS Jr, Preston RJ, et al. The use of mode of action information in risk assessment: quantitative key events/dose-response framework for modeling the dose-response for key events. *Crit Rev Toxicol*. 2014;44(suppl 3):17–43.

Skinner MK. Role of epigenetics in developmental biology and transgenerational inheritance. *Birth Defects Res C Embryo Today*. 2011;93:51–55.

Slikker W Jr, Andersen ME, Bogdanffy MS, et al. Dose-dependent transitions in mechanisms of toxicity. *Toxicol Appl Pharmacol*. 2004a;201:203–225.

Slikker W Jr, Andersen ME, Bogdanffy MS, et al. Dose-dependent transitions in mechanisms of toxicity: case studies. *Toxicol Appl Pharmacol*. 2004b;201:226–294.

Smith LL, Brent RL, Cohen SM, et al. Predicting future human and environmental health challenges: the Health and Environmental Sciences Institute's scientific mapping exercise. *Crit Rev Toxicol*. 2008;38:817–845.

Smith MT, Guyton KZ, Gibbons CF, et al. Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. *Environ Health Perspect*. 2016;124:713–721.

Sordillo JE, Kelly R, Bunyavanich S, et al. Genome-wide expression profiles identify potential targets for gene-environment interactions in asthma severity. *J Allergy Clin Immunol*. 2015;136:885–892, e882.

Sturla SJ, Boobis AR, FitzGerald RE, et al. Systems toxicology: from basic research to risk assessment. *Chem Res Toxicol*. 2014;27:314–329.

Sukata T, Uwagawa S, Ozaki K, et al. Detailed low-dose study of 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane carcinogenesis suggests the possibility of a hormetic effect. *Int J Cancer*. 2002;99:112–118.

Sundseth SS, Alberta JA, Waxman DJ. Sex-specific, growth hormone-regulated transcription of the cytochrome P450 2C11 and 2C12 genes. *J Biol Chem*. 1992;267:3907–3914.

Suntharalingam G, Perry MR, Ward S, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med*. 2006;355:1018–1028.

Suter-Dick L, Alves PM, Blaauboer BJ, et al. Stem cell-derived systems in toxicology assessment. *Stem Cells Dev*. 2015;24:1284–1296.

Szyf M. The dynamic epigenome and its implications in toxicology. *Toxicol Sci*. 2007;100:7–23.

Takeno S, Sakai T. Involvement of the intestinal microflora in nitrazepam-induced teratogenicity in rats and its relationship to nitroreduction. *Teratology*. 1991;44:209–214.

Tarapore P, Ying J, Ouyang B, et al. Exposure to bisphenol A correlates with early-onset prostate cancer and promotes centrosome amplification and anchorage-independent growth in vitro. *PLoS One*. 2014;9:e90332.

Thomas D. Gene–environment-wide association studies: emerging approaches. *Nat Rev Genet*. 2010;11:259–272.

Travis CC, White RK. Interspecific scaling of toxicity data. *Risk Anal*. 1988;8:119–125.

Travis RC, Balkwill A, Fensom GK, et al. Night shift work and breast cancer incidence: three prospective studies and meta-analysis of published studies. *J Natl Cancer Inst*. 2016;108(12):pii: djw169.

Tubiana M. Dose-effect relationship and estimation of the carcinogenic effects of low doses of ionizing radiation: the joint report of the Academie des Sciences (Paris) and of the Academie Nationale de Medecine. *Int J Radiat Oncol Biol Phys*. 2005;63:317–319.

Uetrecht J. Idiosyncratic drug reactions: current understanding. *Annu Rev Pharmacol Toxicol*. 2007;47:513–539.

Uetrecht J, Naisbitt DJ. Idiosyncratic adverse drug reactions: current concepts. *Pharmacol Rev*. 2013;65:779–808.

UK-ILGRA. The precautionary principle: policy and application. Available at United Kingdom Inter-Departmental Liaison Group on Risk Assessment website at http://www.hse.gov.uk/aboutus/meetings/committees/ilgra/pppa.htm; 2007.

Vandenberg LN, Colborn T, Hayes TB, et al. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev*. 2012;33:378–455.

Villeneuve DL, Crump D, Garcia-Reyero N, et al. Adverse outcome pathway (AOP) development. I: Strategies and principles. *Toxicol Sci*. 2014a;142:312–320.

Villeneuve DL, Crump D, Garcia-Reyero N, et al. Adverse outcome pathway development. II: Best practices. *Toxicol Sci*. 2014b;142:321–330.

Villeneuve DL, Garcia-Reyero N. Vision & strategy: predictive ecotoxicology in the 21st century. *Environ Toxicol Chem*. 2011;30:1–8.

Wallace BD, Wang H, Lane KT, et al. Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. *Science*. 2010;330:831–835.

Wang Y, Bryant SH, Cheng T, et al. PubChem BioAssay: 2017 update. *Nucleic Acids Res*. 2017;45:D955–D963.

Waters MD, Fostel JM Toxicogenomics and systems toxicology: aims and prospects. *Nat Rev Genet*. 2004;5:936–948.

Watson RE, Goodman JI. Epigenetics and DNA methylation come of age in toxicology. *Toxicol Sci*. 2002;67:11–16.

Waxman DJ, Pampori NA, Ram PA, Agrawal AK, Shapiro BH. Interpulse interval in circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450. *Proc Natl Acad Sci U S A*. 1991;88:6868–6872.

Weinstein H, Rabinowitz J, Liebman MN, Osman R. Determinants of molecular reactivity as criteria for predicting toxicity: problems and approaches. *Environ Health Perspect*. 1985;61:147–162.

Weston AD, Hood L. Systems biology, proteomics, and the future of health care: toward predictive, preventative, and personalized medicine. *J Proteome Res*. 2004;3:179–196.

Wild CP. Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. *Cancer Epidemiol Biomarkers Prev*. 2005;14:1847–1850.

Williams DE. The rainbow trout liver cancer model: response to environmental chemicals and studies on promotion and chemoprevention. *Comp Biochem Physiol C Toxicol Pharmacol*. 2012;155:121–127.

Williams ES, Panko J, Paustenbach DJ. The European Union's REACH regulation: a review of its history and requirements. *Crit Rev Toxicol*. 2009;39:553–575.

Wilson NH, Hardisty JF, Hayes JR. Short-term, subchronic and chronic toxicology studies. In: Hayes AW, ed. *Principles and Methods of Toxicology*. New York: Informa Healthcare; 2008;1223–1264.

Wogan GN, Paglialunga S, Newberne PM. Carcinogenic effects of low dietary levels of aflatoxin B1 in rats. *Food Cosmet Toxicol*. 1974;12:681–685.

Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D. Cellular imaging predictions of clinical drug-induced liver injury. *Toxicol Sci*. 2008;105:97–105.

Yoon M, Campbell JL, Andersen ME, Clewell HJ. Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results. *Crit Rev Toxicol*. 2012;42:633–652.

Youngson NA, Whitelaw E. Transgenerational epigenetic effects. *Annu Rev Genomics Hum Genet*. 2008;9:233–257.

Zhang L, Nichols RG, Correll J, et al. Persistent organic pollutants modify gut microbiota-host metabolic homeostasis in mice through aryl hydrocarbon receptor activation. *Environ Health Perspect*. 2015;123:679–688.

Zhang X, Ho SM. Epigenetics meets endocrinology. *J Mol Endocrinol*. 2011;46:R11–R32.

chapter 3

Mechanisms of Toxicity

Lois D. Lehman-McKeeman

Step 1: Delivery to the Target

- Absorption and Presystemic Elimination
- Distribution of Toxicants Occurs by Passive or Active Processes
- Elimination Processes Affect Delivery of Toxicants
- The Balance between Metabolic Activation and Metabolic Detoxification Reactions Contributes to Toxicity
  - *Formation of Electrophiles*
  - *Formation of Free Radicals*
  - *Formation of Nucleophiles*
  - *Formation of Redox-Active Reactants*
  - *Detoxification*
  - *Detoxification of Electrophiles*
  - *Detoxification of Free Radicals*
  - *Detoxification of Nucleophiles*
  - *Detoxification of Toxicants with No Functional Groups*
  - *Detoxification of Protein Toxins*
  - *When Detoxification Fails*

Step 2: Reaction of the Ultimate Toxicant with the Target Molecule

- Attributes of Target Molecules
- Types of Reactions
  - *Noncovalent Binding*
  - *Covalent Binding*
  - *Hydrogen Abstraction*
  - *Electron Transfer*
  - *Enzymatic Reactions*
- Effects of Toxicants on Target Molecules
  - *Dysfunction of Target Molecules*
  - *Destruction of Target Molecules*
  - *Neoantigen Formation*
- Toxicity Not Initiated by Reaction with Target Molecules
- Idiosyncratic Toxicity: When No Mechanism Explains the Outcome

Step 3: Cellular Dysfunction and Resultant Toxicities

- Toxicant-Induced Cellular Dysregulation
  - *Altered Gene Expression*
  - *Dysregulation of Transcription*
  - *Dysregulation of Signal Transduction*
  - *Dysregulation of Extracellular Signal Production*
  - *Dysregulation of Electrically Excitable Cells*
  - *Alteration in Neurotransmitter Levels*
  - *Toxicant–Neurotransmitter Receptor Interactions*
  - *Toxicant–Signal Transducer Interactions*
  - *Toxicant–Signal Terminator Interactions*
  - *Dysregulation of the Activity of Other Cells*
- Toxic Alteration of Cellular Maintenance
- Mechanisms of Toxic Cell Death
  - *Primary Metabolic Disorders Jeopardizing Cell Survival*
  - *Mitochondrial Permeability Transition and Necrotic Cell Death*
  - *Other Mechanisms of Cell Death*
  - *Apoptosis*
  - *Necroptosis*
  - *Ferroptosis*
  - *What Determines the Form of Cell Death?*
  - *Induction of Cell Death by Unknown Mechanisms*

Step 4: Inappropriate Repair and Adaptation

- Mechanisms of Repair
  - *Molecular Repair*
  - *Cellular Repair*
  - *Tissue Repair*
- Mechanisms of Adaptation
  - *Adaptation by Decreasing Delivery to the Target*
  - *Adaptation by Decreasing the Target Density or Responsiveness*
  - *Adaptation by Increasing Repair*
- Final and Irreversible Actions of Repair and Adaptation Failure
  - *When Repair Fails*
  - *When Adaptation Fails*
- Toxicity Resulting from Inappropriate Repair and Adaptation
  - *Tissue Necrosis*
- Fibrosis
- Carcinogenesis

Research Approaches to Determine Mechanisms of Toxicity: A Case Study

Conclusions

- Mechanisms of Toxicity and Adverse Outcome Pathways
- The Importance of Mechanisms of Toxicity

Acknowledgments

References

Mechanisms of toxicity describe how an adverse effect occurs. Such events involve many molecular, biochemical, and cellular processes that may act in isolation or in a complex combination to produce a given response. Given the multitude of cells, organs, and pathways that underlie toxic mechanisms, it is unrealistic to discuss every possible mechanism of toxicity in this chapter. Such detailed information is provided in many of the chapters that follow, where emphasis is on a specific organ and the processes that render that organ sensitive or resistant to toxic insult. Rather, the focus of this chapter is to establish the fundamental concepts for how toxicity occurs, to illustrate how to apply these concepts to understanding mechanisms of toxicity, and to give examples of mechanisms of toxicity. Mechanistic toxicology data are also useful for developing more predictive biomarkers of toxicity, developing approaches to

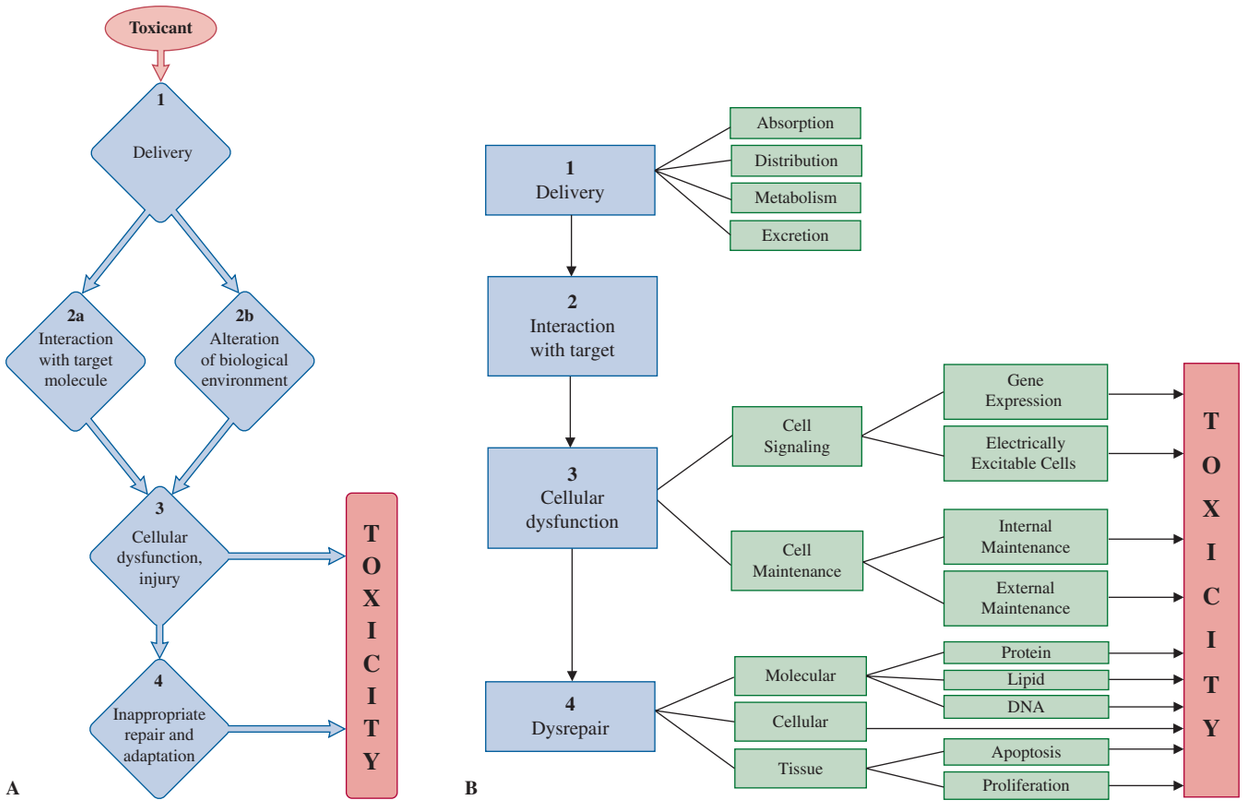

Figure 3-1. *Overview of critical events that contribute to the development of toxicity after chemical exposure.* These fundamental processes serve as the basis for the organization of this chapter. Panel A shows more details of critical events underlying the four major processes outlined in this chapter. Panel B shows more details of the mechanistic events involved in toxic outcomes.

antagonize or prevent toxicity, and gaining insight into fundamental physiologic, biochemical, and molecular processes that underlie normal and abnormal organ function. Knowledge of mechanisms of toxicity is also fundamental for developing risk assessments for chemical exposure, as such data are relevant to determining the likelihood that chemical exposure may cause harmful effects.

Given the sheer number of potential toxicants and the multitude of biological structures and processes that can be impaired, there are a tremendous number of possible toxic effects. Correspondingly, there are many pathways that may lead to toxicity. However, the chapter is structured to demonstrate the course followed from the delivery of the toxicant to its target, to the reactions within the target, and the resultant cellular dysfunction that manifests in toxicity. In this regard, mechanisms of toxicity can be simplified visually to a four-step process depicted in Fig. 3-1, and these steps, encompassing (1) delivery of the toxicant to its target; (2) interactions between the toxicant and its target or the microenvironment; (3) progression to cellular dysfunction; and (4) inappropriate repair or adaptation, serve as the organizational structure of this chapter. As a simple example of this scheme, the toxicity of tetrodotoxin, the puffer fish poison, is manifested by direct interaction with voltage-gated Na^+ channels of neurons, and it causes skeletal muscle paralysis by blocking these channels. In this example, the delivery and interaction of the toxicant with its target are direct, and there are no repair mechanisms that can reduce or prevent the toxicity. In contrast to the tetrodotoxin example, a more complex path to toxicity involves all four steps outlined in Fig. 3-1A and expanded in Fig. 3-1B. Target delivery perturbs cell function and/or structure to initiate repair mechanisms at the molecular, cellular, and/or tissue levels, and adaptive mechanisms may develop to diminish delivery, boost repair capacity, and/or compensate for dysfunction. In the most complex example, even efforts to repair injury can be overwhelmed, with serious, irreversible consequences including cell death or chronic changes like fibrosis or cancer.

STEP 1: DELIVERY TO THE TARGET

Exposure at the target site is an absolute requirement for any mechanism of toxicity. Xenobiotic disposition, defined as the absorption, distribution, biotransformation, and elimination of a toxicant, is a critical determinant of target organ toxicity along with sensitivity of target sites to toxicity. Moreover, the kinetics that describe these processes contributes to the severity of a toxic response. The fundamental principles of disposition, biotransformation, and kinetics are discussed in detail in other chapters (Chaps. 5, 6, and 7, respectively), but an overview is provided here with emphasis on how they contribute to toxic mechanisms. Fig. 3-2 also illustrates how these processes can increase or decrease toxicity.

Absorption and Presystemic Elimination

Absorption is defined as the process by which toxicants cross body membranes to enter the systemic circulation. It can occur from many sites, including the gastrointestinal tract, skin, or lungs, reflecting oral, dermal, or inhalation exposure, respectively. Absorption occurs along the entire length of the gastrointestinal

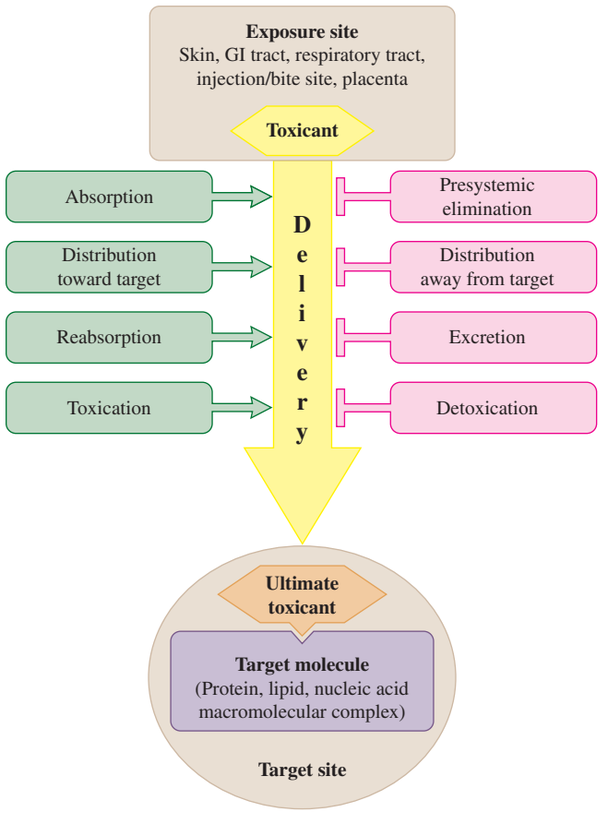

Figure 3-2. *The process of toxicant delivery to the target is the first step in the development of toxicity.* Factors illustrated on the left increase delivery to the target, whereas those on the right denote reduced delivery to the target.

tract, and is usually determined by the extent to which the compound exists in its nonionized, lipid-soluble form. In the skin, the stratum corneum layer of the epidermis is a major barrier to absorption, but once through this barrier, compounds are typically absorbed by diffusion into the venous or lymphatic capillaries to enter the systemic circulation. In the lungs, gases and vapors diffuse quickly through the alveolar space and into the bloodstream. In contrast, aerosols and particles are absorbed throughout the lung based on their size and water solubility. Larger particles will be deposited in the upper regions of the tracheobronchiolar regions, and only the smallest particles can penetrate all the way into the alveolar sac. In all organs, the rate of absorption is determined by the concentration at the site of exposure, rate of dissolution of the chemical, the total area that is exposed, the vascular composition of the region, and physicochemical properties of the toxicant.

Presystemic elimination is a process by which a toxicant is eliminated prior to reaching the systemic circulation. In the case of the gastrointestinal tract, the epithelium may eliminate or modify a compound directly. This is the case for morphine which is conjugated with glucuronic acid in the small intestine. Additionally, many toxicants are absorbed from the gastrointestinal tract directly into the liver from the portal circulation where they are modified by biotransformation enzymes and excreted into bile. This is also referred to as first-pass metabolism, a process that reduces systemic exposure to a toxicant. At the same time, if a toxicant targets the liver, a significant first-pass effect will increase toxicity, as is the case for the mushroom-derived toxicant, α-amanitin. One approach to assessing the contribution of first-pass metabolism to toxicity is to administer the compound by intraperitoneal (IP) injection. Any compound that has a significant first-pass metabolism and targets organs other than liver will be much less toxic when administered by IP injection because it will be taken up by the mesenteric veins and delivered to the liver which will limit systemic exposure. In contrast, toxicants that affect the liver will likely become more toxic when injected by the IP route, whereas those compounds lacking significant first-pass metabolism would not show differential toxicity based on the route of exposure.

There are also examples of presystemic elimination with respect to the skin and lungs. In the skin, the stratum corneum is not only a barrier but also, as a highly lipophilic layer, often limits the absorption of highly lipophilic compounds such as tetrachloro-p-dibenzodioxin (TCDD). In the respiratory tree, the nasal mucosa often absorbs water-soluble compounds to prevent them from reaching the lung. For example, formaldehyde is highly retained in the nasal mucosa so that, despite its overall volatility, the nasal mucosa (rather than the lungs) is the predominant organ of toxicity.

Distribution of Toxicants Occurs by Passive or Active Processes

Tissue distribution is the process by which a toxicant reaches its target site. There are four properties that facilitate distribution to a target. These include the (1) porosity of the capillary endothelium, (2) presence of specialized transport processes, (3) potential for accumulation within cellular organelles, and (4) binding to proteins or other macromolecules.

The porosity of the capillary epithelium enables passive diffusion of toxicants. In particular, the capillaries of the liver (sinusoids) and kidney (peritubular capillaries) have relative large fenestrae (50 to 150 nm) that enable ready passage of even protein-bound compounds. In contrast, the capillaries that form the blood–brain barrier are joined tightly and lack fenestrae to prevent significant distribution of hydrophilic compounds into the brain. Similarly, the seminiferous tubules of the testis are protected by a blood–testis barrier.

There are many specialized ion channels and transporters that influence xenobiotic distribution. For example, voltage-gated Ca^{2+} ion channels enable passage of cations such as lead into cells, and lithium (Li^{+}) can enter cells through both voltage-gated and epithelial Na^{+} channels. Transporter proteins regulate uptake and efflux of many xenobiotics and endogenous substrates. There are at least 52 families of transporters identified in the human genome, representing nearly 400 unique proteins which function in energy-dependent processes to transport organic cations, organic anions, amino acids, nucleosides, and bile acids as substrates (Hediger et al., 2013). The role of these proteins in toxicant disposition is discussed extensively in Chap. 5, but examples are provided here to illustrate how transporters determine target organs of toxicity. The mushroom toxin, α-amanitin, is taken up selectively in the liver by the Na-dependent bile acid transporter (sodium taurocholate cotransporting polypeptide, NTCP), whereas organic anion-transporting polypeptide (Oatp) 1B1/1B3 or its mouse homolog, Oatp1b2, controls hepatic uptake of microcystin, a toxin produced by blue-green algae. Genetically modified Oatp1b2-null mice are resistant to microcystin toxicity because the toxicant is not delivered into the liver efficiently. Additionally, the renal toxicity of ochratoxin is mediated by transport into renal tubules by organic anion transporters (Oat1 and Oat3), and similarly, renal toxicity of the antiviral drug adefovir is determined by OAT1-mediated uptake. Cisplatin and metformin are transported into cells by organic cation transporters (OCTs) including OCT1 in liver and OCT2 in kidney (see Chap. 5).

Efflux transporters often reduce toxicity by removing toxicants from cells. In fact, one of the earliest transporters identified was

the multidrug resistance protein-1 (MRP1), a transporter found to be upregulated and associated with resistance to chemotherapy in cancer cells. MRP1 is an efflux pump that transports chemotherapeutic drugs out of cancer cells, an action that limits toxicity along with its efficacy. However, in brain, P-glycoprotein (MDR1) protects against toxicity by pumping toxicants out of the brain. In a classical example, mice with a spontaneously occurring mutation of the *mdr1* gene exhibited significantly greater toxicity to ivermectin, a neurotoxic pesticide and antihelmintic drug (Schinkel, 1999), because these mice could not efficiently remove the toxicant from the brain.

In the examples above, xenobiotic transporters directly influence toxicity. However, some compounds that inhibit transporter activity may not be directly toxic, but rather alter the disposition of other toxicants or endogenous substrates to cause toxicity. For example, a drug that inhibits OATP1B1 may alter the disposition of other OATP substrates such as statins and increase the potential for statin-induced myopathy (Maeda, 2015). Similarly, inhibition of the bile salt exporting polypeptide (BSEP) can alter the normal biliary excretion of bile acids to increase the risk of cholestasis (Rodrigues et al., 2014).

Physicochemical properties of toxicants often contribute to their accumulation in cells or cellular organelles. For example, amphipathic molecules, defined as those containing hydrophilic and hydrophobic portions, can accumulate in lysosomes or mitochondria to produce toxicity. Accumulation in lysosomes usually occurs by a pH-dependent trapping mechanism leading to lysosomal changes such as phospholipidosis (described below). MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxicant that accumulates in mitochondria. As a highly lipophilic molecule, MPTP can cross the blood–brain barrier where it is metabolized to MPP^+ (1-methyl-4-phenylpyridinium), a cation that is trapped in the mitochondria of dopaminergic neurons. The MPP^+ ion is the ultimate toxicant as it inhibits complex I of the electron transport chain (coenzyme Q:cytocrome c oxidoreductase; see Fig. 3-18) in mitochondrial metabolism resulting in dysfunction and ultimately to cell death and neurodegeneration that resembles Parkinson disease–like symptoms.

Some chemicals accumulate in tissue storage sites that are not the major organ of toxicity and thereby prevent toxicity. For example, lead is deposited in bone where it substitutes for calcium in hydroxyapatite without adversely affecting bone, and highly lipophilic compounds such as chlorinated hydrocarbon insecticides will concentrate in fat (adipocytes), where they are generally nontoxic. Although such storage limits toxicity, any condition that causes these stored toxicants to be released or mobilized from the storage site will increase the likelihood of toxicity. For example, if fat stores are mobilized as occurs with fasting, fat-soluble insecticides that concentrate in adipose can be released and distributed to the nervous system, their ultimate toxic site.

Finally, binding to proteins can influence distribution and target organ toxicity. Keratins are highly abundant proteins in skin and hair, and with a high level of cysteine residues, keratins sequester thiol-reactive metal ions including arsenic and mercury. Often, nail and hair contents of arsenic are used to estimate exposure to this toxic metal, and any release from the keratinocytes may lead to adverse skin lesions. In direct contrast, the metal-binding protein metallothionein binds numerous heavy metals with very high affinity and protects against the toxicity of metals such as cadmium (discussed under Adaptive Repair).

Elimination Processes Affect Delivery of Toxicants

The route and rate of excretion largely depend on the physicochemical properties of the toxicant. The major excretory organs for non-volatile compounds are the liver and kidney, and both organs are most efficient at removing water-soluble, usually ionized compounds. Important considerations for excretion of hydrophilic, ionized compounds include (1) compounds ($<$60 kDa) must be dissolved in plasma water to be filtered through the renal glomerulus; (2) transporters in liver or kidney are highly specialized to secrete and enhance excretion of water-soluble organic anions or cations; and (3) only water-soluble compounds are readily excreted in urine or bile.

The excretion of non-volatile highly lipophilic compounds is much less efficient than that of water-soluble compounds. Lipophilic compounds are readily absorbed by transcellular diffusion, and many lipophilic compounds are eliminated slowly allowing for accumulation in the body with repeated exposure. Examples of such compounds include polyaromatic hydrocarbons, polyhalogenated biphenyls, and chlorinated hydrocarbon insecticides. Biotransformation may facilitate elimination of these compounds, but other processes for excretion of highly lipophilic compounds include excretion in the bile (in a lipid or phospholipid micelle), secretion directly into the intestinal contents, and excretion in milk lipids in mammary glands. In contrast, volatile lipophilic compounds diffuse through pulmonary capillaries and be excreted in expired air.

Whereas excretory pathways reduce toxicant exposure, some toxicants are reabsorbed after initial elimination steps and this reabsorption may increase total exposure to the toxicant. In the kidney, reabsorption mechanisms are highly pH-dependent, as the generally acidic milieu of urine favors reabsorption of weak acids. However, altering urine pH can also be used to enhance excretion and thereby reduce toxicity. Acidification of urine favors the excretion of weak bases, whereas urinary alkalization will favor excretion of weak acids.

Toxicants delivered to the gastrointestinal tract by biliary excretion, intestinal secretion, or secretion from salivary glands and the pancreas are typically absorbed by diffusion across the intestinal mucosal. Enterohepatic circulation is a cycle in which a compound is excreted into bile to enter the intestine where it is modified to facilitate intestinal reabsorption rather than fecal elimination. This initiates a cycle in which the compound is reabsorbed in the intestine, only to return to the liver where it is again excreted into bile, returns to the intestine, and is again reabsorbed. This cycle is frequently observed with glucuronide conjugates because conjugation favors elimination into bile, with subsequent hydrolysis of the glucuronide by β-glucuronidase activity of intestinal microorganisms, and the resulting aglycones are reabsorbed. Enterohepatic circulation is especially important in bile acid homeostasis as nearly 95% of the bile acids in the gut return to the liver, and it is estimated that bile acids recycle about 20 times before being eliminated (Gonzalez, 2012).

Some glutathione (GSH) conjugates that are formed in liver are toxic to the kidney following hydrolysis of the GSH tripeptide by intestinal or pancreatic peptidases to liberate cysteine conjugated metabolites. Ultimately, the cysteine conjugates are further activated in the kidney to highly toxic moieties (Anders, 2004). The renal toxicity of trichloroethylene and hexachlorobutadiene occurs following GSH conjugation and illustrates this mechanism of reabsorption and activation of GSH conjugates (see Chap. 14).

The Balance between Metabolic Activation and Metabolic Detoxification Reactions Contributes to Toxicity

Although some xenobiotics are directly toxic, many others produce toxicity through metabolites formed following exposure. Table 3-1 provides examples of compounds that are either directly toxic or

Table 3-1

Types of Ultimate Toxicants and Their Sources

Direct-Acting Toxicants		
Pb ions		Methylisocyanate
Tetrodotoxin		HCN
TCDD		CO
Toxicants Requiring Metabolic Activation		
Amygdalin	→	HCN
Arsenate	→	Arsenite
Fluoroacetate	→	Fluorocitrate
Ethylene glycol	→	Oxalic acid
D-Limonene	→	D-Limonene-1,2-epoxide
Hexane	→	2,5-Hexanedione
Acetaminophen	→	*N*-Acetyl-*p*-benzoquinoneimine
CCl_4	→	$CCl_3OO^{\bullet}$
Benzo[*a*]pyrene (BP)	→	BP-7,8-diol-9,10-epoxide
4-Ipomeanol	→	Enedial intermeadiate
Toxicants that Generate Reactive Oxygen or Nitrogen species		
Hydrogen peroxide; diquat, doxorubicin, nitrofurantion Cr(V), Fe(II), Mn(II), Ni(II)	→	Hydroxyl radical ($HO^{\bullet}$)
Paraquat $\rightarrow O_2^{\bullet-} + NO^{\bullet} +$	→	Peroxynitrite ($ONOO^-$)
Endogenous Compounds as Ultimate Toxicants		
Sulfonamides displace bilirubin	→	Bilirubin
$CCl_3OO^{\bullet}$ → unsaturated fatty acids	→	Lipid peroxyl or alkoxyl radicals and 4-hydroxynon-2-enal
$HO^{\bullet}$ → proteins	→	Protein carbonyls

require metabolic activation, along with a brief summary of the mechanism of toxicity that develops. Biotransformation of xenobiotics that increases toxicity is referred to as metabolic activation or a toxification process. Metabolic activation is an important mechanism of toxicity because it generates intermediates that adversely affect the microenvironment, alter cellular macromolecules and organelles, and/or stress endogenous protective mechanisms. As examples of these adverse outcomes, oxalic acid formed from ethylene glycol alters the microenvironment by causing systemic acidosis and hypocalcaemia and can obstruct renal tubules if it precipitates as calcium oxalate crystals. The organophosphate insecticide parathion is oxidized to paraoxon and directly alters protein function by inhibiting acetylcholinesterase activity, and the rodenticide fluoroacetate is converted to fluorocitrate, a false substrate that inhibits aconitase. A final example is fialuridine, an antiviral drug that was withdrawn from clinical development because it was metabolized to a triphosphate metabolite that inhibited mitochondrial DNA synthesis by direct inhibition of DNA polymerase-γ. By this mechanism, fialuridine caused profound liver toxicity and in some cases death.

Another major feature of metabolic activation is that it often explains target organ specificity of a toxic outcome or contributes to species differences in toxicity. As an example, the fungal-derived toxicant, 4-ipomeanol, is metabolized to a reactive intermediate that in rodents is catalyzed by a cytochrome P450 enzyme, CYP4B1, an enzyme present in high levels in the lung. As a result, the reactive metabolite is mainly formed in rodent lungs where marked bronchiolar toxicity is observed. In contrast, ipomeanol is hepatotoxic in humans because it is metabolically activated in the liver rather than the lung (Parkinson et al., 2016). In this example, the same reactive metabolite causes toxicity, but species differences in the expression of the enzymes involved in forming the toxic metabolite determine the target organ affected.

Formation of Electrophiles An electrophile is a molecule containing an electron-deficient atom with a partial or full positive charge that allows it to react by accepting electron pairs from electron-rich atoms in nucleophiles. Such reactants are often produced by insertion of an oxygen atom, which withdraws electrons from the atom it is attached to, making that atom electrophilic. This is the case when aldehydes, ketones, epoxides, arene oxides, sulfoxides, nitroso compounds, phosphonates, and acyl halides are formed. Consequently, a major outcome of metabolic activation is that electrophiles are often formed, and these metabolites are commonly involved in mechanisms of toxicity. Formation of many of these metabolites is catalyzed by cytochrome P450s (Chap. 6 provides extensive details and examples). Table 3-2 is also a broad list of toxic electrophilic metabolites, with details on the metabolic enzymes involved in their formation.

Cationic electrophiles may be produced as a result of heterolytic bond cleavage. For example, 7,12-dimethylbenzanthracene and 2-acetylaminofluorene, are hydroxylated to form benzylic alcohols and *N*-hydroxy arylamines (amides), respectively. These metabolites are then esterified, typically by sulfotransferases. Heterolytic cleavage of the C–O or N–O bonds of these esters results in a hydrosulfate anion and the concomitant formation of a benzylic carbonium ion or arylnitrenium ion, respectively (see Chap. 6). The antiestrogen tamoxifen undergoes similar activation by hydroxylation and sulfation to form a carbocationic metabolite. Dimethylnitrosamine (DMN) is C-hydroxylated by CYP2E1, after which it undergoes spontaneous decomposition and heterolytic cleavage of the C–N bond to form a methyl carbonium cation ($^+CH_3$), and this reactive metabolite is the penultimate species involved in carcinogenic effects of DMN. In a similar process, the tobacco-specific nicotine-derived nitrosamine ketone [(methylnitrosamino)-1-(3-pyridyl)-1-butanone; referred to as NNK], the most potent carcinogen in tobacco, may generate either methyl carbonium or piridyloxobutyl carbonium cations, depending on the site of C-hydroxylation that initiates its decomposition.

Formation of Free Radicals A free radical is a molecule or molecular fragment that contains one or more unpaired electrons in its outer orbital. Radicals are formed by three major actions, namely (1) accepting an electron, (2) losing an electron, or (3) homolytic fission of a covalent bond. These three reactions are summarized here.

1. ***Free radicals formed by accepting electrons***: Fig. 3-3 illustrates the cycle by which xenobiotics can accept an electron from cellular reductases (such as cytochrome P450 reductase) to generate a free radical. In this figure, examples of paraquat and nitrofurantoin are presented. Once formed, free radicals typically transfer the extra electron to molecular oxygen, forming a superoxide anion radical ($O_2^{\bullet-}$) and regenerating the parent xenobiotic, which is ready to gain a new electron. This cycle, referred to as "redox cycling," ultimately amplifies the toxic response because one electron acceptor xenobiotic molecule can generate many $O_2^{\bullet-}$ molecules. The toxicity of $O_2^{\bullet-}$ results

Table 3-2

Toxicity Resulting from Formation of Electrophilic Metabolites

ELECTROPHILIC METABOLITE	PARENT TOXICANT	ENZYMES CATALYZING TOXICATION	TOXIC EFFECT
Nonionic electrophiles			
Aldehydes, ketones			
Acetaldehyde	Ethanol	ADH	Hepatotoxicity, cirrhosis
Zomepirac glucuronide (acylglucuronide)	Zomepirac	GT and isomerization	Multiple organs
2,5-Hexanedione	Hexane	CYP	Axonopathy
α,β-Unsaturated aldehydes, ketones			
Acrolein	Allyl alcohol	ADH	Hepatic necrosis
Acrolein	Allyl amine	MAO	Vascular injury
Acrolein	Cyclophosphamide	CYP → sp. cl.	Hemorrhagic cystitis
Muconic aldehyde	Benzene	Multiple	Bone marrow injury
4-Hydroxynon-2-enal	Fatty acids	Lipid peroxidation	Oxidative stress
Quinones, quinoneimines			
DES-4,4′-quinone	Diethylstilbestrol	Peroxidases	Carcinogenesis
N-Acetyl-*p*-benzoquinoneimine	Acetaminophen	CYP, peroxidases	Hepatic necrosis
Epoxides, arene oxides			
Aflatoxin B_1 8,9-epoxide	Aflatoxin B_1	CYP	Carcinogenesis
2-Chlorooxirane	Vinyl chloride	CYP	Carcinogenesis
Bromobenzene 3,4-oxide	Bromobenzene	CYP	Hepatic necrosis
Benzo[*a*]pyrene 7,8-diol 9,10-oxide	Benzo[*a*]pyrene	CYP	Carcinogenesis
Sulfoxides			
Thioacetamide *S*-oxide	Thioacetamide	FMO	Hepatic necrosis
Nitroso compounds			
Nitroso-sulfamethoxazole	Sulfamethoxazole	CYP	Immune reaction
Phosphonates			
Paraoxon	Parathion	CYP	ChE inhibition
Acyl halides			
Phosgene	Chloroform	CYP	Hepatic necrosis
Trifluoroacetyl chloride	Halothane	CYP	Immune hepatitis
Thionoacyl halides			
2,3,4,4-Tetrachlorothiobut-3-enoic acid chloride	Hexachlorobutadiene	Multiple enzymes GST → GGT → DP → CCβL	Renal tubular necrosis
Thioketenes			
Chloro-1,2,2-trichlorovinyl-thioketene	Hexachlorobutadiene	Multiple enzymes GST → GGT → DP → CCβL	Renal tubular necrosis
Cationic electrophiles			
Carbonium ions			
Benzylic carbocation	7,12-Dimethylbenz-anthracene	CYP → ST	Carcinogenesis
Carbonium cation	Diethylnitrosamine	CYP and spontaneous rearrangement	Carcinogenesis
Nitrenium ions			
Arylnitrenium ion	AAF, DMAB	CYP → ST	Carcinogenesis
Sulfonium ions			
Episulfonium ion	1,2-Dibromoethane	GST	Carcinogenesis
Metal ions			
Mercury(II) ion	Elemental Hg	Catalase	Brain injury
Diaquo-diamino platinate(II)	Cisplatinum	Spontaneous rearrangement	Renal tubular necrosis

Abbreviations: AAF, 2-acetylaminofluorene; ADH, alcohol dehydrogenase; CCβL, cysteine conjugate β-lyase; ChE, cholinesterase; CYP, cytochrome P450; DMAB, N,N-dimethyl-4-aminoazobenzene; DP, dipeptidase; FMO, flavin-containing monooxygenase; GT, UDP-glucuronosyltransferase; GGT, gamma-glutamyltransferase; GST, glutathione S-transferase; ST, sulfotransferase.

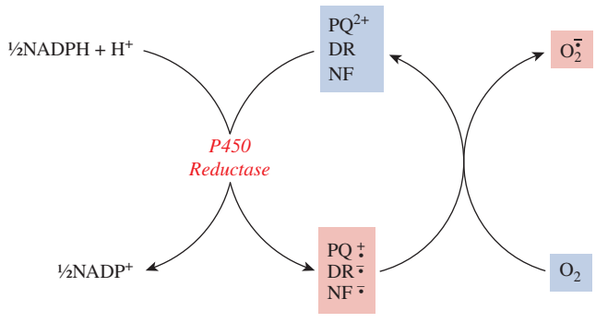

Figure 3-3. *Production of superoxide anion radical ($O_2^{\bullet-}$) by paraquat (PQ^{2+}), doxorubicin (DR), and nitrofurantoin (NF).* Note that formation of $O_2^{\bullet-}$ is not the final step in the toxicity of these xenobiotics, because $O_2^{\bullet-}$ can yield the much more reactive hydroxyl radical (depicted in Fig. 3-4).

from the fact that it is the initial compound in two important toxicity pathways, namely (1) formation of hydrogen peroxide (HOOH) and then hydroxyl radical ($HO^{\bullet}$) and (2) formation of peroxynitrite ($ONOO^-$) and ultimately nitrogen dioxide ($^{\bullet}NO_2$) and carbonate anion radical ($CO_2^{\bullet-}$).

There are also endogenous sources of $O_2^{\bullet-}$. This radical is generated in large quantities by NADPH oxidase (Nox) in activated macrophages and granulocytes during "respiratory burst," and in smaller quantities by many other cells, such as endothelial cells and vascular smooth muscle cells (DiMeo et al., 2016). $O_2^{\bullet-}$ is also produced by the mitochondrial electron transport chain, especially by complexes I and III (coenzyme Q: cytochrome *c* oxidoreductase and NADH:ubiquinone oxidoreductase, respectively).

2. ***Free radicals generated by losing electrons***: Nucleophiles, which generate free radicals when they lose an electron, include compounds such as phenols, hydroquinones, aminophenols, aromatic amines, hydrazines, phenothiazines, and thiols. These structures are prone to losing an electron to form free radicals in a reaction catalyzed by peroxidases. Some chemicals, such as catechols and hydroquinones, may undergo two sequential oxidations, first producing semiquinone radicals by one electron loss followed by a second electron loss to form quinones. Quinones are both reactive electrophiles (Table 3-2) and electron acceptors with the capacity to initiate redox cycling or oxidation of thiols and NAD(P)H. Like peroxidases, oxyhemoglobin (Fe^{2+}) can catalyze the oxidation of aminophenols to semiquinone radicals and quinoneimines. This reaction concurrently oxidizes the Fe^{2+} to Fe^{3+} and forms methemoglobin (MetHb) in the process. This oxidation reaction will increase the likelihood of toxicity because MetHb cannot carry oxygen.
3. ***Free radicals formed by homolytic bond fission***: Reductive fission is a cleavage reaction in which two electrons in a bond are divided equally between the products. The hydroxyl radical ($HO^{\bullet}$) is a free radical generated by homolytic fission. For example, large amounts of $HO^{\bullet}$ are formed from water with ionizing radiation. This mechanism is also exemplified by conversion of CCl_4 to the trichloromethyl free radical ($Cl_3C^{\bullet}$) in a reaction catalyzed by an electron transfer from cytochrome P450 or the mitochondrial electron transport chain (reductive dehalogenation). The Fenton reaction describes the reductive homolytic fission of hydrogen peroxide (HOOH) to the harmful $HO^{\bullet}$ and the harmless HO^- (hydroxide anion; Fig. 3-4). This reaction is a major toxicity mechanism for HOOH and its precursor $O_2^{\bullet-}$ that is catalyzed by transition metal ions, typically Fe(II), Cu(I), Cr(V), Ni(II), or Mn(II). HOOH is a direct or indirect by-product of several enzymatic reactions, including monoamine oxidase, acyl-coenzyme A oxidase, xanthine oxidase, aldehyde oxidase, and CYP2E1. It is also produced in large quantities by spontaneous or superoxide dismutase (SOD)–catalyzed dismutation of $O_2^{\bullet-}$. Importantly, the toxicity of chemicals that chelate transition metals including nitrilotriacetic acid and bleomycin involves Fenton chemistry. Furthermore, the pulmonary toxicity of inhaled mineral particles such as asbestos and silica is caused, at least in part, by the formation of $HO^{\bullet}$ triggered by Fe ions on the particle surface.

Homolytic cleavage is also involved in free radical generation from peroxynitrite ($ONOO^-$; Fig. 3-4). The facile reaction of $ONOO^-$ with CO_2 yields nitroso-peroxycarbonate ($ONOOCO_2^-$), which spontaneously homolyzes into two radicals, namely nitrogen dioxide ($^{\bullet}NO_2$) and the carbonate anion radical ($CO_2^{\bullet-}$). Formation of $ONOO^-$ and the latter radicals are involved in the toxicity of $O_2^{\bullet-}$ and $^{\bullet}NO$. As $^{\bullet}NO$ is the product of nitric oxide synthase (NOS), this mechanism is especially relevant in and around cells that express NOS constitutively (i.e., neurons and endothelial cells) as well as in cells that express the inducible form of NOS.

Formation of Nucleophiles The formation of nucleophiles is a relatively uncommon mechanism for activating toxicants. Cyanide is formed from multiple sources including metabolism of amygdalin (laetrile, found in apricot or apple seeds) by bacterial β-glucosidase (Table 3-1), from the epoxidation of acrylonitrile with subsequent glutathione conjugation, and by thiol-induced decomposition of sodium nitroprusside. Carbon monoxide is a toxic metabolite of dihalomethanes that undergo oxidative dehalogenation. Hydrogen selenide, a strong nucleophile and reductant, is formed from selenite by reaction with glutathione or other thiols.

Formation of Redox-Active Reactants In addition to redox cycling already described, there are specific mechanisms that form redox-active reactants. For example, nitrite is formed from nitrate by bacterial reduction in the intestine or from esters of nitrous or nitric acids in reaction with glutathione, a reaction that can produce MetHb. Additionally, hydroxylated metabolites of dapsone, an antibiotic and primaquine, an antimalarial (dapsone hydroxylamine and 5-hydroxyprimaquine, respectively) produce MetHb by a co-oxidation reaction. Xenobiotic radicals formed in redox cycling (e.g., those depicted in Fig. 3-3) as well as $O_2^{\bullet-}$ can reduce Fe^{3+} bound to ferritin and consequently release it as Fe^{2+} which catalyzes $HO^{\bullet}$ formation (Fig. 3-4).

Overall, the most reactive metabolites are electrophiles and neutral or cationic free radicals. Similarly, free radicals with an extra electron (e.g., those shown in Fig. 3-3) cause damage by giving rise to the neutral $HO^{\bullet}$ radical after the formation and subsequent homolytic cleavage of HOOH.

Detoxification Biotransformation that prevents the formation of a toxic metabolite or eliminates it once formed is an important mechanism that reduces the likelihood of toxicity. Any such process is called detoxification. Detoxification mechanisms are catalyzed mainly by biochemical mechanisms that include phase II biotransformation reactions such as glucuronidation, sulfation, and glutathione conjugation. These reactions, including the enzymes and cofactors required for activity are discussed in detail in Chap. 6. Examples of how detoxification mechanisms reduce the likelihood of toxicity are discussed here. Like many of the reactions involved in metabolic activation, these reactions can differ

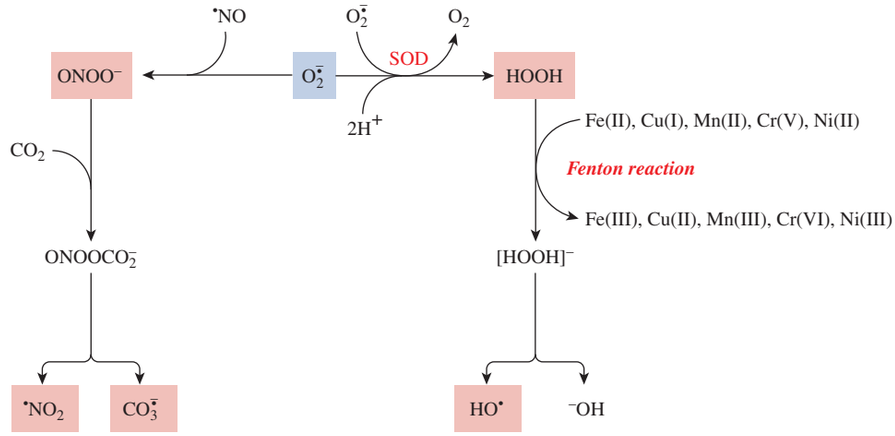

Figure 3-4. *Two pathways for toxicity of superoxide anion radical $O_2^{\bullet-}$ from conversion of $ONOO^-$ and HOOH (nonradical products) to $^{\bullet}NO_2$,$CO_3^{\bullet-}$, and HO• (radicals).* Conversion of $O_2^{\bullet-}$ to HOOH is spontaneous or is catalyzed by SOD. Homolytic cleavage of HOOH to hydroxyl radical and hydroxyl ion (Fenton reaction) is catalyzed by the transition metal ions shown. Hydroxyl radical is the ultimate toxicant for xenobiotics that form $O_2^{\bullet-}$. In the other pathway, $O_2^{\bullet-}$ reacts avidly with nitric oxide (•NO), the product of •NO synthase (NOS), forming peroxynitrite ($ONOO^-$). Spontaneous reaction of $ONOO^-$ with carbon dioxide (CO_2) yields nitrosoperoxy carbonate that with homolytic cleavage forms nitrogen dioxide ($^{\bullet}NO_2$) and carbonate anion radical ($CO_2^{\bullet-}$). All three radical products indicated in this figure are oxidants, whereas $^{\bullet}NO_2$ is also a nitrating agent.

in organs or across species and can be important determinants of target organ toxicity and species differences in sensitivity to toxicity. Furthermore, an important concept in detoxification is that these processes, albeit protective, can be saturated. When protective mechanisms are overwhelmed and saturated, toxicity ensues.

Detoxification of Electrophiles Specific mechanisms for the detoxification of electrophilic chemicals include epoxide hydrolase-catalyzed biotransformation of epoxides and arene oxides to less toxic diols and dihydrodiols, respectively, and carboxylesterase-catalyzed hydrolysis of organophosphate ester pesticides. Quinones are reduced to hydroquinones by NAD(P)H:quinone oxidoreductase (NQO1) and NRH:quinone oxidoreductase (NQO2). α,β-Unsaturated aldehydes (e.g., the lipid peroxidation product 4-oxonon-2-enal) are reduced to alcohols or to their saturated derivative by carbonyl reductase, but they can also be oxidized to acids by aldehyde dehydrogenases.

An extremely important mechanism for the detoxification of electrophilic toxicants is conjugation with glutathione (Forman et al., 2009). Glutathione (GSH) is a tripeptide composed of glycine, cysteine, and glutamic acid, with the glutamic acid linked to cysteine by a γ-carboxyl group (γ-glutamine-cysteinylglycine). It is a major non-protein sulfhydryl in most tissues, with constitutive concentrations of 5 to 10 mM in the liver. Conjugation with GSH occurs spontaneously or can be catalyzed by glutathione *S*-transferases. From a mechanistic perspective, the functional activity of GSTs is a major determinant of species differences in toxicity, the most notable of which is the detoxification of epoxide intermediates of the fungal toxicant, aflatoxin B1 (AFB1). Very low doses of AFB1 are hepatotoxic and tumorigenic in rats but not in mice, even though both species form the reactive epoxide metabolites at similar rates. However, mice conjugate the epoxide metabolite with GSH up to 50-times faster than rats, thereby reducing the toxicity of the epoxide and limiting hepatotoxic and carcinogenic mechanisms. Some metal ions, including Ag^+, Cd^{2+}, Hg^{2+}, and CH_3Hg^+, also readily react with and are detoxified by GSH conjugation.

Beyond biotransformation, covalent binding of electrophiles to proteins is a detoxification reaction provided the bound protein has no critical function and does not become a neoantigen or otherwise harmful. Carboxylesterases, for example, inactivate organophosphates not only by hydrolysis but also by covalent binding.

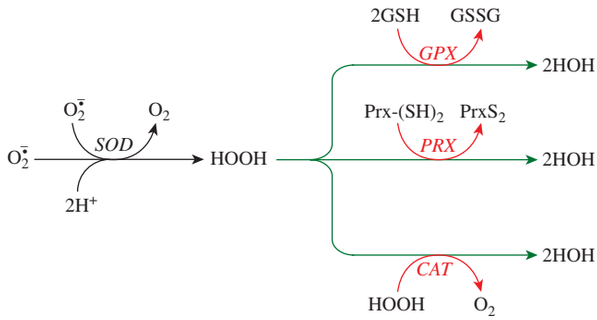

Figure 3-5. *Detoxification of superoxide anion radical ($O_2^{\bullet-}$)and HOOH.* Superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to HOOH, which is further detoxified by glutathione peroxidase (GPX), peroxiredoxin (Prx-$(SH)_2$), and catalase (CAT). When GPX reduces HOOH, it forms glutathione disulfide (GSSG), which is reduced back to GSH by glutathione reductase (requires NADPH; see Fig. 3-6). When Prx-$(SH)_2$ reduces HOOH, its catalytic thiol group (–R–S–H) is oxidized to a sulfenic acid group (–R–S–OH), which in turn reacts with another SH group of Prx, forming HOH and Prx disulfide ($PrxS_2$). Finally, catalase converts HOOH into two moles of water. Catalase is a high-capacity system, whereas Prx and GPX can be saturated at high concentrations of HOOH.

Detoxification of Free Radicals Detoxification of $O_2^{\bullet-}$ is an important mechanism to prevent toxicity because it can otherwise be converted into much more reactive compounds. Detoxification of $O_2^{\bullet-}$ is achieved by the coordinated action that starts with superoxide dismutases (SOD), followed by reactions catalyzed by catalase, glutathione peroxidase, or peroxiredoxin (Fig. 3-5). SODs are high-capacity enzymes located in the cytosol (Cu, Zn-SOD) and the mitochondria (Mn-SOD), which convert $O_2^{\bullet-}$ to HOOH (Fig. 3-5). The HOOH is reduced to water by catalase in peroxisomes (or in the mitochondria in cardiac muscle), by glutathione peroxidases in the cytosol and mitochondria, and by peroxiredoxins in the cytosol, mitochondria, and endoplasmic reticulum (Rhee et al., 2005). In contrast to $O_2^{\bullet-}$, no enzyme eliminates HO•. Furthermore, HO• is

extremely reactive, with such a short half-life (10^{-9} seconds), that affords little time for the HO• to reach and react with antioxidants. Therefore, the only effective protection against HO• is to prevent its formation by elimination of its precursor, HOOH, via conversion to water.

$ONOO^-$ (which is not a free radical oxidant) has a half-life of about 1 second and is significantly more stable than HO•. However, it can react with CO_2 to form reactive free radicals (Fig. 3-4). Glutathione peroxidase and peroxiredoxins can detoxify $ONOO^-$ by reduction to nitrite (ONO^-) the same way they reduce HOOH to water (Fig. 3-5). In addition, $ONOO^-$ reacts with oxyhemoglobin, heme-containing peroxidases, and albumin, all of which could be important sinks for $ONOO^-$. Furthermore, a significant mechanism for preventing $ONOO^-$ buildup is to eliminate its precursors. That is, $ONOO^-$ toxicity can be reduced by scavenging •NO by its reaction with oxyhemoglobin (forming metHb and nitrate) and by decreasing $O_2^{\bullet-}$ levels through the action of SODs.

Peroxidase-generated free radicals are eliminated by electron transfer from glutathione. This results in the oxidation of glutathione, which is reversed by NADPH-dependent glutathione reductase (Fig. 3-6). This pathway illustrates the important role that glutathione plays in the detoxification of both electrophiles and free radicals.

Detoxification of Nucleophiles Nucleophiles are generally detoxified by conjugation at the nucleophilic functional group. Hydroxylated compounds are conjugated by sulfation, glucuronidation, or rarely by methylation, whereas thiols are methylated or glucuronidated and amines and hydrazines are acetylated. These reactions prevent peroxidase-catalyzed conversion of the nucleophiles to free radicals and biotransformation of phenols, aminophenols, catechols, and hydroquinones to electrophilic quinones and quinoneimines. One alternative mechanism for the elimination of thiols, amines, and hydrazines is oxidation by flavin-containing monooxygenases (see Chap. 6). Finally, a specific nucleophile detoxification mechanism is the biotransformation of cyanide to thiocyanate which is catalyzed by either of two sulfurtransferase enzymes, namely rhodanese or mercaptopyruvate sulfurtransferase.

Detoxification of Toxicants with No Functional Groups Biotransformation of compounds such as benzene and toluene occurs in two phases, wherein oxidative reactions introduce functional groups (i.e., hydroxyl or carboxyl moiety), a reaction most often by cytochrome P450 enzymes, after which the oxidative metabolites are conjugated by glucuronidation or sulfation reactions. These processes are referred to as Phase I and Phase II metabolism, respectively. With some exceptions, the final products are inactive, highly hydrophilic organic acids that are readily excreted. Carbonyl reduction of ketones, aldehydes, and esters is catalyzed by at least five enzymes (e.g., carbonyl reductase, 3 members of aldo-keto reductase superfamily, and 11-β-hydroxysteroid dehydrogenase-1). This enzyme reaction initiates detoxification of the potent carcinogen NNK, forming a nicotine-derived nitrosamine alcohol that is readily glucuronidated and excreted into urine. Numerous NNK metabolites are excreted in urine and have been used as biomarkers of NNK exposure and lung cancer risk assessment, thereby demonstrating how understanding detoxification mechanisms can be applied to predicting adverse effects (Yuan et al., 2014).

Detoxification of Protein Toxins It is generally thought that extracellular and intracellular proteases are involved in the inactivation of toxic polypeptides. Several toxins found in venoms, such as α- and β-bungarotoxin, erabutoxin, and phospholipase, contain intramolecular disulfide bonds that are required for their activity. These proteins are inactivated by thioredoxin, an endogenous dithiol protein that reduces the essential disulfide bond.

When Detoxification Fails Although detoxification reactions are generally protective, there are many mechanisms of toxicity that ensue when detoxification reactions are inadequate. The most important reason for such failure is that the exposure to the toxicant overwhelms vital detoxification processes. This will occur typically in a dose-dependent manner and results from saturation of detoxification enzymes, consumption of essential co-substrates, or depletion of cellular antioxidants such as GSH, ascorbic acid, and α-tocopherol. Thus, the dose-response relationship is an important quantitative aspect underlying most toxicity mechanisms. The exception to the fundamental principle that mechanisms of toxicity are dose related is idiosyncratic toxicity (discussed later in this chapter). Species differences in the ability to detoxify reactive compounds can also contribute to marked qualitative differences in toxic outcome even when exposures are similar, and examples of such differences are described throughout this chapter.

Other notable examples of detoxification "failure" occur when a reactive toxicant inactivates a detoxicating enzyme. Some conjugation reactions that detoxify a reactive intermediate, are reversed to initiate toxicity. For example, 2-naphthylamine is *N*-hydroxylated and glucuronidated in liver, with the glucuronide eliminated in urine. However, in the urinary bladder, the glucuronide is hydrolyzed, and the released arylhydroxylamine is converted to the reactive electrophilic arylnitrenium ion. Ultimately, 2-naphthylamine causes bladder cancer because of this local activation of a previously detoxified metabolite. In another example, isocyanates and isothiocyanates form labile GSH conjugates from which they can be released. Thus, methylisocyanate readily forms a GSH conjugate in

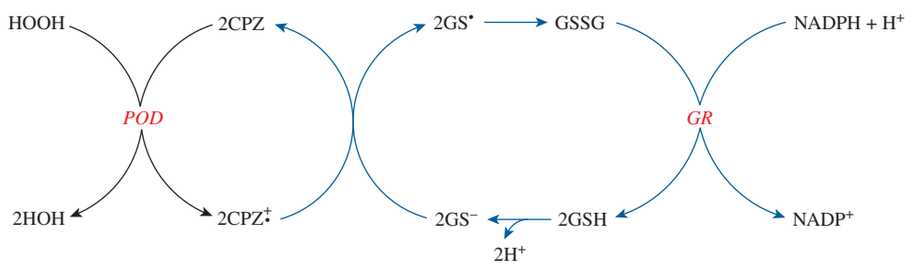

Figure 3-6. *Detoxification of peroxidase (POD)–generated free radicals such as chlorpromazine free radical ($CPZ_•^+$) by glutathione (GSH).* The by-products are glutathione thiyl radical (GS•) and glutathione disulfide (GSSG), from which GSH is regenerated by glutathione reductase (GR), a reaction that requires NADPH.

the lung after inhalation, and once formed, this conjugate is distributed to other tissues, where the reactive electrophilic parent compound may be regenerated.

STEP 2: REACTION OF THE ULTIMATE TOXICANT WITH THE TARGET MOLECULE

Toxicity is initiated by a reaction between the ultimate toxicant and its target molecule (step 2a in Fig. 3-1), with subsequent events leading to dysfunction or injury. The determinants of interaction of the ultimate toxicant with the target molecule include (1) the attributes of target molecules, (2) the types of reactions between ultimate toxicants and target molecules, and (3) the effects of toxicants on the target molecules as illustrated in Fig. 3-7. Additionally, some toxicities result from alteration of the biological (micro)environment (step 2b in Fig. 3-1) in which critical endogenous molecules, cell organelles, cells, and organs function are perturbed.

Attributes of Target Molecules

There are three attributes of a target molecule that determine whether it is associated with or responsible for toxicity, and these critical elements are the ability to (1) react with the target and adversely affect its function, (2) reach an effective concentration at the target site, and (3) alter the target in a way that is mechanistically related to toxicity. This chapter will not present a comprehensive inventory of potential target molecules. However, the most prevalent and toxicologically relevant targets are macromolecules such as nucleic acids (especially DNA), proteins, and membrane lipids.

Although any endogenous compound may be a target, a key requisite is that the target molecule must be accessible to a sufficiently high concentration of the ultimate toxicant. For example, direct interaction with thyroperoxidase, an enzyme involved in thyroid hormone synthesis, also converts some nucleophilic xenobiotics (such as methimazole, amitrole, and resorcinol) into reactive free radicals that inactivate thyroperoxidase. Such compounds reduce thyroxine (T4) levels through this inactivation and cause hypothyroidism. However, reactive metabolites may also diffuse from their site of formation to interact with more distant targets. For example, the arylnitrenium ion metabolite of *N*-methyl-4-aminoazobenzene is formed in the cytoplasm, but readily targets nuclear DNA. Similarly, vinyl chloride epoxide, formed in hepatocytes from vinyl chloride, reacts with DNA in neighboring endothelial cells, and this interaction is an initiating event in vinyl chloride-induced hepatic hemangiosarcoma.

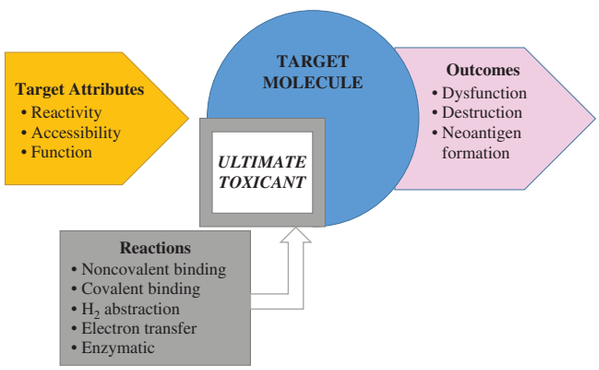

Figure 3-7. *Reaction of the ultimate toxicant with the target molecule: the second step in the development of toxicity.* The figure illustrates that the properties of the toxic molecule can influence the reaction with its target, the diversity of reactions that can occur with the target, and following the interaction of the toxicant with its target, the types of outcomes that are associated with the toxicity.

Not all targets for chemicals contribute to the harmful effects. For example, carbon monoxide (CO) is toxic by preventing oxygen to bind to ferro(Fe^{2+})hemoglobin, but CO also associates with the ferrous iron in cytochrome P450 with little or no consequence. Additionally, covalent binding to proteins, generally considered to be harmful, may be without adverse consequences or may be a form of detoxification if binding spares toxicologically relevant targets. For example, covalent binding of organophosphate insecticides to plasma cholinesterase is a significant protective mechanism that counteracts phosphorylation of acetylcholinesterase. Similarly, although covalent binding of toxicants to various intracellular and structural proteins has been demonstrated, it is often uncertain which modified protein(s) is/are toxicologically relevant. In this regard, comparison of covalent binding profiles between acetaminophen (APAP; 4′-hydroxyacetanilide) and its non-hepatotoxic regioisomer, 3′-hydroxyacetanilide, was a strategy by which modifications of proteins likely to be causally related to liver injury were identified. That is, covalent binding to proteins observed with the non-hepatotoxic isomer were considered to be inconsequential, whereas covalent binding to proteins by APAP that differed from the non-toxic isomer were more likely to contribute to toxicity (Cohen et al., 1997; Jaeschke and Bajt, 2006).

Types of Reactions

The major interactions between the toxicant and target molecules are noncovalent or covalent in nature, but other reactions including hydrogen abstraction, electron transfer, or enzymatic reaction can occur.

Noncovalent Binding Examples of noncovalent binding that can lead to adverse effects include binding of strychnine to glycine receptors on motor neurons in the spinal cord, TCDD to the aryl hydrocarbon receptor (AhR), saxitoxin to sodium channels, and warfarin to vitamin K 2,3-epoxide reductase. Non-covalent binding is attributed to apolar interactions or the formation of hydrogen and ionic bonds between toxicants and their targets including membrane or intracellular receptors, ion channels, and some enzymes. Such forces also are responsible for the intercalation of chemicals such as acridine yellow and doxorubicin into the double helix of DNA. In most cases, noncovalent binding is reversible because of the comparatively low bonding energy. For example, compounds that bind to α2u-globulin to cause renal toxicity (described below), bind in a noncovalent manner by fitting into a hydrophobic binding pocket of this protein.

Covalent Binding Covalent binding is of great toxicological importance because binding is essentially irreversible and permanently alters endogenous molecules (Leung et al., 2012). Covalent adduct formation is common with electrophilic toxicants because they react with nucleophilic atoms in proteins and nucleic acids. In general, soft electrophiles prefer to react with soft nucleophiles (low charge-to-radius ratio in both), whereas hard electrophiles react more readily with hard nucleophiles (high charge-to-radius ratio in both). For example, a hard electrophile such as alkyl carbonium ion binds to similarly hard nucleophiles such as the oxygen in nucleic acids. Metal ions such as silver and mercury are soft electrophiles that react covalently with soft nucleophiles, particularly thiol groups. Conversely, lithium, calcium, and barium metals are hard electrophiles that react preferentially with hard nucleophiles including carboxylate and phosphate anions. Metals

falling between these two extremes, such as chromium, zinc, and lead, exhibit universal reactivity with nucleophiles.

The covalent reaction between HOOH (a soft electrophile) and protein thiol groups is of special biological significance because it produces protein sulfenic acid (Prot-S-OH) as illustrated here:

$$\text{Prot-SH} + \text{HOOH} \rightarrow \text{Prot-S-OH} + \text{HOH}$$

However, the S atom in a protein–sulfenic acid is electrophilic and will further react with another thiol group of the same protein, a different protein or glutathione (GSH), resulting in formation of an intramolecular disulfide, intermolecular disulfide, or a mixed disulfide of the protein and glutathione, respectively:

$$\text{Prot}_1\text{-S-OH} + \text{Prot}_2\text{-SH} \rightarrow \text{Prot}_1\text{-S-S-Prot}_2 + \text{HOH}$$

$$\text{Prot-S-OH} + \text{GSH} \rightarrow \text{Prot-S-SG} + \text{HOH}$$

Hydroperoxides, including lipid hydroperoxides (LOOH; see Fig. 3-8), may also produce protein–sulfenic acids, protein–disulfides, and glutathionylated proteins, which represent posttranslational modifications with a role in redox signaling (Forman et al., 2009).

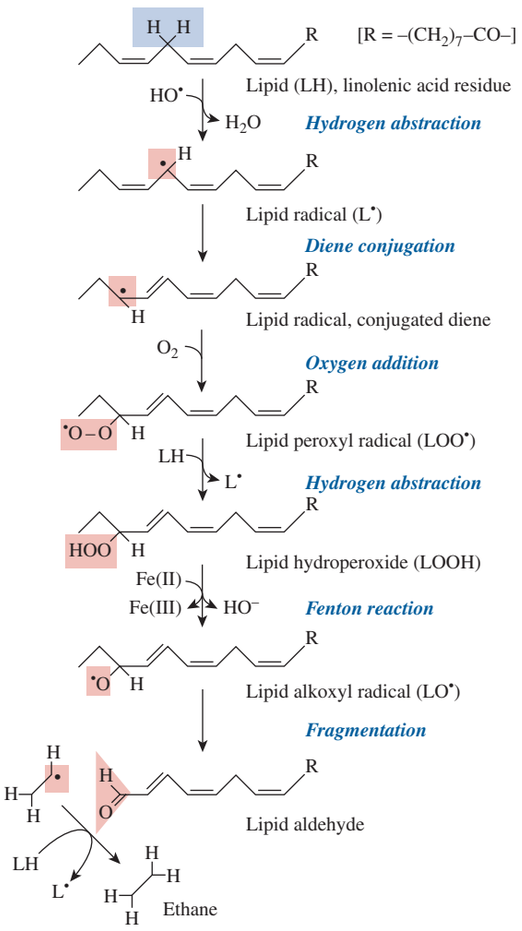

Figure 3-8. *Lipid peroxidation initiated by the hydroxyl radical (HO•), illustrating the many biochemical intermediates formed in this process.* Many of the products, such as the radicals and α,β-unsaturated aldehydes, are reactive, whereas others, such as ethane, are nonreactive indicators of lipid peroxidation.

Hydrogen Abstraction Neutral free radicals, such as those generated in reactions depicted in Fig. 3-4, readily abstract H atoms from endogenous compounds, converting them into radicals. Abstraction of hydrogen from thiols (R-SH) creates thiyl radicals (R-S•), which on recombination with HO• forms sulfenic acids (R-S-OH) as described above. Radicals also remove hydrogen from CH_2 groups of amino acids and convert them to carbonyls. These carbonyls can react covalently with amines, forming cross-links with other proteins or DNA. Abstraction of hydrogen from fatty acids produces lipid radicals and initiates lipid peroxidation.

Electron Transfer As described earlier, some chemicals can oxidize the Fe^{2+} in hemoglobin to Fe^{3+}, producing metHb, which is toxic by its reduced oxygen carrying capacity. Nitrite can oxidize ferrous iron in hemoglobin, whereas dapsone hydroxylamine, 5-hydroxy primaquine, and phenylhydrazine are co-oxidized with oxyhemoglobin, forming metHb and HOOH.

Enzymatic Reactions Snake venoms contain enzymes that cause toxicity by generalized proteolytic degradation of many biomolecules. Many other toxins in nature inhibit important enzymes functions to elicit toxicity. For example, the plant toxins ricin and abrin are *N*-glycosidases, and they hydrolyze a specific glycosidic bond in ribosomal RNA to block protein synthesis. Botulinum toxin is a Zn-protease that hydrolyzes the fusion proteins required for exocytosis of the neurotransmitter acetylcholine preventing its release and leading to paralysis. The lethal factor component of anthrax toxin is also a Zn-protease, which inactivates mitogen-activated protein kinase kinase (MAP2K), an integral kinase involved in many signal transduction pathways (discussed below). A final example of enzymatic reaction-mediated toxicity are bacterial toxins that catalyze the transfer of ADP-ribose from NAD^+ to specific proteins. This mechanism is the basis for how diphtheria toxin blocks the function of elongation factor 2 (EF2) to inhibit protein synthesis.

This brief summary illustrates that chemical reactivity is a major feature by which most toxicants act on endogenous molecules. It is also possible that more than one type of reactivity may occur with target molecules. For example, quinones are electron acceptors and initiate thiol oxidation or free radical reactions that lead to lipid peroxidation, but they may also act as soft electrophiles and bind covalently to protein thiols. Similarly lead ions are soft electrophiles that form coordinate covalent bonds with critical thiol groups in δ-aminolevulinic acid dehydratase to inhibit heme synthesis. However, lead also can be a hard electrophile when it displaces calcium from membrane channels.

Effects of Toxicants on Target Molecules

The three major effects resulting from interaction of the ultimate toxicant with endogenous molecules are dysfunction of the targets, destruction of the target with complete loss of function, or altered immune responses to a modified target.

Dysfunction of Target Molecules Some toxicants activate protein target molecules, mimicking endogenous ligands. This is the case for many drugs that are designed to be agonists of specific functions. Morphine activates opioid receptors, isoproterenol is an agonist of β2 adrenergic receptors, and clofibrate is an agonist of the peroxisome proliferator–activated receptor α (PPARα). Although they are useful clinically, toxicity from these compounds is usually observed as exaggerated pharmacology, and the mechanism of toxicity is a direct effect of receptor activation. Other non-drug examples are phorbol esters and lead ions that stimulate protein kinase C (PKC), an important pathway that regulates DNA replication and cell proliferation.

More commonly, chemicals inhibit the function of target molecules. Several xenobiotics, such as atropine, curare, and strychnine, block neurotransmitter receptors by attaching to the ligand-binding sites, whereas others interfere with the function of ion channels. Tetrodotoxin (described earlier as a direct-acting toxicant) and saxitoxin inhibit opening of the voltage-activated sodium channels in neuronal membranes, whereas DDT and the pyrethroid insecticides inhibit their closure. Some toxicants block ion transporters, others inhibit mitochondrial electron transport complexes, and many inhibit enzymes. Chemicals that bind to tubulin (e.g., vinblastine, colchicine, paclitaxel, trivalent arsenic) or actin (e.g., cytochalasin B or phalloidin) impair the assembly and/or disassembly of these important cytoskeletal proteins.

Protein function is impaired when conformation or structure is altered by interaction with the toxicant. Many proteins possess critical moieties, often thiol groups, which are essential for catalytic activity or correct assembly of macromolecular complexes. Consequently, these proteins are sensitive to covalent and/or oxidative modification of their thiol groups, and dysfunction causes impaired maintenance of the cellular energy and metabolic homeostasis. There are numerous examples of enzymes whose function is inhibited by thiol-reactive compounds including pyruvate dehydrogenase, DNA repair enzymes (O^6-methylguanine-DNA-methyltransferase), ubiquitin-activating and ubiquitin-conjugating enzymes (E1 and E2, respectively), protein tyrosine phosphatases (PTPs), the lipid phosphatase PTEN (phosphatase and tensin homologue), and caspases. Another extremely important thiol-reactive protein is Keap1, an intracellular partner of Nrf2. Covalent and/or oxidative modification of thiol groups in Keap1 triggers the adaptive, cytoprotective electrophile stress response (discussed in detail later in this chapter). Finally, some thiol-reactive and oxidant chemicals (acrolein, methyl isocyanate, and chloroacetophenone) and corrosive gases (phosgene, chloropicrin, and chlorine) react with cysteine thiol groups of the TRPA1 receptor (transient receptor potential cation channel A1) in sensory neurons, and this interaction directly elicits irritation, pain, lacrimation, bronchial secretion, sneezing, coughing, and bronchospasm. If exposure is moderate, these responses are alerting and protective, but they become incapacitating and detrimental when exaggerated at high exposure (Bessac and Jordt, 2010).

An extremely important mechanism of toxicity involves covalent binding of chemicals to DNA that causes nucleotide mispairing during replication. For example, covalent binding of aflatoxin 8,9-oxide to *N*-7 of guanine causes it to pair with adenine rather than cytosine, forming an incorrect codon and consequently an incorrect amino acid in the protein. Such events are involved in aflatoxin-induced mutations of the *Ras* proto-oncogene and *p53* tumor suppressor gene that contribute to liver tumor development (discussed below). 8-Hydroxyguanine and 8-hydroxyadenine are produced by oxidative stress and particularly by binding of $HO^{\bullet}$ to the DNA template, and these mutagenic bases also cause mispairing and codon changes to alter amino acid sequences (Suzuki and Kamiya, 2016).

Destruction of Target Molecules In addition to adduct formation, toxicants alter the primary structure of endogenous molecules by means of cross-linking and fragmentation. Bifunctional electrophiles, such as 2,5-hexanedione, carbon disulfide, acrolein, 4-oxonon-2-enal, 4-hydroxynon-2-enal, and nitrogen mustard alkylating agents, cross-link cytoskeletal proteins, DNA, or DNA with proteins. HOOH and hydroxyl radicals can also induce cross-linking by converting proteins into reactive electrophiles (e.g., protein sulfenic acids and protein carbonyls, respectively), which react with a nucleophilic group (e.g., thiol, amine) in another macromolecule. Cross-linking imposes both structural and functional constraints on the linked molecules to alter function.

Free radicals such as $Cl_3COO^{\bullet}$ and $HO^{\bullet}$ initiate peroxidative degradation of lipids by hydrogen abstraction from fatty acids. The lipid radical ($L^{\bullet}$) is then converted successively to lipid peroxyl radical ($LOO^{\bullet}$) by oxygen fixation, lipid hydroperoxide (LOOH) by hydrogen abstraction, and lipid alkoxyl radical ($LO^{\bullet}$) by the Fenton reaction (Fig. 3-8). Subsequent fragmentation gives rise to hydrocarbons such as ethane and reactive aldehydes such as 4-hydroxynon-2-enal and malondialdehyde (MDA). Thus, lipid peroxidation is an important mechanism of toxicity because it not only destroys lipids in cellular membranes but also generates additional endogenous toxic free radicals (e.g., $LOO^{\bullet}$ and $LO^{\bullet}$) and electrophiles (e.g., 4-oxonon-2-enal and 4-hydroxynon-2-enal). These substances readily react with additional targets, such as membrane proteins, or diffuse intracellularly to more distant molecules including DNA. Similarly, F_2-isoprostanes are stable peroxidation products of arachidonic acid that are not only sensitive markers of lipid peroxidation but also potent pulmonary and renal vasoconstrictors (Basu, 2004). Numerous biomarkers have been identified based on this mechanistic understanding of lipid peroxidation, including end products of the reaction such as MDA along with lipid hydroxides such as hydroxyeicosatetraenoic acid (HETE) and hydroxycholesterols (Niki, 2014).

Several forms of DNA fragmentation are observed in toxicity. Single-strand breaks (SSB) are typically initiated by hydroxyl radicals that cause H abstraction from deoxyribose in DNA and ultimately cleave the phosphodiester bond. Double-strand breaks (DSB), which are typically more toxic or lethal to the affected cell, are caused by multiple hydroxyl radical attacks on a short length of DNA, such as those that occur after ionizing radiation.

Neoantigen Formation Haptens are small molecules that can elicit an immune response only when attached to a larger carrier, usually a protein. The protein carrier, often albumin, does not cause an immune response by itself. Accordingly, an immune-mediated mechanism of toxicity can occur after modification of proteins by toxicants. Some chemicals (e.g., penicillin, dinitrochlorobenzene, and nickel ion) are sufficiently reactive to bind to proteins spontaneously. Others are activated by biotransformation (Sullivan et al., 2015), as exemplified by CYP2E-catalyzed metabolism of halothane. In this example, trifluoroacetyl chloride is formed from halothane and binds as a hapten to microsomal and cell surface proteins in the liver, inducing a hepatitis-like immune reaction in sensitive patients. Another example of hapten-mediated immune activation is urushiol, which is the allergen found in poison ivy. Urushiol undergoes auto-oxidation in the skin to generate a reactive quinone that is the actual hapten.

Haptenized proteins released from cells may evoke antibody-mediated (humoral) and/or T-cell-mediated (cellular) immune response (see Chap. 12). In the humoral response, B cells bind the complete antigen through membrane receptors and associate with T-helper cells (T_h; $CD4^+$). Antigen binding and cell surface costimulatory molecules on T_h cells induce differentiation of B cells into plasma cells that synthesize and secrete antibody. By binding to the antigen, the antibody facilitates destruction of the antigen by phagocytosis. Although protective in nature, this reaction can lead to harmful consequences, as is the case when penicillin-bound haptens react with IgE-type antibodies on the surface of mast cells to trigger release of mast cell mediators (e.g., histamine and leukotrienes), which in turn may cause bronchoconstriction,

vasodilation, and plasma exudation. If not treated quickly, this is a serious adverse response that can lead to anaphylactic shock and possibly death.

An important toxic response seen with mechanisms of neoantigen formation is contact dermatitis. This reaction involves T cells as the main mediators, and there are two major phases of such reactions. In the sensitization phase, T_h cells are activated by signals coming from antigen-presenting cells (APCs), including macrophages and dendritic cells. APCs phagocytose the haptenized protein and then migrate to lymph nodes. APCs process the antigen into peptides, after which the processed antigen is externalized, and transferred to the major histocompatibility complex (MHC) on its surface. On reaching the lymph node, the APC presents the MHC-bound peptide to the T-cell receptor (TCR) of T_h cells, and cell surface coactivator molecules (e.g., CD80 and CD86) on APCs bind to their counterparts (e.g., CD28) on T cells. This signal verifies that the antigen detected is foreign, and if it doesn't occur, the T cell becomes functionally inert (anergic). However, on receiving both signals, the T_h cells become specific to a specific antigen, and the T_h cells express IL-2 to stimulate T-cell proliferation, differentiation, and release into the circulation. The second phase is the elicitation phase which begins on reexposure to the allergen. Antigen-specific T cells enter the exposure site (e.g., the skin), releasing IL-2 and other cytokines to activate cytotoxic T cells ($CD8^+$) along with adhesion molecules at the site of allergen contact. While this response is protective against virus-infected cells and tumor cells, it causes inflammation and injury in cells containing xenobiotic neoantigens. This model also describes how contact allergens, such as nickel and urushiols, cause contact dermatitis. By understanding the mechanism of action, methods to detect and predict compounds that could be contact allergens have been developed (see details of the local lymph node assay; see Chap. 12).

In addition to the above-described classical mechanism of neoantigen-initiated APC-mediated T-cell activation, xenobiotics may cause T-cell activation by more direct ways. For example, Ni ions may not only form coordinate covalent bonds with proteins, making them neoantigens, but also cross-link MHC of APC with the TCR of T cells, activating the latter. Moreover, some drugs (e.g., sulfamethoxazole) are converted into adduct-forming metabolites (e.g., nitroso-sulfamethoxazole) that trigger T-cell activation by APC activation, but also stimulate the TCR directly, without involvement of APCs.

Serious toxicities, including drug-induced lupus and agranulocytosis, are thought to be mediated by immune reactions triggered by drug–protein adducts. The causative chemicals are typically nucleophiles, such as aromatic amines (e.g., aminopyrine, clozapine, procainamide, and sulfonamides), hydrazines (e.g., hydralazine and isoniazid), and thiols (e.g., propylthiouracil, methimazole, and captopril). These substances can be oxidized by myeloperoxidase of granulocytes and monocytes or by the reactive oxygen/nitrogen species (ROS/RNS) system to produce $HO^{\bullet}$, $ONOO^-$, or hypochlorous acid (HOCl) and form reactive metabolites that bind to the surface proteins of these cells, making them antigens (Uetrecht and Naisbitt, 2013). The reactive metabolites may also activate monocytes and thus promote additional immune reactions.

There is increasing evidence that some forms of liver toxicity, particularly those considered to be idiosyncratic in nature, may involve immune-mediated mechanisms (see the section "Idiosyncratic Toxicity"). Briefly, immune cells in liver protect the organ from the many foreign compounds it detoxifies (a concept called immune tolerance). However, activation of the immune system by haptens or other antigens reduces or eliminates immune tolerance and toxicity can ensue. Toxicity observed with clinical use of immunomodulatory checkpoint inhibitors, such as programmed death receptor-1 (PD-1), is associated with activation of the immune system and loss of self-tolerance that develops by blocking the normal inhibitory signal of PD-1. Although these agents are useful in treating various types of cancer, they are also associated with a spectrum of immune-related toxicities, with target organs including liver, lung, and gastrointestinal tract (Day and Hansen, 2016).

Toxicity Not Initiated by Reaction with Target Molecules

Some xenobiotics not only interact with a specific endogenous target molecule to induce toxicity but also may alter the biological microenvironment (see step 2b in Fig. 3-1). There are three important examples of toxicity mediated through changes in the microenvironment. First, solvents and detergents directly alter the lipid phase of cell membranes and destroy transmembrane solute gradients that are essential to cell functions. Second, chemicals that alter H^+ ion concentrations in the aqueous biophase alter the microenvironment by disrupting essential acid–base balance. Examples include acids and substances biotransformed to acids, such as methanol and ethylene glycol. Furthermore, uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol and pentachlorophenol dissociate their phenolic protons in the mitochondrial matrix to dissipate the proton gradient that drives ATP synthesis. Such chemicals cause ATP depletion and death can ensue very quickly. Another important microenvironment change caused by acid–base imbalance is elicited by numerous cationic amphiphilic drugs (e.g., chloroquine and amiodarone) that diffuse into lysosomes and accumulate there after being protonated at their amino groups. These compounds disrupt the normal processing of phospholipids and cause unique vesicular patterns in lysosomes that is the histopathologic hallmark of phospholipidosis. Finally, some xenobiotics cause harm merely by occupying a site or space. For example, some chemicals (e.g., the ethylene glycol–derived calcium oxalate) form water-insoluble precipitates in renal tubules. Another important example of an obstructive mechanism of toxicity is the renal tubular blockage resulting from crystals formed by melamine and its derivative cyanuric acid. Precipitation of melamine and cyanuric acid crystals was established as the mechanism of acute renal failure and death in dogs and cats fed melamine-contaminated pet food, and a similar toxicity occurred in infants fed a melamine-contaminated formula. In this case, the mechanism of toxicity was established by first confirming that acute renal failure could be reproduced in rats dosed with melamine (Dobson et al., 2008). Histopathologic evaluation of the kidney provided microscopic evidence of crystalline inclusions in proximal tubules capable of causing obstruction, and analysis of the crystal content by high-performance liquid chromatography-mass spectrometry (HPLC-MS) and Fourier transform infrared spectroscopy (FTIR) confirmed the presence of melamine and cyanuric acid. This case is also an example of how potential mechanisms can also be excluded as being causally related to toxicity. Specifically, cell culture studies with melamine and cyanuric acid showed that these compounds were not directly cytotoxic to renal proximal tubule cells, thereby giving more credence to the role of precipitation and obstruction in the ensuing toxicity. As such, the mechanistic work confirmed that melamine and cyanuric acid were present in the renal filtrate and re-formed an insoluble complex in the kidney tubules. This produced a physical blockage, leading to renal impairment which progressed rapidly to renal failure and death.

Idiosyncratic Toxicity: When No Mechanism Explains the Outcome

An idiosyncratic toxicity mechanism is one that characterized by its low, frequently rare incidence and its poor predictability. Idiosyncratic toxicity is most often described for adverse reactions to drugs, and the liver and skin tend to be the most frequently affected organs. Idiosyncratic reactions do not follow the general outline of toxic mechanisms illustrated in Fig. 3-1, nor do they follow the general rules regarding disposition and dose dependence outlined above. Rather, idiosyncratic reactions are distinguished from other adverse reactions because they do not show typical dose–response relationships and concentration dependence. Idiosyncratic drug reactions are referred to as type B reactions, whereas more common, concentration-dependent reactions are type A reactions. Examples of drugs known to cause idiosyncratic toxicity include isoniazid (antituberculosis), ximelagatran (anticoagulant), azithromycin (antibiotic), and carbamazepine (antiepileptic).

By their very nature, the mechanism of idiosyncratic reactions is not well-defined. However, there is emerging evidence that nongenetic and genetic factors contribute to susceptibility to such toxicity. Nongenetic factors could include current disease states, including infection, inflammation, and pregnancy. Numerous genetic factors including polymorphisms in drug metabolizing enzymes and drug transporters, enzymes that reduce ROS, and the MHC proteins have also been identified. In fact, there is growing evidence that immune-mediated effects, particularly those involving the adaptive immune system, are highly likely to contribute to idiosyncratic responses, and there is a strong clinical association between specific human leukocyte antigen (HLA) allele polymorphisms and idiosyncratic drug reactions to drugs such as carbamazepine (Uetrecht and Naisbitt, 2013).

Although the low frequency and multifactorial nature of idiosyncratic toxicity makes it difficult explain, there is a growing effort to predict this type of toxicity. However, research efforts have been directed toward developing in vitro and in vivo methods that predict intrinsic immunogenicity of drugs and chemicals, along with models that assess mitochondrial dysfunction, failure to adapt to modest injury, or inflammatory stress factors. Additionally, some mechanistic approaches apply quantitative systems pharmacology (QSP) to evaluate multiple factors and biological processes simultaneously. QSP is an in silico modeling technique that leverages and assimilates known physiology and the results of in vitro experiments in order to make predictions about how drugs affect biological processes. This type of modeling is proposed as a potentially useful tool for predicting and explaining critical events that likely precede immune-mediated idiosyncratic liver injury (Kullack-Ublick et al., 2017).

STEP 3: CELLULAR DYSFUNCTION AND RESULTANT TOXICITIES

Toxicity-induced cellular dysfunction is determined by the function of the target molecule. If the target molecule is involved in cellular signaling and regulation, then altered gene expression and/or cellular function will occur. However, if the target molecule is involved predominantly in essential processes, the resultant dysfunction may compromise cell survival, and the nature of cell death varies with the mechanism of cell dysfunction (Fig. 3-9).

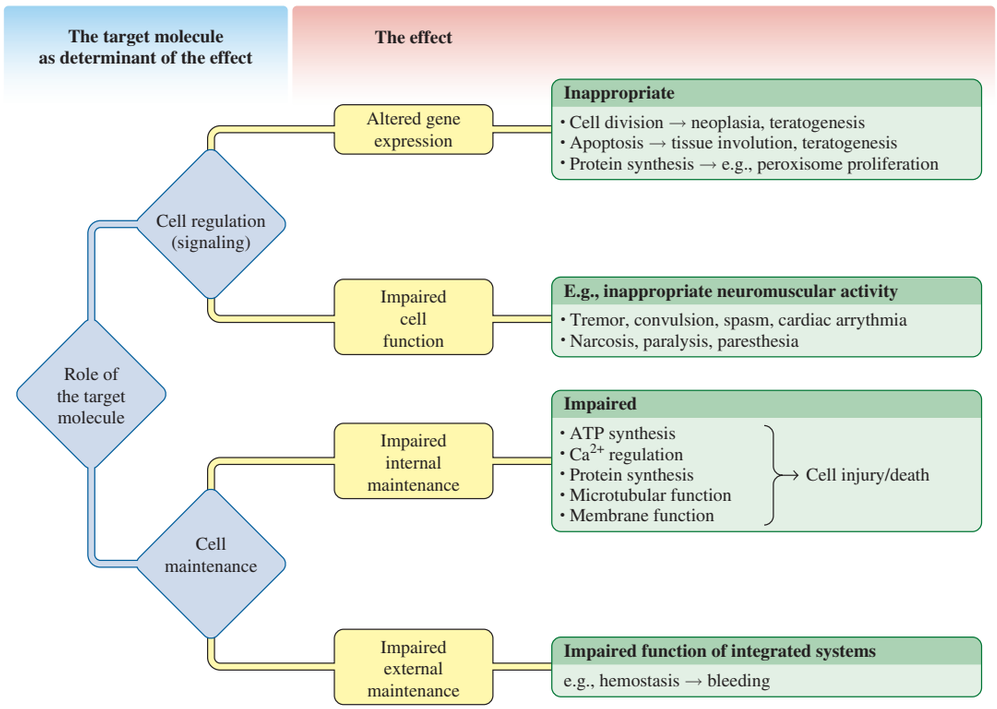

Figure 3-9. *The third step in the development of toxicity: alteration of cell functions.* The major effects are manifested as altered regulation of cell signaling or maintenance of cell function, with specific effects including altered gene expression, impaired cell function, impaired intracellular homeostasis, and extracellular homeostasis. Examples of such changes are included on the right side of the figure.

Toxicant-Induced Cellular Dysregulation

Transcriptional processes as well as numerous pathways involved in signal transduction cascades contribute to toxicant-induced alterations in cell regulation and gene expression. There are many cellular receptors that function to transmit signals to regulatory regions of genes and/or to functional proteins. Receptor activation may alter gene expression to increase or decrease protein expression. Additionally, the phosphorylation state of proteins can be altered, which will activate or inhibit their function. Programs controlling the fate of cells primarily affect gene expression, whereas those regulating the ongoing activities primarily influence the activity of functional proteins. Often, however, one signal evokes changes in both gene expression and cell function responses because of the complex branching and interconnection of many signaling networks.

Altered Gene Expression Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. The genetic information may be transcribed from DNA into messenger RNA (mRNA), which in turn is translated into proteins. There are other noncoding RNAs, including small interfering double-stranded RNA (siRNA) and microRNA (miRNA). miRNAs are short sequences, typically 20 to 25 base pairs in length, derived from regions of RNA transcripts that fold back on themselves to form short hairpins, whereas siRNAs are generated from longer regions of double-stranded RNA (Yokoi and Nakajima, 2013). The biogenesis of miRNAs is summarized in Fig. 3-10. Both siRNAs and miRNAs interfere with complementary nucleotide sequences to repress protein synthesis (discussed in detail below). Chemicals alter gene expression by affecting mRNAs, miRNAs, or both. Altered gene expression may also be affected directly at transcription, at signal transduction pathways, and at the synthesis, storage, or release of the extracellular signaling molecules.

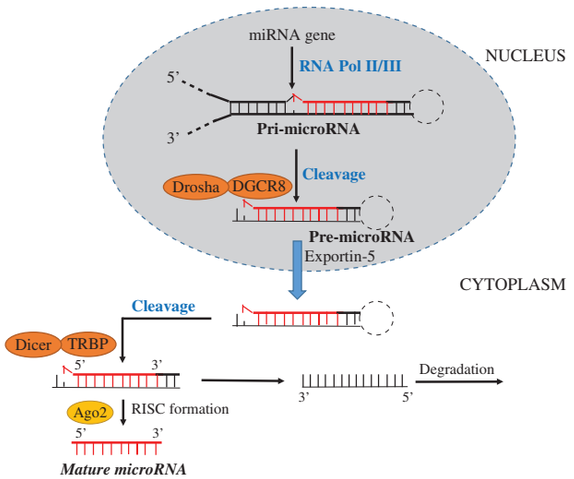

Figure 3-10. *Biogenesis of microRNAs (miRNAs).* Genes encoding for miRNAs are much longer than the processed, mature miRNA. miRNAs are transcribed by RNA polymerases as large precursors termed pri-miRNA, which may be several kilobases long, but are cut shorter by an RNase (Drosha), assisted by its partner protein DiGeorge syndrome critical region in gene 8 (DGCR8), to become pre-miRNA (60 to 70 nucleotides in length). PreRNAs are then exported from the nucleus to the cytoplasm by the karyopherin exportin 5 and Ran-GTP complex (ras-related nuclear protein; not shown) where it is cleaved further by Dicer, another RNAase, and its partner TRBP, and this cytoplasmic cleavage forms the mature miRNA. When a miRNA associates with Argonaute protein (Ago2), it forms the microRNA-induced silencing complex (RISC). The miRNA binds with base pairing to the untranslated region (UTR) of the target mRNA and RISC downregulates its translation and/or enhances mRNA cleavage. Because miRNAs bind to their target mRNAs by partial complementarity over a short sequence, one miRNA may have hundreds of mRNA targets. Therefore, a single miRNA can silence the expression of many proteins.

Dysregulation of Transcription Gene transcription is controlled largely by interplay between transcription factors (TFs) and the regulatory or promoter region of genes coding for mRNA and miRNA. By binding to distinctive nucleotide sequences in the promoter, ligand- and signal-activated TFs facilitate or impede the formation of the preinitiation complex to increase or repress transcription. Xenobiotics may interact with TFs (or other components of the preinitiation complex) and/or may alter the promoter region of the gene.

Several endogenous compounds, such as hormones (e.g., steroids and thyroid hormones) and vitamins (retinoids and vitamin D), influence gene expression by binding to and activating TFs or intracellular receptors (Table 3-3). However, many xenobiotics also mimic these natural ligands to alter cellular processes. For example, clofibrate and other fibric acid lipid-lowering drugs, phthalate esters, and the experimental compound Wy-14,643 ([4-chloro—(2,3-xylidino)-2-pyrimidinylthio] acetic acid) substitute for polyunsaturated fatty acids as ligands for the peroxisome proliferator–activated receptor α (PPARα), whereas xenoestrogens (e.g., DES, DDT, and bisphenol A) substitute for endogenous ligands of estrogen receptors (ERs). The polychlorinated hydrocarbon, tetrachloro-dibenzo-dioxin (TCDD) is a well-established ligand for the aryl hydrocarbon receptor (AhR).

Ligand-activated TFs control many fundamental cellular processes, and accordingly, adverse effects of compounds that act through TFs often result from perturbation of cell proliferation or by inducing cell death (Table 3-3). Glucocorticoid receptor agonists (such as dexamethasone) induce apoptosis of lymphoid cells. Although this is desirable in the treatment of lymphoid malignancies, it is an adverse response in many other conditions. TCDD produces thymic atrophy by inducing thymocyte apoptosis. Estrogens exert mitogenic effects through the ER in female reproductive organs, mammary gland, and liver, and prolonged exposure to estrogen and ER-mediated cell proliferation is involved in tumor formation in these organs. Furthermore, ER activation by diethylstilbestrol (DES) is involved in the induction of vaginal adenocarcinoma characterized by delayed development in late puberty after transplacental exposure to the synthetic estrogen. This is an example wherein stimulation of ERs during a critical prenatal period of differentiation reprograms estrogen target tissues, which increases the risk of benign and malignant abnormalities in the reproductive tract later in life (Al Jishi and Sergi, 2017).

TF-mediated toxicity can be defined mechanistically in genetically modified mouse models. For example, mice overexpressing estrogen receptor α (ERα) are exceedingly sensitive to DES-induced uterine adenocarcinoma, whereas ERα-knockout mice are resistant, demonstrating a role for ERα in tumorigenesis. Similarly, the mitogenic and hepatic tumor–promoting effects of peroxisome proliferators in rodents were confirmed to be receptor-mediated because they are not observed in PPARα-null mice. No hepatocellular and peroxisomal proliferation is observed in human liver after exposure to fibrates, and humans express lower levels of PPARα that is often in nonfunctional forms. Furthermore, humanized mice (i.e., PPARα-null mice engineered to express human PPARα) do not show hepatocellular and peroxisomal proliferation when treated with fibrates, indicating that the human receptor does not signal for proliferation in a manner similar to mice

Table 3-3

Toxicants Acting on Ligand-Activated Transcription Factors: Examples of Ligands and Effects

LIGAND-ACTIVATED TRANSCRIPTION FACTOR (GENE SYMBOL)	ENDOGENOUS LIGANDS	EXOGENOUS LIGAND	EFFECTS OF AGONISTS
Aryl hydrocarbon receptor (AhR)	Kynurenine	TCDD	Thymic atrophy
	Indole-3-carbinol; ITE	PCBs	Wasting syndrome
	Microbial metabolites: 3-methylindole, tryptamine	PAHs	Teratogenesis (cleft palate)
			Hepatocarcinogenesis in rats
			Enzyme induction (e.g., CYP1A1)
Peroxisome proliferator–activated receptor (PPARα; NR1C1)	Fatty acids	Fibrate esters (e.g., clofibrate)	Hepatocarcinogenesis in mice
	Leukotriene B4	Phthalate esters (e.g., DEHP)	Peroxisome proliferation
			Enzyme induction (e.g., CYP4A1, acyl-CoA oxidase)
Farnesoid X receptor (FXR; NR1H4)	Bile acids	Obeticholic acid	Inhibition of CYP7A1; inhibits bile acid synthesis and increases cholesterol
			Modulates fatty liver; involved in liver regeneration
Pregnane X receptor (PXR; NR1I2)	Pregnenolone	PCN, dexamethasone	Enzyme induction (e.g., CYP3A)
	Progesterone	Spironolactone, cyproterone	Transporter induction (e.g., Oatp1a4, Mdr1, Mrp1, Mrp2, Mrp3)
		Rifampicin	
Constitutive androstane receptor (CAR; NR1I3)	3α,5α-Androstenol	TCPOBOP, phenobarbital*	Enzyme induction (e.g., CYP2B, CYP3A)
	3α,5α-Androstanol (inhibitors)		Hepatocarcinogenesis in rodents
Estrogen receptor (ER; NR3A1)	17-β Estradiol	Ethynylestradiol	Mammary and hepatic carcinogenesis
		Diethylstilbestrol	
		Bisphenol A	
		DDT, methoxychlor	
		Tamoxifen, raloxifene	Antagonists (breast cancer prevention)
Glucocorticoid receptor (GR; NR3C1)	Cortisol (human)	Dexamethasone	Apoptosis of lymphocytes
	Corticosterone (rodent)	Prednisone	Teratogenesis (cleft palate)
Androgen receptor (AR; NR3C4)	Testosterone, dihydrotestosterone	DHEA	Muscle hypertrophy and bone growth
		Cyproterone, flutamide (antagonists)	Reduce prostate growth
Retinoic acid receptors (RAR; NR1B1 and RXR; NR2B1)	All-*trans*-retinoic acid	13-*cis*-Retinoic acid	Teratogenesis (craniofacial, cardiac, thymic malformations)

Abbreviations: ITE, 2-(1'H-indole-3'carbonyl)-thiazole-4-carboxylic acid methylester; TCPOBOP, 1,2-bis[2-(3,5-dichloropyridyloxy)]benzene; DHE, dihydroepiandrosterone.

**Phenobarbital indirectly activates CAR, not as a ligand.*

SOURCES: AhR ligands described in Hubbard et al. (2015) and endogenous ligands adapted from Germain et al. (2006).

(Peters et al., 2012). Furthermore, transfection of rat PPARα into human hepatocytes does not render the cells responsive to PPARα ligands, suggesting additional species differences downstream of ligand binding.

In addition to altering the fate of specific cells, compounds that act on ligand-activated TFs can also evoke changes in the metabolism of endobiotics and xenobiotics by inducing overexpression of relevant metabolizing enzymes. For example, PPARα ligands induce lipoprotein lipase and enzymes involved in fatty acid oxidation. Unlike the hepatic proliferative effects of PPARα agonists, these effects occur not only in rodents but also in humans, and are the basis for using fibrates as lipid-lowering drugs. TCDD, phenobarbital, and pregnenolone 16α-carbonitrile (PCN) activate AhR, constitutive androstane receptor (CAR), and pregnane X receptor (PXR), respectively (Table 3-3). This activation induces specific cytochrome P450 enzymes (CYPs), and the major CYPs induced by AhR, CAR, and PXR agonists are the CYP1A, CYP2B, and CYP3A families, respectively. However, rodent and human CARs and PXRs exhibit low degrees of sequence conservation in their ligand-binding domains, and the low sequence homology accounts for marked species differences in the induction potential of their ligands. For example, TCPOBOP activates CAR more effectively in mice than in humans, and rifampicin activates PXR in humans more effectively than in rodents (Gonzalez and Yu, 2006). Furthermore, there is a broad group of xenobiotic-metabolizing enzymes that are induced by these chemicals. For example, TCDD increases the expression of cytochrome P450 lAl, UDP-glucuronosyltransferase-1, and several subunits of mouse and rat glutathione *S*-transferase in an AhR-dependent manner. CYP1A1 is also strongly expressed in epithelial cells of the dioxin-induced cystic lesions in human skin such that immunohistochemical detection of CYP1A1 in skin has been applied as a biomarker of dioxin exposure (Saurat et al., 2012).

The mechanism of transcriptional regulation involves direct engagement with response elements in promoter regions. For example, after TCDD binds to AhR, it is complexed with the nuclear translocator protein Arnt (also called HIF-1β), and the complex binds to the dioxin (or xenobiotic) response element (DRE or XRE) to initiate transcription (Fig. 3-11). The cyclooxygenase-2 (COX-2) gene, whose product is involved in the synthesis of inflammatory prostaglandins, also contains the DRE/XRE so it is transactivated by TCDD-activated AhR. Similarly, the ligand-activated PPARα, after dimerizing with RXR, upregulates the transcription of genes encoding enzymes involved in fatty acid transport and β-oxidation by interacting with the peroxisome proliferator response element (PPRE).

Transcriptional Dysregulation by Chemical Alteration of Regulatory Region of Genes Xenobiotics may alter the regulatory gene regions through direct chemical interaction or by changing methylation patterns. For example, thalidomide causes developmental defects because it intercalates into GC boxes (GGGCGG) that are binding sites for several TFs including specificity protein 1 (Sp1) and early growth response protein 1 (Egr-1; Stephens et al., 2000). Genes that are essential for angiogenesis and normal limb development, including insulin-like growth factor-1 (IGF-1) and fibroblast growth factor-2 (FGF2), contain these GC boxes. Consequently, intercalation of thalidomide into the GC box sequence impairs the function of these target genes and disrupts normal signaling that is required for the critical timing of embryonic development.

Methylation of cytosine bases in CpG islands in the promoter regions of genes is a major epigenetic mechanism, which together with coupled histone modifications influences transcriptional activity of the downstream gene. The operational definition of an epigenetic mechanism is one that affects heritable traits that are not

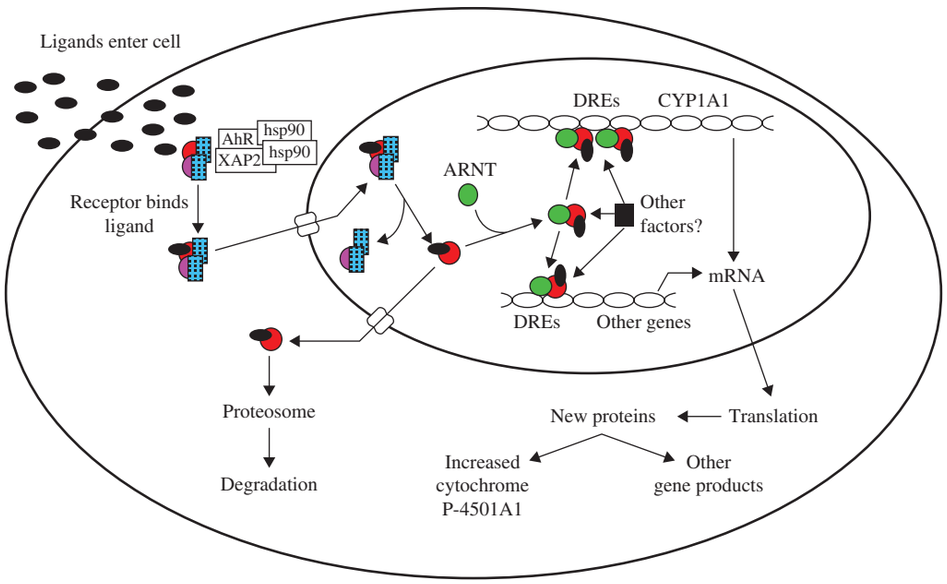

Figure 3-11. *Mechanism of ligand-induced Ah receptor (AhR) activation and its translocation to the nucleus to regulate DRE-mediated gene transcription.* In the absence of ligand, AhR is localized in the cytoplasm bound to a dimer of heat shock protein 90 (Hsp90) and XAP2 (hepatitis B virus X–associated protein 2; also called immunophilin-like AhR interacting protein) and several other proteins which prevent proteolysis. When ligand binds to AhR, the protein is translocated to the nucleus where Hsp 90 dissociates and Arnt (also called hypoxia-inducible factor α; HIF-1α) binds to the receptor. The activated AhR/ARNT heterodimer complex is then capable of interacting with DNA by binding to the dioxin (or xenobiotic)-responsive element (DRE or XRE), with the core sequence 5′-GCGTG-3′ within the consensus sequence 5′-T/GNGCGTGA/CG/CA-3′ in the 5′- regulatory region of a broad class of genes. (From Denison and Nagy [2003], with permission.)

explained by alterations in the DNA sequence. Thus, increased promoter methylation (hypermethylation) usually silences genes, whereas decreased methylation (hypomethylation) permits their activation. Importantly, when DNA replication occurs, the methylation pattern is copied from the parent strand to the daughter strand by DNA methyltransferase-1 (DNMT1), making the pattern heritable. Moreover, environmental conditions can alter promoter methylation to cause heritable changes in gene regulation. Altered promoter methylation has been implicated in chemically induced systemic lupus erythematosus (SLE) in developmental deficiencies manifested postnatally or in subsequent generations, and in cancer. In the case of drug-induced lupus, several drugs, such as procainamide (antiarrhythmic) and hydralazine (antihypertensive), induce SLE that can reverse upon cessation of drug treatment. Both drugs induce global DNA hypomethylation in $CD4^+$ T lymphocytes with concomitant overexpression of proteins important in inflammation. These changes increase T-cell activation that progresses to a systemic and cutaneous autoimmune response (Vedove et al., 2009).

DNA methylation is also an important process in genomic imprinting that takes place during formation of germ cells. Most genes in both sperm and ova similarly undergo or avoid epigenetic alterations. Offspring carry maternal and paternal alleles of most genes. However, some imprinted genes are epigenetically altered (generally methylated and silenced) only in the male or female germ cells, so that only the paternal or the maternal gene copy is expressed in the offspring. This is the case for the insulin-like growth factor-2 gene, which is expressed only from the paternal allele. Because imprinted genes have only a single active copy, they are especially susceptible for epigenetic dysregulation. Prenatal exposure to chemicals such as TCDD, ethanol, and bisphenol A has been implicated in altered genomic imprinting and adverse effects in adulthood including endocrine disruption and obesity (Dolinoy et al., 2007).

Role of miRNAs in Toxicant-Induced Alterations in Gene Expression miRNAs repress translation or increase mRNA degradation to decrease gene and/or protein expression. Accordingly, any toxicant that increases miRNA activity will decrease gene expression, whereas a decrease in miRNA increases gene expression through the loss of repression. These small noncoding RNAs are derived through a multistep process (Fig. 3-10), and overall, they serve to regulate many fundamental cellular functions, resulting in a broad range of potential adverse effects on development, immune responses, metabolism, and diseases, as well as specific toxicological outcomes. There are at least 2500 mature miRNAs that have been identified in the human genome (Murakami and Kawada, 2017).

miRNAs contribute to many mechanisms of toxicity. A major miRNA in liver is miR-122, which comprises about 50% and 70% of the miRNAs in adult mouse and human liver, respectively. miR-122 is regulated by liver-enriched TFs, including hepatocyte nuclear factors 6 (HNF6) and 4a (HNF4a) in a program that controls proliferation and differentiation of both hepatocytes and biliary epithelial cells (cholangiocytes). miR-122 is also implicated to have a central role in the development of fatty liver (steatosis) because genes involved in lipid biosynthesis are upregulated in miR-122 null mice. Similarly, miR-122 is decreased in a carbon tetrachloride-induced model of liver fibrosis in mice. Finally, loss of miR-122 has also been associated with increased incidence of hepatocellular carcinoma. Although the target genes regulated by miR-122 are not fully identified, inflammatory genes such as *Ccl2* (chemokine C-C motif ligand 2; also called macrophage chemoattractant protein 1; MCP1) and the profibrotic Kruppel-like factor 6 (KLF6) are regulated by miR-122. Additionally, validated targets of miR-122 with a role in carcinogenesis include cyclin G1, IGF1R, and Wnt 1 (Bandiera et al., 2015; Murakami and Kawada, 2017).

miRNAs also contribute to the regulation of drug-metabolizing enzymes. For example, CYP3A4, a constitutively abundant hepatic isozyme in most species, and CYP2E1 (involved in the formation of active metabolites of acetaminophen, carbon tetrachloride, and dimethylnitrosamine) are repressed by miR-27b and miR-378, respectively. The complexity of protein expression and regulation is underscored by the observation that although TFs control transcriptional expression of miRNAs, miRNAs can also control the translational regulation of these TFs (Yokoi and Nakajima, 2011).

In kidney, miR-21 has been shown to be upregulated in models of both acute and chronic injury. It is one of the most highly expressed miRNA in healthy kidneys, where it is detected in the renal cortex. It is relatively inactive under normal conditions, but when there is kidney injury or toxicity, miR-21 expression increases markedly. Moreover, this miRNA plays an important role in the development of fibrotic conditions in the kidney (discussed below). PPAR-α, particularly in endothelial cells, is a known target for miR-21, and during renal injury, PPAR-α expression decreases significantly as a result miR-21 targeting (Loboda et al., 2016).

Dexamethasone downregulates the expression of the miRNA cluster miR-17-92, which regulates expression of the proapoptotic protein Bim. The downregulation of miR-17-92 increases transcriptional activation of Bim, and this action contributes to glucocorticoid-induced apoptosis in lymphoid cells (Molitoris et al., 2011). Additional examples regarding the role of miRNAs in mechanisms of toxicity are discussed throughout the chapter, particularly in the sections summarizing fibrosis and carcinogenesis.

Dysregulation of Signal Transduction In its most basic definition, signal transduction is the process by which a chemical or physical signal is transmitted through a cell in a stepwise series of events, often involving kinase or phosphatase activity, which modifies protein activity by phosphorylation status to invoke a response. As such, many extracellular signaling molecules, including growth factors, cytokines, hormones, neurotransmitters, and some secreted proteins, engage intracellular signal transducing networks and ultimately activate TFs.

A major signal transduction pathway is the mitogen-activated protein kinase (MAPK) pathway, which is also referred to as the extracellular signal–regulated kinase (ERK). Signaling in this pathway is initiated by the binding of a signaling molecule to a cell surface receptor such as epidermal growth factor (EGF) binding to epidermal growth factor receptor (EGFR; Fig. 3-12). Mitogen binding causes Ras (a GTPase) to switch to GTP to phosphorylate and activate the kinase activity Raf, which in turn phosphorylates MEK which phosphorylates ERK. Raf, MEK, and ERK are also referred to as MAP3K (i.e., MAP kinase kinase kinase), MAP2K, and MAPK, respectively. There are numerous receptors and ligands that can activate this critical pathway that regulates cell division including IGF, fibroblast growth factor, and transforming growth factor α (TGF-α). This important pathway is shown schematically before presenting the more complex interaction across signaling pathways, and is intended to illustrate how such a series of kinase activities in a cascade can be affected by endogenous ligands or toxicants to perturb feedback regulation and signal amplification. Mutations in Ras and raf are also well described in carcinogenic mechanisms (discussed below).

Fig. 3-13 is a complex illustration of numerous networks with emphasis on those involved in cell cycle regulation, and identifies some signal-activated TFs that control transcriptional activity of genes that determine cell fate, with the MAPK pathway (4) shown in the middle of the figure. Once again, this chapter will not discuss

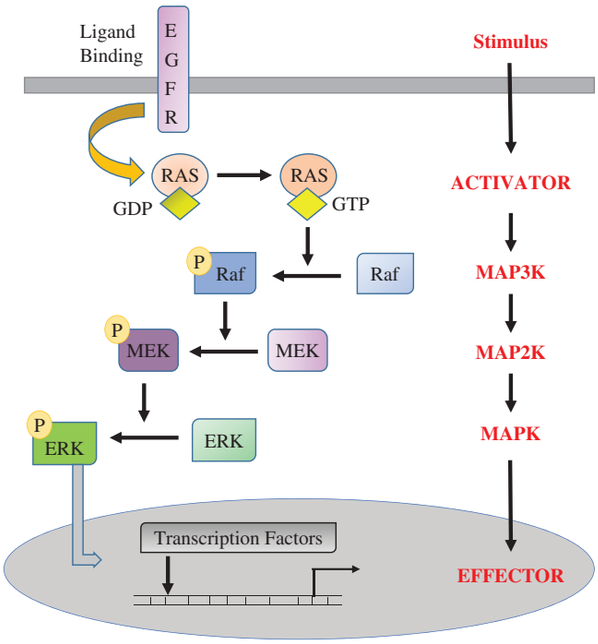

Figure 3-12. *The MAP kinase (MAPK) signal transduction pathway illustrating the cascade from receptor-mediated activation to transcriptional regulation.* The figure shows activation by the binding of the stimulus, epidermal growth factor (EGF) to the EGF receptor which activates Ras by converting it from its inactive GDP-bound form to the active GTP-bound state. This activation initiates a cascade of phosphorylation events (P) in MAP3K, MAP2K, and MAPK proteins (Raf, MEK, and ERK, respectively). The phosphorylated MAPK (ERK) moves into the nucleus and phosphorylates numerous transcription factors thereby enabling them to bind to cognate sequences in the promoter regions to facilitate transcription. In highlighting this cascade, the figure illustrates the cascade from the original stimulus to the activator of the pathway followed by the kinase activity, and finally the effector function. This pathway is a major regulator of cell cycle homeostasis and it is illustrated in other figures. Mutations in this pathway are also involved in carcinogenic mechanisms (discussed in the chapter).

all potential signaling mechanisms that can be altered in a toxic response. However, pathways that are fundamental to understanding adverse effects are summarized here.

The lower portion of Fig. 3-13 indicates important TFs activated by signaling pathways. For example, c-Fos and c-Jun, which dimerize (called AP-1) to bind to the tetradecanoylphorbol acetate (TPA) response element (TRE), play an important role in regulating cell division because they activate cyclin D to drive the G_1/S-phase transition of the cell cycle. There are other signal-activated TFs that also upregulate transcription of cyclin D and induce cell proliferation including c-Myc, NF-κB (nuclear factor kappa–light chain enhancer of activated B cells), CREB (cAMP response element binding protein), and STAT (signal transducer and activator of transcription). Major pathways converging on these TFs are shown in Fig. 3-13.

Signaling through the transforming growth factor β (TGF-β) signaling pathway is also an important pathway that influences cell growth, differentiation, and wound repair, and there are two transmembrane receptors (type I and type II) that function as serine/threonine kinases required for its signal transduction (Fig. 3-14). TGF-β binds to its type II receptor, which recruits and phosphorylates the type I receptor, and this complex in turn phosphorylates and activates Smad proteins (Smad 2, 3) that are the ultimate TFs that regulate target gene expression. TGF-β is a master regulator of numerous programs involved in wound healing, and it plays a central role in the development and progression of fibrotic diseases (discussed below). Altered signaling through TGF-β receptors has been implicated in Loeys–Dietz syndrome (LDS), a genetic disease characterized by serious defects in connective tissue formation and organization that increases the risk of aortic aneurysms particularly in children (Loeys et al., 2006).

The Wnt and hedgehog (Hh) pathways (Figs. 3-15 and 3-16) are also important regulators of cell division and differentiation. Both pathways have critical roles in embryonic development, tissue regeneration, and carcinogenesis. Activation of Wnt and Hh pathways regulate cellular levels of β-catenin and Gli (glioma-associated transcription factor), respectively, which in turn control transcription of numerous genes involved in cell cycle regulation, proteosomal activity, and cell differentiation. Wnt ligands are secreted proteins that bind to the cell surface receptor, frizzled (FZD) and the coreceptor, lipoprotein receptor-related protein (LRP). When Wnt is activated (Fig. 3-15), β-catenin escapes phosphorylation and degradation to translocate to the nucleus where it transcriptionally regulates many genes involved in cell cycle regulation, cell motility, and differentiation. Chemicals that influence Wnt signaling have numerous adverse effects including abnormal embryonic development, cell proliferation, and carcinogenesis.

Hh ligands are modified by palmitate or cholesterol to bind to the membrane receptor Patched (Ptch). In the absence of ligand, Ptch inhibits the activity of Smoothened (Smo) to prevent signal transduction. However, when Hh binds to Ptch, Smo is activated, and downstream effects prevent normal degradation of Gli so that it accumulates and translocates to the nucleus to regulate gene expression (Fig. 3-16). Toxicants that activate or inhibit Smo can have significant adverse effects. For example, cyclopamine is a Smo inhibitor that disrupts normal development to cause birth defects including cyclopia (failure of two ocular orbits to form).

Signal transduction occurs through successive protein–protein interactions and protein phosphorylation reactions at hydroxyl groups in specific serine, threonine, or tyrosine residues. Most cell surface growth factor receptors are kinases that phosphorylate their downstream targets. In addition to the MAPK pathway already described, the PI3K pathway (Fig. 3-13) leads to phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP_2), a lipid commonly found in the plasma membrane, to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 subsequently recruits the protein kinase Akt (or protein kinase B) to the membrane for activation. PI3K signaling is quite complex and includes many branches that regulate intermediary metabolism, protein synthesis, autophagy (discussed below), and cell senescence. In addition, Akt may also indirectly activate the mammalian target of rapamycin (mTOR), a ubiquitous protein kinase, whose phosphorylated products promote the trophic state of cells by boosting protein synthesis and restraining autophagy. Furthermore, Akt catalyzes the inactivating phosphorylation of glycogen synthase kinase 3 (GSK3), a ubiquitous multifunctional constitutionally active protein kinase. Inactivation of GSK3α and GSK2β causes numerous effects ranging from promotion of glycogen synthesis to suppression of apoptosis (see later). Additionally, Akt inactivates the FoxO family of TFs, which prevents protein degradation in the skeletal muscle and promotes proliferation, thereby countering muscle atrophy.

Most intracellular signal transducer proteins exist in the phosphorylated and dephosphorylated state, with protein kinases and phosphatases catalyzing these reactions, respectively. Some growth factor receptors amplify their signals by inducing formation of HOOH. For example, by attacking protein thiolate groups and converting them into sulfenic acid groups (discussed above),

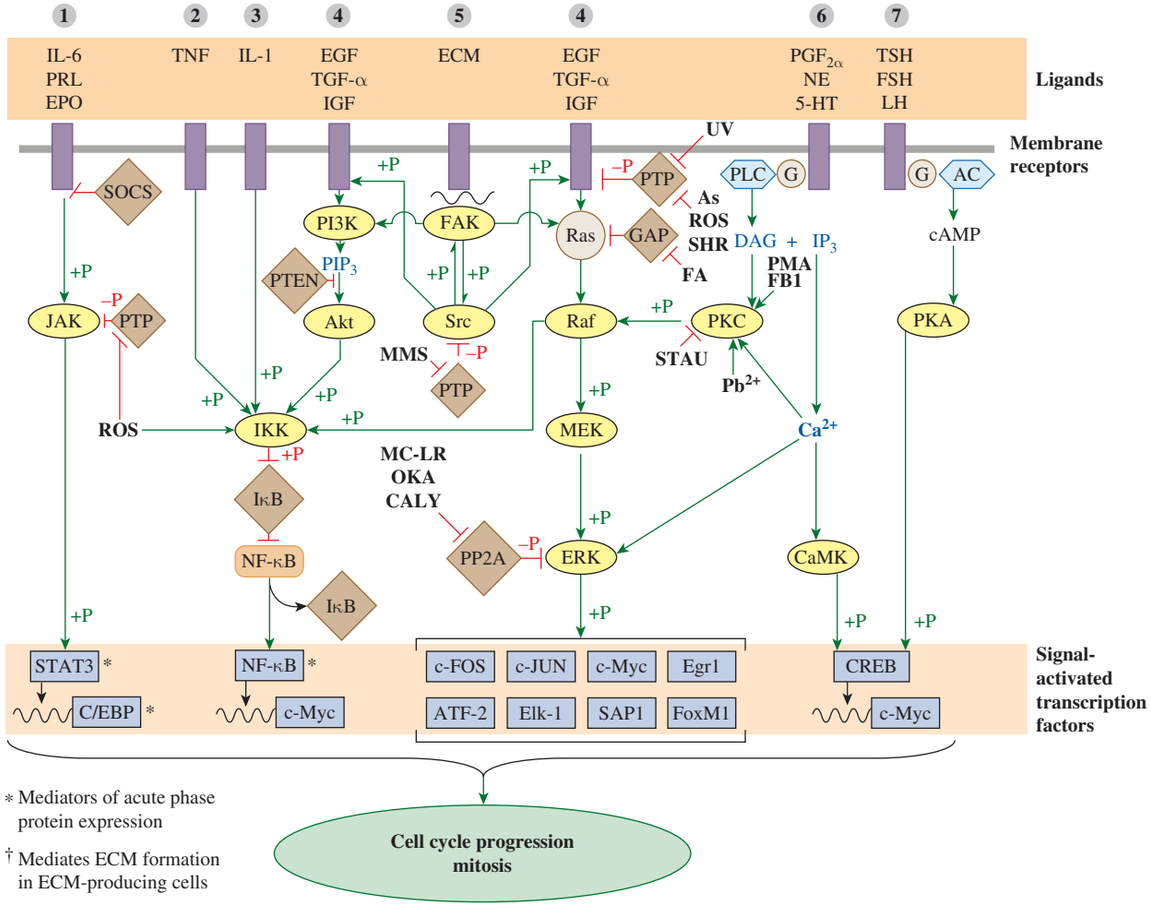

Figure 3-13. *Selected signal transduction pathways to illustrate phosphorylation cascades, potential interactions across pathways, and ultimately transcriptional regulation of the cell cycle.* The MAPK pathway illustrated in Fig. 3-12 is presented as activated by EGF in the middle of this figure (4) along with the numerous transcription factors it activates. The symbols of cell membrane receptors are numbered 1 to 7 and some of their activating ligands are indicated. G protein–coupled receptors are depicted in the numbers at the top of the figure and include the (1) JAK-STAT pathway; (2 and 3) NF-κB activation; (4) phosphatidilinositol-3-kinase (PI3K) and MAPK pathway; (5) integrin receptor–mediated activation of the focal adhesion kinase pathway that influences extracellular matrix (ECM) regulation; (6) phospholipase C activation of the protein kinase C (PKC) pathway; and (7) hormone-mediated activation of the cAMP and protein kinase A (PKA) pathway. The complexity of the figure illustrates the numerous interconnections between the pathways. For example, the G protein–coupled receptor (6) can relay signal into the MAPK pathway via PLC-catalyzed formation of second messengers that activate PKC, whereas signals from TNF and IL-1 receptors (2 and 3) are channeled into the PI3K–Akt–NF-κB pathway by phosphorylating IKK. The integrin receptor (5) can also engage the growth factor receptors and Ras as well as PI3K via FAK and the tyrosine kinase Src. For illustration purposes, the scheme is oversimplified. Virtually all components of the signaling network (e.g., G proteins, PKCs, and MAPKs) are present in multiple and functionally different forms whose distribution may be cell-specific. The pathways depicted are not equally relevant for all cells. In addition, these pathways determine the fate of cells, and they also control certain aspects of the ongoing cellular activity. *Abbreviations and symbols*: Circles represent G proteins, oval symbols protein kinases, rectangles transcription factors, wavy lines genes, and diamond symbols inhibitory proteins, such as protein phosphatases (PTP, PP2A) and the lipid phosphatase PTEN, the GTPase-activating protein GAP, and inhibitory binding proteins such as IκB and suppressor of cytokine signaling (SOCS). Arrowheads indicate stimulation or formation of second messengers (e.g., DAG, IP_3, PIP_3, cAMP, and Ca^{2+}), whereas blunt arrows indicate inhibition. Phosphorylation and dephosphorylation are indicated by +P and −P, respectively. Abbreviations for interfering chemicals are printed in black (As, arsenite; CALY, calyculin A; FA, fatty acids; FB1, fumonisin B; MC-LR, microcystin-LR; OKA, okadaic acid; MMS, methylmethane sulfonate; PMA, phorbol miristate acetate; ROS, reactive oxygen species; SHR, SH-reactive chemicals, such as iodoacetamide; STAU, staurosporin).

HOOH reversibly inactivates protein tyrosine phosphatase (PTP) and phosphatase and tensin homolog (PTEN), a lipid phosphatase that specifically dephosphorylates PIP_3 (see Fig. 3-13; Sies, 2014).

Chemically Altered Signal Transduction and Proliferation Xenobiotics that facilitate phosphorylation of signal transducers often promote mitosis and tumor formation. Examples include phorbol esters and fumonisin B that activate protein kinase C (PKC). The activated PKC promotes mitogenic activity through two major phosphorylation reactions. These are the phosphorylation of Raf, the first protein kinase in the MAPK pathway (Fig. 3-12), and by phosphorylation of a protein phosphatase that dephosphorylates the TF c-Jun at specific sites (Thr 231, Ser 234, and Ser 249), thereby permitting its binding to DNA. Chemicals that modulate PKC activity often mimic diacylglycerol (DAG), an endogenous activator of PKC. The other PKC activator is Ca^{2+}, which can be mimicked by Pb^{2+}. Lead ions increase PKC activity at picomolar concentrations, when Pb^{2+} occupies only high-affinity binding sites on PKC, but Pb^{2+} inhibits PKC activity at high, micromolar concentrations, where the low-affinity sites are also occupied.

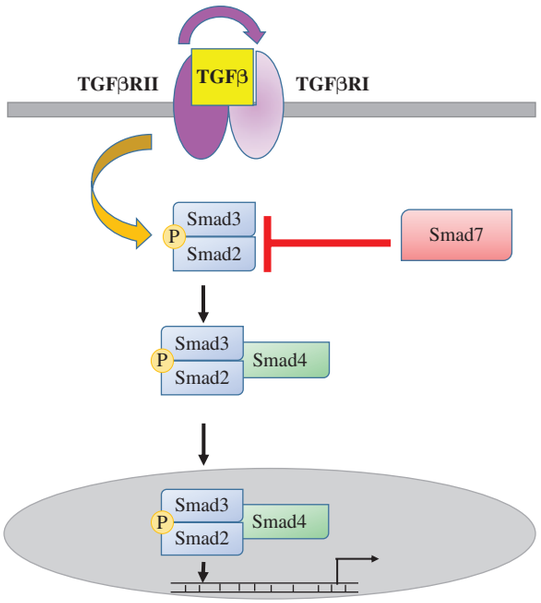

Figure 3-14. *The TGF-β signaling pathway.* Transforming growth factor β (TGF-β) is a major regulator of cell differentiation, and it determines the extent of the epithelial-mesenchymal transition (EMT), a process that is central to the development of fibrosis (discussed later in the chapter). The TGF-β ligand binds to its type II receptor, which recruits a type I receptor dimer forming a heterotetrameric complex with the ligand. These receptors are serine/threonine kinase receptors. The type II receptor phosphorylates serine residues of the type I receptor, which activates the protein. The signal transduction continues as the activated receptor kinase phosphorylates Smad 2/3 which recruits Smad 4, and this complex translocates to the nucleus. Smad 7 is a major negative regulator of this pathway.

Hyperphosphorylation of proteins may result not only from increased phosphorylation by kinases but also from decreased dephosphorylation by phosphatases. Inhibition of phosphatases, including the lipid phosphatase PTEN, appears to be the underlying mechanism of the mitogenic effect of various chemicals, oxidative stress, and ultraviolet (UV) irradiation. PTP and dual-specificity phosphatases (i.e., enzymes that remove phosphate from phosphorylated tyrosine as well as serine and threonine residues) as well as PTEN contain a catalytically active cysteine that is susceptible to inactivation by HOOH via sulfenic acid formation and by covalent reaction with other SH-reactive chemicals (Forman et al., 2004). Indeed, xenobiotics such as the SH-reactive iodoacetamide, the organometal compound tributyltin, arsenite, as well as HOOH cause phosphorylation of the epidermal growth factor (EGF) receptor (Fig. 3-13) by interfering with the PTP that dephosphorylates and inactivates this receptor (Juarez et al., 2008).

Protein phosphatase 2A (PP2A) is the major soluble Ser/Thr phosphatase in cells and is likely responsible, at least in part, for reversing the growth factor–induced stimulation of MAPK, thereby keeping the extent and duration of MAPK activation under control (Juntilla et al., 2008). Several natural toxins are extremely potent inhibitors of PP2A, including the blue-green algae poison microcystin-LR and the dinoflagellate-derived okadaic acid. With chronic, low-dose exposure, both compounds are tumor promoters. However, acute high-dose exposure to microcystin induces severe liver injury, whereas acute exposure to okadaic acid causes gastrointestinal effects associated with shellfish poisoning. In these acute conditions, hyperphosphorylation of proteins other than those involved in proliferative signaling (e.g., hepatocellular microfilaments in microcystin poisoning) is likely responsible for the toxicity.

Apart from phosphatases, there are also inhibitory binding proteins that can keep signaling under control. Such is the case for IκB, which binds to NF-κB, preventing its transfer into the nucleus and its function as a TF (Fig. 3-13). When phosphorylated by its designated IκB kinase (IKK), IκB is degraded by the proteasome to release NF-κB. The released NF-κB is a critical regulator of inflammatory processes. Through its signaling, a broad, pro-inflammatory program is controlled, including the release of cytokines (e.g., TNF and IL-1β), chemokines (e.g., MCP-1), cell adhesion molecules (e.g., ICAM-1, E-selectin, and P-selectin), enzymes producing inflammatory lipid mediators (e.g., PLA2 and COX-2), and acute-phase proteins (e.g., C-reactive protein and α1-acid glycoprotein; Karin, 2006). However, IKK can also be phosphorylated (activated) by other protein kinases, such as Raf (MAPK cascade) and Akt. NF-κB activation via Akt-mediated IKK phosphorylation is involved in the proliferative (and possibly carcinogenic) effect of arsenite, nicotine, and NNK.

Another pathway from which aberrant mitogenic signals may originate is the GTP/GDP-binding protein Ras. Signaling through Ras is normally terminated via stimulation of its own GTPase activity by a GTPase-activating protein (GAP; Fig. 3-13) that returns Ras into its inactive GDP-bound state. Fatty acids, which may accumulate in response to phospholipase A activation and exposure to peroxisome proliferators, inhibit GAP and can delay the turning off of Ras, leading to increased cell proliferation. Finally, genotoxic carcinogens may mutate Ras, and if the mutation leads to loss of its GTPase activity, the result is sustained signaling of the MAPK pathway to increase the likelihood of malignant transformation (discussed below).

Chemically Altered Signal Transduction with Antiproliferative Effect Cell proliferation is generally tightly controlled and is turned off when repair of injured cells is complete. However, an insult that decreases proliferative signaling after cell injury may compromise replacement of injured cells, and can direct a cell to apoptotic death (discussed below). Indeed, inhibitors of PKC (staurosporin), PI3K (wortmannin), and IκB degradation (gliotoxin) induce apoptosis. Similarly, TGF-β and glucocorticoids increase IκB, which in turn decreases NF-κB activation and c-Myc expression, and both mechanisms may contribute to the apoptotic effect of TGF-β and glucocorticoids, particularly in lymphoid cells.

Dysregulation of Extracellular Signal Production Hormones of the anterior pituitary exert mitogenic effects on endocrine organs by acting on cell surface receptors. Pituitary hormone production is under negative feedback control by hormones of the peripheral organs, and perturbation of this circuit adversely affects pituitary hormone secretion and, in turn, endocrine organ function. For example, xenobiotics that inhibit thyroid hormone production (e.g., the herbicide amitrole and the fungicide metabolite ethylenethiourea) or enhance thyroid hormone elimination (e.g., phenobarbital) reduce thyroid hormone levels and increase the secretion of thyroid-stimulating hormone (TSH) because of the reduced feedback inhibition. The increased TSH secretion stimulates cell division in the thyroid gland, which is responsible for the goiters or thyroid tumors caused by such toxicants (see Chap. 20). Decreased secretion of pituitary hormone produces the opposite adverse effect, with apoptosis followed by involution of the target organ. For example, estrogens produce testicular atrophy in males by means of feedback inhibition of gonadotropin secretion.

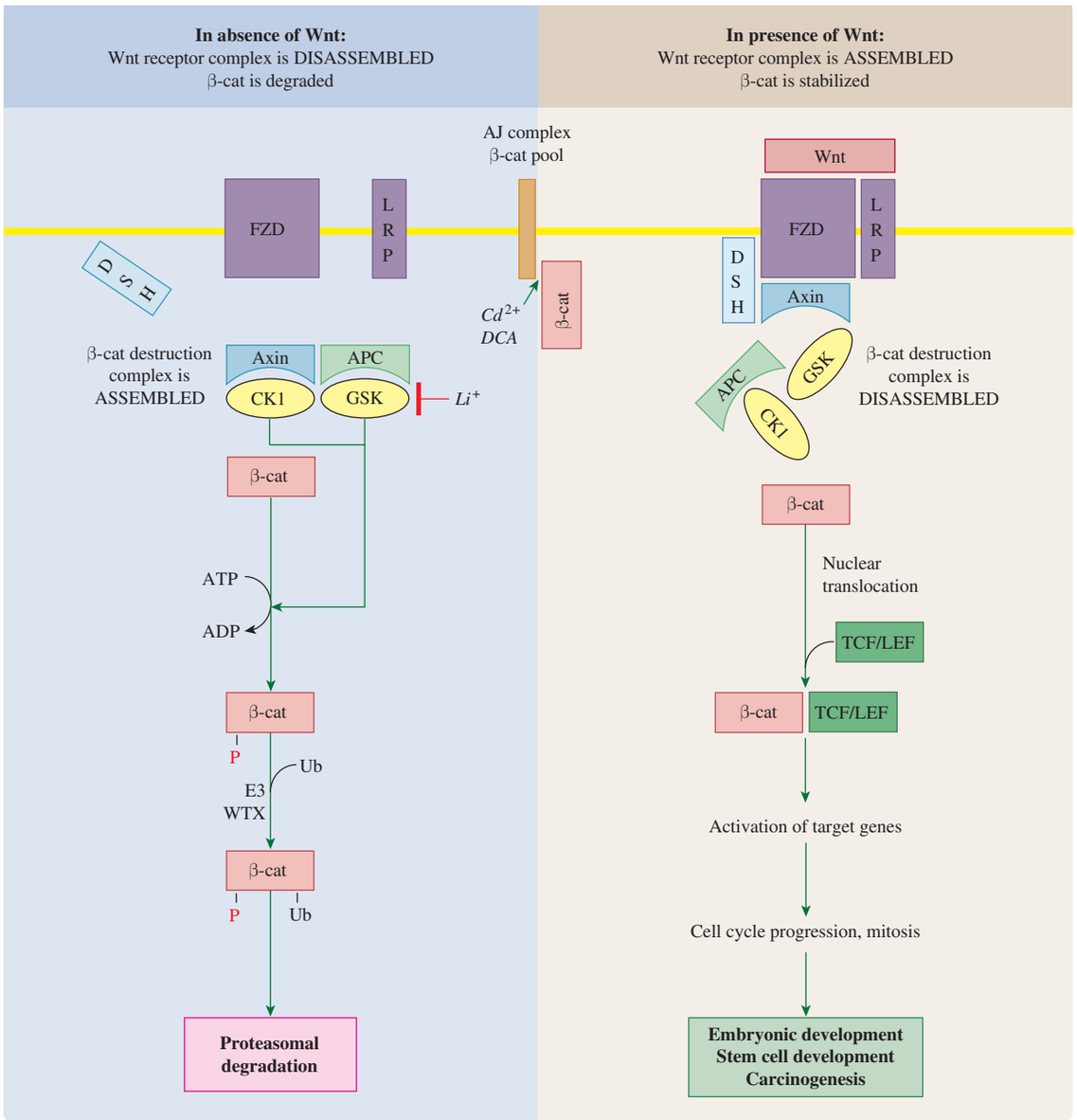

Figure 3-15. *The Wnt signaling pathway.* The pathway is activated when Wnt binds to the cell surface receptor frizzled (FZD) and co-receptor lipoprotein receptor–related protein (LRP). In the absence of Wnt (left panel) intracellular proteins, including Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK) form the β-catenin (β-cat) destruction complex. The kinase members of this complex phosphorylate β-cat, targeting it for ubiquitination by an E3 ubiquitin ligase and proteasomal degradation. On binding of Wnt to FZD and LRP (right panel) the Wnt receptor complex assembles, and with recruitment of Axin into this complex, β-cat escapes phosphorylation, ubiquitination, and degradation. β-cat accumulates, translocates into the nucleus, and is recruited to its target genes by TCF/LEF family of transcription factors. Wnt signaling upregulates the transcription of numerous genes. Chemicals that influence Wnt signaling may interfere with embryonic development, cell proliferation, and carcinogenesis. *Abbreviations*: DSH, disheveled; LEF, lymphoid enhancer–binding factor; TCF, T-cell factor; WTX, Wilms tumor suppressor.

Impaired Ongoing Cellular Activity Ongoing control of specialized cells is exerted by signaling molecules acting on membrane receptors that transduce the signal by regulating Ca^{2+} entry into the cytoplasm or stimulating the enzymatic formation of intracellular second messengers. Ca^{2+} or other second messengers ultimately alter phosphorylation of functional proteins, changing their activity and, in turn, cellular functions almost instantly. Perturbation of ongoing cellular activity by chemicals may be due to an alteration in (1) the concentration of neurotransmitters, (2) receptor function, (3) intracellular signal transduction, or (4) the signal-terminating processes.

Dysregulation of Electrically Excitable Cells Many xenobiotics influence cellular activity in excitable cells, such as neurons and skeletal, cardiac, and smooth muscle cells. Cellular functions such as the release of neurotransmitters and muscle contraction are controlled by transmitters and modulators synthesized and released by adjacent neurons. Mechanistic details concerning altered regulation of excitable cells is provided here as this information is not considered in other chapters of this book. These mechanisms are summarized in Fig. 3-17, and chemicals that interfere with these mechanisms are listed in Table 3-4.

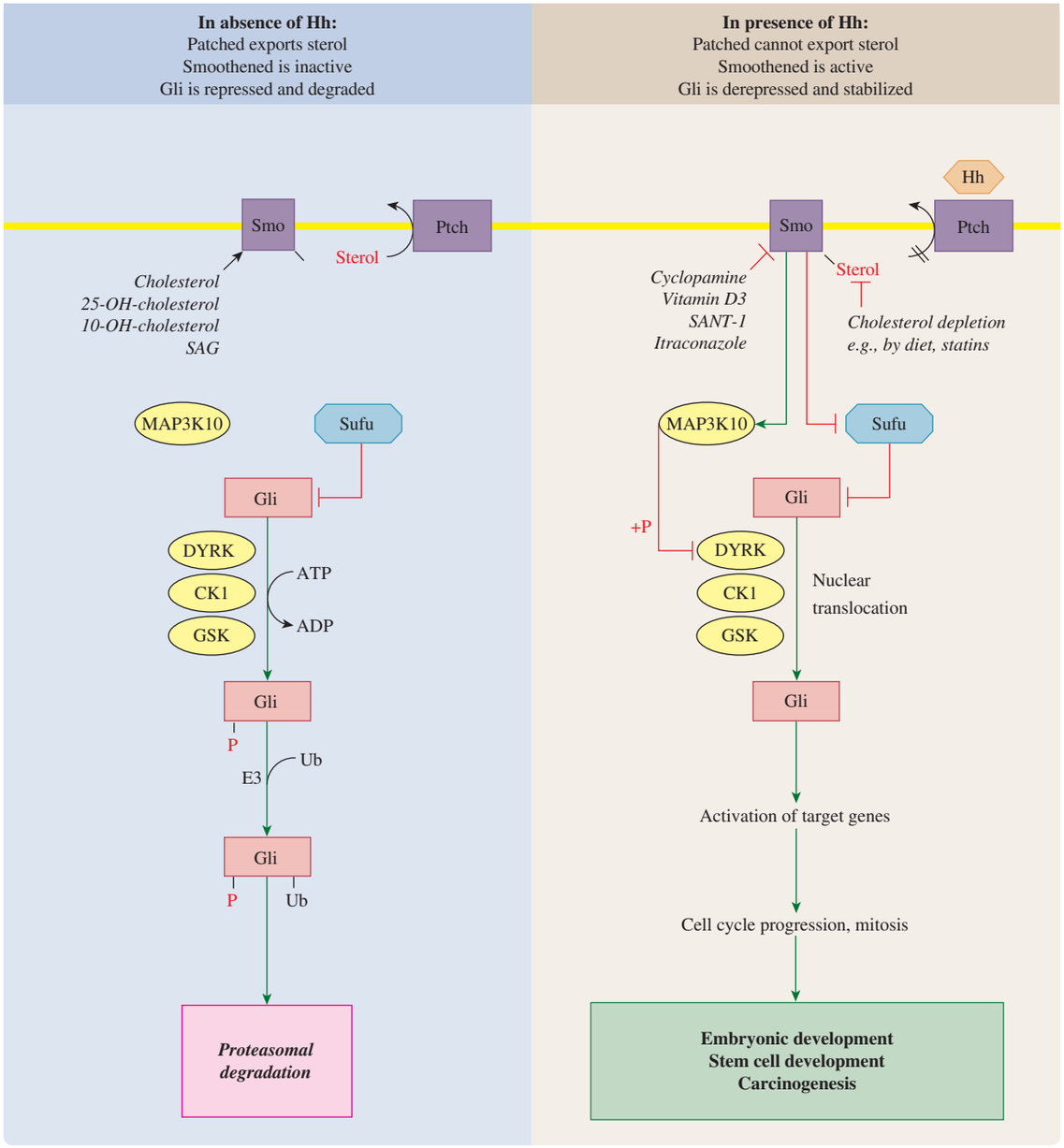

Figure 3-16. *The hedgehog (Hh) signaling pathway.* Hh ligands are secreted proteins covalently modified by palmitate and cholesterol. They act in autocrine, paracrine, or endocrine manner through their membrane receptor Patched (Ptch), which is the key inhibitor of Hh signaling in the absence of ligand. When Hh ligands are absent (left panel), Ptch impedes the pathway by inhibiting the activity of Smoothened (Smo), a positive regulator of signaling. Ptch is a sterol pump that exports sterols (cholesterol or an oxysterol) to remove these activator molecules from Smo and rendering Smo inactive. The repression of Smo by Ptch incapacitates the downstream Hh effectors, the glioma-associated family of transcription factors (Gli). Binding of Hh to Ptch (right panel) abrogates its sterol-exporting activity and Smo is activated. The active Smo suppresses Sufu to enable Gli activation, and activates mitogen-activated protein kinase kinase kinase 10 (MAP3K10), which in turn phosphorylates and inhibits DYRK, to prevent phosphorylation, ubiquitination, and proteasomal degradation of Gli. Active Gli translocates to the nucleus, and binds to its target genes. Hh signaling upregulates the transcription of numerous genes, including Ptch and Gli forming a positive feedback loop. It also induces secreted frizzled-related protein 1 (SFRP1) that binds Wnt proteins and inhibits Wnt signaling (Fig. 3-15), illustrating the interaction between the Wnt and Hh signaling pathways. Chemicals that activate or inhibit Smo may interfere with embryonic development. For example, the plant alkaloid cyclopamine inhibits Smo and causes the birth defect, cyclopia. Smo inhibitors are potentially anticarcinogenic, whereas Smo activators may promote carcinogenesis by increased Hh signaling. *Abbreviations*: SAG, smoothened agonist; SANT-1, smoothened antagonist 1.

Altered regulation of neural and/or muscle activity is the basic mechanism of action of many drugs and is responsible for toxicities associated with drug overdosage, pesticides, and microbial, plant, and animal toxins (Tatti et al., 2017). As neurons are signal-transducing cells, the influence of toxic chemicals is seen not only on the neuron affected by the toxicant but also on downstream cells influenced by neuronal activity. Thus, the action of tetrodotoxin is to block voltage-gated Na^+ channels in motor neurons (item 7 in Fig. 3-17), but the observed effect is skeletal muscle paralysis. Similarly, tarantula venoms, including ceratotoxins (CccTx1-3) and

phrixotoxin 3, also cause paralysis through inhibition of voltage-gated Na^+ channels (Bosmans et al., 2006). In contrast, cyclodiene insecticides, which block GABA receptors in the central nervous system (item 3 in Fig. 3-17), induce neuronal excitation and convulsions (Narahashi, 1992).

Alteration in Neurotransmitter Levels Chemicals may alter synaptic levels of neurotransmitters by interfering with their synthesis, storage, release, or removal from the vicinity of the receptor. These mechanisms manifest toxicity that is associated with the physiologic activity of the neurotransmitter. That is, if synaptic levels of a neurotransmitter are increased, the resulting response is an exaggeration of the normal effects. In contrast, if synaptic levels of a neurotransmitter are decreased, the outcome is usually the opposite of the normal activity. For example, GABA is an inhibitory neurotransmitter, and decreased synthesis of GABA by hydrazine and isoniazid will cause convulsions (Chen et al., 2016). Reserpine inhibits the neuronal storage of norepinephrine, 5-hydroxytryptamine, and dopamine, and depleting these transmitters causes a variety of cardiovascular and neurologic effects. Skeletal muscle paralysis caused by botulinum toxin is due to inhibition of acetylcholine release from motor neurons to prevent stimulation of the acetylcholine receptors at the neuromuscular junction (receptor 1 in Fig. 3-17). In contrast, inhibition of acetylcholinesterase by organophosphate or carbamate insecticides or chemical warfare agents (e.g., soman) prevents the hydrolysis of acetylcholine, resulting in massive stimulation of cholinergic receptors (receptors 1, 5, and 11 in Fig. 3-17) and a cholinergic crisis. Inhibition of the neuronal reuptake of norepinephrine by cocaine or tricyclic antidepressants is responsible for overexcitation of α_1-adrenergic receptors on vascular smooth muscles, resulting in nasal mucosal ulceration and myocardial infarction in heavy cocaine abusers, whereas overstimulation of β_1-adrenergic receptors contributes to life-threatening arrhythmias. Similar cardiac complications may result from amphetamine abuse, because

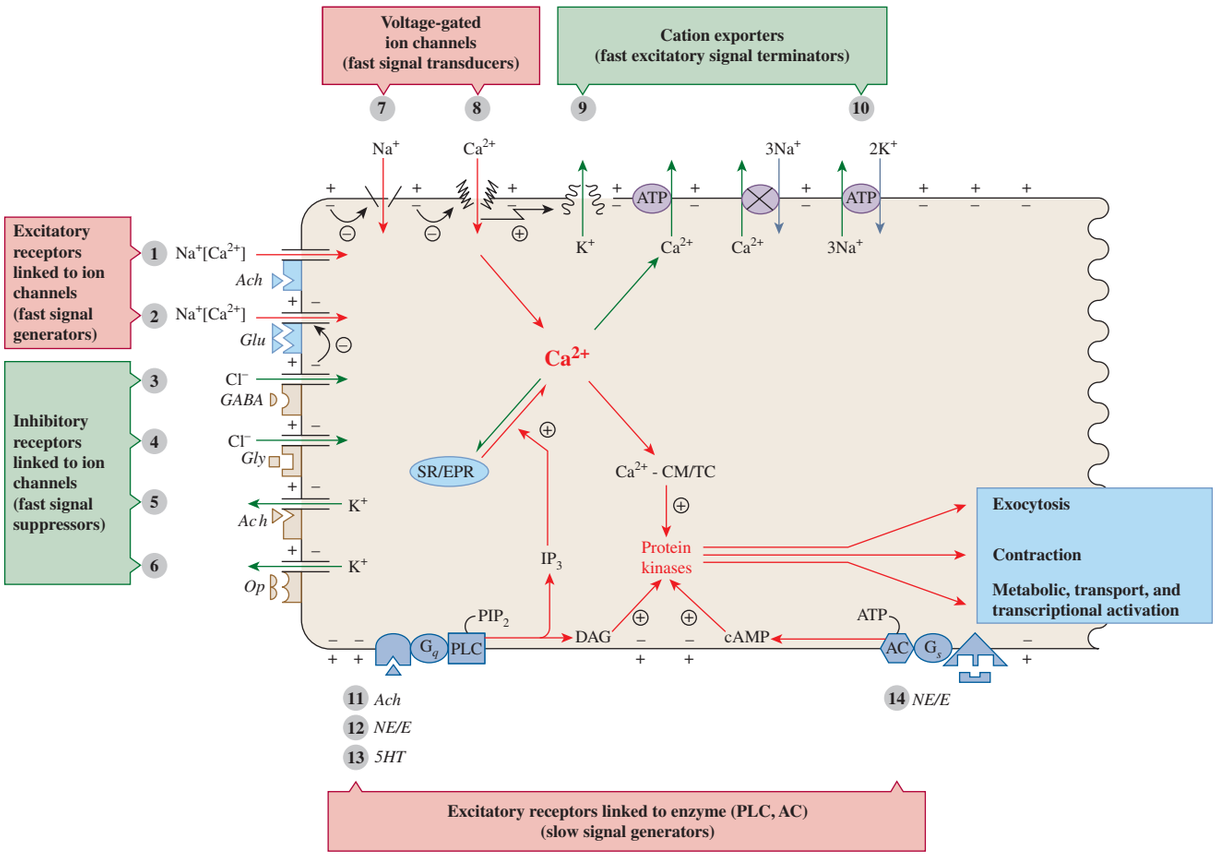

Figure 3-17. *Signaling mechanisms for neurotransmitters.* This simplified scheme depicts major cellular signaling mechanisms in neurons, muscle, and exocrine cells. Chemicals acting on the numbered elements are listed in Table 3-4. Fast signaling is initiated by the opening of ligand-gated Na^+/Ca^{2+} channels (1, 2). The resultant cation influx decreases the inside negative potential (i.e., evokes depolarization) and triggers the opening of the voltage-gated Na^+ and Ca^{2+} channels (7, 8). As a second messenger, Ca^{2+} activates intracellular Ca^{2+}-binding proteins such as calmodulin (CM) and troponin C (TC), which in turn enhance the phosphorylation of specific proteins. The signal is terminated by channels and transporters (9, 10) that remove cations from the cells to reestablish the inside negative resting potential (repolarization) and restore the resting Ca^{2+} level. Fast signaling can be suppressed by opening the ligand-activated Cl^- or K^+ channels (3–6), which increase intracellular hyperpolarization to counteract opening of the voltage-gated Na^+ and Ca^{2+} channels (7, 8). Signal transduction from other receptors (11–13) that are coupled to G_q proteins involves generation of the second messenger inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC), whereas signaling from receptor 14 that is coupled to G_s protein involves production of cyclic AMP (cAMP) by adenylyl cyclase (AC). These second messengers influence cellular activities by mobilizing Ca^{2+} from the sarcoplasmic or endoplasmic reticulum (SR and EPR), as is the case for IP_3, or by activating protein kinases, as is the case for cAMP and DAG activation of PKA and PKC, respectively. The figure does not depict that inhibitory receptors 5 and 6 are G_i protein–coupled that function by opening K^+ channels and inhibit AC. *Abbreviations*: Ach, acetylcholine; Glu, glutamate; GABA, γ-aminobutyric acid; Gly, glycine; Op, opioid peptides; NE, norepinephrine; E, epinephrine; 5HT, 5-hydroxytryptamine; G, G protein; PIP_2, phosphatidylinositol 4,5-bisphosphate. Positive and negative signs (circled) indicate activation and inhibition, respectively.

Table 3-4

Agents Acting on Signaling Systems for Neurotransmitters

RECEPTOR/CHANNEL/PUMP		AGONIST/ACTIVATOR		ANTAGONIST/INHIBITOR	
NAME*	LOCATION	AGENT	EFFECT	AGENT	EFFECT
1. Acetylcholine nicotinic receptor	Skeletal muscle	Nicotine Anatoxin-a Cytisine *Ind*: ChE inhibitors	Muscle fibrillation, and then paralysis	Tubocurarine, lophotoxin (from sea whip) α-Bungarotoxin (banded krait venom) α-Cobrotoxin (cobra venom) α-Conotoxin (marine cone snail) Erabutoxin b (banded krait venom) *Ind*: botulinum toxin	Muscle paralysis
	Neurons	See above	Neuronal activation	Pb^{2+}, general anesthetics	Neuronal inhibition
2. Glutamate receptor	CNS neurons	*N*-Methyl-*d*-aspartate Kainate, domoate Quinolinate Quisqualate *Ind*: hypoxia, HCN → glutamate release	Neuronal activation → convulsion, neuronal injury ("excitotoxicity")	Phencyclidine Ketamine General anesthetics	Neuronal inhibition → anesthesia Protection against "excitotoxicity"
3. $GABA_A$ receptor	CNS neurons	Muscimol, avermectins, sedatives (barbiturates, benzodiazepines), general anesthetics (halothane), alcohols (ethanol)	Neuronal inhibition → sedation, general anesthesia, coma, depression of vital centers	Bicuculline Picrotoxin Pentylenetetrazole Cyclodiene insecticides Lindane, TCAD *Ind*: isoniazid	Neuronal activation → tremor, convulsion
4. Glycine receptor	CNS neurons, motor neurons	Avermectins (?), general anesthetics	Inhibition of motor neurons → paralysis	Strychnine *Ind*: tetanus toxin	Disinhibition of motor neurons → tetanic convulsion
5. Acetylcholine M_2 muscarinic receptor	Cardiac muscle	*Ind*: ChE inhibitors	Decreased heart rate and contractility	Belladonna alkaloids (e.g., atropine), atropine-like drugs (e.g., TCAD)	Increased heart rate
6. Opioid receptor	CNS neurons, visceral neurons	Morphine and congeners (e.g., heroin, meperidine) *Ind*: clonidine	Neuronal inhibition → analgesia, central respiratory depression, constipation, urine retention	Naloxone	Antidotal effects in opiate intoxication
7. Voltage-gated Na^+ channel	Neurons, muscle cells, etc.	Aconitine, veratridine Grayanotoxin (rhododendrons) Batrachotoxin (poison dart frogs) Scorpion toxins Ciguatoxin (fish) DDT, pyrethroids	Neuronal activation → convulsion	Tetrodotoxin (pufferfish), saxitoxin (algae and cyanobacteria) μ-Conotoxin Local anesthetics Phenytoin Quinidine	Neuronal inhibition → paralysis, anesthesia Anticonvulsive action
8. Voltage-gated Ca^{2+} channel	Neurons, muscle cell, etc.	Maitotoxin (dinoflagellates) Atrotoxin (?) Latrotoxin [?] (black widow spider)	Neuronal/muscular activation, cell injury	ω-Conotoxin Pb^{2+}	Neuronal inhibition → paralysis

(*continued*)

Table 3-4

(Continued)

RECEPTOR/CHANNEL/PUMP		AGONIST/ACTIVATOR		ANTAGONIST/INHIBITOR	
NAME*	LOCATION	AGENT	EFFECT	AGENT	EFFECT
9. Voltage/Ca^{2+}-activated K^+ channel	Neurons, smooth and skeletal muscle, cardiac muscle	Pb^{2+}	Neuronal/muscular inhibition	Ba^{2+}, apamin (bee venom), dendrotoxin, 20-HETE, hERG inhibitors (e.g., cisapride, terfenadine)	Neuronal/muscular activation → convulsion/spasm vasoconstriction, PMV tachycardia (torsade de pointes)
10. Na^+, K^+-ATPase	Universal			Digitalis glycosides Oleandrin Chlordecone	Increased cardiac contractility, excitability Increased neuronal excitability → tremor
11. Acetylcholine M_3 muscarinic receptor	Smooth muscle, glands	*Ind*: ChE inhibitors	Smooth muscle spasm, salivation, lacrimation	Belladonna alkaloids (e.g., atropine), atropine-like drugs (e.g., TCAD)	Smooth muscle relaxation → intestinal paralysis, decreased salivation, decreased perspiration
Acetylcholine M_1 muscarinic receptor	CNS neurons	Oxotremorine *Ind*: ChE inhibitors	Neuronal activation → convulsion	See above	
12. Adrenergic α_1 receptor	Vascular smooth muscle	(Nor)epinephrine *Ind*: cocaine, tyramine, amphetamine, TCAD	Vasoconstriction → ischemia, hypertension	Prazosin	Antidotal effects in intoxication with α_1-receptor agonists
13. 5-HT_2 receptor	Smooth muscle	Ergot alkaloids (ergotamine, ergonovine)	Vasoconstriction → ischemia, hypertension	Ketanserine	Antidotal effects in ergot intoxication
14. Adrenergic β_1 receptor	Cardiac muscle	(Nor)epinephrine *Ind*: cocaine, tyramine, amphetamine, TCAD	Increased cardiac contractility and excitability	Atenolol, metoprolol	Antidotal effects in intoxication with β_1-receptor agonists

Abbreviations: ChE, cholinesterase; CNS, central nervous system; 20-HETE, 20-hydroxy-5,8,11, 14-eicosatetraenoic acid; Ind, indirectly acting (i.e., by altering neurotransmitter level); PMV, polymorphic ventricular; TCAD, tricyclic antidepressant.

**Numbering of the signaling elements in this table corresponds to those in Fig. 3-17. This table is simplified. Virtually all receptors and channels listed occur in multiple forms with different sensitivity to the agents. The reader should consult additional literature for more detailed information.*

amphetamine enhances the release of norepinephrine from adrenergic neurons and competitively inhibits neuronal reuptake of this transmitter. A hypertensive crisis can occur with the combined use of tricyclic antidepressants and monoamine oxidase inhibitors, drugs that block different mechanisms of norepinephrine elimination. Concomitant use of drugs that increase the concentration of serotonin (5-HT) by enhancing its neuronal release and decreasing its neuronal reuptake (e.g., fluoxetine) or its biotransformation (e.g., monoamine oxidase inhibitors) induces serotonin syndrome with cognitive and behavioral changes, autonomic dysfunction, and neuromuscular abnormalities. Some cytotoxic antineoplastic drugs (e.g., cisplatin) and radiation cause nausea and emesis by inducing release of 5-HT from enterochromaffin cells of the intestinal mucosa, which stimulates the 5-HT_3 receptors (5-HT-gated cation channel, functionally similar to item 1 in Fig. 3-17) on the adjacent vagal afferent neurons, thereby evoking the vomiting reflex. Intestinal release of 5-HT and stimulation of vagal afferent neurons are also involved in the emetic effect of ipecac syrup. The α_2-adrenergic receptor agonist clonidine induces release in the brain of β-endorphin, an endogenous peptide that stimulates opioid receptors (item 6 in Fig. 3-17). This explains why clonidine intoxication mimics several symptoms of morphine poisoning, including depressed respiration and pinpoint pupils.

Toxicant–Neurotransmitter Receptor Interactions Some chemicals interact directly with neurotransmitter receptors, including (1) agonists that associate with the ligand-binding site on the receptor and mimic the natural ligand, (2) antagonists that occupy the ligand-binding site but cannot activate the receptor, (3) activators, and (4) inhibitors that bind to a site on the receptor that is not directly involved in ligand binding. In the absence of other actions, agonists and activators mimic, whereas antagonists and

inhibitors block, the physiologic responses characteristic of endogenous ligands. For example, muscimol, a mushroom poison, is an agonist at the inhibitory $GABA_A$ receptor (item 3 in Fig. 3-17), whereas barbiturates, benzodiazepines, general anesthetics, and alcohols are activators. Thus, all these chemicals inhibit central nervous system activity, resulting in sedation, general anesthesia, and coma, and may ultimately inhibit the medullary respiratory center, depending on the dose administered. There are also similarities in the responses evoked by agonist/activators on excitatory receptors and those elicited by antagonists/inhibitors on inhibitory sites. Glutamate receptor agonists and muscarinic receptor agonists cause neuronal hyperactivity in the brain and ultimately convulsions, as do inhibitors of $GABA_A$ receptor. Likewise, chemicals acting as agonists/activators on inhibitory receptors and those acting as antagonists/inhibitors on excitatory receptors may exert similar effects. Finally, the numerous subtypes of neurotransmitter receptors may be affected differentially by toxicants. For example, the neuronal nicotinic acetylcholine receptor is extremely sensitive to inhibition by lead ions, whereas the muscular nicotinic receptor subtype is not.

Some sensory neurons have membrane receptors that are stimulated by noxious chemicals and operate as ligand-gated cation channels. The transient receptor potential (TRP) channels open to enable Na^+ and Ca^{2+} influx. TRPV1 is stimulated by capsaicin, the pungent ingredient of hot peppers, and mediates the burning sensation of the tongue and reflex stimulation of the lacrimal gland associated with exposure to pepper spray. However, the TRPA1 receptor is activated by thiol-reactive and oxidant chemicals, such as the lacrimator compounds in tear gas, acrolein, methyl isocyanate, and corrosive gases. As described previously, these chemicals evoke considerable irritation and pain, along with lacrimation, bronchial secretion, sneezing, coughing, bronchospasm, and neurogenic inflammation through this receptor (Bessac and Jordt, 2010).

Toxicant–Signal Transducer Interactions Many chemicals alter neuronal and/or muscle activity by acting on signal transduction processes. Voltage-gated Na^+ channels (item 7 in Fig. 3-17), which transduce and amplify excitatory signals generated by ligand-gated cation channels (receptors 1 and 2 in Fig. 3-16), are activated by a number of toxins derived from plants and animals (Table 3-4) as well as some synthetic chemicals such as DDT, resulting in overexcitation (Narahashi, 1992). In contrast, chemicals that block voltage-gated Na^+ channels (such as tetrodotoxin and saxitoxin) cause paralysis. Na^+ channels are also important in signal transduction in sensory neurons, as Na^+-channel activators evoke an excited state, whereas Na^+-channel inhibitors induce anesthesia.

Toxicant–Signal Terminator Interactions The cellular signal generated by cation influx is terminated by removal of the cations through channels or by transporters (Fig. 3-17). Inhibition of cation efflux by Ba^{2+} may prolong excitation by blockade of Ca^{2+}-activated K^+ channels (item 9 in Fig. 3-17) to cause potentially lethal neuroexcitatory and spasmogenic effects. The arachidonic acid metabolite 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) also blocks Ca^{2+}-activated K^+ channels, causing vasoconstriction. In fact, induction of 20-HETE production by cyclosporine in renal proximal tubular cells and the resultant decrease in renal cortical blood flow may contribute to the nephrotoxicity of cyclosporine (Seki et al., 2005). Blockade of hERG channels (human Ether-a-go-go Related Gene), a voltage-gated K^+ channel, causes prolonged delays in cardiac repolarization, as indicated by prolonged QT time in an ECG (see Chap. 18). Sustained QT prolongation may evoke polymorphic ventricular tachycardia (torsade de pointes), which degenerates into ventricular fibrillation and death (Cubeddu, 2016). Several drugs that show sustained QT prolongation (e.g., astemizole, cisapride, grepafloxacin, and terfenadine) have been withdrawn from clinical use because of serious side effects including death.

Glycosides from digitalis and other plants inhibit Na^+,K^+-ATPase (item 10 in Fig. 3-17) and thus increase the intracellular Na^+ concentration, which, in turn, decreases Ca^{2+} export by Ca^{2+}/Na^+ exchange. The resultant rise in the intracellular concentration of Ca^{2+} enhances the contractility and excitability of cardiac muscle. Failure of Na^+,K^+ pump function is also believed to contribute to neuronal damage resulting from hypoxia, hypoglycemia, and cyanide intoxication. Inasmuch as 70% of the ATP produced in neurons is used to drive the Na^+,K^+ pump; cessation of ATP synthesis causes a cell to become or remain depolarized. The depolarization-induced release of neurotransmitters such as glutamate from such neurons is thought to be responsible for the hypoxic seizures and further amplification of neuronal injury by the neurotoxic actions of glutamate.

Dysregulation of the Activity of Other Cells While many signaling mechanisms also operate in nonexcitable cells, disturbance of these processes is usually less consequential. For example, rat liver cells possess α_1-adrenergic receptors (item 12 in Fig. 3-17) whose activation evokes metabolic changes, such as increased glycogenolysis and glutathione export, through elevation of intracellular Ca^{2+}. However, no role in a toxic mechanism has been ascribed to xenobiotic interaction with these receptors.

Many exocrine secretory cells are controlled by muscarinic acetylcholine receptors (item 11 in Fig. 3-17). Salivation, lacrimation, and bronchial hypersecretion after organophosphate insecticide poisoning are due to stimulation of these receptors. In contrast, blockade of these receptors contributes to the hyperthermia characteristic of atropine poisoning. Kupffer cells, resident macrophages in the liver, secrete inflammatory mediators that can harm neighboring cells. Because Kupffer cells possess glycine-gated Cl^- channels (glycine receptors; item 4 in Fig. 3-17), the secretory function of these macrophages (e.g., secretion of inflammatory mediators) can be blocked by administration of glycine, which induces hyperpolarization via influx of Cl^-. Such intervention alleviates ethanol-induced liver injury (Yamashina et al., 2005).

Toxic Alteration of Cellular Maintenance

The major toxicity manifested by toxicant-induced alterations in cellular maintenance is cell death. This is usually an irreversible process that can disrupt the structural and functional integrity of the organ or organism, and it invokes numerous repair or adaptive processes to compensate for the loss of function. There are several different types of cell death that are distinguished by the morphological and biochemical alterations that accompany them. Understanding mechanisms of cell death provides for deeper recognition of the cellular processes that initiate this toxic outcome along with the compensatory changes that accompany these processes.

Mechanisms of Toxic Cell Death

Despite unique morphologic outcome and initiating events, there are three common, major events underlying cell death. These are ATP depletion, sustained rise in intracellular Ca^{2+}, and overproduction of ROS and RNS. These events and the chemicals that may cause them are individually characterized, after which the features of difference pathways leading to cell death are discussed. Classically,

cell death was considered to occur via necrosis, a process deemed to be irreversible damage. However, there are now additional pathways that lead to cell death, many of which involve more complex signaling mechanisms and a specific, regulated program. These pathways are primarily differentiated by the agents that induce the effects and the mediators of cell death. Mitochondrial changes are at the core of every cell death, regardless of the pathways or mediators involved.

Primary Metabolic Disorders Jeopardizing Cell Survival Cell viability is maintained by ATP as it supports intermediary metabolism and is the major source of cellular energy. ATP is utilized in numerous biosynthetic reactions, activating endogenous compounds by phosphorylation and adenylation, and it is incorporated into cofactors as well as nucleic acids. It is required for muscle contraction and polymerization of the cytoskeleton, fueling cellular motility, cell division, vesicular transport, and the maintenance of cell morphology. ATP drives many different ion transporters, such as the Na^+,K^+-ATPase in the plasma membrane, the Ca^{2+}-ATPase in plasma and ER membranes, and H^+-ATPase in the membrane of lysosomes and neurotransmitter-containing vesicles. These pumps also support essential cell functions. For example, the Na^+ concentration gradient across the plasma membrane generated by the Na^+,K^+ pump drives Na^+-glucose and Na^+–amino acid cotransporters as well as the Na^+/Ca^{2+} antiporter, facilitating the entry of these nutrients and the removal of Ca^{2+}.

Chemical energy is released by hydrolysis of ATP to ADP and AMP. The ADP is rephosphorylated in the mitochondria by ATP synthase (Fig. 3-18). *Oxidative phosphorylation* is the process by which ATP is synthesized when coupled to the oxidation of hydrogen to form water. In addition to ATP synthase, oxidative phosphorylation requires the (1) delivery of hydrogen in the form of NADH to the initial electron transport complex; (2) delivery of oxygen to the terminal electron transport complex; (3) delivery of ADP and inorganic phosphate to ATP synthase; (4) flux of electrons along the electron transport chain to O_2, accompanied by ejection of protons from the matrix space across the inner membrane; and (5) return of protons across the inner membrane into the matrix space down an electrochemical gradient to drive ATP synthase.

Several chemicals impede the various steps in oxidative phosphorylation to disrupt mitochondrial ATP synthesis (Wallace, 2008; Caito and Aschner, 2015). These chemicals are divided into five groups (Table 3-5). Substances in class A interfere with the delivery of hydrogen to the electron transport chain. For example, fluoroacetate inhibits the citric acid cycle and the production of reduced cofactors. Class B chemicals, such as rotenone and cyanide, inhibit the transfer of electrons along the electron transport chain to oxygen. Class C agents interfere with oxygen delivery to the terminal electron transporter, cytochrome oxidase. All chemicals that cause hypoxia ultimately act at this site. Chemicals in class D inhibit the activity of ATP synthase, the key enzyme for oxidative phosphorylation. At this site, the synthesis of ATP may be inhibited in one of four ways: (1) direct inhibition of ATP synthase, (2) interference with ADP delivery, (3) interference with inorganic phosphate delivery, and (4) deprivation of ATP synthase from its driving force, the controlled influx of protons into the matrix space. Protonophoric chemicals (uncouplers) such as 2,4-dinitrophenol and pentachlorophenol import protons into the mitochondrial matrix, dissipating the proton gradient that drives the controlled influx of protons into the matrix, which in turn drives ATP synthase. Finally, chemicals causing mitochondrial DNA injury to impair synthesis of specific proteins encoded by the mitochondrial genome (e.g., subunits of complex I and ATP synthase) are listed in group E. These include many of dideoxynucleoside antiviral drugs such as zidovudine.

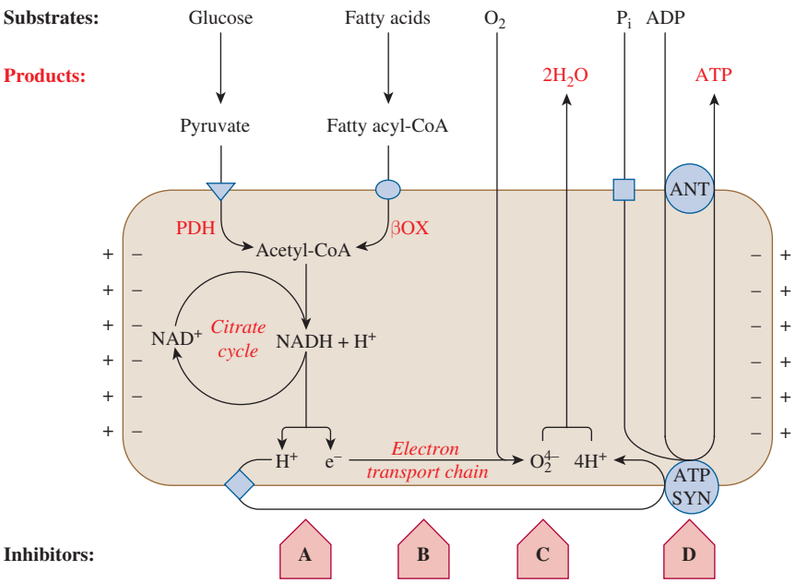

Figure 3-18. *ATP synthesis (oxidative phosphorylation) in mitochondria.* Arrows with letters A to D indicate the ultimate sites of action of four categories of agents that interfere with oxidative phosphorylation (Table 3-5). Toxicants that induce DNA damage (Class E, Table 3-5) are not illustrated. For simplicity, this scheme does not indicate the outer mitochondrial membrane and that protons are extruded from the matrix space along the electron transport chain at three sites. *Abbreviations*: βOX, beta-oxidation of fatty acids; e^-, electron; P_i, inorganic phosphate; ANT, adenine nucleotide translocator; ATP SYN, ATP synthase (F_oF_i-ATPase).

ATP synthase is also present in the outer segment disk of the retinal rods as well as the plasma membrane of certain cells and the concentric membranes that form the myelin sheath around the axon of neurons. In plasma and myelin membranes, ATP is formed in the external membrane by ecto-ATP synthase. ATP supply to axons, through the ecto-ATP synthase in myelin, may explain why demyelinated axons die, that is, they have no ATP source. In contrast, myelinated axons are sensitive to hypoxia but can synthesize ATP, if required.

Impairment of oxidative phosphorylation is detrimental to cells because with failure of ADP rephosphorylation, ATP is depleted and there is accumulation of ADP and its breakdown products. The biochemical changes observed in hepatocytes exposed to KCN and iodoacetate illustrate the events involved in toxicant-induced impairment of oxidative phosphorylation. In response to these toxicants, there is a rapid rise in cytosolic H^+ and Mg^{2+} resulting from hydrolysis of adenosine diphosphates and triphosphates (existing as Mg salts) with cellular acidosis resulting from the release of phosphoric acid and Mg^{2+}. Increased conversion of pyruvate to lactate also may contribute to acidosis. The lack of ATP compromises the operation of ATP-requiring ion pumps, leading to the loss of ionic and volume-regulatory controls. Furthermore, there is an increase

Table 3-5

Agents That Impair Mitochondrial ATP Synthesis*

Class A. Inhibitors of hydrogen delivery to the electron transport chain

1. Glycolysis (critical in neurons): hypoglycemia; iodoacetate, koningic acid, and NO^+
2. Gluconeogenesis (critical in renal tubular cells): coenzyme A depletors (see below)
3. Fatty acid oxidation (critical in cardiac muscle): hypoglycin, 4-pentenoic acid, 4-ene-valproic acid
4. Pyruvate dehydrogenase: arsenite, DCVC, *p*-benzoquinone
5. Citrate cycle
   (a) Aconitase: fluoroacetate, $ONOO^-$
   (b) Isocitrate dehydrogenase: DCVC
   (c) Succinate dehydrogenase: malonate, DCVC, PCBD-Cys, 2-bromohydroquinone, 3-nitropropionic acid, *cis*-crotonalide fungicides
6. Depletors of TPP (inhibit TPP-dependent PDH and α-KGDH): ethanol (when chronically consumed)
7. Compounds that deplete coenzyme A (CoA)
   (a) Thiol-reactive electrophiles: 4-(dimethylamino)phenol, *p*-benzoquinone
   (b) Drugs enzymatically conjugated with CoA: salicylic acid (the metabolite of aspirin), valproic acid
8. Compounds that deplete NADH
   (a) Alloxan, t-butylhydroperoxide
   (b) Activators of poly(ADP-ribose) polymerase (PARP): agents causing DNA damage (e.g., MNNG, hydrogen peroxide, $ONOO^-$)

Class B. Inhibitors of electron transport

1. Inhibitors of electron transport complexes
   (a) NADH–coenzyme Q reductase (complex I): rotenone, amytal, MPP^+, paraquat
   (b) Coenzyme Q–cytochrome *c* reductase (complex III): antimycin-A, myxothiazole
   (c) Cytochrome oxidase (complex IV): cyanide, hydrogen sulfide, azide, formate, $^{\bullet}NO$, phosphine (PH_3)
   (d) Multisite inhibitors: dinitroaniline and diphenylether herbicides, $ONOO^-$
2. Electron acceptors: CCl_4, doxorubicin, menadione, MPP^+

Class C. Inhibitors of oxygen delivery to the electron transport chain

1. Chemicals causing respiratory paralysis: CNS depressants (e.g., opioids), convulsants
2. Chemicals impairing pulmonary gas exchange: CO_2, NO_2, phosgene, perfluoroisobutene
3. Chemicals inhibiting oxygenation of Hb: carbon monoxide, methemoglobin-forming chemicals
4. Chemicals causing ischemia: ergot alkaloids, cocaine

Class D. Inhibitors of ADP phosphorylation

1. ATP synthase: oligomycin, cyhexatin, DDT, chlordecone
2. Adenine nucleotide translocator: atractyloside, DDT, free fatty acids, lysophospholipids
3. Phosphate transporter: *N*-ethylmaleimide, mersalyl, *p*-benzoquinone
4. Chemicals dissipating the mitochondrial membrane potential (uncouplers)
   (a) Cationophores: pentachlorophenol, dinitrophenol, benzonitrile, thiadiazole herbicides, salicylate, CCCP, cationic amphiphilic drugs (bupivacaine, perhexiline), valinomycin, gramicidin, calcimycin (A23187)
   (b) Chemicals permeabilizing the mitochondrial inner membrane: PCBD-Cys, chlordecone
5. Multisite inhibitor drugs: phenformin, propofol, salicylic acid (when overdosed)

Class E. Chemicals causing mitochondrial DNA damage

1. Antiviral drugs: zidovudine, zalcitabine, didanosine, fialuridine
2. Antibiotics: chloramphenicol (when overdosed), linezolid
3. Ethanol (when chronically consumed)

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DCVC, dichlorovinyl-cysteine; α-KGDH, α-ketoglutarate dehydrogenase; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MPP^+, 1-methyl-4-phenylpyridinium; PCBD-Cys, pentachlorobutadienylcysteine; PDH, pyruvate dehydrogenase; TPP, thiamine pyrophosphate.

**The ultimate sites of action of these agents are indicated in Fig. 3-18.*

in intracellular Na^+ that follows acidosis and hypermagnesemia. The increase in Na^+ likely results from failure of the ATP-requiring Na^+ pumps, and this effect leads to the formation of plasma membrane blebs, an early morphologic change in cell death. Although it contributes to acidosis, increased levels of intracellular phosphoric acid may be beneficial because it counteracts or prevents the rise in cytosolic Ca^{2+} by forming calcium phosphate. However, as discussed below, increased intracellular Ca^{2+} levels are generally very detrimental to cell viability. Biochemically, cellular acidosis directly decreases the activity of phospholipases and inhibits mitochondrial permeability transition (MPT; discussed in the section "Mitochondrial Permeability Transition and Necrotic Cell Death"). Finally, in its terminal stages, the intracellular pH rises, and this increases phospholipase activity, which enhances degradation of membrane phospholipids to initiate irreversible membrane damage.

Sustained Rise of Intracellular Ca^{2+} Intracellular Ca^{2+} levels are highly regulated. There is a 10,000-fold difference between extracellular and cytosolic Ca^{2+} concentration that is maintained by the impermeability of the plasma membrane to Ca^{2+} and by transport mechanisms that remove Ca^{2+} from the cytoplasm. Ca^{2+} is actively pumped out of the cytosol across the plasma membrane and it is sequestered in the ER and mitochondria. Importantly, mitochondria express a low-affinity Ca^{2+} transporter that plays a significant role in Ca^{2+} sequestration when the cytoplasmic concentrations rise into the micromolar range. Under such conditions, a large amount of Ca^{2+} accumulates in the mitochondria, where it is deposited as calcium phosphate. If toxicants open ligand- or voltage-gated Ca^{2+} channels or damage plasma membranes, Ca^{2+} readily moves down its concentration gradient from extracellular fluid into the cytoplasm. Similarly, toxicants may increase cytosolic Ca^{2+} by causing it to leak from the mitochondria or the endoplasmic reticulum (EPR) into the cytoplasm. They also diminish Ca^{2+} efflux through inhibition of Ca^{2+} transporters or depletion of their driving forces. Examples of chemicals that can cause a sustained rise in cytoplasmic Ca^{2+} levels are listed in Table 3-6.

Sustained elevation of intracellular Ca^{2+} is harmful because it will deplete energy reserves, alter microfilament structure, activate hydrolytic enzymes, and generate ROS and RNS. Increased intracellular Ca^{2+} levels alter cellular energy balance because high cytoplasmic Ca^{2+} concentrations increase mitochondrial Ca^{2+} uptake by the Ca^{2+} uniporter. In doing so, the mitochondrial membrane potential ($\Delta\Psi m$) is dissipated, and ATP synthesis is reduced. Ca^{2+} may also impair ATP synthesis by causing oxidative injury to the inner membrane by mechanisms described later. Furthermore, a sustained rise in cytoplasmic Ca^{2+} increases ATP consumption by forcing Ca^{2+}-ATPases to work to eliminate the excess Ca^{2+}.

A second mechanism by which an uncontrolled rise in cytoplasmic Ca^{2+} causes cell injury is microfilamental dissociation (Orrenius et al., 2015). The cell-wide network of actin filaments maintains cellular morphology by attachment of the filaments to actin-binding proteins in the plasma membrane. An increase of cytoplasmic Ca^{2+} causes dissociation of actin filaments from α-actinin and fodrin, proteins involved in anchoring the filaments to the plasma membrane. This mechanism leads to bleb formation in plasma membranes, a condition that predisposes the membrane to rupture.

Table 3-6

Agents Causing Sustained Elevation of Cytosolic Ca^{2+}

A. Chemicals inducing Ca^{2+} influx into the cytoplasm
- I. Via ligand-gated channels in neurons
  1. Glutamate receptor agonists ("excitotoxins"): glutamate, kainate, domoate
  2. TRPV1 receptor (capsaicin receptor) agonists: capsaicin
  3. TRPA1 receptor agonists: SH-reactive electrophiles, such as lacrimators (e.g., chlorobenzalmalonitrile), acrolein, methyl isocyanate, phosgene, chloropicrin
- II. Via voltage-gated channels: maitotoxin, $HO^{\bullet}$
- III. Via "newly formed pores": maitotoxin, amphotericin B, chlordecone, methylmercury, alkyltins
- IV. Across disrupted cell membrane
  1. Detergents: exogenous detergents, lysophospholipids, free fatty acids
  2. Hydrolytic enzymes: phospholipases in snake venoms, endogenous phospholipase A_2
  3. Lipid peroxidants: carbon tetrachloride
  4. Cytoskeletal toxins (by inducing membrane blebbing): cytochalasins, phalloidin
- V. From mitochondria
  1. Oxidants of intramitochondrial NADH: alloxan, t-BHP, NAPBQI, divicine, fatty acid hydroperoxides, menadione, MPP^+
  2. Others: phenylarsine oxide, gliotoxin, $^{\bullet}NO$, $ONOO^-$
- VI. From the endoplasmic reticulum
  1. IP_3 receptor activators: γ-HCH (lindane), IP_3 formed during "excitotoxicity"
  2. Ryanodine receptor activators: δ-HCH

B. Chemicals inhibiting Ca^{2+} export from the cytoplasm (inhibitors of Ca^{2+}-ATPase in cell membrane and/or endoplasmic reticulum)
- I. Covalent binders: acetaminophen, bromobenzene, CCl_4, chloroform, DCE
- II. Thiol oxidants: cystamine (mixed disulfide formation), diamide, *t*-BHP, $O_2^{\bullet-}$, and HOOH generators (e.g., menadione, diquat)
- III. Others: vanadate, Cd^{2+}, thapsigargin (specific SERCA inhibitor)
- IV. Chemicals impairing mitochondrial ATP synthesis (see Table 3-5)

Abbreviations: DCE, 1,1-dichloroethylene; HCH, hexachlorocyclohexane; MPP^+, 1-methyl-4-phenylpyridinium; NAPBQI, N-acetyl-p-benzoquinoneimine; SERCA, sarco/endoplasmic reticulum calcium ATPase; t-BHP, t-butyl hydroperoxide.

A third event whereby high Ca^{2+} concentrations are deleterious to cells is activation of hydrolytic enzymes that degrade proteins, phospholipids, and nucleic acids. Many integral membrane proteins are targets for Ca^{2+}-activated neutral proteases, or calpains (Orrenius et al., 2015). Calpain-mediated hydrolysis of actin-binding proteins may also initiate formation of membrane blebs. Other enzymatic alterations include the activation of a Ca^{2+}-Mg^{2+}-dependent endonuclease to cause chromatin fragmentation and alteration of topoisomerase II activity to disrupt DNA cleavage. Changes in phospholipase activity increase the formation of fatty acid intermediates that have detergent-like activity to further disrupt the integrity of the cell membrane.

Overproduction of ROS and RNS There are a number of xenobiotics that can directly generate ROS and RNS, such as chemicals that cause redox cycling and transition metals (refer to Figs. 3-3 and 3-4). In addition, overproduction of ROS and RNS can be secondary to intracellular hypercalcemia, as Ca^{2+}activates enzymes that generate ROS and/or RNS by activating dehydrogenases in the citric acid cycle which accelerate hydrogen output and in turn increases the flux of electrons along the electron transport chain (see Fig. 3-18). This action increases the formation of $O_2^{\cdot-}$ by the mitochondrial electron transport chain. Ca^{2+} also activates proteases that convert xanthine dehydrogenase into xanthine oxidase, which generates $O_2^{\cdot-}$ and HOOH. Finally, neurons and endothelial cells constitutively express NOS that is activated by Ca^{2+}. Given the extremely high reactivity of $^{\cdot}NO$ with $O_2^{\cdot-}$, coproduction of these radicals will inevitably lead to formation of $ONOO^-$, a highly reactive oxidant (Calcerrada et al., 2011; Fig. 3-4).

An important mechanistic consideration in understanding cell death mechanisms is that the interplay between the three primary derangements in biochemistry discussed above do not remain isolated but interact and amplify each other in a number of ways. This interplay, depicted in Fig. 3-19, includes the following:

- Depletion of cellular ATP reserves that deprive the endoplasmic and plasma membrane Ca^{2+} pumps of fuel, causing elevation of Ca^{2+} in the cytoplasm. With the influx of Ca^{2+} into the mitochondria, the mitochondrial membrane potential ($\Delta\Psi m$) declines, hindering ATP synthase.
- Intracellular hypercalcemia that facilitates formation of ROS and RNS, which inactivates thiol-dependent Ca^{2+} pumps by oxidative damage to further exacerbate hypercalcemia.
- Formation of ROS and RNS inhibits ATP synthesis. $^{\cdot}NO$ is a reversible inhibitor of cytochrome oxidase; NO^+ (nitrosonium cation, a product of $^{\cdot}NO$) *S*-nitrosylate and inactivate glyceraldehyde 3-phosphate dehydrogenase, impairing glycolysis, whereas $ONOO^-$ irreversibly inactivates respiratory chain complexes I, II, III and aconitase (by reacting with their Fe-S center; Calcerrada et al., 2011).
- Formation of $ONOO^-$ that induces DNA single-strand breaks, which activates poly(ADP-ribose) polymerase (PARP). As part of the repair strategy, activated PARP transfers multiple ADP-ribose moieties from NAD^+ to nuclear proteins and PARP itself. This consumption of NAD^+ severely compromises ATP synthesis (see Fig. 3-18), increasing the need to resynthesize NAD^+ further consumes ATP to ultimately cause a significant cellular energy deficit (Calcerrada et al., 2011).

However, the chain of events that contribute to the worsening metabolic conditions are somewhat cell- and toxicant-specific. For example, cyanide toxicity in neurons is associated with rapid depolarization and glutamate release, followed by Ca^{2+} influx through voltage-gated as well as glutamate-gated channels (see items 8 and 2, respectively, in Fig. 3-17). In contrast, in KCN- and iodoacetate-poisoned liver cells, early events center on cellular acidosis, and the increase in cytoplasmic Ca^{2+} occurs somewhat later. Finally, a nitrosative stress also contributes to propagation of acetaminophen-induced hepatocellular injury. In this example, the initiating event is covalent binding of *N*-acetyl-*p*-benzoquinoneimine (NAPBQI) to mitochondrial proteins. This purportedly causes a surge in mitochondrial ($O_2^{\cdot-}$) generation, followed by in situ formation of $ONOO^-$ which modifies mitochondrial proteins. With depletion of glutathione, covalent binding incapacitates the plasma membrane Ca^{2+} pump to increase intracellular Ca^{2+} levels which further reduces mitochondrial function and ATP production (Jaeschke and Bajt, 2006). The interplay of ATP depletion, intracellular hypercalcemia, and overproduction of ROS and RNS involves multiple cycles that progressively aggravate biochemical dysfunction until the cell dies.

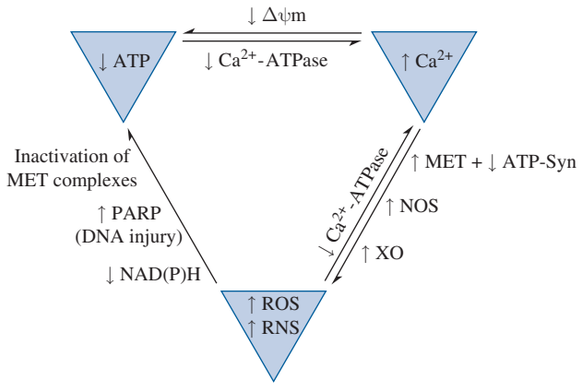

Figure 3-19. *Interrelationship between the primary metabolic disorders (ATP depletion, intracellular hypercalcemia, and overproduction of ROS/RNS) that ultimately cause necrosis or apoptosis. Abbreviations*: ATP-Syn, ATP synthase; MET, mitochondrial electron transport; NOS, nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; RNS, reactive nitrogen species; XO, xanthine oxidase; $\Delta\Psi m$, mitochondrial membrane potential.

Mitochondrial Permeability Transition and Necrotic Cell Death Mitochondrial permeability transition (MPT) is an abrupt change in mitochondrial membranes that results in formation of a nonspecific pore that opens when mitochondrial Ca^{2+} levels are high, especially when accompanied by decreased $\Delta\Psi m$, generation of ROS and RNS, and depletion of ATP (Hurst et al., 2017). Formation of the pore, which results from misfolded proteins in both the inner and outer mitochondrial membranes, enables solutes $<$1500 Da, and protons to readily enter into the mitochondrial matrix space, which in turn causes rapid and complete dissipation of $\Delta\Psi m$, cessation of ATP synthesis, and osmotic influx of water. Collectively, these events produce biochemical dysfunction and mitochondrial swelling. Ca^{2+} in the mitochondrial matrix effluxes out of mitochondria through the pore to increase cytoplasmic Ca^{2+} concentrations. In the face of these changes, mitochondria are incapable of synthesizing ATP and the organelle further wastes any remaining ATP because depolarization of the inner membrane converts ATP synthase to an ATPase that hydrolyzes ATP. At this point, even glycolysis is compromised by the insufficient ATP supply to the ATP-requiring glycolytic enzymes (hexokinase and phosphofructokinase). If extensive, a complete bioenergetic catastrophe develops that culminates in necrotic cell death.

The histopathologic presentation of hepatic centrilobular necrosis is illustrated in Fig. 3-20. Morphologically, there is obvious loss of the nucleus, and individual cells are no longer obvious in the necrotic region. Such cellular loss is usually irreversible unless the organ can

mount a repair process (discussed below). However, this final outcome is preceded by a continuum of changes that begin with mitochondrial swelling (which can only be seen by electron microscopy) followed by a continuum of nuclear changes. The nuclear changes noted in necrotic cell death include (1) pyknosis characterized by shrunken and abnormally dark basophilic staining of the nucleus; (2) karyolysis, in which nuclei appear swollen and abnormally pale basophilic staining, and (3) karyorrhexis, the rupture and fragmentation of the nucleus (Table 3-7). Numerous imaging methods, including confocal microscopy, can be used to detect the onset of the MPT in individual mitochondria within cultured living cells. Selective flurophores that distribute to the cytoplasm such as calcein will redistribute into the mitochondria when MPT occurs. Similarly, fluorophores that monitor membrane potential, such as tetramethylrhodamine methylester, will be simultaneously released from the mitochondria. In this way, MPT is reflected by the mitochondrial presence of calcein and the cytoplasmic appearance of the rhodamine methylester. Cyclosporin A (CsA) and trifluoperazine (TFP) have been shown to block the MPT in numerous models of cell death. Co-treatment or pretreatment with CsA or TFP prevents cell killing, thereby proving that the MPT is a causative factor in necrotic cell death.

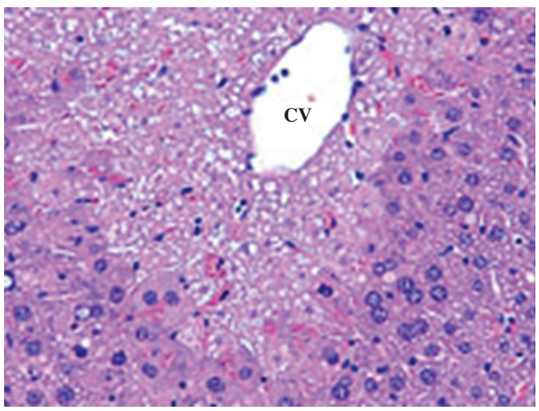

Figure 3-20. *Photomicrograph depicting the morphological presentation of hepatocellular necrosis.* There is a focal area of necrosis immediately surrounding the central vein (CV) with evidence for loss of cell integrity and complete absence of nuclei in this region. The region outside this focal area of necrosis is generally normal. An apoptotic lesion would be more eosinophilic in nature and nuclei would still be present but potentially fragmented. (From https://www.niehs.nih.gov/research/resources/visual-guides/liverpath/assets/images/i_k/jw103.jpg.)

Other Mechanisms of Cell Death Although necrotic cell death is a common mechanism by which toxicants act, there are numerous additional mechanisms by which cell death occurs. In the case of necrosis, the common steps outlined above occur in a somewhat uncontrolled cascade. However, other forms of cell death typically involve a program or more ordered cascade that ultimately triggers irreversible damage. These programs include apoptosis, necroptosis, ferroptosis, and autophagy, and the general morphologic, biochemical, and molecular features of these pathways are summarized in Table 3-7. While the necrotic cell swells and lyses, the apoptotic cell shrinks. Accordingly, apoptotic changes are manifested as condensation of nuclear and cytoplasmic materials, after which the cell breaks into membrane-bound fragments (apoptotic bodies) that are phagocytosed (Zhivotovsky and Orrenius, 2001; Orrenius et al., 2011).

Table 3-7

Morphological, Biochemical, and Molecular Features of Cell Death Mechanisms

	MORPHOLOGICAL FEATURES			BIOCHEMICAL FEATURES	CORE REGULATORS	
TYPE	CELL MEMBRANE	NUCLEUS	CYTOPLASM		POSITIVE	NEGATIVE
Autophagy	No change	No chromatin condensation	Accumulation of autophagic vacuoles	Substrate degradation	ATG5 ATG7 Beclin	
Necrosis	Blebbing and rupture	Pyknosis, karyorrhexia, and karyolysis	Swelling	Lysosomal enzyme release	None: Pathologic stimulation	
Apoptosis	Blebbing and rounding up	Pyknosis, fragmentation, and chromatin condensation	Reduction of cell volume	Phosphatidylserine exposure, activation of caspases, DNA fragmentation	p53, Bax, Bak	Bcl-2, Bcl-X_L
Necroptosis	Rupture	Moderate chromatin condensation	Swelling of cell and cytoplasmic organelles	Reduced ATP levels, activation of RIPK1, RIPK3, and MLKL, release of DAMPS	RIPK1 RIPK3 MLKL	Necrostatin Caspase-8
Ferroptosis	No blebbing or rupture	No chromatin changes and normal size maintained	Small mitochondria with reduction and vanishing of mitochondria cristae	Iron and ROS accumulation, GSH depletion and NADPH oxidation, release of 11-HETE and 15-HETE	VDAC2 Ras NOX TFR1 p53	Gpx4 SLC7A11 Nrf2

Abbreviations: ATG, autophagy protein; MLKL, mixed lineage kinase domain like; RIPK, receptor interacting protein kinase; VDAC2, voltage-dependent anion channel 2.

Apoptosis A scheme of the apoptotic pathways is presented in Fig. 3-21. Apoptosis is characterized by pyknotic change, cytoplasmic shrinkage, nuclear fragmentation, and blebbing of cell membranes. Ultimately, apoptotic cells disintegrate into membrane-enclosed fragments called apoptotic bodies. Like necrosis, mitochondrial dysfunction and MPT contribute to apoptosis. However, a critical first step in apoptosis is the release of mitochondrial cytochrome *c* (cyt *c*) from the mitochondria into the cytoplasm. Cyt *c* is a small, positively charged heme protein that normally resides in the mitochondrial intermembrane space where it is attached electrostatically to cardiolipin. Peroxidation of cardiolipin by HOOH, a process catalyzed by cyt *c*, results in detachment of cyt *c* from the lipid, a critical first step for cyt *c* to be released into the cytoplasm (Orrenius et al., 2011). This release, which adversely affects ATP synthesis in the mitochondria, initiates the apoptotic cascade by causing the cytoplasmic adapter protein apoptotic protease activating factor (Apaf-1) to oligomerize and bind latent procaspase-9, forming a complex called the apoptosome, and facilitating its conversion to active caspase-9.

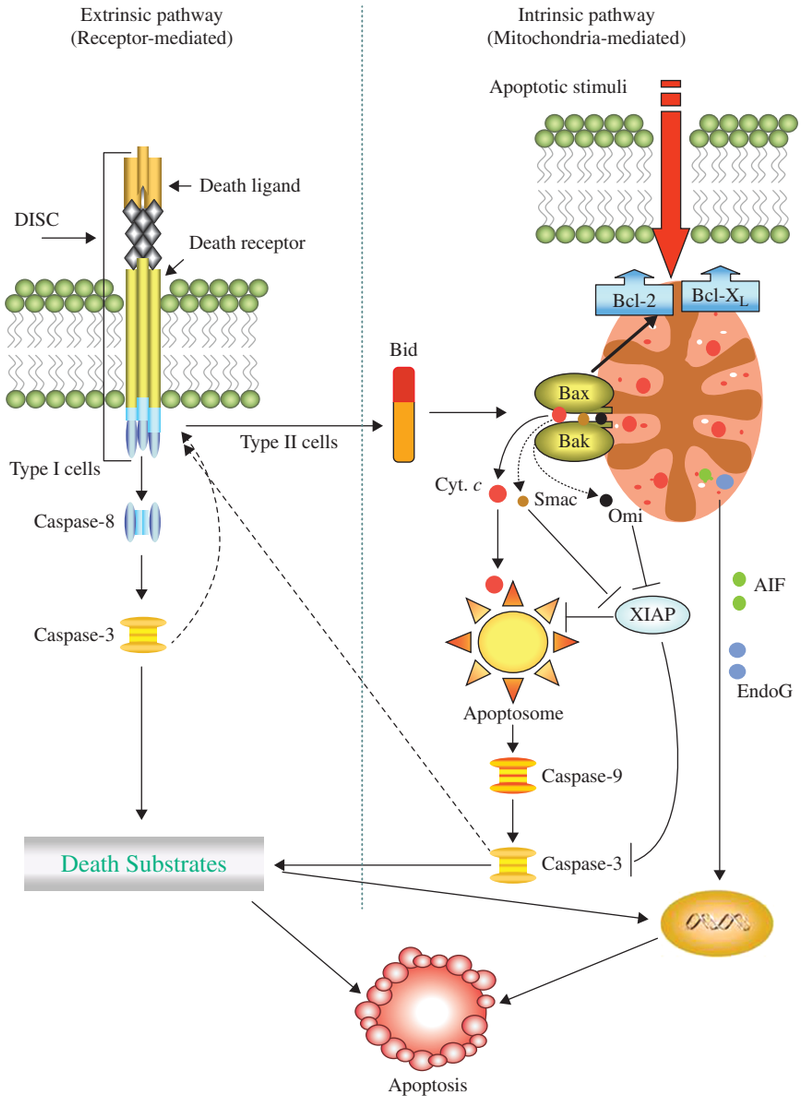

Figure 3-21. *The extrinsic and intrinsic pathways of apoptosis.* In the intrinsic pathway (right) mitochondrial-mediated changes open the permeability transition pore spanning both mitochondrial membranes and/or cause release of cytochrome *c* (Cyt *c*) and other proapoptotic and antiapoptotic proteins. Cyt *c* release is facilitated by Bax and opposed by Bcl-2 protein. The released Cyt *c* forms a multi-protein complex known as the apoptosome and initiates activation of the caspase cascade through caspase-9. In the extrinsic pathway, apoptosis is initiated by receptor activation, and binding of TNF-α to TNF receptor 1 is the major extrinsic mediator of apoptosis. Additionally, activation of the Fas receptor (first apoptosis signal) which binds the Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) receptor also activate the extrinsic pathway. The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the Fas-associated death domain protein (FADD), caspase-8, and caspase-10. Caspase-8 is the major caspase involved in the extrinsic apoptotic pathway. The ligand-bound death receptors and the released Cyt *c* interact with specific adapter proteins (i.e., FADD, TRADD, and Apaf-1) through which specific initiator procaspases (PC, e.g., PC-8 and PC-9) become active caspases (C). The latter in turn cleave and activate other proteins, including Bid and the main effector procaspase-3 (PC-3). The active effector caspase-3 activates other effector procaspases (PC-6 and PC-7). Finally, C-3, C-6, and C-7 cleave specific cellular proteins, leading to morphological and biochemical features of apoptosis. (From Orrenius et al., 2011, with permission.)

There are two major pathways that lead to apoptosis in mammalian cells referred to as the extrinsic and intrinsic pathways (Fig. 3-21), and caspase activation is the hallmark of both pathways. The intrinsic pathway is initiated by signals for cell death acting directly on mitochondria and stimulate the release of proapoptotic proteins including cyt *c*, with subsequent activation of procaspase-9 and activation of caspase-3. The extrinsic pathway is a receptor-mediated pathway in which activation of caspases, particularly procaspase-8, occurs after ligation of membrane receptors (Fas receptor [CD95] or tumor necrosis factor receptor-1 [TNFR1]). Procaspase-8 is activated directly from receptor ligation through formation of the death-inducible signaling complex (DISC), and caspase-8 activates caspase-3, which in turn cleaves target proteins leading to apoptosis. As such, although the intrinsic and extrinsic pathways may be activated by different signals, they converge on caspase-3 activation.

Caspases are cysteine-aspartic proteases with a cysteine in the active site that cleaves target proteins only after an aspartate residue. They reside mostly in the cytoplasm in inactive forms (procaspases), which are activated by either dimerization (initiators) or proteolytic cleavage (effectors; Tummers and Green, 2017). Caspase activity is controlled by cytoplasmic proteins termed inhibitors of apoptosis proteins (IAPs; Finlay et al., 2017). The best characterized IAP is XIAP (X-linked inhibitor of apoptosis), which binds caspase-9, caspase-3, and caspase-7 to inhibit their activation. Accordingly, the release of cyt *c* occurs in concert with activation of proteins that physically remove the IAPs from the caspases just being activated. Activity of XIAP is blocked by binding to DIABLO (Smac) and HTRA2 (Omi) proteins released from mitochondria after proapoptic stimuli. In addition to their role in facilitating cyt *c* activity, Smac and Omi, as residents of the mitochondrial intermembrane space, are jointly mobilized to promote the caspase cascade.

Caspases-8 and -9 are initiator caspases that carry the activation wave to the downstream effector caspases (caspases-3, -6, and -7), which cleave specific cellular proteins. It is the hydrolysis of these specific proteins that accounts directly or indirectly for the morphological and biochemical alterations in apoptotic cells. For example, proteolytic inactivation of poly (ADP-ribose) polymerase (PARP) prevents futile DNA repair and wasting of ATP, and cleavage of Beclin 1 aborts autophagy (discussed below). Hydrolytic activation of sterol regulatory element–binding proteins contributes to the externalization of phosphatidylserine (PS) onto the outer leaflet of the plasma membrane, and this change in PS (normally in the inner leaflet of the cell membrane) identifies the apoptotic cell to phagocytes. This switch in PS location also enables experimental detection of apoptotic cells with fluorescently labeled annexin V.

Apoptotic signaling is controlled by the Bcl-2 (B-cell lymphoma-2) family of proteins, which serves as a rheostat of sorts for apoptosis because its members either facilitate or inhibit apoptotic processes. Members that facilitate apoptosis include Bax (Bcl-2-associated X protein) along with Bak, Bad, Bid, Bim, BNIP3, Puma, and Noxa, whereas those that inhibit are Bcl-2 and Bcl-X_L (Walensky and Gavathiotis, 2011). Of these, Bax is a pivotal proapoptotic protein. It acts through oligomerization that forms pores in the mitochondrial outer membrane (MOM), thereby facilitating release of cyt *c* and other intermembrane proapoptotic proteins via MPT. Moreover, mitochondrial outer membrane permeabilization (MOMP) by Bax and its congeners is sufficient to evoke egress of cyt *c* from the mitochondria, and this action is responsible for cyt *c* release in apoptosis initiated at extramitochondrial targets, including death receptors and nuclear DNA (see below). Bax and Bid proteins are also involved in cell death programs that are initiated by stimulation of death receptors (CD95 and TNFR1) at the cell surface or in response to DNA damage. In contrast, the antiapoptotic proteins Bcl-2 and Bcl-X_L are the dominant negative regulators of programmed cell death, and they act by forming dimers with death-inducing counterparts to neutralize them. In simplest terms, Bax and Bcl-2 serve as the major proapoptotic and antiapoptotic regulatory proteins, such that relative levels and activity of these two proteins dictate the balance between cell survival and death.

DNA damage can also activate apoptotic processes through activation of the tumor suppressor gene *p53*. In the face of DNA damage, p53 is translocated to the nucleus where it regulates gene expression in a pleiotropic manner. In the case of apoptosis, p53 increases expression of proapoptotic members of the Bcl-family of proteins. Cytoplasmic p53 can also abrogate the function of antiapoptotic proteins to favor apoptotic cell death (Green and Kroemer, 2009). DNA damage can cause mutations and cancer such that apoptotic death in the face of DNA damage is an important self-defense mechanism against cancer. Furthermore, many antitumor drugs damage nuclear DNA and induce apoptosis primarily via a p53-dependent mechanism. At the same time, drug-induced apoptosis is responsible for the cytotoxicity in rapidly dividing cells, which is observed with cancer chemotherapeutic regimens.

Necroptosis Another form of programmed cell death is necroptosis, which is an inflammation-related cell death. It is a programmed form of necrosis triggered by ligands of the death receptor family, particularly members of the TNF receptor superfamily, and its development is regulated by receptor-interacting serine/threonine protein kinases 1 and 3 (RIPK1 and RIPK3) and the mixed lineage kinase domain-like protein (MLKL). Necroptosis is distinguished from necrosis in that it is a programmed process and although the cell membrane is disrupted, this process is also tightly regulated (Table 3-7). Furthermore, necroptosis can be initiated by the same death receptors that trigger the extrinsic apoptotic pathway, with a major distinction being that necroptosis occurs when caspase-8 is inhibited. In fact, basal levels of caspase-8 can suppress necroptosis (Zhou and Yuan, 2014).

Necroptosis is initiated by activation of TNFR1, Fas, and toll-like receptors (Weinlich et al., 2017). In the case of TNFα, binding to TNFR1 initiates assembly of the molecular complex consisting of the TNF receptor–associated death domain (TRADD) along with TNF receptor–associated factor 2 (TRAF2) and cIAP1/2 that is associated with the intracellular domain of TNFR, and this in turn recruits RIPK1 (Fig. 3-22). Ultimately, the kinase activity of RIPK1 recruits and phosphorylates RIPK3 into the signaling complex and this dimer subsequently phosphorylates the pronecroptotic protein MLKL to form the necrosome. Necroptosis is ultimately mediated by the complex of phosphorylated RIPK3 and oligomers of MLKL, with phospho-MLKL the most downstream effector of necroptosis. MLKL induces cell death by inserting into the lipid bilayer of the plasma membrane leading to permeabilization, rupture, and the release of damage-associated molecular patterns (DAMPs) that can elicit immune responses. Activated MLKL can also translocate into intracellular membranes to cause similar rupture of the endoplasmic reticulum, lysosomes, or mitochondria. Although cell death is mediated by MLKL, the activity of RIPK1 is required for necroptosis to occur because necrostatin-1, an endogenous protein that specifically inhibits the kinase activity of RIPK1, can completely block necroptotic cell death.

Necroptosis has been implicated in the pathology of many types of acute tissue damage, including myocardial infarction and ischemia-reperfusion injury. It is also implicated in many inflammation-related processes, including atherosclerosis, inflammatory bowel disease, and neurodegeneration (Pasparakis and Vandenabeele, 2015). There is also evidence that necroptosis plays a role in inflammatory-related liver toxicity (see Chap. 13).

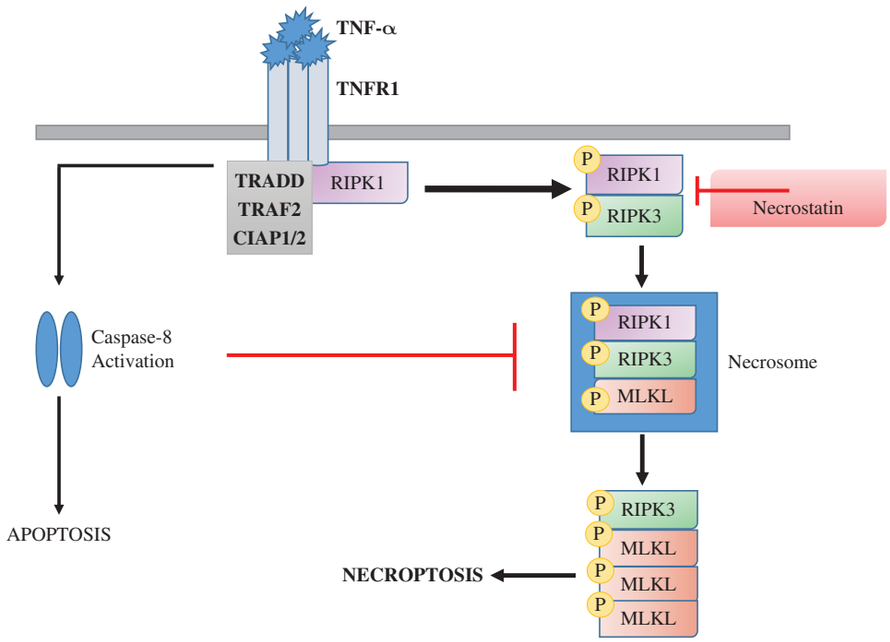

Figure 3-22. *The pathway regulating necroptosis.* TNF-α-mediated necroptosis is initiated by binding of TNF-α to TNFR1. Under conditions of reduced or absent caspase-8 activity, phosphorylation of RIPK1 recruits RIPK3 which undergoes auto-phosphorylation and then recruits and phosphorylates MLKL to form an active complex termed the necrosome. RIPK3 and MLKL ultimately translocate to and destabilize the plasma membrane and initiate necroptotic cell death. Necroptosis has some features of necrosis, but this process causes release of intracellular contents, including damage-associated molecular patterns (DAMPs) that produce pro-inflammatory responses. It is distinguished from apoptosis because it is caspase-independent. The endogenous small molecule necrostatin is an inhibitor of necroptosis. Although a TNFα-mediated pathway is illustrated, other stimuli, including Fas ligand, lipopolysaccharide (LPS), Toll-like receptors (TLRs), and genotoxic stress can induce necroptosis through the activation of RIPK1. *Abbreviations*: RIPK, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like protein.

Ferroptosis Another recently recognized form of regulated cell death is ferroptosis, a process characterized by the accumulation of lipid peroxidation products and ROS that are derived specifically from iron metabolism (Xie et al., 2016). Accordingly, along with activation of the mitogen-activated protein kinase (MAPK) pathway, iron metabolism and lipid peroxidation signaling are the central mediators of ferroptosis (Dixon and Stockwell, 2014). Excessive iron generates ROS by the Fenton reaction (Fig. 3-4), whereas other sources of ROS, including NADPH-dependent lipid peroxidation (Fig. 3-9) and depletion of GSH, contribute to ferroptotic cell death. Ferroptotic cell death is distinguished from necrosis, apoptosis, and necroptosis in that there is no change in nuclear size and no chromatin condensation, nor does the plasma membrane show frank rupture. Rather, ferroptosis is characterized by small mitochondria with reduction or complete loss of mitochondrial cristae along with rupture of the outer mitochondrial membrane (Table 3-7). Although the network of signaling pathways(s) controlling ferroptosis is not fully understood, ferroptosis is recognized as a nonapoptotic form of cell death for which toxicants that inhibit glutathione biosynthesis or the glutathione-dependent antioxidant enzyme glutathione peroxidase 4 (GPX4) are integrally involved. This process is also characterized by activation of the mitochondrial voltage-dependent anion channel (VDAC), particularly VDAC2/3, increased activity of NADPH oxidase activity that forms superoxide (O_2^-), and inhibition of the cysteine/glutamate antiporter (SLC7A11). These three effects act individually or in concert to increase sensitivity to ferroptosis. In contrast, GPX4 activity has been shown to protect against ferroptosis (Xie et al., 2016).

What Determines the Form of Cell Death? The mode of cell death has consequences for the surrounding tissue. Moreover, many toxicants exert metabolic disturbances that involve MPT and progress to apoptotic, necroptotic, and ferroptotic changes. However, in general, the severity of the insult determines the mode of cell death. In particular, it appears that a severe toxic insult causes necrotic cell death rather than apoptosis because it incapacitates the cell to prevent it from a more deliberate form of programmed cell death. This incapacitation may result from the increasing number of mitochondria undergoing MPT, depletion of ATP, and lack of caspase activation.

In experimental models as distinct as Ca^{2+}-exposed hepatocytes, Fas-stimulated T lymphocytes, and HOOH-exposed endothelial cells, necrosis occurs instead of apoptosis when cells are depleted of ATP, whereas apoptosis takes place rather than necrosis when ATP depletion is alleviated by providing substrates for ATP generation (Lelli et al., 1998). Lack of ATP can prevent execution of the apoptotic program because apoptosis involves ATP-requiring steps such as activation of procaspase-9 in the apoptosome complex.

Failure of caspase activation can also result from direct action of reactive toxicants on these enzymes. The active site of caspases is composed of a pentapeptide with a reactive cysteine in it (QACXG). At high concentrations, soft electrophiles, disulfides (e.g., glutathione disulfide), and oxidants can react with this cysteine, causing caspase inactivation and ablation of the apoptotic program, thereby favoring necrosis as the final outcome. Such a scenario has been demonstrated for cell death evoked by tributyltin, pyrrolidine dithiocarbamate, and arsenic trioxide (Orrenius, 2004). An important concept in considering necrotic or apoptotic mechanisms of cell

death is that the number of mitochondria undergoing MPT (which probably depends on the degree of chemical exposure) determines the fate of the cell. According to this model, when only a few mitochondria develop MPT, they are removed by selective autophagy (mitophagy; see the section "Cellular Repair") and the cell survives. When more mitochondria suffer MPT, the autophagic mechanism becomes overwhelmed, and proapoptotic factors are released to initiate caspase activation, and when MPT involves virtually all mitochondria, necrosis occurs (Orrenius et al., 2011). Based on the mechanistic understanding of necroptosis and ferroptosis, cell death mediated by these pathways involves triggering mechanisms that differ from necrosis or apoptosis, but they still converge on altered mitochondrial function.

Induction of Cell Death by Unknown Mechanisms Some toxicants cause cell death by mechanisms other than necrosis or programmed cell death pathways. For example, chemicals that directly damage the plasma membrane, such as lipid solvents, detergents, and venom-derived hydrolytic enzymes will cause direct cell rupture (discussed earlier). Additionally, xenobiotics that damage the lysosomal membrane lead to tissue degradation by release of lysosomal hydrolases, as is the case for aminoglycoside antibiotics and hydrocarbons binding to α_{2u}-globulin that injure renal tubular cells. Other mechanisms of cell death include destruction of the cytoskeleton, such as that observed with microfilament toxins (e.g., phalloidin and cytochalasins) and microtubule toxins (e.g., colchicine and 2,5-hexanedione), the protein phosphatase inhibitor, microcystin, and toxicants that disrupt protein synthesis (e.g., α-amanitin and ricin).

Impairment of External Cellular Maintenance Toxicants may also interfere with cells that are specialized to provide support to other cells, tissues, or the whole organism. Chemicals acting on the liver illustrate this type of toxicity. Hepatocytes produce and release into the circulation a number of proteins and nutrients. They remove cholesterol and bilirubin from the circulation, converting them into bile acids and bilirubin glucuronides, respectively, for subsequent excretion into bile. Interruption of these processes may be harmful to the organism, the liver, or both. For example, inhibition of hepatic synthesis of coagulation factors by dicoumarols (warfarin) does not harm the liver, but may cause death by hemorrhage. Although used clinically in anticoagulant therapy, this same mechanism is responsible for its rodenticidal action. In the fasting state, inhibitors of hepatic gluconeogenesis, such as hypoglycin (the ackee fruit–derived causative agent of Jamaican vomiting sickness; methylene cyclopropyl alanine), may be lethal by limiting the supply of glucose to the brain. Similarly, Reye syndrome, considered to result from hepatic mitochondrial injury caused by a combination of a viral disease (which may induce hepatic NOS) and intake of salicylate (Pugliese et al., 2008), causes not only hepatocellular injury but also severe metabolic disturbances (hypoglycemia and hyperammonemia) that affect other organs. Chemical interference with the β-oxidation of fatty acids or the synthesis, assembly, and secretion of lipoproteins overloads the hepatocytes with lipids, causing steatosis and hepatic dysfunction (Pessayre et al., 2012).

STEP 4: INAPPROPRIATE REPAIR AND ADAPTATION

The final steps in the development of toxicity are inappropriate repair and adaptation (Fig. 3-1). Progression of toxic lesions can be intercepted by repair mechanisms operating at molecular, cellular, and tissue levels as illustrated in Fig. 3-23. Cells and organisms also adapt to toxicity, which renders them more resistant to anticipated adverse effects.

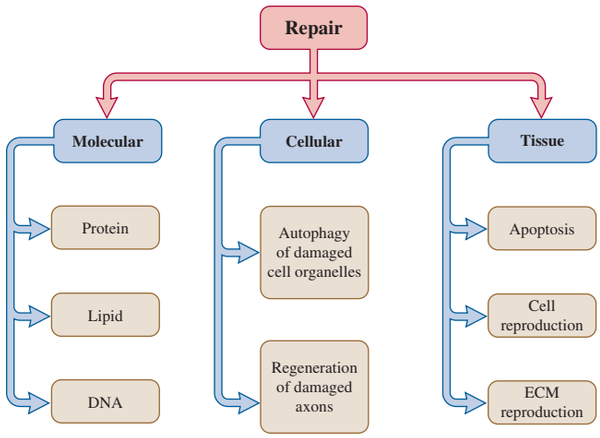

Figure 3-23. *Overview of cellular repair mechanisms.* Dysfunction of these mechanisms results in disrepair, the fourth step in the development of numerous toxic injuries. *Abbreviation*: ECM, extracellular matrix.

Mechanisms of Repair

Molecular Repair Damaged molecules may be repaired in numerous ways. Some chemical alterations, such as oxidation of protein thiols and methylation of DNA, are simply reversed. Hydrolytic removal of the damaged unit and insertion of a newly synthesized, correct unit often occur with chemically altered DNA or protein and peroxidized lipids. In some instances, the damaged molecule is totally degraded and resynthesized. This process is time-consuming but unavoidable in cases such as the regeneration of cholinesterase after organophosphate intoxication.

Repair of Proteins: Thiol Groups As discussed earlier, thiol groups are essential for the function of many proteins. Oxidation of protein thiols, protein disulfides, protein–glutathione mixed disulfides, and protein sulfenic acids is reversed by reduction (Ray et al., 2012) (Fig. 3-24), and thioredoxins and glutaredoxins, small proteins that feature active cysteine residues, are the major endogenous protein-reducing enzymes. There are two isoenzymes of these proteins and thioredoxin reductase that are distinguished by intracellular location (cytosolic and mitochondrial, respectively) (Sies et al., 2017). When the catalytic thiol groups in these proteins are oxidized, they are reduced by NADPH generated by isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. ROS may also oxidize methionines in proteins into sulfoxides (protein-Met-S=O), forming both the S and R epimers, which can be reduced by methionine sulfoxide reductase (Msr) A and B enzymes, respectively (Moskovitz, 2005). Msr enzymes reduce oxidized methionines with concurrent oxidation of their catalytic cysteine to sulfenic acid (Msr-Cys-S-OH), and the modified Msr reacts with a neighboring thiol to form an intramolecular disulfide or with glutathione to form a protein–glutathione disulfide. The activity of the oxidized enzyme is restored by reduction catalyzed by thioredoxin or glutaredoxin, with subsequent steps depicted in Fig. 3-24. Reduction of methionine sulfoxides in lens proteins (e.g., α-crystallin) is especially critical for maintenance of the transparency of the eye lens, and it has been shown that MsrA knockout mice develop cataract on repeated exposure to hyperbaric oxygen (Kantorow et al., 2012). Repair of oxidized hemoglobin (metHb) occurs by means of electron transfer from

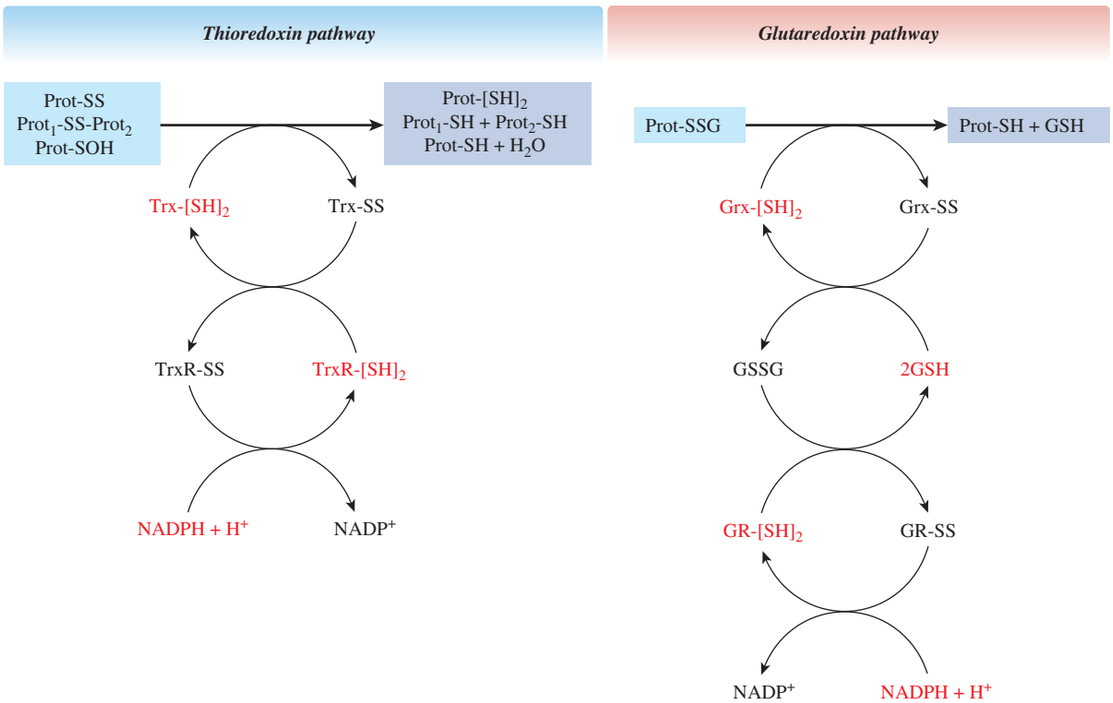

Figure 3-24. *Repair of proteins oxidized at their thiol groups.* Protein disulfides (Prot-SS and $Prot_1$-SS-$Prot_2$) and protein sulfenic acids (Prot-SOH) are reduced by thioredoxin (Trx-$[SH]_2$) or thioredoxin-like proteins. Protein–glutathione-mixed disulfides (Prot-SSG) are reduced by glutaredoxin (Grx-$[SH]_2$), which is also called thioltransferase. The figure also indicates how Trx-$[SH]_2$ and Grx-$[SH]_2$ are regenerated from their disulfides (Trx-SS and Grx-SS, respectively). *Abbreviations*: GSH, glutathione; GSSG, glutathione disulfide; GR-$[SH]_2$ and GR-SS, glutathione reductase (dithiol and disulfide forms, respectively); TrxR-$[SH]_2$ and Trx-SS, thioredoxin reductase (dithiol and disulfide forms, respectively).

cytochrome b_5, which is then regenerated by a NADH-dependent cytochrome b_5 reductase (metHb reductase). Species differences in this enzyme contribute to differences in sensitivity to metHb formation and hypoxia, with cats being most sensitive to this toxicity.

Repair of Proteins: Heat Shock Chaperones Soluble intracellular proteins such as cytosolic enzymes are typically folded into a globular form with hydrophobic amino acid residues folded internally, hydrophilic residues located externally, and a ligand (substrate) binding site that resides in a hydrophobic cleft. Physical or chemical insults may evoke an unduly large opening of this cleft, leading to protein denaturation and aggregation. Molecular chaperones such as the heat-shock proteins (Hsp; e.g., Hsp90, Hsp70, and Hsp40) can prevent protein unfolding by "clamping down" onto the exposed hydrophobic region of their client protein, using energy of ATP hydrolysis to execute a conformational change that is required for this maneuver. Hsp90 is critical for stabilizing the cleft and impeding further unfolding, which would eventually lead to degradation of the protein by the ubiquitin–proteasome system (UPS) described below (Schopf et al., 2017). This important function can be blocked by compounds such as geldanamycin and herbimycin that promote degradation of Hsp90-bound proteins by the UPS.

The process of ubiquitination and the interaction with the heat-shock protein response is illustrated in Fig. 3-25. Hsp 90 and Hsp70 have opposing effects on protein stability. Hsp90 stabilizes proteins and prevents ubiquitination, whereas Hsp70 promotes ubiquitination and proteosomal degradation. Ubiquitin (Ub) is a small (8.6 kDa) protein that directs target proteins to proteosomal degradation when covalently linked to the target. Ubiquitination is a posttranslational protein modification that takes place in an enzymatic cascade composed of three types of proteins—the E1 Ub-activating enzyme, the E2 Ub-conjugating enzyme, and the E3 Ub ligase. E1 first activates Ub by adenylating its terminal carboxyl group to form Ub-AMP, and then it transfers Ub from Ub-AMP to its catalytic SH group, forming a high-energy thioester bond between E1 and Ub. In the subsequent transthiolation reaction, Ub is transferred from E1 to the catalytic SH group of E2. Thereafter, E3 binds both the target protein and the Ub-charged E2 and catalyzes the transfer of Ub from E2 to the target protein, forming an isopeptide bond between the terminal carboxyl group of Ub and an ε-amino group of a lysine residue in the substrate. Multiple rounds of this process generate polyubiquitin chains. In general, mono-ubiquitination triggers endocytosis or autophagy of the protein, whereas substrates that are tagged with Lys48-linked Ub chains are recognized and degraded by the 26S proteasome into small peptides. In contrast, proteins with Lys63-linked Ub chains are essential components of signaling pathways, functioning as scaffolds to assemble signaling complexes, as is the case for NF-κB.

In the case of Hsp functions, dissociation of Hsp90 from the unfolding protein allows Hsp70 and its co-chaperone Hsp40 to recruit the E3 Ub ligases (e.g., CHIP and Parkin) to tag the protein with a polyubiquitin chain (Bedford et al., 2011) so that the protein is degraded in proteasomes. The 26S proteasome is large barrel-shaped multiprotein complex that binds the ubiquitinated protein, deubiquitinates and unfolds it using ATP, and finally hydrolyzes the protein with its threonine-containing protease active sites into small peptides (Fig. 3-25). The UPS controls the cellular level of numerous regulatory proteins (e.g., p53, IκB, Nrf2, β-catenin, and cyclins) and has a prominent role in eliminating oxidized or otherwise damaged and unfolded intracellular proteins (Kriegenburg et al., 2011). For example, the reductive dechlorination of CCl_4

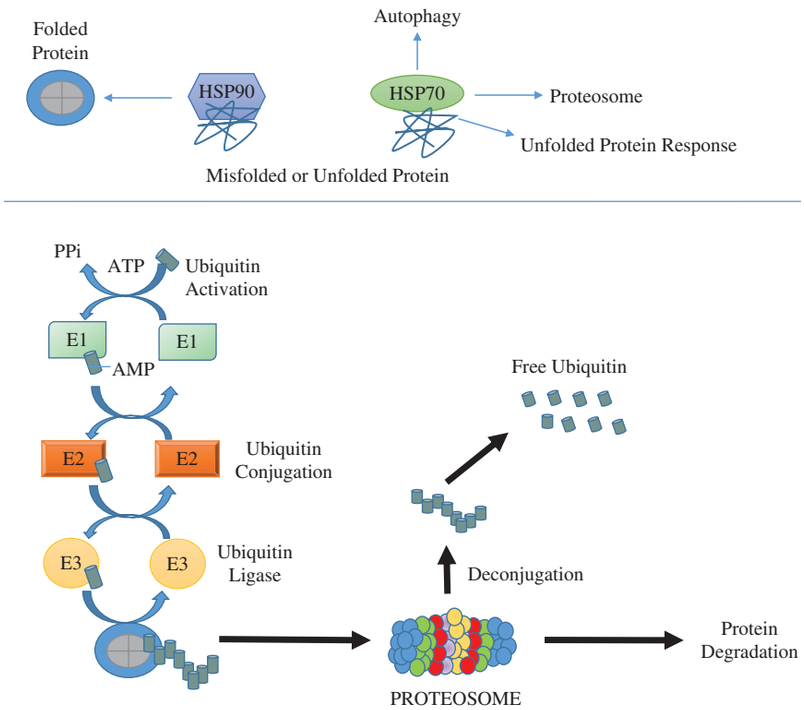

Figure 3-25. *The role of heat-shock proteins (Hsps) in the unfolded protein response and the process of ubiquitination.* Hsp90 and Hsp70 are central to recognizing damaged or misfolded proteins. Hsp90 associates with misfolded proteins and acts as a protective chaperone, whereas binding of Hsp70 directs the misfolded protein for degradation through autophagy (Fig. 3-27), the unfolded protein response (Fig. 3-31), or proteasomal degradation. Proteins are marked for proteasomal destruction by the polyubiquitination pathway. Ubiquitin (Ub) is a small protein (8.6 kDa) that covalently links to a target protein (as a monomer or as a polymer of Ub units) to target it for degradation. Ubiquitination is a posttranslational protein modification that takes place in an enzymatic cascade composed of three types of proteins—the E1 Ub-activating enzyme, the E2 Ub-conjugating enzyme, and the E3 Ub ligase. As shown in the figure, E1 first activates Ub in an ATP-consuming reaction by adenylating the terminal carboxyl group of Ub. In the subsequent transthiolation reaction, Ub is transferred from E1 to the catalytic SH group of E2. Thereafter, E3 binds both the target protein and the Ub-charged E2 and catalyzes the transfer of Ub from E2 to the target protein, forming an isopeptide bond between the terminal carboxyl group of Ub and the ε-amino group of a lysine in the substrate. Multiple rounds of ubiquitination generate polyubiquitin chains. In general, monoubiquitination triggers endocytosis or autophagy of the protein. Substrates that are tagged with Lys48-linked Ub chains are recognized and degraded by the 26S proteasome into small peptides, with the Ubs released for reuse. Proteins polyubiquitinated with Lys63-linked Ub chains are generally not degraded but are essential components of signaling pathways, functioning as scaffolds to assemble signaling complexes.

by CYP2E1 causes the heme in the cytochrome to become cross-linked to the trichloromethyl free radical it forms, and this inactive enzyme is eliminated by proteasomal degradation. Oligomerization and aggregation of damaged and unfolded proteins preclude the proteasome from degrading them. Such substrates can even trap proteasomes, rendering them nonfunctional. After ubiquitination, protein aggregates can also be eliminated by autophagy, a process described in more detail in the section "Cellular Repair."

Repair of Lipids Phospholipids containing fatty acid hydroperoxides are preferentially hydrolyzed by phospholipase A2, with the peroxidized fatty acids replaced by normal fatty acids. Peroxidized lipids (e.g., fatty acid hydroperoxides and phospholipid-associated hydroperoxides) may be reduced by the glutathione peroxidase–glutathione–glutathione reductase system (see Fig. 3-26) or by the peroxiredoxin–thioredoxin–thioredoxin reductase system. NADPH is needed to repair the reductants that are oxidized in the process.

Repair of DNA Damage to DNA is inevitable, as it occurs spontaneously during replication and it is frequently targeted by electrophiles and free radicals. However, nuclear DNA is remarkably stable, in part because it is packaged tightly in chromatin and because complex repair mechanisms have evolved to correct adverse alterations. Mitochondrial DNA, however, lacks histones and efficient repair mechanisms and therefore is more prone to damage. The contribution of DNA damage and DNA repair mechanisms are central to toxicity culminating in carcinogenic and mutagenic effects (see Chaps. 8 and 9). However, the mechanisms of DNA repair, or the disruption of normal DNA repair mechanisms, remain an important aspect of understanding general mechanisms of toxicity and are summarized here.

Direct Repair Certain covalent DNA modifications are directly reversed by enzymes such as DNA photolyase, which cleaves adjacent pyrimidines dimerized by UV light. The enzyme uses the energy of visible light to correct damage, so its function is restricted to light-exposed cells. Minor DNA adducts, such as methyl groups attached to the O^6 position of guanine, are cleaved by O^6-methyguanine-DNA-methyltransferase (MGMT). While repairing the DNA, this alkyltransferase inactivates itself by transferring the adduct onto one of its cysteine residues, after which the enzyme is subjected to ubiquitination and proteasomal degradation. Thus, like glutathione, which is depleted during detoxification of electrophiles, MGMT is consumed during the repair of DNA.

Excision Repair Base excision and nucleotide excision are two major mechanisms for removing damaged bases from DNA (see Chaps. 8 and 9). Lesions that do not distort the helix typically are removed by base excision, in which the altered base is recognized by DNA glycosylase that hydrolyzes the

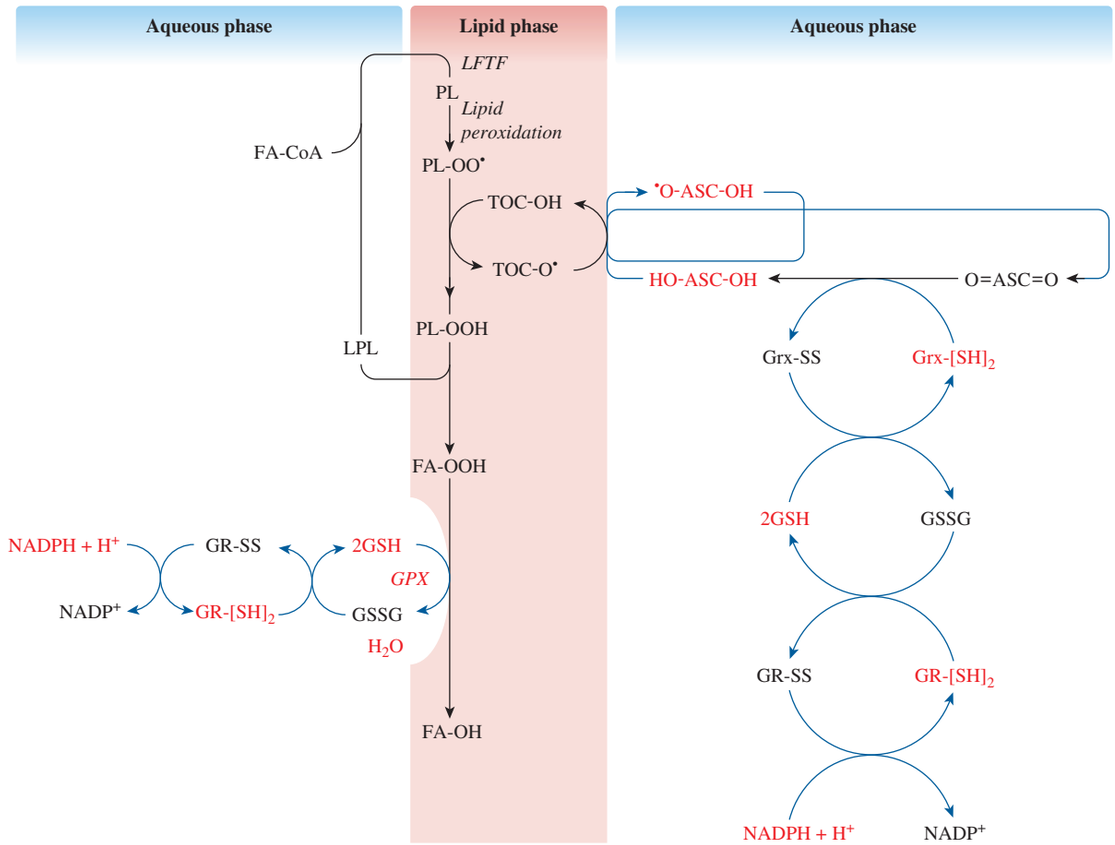

Figure 3-26. *Repair of peroxidized lipids.* Phospholipid peroxyl radicals (PL-OO•) from lipid peroxidation may abstract hydrogen from α-tocopherol (TOC-OH) and yield phospholipid hydroperoxide (PL-OOH). From the latter, the fatty acid carrying the hydroperoxide group is eliminated via hydrolysis catalyzed by phospholipase (PLase), yielding a fatty acid hydroperoxide (FA-OOH) and a lysophospholipid (LPL). The former is reduced to a hydroxy fatty acid (FA-OH) by glutathione peroxidase (GPX), utilizing glutathione (GSH), or peroxiredoxins (not shown), whereas the latter is reacylated to phospholipid (PL) by lysophosphatide fatty acyl-coenzyme A transferase (LFTF), utilizing long-chain fatty acid–coenzyme A (FA-CoA). The figure also indicates regeneration of TOC-OH by ascorbic acid (HO-ASC-OH), regeneration of ascorbic acid from dehydroascorbic acid (O-ASC-O) by glutaredoxin (Grx-$[SH]_2$), and reduction of the oxidized glutaredoxin (Grx-SS) by GSH. Oxidized glutathione (GSSG) is reduced by glutathione reductase (GR-$[SH]_2$), which is regenerated from its oxidized form (GR-SS) by NADPH, the ultimate reductant. *Abbreviations*: TOC-O•, tocopheroxyl radical; •O-ASC-OH, ascorbyl radical.

N-glycosidic bond, releasing the modified base and creating an apurinic or apyrimidinic (AP) site in the DNA. For example, 8-hydroxyguanosine, a major mutagenic product of oxidative stress, is removed from the DNA by a specific DNA glycosylase. The AP site is recognized by the AP endonuclease, which hydrolyzes the phosphodiester bond adjacent to the abasic site. After its removal, the abasic sugar is replaced with the correct nucleotide by a DNA polymerase and is sealed in place by a DNA ligase.

DNA adducts are bulkier lesions, often produced by reactive intermediates (such as those from formaldehyde, aflatoxins, or aminofluorene derivatives) and dimers caused by UV radiation. These adducts are removed by the nucleotide excision repair system (Yu et al., 2015). In this mechanism, proteins that recognize the distorted double helix at the lesion unwind the DNA and excise a number of intact nucleotides on both sides of the lesion along with the one containing the adduct. The excised section of the strand is restored by insertion of nucleotides into the gap of the actions of DNA polymerase and ligase, using the complementary strand as a template. In contrast, lesions in the transcribed DNA strand that block RNA polymerase activity are removed by the transcription-coupled repair system. This involves assembly of repair proteins to remove the stalled RNA polymerase before excising the damage and filling the gap. Resynthesis of the removed section of the strand is designated as unscheduled DNA synthesis. Overall, excision repair has a remarkably low error rate of less than one mistake in 10^9 bases repaired.

PARP (poly-[ADP-ribose] polymerase) is another important contributor to excision repair. PARP binds to damaged DNA caused by base damage or single-strand breaks (SSBs) and becomes activated. The active PARP cleaves NAD^+ to use the ADP-ribose moiety of this cofactor for attaching long chains of polymeric ADP-ribose to nuclear proteins, such as histones. The ADP-ribose unit contains two negative charges, so the poly(ADP-ribosyl)ated proteins are also negatively charged, and the resultant electrorepulsive force between the charged proteins and DNA causes decondensation of the chromatin structure. This opens tightly packed chromatin and allows the repair enzymes to access and repair the damaged DNA. Thereafter, poly(ADP-ribose) glycohydrolase gains access to the nucleus from its perinuclear localization and reverses the PARP-mediated modification of nuclear proteins.

Nonhomologous End Joining This process repairs double-strand breaks (DSBs) that may be formed when two SSBs occur in close proximity, or when DNA with SSBs undergoes replication. This repair system directly ligates the broken ends without the need for a homologous template. DSBs are recognized by the Ku protein

(a heterodimer of Ku70 and Ku80) that binds to the DNA end. Ku then binds and activates DNA-dependent protein kinase catalytic subunit (DNA-PKcs), leading to recruitment and activation of DNA polymerases and DNA ligase. Although the nonhomologous end joining is error-prone, it can operate in any phase of the cell cycle and is the mechanism for DSB repair in nondividing terminally differentiated cells including neurons.

Recombination (or Postreplication) Repair Homologous recombination can be used to repair postreplication gaps and DSBs (Li and Heyer, 2008). Postreplication gaps form when excision of a bulky adduct or an intrastrand pyrimidine dimer does not occur before DNA replication. At replication, two homologous ("sister") yet dissimilar DNA duplexes are formed; one has a large postreplication gap in its daughter strand and an intact duplex synthesized at the opposite leg of the replication fork. This intact duplex is utilized to complete the postreplication gap in the damaged duplex, which is accomplished by recombination of the appropriate strands of the two homologous duplexes. After separation, the sister duplex that originally contained the gap carries in its daughter strand a section originating from the parent strand of the intact sister, which in turn carries in its parent strand a section originating from the daughter strand of the damaged sister. This strand recombination explains the phenomenon of "sister chromatid exchange," which is indicative of DNA damage corrected by recombinational repair. Given this mechanism, homologous recombination can function only after replication (in S and G2 phases), when sister chromatid sequences are available for use as templates (see below), and accordingly, recombination is not an option in nondividing cells.

Proliferating cell nuclear antigen (PCNA) is a central player in postreplication repair. PCNA binds to chromatin and slides along the DNA strand to tether the polymerase to the DNA template. PCNA is a platform for a wide variety of proteins that participate in DNA replication and damage. These include the MRN complex (the trimer of Mre11, Rad50, and Nbs1 proteins), which binds to the DSB and is involved in end processing by generation of single-stranded DNA, and the breast cancer susceptibility proteins BRCA1 and BRCA2, which assist the single-stranded DNA in invading the undamaged template in an ATP-dependent process (Gao et al., 2017).

In general, there is a limited role for RNA in DNA repair processes. However, there is emerging evidence that the machinery involved in miRNA formation, beyond its role in translational repression (discussed above), is also involved in DNA damage repair (Hawley et al., 2017). In human cells, there are novel miRNAs that appear to be derived from the vicinity of DSBs. Moreover, small RNAs, termed DNA damage-induced RNAs (diRNAs), are generated only after DNA damage, and although their precise function is not established, they are required for the proficient repair of such damage. The role of small RNAs in repairing DNA damage is an ongoing area of research, and it will be important to determine whether, and if so how, they are directly involved in DNA repair.

Cellular Repair The ability to repair or remove damaged cells or organelles is important for preventing cellular dysfunction. Autophagic removal of damaged cell organelles may be viewed as a universal mechanism of cellular repair, whereas clearance and regeneration of damaged axons is a mechanism specific for nerve cells. Autophagy is a lysosomal process in which intracellular substrates are degraded within the lysosomal compartment. It is a type of cell death mechanism, but one that is unique in its characteristic presence of cytoplasmic vacuoles resulting from engulfment of cellular components (Table 3-7). Autophagy is a constitutive and rapid process, and most autophagosomes are very short-lived, with a half-life of no more than 10 minutes. It is also distinct from apoptosis and necrosis in that the clearance of "debris" is an intracellular process, and there is no involvement of other phagocytic cells. Autophagy occurs by the engulfment of cellular constituents (macroautophagy), membrane uptake of smaller amounts of cytoplasm and organelles (microautophagy), or chaperone-mediated events that enable specific protein substrates to be taken into the lysosome for degradation. Autophagy is also an important mechanism for survival in the fasting state.

With respect to toxicity, autophagy is an important mechanism for removing misfolded proteins from cells resulting from oxidative stress or ER stress. ER stress is an adaptive mechanism that is designed to trigger transcriptional activation of a genetic program that will enhance autophagy and the capacity for normal protein folding in the ER. However, if excessive or prolonged, ER stress ultimately triggers cell death, typically in the form of apoptosis. While autophagy serves the purpose of cell restitution in all cells, it is particularly important in terminally differentiated cells, such as neurons and myocytes, where cell renewal by cell replication is not possible.

Autophagic removal of damaged cell organelles is a process that supports their quality control, disposal, and recycling. For example, deletion of depolarized mitochondria occurs through selective autophagy or mitophagy in cells exposed to mitochondrial poisons, such as the protonophoric uncoupler CCCP, valinomycin, and paraquat (see Table 3-6). There are five stages of autophagy starting with the initiation stage, followed by elongation, membrane closure, maturation and finally, degradation of cytoplasmic cargo (Fig. 3-27). Autophagy is mediated by the actions of a core group of kinases and other proteins including (a) the un-51-like kinase (ULK) complex, (b) the class II PI3K complex, (c) a ubiquitin-like conjugation system that requires autophagy-related proteins (ATGs) such as ATG7, ATG3, and the ATG12-ATG 5:Atg16L complex, (d) the ATG9 transmembrane protein involved in vesicle trafficking, and (e) vacuole membrane protein 1 (VMP1) that binds with Beclin-1 (Bcl-2-interacting protein-1; Petibone et al., 2017). Removal of damaged mitochondria by autophagy is important not only for maintaining a functional mitochondrial pool but also for limiting oxidative cell damage and preventing apoptosis, because injured mitochondria may be sources of ROS and apoptotic factors, such as cyt *c*, Smac, and AIF. Autophagy also contributes to structural restitution of liver cells as is the case for removal of peroxisomes after exposure to peroxisome proliferators and elimination of lipid droplets in hepatic steatosis.

Regeneration of Damaged Axons It is generally recognized that neurons in the central nervous system have limited capacity to regenerate. In contrast, axonal damage in peripheral neurons can be repaired by axonal regeneration, a process that requires macrophages and Schwann cells. Macrophages remove debris by phagocytosis and produce cytokines and growth factors, which activate Schwann cells to proliferate and transdifferentiate to facilitate and support axonal regrowth. Distal to the injury, Schwann cells play an indispensable role in promoting axonal regeneration by increasing their synthesis of cell adhesion molecules (e.g., N-CAM), elaborating proteins for basement membrane reconstruction, and by producing an array of neurotrophic factors (e.g., brain-derived neurotrophic factor, glial cell line–derived neurotrophic factor, and nerve growth factor) and their receptors (Wong et al., 2017). While comigrating with the regenerating axon, Schwann cells guide neuronal regrowth and provide trophic support to the axon to reinnervate the target cell.

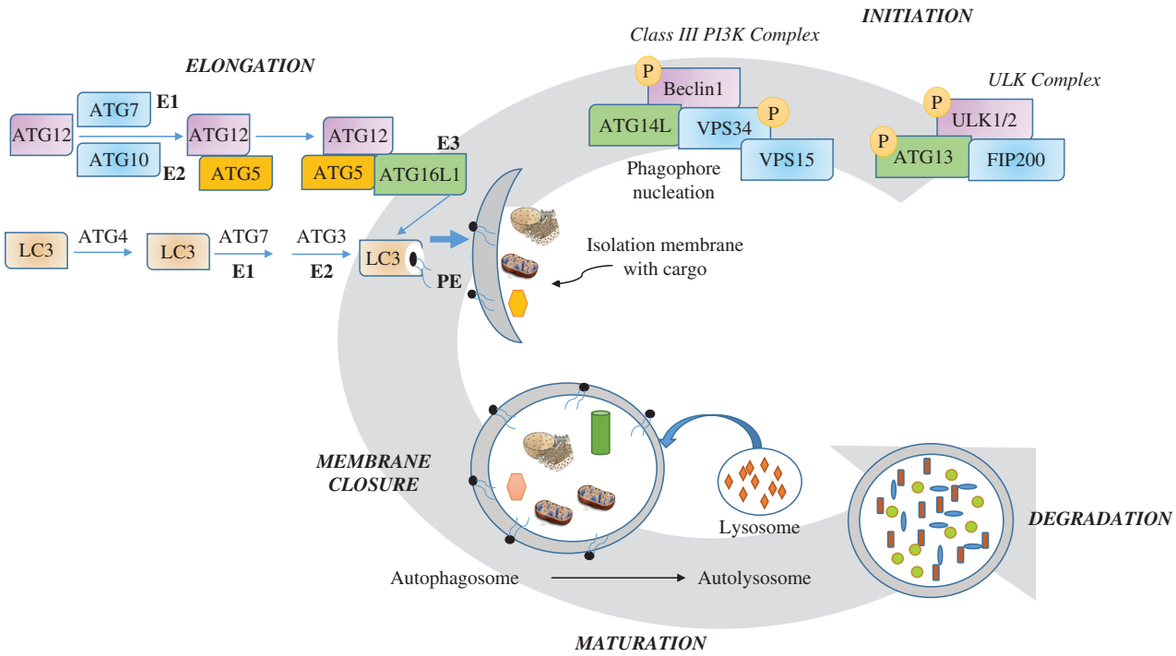

Figure 3-27. *The process of autophagy including the five major stages (initiation, elongation, membrane closure, maturation, and degradation).* Autophagy is initiated by activation of the unc-51-like kinase (ULK) in a complex composed of ULK1, ULK2, autophagy-related proteins (ATG13), and FIP200, a scaffold protein. Additionally, phosphorylation of Beclin 1 is necessary for initiating the PI3 kinase complex, which is required for phagophore nucleation. The phagophore elongates around cytoplasmic components, including polyubiquitinated cargo, after which it closes to form the autophagosome. When the autophagosome fuses with a lysosome, the autolysosome is formed, after which the autophagosome inner membrane and its cytoplasmic cargo are degraded by acid proteases and hydrolases. The signal transduction pathways along with the cycle from initiation to degradation is illustrated. *Abbreviations*: PE, phosphatidylethanolamine; E1, E2, and E3, ubiquitin conjugating enzymes; LC3, microtubule-associated protein 1A/1B-light chain 3; VPS, vacuolar protein sorting.

In contrast to the peripheral nervous system, neurons in the central nervous system lose their intrinsic growth capacity postnatally, which occurs simultaneously with increased expression of some neuronal growth inhibitory Krüppel-like TFs, such as KLF4. Accordingly, damage to central neurons is irreversible but is compensated for in part by the large number of reserve nerve cells that can take over the functions of lost neurons. For example, in Parkinson disease, symptoms are not observed until there is at least an 80% loss of nigrostriatal neurons.

Tissue Repair In tissues with cells capable of multiplying, damage is reversed by deletion of the injured cells and regeneration of the tissue by proliferation. However, it is important to remove the damaged cells, and often, apoptotic processes contribute. As described above, apoptosis is a controlled, orderly process in which dead cells are removed without inflammation. This is in contrast to necrosis which is a less orderly process that ends with cell debris in the extracellular environment. The constituents of the necrotic cells attract inflammatory cells, and the ensuing inflammation amplifies cell injury (described below). However, apoptosis may support tissue restoration only for tissues that are made up of constantly renewing cells (e.g., the bone marrow, the respiratory and GI epithelium, and the epidermis of the skin), or of conditionally dividing cells (e.g., hepatic and renal parenchymal cells). The role of apoptosis as a tissue repair strategy is markedly lessened in organs containing nonreplicating and nonreplaceable cells, such as the neurons, cardiac muscle cells, and female germ cells, because deletion of such cells, if extensive, will decrease organ function.

Proliferation: Regeneration of Tissue Repair of tissue injury involves regeneration of lost cells, reconstitution of the extracellular matrix (ECM), and reintegration of the newly formed elements. In organs such as liver, kidney, and lung, various types of cells are involved in the process of tissue restoration. Nonparenchymal cells of mesenchymal origin residing in the tissue, such as resident macrophages and endothelial cells, and those migrating to the site of injury, such as monocytes, produce factors that stimulate parenchymal cells to divide and stimulate some specialized cells (e.g., the stellate cells in the liver) to synthesize ECM molecules.

Replacement of Lost Cells by Mitosis After tissue injury, repair is frequently mediated by cell proliferation (Fig. 3-28). DNA synthesis is increased, and this response is detected experimentally as an increase in the expression of proteins associated with S-phase of the cell cycle (including immunohistochemical detection of Ki-67 and PCNA or by incorporation of substrates into the DNA template during S-phase such as bromodeoxyuridine [BrdU] or ^{3}H-thymidine). Also, mitotic figures can be seen microscopically in cells. As early as 2 to 4 hours after administration of a low dose of carbon tetrachloride to rats, the mitotic index in the liver increases dramatically, indicating that cells already in the G_2 phase progress rapidly to mitosis (M-phase). The mitotic activity of the hepatocytes culminates at 36 to 48 hours, after a full transit through the cycle, indicating that quiescent cells residing in G_0 enter and progress to mitosis. In the CCl_4 model, mitosis of nonparenchymal cells occurs later, after activation and replication of parenchymal cells. In severe toxic liver injury, when hepatocyte replication is impaired (e.g., in rats dosed with high dosages of galactosamine or acetylaminofluorene, and in humans intoxicated with acetaminophen), restoration of the liver may depend on stem cell–derived cells, called oval cells, which are located in the terminal bile ductules. These cells proliferate and differentiate into both hepatocytes

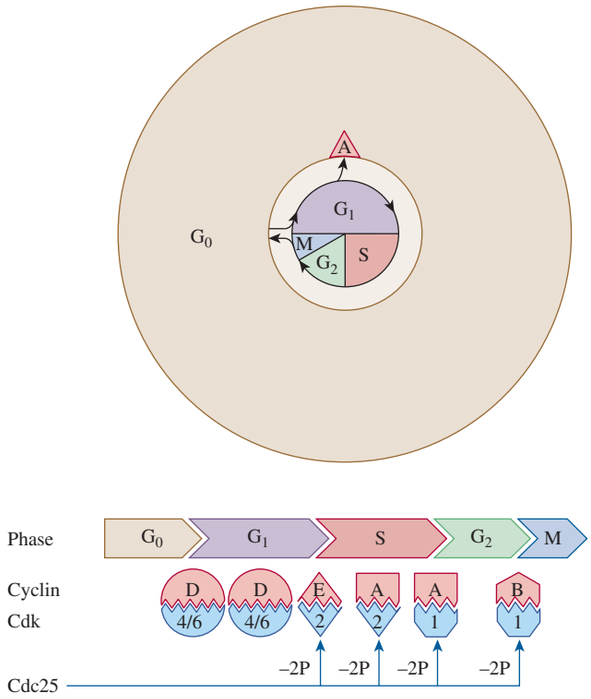

Figure 3-28. *The cell division cycle and participating cyclins and cyclin-dependent protein kinases.* Most cells are normally in G_0 phase, a differentiated and quiescent state. After receiving signals to divide, they progress into the G_1 phase of the cell division cycle. G_0/G_1 transition involves activation of immediate early genes for cells to acquire replicative competence, and the cells progress to the phase of DNA synthesis (S). After DNA replication, cells prepare further for mitosis in the G_2 phase. Mitosis (M) is the shortest phase of the cell cycle (approximately 40 minutes out of the 40-hour-long cycle of hepatocytes) and most likely requires the largest energy expenditure per unit of time. The daughter cells produced may differentiate and enter the pool of quiescent cells (G_0), substituting for those which had been lost. Also illustrated are the cyclins and cyclin-dependent protein kinases (Cdk) that are required for DNA replication and cell division.

and biliary epithelial cells (cholangiocytes) (Fausto et al., 2006; Shin and Kaestner, 2014). As these hepatic progenitor cells produce α-fetoprotein, the increase in serum α-fetoprotein is predictive of an improved outcome of acetaminophen-induced injury.

Hepatic sinusoidal endothelial cells originate from bone marrow progenitors, and when they are chemically injured (e.g., by monocrotaline) bone marrow–derived precursor cells move into the sinusoids and contribute to their replacement, as demonstrated in a rat model of hepatic sinusoidal obstruction syndrome (Harb et al., 2009). Accordingly, bone marrow infusion ameliorates, whereas myelosuppression aggravates, such liver injury. In an ozone-exposed lung, the nonciliated, bronchiolar Clara cells and alveolar type II pneumocytes proliferate and differentiate to replace damaged ciliated bronchial epithelial cells and type I pneumocytes, respectively (Tam et al., 2011). In some tissues, such as intestinal mucosa and bone marrow, stem cells first divide to provide self-renewal and then differentiate to replace more mature cells lost through injury.

The liver is uniquely capable of regeneration, and it can fully regenerate in a matter of days after partial hepatectomy. In this model, there is a pattern of gene expression that programs this regeneration. Genes that are upregulated include those that code for TFs important in proliferative signaling, such as c-*fos*, c-*jun*, *Egr1*, and c-*myc* (see Fig. 3-13), the antiapoptotic protein Bcl-X_L, and mdm2, a ubiquitin ligase that limits p53-dependent inhibition of the cell cycle (see Fig. 3-33). Some genes involved in cell cycle inhibition are temporarily overexpressed (e.g., the cyclin-dependent kinase inhibitor p21 and gadd45), providing an inhibitory signal that keeps tissue regeneration precisely regulated. Nevertheless, gene expression is reprogrammed so that DNA synthesis and mitosis gain priority over specialized cellular activities. For example, as a result of dedifferentiation, regenerating hepatocytes have reduced levels of cytochrome P450, some phase II conjugating enzymes, and PPARα. Similarly, hepatic stellate cells showed decreased accumulation of fat and vitamin A.

The regenerative process is initiated by the release of numerous chemical mediators from damaged cells. Nonparenchymal cells, such as resident macrophages and endothelial cells, release cytokines and growth factors that promote and propagate the regenerative process. In the partial hepatectomy model, the initial or priming phase of liver regeneration is controlled by TNF-α and IL-6, whose hepatic mRNA and serum levels increase. TNF, secreted from Kupffer cells, acts on the macrophages in an autocrine manner, activating its receptor and the coupled signal transduction network. This in turn activates NF-κB, which increases IL-6 expression. Secreted IL-6 exerts its action on the hepatocytes and through its receptor (Fig. 3-13) activates Janus kinase (JAK) and induces TFs (e.g., Stat3 and C/EBPβ) to activate several downstream target genes. This cytokine network promotes transition of the quiescent liver cells (G_0) into cell cycle (G_1) and makes them receptive to growth factors ("priming"). Growth factors, especially hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), and heparin-binding epidermal growth factor–like growth factor (HB-EGF), initiate the progression of the primed cells in the cycle toward mitosis (Fausto et al., 2006; Michalopoulos, 2013). Despite its name, neither the formation nor the action of HGF is restricted to the liver. It is produced by resident macrophages and endothelial cells of various organs, including liver, lung, and kidney, and in a paracrine manner activates receptors on neighboring parenchymal cells.

The communication between parenchymal and nonparenchymal cells during tissue repair is mutual. For example, transforming growth factor-α (TGF-α), a mitogen produced by regenerating hepatocytes, acts as an autocrine and a paracrine mediator on liver cells as well as on adjacent nonparenchymal cells. The mitogenic growth factors initiate signaling through the MAPK pathway and the PI3K–Akt pathway (Fig. 3-13), thereby activating numerous TFs (including c-Jun, c-Fos, c-Myc, FoxM1B, NF-κB, and Stat3), which ultimately induce cyclins and the protein phosphatase cdc25. Cyclins also activate Cdks by associating with them (Fig. 3-28), whereas cdc25 activates Cdk1 and Cdk2 by dephosphorylating two amino acid residues (Thr14 and Tyr15). The activated Cdks accelerate the cell cycle mainly by phosphorylation of retinoblastoma protein (pRb), which when phosphorylated is inactivated to enable cell cycle progression.

Although the regulation of proliferation by cytokines and growth factors is considered an essential part in the repair of most tissues, there are also tissue-specific features of repair. For example, in the liver, bile acids stimulate hepatocyte proliferation through activation of FXR (Huang et al., 2006). In mice subjected to partial hepatectomy, liver regrowth is accelerated by bile acid feeding, but is markedly delayed when FXR (Table 3-3) is deleted or recirculation of bile acids from the intestine to the liver is prevented.

In addition to proliferation, cell migration also contributes to tissue repair. For example, when the mucosa of the GI tract is damaged, cells of the residual epithelium rapidly migrate to the site of injury where they elongate to reestablish the continuity of

the surface even before this can be achieved by cell replication. Cell movement involves orderly dissociation of cadherin-mediated cell–cell contacts (involving β-catenin phosphorylation) and integrin-mediated cell–ECM contacts at focal adhesions (involving phosphorylation of focal adhesion kinase [FAK]), assembly of actin stress fibers, and formation of lamellopodia.

Mucosal repair is dictated not only by growth factors and cytokines operative in tissue repair elsewhere but also by trefoil factors (TFFs). TFFs are small (7 to 12 kDa) protease-resistant proteins that are abundantly secreted from specific mucosal cells (e.g., intestinal goblet cells) and are associated with the mucous layer of the GI tract (Aihara et al., 2017). TFF expression is rapidly upregulated at the margins of mucosal injury by cytokine and growth factor signaling. Some growth factors (e.g., HGF and EGF) influence motility (motogens) and proliferation (mitogens) of enterocytes, whereas TFFs are potent motogens. Although TFFs do not act alone, they are the only peptides shown to be essential for restitution of the injured intestinal mucosa. For example, dextran sodium sulfate (DSS) causes marked and diffuse colonic mucosal injury, but in TFF-3 null mice, an otherwise nonlethal dose of DSS elicits severe toxicity that can be lethal.

Replacement of the Extracellular Matrix The ECM is a biochemical and structural support composed of a variety of proteins (such as collagen and elastin), proteoglycans (such as chondroitin sulfate), and glycosaminoglycans (such as hyaluronic acid). Each tissue has its own specific ECM composition, but in liver, stellate cells located in the space of Disse are major contributors to ECM production. Stellate cells store vitamin A and are generally at rest. However, these cells are activated during liver regeneration, when they undergo mitosis and transdifferentiate into myofibroblast-like contractile and secretory cells (Tsuchida and Friedman, 2017). Activation of stellate cells is mediated chiefly by two growth factors, namely platelet-derived growth factor (PDGF) and TGF-β. Both growth factors may be released from platelets which accumulate and degranulate at sites of injury, and TGF-β is also secreted from activated stellate cells. TGF-β is a major regulator of stellate cell transdifferentiation and stimulates the synthesis of ECM components and α-smooth muscle actin. As previously described, TGF-β acts through its Ser/Thr kinase receptor (Fig. 3-14), which phosphorylates the TFs Smad2 and 3 (Fabregat et al., 2016). TGF-β also plays a central role in ECM formation in other tissues. In kidney and lung, for example, TGF-β targets the mesangial cells and the septal fibroblasts, respectively. Remodeling of the ECM is aided by matrix metalloproteinases, which hydrolyze specific components of the matrix, as well as by tissue inhibitors of matrix metalloproteinases.

The way in which tissue regeneration is terminated after repair is unclear, but the gradual dominance of TGF-β, a potent antimitogen, is a contributing factor in the termination of cell proliferation. Additionally, ECM production may be halted by induction of Smad7 (an inhibitory Smad) (Fig. 3-14), which competitively inhibits phosphorylation of Smad2 and 3. However, although repair of the ECM helps to regenerate injured tissue, chronic stimulation by TGF-β also plays a role in fibrosis (discussed below).

Other Reactions to Tissue Injury In addition to mediators that aid in the replacement of lost cells and the ECM, resident macrophages and endothelial cells activated by cell injury produce other mediators that induce ancillary reactions with uncertain benefit or harm to tissues. Such reactions include inflammation, altered production of acute-phase proteins, and generalized reactions such as fever.

Inflammation—Leukocyte Invasion Alteration of the microcirculation and accumulation of inflammatory cells are the hallmarks of inflammation. These processes are largely initiated by resident macrophages secreting cytokines, such as TNF-α and interleukin-1 (IL-1), which increase capillary permeability through release of mediators from endothelial cells and fibroblasts (Brenner et al., 2013). In the face of toxicity, activated endothelial cells facilitate the egress of circulating leukocytes into the injured tissue by releasing chemoattractants and simultaneously express selectins, a group of cell adhesion molecules that interact with ligands on the surface of leukocytes, causing them to "roll" onto the capillary surface to facilitate transmigration into the damaged tissue. A strong interaction develops between intercellular adhesion molecules (ICAM-1) expressed on endothelial cell membranes and integrins expressed on leukocyte membranes. This interaction is also essential for the subsequent transendothelial migration of leukocytes. Chemoattractants involved in this response include cytokines (or chemokines), such as the MCP-1 and IL-8, as well as lipid-derived compounds, such as platelet-activating factor (PAF) and leukotriene B_4 (LTB_4). Ultimately, all types of cells near the injury express ICAM-1, thus promoting leukocyte invasion, and the invading leukocytes synthesize additional chemotactic mediators to propagate the inflammatory response. The major regulators of this entire response include the proinflammatory mediators TNF-α and IL-1, which activate NF-κB and C/EBP (Brenner et al., 2013).

Inflammation: ROS and RNS Production Macrophages, as well as leukocytes, recruited to the site of injury undergo a respiratory burst, discharging free radicals and enzymes into the damaged tissue. The respiratory burst is a multifactorial and robust production of ROS in the inflamed tissue, with NADPH oxidase (NOX2) responsible for the production of superoxide anion from NADPH to increase ROS (El-Benna et al., 2016). The superoxide anion generated by NOX2 activity can give rise to the hydroxyl radical ($HO^{\bullet}$) through the Fenton reaction. Moreover, nitric oxide synthase (NOS) also contributes to the respiratory burst by catalyzing the formation of the nitric oxide free radical ($^{\bullet}NO$) via oxidation of arginine to citrulline. Finally, myeloperoxidase (MPO) in granulocytes catalyzes for the formation of HOCl, a powerful oxidizing agent, from HOOH and chloride ion. Like HOOH, HOCl can also form $HO^{\bullet}$ resulting from electron transfer from Fe^{2+} or from $O_2^{\bullet-}$ to HOCl. All these reactive intermediates exert beneficial antimicrobial activity at the site of microbial invasion, but because they are so reactive, they can also damage the adjacent healthy tissues and thus contribute to propagation of tissue injury.

In some chemical-induced injuries, inflammation plays the leading role. For example, alpha-naphthylisothiocyanate (ANIT), a cholestatic chemical, causes neutrophil-dependent hepatocellular damage. ANIT acts on bile duct epithelial cells, causing them to release chemoattractants for neutrophils, which on invading the liver, injure hepatocytes. Kupffer cell activation, TNF release, and subsequent inflammation are also prominent and causative events in galactosamine-induced liver injury in rats (Stachlewitz et al., 1999).

Altered Protein Synthesis: Acute-Phase Proteins Interleukins, including IL-6, IL-1, and IL-8 along with TNF-α, alter the transcriptional activity of genes encoding a group of proteins referred to as acute-phase reactants, and these can be increased or decreased (positive and negative acute-phase proteins, respectively; Table 3-8). Many of the hepatic acute-phase proteins, such as C-reactive protein and hepcidin, are secreted into the circulation, where elevated levels in serum are diagnostic of tissue injury, inflammation, or neoplasm. Increased sedimentation of red blood cells, which is also indicative of these conditions, is due to enrichment of blood plasma with positive acute-phase proteins such as fibrinogen.

Apart from their diagnostic value, positive acute-phase proteins may play roles in minimizing tissue injury and facilitating

Table 3-8

Overview of Acute-Phase Proteins and their General Function

Positive Acute-Phase Proteins	Generalized function or Role in Acute-Phase Response
C-reactive protein	Facilitated phagocytosis of bacteria
Ceruloplasmin	Copper transport protein with antioxidant activity
α2-macroglobulin	Inhibits thrombin and plasmin to reduce coagulation and fibrinolysis
α1-acid glycoprotein	Binds steroids, lipophilic, and acidic drugs; anti-inflammatory agent
Haptoglobin	Binds free hemoglobin to limit systemic iron availability
Hepcidin	Prevents release of iron bound by ferritin in the intestinal tract and in macrophages; increases internalization of ferritin
Ferritin	Iron-binding protein
Serum amyloid A	Chemotactic activity and inhibition of myeloperoxidase release
α1-antitrypsin	Downregulates inflammation
Plasminogen activator inhibitor-1	Prevents plasminogen-mediated degradation of blood clots
Coagulation factors: fibrinogen, prothrombin, von Willebrand factor	Role in coagulation
Negative Acute-Phase Proteins	
Albumin	Major hepatic protein; decreased synthesis saves amino acids for synthesizing positive acute-phase reactants
Transferrin	Contributes to the regulation of free iron levels
Transthyretin	Binds and transports thyroid hormone
Retinol-binding protein	Binds and transports retinol
Antithrombin	A serine protease inhibitor (serpin) that inactivates clotting factors, kallikrein, plasmin, and trypsin

repair. For example, many of them, such as α_2-macroglobulin and α_1-antiprotease, inhibit lysosomal proteases released from the injured cells and recruited leukocytes. Haptoglobin binds hemoglobin in blood, MT complexes metals in cells, heme oxygenase oxidizes heme to biliverdin, and opsonins facilitate phagocytosis. Upregulated acute-phase proteins may also be involved in the clearance of substances released on tissue injury. For example, induction of hepcidin triggers degradation of the iron efflux channel to decrease duodenal Fe^{2+} absorption and Fe^{2+} export from macrophages and which limits systemic availability of iron.

Downregulated acute-phase proteins include some plasma proteins, such as albumin, transthyretin, and transferrin. Furthermore, hepatic enzymes (e.g., several forms of cytochrome P450 and glutathione *S*-transferases), ligand-activated TFs (e.g., PPARα and the bile acid receptor FXR), and transporters such as bile acid transporters on sinusoidal and canalicular membrane of hepatocytes and the bile canalicular export pump Mrp2 are also downregulated. The downregulation of hepatic metabolizing enzymes and transporters often alters the disposition and toxicity of bile acids and xenobiotics during the acute phase of tissue injury.

Although the acute-phase response is phylogenetically preserved, some of the acute-phase proteins are somewhat species specific. For example, during the acute phase of tissue injury or inflammation, C-reactive protein and serum amyloid A levels dramatically increase in humans but not in rats, whereas the concentrations of α_1-acid glycoprotein and α_2-macroglobulin increase markedly in rats but only moderately in humans.

Mechanisms of Adaptation

Adaptation is a biological process by which an organism develops increased tolerance to the harm itself. It involves responses acting to preserve or regain the biological homeostasis in the face of increased harm. Theoretically, adaptation to toxicity may result from biological changes causing (1) diminished delivery of the causative chemical(s) to the target, (2) decreased size or susceptibility of the target, (3) increased capacity of the organism to repair itself, and (4) mechanisms to compensate for the toxicant-inflicted dysfunction. Mechanistically, adaptation involves sensing the noxious chemical and/or the initial damage or dysfunction, with a response that limits the toxicity that typically occurs through altered gene expression. Such mechanisms are briefly outlined here.

Adaptation by Decreasing Delivery to the Target Given that the first step in the development of toxicity is delivery of the ultimate toxicant to the target, some adaptive processes include those that decrease absorption, increase sequestration by intracellular binding proteins, enhance their detoxification, or promote cellular export.

Regulation of Iron Absorption Adaptive mechanisms triggered by iron itself regulate intestinal absorption of this essential yet potentially harmful metal ion, with absorption adjusted to meet demand. Fe^{2+} is taken up from the intestinal lumen into enterocytes by the proton-coupled divalent metal transporter 1 (DMT1) localized in the luminal membrane, and it is exported across the basolateral membrane of these cells through the ferroportin iron

channel. High iron intake diminishes expression of both DMT1 and ferroportin in the enterocytes, whereas low intake has the opposite effect. DMT1 is regulated at the translational level by the intracellular iron-regulatory proteins (IRPs) that contain a [4Fe-4S] cluster. Iron supply influences the Fe content of this cluster and in turn affects the capacity of IRP to bind to mRNAs containing the iron response element (IRE). In iron deficiency, loss of Fe from the [4Fe-4S] cluster allows the IRP1 apoprotein to bind to and protect DMT1 mRNA from enzymatic degradation and increase DMT1 protein. Conversely, iron overload saturates IRP1 with Fe so that it cannot bind to and stabilize DMT1 mRNA, which decreases DMT1 and limits Fe^{2+} absorption. DMT1 also mediates the transport of Cd^{2+} such that its overexpression in conditions of iron deficiency can also increase intestinal absorption of this toxic metal ion.

In addition to its uptake, Fe homeostasis is maintained by ferroportin, which allows efflux of iron from enterocytes and other cells that may store iron including macrophages (from phagocytosis) and hepatocytes. Ferroportin in plasma membranes is regulated directly by the acute-phase reactant hepcidin (Table 3-8; Ganz, 2011), which binds to ferroportin and triggers its internalization and lysosomal hydrolysis. Hepcidin is also regulated by iron supply, as iron deficiency decreases hepcidin expression, precluding ferroportin degradation, and in concert with upregulated DMT1, permits increased iron absorption from the duodenum. In contrast, iron overload increases the secretion of hepcidin to increase degradation of ferroportin, and in concert with decreased synthesis of DMT1, shuts down iron absorption from the intestine. This system can also respond to toxicants. For example, the hepatotoxic herbicide diquat undergoes redox cycling and increases hepatic free-iron concentrations. However, an adaptive increase in hepatic hepcidin mRNA decreases plasma iron levels (Higuchi et al., 2011) to provide an appropriate protective response.

Induction of Ferritin and Metallothionein Adaptive cellular accumulation of the binding proteins ferritin and metallothionein (MT) is protective against iron and cadmium ions, respectively. Like DMT1, ferritin is regulated by IRP1 in the face of iron overload. However, apoIRP1 acts oppositely on ferritin mRNA to block its translation. Therefore, in iron overload, ferritin mRNA is markedly increased. In this manner, ferritin is protective as it acts to bind and remove Fe^{2+} systemically.

MT is induced by cadmium, and elevated levels of MT protect the liver by restricting distribution of this toxic metal ion to sensitive intracellular targets. Induction of MT by Cd^{2+} is likely mediated by displacement of Zn ions from the protein which then activate metal-responsive transcription factor 1 (MTF-1) to increase transcription of the MT gene. The adaptive response afforded by MT induction is illustrated by the fact that induction of the metal binding protein subsequently protects against highly toxic exposures to Cd^{2+}.

The Electrophile Stress Response: Induction of Detoxification Adaptive increases in detoxification and cellular export have a major role in limiting toxicity. An important contributor to such adaptation is the Keap1-Nrf2 pathway, a cytosolic complex that senses thiol reactivity. The response is initiated by Nrf2 (NF-E2-related factor-2), a TF that activates genes with an electrophile response element (EpRE) in their regulatory region (Fig. 3-29; Chartoumpekis et al., 2015). Normally Nrf2 is retained in the cytoplasm by Keap1, a cysteine-rich homodimeric protein. Keap1 keeps Nrf2 inactive by anchoring it to the cytoskeleton and maintains low intracellular levels by linking it to cullin 3, a component of E3 ubiquitin ligase that directs it to proteasomal degradation. Many different thiol-sensitive compounds bind to Keap1 at its reactive cysteine thiol groups thereby forcing Keap1 to release Nrf2. This broad group of activators includes electrophiles, α,β-unsaturated aldehydes and ketones, isothiocyanates, thiol-reactive metal ions, and direct and indirect oxidants. On disruption of the Keap1–Nrf2 complex, active Nrf2 translocates into the nucleus, forms a heterodimer with small Maf proteins, and activates genes through binding to EpREs.

There are many genes with EpRE motifs. The broad response is illustrated in Fig. 3-29 and includes (1) enzymes that detoxify xenobiotics, (2) enzymes that eliminate $O_2^{\cdot-}$, (3) proteins that detoxify heme, (4) enzymes involved in the synthesis of GSH and its regeneration from GSSG, and (5) efflux transporters. It should be noted that the list of Nrf2-regulated genes provided here is an incomplete list, and other Nrf2-induced proteins of known toxicological relevance are discussed later.

To provide an example of the broad, pleiotropic response initiated by Nrf2 activation, Fig. 3-30 illustrates Nrf2-mediated adaptive changes that facilitate elimination and detoxification of electrophilic chemicals in response to *t*-butylhydroquinone (*t*BHQ), a well-known inducer of this pathway. Additional compounds that induce Nrf2 through thiol reactivity include butylated hydroxyanisole (BHA), sulforaphane (a compound in broccoli), curcumin (a compound in turmeric-containing 2 α,β-unsaturated ketone groups), resveratrol (a hydroquinone-type compound in red grapes), oltipraz (a dithiolenethione compound), and derivatives of oleanolic acid, such as 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO). These chemicals have been shown to protect from toxicant-induced tissue injury and cancer. For example, treatment with oleanolic acid or CDDO ameliorates acetaminophen-induced hepatotoxicity and aflatoxin-induced hepatocarcinogenesis (Klaassen and Reisman, 2010). Moreover, CDDO has been evaluated clinically for use in chronic kidney disease, and there is growing interest in Nrf2 activation in cancer chemoprevention (Cao et al., 2015).

Finally, the critical role of Nrf2 in adapting to toxicity is confirmed in Nrf2 knockout mice that are more sensitive to many toxicants including the hepatotoxicity of acetaminophen, the pulmonary toxicity of butylated hydroxytoluene or cigarette smoke, the neurotoxicity of 3-nitropropionic acid, and the carcinogenicity of benzo[*a*]pyrene (Klaassen and Reisman, 2010). Conversely, liver-specific deletion of Keap1 constitutively activates Nrf2 in hepatocytes, causing them to overexpress many detoxifying enzymes and become resistant to acetaminophen-induced hepatotoxicity.

Adaptation by Decreasing the Target Density or Responsiveness Decreasing the density and sensitivity of the xenobiotic target is an adaptation mechanism for several receptors. Such alterations underlie the tolerance induced by opioids and drugs of abuse. There are several opioid receptors, but the major receptor for drugs like morphine or heroine is the μ-opioid receptor. This is a G_i protein–coupled inhibitory receptor, and stimulation by an agonist inhibits adenyl cyclase to decrease levels of cyclic AMP levels and PKA activity. Brief stimulation induces adaptation manifested as receptor downregulation by loss of the receptor from the plasma membrane. Receptor downregulation is mediated by receptor internalization that facilitates lysosomal degradation rather than recycling back to the membrane (Bailey and Connor, 2005). Additionally, there is an opening of K^+ channels in neurons with opioid receptors to cause hyperpolarization. Tolerance to opioids leads to the requirement for increasing doses of a drug such as morphine to maintain pharmacologic activity, and the need for higher or more frequent doses enhances abuse potential.

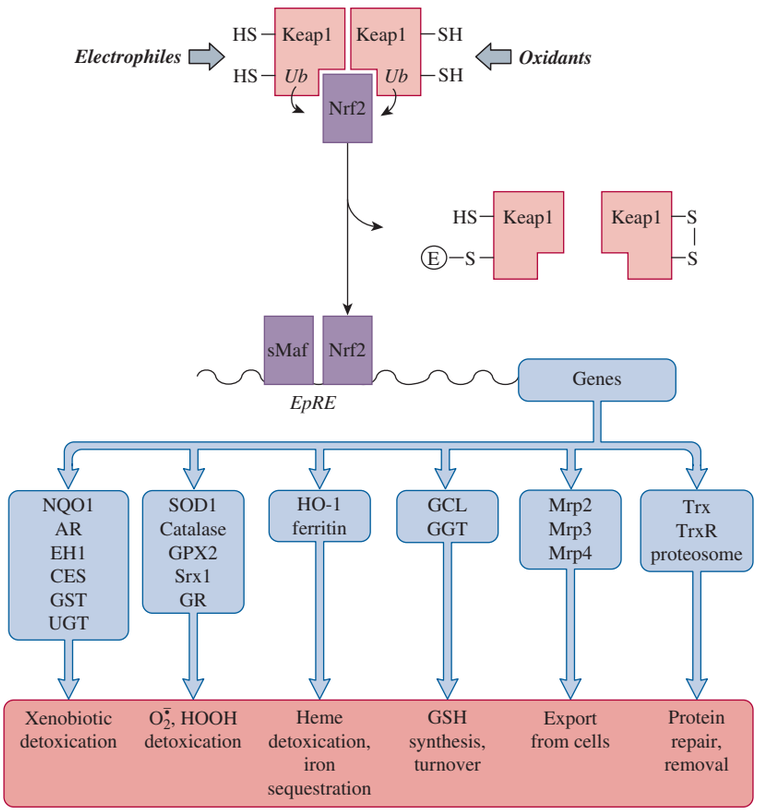

Figure 3-29. *Signaling by Keap1/Nrf2 mediates the electrophile stress response.* Normally NF-E2-related factor 2 (Nrf2) is kept inactive and at a low intracellular level by interacting with Keap1 that promotes its ubiquitination and proteasomal degradation. Electrophiles covalently bind to, whereas oxidants oxidize, the reactive thiol groups of Keap1, causing Keap1 to release Nrf2. Alternatively, Nrf2 release may follow its phosphorylation by protein kinases. After being released from Keap1, active Nrf2 accumulates in the cell, translocates into the nucleus, and forms a heterodimer with small Maf proteins to activate genes that contain electrophile response element (EpRE) in their promoter region. These include enzymes, binding proteins, and transporters functioning in detoxication and elimination of xenobiotics, ROS, and endogenous reactive chemicals, as well as some proteins that can repair or eliminate oxidized proteins (highlighted in blue). Induction of such proteins represents an electrophile stress response that provides protection against a wide range of toxicants. *Abbreviations*: AR, aldose reductase; CES, carboxylesterase; EH1, microsomal epoxide hydrolase; GCL, glutamate–cysteine ligase; GGT, gamma-glutamyl transpeptidase; GPX2, glutathione peroxidase 2; GR, glutathione reductase; GST, glutathione *S*-transferase; HO-1, heme oxygenase 1; NQO1, NAD(P)H:quinone oxidoreductase; Mrp2, Mrp3, and Mrp4, multidrug resistance protein 2, 3, and 4; SOD1, superoxide dismutase 1; Srx1, sulfiredoxin 1; UGT, UDP-glucuronosyltransferase; Trx, thioredoxin; TrxR, thioredoxin reductase.

Adaptation by Increasing Repair There are several repair mechanisms that can be induced after toxicant exposure. Some of these may aid in repairing damaged molecules, proteins, and DNA, others support regeneration of injured tissue.

The Electrophile Stress Response: Induction of Enzymes Repairing Oxidized Protein In addition to enzymes that enhance detoxification, Nrf2 activation induces proteins that mediate protein repair. These include thioredoxin 1 (Trx1) and thioredoxin reductase 1 (TR1), which catalyze reduction of oxidized proteins, and several subunits of the proteasome complex. Trx1 and TR1 are also reduction partners for ribonucleotide reductase, thereby supporting de novo synthesis of deoxyribonucleotides necessary for DNA synthesis. In this manner, induction of Trx1 and TR1 mediate repair of both protein and DNA.

Induction of Chaperones Repairing Misfolded Protein: The Heat-Shock Response The role of heat-shock proteins has been previously described in the section "Repair of Proteins." Regulation of these proteins to support adaptive responses is mediated by heat-shock transcription factors (HSF), mainly HSF1, which transactivate genes that encode Hsp through heat-shock response elements (HSE). This process often takes place simultaneously with the electrophile response discussed above. HSF1, like Nrf2, normally resides in the cytoplasm, where it associates with Hsp90, Hsp70, and Hsp40. On heat- or chemical-induced protein damage, Hsps are sequestered by damaged proteins (Fig. 3-25), allowing HSF1 to translocate to the nucleus, where it forms a trimer and is phosphorylated. The chaperones Hsp90 and Hsp70, together with co-chaperone proteins, are especially important in maintaining the integrity of hundreds of proteins (Schopf et al., 2017) including those carrying out housekeeping functions and those involved in signaling and apoptosis. Therefore, induction of Hsps has pleiotropic effects beyond increased protection from cytotoxicity.

Endoplasmic Reticulum Stress and the Unfolded Protein Response The endoplasmic reticulum (ER) is the primary site where transmembrane and secretory proteins are folded. In this Ca^{2+}-rich, oxidative environment, protein modifications including *N*-glycosylation at asparagine residues, formation of disulfide bonds, and folding assisted by EPR-resident chaperones occur. When there is damage to proteins being processed in the EPR by reactive metabolites (often produced in situ by CYP enzymes), generation of

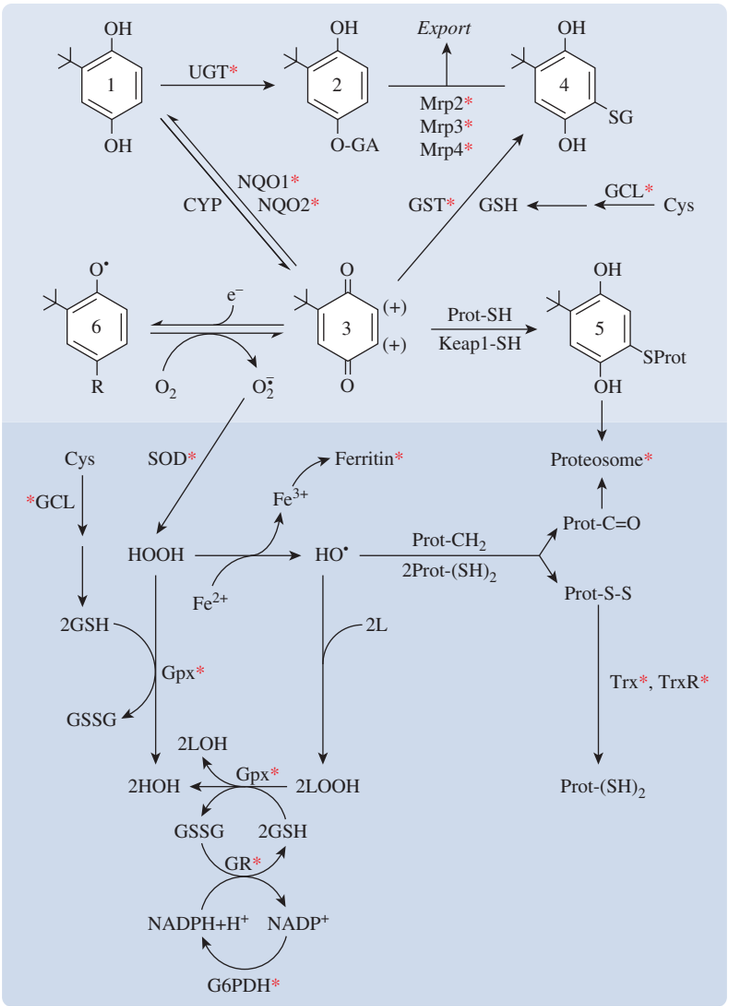

Figure 3-30. *Adaptive changes in response to the t-butylhydroquinone (tBHQ)–induced electrophile stress.* This figure illustrates the numerous proteins (i.e., enzymes, exporters, and repair proteins) that, when induced in response to the *t*BHQ-mediated electrophile stress, facilitate the detoxication and export of *t*BHQ and/or its metabolites, the detoxication of ROS formed during the biotransformation of *t*BHQ, and repair or removal of proteins damaged by the reactive metabolites. Proteins induced by Nrf2 are marked with an asterisk. *t*BHQ (designated as 1 in the aromatic ring) can be detoxified by glucuronidation (2), but cytochrome P450–catalyzed dehydrogenation to *t*-butylquinone (*t*BQ; 3), which contains electrophilic carbon atoms (+), is believed to be the actual inducer of electrophile stress response. *t*BQ can be detoxified by conjugation with glutathione (GSH) to form *t*BHQ-SG (4); it can covalently bind to SH groups in proteins (5), including Keap1, or by accepting an electron (e), *t*BQ can also form the *t*-butylsemiquinone radical (6), which passes the electron to molecular oxygen to form superoxide anion radical ($O_2^{\bullet-}$), completing a redox cycle. In a process catalyzed by superoxide dismutase (SOD), $O_2^{\bullet-}$ can form HOOH (left side of figure), which is detoxified by GSH-peroxidase (GPX), or involved in the Fe^{2+}-catalyzed Fenton reaction to form hydroxyl radical ($HO^{\bullet}$).

ROS, and/or depletion of Ca^{2+} in the EPR lumen, there is accumulation of unfolded or misfolded proteins, a condition known as EPR stress (Malhi and Kaufman, 2011; Carreras-Sureda et al., 2017). EPR stress can be triggered by high secretory activity, along with conditions that perturb protein maturation, calcium homeostasis, or redox balance. Additionally, it can also be induced by compounds that disturb different aspects of EPR homeostasis, including hapsigargin (which blocks SERCA-mediated Ca^{2+} uptake into EPR; see Table 3-6), tunicamycin (which inhibits *N*-glycosylation of proteins), castanospermine (which interferes with maturation of nascent glycoproteins by deglucosylation), and brefeldin A (which inhibits transport of proteins from EPR to Golgi). When the load of unfolded or misfolded proteins in the EPR exceeds the capacity of EPR-resident chaperones, these proteins trigger a complex adaptive response called unfolded protein response (UPR) that is designed to reduce the load of unfolded proteins and restore EPR homeostasis (Fig. 3-31). However, if EPR homeostasis cannot be restored, UPR switches to a proapoptotic pathway that eliminates damaged cells.

In the adaptive mode, UPR includes mechanisms to (1) reduce mRNA translation to decrease the functional load on the EPR, (2) increase transcription of EPR chaperones to boost the folding capacity, and (3) remove aberrant proteins via translocon peptide channels into the cytosol for proteasomal degradation. As noted above, the final option is to eliminate the affected cell via apoptosis if EPR stress is sustained or massive. The eukaryotic translation initiation factor 2α (eIF2α) and TF X-box binding protein 1 (XBP1), ATF6, ATF4, and C/EBP homologous protein (CHOP; also called Gadd153) are the major regulators of this adaptive response.

There are three transmembrane EPR proteins that sense the overload of the EPR lumen with misfolded proteins and transduce

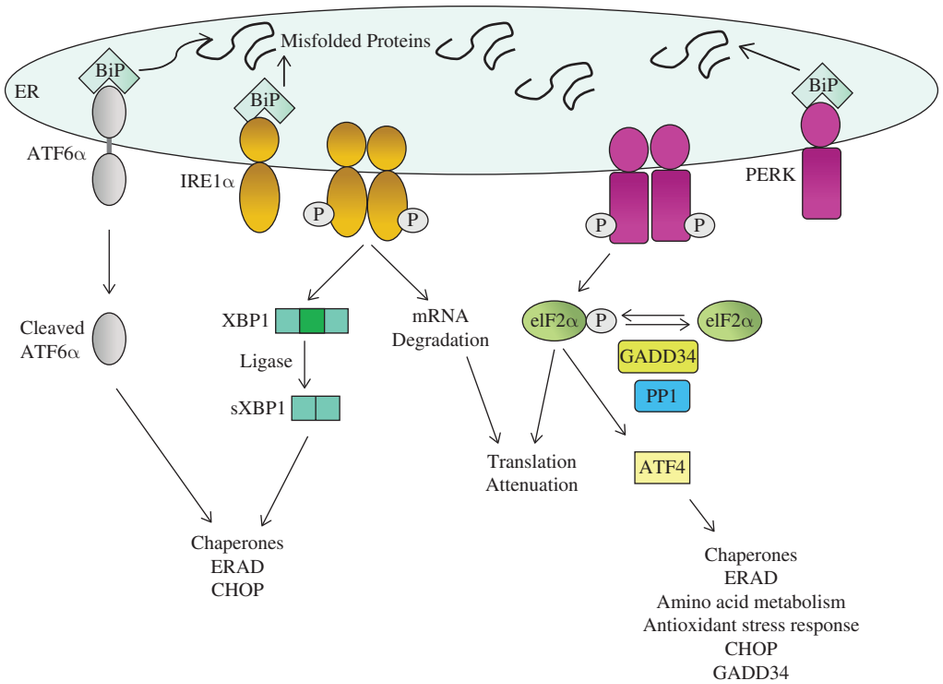

Figure 3-31. *Overview of the unfolded protein response (UPR).* There are three main signaling branches in the UPR. Under normal conditions, BiP (binding immunoglobulin protein), a Hsp70 molecular chaperone, inhibits UPR. However, under endoplasmic reticulum (ER) stress, BiP dissociates from the UPR transducers to chaperone misfolded proteins into the ER lumen and initiate one of three branches of the UPR. Activating factor 6 (ATF6) translocates to the Golgi where it is processed to generate a transcription factor that targets genes involved in ER-associated protein degradation (ERAD). When activated, IRE1α forms an oligomer that processes the mRNA coding for X-box binding protein 1 (XBP1), another transcription factor that activates proteins involved in ERAD, ER chaperones, and lipid biosynthesis. Activation of PERK is associated with active dimer formation that phosphorylates eiF2α (eukaryotic initiation factor 2α), which represses global translation while facilitating the activation of numerous factors including activating transcription factor 4 (ATF4). Activation of ATF4 drives the expression of genes involved in protein folding, amino acid biosynthesis, oxidative stress, autophagy, and apoptosis. (From Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol.* 2011;54:795–809; with permission.)

this signal to initiate the UPR (Fig. 3-31). These include PKR-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), a protein with kinase and endoribonuclease (RNase) activities, and the precursor form ATF6 (activating TF 6). Under normal conditions, the EPR-resident soluble chaperone BiP (or Grp78) inhibits UPR. However, when stressed, the unfolded proteins accumulating in the lumen of the EPR sequester BiP away from these sensors, thereby turning them on to signal for the UPR. PERK catalyzes the phosphorylation of the eIF2α, resulting in attenuation of translation and decreased synthesis of most (but not all) proteins. Upon activation, IRE1α undergoes dimerization and trans-autophosphorylation, which increases its RNase activity, enabling it to cleave and process the mRNA for XBP1 (X-box binding protein-1), and XBP1 upregulates numerous genes involved in secretory function including ER chaperones and lipid synthesis. The third sensor ATF6 resides as a 90-kDa precursor protein in the EPR with its Golgi localization sequences masked by BiP. On dissociation from BiP, ATF6 translocates to the Golgi where it is cleaved by SP1 and SP2 proteases (site-1 and site-2 proteases). This cleavage releases activating factor 6 (ATF6), a potent TF that together with XBP1 activates transcription of ER chaperone proteins that promote protein folding.

Overall, UPR serves to sustain cell survival. However, with excessive EPR stress, PERK, IRE1α, and ATF6 act in concert to initiate apoptosis (Carreras-Sureda et al., 2017). Mitochondria-associated membranes (MAMs) are specialized membranes of the ER that are in close proximity to the mitochondrial outer membrane and facilitate communication between the EPR and mitochondria. Core components of UPR, including PERK and IRE1α, are enriched in MAMs. Moreover, while PERK-catalyzed phosphorylation of eIF2α decreases the synthesis of most proteins, it can initiate cell death programs by complex regulation that ultimately induces the expression of the proapoptotic protein Bim along with the cell surface death receptor TRAIL-2, while inhibiting the expression of the antiapoptotic protein Bcl-2. Activated IRE1α may also initiate cell death by recruiting the adaptor protein TNF receptor–associated factor-2 (TRAF2), with subsequent activation of apoptosis signal-regulating kinase 1 (ASK1) and JNK. After phosphorylation, JNK activates proapoptotic Bim and inactivates antiapoptotic Bcl-2 proteins (Malhi and Kaufman, 2011). The IRE1α–TRAF2 complex may also recruit and activate procaspase 12. Alternatively, Ca^{2+} released from the EPR can activate calpains, which cleave the EPR-associated procaspase-12 into active caspase-12. The latter then engages the caspase cascade by activating effector caspases to initiate apoptosis.

Induction of Enzymes Repairing DNA: The DNA Damage Response Maintaining the fidelity of DNA is critical to cell survival, and DNA damage initiates numerous pathways for repair (discussed above). The tumor suppressor p53, regarded as the "guardian of the genome," is also critical to DNA repair processes. Normally, p53 is kept inactive and at low levels in the cytoplasm by binding protein mdm2 (mouse double minute homolog 2), an E3 ubiquitin ligase that ubiquitinates p53 and facilitates its proteasomal degradation. However, it is activated by several kinase pathways that signal in response to double-stranded DNA breaks. These pathways include the MRN complex (the trimer of Mre11,

Rad50, and Nbs1 proteins) or the Ku protein (the dimer of Ku70 and Ku80; see the section "Repair of DNA") that bind and activate protein kinases including ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), respectively, to phosphorylate and activate p53 (Batchelor et al., 2009). With DNA damage and through the cascade that phosphorylates p53, it escapes from mdm2, allowing its activation, stabilization, and translocation to the nucleus. p53 facilitates DNA repair by several mechanisms. First, it initiates cell cycle arrest at the G_1/S interface by upregulating the cyclin-dependent kinase inhibitor protein p21, thereby allowing more time for DNA repair. Second, it increases expression of numerous proteins directly involved in DNA repair. These include gadd45 (growth arrest and DNA damage inducible) that interacts with histones and facilitates access of proteins (e.g., topoisomerase) to DNA, XPE, and XPC, members of the xeroderma pigmentosum group of proteins important in UV-induced DNA damage recognition, MSH2 (operating in mismatch repair), PCNA to enable excision and the recombination repair processes, and ribonucleotide reductase to synthesize deoxyribonucleotides needed to support DNA synthesis. Beyond their action on p53, the DNA damage–activated protein kinases (ATM, ATR, and DNA-PK) also phosphorylate histone H2AX adjacent to the damage, which then becomes ubiquitinated. These markings in turn recruit repair proteins (including BRCA1) and induce chromatin relaxation for better access of repair enzymes to the lesion.

Overall, DNA repair mechanisms and the more complicated DNA damage response (DDR) protect normal cells from DNA damage and potential transformation to cancer cells. However, these mechanisms may also provide a selective growth advantage by protecting cells from DNA damage associated with radiotherapy or chemotherapy, and contribute to an adaptive response that causes resistance to anticancer treatments (discussed below).

Adaptive Increase in Proliferative Responses to Promote Tissue Repair In association with toxic injury, there is often a resulting signaling cascade to initiate tissue repair via cell replacement. In this situation, phosphatases such as PTP and PTEN, which normally serve as brakes on growth factor receptor-initiated mitogenic signaling, play an active role (refer to Fig. 3-13). These enzymes, which contain reactive cysteine thiols in their active sites, are covalently modified and thereby inactivated by electrophiles. In this manner, the loss of their inhibitory activity on proliferative signaling cascades serves to amplify intracellular signaling for mitosis and survival.

NF-κB is also a converging pathway for proliferative signaling, as it transactivates genes producing cell cycle acceleration (e.g., cyclin D1 and c-Myc) and inhibitors of apoptosis (e.g., antiapoptotic Bcl proteins and the caspase IAPs; Karin, 2006). In addition, NF-κB also transactivates ferritin, GST, SOD1, HO-1, a proteasome subunit, and gadd45, facilitating detoxification and molecular repair. Collectively, these roles of NF-κB explain its broad involvement in tolerance to chemically induced tissue injury and adaptation to ionizing radiation.

Proliferating cells also need to increase protein synthesis, a process controlled by the protein kinase mTOR (mammalian target of rapamycin). mTOR is a major regulator of general metabolic activity. It is activated by the MAPK pathway through phosphorylation of the MAPK isoform Erk and by the PI3K pathway through phosphorylation of Akt (see Fig. 3-13). These pathways are also activated in response to oxidant or electrophile exposure as they are held in check by controlled PTPs and PTEN (Fig. 3-13) such that inhibition of these phosphatases also increases signaling through Erk and Akt. In the cascade, Erk and Akt activate mTOR that in turn phosphorylates and regulates effectors of protein synthesis, such as the translation repressor protein 4EBP1 and the protein kinase S6K, which modifies ribosomes increasing their translational efficiency (Shaw and Cantley, 2006). In contrast to its activation during cell proliferation, it is shutdown in conditions such as hypoxia to reduce ATP synthesis.

Adaptation to Hypoxia: The Hypoxia Response Hypoxia-inducible factor 1α (HIF-1α) is maintained at very low constitutive intracellular levels because of continuous hydroxylation of its two proline residues by HIF-prolyl hydroxylases. Hydroxylation of the proline residues permits a ubiquitin ligase subunit (von Hippel Lindau protein [VHL]) to initiate proteasomal degradation. Under normal, non-stress conditions, HIF-1α is a short-lived protein with a half-life of less than 5 minutes. HIF-1α is also kept transcriptionally inactive by hydroxylation at one of its asparagine residues by HIF-asparagine hydroxylases, which prevents interaction of HIF-1α with transcriptional coactivators, such as p300 and CREB-binding protein (CBP). However, when O_2 delivery is impaired and hypoxia persists for more than a few minutes, HIF-1α increases markedly (Ju et al., 2016). Moreover, HIF hydroxylases have a K_M for O_2 that is very close to ambient O_2 concentration, so that when the O_2 concentration falls, there is decreased hydroxylation of HIF-1α as well as its VHL-mediated ubiquitination for proteasomal degradation, which increase the abundance and transcriptional activity of HIF-1α. HIF hydroxylases are members of the Fe^{2+} and ascorbate-dependent dioxygenases and Fe^{2+} deficiency also impairs their activity. This feature explains why iron chelators (deferoxamine) or Fe^{2+}-mimicking metal ions (Co^{2+} and Ni^{2+}) induce and activate HIF-1α (Maxwell and Salnikow, 2004).

In hypoxic conditions, HIF-1α dimerizes with HIF-1β (also called Arnt, which is the dimerization partner for AhR; Fig. 3-11). The HIF complex transactivates a vast array of genes containing the hypoxia response element (HRE). Many of the activated genes function to enable acclimatization to hypoxia (Harada, 2016). These include (1) erythropoietin (EPO) produced largely in kidney to activate erythropoiesis in bone marrow, (2) proteins involved in iron homeostasis (e.g., transferrin, TFR, ceruloplasmin, and heme oxygenase) to increase availability of iron for erythropoiesis, (3) vascular endothelial growth factor (VEGF) and angiopoietin-2 to stimulate blood vessel growth, (4) proteins facilitating glycolysis including the glucose transporter GLUT1 and some glycolytic enzymes, (5) proteins that correct acidosis caused by glycolytic overproduction of lactate (e.g., a monocarboxylate transporter and a Na^+/H^+ exchanger for export of lactate and H^+), (6) the REDD1 signal transducer protein that initiates a complex signaling pathway that leads to suspension of the ATP-consuming protein synthesis via inactivation of mTOR, and (7) a generalized response to promote ECM remodeling (e.g., matrix metalloproteinase-2) and cell migration and to induce autophagy and/or apoptosis. As an example of an adaptive response to hypoxia, it has been shown that in mice housed in a low O_2 environment (hypoxic preconditioning), HIF-1α is stabilized in the retina to induce hypoxia-responsive genes (EPO and VEGF) upon which the retina becomes resistant to light toxicity (Grimm et al., 2005). Adaptation to hypoxia also occurs in response to high-altitude conditions, chronic cardiorespiratory dysfunction, and ischemic preconditioning. The hypoxia response can develop as a result of toxicities that cause acute or subacute conditions of hypoxia (e.g., respiratory muscle weakness after organophosphate intoxication and diquat-induced pulmonary injury) or as a delayed sequel to conditions of respiratory surface restriction as is seen in hard metal disease (see Chap. 15).

The Energy Stress Response to Adapt to Energy Depletion Cells try to maintain ATP levels at all times. When the rephosphorylation of AMP and ADP to ATP does not keep up with the rate of ATP use, the ratio of AMP to ATP increases.

AMP-activated protein kinase (AMPK) represents an adaptive cellular mechanism that responds to energy deficits to boost ATP production and limit ATP consumption (Hardie et al., 2006). The kinase is strongly activated by AMP, and it is phosphorylated by protein kinase LKB1 (or by the calmodulin-dependent protein kinase kinase [CaMKK] in neurons). Phosphorylated AMPK is activated and targets two sets of proteins that function to increase ATP production either from catabolism of glucose and fatty acids or by promoting mitochondrial ATP biogenesis, while concurrently reducing reactions that consume ATP. For example, to increase ATP levels AMPK increases (a) glucose uptake (via glucose transporters GLUT4 and GLUT1), (b) glycolysis (via phosphorylation and activation of 6-phosphofructo-2-kinase [PFK-2]), and (c) fatty acid oxidation in mitochondria (via phosphorylation and inactivation of acetyl-CoA-carboxylase). Simultaneously, it induces an extensive program to reduce ATP consumption by broadly inhibiting (a) glycogen synthesis by phosphorylation and inactivation of glycogen synthase, (b) lipid synthesis by phosphorylating and inactivating acetyl-CoA-carboxylase, (c) cholesterol synthesis by phosphorylating and inactivating HMG-CoA reductase, (d) glucose synthesis via decreased expression of key gluconeogenetic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase, and (e) protein synthesis by inhibiting mTOR. AMPK-mediated modulation of cellular energy supply and consumption occurs largely through kinase-mediated phosphorylation reactions rather than transcriptional regulation, rendering this pathway a rapid, adaptive response.

In addition to the rapid AMPK-dependent reprogramming of cellular intermediary metabolism, there is an ultimate means for cells to generate fuel for ATP production in case of nutrient shortage. This slower process involves consumption of the cell's own constituents (lipid droplets, glycogen particles, proteins, and organelles) by nonselective or bulk autophagy (Rabinowitz and White, 2010; Petibone et al., 2017) with lysosomal hydrolysis providing essential metabolic intermediates (amino acids, fatty acids, nucleosides, and carbohydrates). As discussed earlier, autophagy is a five-step process that involves a complex program of kinase activity including ULK-1, autophagy-related proteins (Atg), and focal adhesion kinase family-interacting protein of 200 kDa (FIP200), which is an initiator of autophagosome formation (Fig. 3-27). When nutrients are abundant, mTOR phosphorylates and inactivates ULK-1 and Atg13, thereby inhibiting autophagy. However, in conditions of nutrient shortage, mTOR is inactivated by signaling pathways, such as those activated by AMP and hypoxia, which releases inhibition of the ULK-1–Atg13–FIP200 complex, and in turn initiates autophagosome formation (Hosokawa et al., 2009). The important role that mTOR serves in switching autophagy on and off is further evidence of its role as key regulator of cellular energy homeostasis.

In surveying the major cellular adaptation mechanisms to toxicants herein, it should be easy to recognize that one toxic event may initiate several of these adaptive responses. For example, cells exposed to a hypoxic environment can rapidly respond with both AMPK-mediated program of energy stabilization and HIF-1α-directed adaptation to oxygen shortage. Theoretically, an electrophilic toxicant that binds covalently to cellular macromolecules can also generate oxidative stress, and would be expected to induce numerous adaptive processes, including the electrophile response, the heat-shock response, EPR stress, the DDR, and cell proliferation. Furthermore, if severe enough to compromise ATP synthesis, the energy stress response may be elicited. Such complexity illustrates the broad underpinning of almost any mechanism of toxicity.

Adaptation by Neurohumoral Mechanisms There are numerous adaptive responses to dysfunctions of organs or organ systems that are mediated by humoral or neuronal signals between cells located in the same or different organs. For example, the rapid hyperventilation evoked by acute hypoxia or HCN inhalation is mediated by a neural reflex initiated by glomus cells in the carotid body. These chemosensitive cells generate a Ca^{2+} signal via AMPK (Evans et al., 2005). In addition, CN^{-}, mitochondrial electron transport inhibitors (e.g., rotenone, antimycin A, myxothiazole), uncouplers (e.g., 2,4-dinitrophenol), or ATP-synthase inhibitors (e.g., oligomycin) as well as AMPK activators mimic the response to hypoxia in these cells.

Final and Irreversible Actions of Repair and Adaptation Failure

The most important consequences of inappropriate repair are irreversible organ failure, as is the case with necrosis or tissue changes that advance to fibrosis or carcinogenesis. Before discussing these final, irreversible effects, general principles concerning failure to repair or adapt are presented.

When Repair Fails Although repair mechanisms operate at molecular, cellular, and tissue levels, they can fail to provide protection against injury. Toxicity is manifested when repair of the initial injury fails because the repair mechanisms become overwhelmed, exhausted, or impaired. Failure occurs because the fidelity of the repair mechanisms is not absolute, as in the case for DNA repair. However, repair most often fails when damage overwhelms repair mechanisms, by depleting protective elements and exhausting the capacity for repair. For example, alkylation of DNA depletes MGMT, lipid peroxidation depletes α-tocopherol, and overproduction of oxidized or otherwise damaged proteins can exhaust the pool of ubiquitin. Sometimes toxicant-induced injury adversely affects the repair process itself. For example, ethanol generates ROS via CYP2E1 that impairs the proteasomal removal of damaged proteins. Autophagic removal of cell constituents may be compromised by lysosomotropic drugs that increase intralysosomal pH to reduce lysosomal hydrolase activity, a defect that may contribute to the mechanism of chloroquine-induced myopathy in rats. Thiol-reactive chemicals can inactivate ubiquitin-activating E1, E2, and E3 enzymes because each family contains a catalytically active cysteine, as well as lysosomal cysteine proteases (e.g., cathepsins B, H, and L), to compromise the clearance of damaged proteins by both the UPS and autophagy. These repair mechanisms are especially important in neurons, as genetic deletion of proteins mediating UPS and autophagy causes accumulation of intraneuronal inclusions, neuronal loss, or neurodegeneration. In fact, there is growing evidence that human neurodegenerative diseases appear to be directly related to dysfunction of UPS and/or autophagy (Bedford et al., 2011; Menzies et al., 2017).

It is also possible that repair contributes to toxicity. This often occurs in a passive manner, when excessive amounts of NAD^{+} are cleaved by PARP in the process of repairing damaged DNA strands, or when too much NAD(P)H is consumed for the repair of oxidized proteins and endogenous reductants. Either event can compromise oxidative phosphorylation, which is also dependent on the supply of reduced cofactors, and exacerbate ATP depletion. However, repair also may play an active role in toxicity. This is often observed with chronic tissue injury, when repair processes progress to uncontrolled proliferation rather than remodeling. Such proliferation of cells may become neoplastic, whereas overproduction of ECM progresses to fibrosis. A specific case is when the cellular repair processes that normally degrade damaged proteins, such as the UPS

and the autophagy pathway, become inappropriately stimulated by adverse signaling, causing degradation of ordinary intracellular proteins. For example, glucocorticoids can cause dose- and time-dependent muscle atrophy and wasting. The mechanism underlying muscle atrophy is reduced signaling through the IGF-1–PI3K–Akt pathways, which leaves FoxO TFs unchecked to regulate expression of muscle-specific ubiquitin ligases (MAFbx, also called atrogin-1, and MuRF1) that enhance muscle catabolism and wasting (Atherton et al., 2016).

When Adaptation Fails As described in the previous sections, moderate oxidative stress activates NF-κB, AP-1, and Nrf2 to initiate adaptive protection. However, extensive oxidant exposure aborts these programs because it leads to oxidation of thiol groups in the DNA-binding domain of these TFs. Similarly, some otherwise adaptive and protective mechanisms may be harmful under extreme conditions and numerous examples are provided here. For example, tubuloglomerular feedback is important in regulating glomerular filtration. However, with acute renal injury, tubular reabsorption is impaired and causes polyuria, which triggers tubuloglomerular feedback to further reduce glomerular blood flow and filtration. These seemingly protective events may precipitate anuric renal failure. It is also possible that an adaptive mechanism that is beneficial in the short term may become harmful when forced to operate for a prolonged time. For example, bulk autophagy likely contributes to the lethal wasting syndrome induced by TCDD. Chronic inflammation, tissue injury, or cancer may lead to iron deficiency and anemia because IL-6 upregulates hepcidin secretion from the liver, which reduces intestinal iron absorption. As discussed earlier, NF-κB activation is indispensable for repair via proliferation of the acutely injured tissue. However, NF-κB also targets cytokines (e.g., TNF and IL-1β), which in turn further activate NF-κB through receptor signaling (see Fig. 3-13). This vicious cycle may lead to chronic inflammation and cancer when persistent tissue injury maintains NF-κB signaling (Karin, 2006). Sustained cell proliferation is causally related to neoplasia in a variety of models including phenobarbital-induced liver tumors in rodents and lung tumors associated with occupational exposure to silica. Sustained activation of HIF-1α in tumors facilitates invasiveness, in part by increasing VEGF expression and angiogenesis. In the kidney, HIF-1α may also be involved in fibrogenesis, as it targets critical genes that maintain the extracellular matrix (ECM) such as tissue inhibitor of metalloproteinase-1 (TIMP-1).

Toxicity Resulting from Inappropriate Repair and Adaptation

Failure to repair involves molecular, cellular, and tissue dysfunction and can show species- and age-related differences. Some toxicities involve dysrepair at an isolated level. For example, hypoxia and dyspnea develop after exposure to metHb-forming chemicals if the amount of metHb produced overwhelms the capacity of metHb reductase. Because this repair enzyme is deficient at early ages, neonates are especially sensitive to chemicals that cause methemoglobinemia. Ineffective reduction of metHb also contributes to the formation of Heinz bodies, which are protein aggregates formed in red blood cells as a function of oxidative stress and aging. As noted earlier, there are also species differences in metHb reductase activity that are responsible for differences in sensitivity to metHb formation.

Tissue Necrosis Although cell death ensues when repair mechanisms are inefficient or the molecular damage is not readily reversible, progression to tissue necrosis can be interrupted by apoptosis and cell proliferation. Injured cells can initiate apoptosis, which counteracts the progression of the toxic injury. Apoptosis does this by preventing necrosis of injured cells and the consequent inflammatory response, which may cause additional injury by releasing cytotoxic mediators. A classic example illustrating the role of the inflammatory response in tissue necrosis is activation of Kupffer cells with administration of bacterial LPS (endotoxin) which greatly exacerbates the hepatotoxicity of galactosamine. In contrast, when the Kupffer cells are selectively eliminated by pretreatment of rats with gadolinium chloride, the necrotic effect of numerous hepatotoxicants is markedly alleviated (Nolan, 2010). Blockade of Kupffer cell function with glycine also protects the liver from numerous hepatotoxicants and is associated with decreased levels of pro-inflammatory cytokines (TNF-α) and increased levels of anti-inflammatory mediators such as IL-10 (Bruck et al., 2003).

Cell proliferation also serves to negate the loss of organ function when necrosis occurs. Early cell division is instrumental in the rapid and complete restoration of injured tissue and the prevention of necrosis. For example, if cell proliferation is blocked in liver, a normally non-necrogenic dose of a toxicant such as carbon tetrachloride causes hepatic necrosis (Mehendale, 2005).

The dose–response relationship that describes toxicant-induced injury also contributes to the dose response for irreversible injury. In general, tissue repair increases up to a threshold dose that can limit or prevent irreversible injury. However, once that threshold is reached, toxicity and cell death progress rapidly. For example, with hepatotoxicants, apoptosis and cell proliferation are operative with latent tissue injury caused by low (non-necrogenic) doses of toxicants, but are inhibited with severe injury induced by high (necrogenic) doses. For example, 1,1-dichloroethylene, carbon tetrachloride, and thioacetamide induce apoptosis in the liver at low doses, but cause hepatic necrosis after high-dose exposure. Similarly, there is an early mitotic response in the liver to low-dose carbon tetrachloride, but this response is not observed with higher doses of the solvent.

Fibrosis

Fibrosis is characterized by excessive formation and deposition of excess connective tissue with abnormal composition of the ECM. In general, fibrotic conditions can develop in any organ, but those of note include liver, lung, kidney, and heart. Hepatic fibrosis, or cirrhosis, is associated with fatty liver disease as well as toxicants including chronic consumption of ethanol, treatment with methotrexate, and exposure to thioacetamide, carbon tetrachloride, and high levels of iron. Chronic fibrotic changes in the renal glomerulus are associated with diabetic kidney disease, and pulmonary fibrosis is induced by drugs such as bleomycin and amiodarone and associated with prolonged inhalation of oxygen or mineral particles. Finally, drugs including doxorubicin may cause generalized cardiac fibrosis, whereas drugs acting as agonists on 5-HT_{2B} receptors, such as fenfluramine, bromocriptine, and methysergide, cause valvular interstitial cell proliferation and heart valve fibrosis. Exposure to high doses of ionizing radiation induces fibrosis in many organs.

Physiologically, fibrosis is an exaggerated wound healing response which interferes with normal organ architecture and function. The epithelial-mesenchymal transition (EMT) is a fundamental process in fibrotic disease. This response is characterized by the phenotypic transformation of an epithelial cell into a fibroblast or myofibroblast. Although EMT is necessary for organ development and wound healing, it is uncontrolled in fibrosis, and cells that manufacture the ECM during tissue repair overproduce the matrix in an uncontrolled manner.

As an end-stage disease, fibrosis is detrimental because (1) scars can compress and ultimately obliterate parenchymal cells and blood vessels; (2) deposition of basement membrane components between the capillary endothelial cells and the parenchymal cells presents a diffusional barrier that contributes to malnutrition of the tissue cells; (3) an increased amount of ECM alters the elasticity and flexibility of the whole tissue, compromising the mechanical function of organs such as the heart and lungs, and (4) fibrotic disease can change basic aspects of cell behavior, including cell polarity, motility, and gene expression.

TGF-β is a central mediator of fibrotic disease. Its role as a mediator of fibrosis is well recognized, but additional factors including connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), and IL-4 also contribute to the process. TGF-β is highly overexpressed in several experimental models of fibrosis and in patients with active liver cirrhosis. Moreover, there is experimental evidence that subcutaneous injection of TGF-β induces local fibrosis, and transgenic mice engineered to overexpress TGF-β develop severe hepatic fibrosis.

The fibrotic action of TGF-β is due to increased production and decreased degradation of ECM components. TGF-β stimulates the synthesis of individual ECM components in target cells via the Smad pathway (see Fig. 3-14). TGF-β also augments ECM formation by inhibiting ECM degradation by increasing the expression of inhibitor proteins that antagonize ECM-degrading enzymes, such as TIMP-1 and plasminogen activator inhibitor-1 (PAI-1). TGF-β also induces transcription of its own gene in target cells, suggesting that the TGF-β produced by these cells can amplify in an autocrine manner the production of the ECM (Fabregat et al., 2016). There are also changes to the composition of the ECM in fibrotic diseases. Basement membrane components, such as collagen IV and laminin, as well as the fibrillar-type collagens (collagens I and III), which confer rigidity to tissues, increase disproportionately during fibrogenesis.

In general, miRNAs contribute to fibrosis by affecting the EMT, and numerous miRNAs contribute to this process. Three major miRNAs involved in fibrotic disease are miR-21, miR-29, and miR-200, which are directly regulated by TGF-β activation of pSmad3 (Zou et al., 2017). Constitutively, miR-21 is highly expressed in the healthy kidney, but it is markedly overexpressed in idiopathic pulmonary fibrosis (IPF) and renal fibrosis. Major targets for miR-21 include matrix metalloprotease-9 (MMP-9) and Smad-7, and downregulation of these targets prevents ECM degradation and inhibition of TGF-β signaling, respectively (Loboda et al., 2016). miR-29, which acts to repress collagen translation, is downregulated by TGF-β and in doing so, α-smooth muscle actin and collagen I are increased, particularly in the kidney (Roderburg et al., 2011). miR-200 is expressed in lung fibroblasts and is downregulated in fibrotic conditions. Decreased expression of miR-200 exerts a profibrotic action by increasing expression of numerous mesenchymal markers.

Carcinogenesis

As there is an entire chapter dedicated to carcinogenesis (see Chap. 8), only those aspects relating directly to mechanisms associated with repair and adaptive processes are discussed here. A central feature of carcinogenic mechanisms is altered expression of proteins that function as proto-oncogenes and/or suppressor proteins. These important proteins can be altered by mutation resulting from direct DNA damage or by altered transcription leading to overexpression of proto-oncogenes or decreased expression of tumor suppressors.

Another important consideration is that genetic or epigenetic mechanisms are involved in carcinogenesis. DNA-reactive or genotoxic mechanisms bring about a *qualitative* change in gene expression, with expression of an altered gene product, a mutant protein or miRNA, and gain or loss in activity. In contrast, epigenetic mechanisms are non-DNA reactive (or non-genotoxic) and do not alter gene sequence, but rather, cause *quantitative* change in gene expression resulting in more or less gene product. Regardless of mechanism, cancer ultimately develops from cellular failures in apoptosis and/or from uncontrolled cell proliferation.

Genotoxic Mechanisms of Carcinogenesis Chemicals that react with DNA may cause damage such as adduct formation, oxidative alteration, and strand breakage. As described above, these lesions are repaired or the injured cells are eliminated. If neither event occurs, a lesion in the parental DNA strand may induce a mutation in the daughter strand during replication. The mutation may remain silent if it does not alter the protein encoded by the mutant gene or if the mutation causes an amino acid substitution that does not affect the function of the protein. Alternatively, the genetic alteration may be incompatible with cell survival. The worst scenario occurs when the altered genes express mutant proteins that reprogram cells for multiplication and escaping apoptosis (i.e., immortalization). This trait is passed to daughter cells during mitosis, enabling sustained proliferation. Moreover, enhanced DNA replication and cell division increases the likelihood of additional mutations that may further augment their growth advantage. Ultimately, tumors arise from the nidus of rapidly proliferating transformed cells (Fig. 3-32).

The critical role of DNA repair in preventing carcinogenesis is exemplified by the human heritable disease xeroderma pigmentosum (XP) in which affected individuals lack excision repair proteins. These people are at high risk for developing sunlight-induced skin cancers, and they are also more prone to chemically induced DNA damage from recognized carcinogens including aflatoxin B_1, aromatic amines, and polycyclic hydrocarbons (Giordano et al., 2016).

Mutation of Proto-oncogenes Proto-oncogenes are highly conserved genes that encode proteins that stimulate the progression of cells through the cell cycle or oppose apoptosis (Jonckheere et al., 2017). Under normal conditions, these important proteins are required for regulating growth, with essential roles in supporting embryogenesis, tissue regeneration, and growth factor responses. Mutations in proto-oncogenes can cause permanent activation and/or overexpression of these proteins to favor neoplastic transformation. The altered gene is then referred to as an oncogene. Fig. 3-33 depicts several proto-oncogene products that are involved in initiating the cell division cycle.

An example of mutational activation of an oncogene is the Ras family of proteins (H-Ras, K-Ras, and N-Ras). Ras proteins are G-coupled proteins with GTP/GDP-binding capacity as well as GTPase activity. Ras is activated downstream from growth factor receptors and protein tyrosine kinases and upstream from the mitogen-activated protein kinase (MAPK) cascade (Figs. 3-12 and 3-13). Ras functions as a molecular switch that is only active in the GTP-bound form. Mutations in Ras genes occur mainly in codons 12, 13, or 61 and represent a dominant gain of function because they lock the protein in the permanently active GTP-bound form to drive uncontrolled cell division and transformation (Fig. 3-33). Activating mutations in Ras genes occur in approximately 20% of all human cancers, with K-Ras mutations being the most prevalent (Chang et al., 2016). Mutation of the *K-ras* oncogene is also mechanistically linked to pancreatic cancer (Jonckheere et al., 2017).

Activating mutations in the proto-oncogene b-Raf is another important example of genotoxic mechanisms of carcinogenesis. Raf proteins are protein kinases just downstream from Ras and are the first signal transducers in the MAP kinase pathway (see Fig. 3-12). These mutations are expressed in 15% to 30% of all

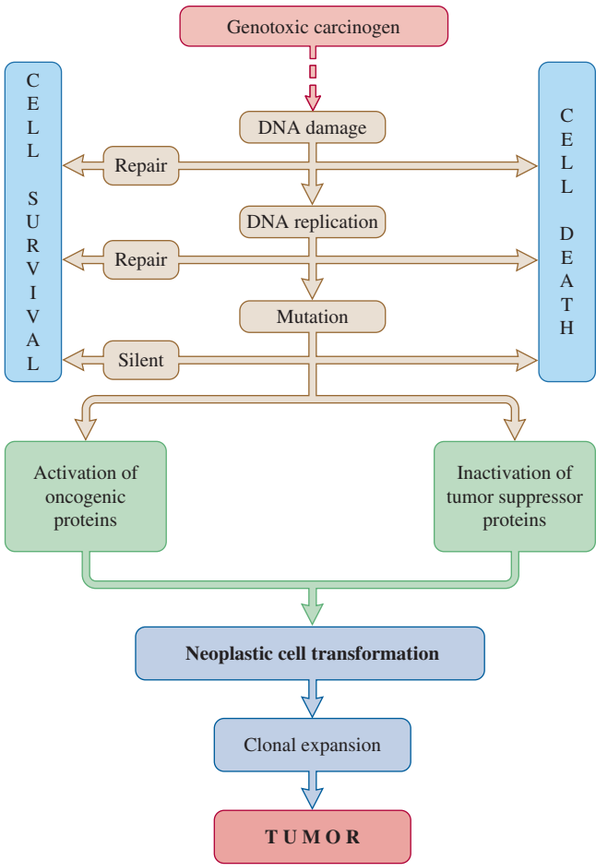

Figure 3-32. *The process of carcinogenesis initiated by genotoxic carcinogens.* Genotoxic mechanisms require some form of DNA damage. Activating mutations of proto-oncogenes that encode permanently active oncoproteins and inactivating mutations of tumor suppressor genes that encode permanently inactive tumor suppressor proteins can cooperate in neoplastic transformation of cells.

human tumors, and are particularly prevalent in malignant melanoma (with a frequency of nearly 60% of these tumors). The most common mutation (V600E) occurs within the activation segment of b-Raf. The increased kinase activity of the mutant b-Raf continually sends a Ras-independent proliferative signal down the MAPK pathway to promote neoplastic transformation (Roskoski, 2010).

While constitutive activation of oncogenes is a common initiator of chemical carcinogenesis, overexpression resulting from gene amplification also can contribute to neoplastic cell transformation. Such an event can occur spontaneously or may be initiated by DNA strand breaks.

Mutation of Tumor Suppressor Genes Tumor suppressor genes encode proteins that inhibit the cell cycle progression or promote DNA repair or apoptosis when there is DNA damage. Fig. 3-33 depicts such proteins, which include cyclin-dependent protein kinase inhibitors (e.g., p15, p16, and p21), TFs including p53 and Smads, ATM and ATR that sense DNA damage to signal p53 activation, BRCA1 and BRCA2 that contribute to recombinational DNA repair, PTEN phosphatase that turns off the PI3K–Akt pathway-mediated proliferative signaling, and the tuberous sclerosis complex-2 (TSC2) that prevents activation of mTOR. Uncontrolled proliferation occurs when the mutant tumor suppressor gene can no longer suppress cell division. Inactivating mutations of specific tumor suppressor genes in germ cells are responsible for the inherited predisposition to cancer, as in familial retinoblastoma (pRb), Wilms tumor, familial polyposis, and Li–Fraumeni syndrome. Mutations of tumor suppressor genes in somatic cells contribute to nonhereditary cancers, with p53, p16, PTEN, and pRb showing high frequency of mutation in human cancer.

As described earlier, p53, otherwise referred to as the guardian of the genome, controls cell cycle arrest to enable DNA repair (Fig. 3-34). The critical and essential role of p53 function is further illustrated in conditions in which it is deficient or defective. Cells that have no p53 are about a million times more likely to permit DNA amplification than are cells with a normal level of this suppressor gene. Furthermore, mice with the *p53* gene deleted develop tumors in 6 to 9 months. Mutations in the *p53* gene are found in approximately 50% of human tumors and in a variety of induced cancers. The majority are missense mutations that alter amino acid sequence to yield an aberrant that cannot activate the essential tumor suppressor transcriptional response (Joerger and Fersht, 2016). A classic example of carcinogen-mediated p53 mutation is the point mutation in codon 249 that predominates in human hepatocellular carcinomas arising from exposure to aflatoxin B_1. There is compelling evidence that this mutation is causally related to aflatoxin-induced liver cancer in humans (Hussain et al., 2007; Wogan et al., 2012). However, this same mutation is not observed in rat liver following aflatoxin exposure.

The contribution of miRNA regulation to cancer is complex (Wang and Taniguchi, 2013). For example, p53 promotes the processing of numerous miRNAs and specifically upregulates transcription of miRNA34a-c after DNA damage. The miRNA34 family regulates the G_1/S cell cycle checkpoint, so in the presence of p53 mutation, this important checkpoint in cell cycle control is lost. Furthermore, direct damage to genes coding for miRNAs may also contribute to carcinogenesis. For example, amplification of miR-21, which normally represses the translation of PTEN, and deletion of miR-15 and miR-16, which normally represses translation of cdc25A, occur in spontaneous human malignancies. Loss of the repressive function of these miRNAs serves to disrupt cell cycle regulation.

Non-genotoxic Mechanisms in Carcinogenesis Non-genotoxic carcinogens often stimulate sustained cell proliferation. This can occur by direct activity on proliferative signaling, such as PKC activation by phorbol esters or by protein phosphatase inhibition by okadaic acid. Additionally, sustained cell proliferation can be evoked by endogenous mitogens and hormones, including estrogens on mammary gland or liver cells, TSH on the follicular cells of the thyroid gland, and luteinizing hormone on Leydig cells in testes, and some toxicants that cause sustained cell injury (such as chloroform and D-limonene). There are also numerous xenobiotics, often microsomal enzyme inducers, that cause sustained proliferation, particularly in liver, to cause tumors in rodents but not in humans. Such compounds include phenobarbital and DDT along with peroxisome proliferators (e.g., fibrates, Wy-14643, and dihalogenated and trihalogenated acetic acids).

Role of Signaling in Carcinogenesis As described under tissue repair, cell injury evokes the release of mitogenic growth factors such as HGF and TGF-α from tissue macrophages and endothelial cells. Although these growth factors are instrumental in tissue repair after acute cell injury, their continuous presence is potentially harmful because they may ultimately transform the affected cells into neoplastic cells. Indeed, transgenic mice that overexpress TGF-α develop hepatomegaly at a young age and tumors by 12 months (Fausto et al., 2006).

Role of DNA Methylation and Histone Modification in Carcinogenesis Some non-genotoxic carcinogens alter DNA methylation. As discussed earlier, specific cytosine residues located

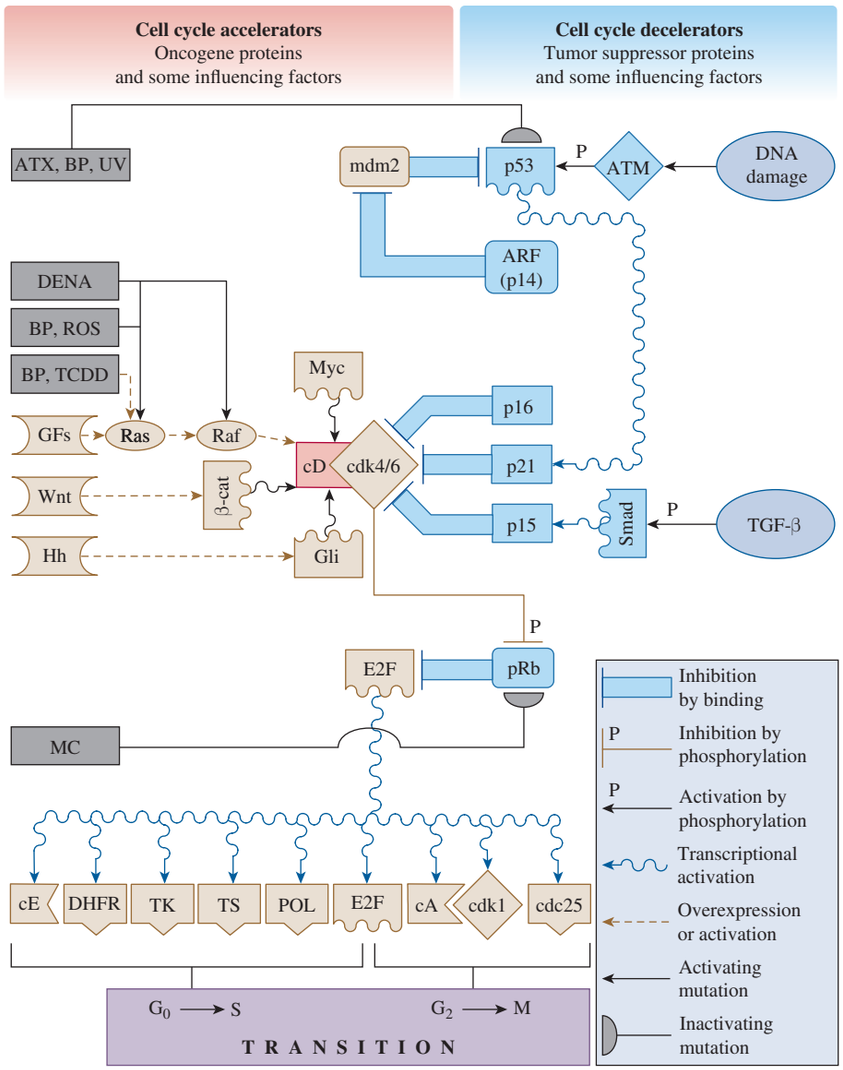

Figure 3-33. *Key regulatory proteins controlling the cell division cycle along with relevant effects in the signaling cascades that regulate the cell cycle.* The left side of the figure highlights proteins that accelerate the cell cycle and are oncogenic if permanently active or expressed at high level. In contrast, proteins on the right, represented by blue symbols, decelerate or arrest the cell cycle to suppress oncogenesis, unless they are inactivated (e.g., by mutation). Expression of cyclin D (cD) is increased, for example, by growth factors signaling through Ras proteins and the MAPK pathway as well as by Wnt and Hedgehog (Hh) ligands that ultimately signal through β-cat and Gli transcription factors, respectively. Carcinogens such as benzo[a]pyrene (BP), reactive oxygen species (ROS), and diethylnitrosamine (DENA) may cause mutation of the Ras or Raf gene that results in permanently active mutant proteins.

in CpG islands in promoter regions can be methylated by DNA cytosine methyltransferases using *S*-adenosyl methionine as the methyl donor. Promoter methylation silences genes by weakening TF binding to the promoter and triggering secondary alterations in histone proteins, which renders the histone more compact, thereby reducing access of TFs and other proteins involved in transcription initiation at the gene promoter. It is well documented that the normal methylation pattern of DNA is disrupted in cancer cells. Paradoxically, global DNA hypomethylation is observed in the face of hypermethylation of promoter regions of numerous tumor suppressor genes (Esteller, 2005), such as Cdk inhibitors (p15 and p16), the DNA repair enzyme MGMT, and the lipid phosphatase PTEN. Importantly, both global hypomethylation and tumor suppressor gene hypermethylation intensify with increased malignancy of the tumor. The relevance of hypermethylation-induced silencing of tumor suppressor genes in carcinogenesis is supported by the finding that inhibitors of DNMTs (such as 5-aza-2′-deoxycytidine) can stop the growth of cancer cells and induce their differentiation by demethylating the dormant tumor suppressor genes to restore their expression (Esteller, 2005).

DNA methylation status can also be altered by non-genotoxic carcinogens. This is the case for ethionine, which depletes *S*-adenosyl-methionine (the methyl donor for DNMTs), and diethanolamine, which inhibits cellular utilization of choline, a dietary source for methyl groups needed to convert homocysteine to methionine. Additionally, on prolonged administration, the methylation status of Ras or Raf is altered by phenobarbital, whereas methylation of c-Jun, c-Myc, and IGF-2 is affected by treatment with dichloroacetic acid, dibromoacetic acid, or trichloroacetic acid.

Role of MicroRNAs in Non-genotoxic Mechanisms of Carcinogenesis miRNAs can affect carcinogenic mechanisms through effects on DNA repair (described above) as well on

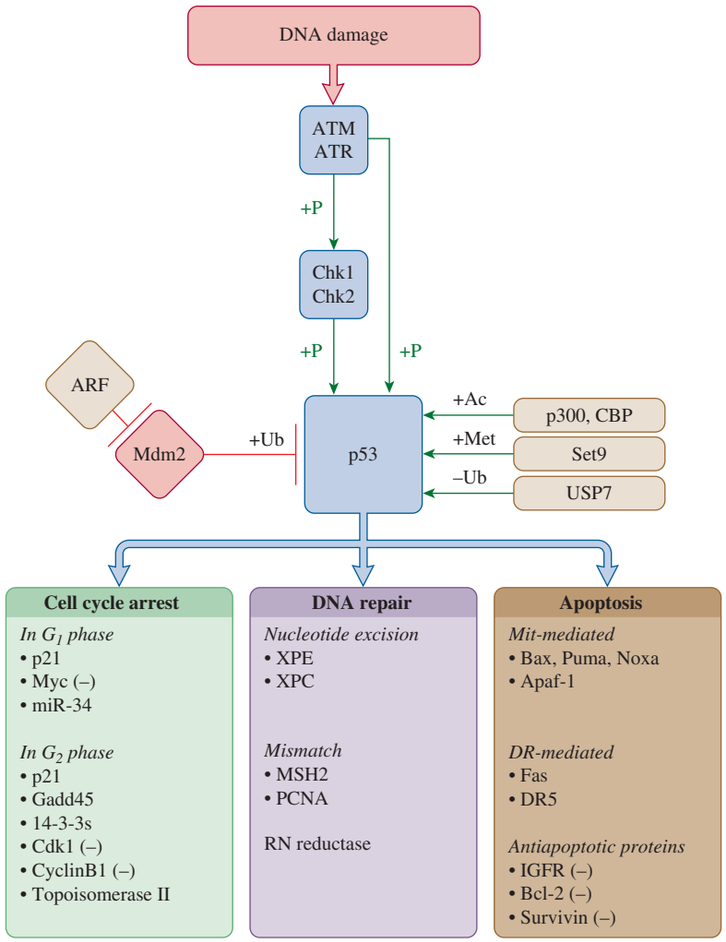

Figure 3-34. *The role and regulation of the guardian of the genome: p53 tumor suppressor protein.* When activated by DNA damage, p53 mediates cell cycle arrest, DNA repair, and apoptosis. p53 acts chiefly as a transcription factor that can activate the transcription of most target genes, while repressing some of others, such as those marked with (–) in the figure. For example, p53 transactivates *p21* and *gadd45* genes, but it represses the *Cdk1* and *cyclin B1* genes, p53 also represses the genes of antiapoptotic proteins (e.g., Bcl-2 and IGF-1 receptor), to promote apoptosis. These (and other) p53-induced proapoptotic mechanisms may be cell-specific, such that they are not necessarily occurring in the same cell at the same time. The intracellular level and activity of p53 depends primarily on the presence of mdm2 which inactivates p53 by ubiquitinating it. In fact, overexpression of mdm2 can lead to constitutive inhibition of p53 and thereby promotes oncogenesis even if the *p53* gene is unaltered.

pathways considered to be non-genotoxic. The miR-17-92 cluster is considered oncogenic because its members promote mitosis by repressing the translation of PTEN and p21, and they inhibit apoptosis by repressing the proapoptotic protein Bim. miR-29b also targets the mRNAs of p21 and Bim, and miR-221/222 targets PTEN, TIMP-3 (metalloproteinase inhibitor 3), and Bim (Garofalo and Croce, 2011). The role of miRNAs in tumorigenesis induced by non-genotoxic carcinogens is well exemplified by PPARα-induced rodent hepatocarcinogenesis. In this example, treatment with Wy-14643 downregulates let-7c miRNA, which normally represses translation of c-Myc. Accordingly, the reduced levels of let-7c in response to Wy-14,643 increases c-Myc protein synthesis, which drives hepatocellular proliferation. Furthermore, c-Myc upregulates the miR-17-92 cluster which in turn represses the translation of p21, an antimitotic cyclin-dependent kinase, and Bim, a proapoptotic protein, and together, these actions permit mitosis while opposing apoptosis. In contrast to normal mice, PPARα-humanized mice (expressing the human PPARα) do not show downregulation of let-7c or hepatocellular proliferation with Wy-14,643 treatment, supporting a causative relationship between these events (Gonzalez and Shah, 2008).

Genotoxic and non-genotoxic mechanisms of carcinogenesis may not be mutually exclusive, but rather, they can complement or amplify each other. Increased mitotic activity, regardless of mechanism or pathway involved, increases the likelihood of carcinogenicity for two major reasons. First, increased mitotic activity increases the probability that mutations may occur because the shortened G_1 phase allows less time for DNA repair. Although repair still may be feasible after replication, postreplication repair is error-prone. Second, increased proliferation enables clonal expansion of the initiated cells, and this facilitated growth contributes to tumor formation and progression. The action of estrogen-induced tumors exemplifies the interaction between DNA-reactive and non-DNA-reactive mechanisms. Estrogen metabolites include reactive quinone and hydroquinone intermediates that can produce mutagenic free radicals via redox cycling. Concurrently, estrogens induce receptor-mediated cell proliferation. Similarly, genotoxic carcinogens can also cause epigenetic alterations. For example, 2-acetylaminofluorene forms DNA adducts, but also increases DNA methylation in numerous genes that silences a variety of tumor suppressor proteins (Pogribny et al., 2011). p16, ARF, and MGMT are also hypermethylated in aflatoxin B_1–induced

mouse lung tumor and in 7,12-dimethylbenzanthracene-induced skin tumor. Thus, epigenetic alterations evoked by genotoxic carcinogens may contribute to carcinogenesis after the initiating genotoxic event (Pogribny et al., 2011). Conversely, global DNA hypomethylation and tumor suppressor gene hypermethylation may increase the occurrence of mutations in cells exposed to nongenotoxic carcinogens.

Failure to Execute Apoptosis Promotes Mutation and Clonal Growth In many cases, initiated preneoplastic cells have much higher apoptotic activity than do normal cells (Fox and MacFarlane, 2016), a mechanism designed to counteract clonal expansion. It follows then that inhibition of apoptosis is detrimental because it facilitates the fixing of mutations and allows for clonal expansion of preneoplastic cells. This is the case for the pathogenesis of human B-cell lymphomas, in which there is chromosomal translocation associated with aberrantly increased expression of Bcl-2 protein to override programmed cell death by inactivating the proapoptotic Bax protein. Increased levels of Bcl-2 are also detected in other types of cancer, and a high Bcl-2/Bax ratio in a tumor is a marker for poor prognosis.

RESEARCH APPROACHES TO DETERMINE MECHANISMS OF TOXICITY: A CASE STUDY

Understanding how all of the processes outlined in this chapter influence toxic outcome is fundamental to understanding mechanisms of toxicity. However, understanding how to apply these concepts to defining a mechanism of toxicity requires incorporating them into actual cases. Throughout this chapter, examples demonstrating how disposition influences toxicant exposure, how a toxicant interacts with and alters important cellular functions, how biochemical, cellular, and molecular processes dictates sensitivity to toxicity and likelihood of repair and adaptation, and how species differences govern susceptibility to toxicity have been provided. As such, the examples provide specific cause–effect relationships, but in isolation, they are not an integrated example that illustrates a mechanism of toxicity as a continuum from the key events that initiate the toxic process to the final outcome of cell dysfunction or disease process. Throughout this textbook, there are many examples of toxic mechanisms focused on specific organ systems. However, to conclude this chapter, an example of a comprehensive mechanism of toxicity and the research approaches used to define the mechanism are summarized. The case example is D-limonene-induced nephrotoxicity and carcinogenicity, a classic and comprehensive example of a well-defined mechanism of toxicity that helped to establish how information on mechanisms of toxicity can inform human risk assessment (Lehman-McKeeman, 2018).

D-Limonene is an oil found in citrus, primarily oranges, and it is widely used in commerce as a flavor and fragrance. After oral dosing to rats, it produced evidence of renal toxicity, although only in males, and with chronic dosing, renal toxicity progressed to renal cell injury, cell proliferation, and ultimately to the development of renal tubular tumors. The first clue regarding toxic mechanisms was histopathologic evaluation of kidneys after single dosages of the compound. These studies showed evidence of droplets in the renal cortex, and specifically in proximal tubule cells, when evaluated by routine hematoxylin and eosin (H&E) staining. More specific staining methods for protein deposits (Mallory–Heidenhain staining) confirmed that these droplets were proteinaceous. Based on histopathologic evidence of protein accumulation, the renal cortices from D-limonene-treated rats were analyzed to determine whether the protein accumulation was generalized to all filtered proteins or more specific to a few proteins. Based on two-dimensional gel electrophoresis, only one protein was accumulating in male rat kidneys, and this protein was sequenced to confirm its identity as α2u-globulin.

The identification of α2u-globulin provided a foundation to understanding why renal toxicity was only seen in male rats because this protein is regulated by numerous hormones and is deemed to be a male rat-specific protein. Female rats have the α2u-globulin gene, but its expression is effectively repressed by estrogen. The next step in deducing the mechanism of toxicity was to determine how D-limonene caused α2u-globulin to accumulate in the renal cortex and how this protein could lead to renal toxicity. To address these issues, research efforts were directed toward establishing the fate of D-limonene and whether D-limonene or its metabolites interacted with α2u-globulin.

The fate of D-limonene had been characterized based on metabolism profiles obtained from urine or in hepatocyte cultures. These metabolism studies suggested that there were no likely reactive metabolites or metabolites that would be expected to be directly toxic. The critical question was whether D-limonene or its metabolites were detected in the kidney, and if so, whether any of them bound to α2u-globulin. Such studies were facilitated by the use of radiolabeled D-limonene, and equilibrium dialysis experiments showed that radioactivity was detected in a high-molecular-weight fraction (protein) of male rat kidney homogenates. Based on evidence of protein binding, additional studies, gas-chromatography–mass spectrometry (GC-MS) analyses were used to identify a D-limonene metabolite in the protein fraction, and the metabolite was one that had not previously been reported. Specifically, D-limonene-1,2-epoxide was identified in the dialyzed protein fraction of male rat kidney homogenates, but this metabolite was not detected in kidney homogenates from D-limonene-treated female rats. Although it is generally considered that epoxides are unstable metabolites that either react covalently with macromolecules or are hydrolyzed to a corresponding diol metabolite by epoxide hydrolase (described previously; see Chap. 6), D-limonene-1,2-epoxide was an exception to this general rule because it is very stable, bound reversibly to renal proteins, and did not hydrolyze to its corresponding diol.

To address questions regarding how D-limonene-1,2-epoxide might cause α2u-globulin to accumulate, the protein was purified, and in vitro studies confirmed that the epoxide bound reversibly to α2u-globulin with a dissociation constant of approximately 10^{-7} M. Additional studies established that binding of D-limonene-1,2-epoxide to α2u-globulin reduced the lysosomal degradation of the protein relative to the native protein. Specifically, the rate of degradation by renal cortical lysosomal lysates decreased by about 30%, and this change was specific to α2u-globulin because the lysosomal degradation of other proteins such as albumin was not altered by the presence of the epoxide and there were no changes in lysosomal cathepsin activity in D-limonene-treated rat kidneys.

Although the ligand–protein complex can be isolated from the kidney in vivo, one part of the mechanism that is not defined is where the interaction between D-limonene-1,2-epoxide and α2u-globulin occurs. However, given that the complex appears to be localized in the renal phagolysosomal compartment, it is likely that the complex does not form in the kidney. Rather, the interaction probably occurs outside the kidney (in liver or blood), and following glomerular filtration, the complex enters the proximal tubule cells by endocytosis and accumulates in the phagolysosomes after lysosomal fusion.

Collectively, the data summarized thus far indicate that formation of D-limonene-1,2-epoxide and its ability to bind to

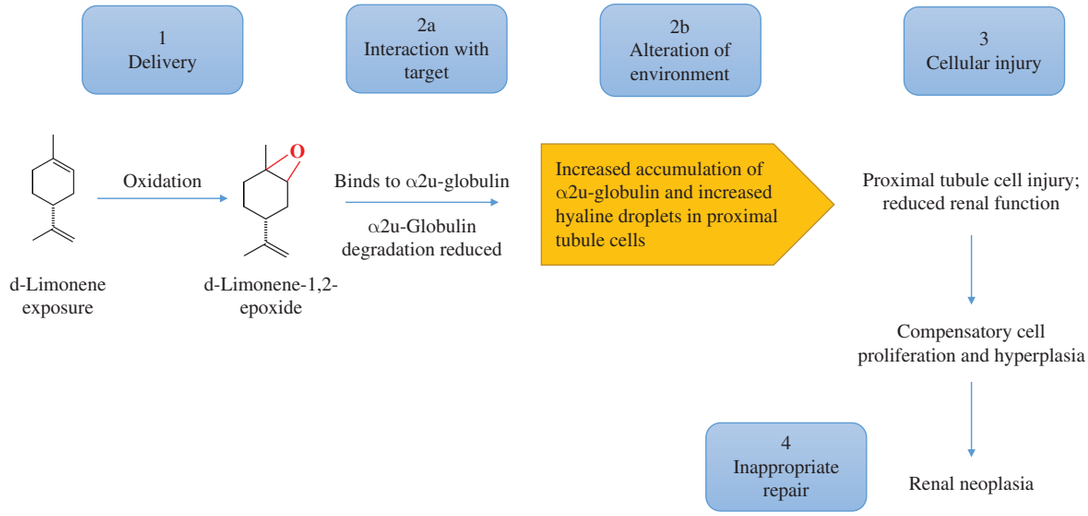

Figure 3-35. *The mode of action for D-limonene-induced hyaline droplet nephropathy and renal carcinogenesis.* The key events in the mechanism of action by which D-limonene, a naturally occurring terpene (oil of orange), causes kidney cancer in male rats. The mechanism requires biotransformation to D-limonene-1,2-epoxide which binds reversibly to α2u-globulin in male rats, and the interaction with the protein reduces its normal degradation in renal lysosomes leading to marked accumulation of the protein and histological evidence of hyaline droplets. With chronic exposure, there is sustained injury in the proximal tubules, with compensatory cell proliferation, which ultimately increases the risk of renal cancer. This syndrome is unique to male rats because only male rats synthesize α2u-globulin, and the renal syndrome described only occurs with this male rat-specific protein. In summarizing this mechanism, the components of the critical events involved in mechanisms of toxicity (Fig. 3-1) are also shown to illustrate how every step discussed in the chapter can be represented by the example of hyaline droplet nephropathy (also called α2u-globulin nephropathy).

α2u-globulin are key events that lead to the accumulation of protein and initiate D-limonene-induced renal toxicity (Fig. 3-35). However, as the duration of treatment continues, the lysosomes become enlarged, engorged with protein and polyangular in shape with ensuing cytotoxicity presenting as single cell necrosis in the P2 segment of the proximal tubule. Thus, necrosis is likely to be the mechanism of cell death, but it is unclear as to how an engorged lysosomal compartment is cytotoxic. Commensurate with cell necrosis, there is a dose- and time-dependent loss in renal function. Renal functional changes observed include reduced uptake of organic anions, cations, and amino acids, a mild proteinuria and increased levels of renal toxicity biomarkers, particularly glutathione *S*-transferases (α and μ isoforms), have been reported. In response to cell death and functional changes, there is cell proliferation in the kidney, most notably in the P2 segment of the proximal tubules, the site of the protein accumulation. With continued treatment, the cell proliferation continues, but does not fully restore renal function. The sustained increase in renal cell proliferation is believed to exert a promotional influence on the kidney, such that sustained cell turnover is mechanistically linked to the development of renal tubular tumors. Thus, α2u-globulin nephropathy begins as protein accumulation, but represents a continuum of changes that ultimately progress to renal tumors. The major events in this continuum are summarized in Fig. 3-35, with application and illustration of the four-component framework outlined for mechanisms of toxicity developed in this chapter (Lehman-McKeeman, 2018).

CONCLUSIONS

Mechanisms of Toxicity and Adverse Outcome Pathways

A "real world" application of mechanisms of toxicity is the development of adverse outcome pathways (AOPs) to inform and facilitate toxicity testing and hazard identification. Basically, an AOP is a systematic approach that organizes and integrates a series of biological events such as those described throughout this chapter that are likely to lead to an adverse outcome. AOPs place emphasis on the key events, often called molecular initiating events that link a causal chain of endpoints to the final toxicity. These events are also organized into biological levels ranging from macromolecular effects to those observed at the cellular, organ, and organism level (Burden et al., 2015; Wittwehr et al., 2017). Overall, an AOP is a general pathway that is somewhat similar to that outlined at the beginning of this chapter (Fig. 3-1), and the molecular initiating events include many of the fundamental mechanistic effects outlined in this chapter. However, the AOP approach provides for a systematic integration of existing knowledge on chemical mode of action, starting with a molecular initiating event and continuing through numerous key events that culminate in a toxic effect (Fig. 3-36). Additionally, integration of mechanisms of toxicity into AOPs may be beneficial for prioritizing compounds for more defined toxicity testing in animals or as a stand-alone system for predicting toxicity. Currently, the proposed applications and utility of AOPs focus on their potential to (1) advance the use of in vitro, nonanimal methods in safety evaluation; (2) extrapolate across species, with emphasis on increasing confidence of human safety; and (3) identify potential mechanism-based biomarkers of toxicity. While these approaches may bring more integrated systems biology approaches to assessing mechanisms of toxicity, the tools must be able to broadly integrate all aspects of toxic mechanisms presented in this chapter. That is, a useful tool will have to encompass the broad aspects of chemical disposition and biotransformation, and inform quantitative relationships describing when cellular protective mechanisms are overwhelmed thereby initiating or propagating the chain of key events that lead to toxicity. The application of AOPs in mechanistic and predictive toxicology is an emerging area, but it is not yet clear whether, and if so how, these approaches will advance the application of mechanism of toxicity into hazard identification and risk assessment.

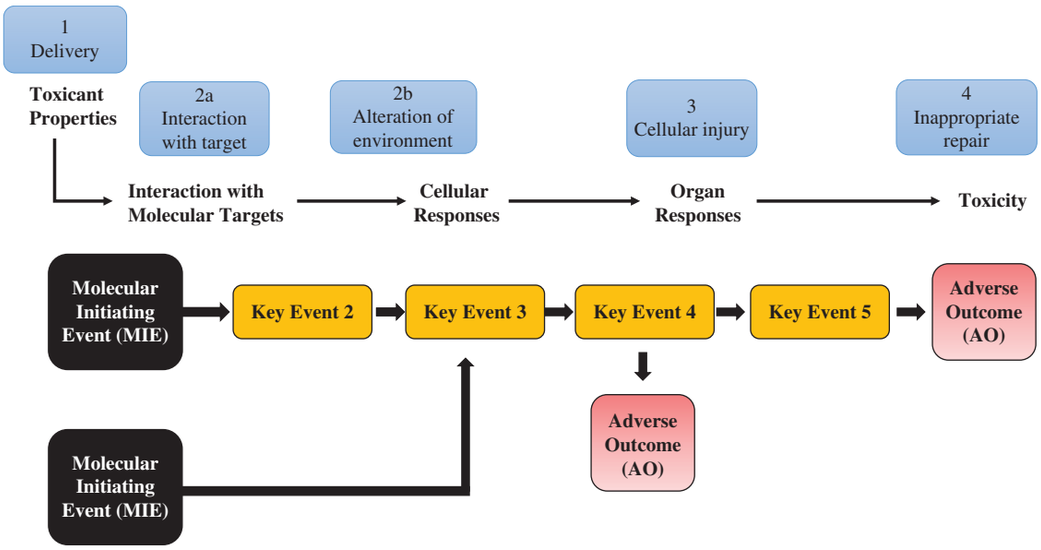

Figure 3-36. *Overview of adverse outcome pathways.* Adverse outcome pathways (AOPs) are a systematic organization of events that can lead to a toxic effect. Key events are those that are causally related to the final toxicity, and as illustrated can be organized into those observed at the molecular, cellular, and organ levels. Overall, an AOP is very similar to that outlined in Fig. 3-1, as highlighted in the figure.

The Importance of Mechanisms of Toxicity

In this chapter, a simplified scheme has been used as an overview of the development of toxicity (Fig. 3-1 A and B), and the potential events that follow toxicant exposure and contribute to toxicity have been presented. In completing the chapter, it should be readily apparent that the route to toxicity can be considerably diverse and complicated, as described by the many examples throughout the chapter and by the comprehensive case study of D-limonene. This chapter is a compendium of the fundamental processes that determine toxicity and provides the foundation for more in-depth understanding of specific toxicities that are discussed in detail throughout other chapters. The basic concepts of any toxic mechanism are essential to identifying key events associated with toxic outcome and inform species differences in toxicity, with particular emphasis on informing the human relevance of toxicity. Moreover, understanding mechanisms of toxicity provides opportunities and direction to identifying biomarkers of toxicity, tools to predict toxicity, and potential ways to mitigate adverse effects.

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REFERENCES

Aihara E, Engevik KA, Montrose MH. Trefoil factor peptides and gastrointestinal function. *Annu Rev Physiol*. 2017;79:357–380.

Al Jishi T, Sergi C. Current perspective of diethylstilbestrol (DES) exposure in mothers and offspring. *Reprod Toxicol*. 2017;71:71–77

Anders MW. Glutathione-dependent bioactivation of haloalkanes and haloalkenes. *Drug Metab Rev*. 2004;36:583–594.

Atherton PJ, Greenhaff PL, Phillips SM, et al. Control of skeletal muscle atrophy in response to disuse: clinical/preclinical contentions and fallacies of evidence. *Am J Physiol Endocrinol Metab*. 2016;311:E594–E604.

Bailey CP, Connor M. Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol*. 2005;5:60–68.

Bandiera S, Pfeffer S, Baumert TF, et al. miR-122—a key factor and therapeutic target in liver disease. *J Hepatol*. 2015;62:448–457.

Basu S. Isoprostanes: novel bioactive products of lipid peroxidation. *Free Radic Res*. 2004;38:105–122.

Batchelor E, Loewer A, Lahav G. The ups and downs of p53: understanding protein dynamics in single cells. *Nat Rev Cancer*. 2009;9:371–377.

Bedford L, Lowe J, Dick LR, Mayer RJ, Brownell JE. Ubiquitin-like protein conjugation and the ubiquitin–proteasome system as drug targets. *Nat Rev Drug Discov*. 2011;10:29–46.

Bessac BF, Jordt SE. Sensory detection and responses to toxic gases: mechanisms, health effects, and countermeasures. *Proc Am Thorac Soc*. 2010;7:269–277.

Bosmans F, Rash L, Zhu S, et al. Four novel tarantula toxins as selective modulators of voltage-gated sodium channel types. *Mol Pharmacol*. 2006;69:419–429.

Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. *J Hepatology*. 2013;59:583–594.

Bruck R, Wardi J, Aeed H, et al. Glycine modulates cytokine secretion, inhibits hepatic damage and improves survival in a model of endotoxemia in mice. *Liver Int*. 2003;23:276–82.

Burden N, Sewell F, Andersen ME, et al. Adverse outcome pathways can drive non-animal approaches for safety assessment. *J Appl Toxicol*. 2015;35:971–975.

Caito SW, Aschner M.Mitochondrial redox dysfunction and environmental exposures. *Antioxid Redox Signal*. 2015;23:578–95.

Calcerrada P, Peluffo G, Radi R. Nitric oxide-derived oxidants with a focus on peroxynitrite: molecular targets, cellular responses and therapeutic implications. *Curr Pharm Des*. 2011;17:3905–32.

Cao M, Onyango EO, Williams CR, et al. Novel synthetic pyridyl analogues of CDDO-imidazolide are useful new tools in cancer prevention. *Pharmacol Res*. 2015;100:135–47.

Carreras-Sureda A, Pihan P, Hetz C. The unfolded protein response: at the intersection between endoplasmic reticulum function and mitochondrial energetics. *Front Oncol*. 2017;7:55.

Chang Y-Y, Pei-Ching L, Hung-Hsin L, et al. Mutation spectra of RAS gene family in colorectal cancer. *Am J Surgery*. 2016;212:537–544.

Chartoumpekis DV, Wakabayashi N, Kensler TW. Keap1/Nrf2 pathway in the frontiers of cancer and non-cancer cell metabolism. *Biochem Soc Trans*. 2015;43:639–44.

Chen HY, Albertson TE, Olson KR. Treatment of drug-induced seizures. *Br J Clin Pharmacol*. 2016;81412–81419.
Cohen SD, Pumford NR, Khairallah EA, et al. Contemporary issues in toxicology: selective protein covalent binding and target organ toxicity. *Toxicol Appl Pharmacol*. 1997;143:1–2.
Cubeddu LX. Drug-induced inhibition and trafficking disruption of ion channels: pathogenesis of QT abnormalities and drug-induced fatal arrhythmias. *Curr Cardiol Rev*. 2016;12:141–154.
Day D, Hansen AR. Immune-related adverse events associated with immune checkpoint inhibitors. *BioDrugs*. 2016;30:571–584.
Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol*. 2003;43:309–334.
DiMeo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS sources in physiological and pathological conditions. *Oxid Med Cell Longev*. 2016;2016:1245049.
Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nat Chem Biol*. 2014;10:9–17.
Dobson RL, Motlagh S, Quijano M, et al. Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. *Toxicol Sci*. 2008;106:251–262.
Dolinoy DC, Das R, Weidman JR, Jirtle RL. Metastable epialleles, imprinting, and the fetal origins of adult diseases. *Pediatr Res*. 2007;61:30R–37R.
El-Benna J, Hurtado-Nedelec M, Marzaioli V, et al. Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Immunol Rev*. 2016;273:180–193.
Esteller M. Aberrant DNA methylation as a cancer-inducing mechanism. *Annu Rev Pharmacol Toxicol*. 2005;45:629–656.
Evans AM, Mustard KJ, Wyatt CN, et al. Does AMP-activated protein kinase couple inhibition of mitochondrial oxidative phosphorylation by hypoxia to calcium signaling in O_2-sensing cells? *J Biol Chem*. 2005;280:41504–41511.
Fabregat I, Moreno-Caceres J, Sanchez A, et al. TGF-beta signaling and liver disease. *FEBS J*. 2016;283:2219–2232.
Fausto N, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology*. 2006;43:S45–S53.
Finlay D, Teriete P, Vamos M, Cosford NDP, Vuori K. Inducing death in tumor cells: roles of the inhibitor of apoptosis proteins. *F1000Res*. 2017;6:587.
Forman HJ, Fukuto JM, Torres M. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol*. 2004;287:C246–C256.
Forman HJ, Zhang H, Rinna A. Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med*. 2009;30:1–12.
Fox JL, MacFarlane M. Targeting cell death signalling in cancer: minimising "collateral damage." *British J Cancer*. 2016;115:5–11.
Ganz T. Hepcidin and iron regulation, 10 years later. *Blood*. 2011;117:4425–4433.
Gao Y, Mutter-Rottmayer E, Zlatanou A, Vaziri C, Yang Y. Mechanisms of post-replication DNA repair. *Genes (Basel)*. 2017;8(2):pii: E64.
Garofalo M, Croce CM. MicroRNAs: master regulators as potential therapeutics in cancer. *Annu Rev Pharmacol Toxicol*. 2011;51:25–43.
Germain P, Staels B, Dacquet C, et al. Overview of nomenclature of nuclear receptors. *Pharm Rev*. 2006;58:685–704.
Giordano CN, Yew YW, Spivak G, Lim HW. Understanding photodermatoses associated with defective DNA repair: syndrome with cancer predisposition. *J Am Acad Dermatol*. 2016;75:855–870.
Gonzalez FJ. Nuclear receptor control of enterohepatic circulation. *Compr Physiol*. 2012;2:2811–2828.
Gonzalez FJ, Shah YM. PPARalpha: mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. *Toxicology*. 2008;246:2–8.
Gonzalez FJ, Yu AM. Cytochrome P450 and xenobiotic receptor humanized mice. *Annu Rev Pharmacol Toxicol*. 2006;46:41–64.
Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. *Nature*. 2009;458:1127–1130.
Grimm C, Hermann DM, Bogdanova A, et al. Neuroprotection by hypoxic preconditioning: HIF-1 and erythropoietin protect from retinal degeneration. *Semin Cell Dev Biol*. 2005;16:531–538.
Harada H. Hypoxia-inducible factor 1–mediated characteristic features of cancer cells for tumor radioresistance. *J Rad Res*. 2016;57 (S1): i99–i105.
Harb R, Xie G, Lutzko C, et al. Bone marrow progenitor cells repair rat hepatic sinusoidal endothelial cells after liver injury. *Gastroenterology*. 2009;137:704–712.
Hardie DG, Hawley SA, Scott JW. AMP-activated protein kinase—development of the energy sensor concept. *J Physiol*. 2006;574:7–15.
Hawley BR, Lu W-T, Wilczynska A, Bushell M. The emerging role of RNAs in DNA repair. *Cell Death Differ*. 2017;24:580–587.
Hediger MA, Clemencon B, Burrier RE, Bruford EA. The ABCs of membrane transporters in health and disease (SLC series): introduction. *Mol Aspects Med*. 2013;34:95–107.
Higuchi M, Yoshikawa Y, Orino K, Watanabe K. Effect of diquat-induced oxidative stress on iron metabolism in male Fischer-344 rats. *Biometals*. 2011;24:1123–1131.
Hosokawa N, Hara T, Kaizuka T, et al. Nutrient-dependent mTORC1 association with the ULK-1–Atg13–FIP200 complex required for autophagy. *Mol Biol Cell*. 2009;20:1981–1991.
Huang W, Ma K, Zhang J, et al. Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Science*. 2006;312:233–236.
Hubbard TD, Murray IA, Perdew GH. Indole and tryptophan metabolism: endogenous and dietary routes to Ah receptor activation. *Drug Metab Dispo*. 2015;43:1522–1535.
Hurst S, Hoek J, Sheu SS.Mitochondrial Ca2+ and regulation of the permeability transition pore. *J Bioenerg Biomembr*. 2017;49:27–47.
Hussain SP, Schwank J, Staib F, et al. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*. 2007;26:2166–2176.
Jaeschke H, Bajt ML. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol Sci*. 2006;89:31–41.
Joerger AC, Fersht AR. The p53 pathway: origins, activation in cancer and emerging therapeutic approaches. *Annu Rev Biochem*. 2016;85:375–404.
Jonckheere N, Vasseur R, Van Seuningen I. The cornerstone K-RAS mutation in pancreatic adenocarcinoma: from cell signaling network, target genes, biological processes to therapeutic targeting. *Crit Rev Oncol Hematol*. 2017;111:7–19.
Ju C, Colgan SP, Eltzschig HK. Hypoxia-inducible factors as molecular targets for liver diseases. *J Mol Med*. 2016;94:613–27
Juarez JC, Manuia M, Burnett ME, et al. Superoxide dismutase 1 (SOD1) is essential for H2O2-mediated oxidation and inactivation of phosphatases in growth factor signaling. *Proc Natl Acad Sci U S A*. 2008;105:7147–7152.
Juntilla MR, Li S-P, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB J*. 2008;22:954–965.
Kantorow M, Lee W, Chauss D. Focus on molecules: methionine sulfoxide reductase A. *Exp Eye Res*. 2012;100:110–111.
Karin M. Nuclear factor-κB in cancer development and progression. *Nature*. 2006;441:431–436.
Klaassen CD, Reisman SA. Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. *Toxicol Appl Pharmacol*. 2010;244:57–65.
Kriegenburg F, Poulsen EG, Koch A, et al. Redox control of the ubiquitin-proteasome system: from molecular mechanisms to functional significance. *Antioxid Redox Signal*. 2011;15:2265–99.
Kullack-Ublick GA, Andrade RJ, Merz M, et al. Drug-induced liver injury: recent advances in diagnosis and risk assessment. *Gut*. 2017;66(6):1154–1164.
Lehman-McKeeman LD. α2u-Globulin nephropathy. In: McQueen C, ed. *Comprehensive Toxicology*. 3rd ed. Vol 14. Amsterdam: Elsevier; 2018:436–451.
Lelli JL, Becks LL, Dabrowska MI, Hinshaw DB. ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. *Free Radic Biol Med*. 1998;25:694–702.
Leung L, Kalgutkar AS, Obach RS. Metabolic activation in drug-induced liver injury. *Drug Metab Rev*. 2012;44:18–33.
Li X, Heyer WD. Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res*. 2008;18:99–113.

Loboda A, Sobczak M, Jozkowicz A, Dulak J. TGFβ1/Smads and miR-21 in renal fibrosis and inflammation. *Mediators Inflamm*. 2016; 2016:8319283.

Loeys BL, Schwarze U, Holm T, et al. Aneurysm syndromes caused by mutations in the TGF-beta receptor. *N Engl J Med*. 2006;355(8): 788–798.

Maeda K. Organic anion transporting polypeptide (OATP)1B1 and OATP1B3 as important regulators of the pharmacokinetics of substrate drugs. *Biol Pharm Bull*. 2015;38:155–168.

Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol*. 2011;54:795–809.

Maxwell P, Salnikow K. HIF-1, an oxygen and metal responsive transcription factor. *Cancer Biol Ther*. 2004;3:29–35.

Mehendale HM. Tissue repair: an important determinant of final outcome of toxicant-induced injury. *Toxicol Pathol*. 2005;33:41–51.

Menzies FM, Fleming A, Caricasole A, et al. Autophagy and neurodegeneration: pathogenic mechanisms and therapeutic opportunities. *Neuron*. 2017;93:1015–1034.

Michalopoulos GK. Principles of liver regeneration and growth homeostasis. *Comp Physiol*. 2013;3:48–513.

Molitoris JK, McColl KS, Distelhorst CW. Glucocorticoid-mediated repression of the oncogenic microRNA cluster miR-17~92 contributes to the induction of Bim and initiation of apoptosis. *Mol Endocrinol*. 2011;25:409–420.

Moskovitz J. Methionine sulfoxide reductases: ubiquitous enzymes involved in antioxidant defense, protein regulation, and prevention of aging-associated diseases. *Biochim Biophys Acta*. 2005;1703:213–219.

Murakami Y, Kawada N. MicroRNAs in hepatic pathophysiology. *Hepatology Res*. 2017;47:60–69.

Narahashi T. Nerve membrane Na^+ channels as targets of insecticides. *Trends Pharmacol Sci*. 1992;13:236–241.

Niki E. Biomarkers of lipid peroxidation in clinical material. *Biochim Biophys Acta*. 2014;1840:809–817.

Nolan JP. The role of intestinal endotoxin in liver injury: a long and evolving history. *Hepatology*. 2010;52:1829–1835.

Orrenius S. Mitochondrial regulation of apoptotic cell death. *Toxicol Lett*. 2004;149:19–23.

Orrenius S, Gogvadze V, Zhivotovsky B. Calcium and mitochondria in the regulation of cell death. *Biochem Biophys Res Commun*. 2015;460: 72–81.

Orrenius S, Nicotera P, Zhivotovsky B. Cell death mechanisms and their implications in toxicology. *Toxicol Sci*. 2011;119:3–19.

Parkinson OT, Teitelbaum AM, Whittington D, et al. Species differences in microsomal oxidation and glucuronidation of 4-ipomeanol: relationship to target organ toxicity. *Drug Metab Dispos*. 2016;44:1598–1602.

Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. *Nature*. 2015;517:311–320.

Pessayre D, Fromenty B, Berson A, et al. Central role of mitochondria in drug-induced liver injury. *Drug Metab Rev*. 2012;44:34–87.

Peters JM, Shah YM, Gonzalez FJ. The role of peroxisome proliferator-activated receptors in carcinogenesis and chemoprevention. *Nat Rev Cancer*. 2012;12:181–195.

Petibone DM, Majeed W, Casciano DA. Autophagy function and its relationship to pathology, clinical applications drug metabolism and toxicity. *J Appl Toxicol*. 2017;37:23–37.

Pogribny IP, Muskhelishvili L, Tryndyak VP, Beland FA. The role of epigenetic events in genotoxic hepatocarcinogenesis induced by 2-acetylaminofluorene. *Mutat Res*. 2011;722:106–113.

Pugliese A, Beltramo T, Torre D. Reye's and Reye's-like syndromes. *Cell Biochem Funct*. 2008;26:741–746.

Rabinowitz JD, White E. Autophagy and metabolism. *Science*. 2010;330:1344–1348.

Ray PD, Huang B-W, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal*. 2012;24: 981–990.

Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med*. 2005;38:1543–1552.

Roderburg C, Urban GW, Bettermann K, et al. Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. *Hepatology*. 2011;53:209–218.

Rodrigues AD, Lai Y, Cvijic ME, et al. Drug-induced perturbations of the bile acid pool, cholestasis, and hepatotoxicity: mechanistic considerations beyond direct inhibition of the bile salt export pump. *Drug Metab Dispos*. 2014;42:566–674.

Roskoski R Jr. RAF protein-serine/threonine kinases: structure and regulation. *Biochem Biophys Res Commun*. 2010;399:313–317.

Saurat JH, Kaya G, Saxer-Sekulic N, et al. The cutaneous lesions of dioxin exposure: lessons from the poisoning of Victor Yushchenko. *Toxicol Sci*. 2012;125:310–317.

Schinkel AH. P-glycoprotein, a gatekeeper in the blood–brain barrier. *Adv Drug Deliv Rev*. 1999;36:179–194.

Schopf FH, Biebl MM, Buchner J. The HSP90 chaperone machinery. *Nature Rev Mol Cell Biol*. 2017;18:345–360.

Seki T, Ishimoto T, Sakurai T, et al. Increased excretion of urinary 20-HETE in rats with cyclosporine-induced nephrotoxicity. *J Pharmacol Sci*. 2005;97:132–137.

Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*. 2006;441:424–430.

Shin S, Kaestner KH. The origin, biology, and therapeutic potential of facultative adult hepatic progenitor cells. *Curr Top Dev Biol*. 2014;107: 269–92.

Sies H. Role of metabolic H_2O_2 generation redox signaling and oxidative stress. *J Biol Chem*. 2014;289:8735–8741.

Sies H, Berndt C, Jones DP. Oxidative stress. *Annu Rev Biochem*. 2017;86:715–748.

Stachlewitz RF, Seabra V, Bradford B, et al. Glycine and uridine prevent D-galactosamine hepatoxicity in the rat: role of the Kupffer cells. *Hepatology*. 1999;29:737–745.

Stephens TD, Bunde CJ, Fillmore BJ. Mechanism of action in thalidomide teratogenesis. *Biochem Pharmacol*. 2000;59:1489–1499.

Sullivan A, Gibson A, Park BK, Naisbitt DJ. Are drug metabolites able to cause T-cell-mediated hypersensitivity reactions? *Expert Opin Drug Metab Toxicol*. 2015;11:357–366.

Suzuki T, Kamiya H. Mutations induced by 8-hydroxyguanine (8-oxo-7,8-dihydroguanine), a representative oxidized base, in mammalian cells. *Genes Environ*. 2016;39:2. eCollection 2017.

Tam A, Wadsworth S, Dorscheid D, Man SF, Sin DD. The airway epithelium: more than just a structural barrier. *Ther Adv Respir Dis*. 2011;5: 255–273.

Tatti R, Haley MS, Swanson OK, Tselha T, Maffei A.Neurophysiology and regulation of the balance between excitation and inhibition in neocortical circuits. *Biol Psychiatry*. 2017;81:821–831.

Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastorenterol Hepatol*. 2017;14(7):397–411.

Tummers B, Green DR. Caspase-8: regulating life and death. *Immunol Rev*. 2017;277:76–89.

Uetrecht J, Naisbitt DJ. Idiosyncratic adverse drug reactions: current concepts. *Pharmacol Rev*. 2013;65:779–808.

Vedove CD, Del Giglio M, Schena D, Girolomoni G. Drug-induced lupus erythematosus. *Arch Dermatol Res*. 2009;301:99–105.

Walensky LD, Gavathiotis E. BAX unleashed: the biochemical transformation of an inactive cytosolic monomer into a toxic mitochondrial pore. *Trends Biochem Sci*. 2011;36:642–652.

Wallace KB. Mitochondrial off targets of drug therapy. *Trends Pharmacol Sci*. 2008;29:361–366.

Wang Y, Taniguchi T. MicroRNAs and DNA damage response: implications for cancer therapy. *Cell Cycle*. 2013;12:32–42.

Weinlich R, Oberst A, Beere HM, Green DR. Necroptosis in development, inflammation and disease. *Nat Rev Mol Cell Biol*. 2017;18: 127–136.

Wittwehr C, Aladjov H, Ankley G, et al. How adverse outcome pathways can aid the development and use of computational prediction models for regulatory toxicology. *Toxicol Sci*. 2017;155:326–336.

Wogan GN, Kensler TW, Groopman JD. Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A

review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2012;29:249–257.

Wong KM, Babetto E, Beirowski B. Axon degeneration: make the Schwann cell great again. *Neural Regen Res*. 2017;12:518–524.

Xie Y, Hou W, Song X, et al. Ferroptosis: process and function. *Cell Death Differ*. 2016;23:369–379.

Yamashina S, Ikejima K, Enomoto N, et al. Glycine as a therapeutic immune-nutrient for alcoholic liver disease. *Alcohol Clin Exp Res*. 2005;29:162S–165S.

Yokoi T, Nakajima M. Toxicological implications of modulation of gene expression by microRNAs. *Toxicol Sci*. 2011;123:1–14.

Yokoi T, Nakajima M. microRNAs as mediators of drug toxicity. *Annu Rev Pharmacol Toxicol*. 2013;53:377–400.

Yu R, Lai Y, Hartwell HJ, Moeller BC, et al. Formation, accumulation and hydrolysis of endogenous and exogenous formaldehyde-induced DNA damage. *Toxicol Sci*. 2015;146:170–182.

Yuan JM, Butler LM, Stepanov I, Hecht SS. Urinary tobacco smoke-constituent biomarkers for assessing risk of lung cancer. *Cancer Res*. 2014;74:401–11.

Zhivotovsky B, Orrenius S. Current concepts in cell death. *Curr Protoc Cell Biol*. 2001;chap 18:unit 18.1.

Zhou W, Yuan J. Necroptosis in health and diseases. *Semin Cell Dev Biol*. 2014;35:14–23.

Zou YX-Z, Liu T, Gong Z-C, et al. MicroRNAs-mediated epithelial-mesenchymal transition in fibrotic diseases. *Eur J Pharmacol*. 2017;796:190–206.

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chapter 4

Risk Assessment

Elaine M. Faustman

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- Assessing Toxicity of Chemicals: Approaches
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Acknowledgments

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INTRODUCTION AND HISTORICAL CONTEXT

In the 1970s U.S. Congress established a basic plan for environmental laws that authorized regulatory actions to protect public health and the environment. These science-based actions provided the foundation for environmental and human health risk assessment. Recent updates and revisions of these laws including the 2016 Frank R. Lautenberg Chemical Safety from the 21st Century Act, which modified the 1976 Toxic Substance Control Act, have reinforced the need for toxicology evaluation. Toxicological research and toxicity testing constitute the scientific core of risk assessment, which is used for evaluating potential adverse health impacts from chemical exposures. Considerations for risk evaluation were not new, since for decades the American Conference of Governmental Industrial Hygienists (ACGIH) set threshold limit values for occupational exposures and the U.S. Food and Drug Administration (FDA) established acceptable daily intakes (ADIs) for pesticide residues and food additives. In 1958, the U.S. Congress instructed the FDA in the Delaney Clause to prohibit the addition of all substances found to cause cancer in animals or humans to the food supply. Pragmatically, this policy allowed food sources that had nondetectable levels of these additives to be declared "safe." As advances in analytical chemistry revealed that "nondetects" were not equivalent to "not present," regulatory agencies were forced to develop "tolerance levels" and "acceptable risk levels." Risk assessment methodologies blossomed in the 1970s with the rising need to address these issues and to provide a common framework for considering human and ecological health effects (Albert, 1994).

Risk assessment was defined as a specific activity in 1983 in the National Research Council (NRC) publication *Risk Assessment in the Federal Government: Managing the Process* (*Red Book*). This book detailed steps for hazard identification, dose–response assessment, exposure analysis, and characterization of risks (NRC, 1983). These four basic framework steps now form the foundation framework for risk assessment and risk management approaches for many contexts such as children's health and ecological health, and they provide a consistent context for risk assessment frameworks across agencies nationally and worldwide. Risk assessment frameworks have been used by the Health and Safety Executive Risk Assessment Policy Unit of the United Kingdom (Great Britain and Health and Safety Executive, 1999) and by the World Health Organization (WHO) (NRC, 2009; WHO, 2010) and build from these common components. Fig. 4-1 illustrates a framework with bidirectional arrows demonstrating an ideal situation where mechanistic research feeds directly into risk assessments and critical data uncertainty drives research. The common elements of the basic risk assessment framework are shown in red highlight. Initially, attention in risk assessment was focused on cancer risks; in subsequent years, noncancer endpoints were examined with similar methods and risk assessments were performed across life stages and for ecosystems. Continuing advances in toxicology, epidemiology, exposure assessment, biologically based modeling of adverse responses, and modeling of variability and uncertainty, as well as the rapidly growing areas of "-omics" including genomics, transcriptomics, proteomics, metablomics, etc., have contributed to refinements in risk assessment. Interest continues in biomarkers of exposure, effect, and susceptibility to link exposure to disease prediction (NRC, 1989a, 1989b, 1992, 2017). Risk assessment links with the public policy

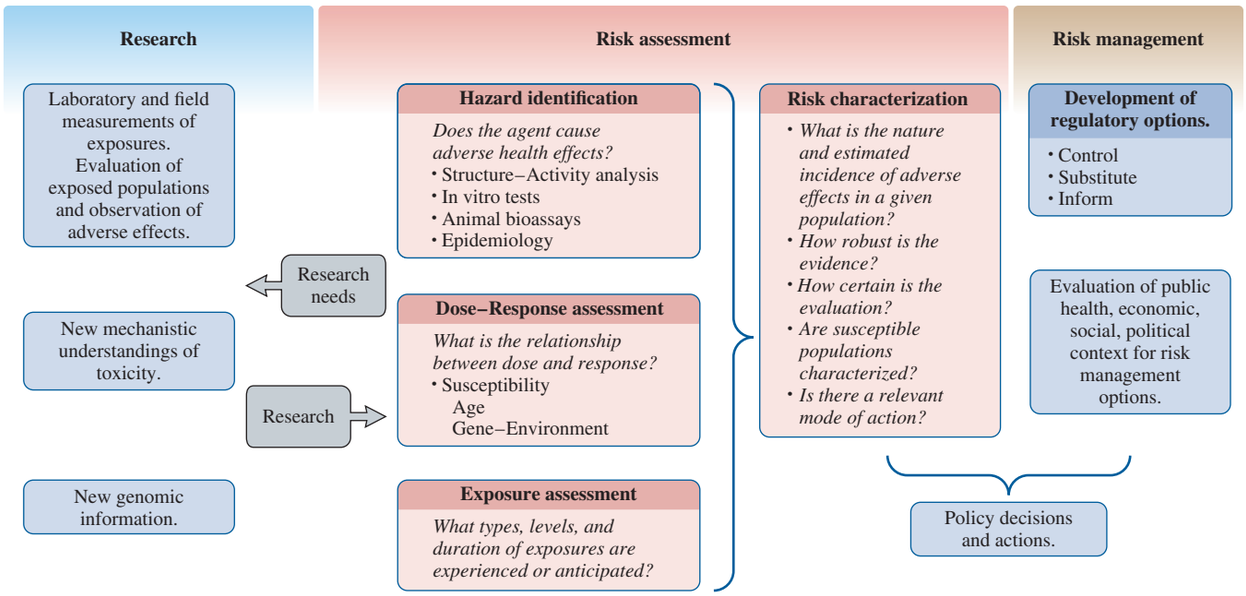

Figure 4-1. *Risk assessment/risk management framework.* This framework shows, under the red highlight, the four key steps of risk assessment: hazard identification, dose–response assessment, exposure assessment, and risk characterization. It shows an interactive, two-way process where research needs from the risk assessment process drive new research, and new research findings modify risk assessment outcomes. At the start of the risk assessment process, a risk problem is formulated and provides context for the assessment (Ecology RA). (Adapted from Calkins et al. [1980], NRC [1993, 1994a], Risk Commission [1997], and Gargas et al. [1999]; reprinted with permission from the National Academies Press, National Academy of Sciences.)

objectives from risk management often require extrapolations that go far beyond the observation of actual effects. Extrapolating data to address risks continues to generate controversy.

The National Academy of Sciences (NAS) 1994 report entitled *Science and Judgment in Risk Assessment* captured in its title the combination of qualitative and quantitative approaches essential for effective assessment of risks (NRC, 1994). The report discussed in detail the challenges and provided approaches for incorporating new scientific findings into the risk assessment process. It also highlighted ways to deal with uncertainty when insufficient scientific information was available. To address these challenges, the Presidential/Congressional Commission on Risk Assessment and Risk Management (Risk Commission, 1997) formulated a comprehensive framework that (1) placed each environmental problem or issue into public health and/or ecological context and (2) proactively called for engaging relevant stakeholders, often affected or potentially affected community groups, from the very beginning of the six-stage process shown in Fig. 4-2. This report emphasized that particular exposures and potential health effects must be evaluated across sources and exposure pathways and in light of multiple endpoints, not just one chemical, in one environmental medium (air, water, soil, food, products), for one health effect at a time. The single-chemical, single-exposure approach had been the general approach up to this time. The importance of defining the risk problem based on this initial input is critical to risk assessment. Problem formulation and scoping has now been defined as Phase 1 for risk-based decision making and has been outlined by the U.S. Environmental Protection Agency (EPA) (U.S. EPA, 1992a) and reinforced by *Science and Decisions*, the *Silver Book* (NRC, 2009).

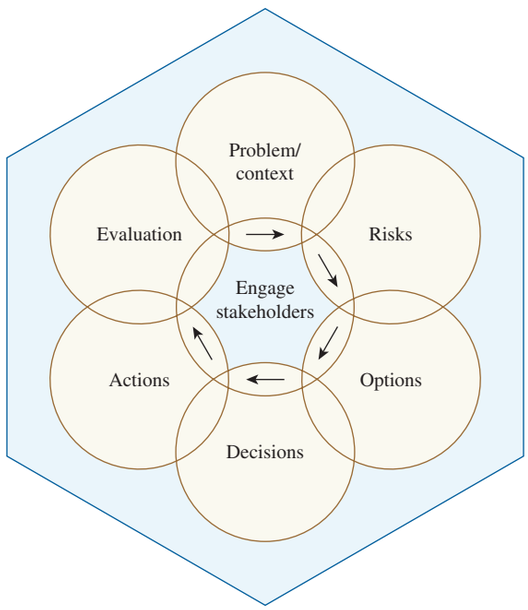

Figure 4-2. *Risk management framework for environmental health from the U.S. Commission on Risk Assessment and Risk Management.* The framework comprises six stages: (1) formulating the problem in a broad public health context; (2) analyzing the risks; (3) defining the options; (4) making risk reduction decisions; (5) implementing those actions; and (6) evaluating the effectiveness of the actions taken. Interactions with stakeholders are critical across all the stages and thus have been put at the center of the framework (Charnley and Omenn, 1997; Risk Commission, 1997).

DEFINITIONS

Risk assessment is the systematic scientific evaluation of potential adverse health effects resulting from human exposures to hazardous agents or situations (NRC, 1983, 1994; Omenn, 2000). Risk is defined as the probability of an adverse outcome based on the exposure and potency of the hazardous agent(s). Although historically there have been differences in how the term hazard has been used, international risk harmonization activities have allowed for

Table 4-1

Example Objectives of Risk Assessment

1. Protect human and ecological health
   - Toxic substances
2. Prioritize testing needs
3. Balance risks and benefits
   - Drugs
   - Pesticides
4. Set target levels of risk
   - Food contaminants
   - Water pollutants
5. Set priorities for program activities
   - Regulatory agencies
   - Manufacturers
   - Environmental/consumer organizations
6. Inform green chemistry, life cycle analysis, and clinical alternatives
7. Estimate residual risks and extent of risk reduction after steps are taken to reduce risks

current consensus on hazard as reference to intrinsic toxic properties, whereas exposure becomes an essential consideration along with hazard for risk. Risk assessment requires an integration of both qualitative and quantitative scientific information. For example, qualitative information about the overall evidence and nature of the endpoints and hazards is integrated with quantitative assessment of the exposures, host susceptibility factors, and the magnitude of the hazard. A description of the uncertainties and variability in the estimates is a significant part of risk characterization and an essential component of risk assessment. Analogous approaches are applied to ecological risks, as demonstrated by EPA's ecological risk assessment guidelines (U.S. EPA, 1992a, 2004). Example objectives of risk assessment are outlined in Table 4-1.

The phrase "characterization of risk" reflects the combination of qualitative and quantitative analysis. The term "hazard characterization" versus "hazard identification" has also been seen in both U.S. EPA (2012b) and WHO (1999) documents. This addition recognizes the difficulties in evaluating only hazard information without dose–response information. Unfortunately, many users equate risk assessment with quantitative risk assessment, generating a number for an overly precise risk estimate, while ignoring crucial information about the uncertainties of the risk assessment, mode of action (MOA), and type of effect across species or context.

Risk management refers to the process by which policy actions are chosen to control hazards identified in the risk assessment stage of the framework (Fig. 4-2). Risk managers consider scientific evidence and risk estimates—along with statutory, engineering, economic, social, and political factors—in evaluating alternative options and choosing among those options (Risk Commission, 1997).

Risk communication is the challenging process of making risk assessment and risk management information comprehensible to the public, community groups, lawyers, local elected officials, judges, business people, labor, environmentalists, etc. (NRC, 1989a, 1996, 2004; Morgan, 1993; Sandman, 1993; Fischhoff et al., 1996). Such communication requires an understanding of the level of risk response needed, how and which individuals and organizations should be informed, and what the audience hears versus what was intended as the risk communication message. Involving stakeholders in the initial problem formulation as well as in the translation of the bottom line has become an essential part of risk management. Part of this analytical–deliberative process is the crucial, too-often neglected or delayed need for considering the fears, perceptions, priorities, and proposed remedies of these stakeholders (Risk Commission, 1997; Drew et al., 2003, 2006; Judd et al., 2005; Kramer et al., 2006; Cullen et al., 2008). Underestimating potential public and media attitudes toward local polluters, other responsible parties, and relevant government agencies may lead to what has been labeled "the outrage factor" (Sandman, 1993), greatly influencing the communication process and the choices for risk management. Sometimes the decision makers and stakeholders simply want to know the "bottom line": whether a substance or a situation is "safe" or not. Others will be keenly interested in knowing how and why the risk estimates are uncertain and may be well prepared to challenge underlying assumptions about context and methodology. Stakeholders can also be part of a risk management solution (Judd et al., 2005; Drew et al., 2006). Perception of risk is further discussed at the end of this chapter.

DECISION MAKING

Risk management decisions are reached under diverse statutes in the United States. Table 4-2 lists examples of major environmental statutes and the year of initial enactment. Some statutes specify reliance on risk alone, whereas others require a balancing of risks and benefits of the product or activity. Risk assessments provide a valuable framework for priority setting within regulatory and health agencies, in the chemical development process within companies, and in resource allocation by environmental organizations. Similar approaches for risk assessment have been developed in many other countries and through such international organizations as the International Programme on Chemical Safety (IPCS) within the WHO. Within the IPCS, there are significant efforts toward global harmonization of risk assessment methodology (WHO, 2000, 2006; WHO, 2004). The *WHO Human Health Risk Assessment Toolkit: Chemical Hazards* is an example (WHO, 2010). Their goals include use of a common risk assessment framework with acceptance of different risk assessment approaches through this common understanding and agreement on basic principles of testing and evaluation. Table 4-2 lists the recent Occupational Safety and Health Administration (OSHA) Hazard Communication Standard 2015, which now uses the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). This standard represents an arduous yet highly meritorious effort to harmonize global labeling of chemicals. This GHS labeling has provided a common language for countries to use in trade and manufacturing that facilitates safety in the workplace and for chemicals in transit. An emphasis on hazard and not risk has been one of the more controversial issues across nations.

A major challenge for risk assessment, risk communication, and risk management is to work across disciplines to demonstrate the biological plausibility and clinical significance of the conclusions from epidemiologic studies, animal bioassays, short-term in vitro and in vivo tests, and structure–activity studies of chemicals thought to have potential adverse effects. Biomarkers of exposure, effect, or individual susceptibility can link the presence of a chemical in various environmental compartments to specific sites of potential action in target organs and to host health impacts and disease (NRC, 1989a, 1989b, 1989c, 1992, 2017; Adami et al., 2011). Mechanistic investigations of the actions of specific chemicals can help us penetrate the "black box" approach of simply counting tumors, for example, in exposed animals in routine bioassays. Greater appreciation of the mechanisms and extent of individual variation in susceptibility to adverse health impacts among humans can improve protection of susceptible populations and better relate

Table 4-2

Examples of Major Toxic Chemical Laws in the United States by Responsible Agency and Year of Initial Enactment

EPA	Air pollutants	Clean Air Act, 1970
	Water pollutants	Federal Water Pollution Control Act, 1948
	Drinking water	Safe Drinking Water Act, 1974
	Pesticides	Fungicides, Insecticides & Rodenticides Act (FIFRA), 1947; Food Quality Protection Act (FQPA), 1996
	Ocean dumping	Marine Protection, Research and Sanctuaries Act (MPRSA) or Ocean Dumping Act, 1972
	Toxic chemicals	Toxic Substances Control Act (TSCA), 1976; The Frank R. Lautenberg Chemical Safety for the 21st Century Act (2016)
	Hazardous wastes	Resource Conservation and Recovery Act (RCRA), 1976
	Abandoned hazardous wastes	Superfund (CERCLA), 1980
CEQ	Environmental impacts	National Environmental Policy Act (NEPA), 1969
OSHA	Workplace	Occupational Safety and Health (OSH) Act, 1970; Hazard Communication Standard (HCS) and Globally Harmonized Systems of Classification and Labelling of Chemicals (GHS, 2015).
FDA	Foods, drugs, and cosmetics	Food and Drugs Act, 1906; Food, Drugs and Cosmetics Act (FDC), 1938; FDA Modernization Act, 1997
CPSC	Dangerous consumer products	Consumer Product Safety Act (CPSA), 1972*
DOT	Transport of hazardous materials	Hazardous Materials Transportation Act (HMTA), 1975

Abbreviations: CEQ, Council for Environmental Quality (now Office of Environmental Policy); CPSC, Consumer Product Safety Commission; DOT, Department of Transportation; EPA, Environmental Protection Agency; FDA, Food and Drug Administration; OSHA, Occupational Safety and Health Administration.

**Note that numerous states have enacted reporting requirements related to toxic chemicals in children's consumer products. The Washington Children's Safe Product Act of 2008 is one example.*

findings in animals to the characterization of risk for humans. Identifying individual behavioral and social risk factors can be critically important both to the characterization of risk and to the reduction of risk.

HAZARD IDENTIFICATION

Assessing Toxicity of Chemicals—Introduction

Throughout this book, a common toxicology paradigm (Fig. 4-3) forms the basis for our toxicity assessments. For risk assessment this paradigm is a critical underpinning where delivery represents exposure and absorption, distribution, metabolism, and excretion (ADME) determining the amount, rate, form, distribution, and fate of the chemical. The key initial impacts of the chemical interacting with the target link the early cellular dysfunction with early biomarkers effect. Dysrepair is identified as endpoint impacts across levels of biological investigation with observations at the molecular, cellular, and tissue levels.

In order to assess the toxicity of chemicals, information from four example types of studies is used: structure–activity relationships (SAR), in vitro or short-term studies, in vivo animal bioassays, and human epidemiologic studies. In many cases, toxicity information for chemicals is limited. For example, in 1998, the EPA evaluated high production volume (HPV) chemicals (those produced in excess of one million pounds per year) to ascertain the availability of chemical hazard data. The EPA study found that for 43% of these HPV chemicals, there were no publicly available studies for any of the basic toxicity endpoints (U.S. EPA, 1998a). In response, the EPA established a voluntary program called the HPV Challenge Program (U.S. EPA, 2011b). Industry participants in this program committed to filling the data gaps for HPV chemicals. Since its inception, there have been over 2200 chemicals sponsored for additional testing. International efforts such as the Organization for Economic Cooperation and Development (OECD) and screening information data set (SIDS) program are also addressing data needs related to HPV chemicals, highlighted by the publication of their *Manual for Investigation of HPV Chemicals* (OECD, 2013).

Data requirements for specific chemicals can vary greatly by compound type, use, and applicable regulatory statutes. Introduced in 2003 and approved in 2006, the European Union released a regulatory framework for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (REACH, 2011). Under the REACH framework, since 2007, all stakeholders that sell or produce compounds in the European Union submit dossiers that include physical, chemical, and toxicological data as well as environmental fate and transport information to inform risk assessment studies for all chemicals in use in Europe. The REACH data is available via the European Chemicals Agency registered substances list (ECHA, 2017). Chemical safety assessments are submitted prior to approval in an approach similar to premanufacturing notices (PMNs) in the United States for the EPA (U.S. EPA, 2011c).

Table 4-3 shows requirements and costs for one example class of chemicals, pesticides (Stevens, 1997; U.S. EPA, 1998b, 2000b), in the United States (40 CFR 158.340). It also illustrates current international efforts to align these testing guidelines by listing examples of the harmonized 870 test guidelines (U.S. EPA, 2010a; Patlewicz et al., 2013). The emphasis in REACH is on non–in vivo animal tests. Also, REACH has utilized the international globally harmonized labeling laws for hazard identification (GHS). Some of the successes from REACH have stimulated new changes in the

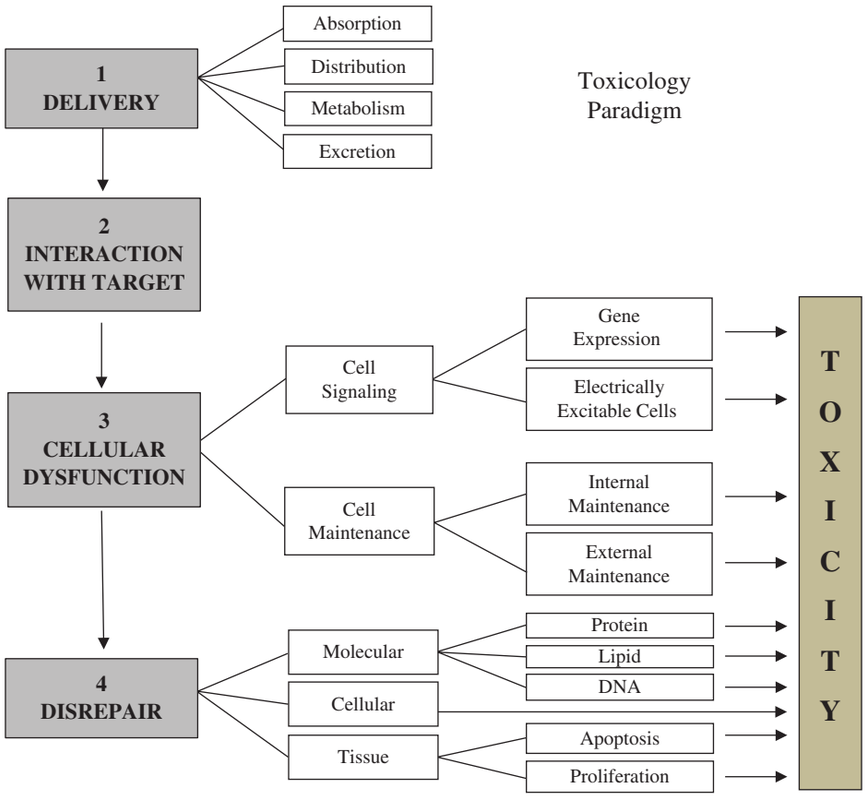

Figure 4-3. *Toxicology paradigm.* A common paradigm forms the basis for our toxicology assessments and subsequent translation for assessment of health risks. For risk assessment this paradigm is a critical underpinning where delivery represents exposure and absorption, distribution, metabolism, and elimination (ADME) determining the amount, the rate, the form and distribution of the chemical and its metabolites. The key initial impacts of the chemical interacting with the target links the early cellular dysfunction with early biomarkers effect. Disrepair is identified as endpoint which impacts across levels of biological investigation and observation at the molecular, cellular, and tissue levels.

United States via amendments for the Toxic Substances Control Act (TSCA). Recent release of the Integrated Chemical Environment (ICE) reference database provides a welcome integrated access to in vivo and in vitro data to support Tox21 activities (Bell et al., 2017).

Assessing Toxicity of Chemicals: Approaches

Structure–Activity Relationships

Given the cost of two to four million dollars and the 3 to 5 years required for testing a single chemical in a lifetime rodent carcinogenicity bioassay, initial decisions on whether to continue development of a chemical, to submit a premanufacture notice (PMN), or to require additional testing may be based largely on results from structure–activity relationships (SARs) and limited short-term assays.

A chemical's structure, solubility, stability, pH sensitivity, electrophilicity, volatility, and chemical reactivity can be important information for hazard identification. Historically, certain key molecular structures have provided regulators with some of the most readily available information on the basis of which to assess hazard potential. For example, 8 of the first 14 occupational carcinogens were regulated together by the OSHA as belonging to the aromatic amine chemical class. The EPA Office of Toxic Substances relies on SARs to meet deadlines to respond to PMN for new chemical manufacture under the Toxic Substances Control Act (TSCA). Structural alerts such as *N*-nitroso or aromatic amine groups, amino azo dye structures, or phenanthrene nuclei are clues to prioritize chemicals for additional evaluation as potential carcinogens. SAR information for specific noncancer health endpoints can be challenging. The database of known developmental toxicants limits SARs to a few chemical classes, including chemicals with structures related to those of valproic acid, retinoic acid, phthalate esters, and glycol ethers (NRC, 2000). More recently, -omic technologies have been used to supplement SAR relationship databases, as seen with the creation of the National Cancer Institute's (NCI) gene expression database (NCI, 2017) and the U.S. EPA Computational Toxicity Program Screening Database (ToxCast™) (U.S. EPA, 2017b).

SARs have been used for assessment of complex mixtures of structurally related compounds. Prominent applications have been the assessment of risks associated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), related chlorinated and brominated dibenzo-*p*-dioxins, dibenzofurans, and planar biphenyls, and chemicals generally present as mixtures in the environment. Toxicity equivalence factors (TEFs) are used to evaluate health risks associated with closely related chemicals. For the TCDD class, this is based on a common mechanism of aryl hydrocarbon (Ah) receptor induction (U.S. EPA, 1994a). The estimated toxicity of environmental mixtures containing these chemicals is calculated as the sum of the product of the concentration of each chemical multiplied by its TEF value. The WHO has organized efforts to reach international consensus on the TEFs used for polychlorinated biphenyls (PCBs),

Table 4-3

Example EPA/FIFRA Requirements for Hazard Evaluation of Pesticides

REVISED 870 GUIDELINE	TYPE OF TOXICITY STUDY	TEST SYSTEM	OBJECTIVE	APPROXIMATE COST/STUDY* (US$)
1100	Acute oral	Rats	Define toxic dose by ingestion	2,700
1200	Acute dermal	Rabbits	Define toxic dose by absorption through skin	2,000
1300	Acute inhalation	Rats	Define toxic dose by inhalation	6,800
2400	Ocular	Rabbits	Assess eye irritation/injury	2,000
2500	Skin irritation	Rabbits	Assess skin irritation/injury	130
2600	Sensitization	Guinea pigs	Assess allergic potential	4,000
6100–6855	Neurotoxicity†,‡	Hens/rats	Assess nervous system injury	34,000‡
5100–5915	Mutagenicity§	In vivo/in vitro	Determine genotoxic potential; screen for carcinogenicity	6,800
3050–3465	Range-finding§	Rats	Determine effects following repeated doses; set dose level	95,000
	Subacute (28- to 90-day*)	Mice		95,000
		Dogs	For longer studies	136,000
		Rabbits		101,000
		Rats	Identify target organs; set dose	258,000
		Mice	Levels for chronic studies	258,000
4200–4300	Carcinogenicity/ chronic toxicity	Rats	Determine potential to induce tumors; define dose–response relationships (lifetime)	1,900,000
		Mice		1,087,000
		Dogs	Determine long-term toxic effects (1 year)	543,000
3550–3800	Reproduction and teratogenicity	Rats	Determine potential to cause fetal abnormalities and effects on development, fertility, pregnancy, and development of offspring over at least two generations	686,000
		Rabbits		
7485	Toxicokinetics	Rats, mice	Determine and quantitate the metabolic fate of a pesticide	135,000
8600	Developmental neurotoxicity	Rats	Determine potential to cause adverse developmental outcomes following exposure during gestation and lactation on gross neurological behavioral abnormalities and function, motor activity, neuropathology and brain weight measurements throughout post-natal development and adulthood	700,000

**Indicates per assay cost using Consumer Price Index (CPI) conversion factors to determine 2010 value (oregonstate.edu/cla/polisci/sahr/sahr). Estimates were provided using guideline 81 series.*

†Required for organophosphate insecticides only.

‡Additional neurotoxicity tests 81-7, 81-8, 82-6, 82-7, and 83-6 have been added to requirements for certain materials and can include tests such as functional observational battery, motor activity, developmental landmarks, and learning and memory assessments (Sette, 1991). Costs listed for this type of study are only those for the initial study, not additional testing.

§Range-finding studies are not required, but provide justification for setting dose levels in required studies. EPA-required studies can include reverse mutation assays in Salmonella, forward mutation assays in mammalian cells—for example, Chinese hamster ovary cells and mouse lymphoma L5178Y (the locus cells)—and in vivo cytogenetics (Dearfield, 1990).

SOURCES: Adapted from Stevens (1997), and updated with revised EPA 870 guideline information (U.S. EPA, 2010a, 2017). For details on changes in the health effects test guidelines reflective of the harmonization of the toxicology guidelines between the Office of Pollution Prevention and Toxics (OPPT) and the Office of Pesticide Programs (OPP), within the EPA and with the Organization for Economic Cooperation and Development (OECD) guidelines, see EPA (U.S. EPA, 2010a, 2017) (https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-870-health-effects-test-guidelines).

polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) for both humans and wildlife, and has updated its values and published the supporting database (Van den Berg et al., 1998, 2006; Haws et al., 2006). Under the auspices of the WHO, the dioxin-like PCB congeners have been assigned TEFs reflecting their toxicity relative to TCDD, which itself has been assigned a TEF of 1.0.

Computerized SAR methods have, in general, given disappointing results in the U.S. National Toxicology Program (NTP) rodent carcinogenicity prediction challenges (Ashby and Tennant, 1994; Omenn et al., 1995; Benigni and Zito, 2004). More successful are the efforts of pharmaceutical companies using combinatorial chemistry and three-dimensional (3D) molecular modeling approaches to design ligands (new drugs) that can sterically fit into the "receptors of interest." However, for environmental pollutants where selective binding to specific receptors is rare, these applications of SAR have had limited success within risk assessment. Applications for endocrine disrupting chemicals and evaluation of their binding to hormone receptors have been a recent success from ToxCast, with a proposal for using receptor-binding assays for screening endocrine disruptor potential and as an acceptable approach without the additional need for assays such as the Hershberger assay (U.S. EPA, 2015). A renewed interest in quantitative SAR (QSAR) approaches has also resulted from the need to evaluate engineered nanomaterials where the tremendous number of unique new products has highlighted the necessity of using QSAR approaches to handle the avalanche of novel untested materials (Maynard et al., 2006; Liu et al., 2011; Nel et al., 2013).

Efforts within REACH have also emphasized the potential for use of SAR as similar chemicals are collectively evaluated using a concept of "read-across." Substances whose physicochemical, toxicological, and ecotoxicological properties are similar can be grouped as a "category" of substances when they have a common functional group, common precursor or breakdown product, or a common pattern of potency.

In Vitro and Short-Term Tests

The next level of biological information obtained within the hazard identification process includes assessment of the test chemical in in vitro or short-term tests, ranging from bacterial mutation assays performed entirely in vitro to more elaborate short-term tests, such as skin painting studies in mice or altered rat liver foci assays conducted in vivo. For example, EPA mutagenicity guidelines call for assessment of reverse mutations using the Ames *Salmonella typhimurium* assay; forward mutations using mammalian cells, mouse lymphoma L5178Y, Chinese hamster ovary, or Chinese hamster lung fibroblasts; and in vivo cytogenetics assessment (bone marrow metaphase analysis or micronucleus tests) (U.S. EPA,1986, 2005a). Chap. 8 discusses uses of these assays for identifying chemical carcinogens and Chap. 9 describes in detail various assays of genetic and mutagenic endpoints. Other assays evaluate specific health endpoints such as developmental toxicity (Chap. 21) (Faustman, 1988; Whittaker and Faustman, 1994; Brown et al., 1995; Lewandowski et al., 2000; NRC, 2000; Spielmann et al., 2006), reproductive toxicity (Chap. 10) (Gray, 1988; Harris et al., 1992; Shelby et al., 1993; Yu et al., 2009), neurotoxicity (Chap. 18) (Atterwill et al., 1992; Costa, 2000), and immunotoxicity (Chap. 12) (ICCVAM, 1999, 2011a). Less information is available on the extrapolation of these test results for noncancer risk assessment than for the mutagenicity or carcinogenicity endpoints; however, mechanistic information obtained in these systems has been applied to risk assessment (Abbott et al., 1992; U.S. EPA, 1994b; Leroux et al., 1996; NRC, 2000; Smith et al., 2016a, 2016b).

Overall, progress in developing and validating new in vitro assays has been episodic with recent encouraging efforts especially due to U.S. EPA's ToxCast program (Kavlock et al., 2012). In the late 1990s, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) of the NTP reinvigorated the validation process in the United States as a result of Public Law 103-43 and coordinated cross-agency issues relating to development, validation, acceptance, and national/international harmonization of toxicological test methods for use in risk assessments (ICCVAM, 2017). The committee has put forth recommendations for over 40 alternative safety testing methods using various short-term/in vitro assays such as the cell-free corrosivity test, and the mouse local lymph node assay for assessing chemical potential to elicit allergic reactions (ICCVAM, 1999, 2011a; NIEHS, 1999a, 1999b). In 2006, the committee released a document that reviewed in vitro acute toxicity methods.This review was led by the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (United States) and Interagency Coordinating Committee on the Validation of Alternative Methods (United States) (NIEHS, 2006). The European Centre on the Validation of Alternative Methods (ECVAM) is now known as the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM, available at https://eurl-ecvam.jrc.ec.europa.eu/).

EURL-ECVAM has been very active given the visibility of animal rights issues in the European Union, and this Center was originally formed to "support the development, validation, and acceptance of methods that could reduce, refine, or replace [3 Rs] the use of laboratory animals." Early successes of ECVAM are described in Hartung et al. (2003). However, a recent report for the Center for Alternatives for Animal Testing highlights the slow progress on full replacement of animal testing, especially for repeated dose toxicity testing, carcinogenicity, or reproductive toxicity testing (Adler et al., 2011; Hartung et al., 2011). It does highlight numerous successes for evaluating sensitization and toxicokinetics (TK). Most recent successes have been driven by the European Union–conceived free legislation for cosmetic testing. International efforts to reduce the number of animals required for chemical safety testing have been established between ICCVAM, Korea, Japan, Canada, and ECVAM (Table 4-4).

Table 4-4

Example Assays Used for Evaluation of Cosmetics: Multiple OECD Guideline Studies Reviewed and Designed for These Endpoints

OECD GUIDELINES	TYPES OF TOXICITY STUDY	TEST SYSTEM	OBJECTIVE
OECD 439	Irritation	Skin Ethic EpiSkin®	Skin
OECD 492	Irritation	MatTek Epi Ocular®	Eye
OECD 437	Irritation Opacity, Permeability	Bovine Cornea and Opacity Permeability (BCOP)	Eye Cornea
OECD 431	Corrosion	Skin Ethic EpiSkin®	Skin
OECD 435	Corrosion	Corrositex®	Skin

The validation and application of short-term assays are particularly important to risk assessment because such assays can be designed to provide information about mechanisms of effects, and, moreover, they are fast and inexpensive compared with lifetime bioassays (McGregor et al., 1999; Collins et al., 2008; NRC, 2017). Validation considerations for in vitro assays, like other kinds of tests, require determination of their sensitivity (e.g., ability to identify true carcinogens), specificity (e.g., ability to recognize noncarcinogens as noncarcinogens), and predictive value for the toxic endpoint under evaluation. However, the recent NRC publication proposes a series of modified validation considerations that can further define pathways to regulation acceptance (NRC, 2017). The societal costs of relying on such tests, with false positives (noncarcinogens classified as carcinogens) and false negatives (true carcinogens not detected), are the subject of a value-of-information model for testing in risk assessment and risk management (Lave and Omenn, 1986; Lave et al., 1988; Omenn and Lampen, 1988).

Efforts to improve our ability to utilize short-term tests for carcinogenicity prediction include increased attention to improving the mechanistic basis of short-term testing. Examples of this approach include the development and application of several knockout transgenic mouse models as shorter-term in vivo assays to identify carcinogens (Nebert and Duffy, 1997; Tennant et al., 1999). Specific assays for evaluating mechanisms such as the function of the estrogen receptor are an example where in vitro test methods are used to predict endocrine disruptor actions (ICCVAM, 2011b, 2012). The primary use of short-term tests in the United States continues to be for mechanistic evaluations with the hope of informing chemical-specific information or overall MOA. In that context, results from short-term assays have impacted risk assessments. For example, evidence of nonmutagenicity in both in vitro and in vivo short-term assays have played an essential role, allowing regulators to consider nonlinear cancer risk assessment paradigms for nongenotoxic carcinogens (U.S. EPA, 1999). Mechanistic information from short-term in vitro assays can also be used to extend the range of biological observations available for dose–response assessment. In addition, for developmental toxicity assessment, assay methods that acknowledge the highly conserved nature of developmental pathways across species have accelerated the use of a broader range of model organisms and assay approaches for noncancer risk assessments (NRC, 2000). *Toxicity Testing in the 21st Century* and *Toxicity Pathway-Based Risk Assessment* emphasized such approaches for using model organisms and tiered toxicity strategies for risk assessment (NRC, 2007a, 2010, 2017).

Animal Bioassays

Animal bioassays have been a key component of the hazard identification process. See example guidelines for organ/ system-specific endpoints (U.S. EPA, 1991, 1988, 1996a, 1998c). A basic premise of risk assessment is that chemicals that cause tumors in animals can cause tumors in humans. All human carcinogens that have been adequately tested in animals produce positive results in at least one animal model. Thus, "although this association cannot establish that all chemicals and mixtures that cause cancer in experimental animals also cause cancer in humans, nevertheless, in the absence of adequate data on humans, it is biologically plausible and prudent to regard chemicals and mixtures for which there is sufficient evidence of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans" (IARC, 2000)—a reflection of the "precautionary principle." The U.S. EPA cancer guidelines (U.S. EPA, 2005a) also assume relevance of animal bioassays unless lack of relevance for human assessment is specifically determined. In general, the most appropriate rodent bioassays are those that test exposure and biological pathways of most relevance to predicted or known human exposure pathways. Bioassays for reproductive and developmental toxicity and other noncancer endpoints have a similar rationale. The NTP serves as a resource for conducting, designing, and evaluating bioassays for cancer as well as noncancer evaluation. The NTP Office of Human Health Assessment and Translation serves as a resource to the public and regulatory agencies regarding the interpretation and assessment of adverse effects of chemicals (NTP, 2011b). The WHO International Agency for Research on Cancer (IARC) has evaluated over 900 agents "of which more than 400 have been identified as carcinogenic, probably carcinogenic or possibly carcinogenic to humans" (WHO, 2011b).

Consistent features in the design of standard cancer bioassays include testing in two species and both sexes, with 50 animals per dose group and near-lifetime exposure. Important choices include the strains of rats and mice, the number of doses, and dose levels (typically 90%, 50%, and 10–25% of the maximally tolerated dose [MTD]), and details of the required histopathology (number of organs to be examined, choice of interim sacrifice pathology, etc.). The NTP Web site lists details on study designs and protocols (NTP, 2011a, 2017a). Positive evidence of chemical carcinogenicity can include increases in number of tumors at a particular organ site, induction of rare tumors, earlier induction (shorter latency) of commonly observed tumors, and/or increases in the total number of observed tumors. The NTP has added a perinatal exposure phase that includes gestation (in utero exposure via placenta) and postnatal lactation (via the mother's milk) to more directly evaluate the significance of early-life exposures for cancer incidence (NTP, 2017b).

The cancer bioassay, originally designed for hazard identification, is frequently used to evaluate dose–response. The relatively limited number of evaluated doses and the use of high doses have caused issues for low-dose extrapolations and have limited the use of cancer bioassays as a "gold standard" for prediction of human carcinogenicity risk (McClain, 1994; Cohen, 1995; Risk Commission, 1997; Rodericks et al., 1997; Capen et al., 1999; Rice et al., 1999). Tumors may be increased only at the highest dose tested, which is usually at or near a dose that causes systemic toxicity (Ames and Gold, 1990). Second, even without toxicity, the high dose may trigger different events than do low-dose exposures and high doses can saturate important metabolism and elimination pathways.

Rats and mice give concordant positive or negative results in approximately 70% of bioassays, so it is unlikely that rodent/human concordance would be higher (Lave et al., 1988). Haseman and Lockhart (1993) concluded that most target sites in cancer bioassays showed a strong correlation (65%) between males and females—especially for forestomach, liver, and thyroid tumors—so they suggested, for efficiency, that bioassays could rely on a combination of male rats and female mice. Even when concordant, positive results are observed, there can still be large differences in potency, as observed in aflatoxin-induced tumors in rats and mice. In this example, an almost 100,000-fold difference in susceptibility to aflatoxin B_1 (AFB_1)–induced liver tumors is seen between the sensitive rat and trout species and the more resistant mouse strains. Genetic differences in the expression of cytochrome P450 and glutathione *S*-transferases explain most of these species differences and suggest that humans may be as sensitive to AFB_1-induced liver tumors as rats (Eaton and Gallagher, 1994; Eaton et al., 1995, 2001). These species differences have been supported by research results (Groopman and Kensler, 1999; Kensler et al., 2011) and have been extended within epidemiologic studies to demonstrate the interaction of hepatitis C infection with AFB_1 exposure to more fully explain elevated human liver cancer risks.

Table 4-5

Examples of Mechanistic Considerations for Carcinogens: Explanation for Special Cases of Rodent Bioassay Data Lacking Relevance for Human Risk Evaluation

SYSTEM	TARGET ORGAN	MECHANISM FOR SUSCEPTIBLE SPECIES	SPECIES DIFFERENCES	ILLUSTRATIVE CHEMICAL AGENTS
Urinary tract	Renal tumors in male rats	Chemicals bind to α_{2U} globulin, accumulation in target kidney cells, increased necrosis, increased regenerative hyperplasia, renal tubular calcification neoplasia	α_{2U}-Globulin male rat-specific low-molecular-weight protein not found in female rats, humans, mice, monkeys	Unleaded gasoline, 1,4-dichlorobenzene, D-limonene, isophorone, dimethyl-methyl phosphonate, perchloroethylene, pentachloroethane, hexachloroethane
	Bladder	Reactive hyperplasia from cytotoxic precipitated chemicals	Rodent exposure levels exceed solubility, not relevant for human exposure	Saccharin, melamine, nitrilotriacetic acid, fosetyl-A2
Gastric	Forestomach	Direct oral gavage, local cytotoxicity, hyperplasia	Rodent gavage treatment, exposure conditions not relevant for human exposure	BHA, propionic acid, ethyl acrylate
Endocrine	Thyroid gland tumors	Alteration in thyroid homeostasis, decreased thyroid hormone production, sustained increase in thyroid stimulating hormone (TSH), thyroid tumors	Lack of thyroid-binding protein in rodents versus humans, decreased $t_{1/2}$ for T_4, increased TSH levels in rodents	Ethylene bisdithiocarbamate, fungicides, amitrol, goitrogens, sulfamethazine
Respiratory	Rat lung	Overwhelming clearance mechanisms	High-dose effects seen with rodent models	Various particles, titanium dioxide

SOURCES: McClain (1994), Neumann and Olin (1995), Oberdorster (1995), Omenn et al. (1995), Risk Commission (1997), Rodericks et al. (1997), Da Rocha et al. (2014), and Yamada et al. (2017).

Lifetime bioassays have been enhanced with the collection of such additional mechanistic data and with the assessment of multiple noncancer endpoints. It is feasible and desirable to integrate such information together with data from mechanistically oriented short-term tests and biomarker and genetic studies in epidemiology (Perera and Weinstein, 2000). In the example of AFB_1-induced liver tumors, AFB_1–DNA adducts have proved to be an extremely useful biomarker. A highly linear relationship was observed between liver tumor incidence (in rats, mice, and trout) and AFB_1–DNA adduct formation over a dose range of five orders of magnitude (Eaton and Gallagher, 1994). Such approaches may allow for an extension of biologically observable phenomena to doses lower than those leading to frank tumor development, and help to address the issues of extrapolation over multiple orders of magnitude to predict response at environmentally relevant doses.

Table 4-5 presents some mechanistic details about rodent tumor responses that are no longer thought to be predictive of cancer risk for humans. This table lists examples of both qualitative and quantitative considerations useful for determining relevance of rodent tumor responses for human risk evaluations. An example of qualitative considerations is the male rat kidney tumors observed following exposure to chemicals that bind to α_{2u}-globulin (e.g., unleaded gasoline, 1,4-dichlorobenzene, D-limonene). The α_{2u}-globulin is a male-rat-specific low-molecular-weight protein not found in female rats, humans, or other species, including mice and monkeys (McClain, 1994; Neumann and Olin, 1995; Oberdorster, 1995; Omenn et al., 1995; Risk Commission, 1997; Rodericks et al., 1997).

Table 4-5 also illustrates quantitative considerations important for determining human relevance of animal bioassay information. For example, doses of compounds so high as to exceed solubility in the urinary tract outflow lead to tumors of the urinary bladder in male rats following crystal precipitation and local irritation leading to hyperplasia. Such precipitates are known to occur following saccharin or nitrilotriacetic acid bioassay exposures (Cohen et al., 2000). The decision to exclude saccharin from the NTP list of suspected human carcinogens reaffirms the nonrelevance of such high-dose responses for likely human exposure considerations (Neumann and Olin, 1995; NTP, 2016). A gross overloading of the particle clearance mechanism of rat lungs via directly administered particles, as was seen in titanium dioxide (TDO) exposures, resulted in the EPA's delisting of TDO as a reportable toxicant for the Clean Air Act Toxic Release Inventory (Oberdorster, 1995; U.S. EPA, 2016).

Other rodent responses not likely to be predictive for humans include localized forestomach tumors after gavage. Ethyl acrylate, which produces such tumors, was delisted on the basis of extensive mechanistic studies (NTP, 2016). In general, for risk assessment, it is desirable to use the same route of administration as the likely exposure pathway in humans to avoid such extrapolation issues. Despite the example of forestomach tumors, tumors in unusual sites—such as the pituitary gland, the eighth cranial nerve, or the Zymbal gland—should not be immediately dismissed as irrelevant, because organ-to-organ correlation is often lacking (NRC, 1994). The EPA cancer guidelines provide a good list of considerations for evaluating relevance in the sections on evaluating weight of evidence (U.S. EPA, 2005a).

Table 4-6

Example of Three Types of Epidemiologic Study Designs

METHODOLOGICAL ATTRIBUTES	TYPE OF STUDY		
	COHORT	CASE–CONTROL	CROSS-SECTIONAL
Initial classification	Exposure–nonexposure	Disease–nondisease	Either one
Time sequence	Prospective	Retrospective	Present time
Sample composition	Nondiseased individuals	Cases and controls	Survivors
Comparison	Proportion of exposed with disease	Proportion of cases with exposure	Either one
Rates	Incidence	Fractional (%)	Prevalence
Risk index	Relative risk–attributable risk	Relative odds	Prevalence
Advantages	Lack of bias in exposure; yields incidence and risk rates	Inexpensive, small number of subjects, rapid results, suitable for rare diseases, no attrition	Quick results
Disadvantages	Large number of subjects required, long follow-up, attrition, change in time of criteria and methods, costly, inadequate for rare diseases	Incomplete information, biased recall, problem in selecting control and matching, yields only relative risk—cannot establish causation, population of survivors	Cannot establish causation (antecedent consequence), population of survivors, inadequate for rare diseases

SOURCES: Gamble and Battigelli (1978, 1991).

In an attempt to improve the prediction of cancer risks to humans, transgenic mouse models have been developed as possible alternative to the standard 2-year cancer bioassay. Transgenic models use knockout or transgenic mice that incorporate or eliminate a gene that has been linked to human cancer. The NTP evaluated some of these models and found the p53-deficient (p53+/− heterozygous) and Tg.AC (v-Ha-ras transgene) models to be particularly useful in identifying carcinogens and mechanisms of action (Bucher, 1998; Chhabra et al., 2003). The use of transgenic models has the power to improve the characterization of key cellular and MOA of toxicological responses (Mendoza et al., 2002; Gribble et al., 2005). However, these studies have been used primarily for mechanistic characterization rather than for hazard identification. Transgenic models have been shown to reduce cost and time as compared with the standard 2-year assay, but they have also been shown to be somewhat limited in their sensitivity (Cohen, 2001). As stated in the current EPA cancer guidelines, transgenic models should not be used to replace the standard 2-year assay, but can be used in conjunction with other types of data to assist in the interpretation of additional toxicological and mechanistic evidence (U.S. EPA, 2005a). An especially promising emerging area of research has been with the Collaborative Cross. A series of genetically defined (fully sequenced genomes, 20× coverage) mice referred to as the Collaborative Cross have been established to investigate genetic and environmental influences on toxicological response in mice (Chesler et al., 2008) and these strains have already improved our understanding of mammalian genes that predispose to cancer (Koturbash et al., 2011). In a similar manner, human cells have also provided a method to explore human diversity in response directly in vitro (Eduati et al., 2015).

Use of Epidemiologic Data in Risk Assessment

The most convincing lines of evidence for human risk are well-conducted epidemiologic studies in which a positive association between exposure and disease has been observed (NRC, 1983). Environmental and occupational epidemiologic studies are frequently opportunistic. Studies begin with known or presumed exposures, comparing exposed with nonexposed individuals, or with known cases, compared with persons lacking the particular diagnosis.

Table 4-6 shows examples of epidemiologic study designs and provides clues on types of outcomes and exposures evaluated. Although convincing, there are important limitations inherent in epidemiologic studies. Robust exposure estimates are often difficult to obtain as they are frequently done retrospectively (e.g., through retrospective job history records). Also, because many important health effects have long latency before clinical manifestations appear, reconsideration of relevant populations can be challenging. Another challenge for interpretation is that there are often exposures to multiple chemicals, especially when a lifetime exposure period is considered. There is frequently a trade-off between detailed information on relatively few persons and very limited information on large numbers of persons. Contributions from lifestyle factors, such as smoking and diet, are important to assess as they can have a significant impact on cancer development. Human epidemiologic studies can provide both very useful information for hazard assessment and quantitative information for data characterization. Good illustrations of epidemiologic studies and their interpretation for toxicological evaluation are available (Regalado et al., 2006; Gill et al., 2011).

Three example types of epidemiologic study designs—cross-sectional studies, cohort studies, and case–control studies—are detailed in Table 4-6. Cross-sectional studies survey groups of humans to identify risk factors (exposure) and disease, but are not useful for establishing cause and effect. Cohort studies can evaluate individuals selected on the basis of their exposure to a chemical under study. Thus, based on exposure status, these individuals are monitored for development of disease. These prospective studies monitor over time individuals who initially are disease-free to determine the rates at which they develop disease. In case–control

studies, subjects are selected on the basis of disease status: disease cases and matched cases of disease-free individuals. Exposure histories of the two groups are compared to determine key consistent features in their exposure histories. All case–control studies are retrospective studies.

In risk assessment, epidemiologic findings can be judged by the following criteria: strength of association, consistency of observations (reproducibility in time and space), specificity (uniqueness in quality or quantity of response), appropriateness of temporal relationship (did the exposure precede responses?), dose–response, biological plausibility and coherence, verification, and analogy (biological extrapolation) (Hill, 1965; Faustman et al., 1997; WHO, 1999; Adami et al., 2011). These same criteria have been used for evaluating MOAs where integrated considerations of both human and animal studies are done.

Epidemiologic study designs should also be evaluated for their power of detection, appropriateness of outcomes, verification of exposure assessments, completeness of assessing confounding factors, and general applicability of the outcomes to other populations at risk. Power of detection is calculated using study size, variability, accepted detection limits for endpoints under study, and a specified significance level (Healey, 1987; EGRET, 1994; Dean et al., 1995). Meta-analysis is used with epidemiologic studies to combine results from different studies using weighting of results to account for sample size across studies. The importance and complexity of human studies for risk assessment are shown in evaluations with arsenic and dioxin (U.S. EPA, 2010b, 2010d).

Advances from the human genome project have increased sophistication of molecular biomarkers and have improved the mechanistic bases for epidemiologic hypotheses. This has allowed epidemiologists to get within the "black box" of statistical associations and forward our understanding of biological plausibility and clinical relevance. "Molecular epidemiology," the integration of molecular biology into traditional epidemiologic research, is an important focus of human studies where improved molecular biomarkers of exposure, effect, and susceptibility have allowed investigators to more effectively link molecular events in the causal disease pathway. Epidemiologists can now include the contribution of potential genetic factors with environmental risk factors for the determination of the etiology, distribution, and prevention of disease. Highlighting the potential power of genetic information to epidemiologic studies, the Human Genome Epidemiology (HuGE) Network was launched in 1998, providing a literature database of published, population-based epidemiologic studies of human genes (Khoury, 1999).

With the advance of genomics, the range of biomarkers has grown dramatically and includes identification of single-nucleotide polymorphisms (SNPs), genomic profiling, transcriptome analysis, and proteomic analysis (Simon and Wang, 2006). Implications of these improvements for risk assessment are tremendous, as they provide an improved biological basis for extrapolation across the diversity of human populations and allow for improved cross-species comparisons with rodent bioassay information because of evolutionarily conserved response pathways and ontologies (NRC, 2000). In addition, genomics allows for "systems-based" understanding of disease and response, moving risk assessment away from a linear, single-event-based concept and improving the biological plausibility of epidemiologic associations (Toscano and Oehlke, 2005; NRC, 2010). Human databases such as SNP Track from the FDA provide current information on genes and proteins that contain polymorphisms that can affect human response, especially for drug response (U.S. FDA, 2017a, 2017b). An extensive battery of tools to link phenotypic and functional changes as well as drug toxicity and interaction information to genetics is available.

Integrating Qualitative Aspects of Risk Assessment

Qualitative assessment of hazard information should include a consideration of the consistency and concordance of findings, including a determination of the consistency of the toxicological findings across species and target organs, an evaluation of consistency across duplicate experimental conditions, and a determination of the adequacy of the experiments to consistently detect the adverse endpoints of interest.

Qualitative assessment of animal or human evidence is done by many agencies, including the EPA and IARC. Similar evidence classifications have been used for both the animal and human evidence categories by both agencies. These evidence classifications have included levels of "sufficient, limited, inadequate, and no evidence" (U.S. EPA, 1999, 1994b, 2005a), or "evidence suggesting lack of carcinogenicity" (IARC, 2000). For both agencies, these classifications are used for an overall weight-of-evidence approach for carcinogenicity classification. Current categories expand on these initial categories including "Group 4 probably not carcinogenic to humans" (IARC, 2017) or "not likely to be carcinogenic to humans" (U.S. EPA, 2005a).

Weight of evidence is an integrative step used by the EPA to "characterize the extent to which the available data support the hypothesis that a chemical causes cancer in humans" (U.S. EPA, 1999, 2005a). It is the process of "weighing" all of the evidence to reach a conclusion about carcinogenicity. The weight of evidence can consider both the quality and quantity of data as well as any underlying assumptions. The evidence includes data from all of the hazard assessment and characterization studies such as SAR data, in vivo and/or in vitro studies, and epidemiologic data. Using this type of information and weight-of-evidence approach, the EPA includes hazard descriptors to define carcinogenic potential and to provide a measure of clarity and consistency in the characterization narrative: "carcinogenic to humans," "likely to be carcinogenic to humans," "suggestive evidence of carcinogenic potential," "inadequate information to assess carcinogenic potential," and "not likely to be carcinogenic to humans." In this section, approaches for evaluating cancer endpoints are discussed for carcinogens. Similar weight-of-evidence approaches have been proposed for reproductive risk assessment (refer to sufficient and insufficient evidence categories in the EPA's guidelines for reproductive risk [U.S. EPA, 1996a] and considerations by NTP).

The Institute for Evaluating Health Risks defined an "evaluation process" by which reproductive and developmental toxicity data can be consistently evaluated and integrated to ascertain their relevance for human health risk assessment (Moore et al., 1995; Faustman et al., 2011). This evaluation process supported the implementation of EPA's guidelines for developmental toxicity risk assessment (U.S. EPA, 1991) and for the NTP's Office of Health Assessment and Translation (formerly CERHR) (NTP, 2011b). Application of such carefully deliberated approaches for assessing noncancer endpoints has helped avoid the tendency to list chemicals as yes or no (positive or negative) without human relevancy information. Application of systematic review methods for evaluation of chemicals at the NTP (Birnbaum et al., 2013; Rooney et al., 2014; NTP, 2017), EPA, and WHO is under assessment and modification to facilitate the methodological review of the ever-widening body of literature on chemical substructures and nonhuman studies (NTP, 2017).

Mode of Action and Adverse Outcome Pathways

In the 2005 EPA cancer guidelines the importance of using "weight of evidence" to arrive at insights for possible mode of action (MOA) was identified (U.S. EPA, 2005a). MOA information describes key events and processes leading to molecular and functional effects

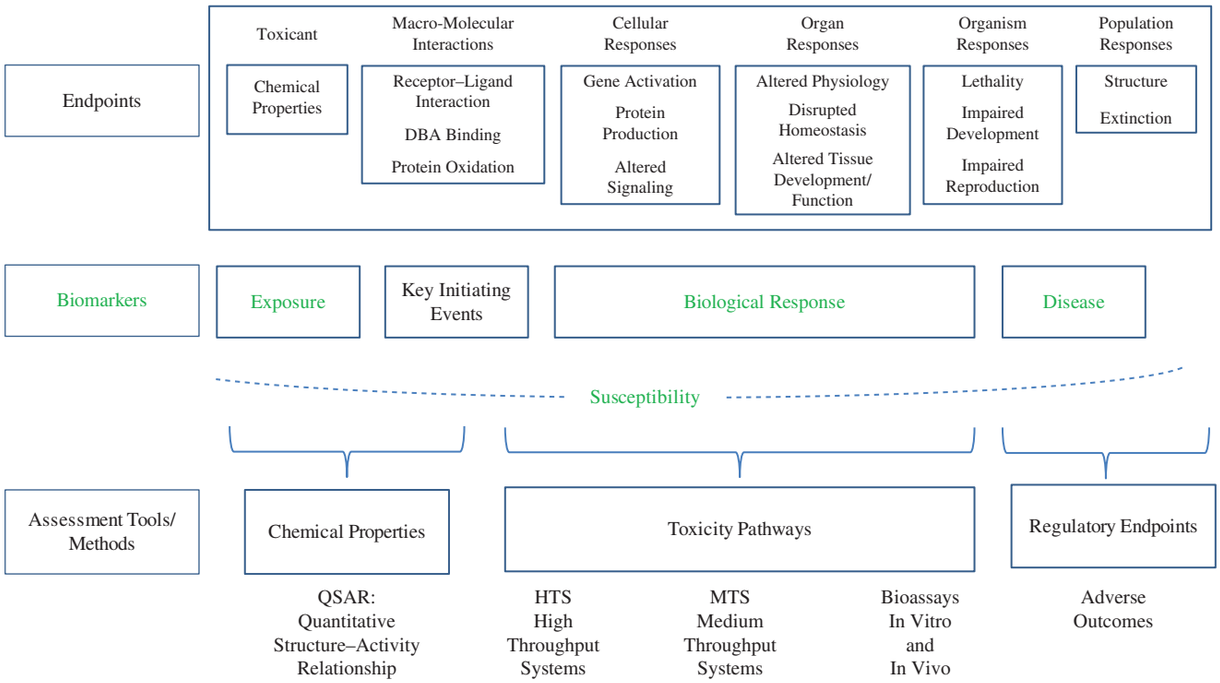

Figure 4-4. *Adverse outcome pathways (AOPs) and biomarkers link responses and describe disease pathogenesis.* Relationships between AOPs and biomarkers of exposure, effect, susceptibility, and disease are illustrated across disease response. Methods used to collect information along the disease continuum include quantitative SAR (QSAR), high and medium throughput in vitro systems, and bioassays (other in vivo and in vitro assessments).

that would in general explain the overall process of cancer development. In many cases these could be plausible hypothesized MOAs for specific toxicity endpoints, but the detailed mechanistic nuances of the pathway might not yet be fully known. The EPA has used such MOA information to suggest nondefault approaches for cancer risk assessments and for evaluating toxicity of compounds with common MOAs in cumulative risk assessments (U.S. EPA, 1996b, 1998a).

Within the EPA's carcinogenic risk assessment guidelines, the MOA framework considers evidence from animal studies, relevance to humans, and life stage or population susceptibility (U.S. EPA, 2005a). Chemical-specific adjustment factors for interspecies differences and human variability have been proposed and build upon guidance developed by the WHO's International Programme on Chemical Safety Harmonization Project (WHO, 2000). Critical to the MOA development is the use of "criteria of causality" considerations, which build upon the Hill criteria used in epidemiology (Hill, 1965; Faustman et al., 1997; U.S. EPA, 1999; Klaunig et al., 2003), and considerations of dose–response relationships and temporal associations, as well as the biological plausibility, coherence, strength, consistency, and specificity of the postulated MOA.

Building on concepts from MOA analyses, Ankley proposed a structured framework for ecological risk assessment entitled *Adverse Outcome Pathways* that links biological events with adverse outcomes and disease (Ankley et al., 2010). This has gained applicability for human risk assessment as well and the OECD has developed a program to facilitate its development and distribution using a knowledge base and wiki facilitated IT site (OECD, 2017).

Fig. 4-4 shows how biomarkers and adverse-outcome pathways (AOPs) can be linked to describe adverse outcomes and disease pathogenesis. In this figure the initial exposure to a chemical is linked through a series of events including early molecular events known as molecular initiating events (MIEs) through responses at the cellular, organ, and ultimately to organism and population level adverse outcomes. By linking these events the AOP approach defines key event relationships that resemble the causal relationships identified using MOA approaches. Perhaps most importantly for risk assessors is that these approaches allow for the identification of a temporal series of events that can be evaluated in vivo and in vitro and across species using related biomarkers of exposure, biological effect, and response. It also can incorporate biomarkers of susceptibility. Fig. 4-4 shows these linkages and illustrates how information from methods such as structure–activity relationship assessments can link high- and medium-throughput assays with high content assay outcomes across in vitro assessments with biomarkers of effect seen at the organ, organism, and population levels with adverse outcomes and disease. These useful concepts can provide both a qualitative and quantitative frame for risk assessment.

DOSE–RESPONSE ASSESSMENT

Integrating Quantitative Aspects of Risk Assessment

Quantitative considerations in risk assessment include dose–response assessment, exposure assessment, variation in susceptibility, and characterization of uncertainty. For dose–response assessment, varying approaches have been proposed including threshold versus nonthreshold endpoints. Traditionally, in the United States, threshold approaches have been applied for assessment of noncancer endpoints, and nonthreshold approaches have been used for cancer endpoints. As we have learned more about

nongenotoxic mechanisms of carcinogenicity, these processes have been evaluated using threshold approaches. Each approach and its inherent assumptions are discussed below, as are efforts to include more detailed mechanistic considerations to harmonize these approaches (Bogdanffy et al., 2001).

In general, human exposure data for prediction of human response to environmental chemicals are quite limited; thus, animal bioassay data have primarily served as the basis for most quantitative risk assessments and have required extrapolation for human health risk prediction. The risk assessor, however, is normally interested in low environmental exposures when considering human risk, exposures that are well below the experimentally observable range of responses in most animal assays. Thus, methods for extrapolating from high dose to low dose as well as extrapolating from animal risk to human risk are required and comprise a major emphasis of dose–response assessment.

The fundamental basis of the quantitative relationships between exposure to a chemical and the incidence of an adverse response is the dose–response assessment. Analysis of dose–response relationships must start with the determination of the critical effects to be quantitatively evaluated. It is usual practice to choose the most robust data sets with adverse effects occurring at the lowest levels of exposure from studies using the most relevant exposure routes. The "critical" adverse effect is defined as the significant adverse biological effect that occurs at the lowest exposure level (Barnes and Dourson, 1988). The EPA has issued toxicity-specific guidelines that are useful in identifying such critical effects (for developmental toxicity [U.S. EPA, 1991], reproductive toxicity [U.S. EPA, 1996a], neurotoxicity [U.S. EPA, 1998c], and cancer [U.S. EPA, 1994b, , 1999, 2005a]). International Programme on Chemical Safety (IPCS) has compiled a document cited as the *Principles for Modeling Dose–Responses for the Risk Assessment of Chemicals* (WHO, 2006b). It outlines key concepts and considerations for dose–response evaluations within the context of a risk assessment.

Threshold Approaches

Approaches for characterizing threshold dose–response relationships include identification of "no observed adverse effect level" (NOAEL) or "lowest observed adverse effect level" (LOAEL). On the dose–response curve illustrated in Fig. 4-5, the doses tested in the bioassay are given as F, G, H, and I. The statistical significance of points G, H, and I is indicated using an asterisk (*). The NOAEL (F) is identified as the highest nonstatistically significant dose tested; in this example the NOAEL occurs at approximately 2 mg/kg body weight. Point G is the LOAEL (~2.3 mg/kg body weight), as it is the lowest dose tested with a statistically significant effect. Lines A to D represent possible extrapolations below the point of departure (POD), which is represented on this figure as a square (■) and is labeled as point E. The POD is used to specify the estimated dose near the lower end of the observed dose range, below which extrapolation to lower exposures is necessary (U.S. EPA, 2005a). In Fig. 4-5, the POD occurs at 10% effective dose or ED_{10}. The type of extrapolation below the POD is depending on the type of data available. The importance of choosing an appropriate POD and extrapolation type is discussed further in the following sections of this chapter. From these modeled approaches, model D shows a model where the threshold (T) represents the dose below which no additional increase in response is observed.

In general, most animal bioassays are constructed with sufficient numbers of test animals to detect biological responses at the 10% response range; however, this is also dependent on endpoint and the background rate of the endpoint in control animals. The risk assessor should always understand the biological significance of

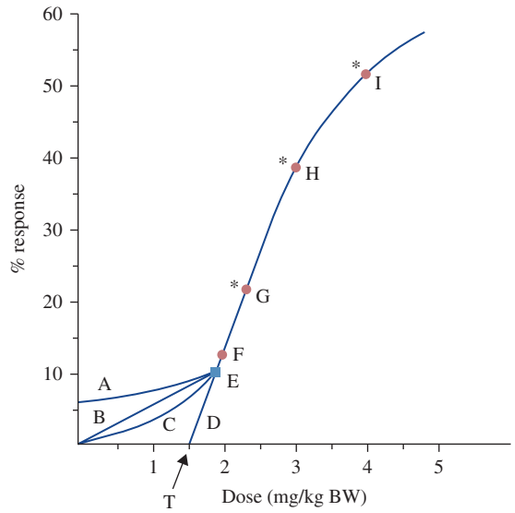

Figure 4-5. *Dose–response curve.* This figure is designed to illustrate a typical dose–response curve with points F to I indicating the biologically determined responses. Statistical significance of these responses is indicated with the symbol "*." Point E (■) represents a dose near the lower end of the observed dose–response range, below which extrapolation to lower doses can occur for cancer risk estimates (U.S. EPA, 2005a). Point F is the highest nonstatistically significant response point; hence, it is the "no observed adverse effect level" (NOAEL) for this example. Point G is the "lowest observed adverse response level" (LOAEL). Point T represents a threshold for response curve D. Curves A to D show some options for extrapolating the dose–response relationship below the range of biologically observed data points and POD.

the responses being evaluated in order to place statistical observations in context. Significance thus usually refers to both biological and statistical criteria (Faustman et al., 1994) and is dependent on the number of dose levels tested, the number of animals tested at each dose, and background incidence of the adverse response in the nonexposed control groups. The NOAEL should not be perceived as risk-free, as several reports have shown that the response of NOAELs for continuous endpoints averages 5% risk, and NOAELs based on quantal endpoints can be associated with risk of greater than 10% (Allen et al., 1994a; Faustman et al., 1994).

As described in Chap. 2, approaches for characterizing dose–response relationships include identification of effect levels such as LD_{50} (dose producing 50% lethality), LC_{50} (concentration producing 50% lethality), ED_{10} (dose producing 10% response), as well as NOAELs. NOAELs have traditionally served as the basis for risk assessment calculations, such as reference doses (RfDs) or acceptable daily intake (ADI) values. RfDs or concentrations (RfCs) are estimates of daily exposure (oral or inhalation, respectively) to a chemical that is assumed to be without adverse health impact in humans. The ADIs are used by the WHO for pesticides and food additives to define "the daily intake of chemical, which during an entire lifetime appears to be without appreciable risk on the basis of all known facts at that time" (WHO, 1962; Dourson et al., 1985). RfDs (first introduced in Chap. 2) and ADI values typically are calculated from NOAEL values by dividing by uncertainty factor (UF) and/or modifying factor (MF) (Dourson and Stara, 1983; Dourson and DeRosa, 1991; U.S. EPA, 1991):

$$\text{RfD} = \frac{\text{NOAEL}}{\text{UF} \times \text{MF}},$$

$$\text{ADI} = \frac{\text{NOAEL}}{\text{UF} \times \text{MF}}.$$

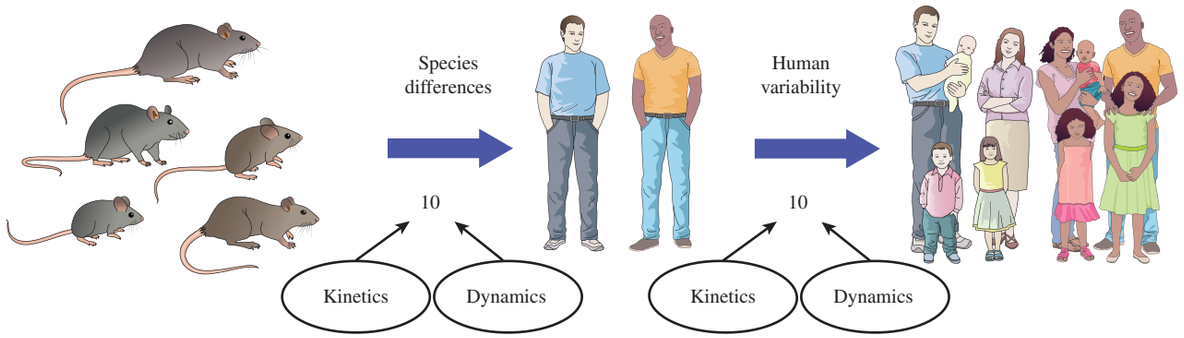

Figure 4-6. *Toxicokinetic (TK) and toxicodynamic (TD) considerations inherent in interspecies and interindividual extrapolations. TK* refers to the processes of absorption, distribution, elimination, and metabolism of a toxicant. *TD* refers to the actions and interactions of the toxicant within the organism and describes processes at organ, tissue, cellular, and molecular levels. This figure shows how uncertainty in extrapolation both across and within species can be considered as being due to two key factors: a kinetic component and a dynamic component. Challenges remain for extrapolating information on human variation to specific populations (NRC, 2009). Refer to the text for detailed explanations.

Tolerable daily intakes (TDIs) can be used to describe intakes for chemicals that are not "acceptable" but are "tolerable" as they are below levels thought to cause adverse health effects. These are calculated in a manner similar to ADI. Historically, dividing by the UFs allows for interspecies (animal-to-human) and intraspecies (human-to-human) variability with default values of 10 each. An additional UF is used to account for experimental inadequacies—for example, to extrapolate from short-exposure-duration studies to a situation more relevant for chronic study or to account for inadequate numbers of animals or other experimental limitations. If only a LOAEL value is available, then an additional 10-fold factor is commonly used to arrive at a value more comparable to a NOAEL. For developmental toxicity endpoints, it has been demonstrated that the application of the 10-fold factor for LOAEL-to-NOAEL conversion is too large (Allen et al., 1994a). Traditionally, a safety factor of 100 would be used for RfD calculations to extrapolate from a well-conducted animal bioassay (10-fold factor for animal-to-human variability) and to account for human variability in response (10-fold factor for human-to-human variability).

MFs are values that can be used to adjust the UFs if data on mechanisms, pharmacokinetics, or relevance of the animal response to human risk are available. For example, if there is kinetic information suggesting there is very similar metabolism for a particular compound in rats and humans, producing the same active target metabolite, then—rather than using a 10-fold UF to divide the NOAEL from the animal toxicity study to obtain a human relevant RfD—a factor of 3 for that UF might be used. Of particular interest is the addition of an extra 10-fold factor through the Food Quality Protection Act (FQPA) to ensure protection of infants and children (U.S. EPA, 1996b). Under this law an additional UF is added to ensure protection of children's health and it is currently being used for determining allowable pesticide chemical residues. This factor is designed to take into account potential prenatal and postnatal toxicity and to overcome the incompleteness of toxicity and exposure data (FQPA, PL 104-170). Illustrative discussions on how such a legislatively mandated UF might be applied are available for chlorpyrifos (Schardein and Scialli, 1999; U.S. EPA, 2000b, 2016; Zhao et al., 2006).

To reduce uncertainty in calculating RfDs and ADIs, there has been a transition from the use of traditional 10-fold UFs to the use of data-derived and chemical-specific adjustment factors. Such efforts have included reviewing the human pharmacologic literature from published clinical trials (Silverman et al., 1999) and developing human variability databases for a large range of exposures and clinical conditions (Renwick, 1991, 1999; Johnson et al., 1997). Toward this goal, Renwick has separated the intraspecies and interspecies UFs into two components: toxicokinetics (TK) and toxicodynamic (TD) aspects (Renwick, 1991, 1999; Johnson et al., 1997). Fig. 4-6 shows these distinctions. A key advantage of this approach is that it provides a structure for incorporating scientific information on specific aspects of the overall toxicological process into the RfD calculations; thus, relevant data can replace a portion of the overall "uncertainty" surrounding these extrapolations. Current WHO guidance uses a 4.0- and 2.5-fold factor for the TK and TD interspecies components, respectively (WHO, 2005), and interindividual TK and TD factors of 3.16 (Renwick and Lazarus, 1998). The NAS publications (NRC, 2009, 2017) have emphasized the need to consider population variability and context in risk assessment. In examples described in this NRC report, high background incidence of cancers or other adverse health outcomes in the population should be considered before setting "acceptable" levels of additional risks (NRC, 2009).

NOAEL values have also been utilized for risk assessment by evaluating a "margin of exposure" (MOE), where the ratio of the NOAEL determined in animals and expressed as mg/kg per day is compared with the level to which a human may be exposed. For example, consider the case where human exposures to a specific chemical are calculated to be solely via drinking water, and the total daily intake of the compound is 0.04 mg/kg per day. If the NOAEL for neurotoxicity is 100 mg/kg per day, then the MOE would be 2500 for the oral exposure route for neurotoxicity. Such a large value is reassuring to public health officials. Low values of MOE indicate that the human levels of exposure are close to levels for the NOAEL in animals. There is usually no factor included in this calculation for differences in human or animal susceptibility or animal-to-human extrapolation; thus, MOE values of less than 100 have been used by regulatory agencies as flags for requiring further evaluation.

The NOAEL approach has been criticized on several points, including that (1) the NOAEL must, by definition, be one of the experimental doses tested and (2) once this is identified, the rest of the dose–response curve is ignored. Because of these limitations, an alternative to the NOAEL approach, the benchmark dose (BMD) method, was proposed (Crump, 1980). In this approach, the dose–response is modeled and the lower confidence bound for a dose at a specified response level (benchmark response [BMR]) is calculated. The BMR is usually specified at 1%, 5%, or 10%. Fig. 4-7 shows the BMD using a 10% BMR (BMD_{10}) and a 95% lower confidence

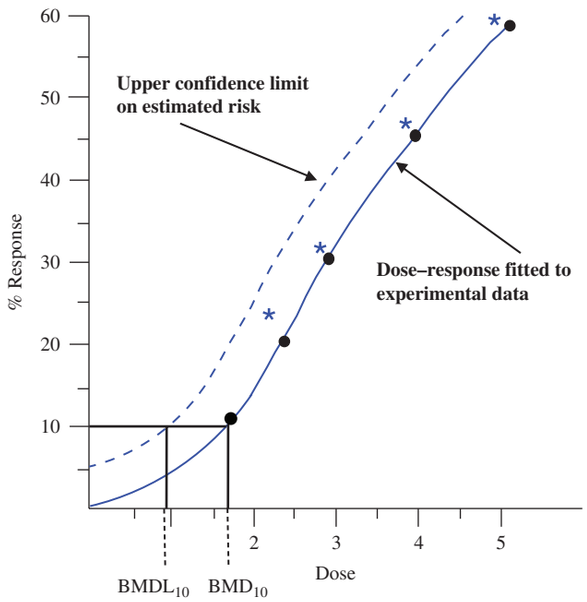

Figure 4-7. *Illustration of benchmark dose (BMD) approach.* This figure shows the $BMDL_{10}$ and BMD_{10}, the lower confidence limit and dose (ED_{10}) associated with a 10% incidence of adverse response, respectively (Kavlock et al., 1995; U.S. EPA, 2005a, 2012a). For reference, points are labeled as described in the legend for Fig. 4-5.

bound on dose ($BMDL_{10}$). The BMD_x (with x representing the percent BMR) is used as an alternative to the NOAEL value for RfD calculations. Thus, the RfD would be:

$$\text{RfD} = \frac{\text{BMD}_x \text{ or BMDL}_x}{\text{UF} \times \text{MF}^{\bullet}}.$$

The EPA has developed software for the application of BMD methods and a technical guidance document to provide guidelines for application of BMDs for both cancer and noncancer endpoints (U.S. EPA, 2000a, 2012a). The final guidance can be found at https://www.epa.gov/risk/benchmark-dose-technical-guidance. The EPA recently released an updated version (2.7) of its BMD software (U.S. EPA, 2017a).

The 2009 WHO guidelines for dose–response modeling for risk assessment also discuss BMD approaches (WHO, 2006b). Both the EPA and WHO guidelines distinguish NOAEL versus BMD-based approaches. Harmonization of approaches available for assessments is discussed in the WHO documents.

The BMD approach has been applied to study noncancer endpoints, including developmental (Allen et al., 1994a) and reproductive toxicity (Auton, 1994). The most extensive studies with developmental toxicity have shown that BMD_{05} values were similar to a statistically derived NOAEL for a wide range of developmental toxicity endpoints and that results from using generalized dose–response models were similar to biologically based dose–response (BBDR) models designed specifically to represent unique features of developmental toxicity testing (Faustman et al., 1999).

Advantages of the BMD approach include (1) the ability to take into account the dose–response curve; (2) the inclusion of a measure of variability (confidence limit); and (3) the use of a consistent BMR level for RfD calculations across studies. Limitations in the animal bioassays include minimal number of test doses for evaluation, shallow dose responses, and use of study designs with widely spaced test doses will limit the utility of these assays for any type of quantitative assessments, whether NOAEL- or BMD-based approaches.

Nonthreshold Approaches

As Fig. 4-5 shows, numerous dose–response curves can be proposed in the low-dose region of the dose–response curve if a threshold assumption is not made. Because the risk assessor may need to extrapolate beyond the region of the dose–response curve for which experimentally observed data are available, the choice of models to generate curves in this region has received lots of attention. For nonthreshold responses, methods for dose–response assessments have models for extrapolation to de minimus (10^{-4} to 10^{-6}) risk levels at very low doses, far below the biologically observed response range and far below the effect levels evaluated for threshold responses.

The EPA cancer guidelines define the dose–response methods as requiring two steps: (1) defining the "POD" or the lowest dose associated with adverse effects within the range of the experimental data and (2) the extrapolation from the POD to low environmentally relevant exposure levels based on experimental data. The extrapolation can be done with a linear model or a nonlinear model with this choice dependent on the amount and type of experimental data available. Risk estimates using the linear model (biological response increases proportionally with level of exposure) are generally higher than those using nonlinear models. For example, in 2003, the EPA released the Dioxin Reassessment and used the BMD dose–response method. The reassessment used a POD based on a 1% response with a linear extrapolation model based on the position that the scientific data were inadequate to rule out the EPA's standard default linear assumption. The EPA Dioxin Reassessment was evaluated in a National Academies Report in 2006 (National Academy of Science, 2006) and this review discussed remaining extrapolation issues. In the EPA's recent draft of dioxin risk assessment (U.S. EPA, 2010b) a revised TCDD cancer risk assessment is presented using the upper bound on the regression slope for estimating cancer mortality risk from the epidemiology evaluation by Cheng et al. (2006). The top 5% of exposure estimates from this epidemiologic study were excluded as the dose-related changes in cancer mortality plateaued at high doses. The EPA lagged exposures by 15 years and adjusted cancer risk based on cumulative TCDD fat concentrations. Their analysis included an estimate of TCDD cancer risks above the TCDD background levels found in the NIOSH worker cohort analyzed by Cheng et al. (2006). Below the POD, the EPA assumed a default linear slope value. This document represents an example recent interpretation of cancer risk assessment by the EPA for data-rich compounds with multiple cancer risk estimates for animal and human studies as well as how extensive mechanistic and kinetic information is considered. This draft report provides insight into recent interpretations of the EPA cancer guidelines (U.S. EPA, 2005a). Increased attention has been given to the use of mechanistic data (such as biomarker data) to extend the POD to lower regions of the dose–response curve.

Statistical or Probability Distribution Models

Two general types of dose–response models exist for extrapolation: statistical (or probability distribution) and mechanistic models (Krewski and Van Ryzin, 1981). The distribution models are based on the assumption that each individual has a tolerance level for a test chemical and that this response level is a variable following a specific probability distribution function. These responses can be modeled using a cumulative dose–response function. Chap. 2 discusses the common normal distribution pattern (see Fig. 2-5). A log-probit model estimates the probability of response at a specified

dose (d); thus, $P(d) = \phi\,[a + \beta \log d]$, where ϕ is the cumulative function for a standard normal distribution of the log tolerances with standard deviations σ and mean μ, a equals μ/σ, and β equals the slope of the probit line ($-1/\sigma$). The probit curve at low doses usually assumes an S shape. Chap. 2 discusses determination of the LD_{50} value from such a curve. However, extrapolation of the experimental data from 50% response levels to a "safe," "acceptable," or "de minimus" level of exposure—for example, one in a million risk above background—illustrates the huge gap between scientific observations and highly protective risk limits (sometimes called virtually safe doses [VSDs] or those corresponding to a 95% upper confidence limit on adverse response rates).

The log-logistic model was derived from chemical kinetic theory. The probability of response at dose d is defined as $P(d) = [1 \exp(a + \beta \log d)]^{-1}$. Like the probit model, this model defines sigmoidal curves that are symmetrical around the 50% response level; however, the log-logistic curves approach the 0% and 100% response levels with a shallow curve shape. The logit and probit curves are indistinguishable in fitting the data in the region of the response curve where experimentally derived data are present (Brown, 1984; Hartung, 1987; Allen et al., 1994b).

Models Derived from Mechanistic Assumptions

This modeling approach designs a mathematical equation to describe dose–response relationships that are consistent with postulated biological mechanisms of response. These models are based on the idea that a response (toxic effect) in a particular biological unit (animal, human, pup, etc.) is the result of the random occurrence of one or more biological events (stochastic events).

Radiation research spawned a series of such "hit models" for cancer modeling, where a hit is defined as a critical cellular event that must occur before a toxic effect is produced. The simplest mechanistic model is the one-hit (one-stage) linear model in which only one hit or critical cellular interaction is required for a cell to be altered. For example, based on somatic mutation theory, a single mutational change would be sufficient for a cell to become cancerous through a transformational event and dose-independent clonal expansion. The probability statement for these models is $P(d) = 1 - \exp^{(-\lambda d)}$, where λd equals the number of hits occurring during a time period. Using this approach, a single molecule of a genotoxic carcinogen would have a minute but finite chance of causing a mutational event.

As theories of cancer have grown in complexity, so too have these hit-based mechanistic models. Multihit models have been developed that can describe hypothesized single-target multihit events, as well as multitarget, multihit events in carcinogenesis. The probability statements for these models are $P(d) = \int^{\lambda d} x^{k-1} \exp(-x) \,/\, \Gamma(k)\mathrm{d}x$, where $\Gamma(k)$ denotes the gamma function with k being the critical number of hits for the adverse response. The Weibull model has a dose–response function with characteristics similar to those of the multihit models, where the response equation is $P(d) = 1 - \exp[-\lambda d^k]$. Here again, k is the critical number of hits for the toxic cellular response.

Armitage and Doll (1957) developed a multistage model for carcinogenesis that was based on these equations and on the hypothesis that a series of ordered stages was required before a cell could undergo mutation, initiation, transformation, and progression to form a tumor. This relationship was generalized by Crump (1984) by maximizing the likelihood function over polynomials, so that the probability statement is:

$$P(d) = 1 - \exp[-(\lambda_0 + \lambda_1 d^1 + \lambda_2 d^2 + \cdots + \lambda_k d^k)].$$

If the true value of λ_1 is replaced with λ_1^* (the upper confidence limit of λ_1), then a linearized-multistage model can be derived where the expression is dominated by d at low doses. The slope on this confidence interval, q_1^*, has been used by the EPA for quantitative cancer assessment. To obtain an upper 95% confidence interval on risk, the q_1^* value (risk/Δ dose in mg/kg per day) is multiplied by the amount of exposure (mg/kg per day). Thus, the upper bound estimate on risk (R) is calculated as:

$$R = q_1^*[\text{risk}(\text{mg/kg per day})^{-1}] \times \text{exposure}(\text{mg/kg per day})$$

This relationship has been used to calculate a "VSD," which represents the lower 95% confidence limit on a dose that gives an "acceptable level" of risk (e.g., upper confidence limit for 10^{-6} excess risk). In the past the Integrated Risk Information System (IRIS) (U.S. EPA, 2011d) developed by the EPA has given q^* values for many environmental carcinogens (U.S. EPA, 2011d). Because both the q_1^* and VSD values are calculated using 95% confidence intervals, the values are believed to represent conservative, protective estimates.

The EPA has utilized the LMS model to calculate "unit risk estimates" in which the upper confidence limit on increased individual lifetime risk of cancer for a 70-kg human breathing 1 μg/m^3 of contaminated air or drinking 2 L per day of water containing 1 ppm (1 mg/L) is estimated over a 70-year life span. The example given in Fig. 4-8 shows the calculation of incremental lifetime cancer risk (ILCR) of skin cancer using soil exposure and q^* values for inorganic arsenic. If a POD-based approach is used for low-dose extrapolation, the model would be used to extrapolate from the POD or upper confidence limit and these slopes would be considered for the q^* values.

Toxicological Enhancements of the Models

Three exemplary areas of research that have improved the models used in risk extrapolation are time to tumor information, physiologically based TK modeling, and BBDR modeling (Albert, 1994). Chap. 7 discusses in detail improvements in our estimation of exposure and offers approaches on how to model "target internal effective dose" in risk assessment rather than just using single-value "external exposure doses." In this chapter we discuss the BBDR modeling.

BBDR modeling aims to make the generalized mechanistic models discussed in the previous section more clearly reflect specific biological processes. Measured rates are incorporated into the mechanistic equations to replace default or computer-generated values. For example, the Moolgavkar–Venson–Knudson (MVK) model is based on a two-stage model for carcinogenesis, where two mutations are required for carcinogenesis and birth and death rates of cells are modeled through clonal expansion and tumor formation. This model has been applied effectively to human epidemiologic data on retinoblastoma. In animal studies, kidney and liver tumors in the 2-acetylaminofluorene (2-AAF) "mega mouse" study, rat lung tumors following radiation exposure, rat liver tumors following *N*-nitrosomorpholine exposure, respiratory tract tumors following benzo[*a*]pyrene exposure, and mouse liver tumors following chlordane exposure have been modeled (Cohen and Ellwein, 1990; Moolgavkar and Luebeck, 1990).

There have been concerted research efforts to improve our mechanistic understanding of response at lower exposures. Two excellent examples of the use of biomarkers to supplement our exploration of these dose–response relationships are seen in Bailey et al. (2009) and Vlaanderen et al. (2011). Hormetic responses are U-shaped dose–response relationships where small doses of

A. Ingestion of arsenic from soil—point estimation method

$$\frac{\text{Soil concentration} \times \text{Ingestion rate} \times \text{Exposure duration} \times \text{Exposure frequency}}{\text{Body weight} \times \text{Averaging time}} \times \text{Bioavailability} \times q^* = \text{Lifetime cancer risk (ILCR) from skin cancer}$$

$$\frac{2300 \text{ mg/kg} \times 100 \text{ mg per day} \times 30 \text{ years} \times 350 \text{ days per year}}{70 \text{ kg} \times 25{,}550 \text{ days}} \times 0.09 \times 1.50 \text{ (mg/kg per day)}^{-1} = 1.8 \times 10^{-4}$$

B. Ingestion of arsenic from soil—probabilistic methods

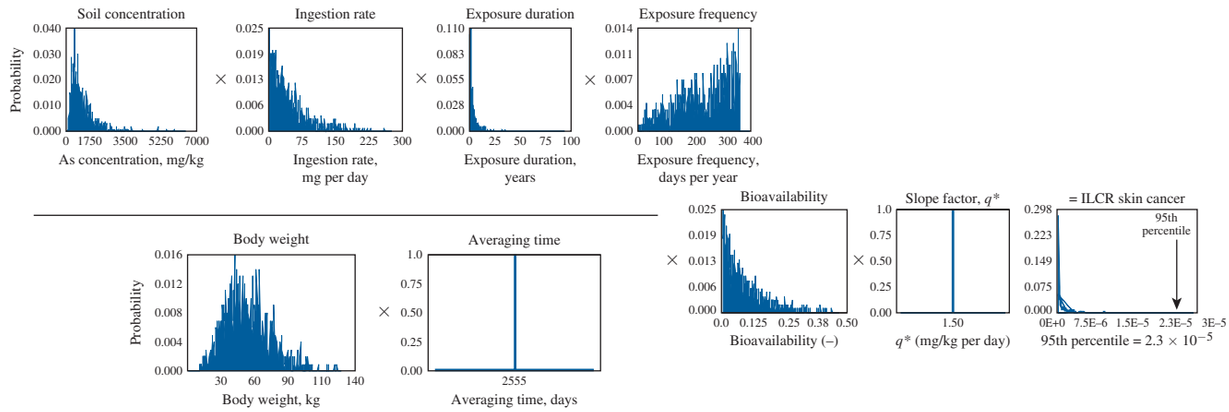

Figure 4-8. *Example of risk calculations for incremental lifetime cancer risk (ILCR) of skin cancer due to ingestion of arsenic in soil.* (**A**) Point exposure estimation method for calculation of ILCR. Point estimates for arsenic exposure input parameters are used in this example to calculate the ILCR. This exposure estimate is multiplied by the bioavailability of arsenic in soil to calculate the absorbed dose. Multiplication of the dose by the slope factor (q^*) yields the lifetime risk. (**B**) Probabilistic exposure methods for calculating the ILCR from arsenic ingestion. In this example, the soil concentration, ingestion rate, exposure duration and frequency, body weight, and bioavailability are modeled as distributions. Note that q^* and averaging time (years) are given as single-point estimations. This method yields a distribution of ILCR, with a 95th percentile upper confidence interval of 2.3×10^{-5}. (Data from Calabrese et al. [1989], U.S. EPA [1989, 1992b, 1999], Davis et al. [1990], Israeli and Nelson [1992], Brorby and Finley [1993], and Agency for Toxic Substances and Disease Registry [2007].)

a chemical can be beneficial but high doses are toxic. These are discussed in Chap. 2. These responses pose challenges to risk assessors. In general, science-based mechanistic considerations of low-dose response are always critical. (See approaches used by the Institute of Medicine for vitamins and essential elements as these compounds consistently exhibit hormesis [Institute of Medicine, 2008]).

Development of BBDR models for endpoints other than cancer is limited; however, several approaches have been explored in developmental toxicity, utilizing MOA information on cell cycle kinetics, enzyme activity, and cytotoxicity as critical endpoints (Faustman et al., 1989, 2005; Shuey et al., 1994; Leroux et al., 1996; Gohlke et al., 2002, 2004, 2005, 2007; Daston et al., 2004). Approaches have been proposed that link pregnancy-specific TK models with temporally sensitive TD models for developmental impacts (Faustman et al., 1999). Unfortunately, there is a lack of specific, quantitative biological information on both kinetics and dynamics for most toxicants and endpoints (NRC, 2000; Faustman et al., 2005). The new NRC report titled *Using 21st Century Science to Improve Risk-related Evaluations* (NRC, 2017) may provide new ideas on how to identify mechanistic findings that link epidemiology with mechanistic observations in in vitro and in vivo assays, hence new attention to mechanistic models may be forthcoming.

EXPOSURE ASSESSMENT

The primary objectives of exposure assessment are to determine source, type, magnitude, and duration of contact with the chemical(s) of interest. This is a critical element of the risk assessment process, as risk does not occur in the absence of exposure. However, it is also frequently identified as the key area of uncertainty in the overall risk determination. Here, the primary focus is on uses of exposure information in quantitative risk assessment.

The primary goal of exposure calculations is not only to determine the type and amount of total exposure but also to find out specifically who may be exposed and how large a dose may be reaching target tissues. A key step in making an exposure assessment is determining what exposure pathways are relevant for the risk scenario under development. Fig. 4-9 shows an example exposure diagram used to illustrate possible exposure pathways from a hazardous chemical release. The subsequent steps entail quantitation of each pathway identified as a relevant exposure and then summarizing these pathway-specific exposures for calculation of overall exposure. Such calculations can include an estimation of total exposures for a specified population as well as calculation of exposure for highly exposed individuals. The EPA has published numerous documents that provide detailed definitions and guidelines for determining such exposures (U.S. EPA, 1992b, 2008, 2011a), with the recognition that special considerations need to be adopted when assessing childhood exposures (U.S. EPA, 2005b, 2005c, 2008).

Conceptually, calculations are designed to represent "a plausible estimate" of exposure of individuals in the upper 90th to 95th percentile of the exposure distribution. Upper bound estimations would be "bounding calculations" designed to represent exposures at levels that exceed the exposures experienced by all individuals in the exposure distribution and are calculated by assuming upper limits for all exposure variables. A calculation for individuals exposed at concentrations near the middle of the exposure distribution is a

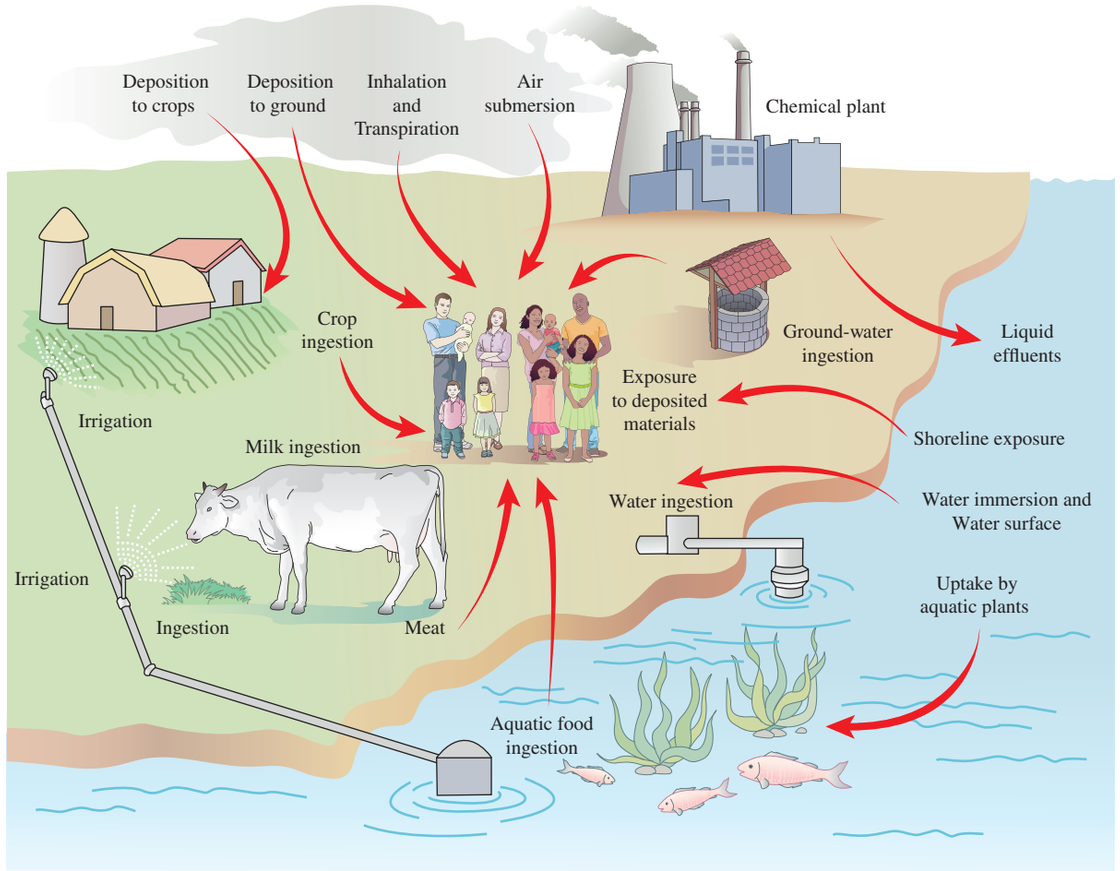

Figure 4-9. *Exposure pathway schematic.* Within the risk assessment process, a critical early step is the identification of the exposure pathway(s). To fully characterize exposure, the site- and chemical-specific exposure pathways must be identified. This hypothetical exposure pathway schematic illustrated the various ways in which contaminants can move from the source through media to points of exposure. (Adapted from Sexton et al. [1992]; reproduced with permission from Taylor & Francis Group, LLC, http://www.taylorandfrancis.com.)

central estimate. Fig. 4-8 gives example risk calculations using two types of exposure estimation procedures (U.S. EPA, 1989, 1992b, 2011a). Part A shows an example point estimation method for the calculation of arsenic (As) exposure via a soil ingestion route. In this hypothetical scenario, As exposure is calculated using point estimates for the upper 95th percent confidence limit of the arithmetic mean for each value in the lifetime average daily dose (LADD) equation. The LADD is calculated as follows:

$$\text{LADD} = \frac{\text{Concentration of the toxicant in soil} \times \text{Contact rate} \times \text{Exposure duration}}{\text{Bodyweight} \times \text{Lifetime}}.$$

In this scenario, the average time (AT) is lifetime (25,550 days) and the contact or ingestion rate (CR) is equal to 100 mg soil per day. The exposure frequency and duration (EDF) is equal to the exposure frequency (daily exposure = 365 days per year) times the exposure duration (30 years). To obtain the ILCR, the LADD must be multiplied by the bioavailability to obtain an absorbed dose and q^* where the upper 95% CLM slope (q^*) is 1.5× the change in risk per mg/kg body weight per day in this example.

Many exposures are now estimated using probability distributions for exposures rather than single-point estimates for the factors within the LADD equation (Finley et al., 1994; Cullen and Frey, 1999). Such approaches can provide a reality check and can be useful for generating more realistic exposure profiles reflective of population exposures. Good sources for U.S. exposures can be obtained from the EPA *Exposure Factors Handbook* (U.S. EPA, 2011a). Fig. 4-8 (Part B) shows how this is done using an example As risk scenario with soil As concentration, ingestion rate, exposure duration, frequency, body weight, and bioavailability modeled as distributed variables. Using Monte Carlo simulation techniques, an overall ILCR distribution can be generated and a 95th percentile for population risk obtained.

The FQPA of 1996 has highlighted the need for considerations of potential special risks for children and the need to evaluate child-specific exposures as well as cumulative exposures and risks (U.S. EPA, 1996b). The EPA released a supplemental document with child-specific exposure factors to address the special considerations necessary when evaluating childhood exposures (U.S. EPA, 2008). Table 4-7 shows example exposure factors for drinking water intake for children and adults. The EPA *Child-Specific Exposure Factors Handbook*, which is online, provides useful information about such exposure distributions. The FQPA also identified the need to evaluate total exposures by determining aggregate exposure measures for all exposures to a single substance. Cross-media exposure analyses,

Table 4-7

Example Exposure Factor Handbook Information: Drinking Water Intake for Children

AGE GROUP	INTAKE (ML PER DAY), MEAN	INTAKE (ML PER DAY), 95TH PERCENTILE	INTAKE (ML/KG PER DAY), MEAN	INTAKE (ML/KG PER DAY), 95TH PERCENTILE
Newborn (<1 month)	184	839	52	232
Infants (1 to <3 months)	227	896	48	205
Infants (3 to <6 months)	362	1056	52	159
Infants (6 to <12 months)	360	1055	41	126
Child (1 to <2 years)	271	837	23	71
Child (2 to <3 years)	317	77	23	60
Child (3 to <6 years)	380	1078	22	61
Child (6 to <11 years)	447	1235	16	43
Child (11 to <16 years)	606	1727	12	34
Child (16 to <18 years)	731	1983	11	31
Adult (18 to <21 years)	826	2540	12	35

SOURCES: Ershow and Cantor (1989) and U.S. EPA (2008, 2011a).

such as those conducted for lead and mercury, are good examples of the value of looking at such total exposures in evaluating human risks, and complex models are available for some compounds (see the EPA's integrated exposure uptake biokinetic [IEUBK] model for lead in children; U.S. EPA, 2010c). Cumulative exposures and cumulative risk refer to the total exposure and risks from a group of compounds. In the FQPA, chemicals with similar modes of toxicity are evaluated using cumulative exposure and risk estimates. For example, the EPA is identifying and categorizing pesticides that act by a common MOA, and cumulative exposures to organophosphates with similar MOAs (cholinesterase inhibition) have been used as an example of a class of pesticides for which cumulative exposure and cumulative risk estimates are needed (U.S. EPA, 1996b, 1998b; ILSI, 1999; Ryan, 2010). The WHO has recently published a framework for combined exposures (Meek et al., 2011).

Additional considerations for exposure assessments include how time and duration of exposure are evaluated in risk assessments. In general, estimates for cancer risk use average exposure over a lifetime, as lifetime average daily dose (see LADD example above). In a few cases, short-term exposure limits (STELs) are required (e.g., ethylene oxide and occupational exposure limits) and characterization of brief but high levels of exposure is significant. In these cases exposures are not averaged over the lifetime and the effects of high, short-term doses are directly estimated. With developmental toxicity, a single exposure can be sufficient to produce an adverse developmental effect if exposures occur during a window of developmental susceptibility; thus, daily doses are used, rather than lifetime weighted averages (U.S. EPA, 1991; Weller et al., 1999). ExpoCast, a part of the EPA's suite of computational tools, provides new insights on how to estimate population exposures and characterize exposure variability (Wambaugh et al., 2013).

RISK CHARACTERIZATION

Risk characterization is a summary of the risk assessment components and serves to outline the key findings and inform the risk manager in public health decisions. It is an analysis and integration of the conclusions from the hazard assessment, the dose–response assessment, and the exposure assessment. The EPA outlines the science policy and elements for completing a risk characterization in its *Risk Characterization Handbook* (U.S. EPA, 2000d). As shown in Fig. 4-1, risk characterization considers the nature, estimated incidence, and reversibility of adverse effects in a given population; how robust the evidence is; how certain the evaluation is; if susceptible populations are characterized; and if there is a relevant MOA.

For many years there has been an information sharing process aimed at harmonization of chemical testing regimes and clinical trial methodologies, so that data might be accepted in multiple countries. Efforts by the WHO and IPCS have included the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals Project (WHO, 2011a). This project has the goal of globally harmonizing but not standardizing the approaches to risk assessment by increasing understanding and developing basic principles and guidance on specific chemical risk assessment issues. The WHO has worked to harmonize dose–response methodologies (DRM) and released a report providing descriptive guidance for risk assessors in using DRM in hazard characterization (WHO, 2006b). Examples of other reports include those on terminology (WHO, 2004) and exposure assessment (WHO, 2006a) and *Uncertainty and Data Quality in Exposure Assessment* (WHO, 2008).

Uncertainty analysis is an essential component in our final risk characterization and includes factors such as variability and lack of knowledge (NRC, 1994). Variability refers to true differences such as is reflected in temporal, spatial, or interindividual differences. It usually cannot be reduced with further study. Lack of knowledge can also be discussed as a source of uncertainty and these factors may be reduced with further study. For example, if only a few environmental samples are taken from a contaminated site for analysis, then uncertainty can be reduced by taking further samples. Formal methods for dealing with uncertainty analysis are described in several references including IPCS (WHO, 2008) and Bogen et al. (2009). Using probabilistic-based approaches (PRA) for exposure assessment and assuming distributional versus dichotomous data for exposure factors are one way of addressing uncertainty that is commonly applied.

Variation in Susceptibility

Risk assessment methodologies incorporating human variability have been slow to develop. Generally, assay results utilize means and standard deviations to measure variation, or even standard errors of the mean. This ignores variability in response due to specific differences in age, sex, health status, and genetics. Default factors of 10× are overutilized to describe cross- and between-species differences and TK and TD data are rare. Nevertheless, EPA and OSHA are expected under the Clean Air Act and the Occupational Safety and Health Act to promulgate standards that protect the most susceptible subgroups or individuals in the population (Omenn et al., 1990; Cullen and Frey, 1999; Faustman and Omenn, 2006; Kramer et al., 2006; Cullen et al., 2008). Sensitive or susceptible populations have been defined and interpreted for the Clean Air Act and in this context "particularly sensitive citizens, such as bronchial asthmatics and emphysematics" are to be protected by the standards. This illustrates not only the importance but also the challenge to risk assessors to ensure protection for human populations.

Ecogenetics has been defined as the study of critical genetic determinants that define susceptibility to environmentally influenced adverse health effects (Costa and Eaton, 2006). Ecogenetic variation can affect biotransformation systems that activate and detoxify chemicals or alter the response in target tissues. With the completion of the Human Genome Project in 2003, the identification of human polymorphisms has greatly expanded our potential for understanding how genetic variability can impact biological response and susceptibility. There have been numerous activities initiated with the goal of understanding the linkage between genes and the environment. The following databases and initiatives are designed to identify polymorphisms, including single-nucleotide polymorphisms (SNP), insertions, deletions, amplifications, and alternative splicing. These include the International HapMap Project (HapMap, 2009), the DNA Polymorphism Discovery Resource (National Human Genome Research Institute, 2010), the National Center for Biotechnology Information (NCBI) SNP databases (NCBI, 2011a, 2011b), and the NIH GAIN program (National Human Genome Research Institute, 2010). Using the information from such databases, researchers can identify genetic similarities and differences in human beings with the goal of identifying genes that affect health, disease, and individual responses to medications and environmental factors (Omenn, 2012). Recent observations now demonstrate that not only genetics but also epigenetics is significant (Sartor et al., 2012). Databases such as ENCODE provide detailed information on cross-species epigenetic signals for a range of conditions (Fingerman et al., 2010; NHGRI, 2017). SNP arrays are available that can easily identify and characterize approximately 1×10^6 SNPs in the human population. Arrays also exist that evaluate SNPs in pathways significant for drug metabolism enzymes and transporters. These are in FDA-approved pathways and have been used for evaluation of appropriate drug therapies. However, they are just starting to be used for environmental risk assessment (Guerrette et al., 2011). Next-generation sequencing, where coverage of the genome can be extended to 40×, has revealed rare copy number variants that can be linked to disease and provide another level of understanding concerning human variation (Sudmant et al., 2010). It should be noted that this genetic information on individuals can raise ethical, social, and legal concerns regarding protections of individuals and their rights (Hsieh, 2004). Recent legislation that protects individuals from discrimination on the basis of genetic variation helps to ensure protection and not exclusion for all workers (Human Genome Program, 2008; United States Act, 2008).

One of the key challenges for toxicologists doing risk assessments will be the interpretation and linking of observations from highly sensitive molecular and genome-based methods with the overall process of toxicity (Eisen et al., 1998; Limbird and Taylor, 1998; Andersen and Barton, 1999; NRC, 2000). The basic need for linkage of observations was highlighted in early biomarker work. The NRC reports on biomarkers (NRC, 1989a, 1989b, 1992) drew distinctions for biomarkers of effect, exposure, and susceptibility across a continuum of exposure, effect, and disease/toxicity. Biomarkers of early effects, such as frank clinical pathology, arise as a function of exposure, response, and time. Early, subtle, and possibly reversible effects can generally be distinguished from irreversible disease states. Chemical-specific biomarkers have the potential to provide critical information, but there is an inherent complexity in pulling the information together. If biomarkers are considered a reflection of exposure or disease state, then considerations have to be made regarding the interactions between genes and the environment and the difference between a population-based assessment and individual assessment (Groopman and Kensler, 1999; NRC, 2017).

Nowhere is the challenge for interpretation of early and highly sensitive response biomarkers clearer than in the complicated data from gene expression arrays (toxicogenomics). Our continued ability to monitor changes in response in thousands of genes has proven challenging for toxicologists. Emphasis on toxicity-based risk assessment methods and analysis has been strongly encouraged by several significant NAS reports (NRC, 2000, 2007a, 2007b, 2010), as well as development and application of pathway-based methods (Yu et al., 2006). Toxicogenomics projects have confirmed the repeatability and cross-platform concordance of microarray data (Bammler et al., 2005; Guo et al., 2006; Nature Biotechnology Editorial, 2006; Patterson et al., 2006) and standards for data interpretation (minimum information about a microarray experiment [MIAME]) have been accepted (Brazma et al., 2001).

Microarray analysis for risk assessment requires analyses beyond basic cluster analysis (Eisen et al., 1998) to arrive at a functional interpretation and linkage to conventional toxicological (apical) endpoints. Because of the vast number of measured responses with gene expression arrays, pattern analysis techniques are being used through pathway mapping platforms such as MAPPFinder and GenMAPP (Moggs et al., 2004; Currie et al., 2005) as well as big data approaches (Conesa et al., 2016). The Gene Ontology Consortium has developed a controlled vocabulary (ontology) for sharing biological information across species. Database users can then annotate their gene products with gene ontology (GO) terms, establishing a consistent description and definition across databases and studies, and this facilitates risk assessment. Through the power of GO terms, analysis methods are being developed to allow for a quantitative time- and dose-dependent interpretation of genomic response using systems-based approaches to integrate responses at the biological and cellular levels (Yu et al., 2006). An example of integrated analysis of multiple -omics data sets is Conceptgen (Sartor et al., 2010). The 2017 NRC report provides a new framework for incorporating this information across various levels of biological observation.

Both the EPA and FDA have formally recognized the power of genomic information as well as its potential limitations. Both agencies have issued interim policies guiding how to include genomic information into risk assessments and they have agreed to use genomic information in conjunction with (but not in isolation of) standard risk assessment data (U.S. EPA, 2011e; U.S. FDA, 2011). Extensive research efforts by the EPA Computational Toxicology program will facilitate future risk assessment uses (Kavlock et al., 2012; U.S. EPA, 2012c).

INFORMATION RESOURCES

There are numerous information resources available for risk assessment and a few are listed below to provide the reader with examples, but not a comprehensive list of risk assessment resources and databases. Such resources include the Toxicology Data Network (NLM, 2017) from the U.S. National Library of Medicine; the NTP (NTP, 2011a), including the *Report on Carcinogens* and the *Office of Health Assessment and Translation*; the WHO IPCS (WHO, 2010); and the IARC (WHO, 2011b). The WHO Toolkit for Risk Assessment is especially user friendly for new risk assessors (WHO, 2010). The WHO, through its IPCS, produces useful risk documents entitled *Concise International Chemical Assessment Documents* that cover a variety of chemical substances (WHO, 2011c). The Codex Alimentarius Commission created by the Food and Agriculture Organization of the UN (FAO) and the WHO produces guidelines and reports relevant for food safety and standards (FAO and WHO, 2011). The Integrated Risk Information Service (IRIS) is the EPA's human health assessment program that evaluates health risks for over 500 chemical substances (U.S. EPA, 2011d). IRIS lists specific risk values used by the U.S. EPA. Additional databases are available from REACH.

There is a need for a note of caution for users of these risk-relevant databases, as with most databases there is a need to consider multiple contributions to differences between recommended values. These differences can be due to variations in how and when new scientific information is incorporated in the evaluations. Also, different regulatory drivers for national values or issues related to legal priorities or delays in implementing draft to final recommended values can occur. Such factors have contributed to differences in arsenic and dioxin risk estimates in the United States, for example, versus other observational values.

Recently, new toxicogenomic databases that identify and, in some cases, provide characterization of chemicals have become available. The NCBI (2011a, 2011b) provides access to an enormous set of biomedical and genomic information. The NCBI portfolio of comprehensive data and tools can be valuable for risk assessment and the aim to integrate with toxicologically relevant endpoints and disease is laudable. It contains information on chemicals and bioassays, data and software, DNA and RNA, protein domains and structures, genes and expression, genetic information databases linked with decision outcomes, genomes and gene maps, and cross-species homology. Resources covering databases and tools help in the study of SARs. A publicly available functional genomics data repository (Gene Expression Omnibus [GEO] database) supports MIAME-compliant data submission, and allows for easily accessible microarray- and sequence-based data, which are searchable for specific profiles of interest based on gene annotation or precomputed profile characteristics. Also, variations such as data on genomic variation, interaction of genotypes and phenotypes, and the single-nucleotide polymorphisms (dbSNP) can be obtained through the NCBI. ACToR (U.S. EPA, 2017b), the EPA's online database on chemical toxicity data and potential chemical risks to human health and the environment, is another useful resource for risk assessments (Judson et al., 2012). It allows users to search and query data including Toxicity Reference Database (ToxRefDB) (30 years and $2 billion worth of animal toxicity studies), ToxCastDB (data from screening 1000 chemicals in over 500 high-throughput assays), ExpoCastDB, and DSSTox (provides high-quality chemical structures and annotations). The ToxRefDB captures thousands of in vivo animal toxicity studies on hundreds of chemicals. Another curated Comparative Toxicogenomics Database (Comparative Toxicogenomics Database, 2017) includes data describing cross-species chemical and gene/protein interactions and chemical–disease and gene–disease relationships to illuminate molecular mechanisms underlying variable susceptibility and environmentally influenced diseases. These data provide insight into complex chemical–gene/protein interaction networks (Davis et al., 2013). These databases can be especially useful for hazard identification and mechanistic information; few emphasize exposure information.

RISK PERCEPTION AND COMPARATIVE ANALYSES OF RISK

It is well known that individuals respond differently to information about hazardous situations and products (Fischhoff, 1981; Fischhoff et al., 1993, 1996; Sandman, 1993; NRC, 1996; Risk Commission, 1997; Institute of Medicine, 1999; Slovic, 2010). Understanding these behavioral responses at the individual, community, and population levels is critical in stimulating constructive risk communication and evaluating potential risk management options for risk assessment issues. In a classic study, students, League of Women Voters members, active club members, and scientific experts were asked to rank 30 activities or agents in order of their annual contribution to deaths (Slovic et al., 1979, 2005). Club members ranked pesticides, spray cans, and nuclear power as safer than did other laypersons. Students ranked contraceptives and food preservatives as riskier and mountain climbing as safer than did others. Experts ranked electric power, surgery, swimming, and x-rays as more risky, but nuclear power and police work as less risky than did laypersons. From studies like these, we now know that there are cultural and gender differences in perception of risks. There are also group differences in perceptions of risk from chemicals among toxicologists, correlated with their employment in industry, academia, or government (Neal et al., 1994). Recent risk perception research has emphasized the importance of knowing the balance between analytical thinking and "affect." Our immediate emotional reaction can shape our initial risk perceptions ("affect"), while our thoughtful, deliberate "analytical" thinking also impacts our risk understanding but the latter is a slower response. Understanding this balance can help us explain why there is a complex relationship between perceived risk and benefits (Slovic, 2010).

Psychological factors such as dread, perceived uncontrollability, and involuntary exposure interact with factors that represent the extent to which a hazard is familiar, observable, and "essential" for daily living (Lowrance, 1976; Morgan, 1993). Fig. 4-10 presents a grid on the parameters controllable/uncontrollable and observable/not observable for a large number of risky activities; for each of the two-paired main factors, highly correlated factors are described in the boxes.

Public demand for government regulations often focuses on involuntary exposures (especially in the food supply, drinking water, and air) and unfamiliar hazards, such as radioactive waste, electromagnetic fields, asbestos insulation, and genetically modified crops and foods. The public can respond negatively when they perceive that information about hazards or new technologies has been withheld or under-rated. This can explain some of the very strong responses to genetically modified foods, HIV-contaminated blood transfusions in the 1980s, or hazardous chemical and radioactive wastes. Loss of trust is exemplified by Japanese reactions following the Fukushima Daiichi nuclear reactor meltdown (March 2011), where initial perceived benefits of nuclear power were transformed to distrust during follow-up actions and responses to the earthquake and tsunami.

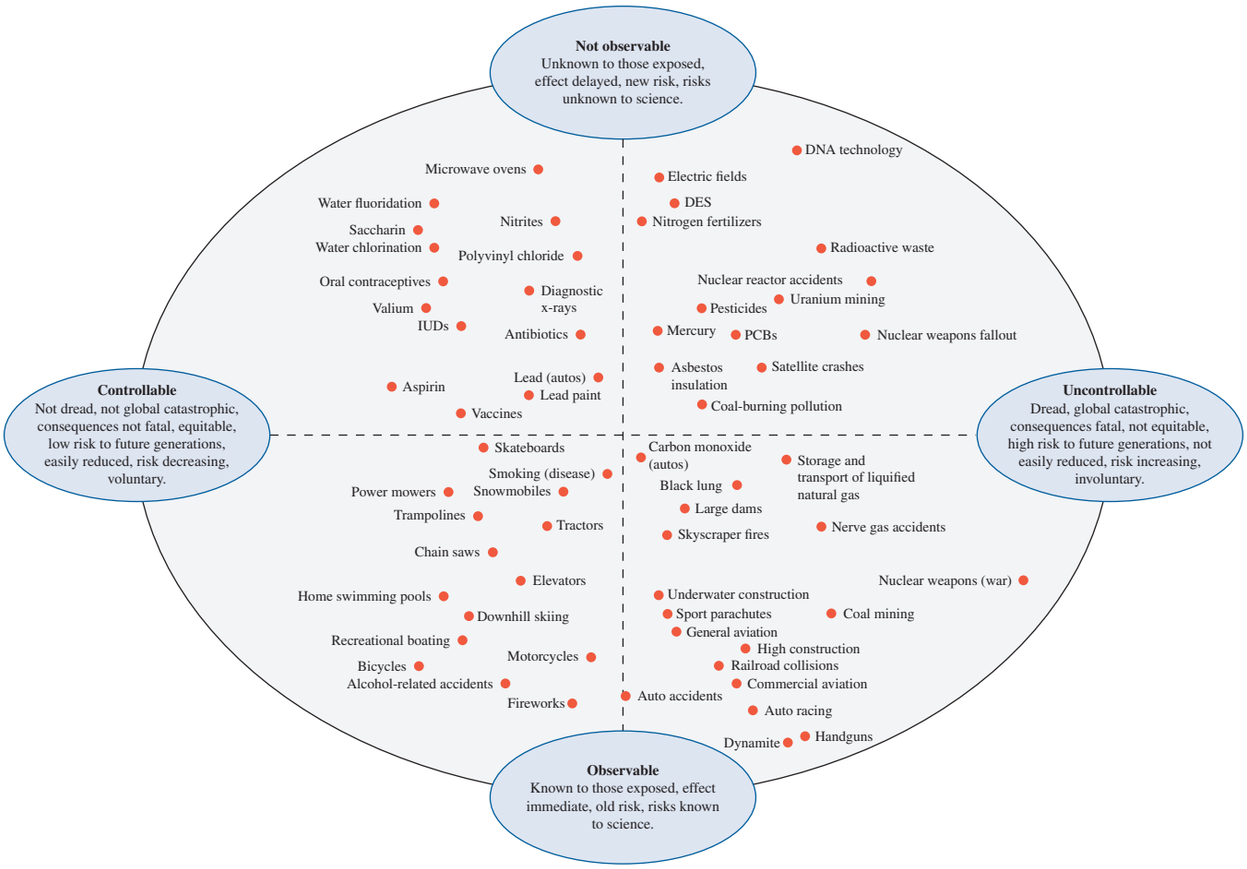

Figure 4-10. *Perceptions of risk illustrated using a "risk space" axis diagram.* Risk space has axes that correspond roughly to a hazard's perceived "dreadedness" and to the degree to which it is familiar or observable. Risks in the upper right quadrant of this space are most likely to provoke calls for government regulation.

Perceptions of risk led to the addition of an extra safety factor (default value 10) for children in the FQPA of 1996. Engineering-based "as low as reasonably achievable" (ALARA) approaches also reflect the general "precautionary principle," which is strongly favored by those who, justifiably, believe we are far from knowing all risks given frequently limited toxicity testing data (Roe et al., 1997).

EMERGING CONCEPTS

In order for risk assessment to inform environmental risk management, there is a need to ensure that the initial "problem formulation" of the risk question(s) is succinctly framed to answer questions in the real world. Environmental health is very dynamic and many divergent emerging environmental challenges such as climate change, energy shortages, and engineered nanoparticles will require an expansion of our context well beyond single-chemical, single-exposure scenarios. In order to accomplish this goal, several factors need to be considered. These factors include defining not only health but also well-being and sustainability and will require a context of global and international scale.

Well-being is increasingly being used to describe human health and the goal of sustainable environmental risk management. The WHO has championed the use of well-being rather than health to describe the Millennium Development Goals (WHO, 2011d). Well-being goes beyond "disease-free" existence to freedom from want (including food and water security) and fear (personal safety) and sustainable futures. Concepts such as food security (abundance and quality of foods), water security (plentiful supplies and high quality of water), and sustainability form internationally recognized environmental and developmental goals. Sustainability embraces the risk management concept that "development that meets the needs of the present, without compromising the ability of future generations" to thrive and hence well-being is one of the goals of environmental actions and decisions. Recognition that environmental problems are global is essential to our understanding of how we manage risks and how we address sustainability (Leiserowitz et al., 2006; Gohlke et al., 2008; Kite-Powell et al., 2008). Ocean health and air pollution are excellent examples of the need for understanding the global context where pollutants do not honor country and national borders. World Trade Organization (WTO) agreements have also placed risk assessment approaches in the forefront as tools for managers working to meet the global goals for sustainable trade and economic health. At the most basic level new risk assessments are being done with a new global emphasis (Carruth and Goldstein, 2004; EPA, 2011f). Reorganization of the EPA along management lines of sustainability now provides an integrative framework for working toward sustainability goals. As a response for this broader framing of health impacts, the EPA Office of Research and Development realigned its research within

a structure of Chemical Safety for Sustainability; Sustainable and Healthy Communities; Safe and Sustainable Water Resources; and Air, Climate and Energy. Risk assessment is viewed as one of the critical approaches for integrating these goals and toxicology is one of the tools used within that approach (Teichman and U.S. EPA, 2011). Further applications for alternatives assessment, life cycle analysis, and green chemistry open up new opportunities to enlarge the framing of our assessments (NRC, 2014a, 2014b).

PUBLIC HEALTH RISK MANAGEMENT

Associated with concepts of well-being and sustainability is a public health orientation to risk assessment and risk management. Public health as a discipline is very compatible with toxicology, where toxicological tests are performed to identify and characterize potential health risks and to prevent the unsafe use of such agents. Public health also has an emphasis on approaches for identifying, characterizing, and preventing risks.

Within public health risk management, there are three stages of prevention: *primary*, whose goal is prevention and risk or hazard avoidance; *secondary*, whose goal is mitigation or preparedness including risk or vulnerability reduction and risk transfer; and *tertiary*, where prompt response or recovery is an approach for decreasing residual risk or risk reduction (Frumkin, 2016). These three prevention stages frame many risk management actions. For example, in occupational health, a prevention hierarchy is applied that is related to these stages where initial preferred risk management actions are substitution where the hazardous chemical or activities is removed and substituted with a safer chemical or process option. Engineering controls with a focus on reducing workplace exposures is a subsequent action. Administrative controls can be used to change job tasks and methods. The use of personal protective equipment (PPE) such as gloves, hats, protective gloves, and work clothes is usually considered after all other management efforts fail to meet occupationally safe levels. In a similar manner, pollution prevention can be related to these stages of prevention with initial elimination of the source of hazardous chemical.

The context for prevention actions can be supported by source reduction or a life-cycle analysis or alternatives analysis. If the former, the prevention activities would consider potential for adverse outcomes along the life of the product including manufacture as well as end-of-life disposal options. Sometimes when an alternatives analysis is performed, only one stage of the life of a product is examined and a "regrettable substitution" can occur where the alternative is either less well studied or knowledge about use and fate and transport is missing (Goldman, 2016).

Hazard analysis frequently used in public health framework refers to both hazard identification and probability-based frequency of anticipated events. Capacity assessment has been used for identifying strengths and resiliency of a system to impact. Recent disasters such as Hurricane Katrina, the Gulf Oil Spill, and the Fukushima Daiichi nuclear reactor meltdown all point to the need for environmental risk assessment to be considered as a part of an even larger context in order to understand critical infrastructure for determining human and environmental risks. Life-cycle analysis, green chemistry, and built environment all require such a broad context for evaluation (Keim, 2002; NRC, 2009). Understanding population variability and vulnerability in a context of "built environment" is also important. The "built environment" encompasses all man-made resources and infrastructure, including buildings, spaces, and transportation systems, which support human activity and have a strong influence on public health (Perdue et al., 2003). These types of frameworks can allow for easier consideration of public health concepts for sustainability, environmental disaster response, and life-cycle systems analysis than many of our traditional frameworks for environmental chemical risk assessment, which can be done in relative isolation.

SUMMARY

The NRC and Risk Commission frameworks for risk assessment and risk management continue to provide a consistent framework-based approach for evaluating risks and taking action to reduce risks. The objectives of risk assessments vary with the issues, risk management needs, and statutory requirements. Hence, setting the context and problem formation for risk evaluation is essential (NRC, 2009). The frameworks are sufficiently flexible to address various objectives and to accommodate new knowledge while also providing guidance for priority setting in industry, environmental organizations, and government regulatory and public health agencies. Risk assessment analyzes the science and, if incomplete, identifies uncertainty and provides approaches for moving forward with decisions (NRC, 2009). Toxicology, epidemiology, exposure assessment, and clinical observations can be linked with biomarkers, cross-species investigations of mechanisms of effects, and systematic approaches to risk assessment, risk communication, and risk management. New insights from the NRC "Using 21st century science to improve risk-related evaluations" provide suggestions on how to achieve integration (NRC, 2017). Advances in toxicology are certain to improve the quality of risk assessments for a broad array of health endpoints as scientific findings substitute data for assumptions and help to describe and model uncertainty more credibly.

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REFERENCES

Abbott BD, Harris MW, Birnbaum LS. Comparisons of the effects of TCDD and hydrocortisone on growth factor expression provide insight into their interaction in the embryonic mouse palate. *Teratology*. 1992;45:35–53.

Adami HO, Berry SC, Breckenridge CB, et al. Toxicology and epidemiology: improving the science with a framework for combining toxicological and epidemiological evidence to establish causal inference. *Toxicol Sci*. 2011;122:223–234.

Adler S, Basketter D, Creton S, et al. Alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010. *Arch Toxicol*. 2011;85:367–485.

Agency for Toxic Substances and Disease Registry. *Toxicological Profile for Arsenic*. Atlanta, GA: Department of Health and Human Services; 2007.

Albert RE. Carcinogen risk assessment in the US Environmental Protection Agency. *Crit Rev Toxicol*. 1994;24:75–85.

Allen BC, Kavlock RJ, Kimmel CA, Faustman EM. Dose-response assessments for developmental toxicity: II. Comparison of generic benchmark dose estimates with no observed adverse effect levels. *Fundam Appl Toxicol*. 1994a;23:487–495.

Allen BC, Kavlock RJ, Kimmel CA, Faustman EM. Dose-response assessment for developmental toxicity. 3. Statistical models. *Fundam Appl Toxicol*. 1994b;23(4):496–509.

Ames BN, Gold LS. Too many rodent carcinogens: mitogenesis increases mutagenesis. *Science*. 1990;247:970–971.

Andersen ME, Barton HA. Biological regulation of receptor–hormone complex C in relation to dose–response assessments of endocrine-active compounds. *Toxicol Sci*. 1999;48:38–50.

Ankley GT, Bennett RS, Erickson RJ, et al. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem*. 2010;29(3): 730-741.

Armitage P, Doll R. A two-stage theory of carcinogenesis in relation to the age distribution of human cancer. *Br J Cancer*. 1957;11:161–169.

Ashby J, Tennant RW. Prediction of rodent carcinogenicity of 44 chemicals: results. *Mutagenesis*. 1994;9:7–15.

Atterwill CK, Johnston H, Thomas SM. Models for the in vitro assessment of neurotoxicity in the nervous system in relation to xenobiotic and neurotrophic factor-mediated events. *Neurotoxicology*. 1992;13: 39–54.

Auton TR. Calculation of benchmark doses from teratology data. *Regul Toxicol Pharmacol*. 1994;19:152–167.

Bailey GS, Reddy AP, Pereira CB, et al. Nonlinear cancer response at ultralow dose: a 40800-animal ED(001) tumor and biomarker study. *Chem Res Toxicol*. 2009;22:1264–1276.

Bammler T, Beyer RP, Bhattacharya S, et al. Standardizing global gene expression analysis between laboratories and across platforms. *Nat Methods*. 2005;2:351–356.

Barnes DG, Dourson MJ. Reference dose (Rfd): description and use in health risk assessment. *Regul Toxicol Pharmacol*. 1988;8:471–486.

Bell SM, Phillips J, Sedykh A, et al. An integrated chemical environment to support 21st-century toxicology. *Environ Health Perspect*. 2017;125(5):054501.

Benigni R, Zito R. The second National Toxicology Program comparative exercise on the prediction of rodent carcinogenicity: definitive results. *Mutat Res*. 2004;566:49–63.

Birnbaum LS, Thayer KA, Bucher JR, Wolfe MS. Implementing systematic review at the National Toxicology Program: status and next steps. *Environ Health Perspect*. 2013;121(4):a108–a109.

Bogdanffy MS, Daston G, Faustman EM, et al. Harmonization of cancer and noncancer risk assessment: proceedings of a consensus-building workshop. *Toxicol Sci*. 2001;61:18–31.

Bogen KT, Cullen AC, Frey HC, Price PS. Probabilistic exposure analysis for chemical risk characterization. *Toxicol Sci*. 2009;109:4–17.

Brazma A, Hingamp P, Quackenbush J, et al. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. *Nat Genet*. 2001;29:365–371.

Brorby G, Finley G. Standard probability density functions for routine use in environmental health risk assessment. In: *Society for Risk Analysis Annual Meeting*. Savannah, GA; 1993.

Brown CC. High-to-low-dose extrapolation in animals. In: Rodricks JV, Tardiff RG, eds. *Assessment and Management of Chemical Risks*. Washington, DC: American Chemical Society; 1984:57–79.

Brown NA, Spielmann H, Bechter R. Screening chemicals for reproductive toxicity: the current alternatives: the report and recommendations of an ECVAM/ETS workshop. *Altern Lab Anim*. 1995;23:868–882.

Bucher JR. Update on National Toxicology Program (NTP) assays with genetically altered or "transgenic" mice. *Environ Health Perspect*. 1998;106:619–621.

Calabrese EJ, Pastides H, Barnes R. How much soil do young children ingest: an epidemiologic study. In: Kostecki PT, Calabrese EJ, eds. *Petroleum Contaminated Soils*. Chelsea, MI: Lewis Publishers; 1989:363–397.

Calkins DR, Dixon RI, Gerber CR. Identification, characterization, and control of potential human carcinogens: a framework for federal decision-making. *J Natl Cancer Inst*. 1980;61:167–175.

Capen CC, Dybing E, Rice JM, Wilbourn JD, eds. *Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis*. IARC Scientific Publications No. 147. International Agency for Research on Cancer and World Health Organization; 1999.

Carruth RS, Goldstein BD. The asbestos case: a comment on the appointment and use of nonpartisan experts in World Trade Organization dispute resolution involving health risk. *Risk Anal*. 2004;24:471–481.

Charnley G, Omenn GS. A summary of the findings and recommendations of the commission on risk assessment and risk management (and accompanying papers prepared for the commission). *Hum Ecol Risk Assess*. 1997;3:701–711.

Cheng H, Aylward L, Beall C, et al. TCDD exposure-response analysis and risk assessment. *Risk Anal*. 2006;26(4):1059–1071.

Chesler EJ, Miller DR, Branstetter LR, et al. The Collaborative Cross at Oak Ridge National Laboratory: developing a powerful resource for systems genetics. *Mamm Genome*. 2008;19:382–389.

Chhabra RS, Bucher JR, Wolfe M, Portier C. Toxicity characterization of environmental chemicals by the US National Toxicology Program: an overview. *Int J Hyg Environ Health*. 2003;206:437–445.

Cohen SM. Human relevance of animal carcinogenicity studies. *Regul Toxicol Pharmacol*. 1995;21:75–80 [discussion 81–86].

Cohen SM. Alternative models for carcinogenicity testing: weight of evidence evaluations across models. *Toxicol Pathol*. 2001;29(suppl): 183–190.

Cohen SM, Arnold LL, Cano M, Ito M, Garland EM, Shaw RA. Calcium phosphate-containing precipitate and the carcinogenicity of sodium salts in rats. *Carcinogenesis*. 2000;21:783–792.

Cohen SM, Ellwein LB. Proliferative and genotoxic cellular effects in 2-acetylaminofluorene bladder and liver carcinogenesis: biological modeling of the EDO1 study. *Toxicol Appl Pharmacol*. 1990;104:79–93.

Collins FS, Gray GM, Bucher JR. Toxicology. Transforming environmental health protection. *Science*. 2008;319(5865):906-907.

Comparative Toxicogenomics Database. Available at: http://ctd.mdibl.org/. 2017.

Conesa A, Madrigal P, Tarazona S, et al. A survey of best practices for RNA-seq data analysis. *Genome Biol*. 2016; 17: 13.

Costa L, Eaton D. Introduction. In: Costa L, Eaton D, eds. *Gene–Environment Interactions: Fundamentals of Ecogenetics*. Hoboken, NJ: Wiley; 2006.

Costa LG. Biochemical and molecular neurotoxicology. In: Maines MD, Costa LG, Reed DJ, eds. *Current Protocols in Toxicology*. New York: Wiley; 2000.

Crump KS. An improved procedure for low-dose carcinogenic risk assessment from animal data. *J Environ Pathol Toxicol*. 1980;5:339–348.

Crump KS. A new method for determining allowable daily intakes. *Fundam Appl Toxicol*. 1984;4:854–871.

Cullen AC, Corrales MA, Kramer CB, Faustman EM. The application of genetic information for regulatory standard setting under the Clean Air Act: a decision-analytic approach. *Risk Anal*. 2008;28:877–890.

Cullen AC, Frey HC. *Probabilistic Techniques in Exposure Assessment: A Handbook for Dealing with Variability and Uncertainty in Models and Inputs*. New York: Plenum Press; 1999.

Currie RA, Bombail V, Oliver JD, et al. Gene ontology mapping as an unbiased method for identifying molecular pathways and processes affected by toxicant exposure: application to acute effects caused by the rodent non-genotoxic carcinogen diethylhexylphthalate. *Toxicol Sci*. 2005;86:453–469.

Da Rocha MS, Arnold LL, De Oliveira ML, et al. Diuron-induced rat urinary bladder carcinogenesis: Mode of action and human relevance evaluations using the International Programme on Chemical Safety framework. *Crit Rev Toxicol*. 2014;44(5):393-406.

Daston G, Faustman EM, Ginsberg G, et al. A framework for assessing risks to children from exposure to environmental agents. *Environ Health Perspect*. 2004;112:238–256.

Davis AP, Murphy CG, Johnson R, et al. The comparative toxicogenomics database: update 2013. *Nucleic Acids Res*. 2013;41(database issue):D1104–D1114.

Davis S, Waller P, Buschborn R. Quantitative estimates of soil ingestion in normal children between the ages of 2 and 7 years: population-based estimates using aluminum, silicon, and titanium as soil tracer elements. *Arch Environ Health*. 1990;45:112–122.

Dean AG, Dean JA, Coulombier D. *Epi Info, Version 6: A Word Processing, Database, and Statistics Program for Public Health on IBM-Compatible Microcomputers*. Atlanta, GA: Centers for Disease Control and Prevention (CDC); 1995.

Dearfield KL. *Pesticide Assessment Guidelines. Subdivision F, Hazard Evaluation: Humans and Domestic Animals*. Series 83-3. Washington, DC: Environmental Protection Agency; 1990.

Dourson MJ, Hertzberg RC, Hartung R, Blackburn K. Novel methods for the estimation of acceptable daily intake. *Toxicol Ind Health*. 1985;1: 23–41.

Dourson ML, DeRosa CT. The use of uncertainty factors in establishing safe levels of exposure. In: Krewski D, Franklin C, eds. *Statistics in Toxicology*. New York: Gordon & Breach; 1991:613–627.

Dourson ML, Stara JF. Regulatory history and experimental support of uncertainty (safety factors). *Regul Toxicol Pharmacol*. 1983;3:224–238.

Drew CH, Grace DA, Silbernagel SM, et al. Nuclear waste transportation: case studies of identifying stakeholder risk information needs. *Environ Health Perspect*. 2003;111:263–272.

Drew CH, Kern M, Martin T, Blozek ML, Power M, Faustman EM. The Hanford Openness Workshops: fostering open and transparent long-term decision making at the department of energy. In: Leschine TM, ed. *Long-Term Management of Contaminated Sites (Research in Social Problems and Public Policy, Volume 13)*. Emerald: Bingley, West Yorkshire UK; 2006;13–48.

Eaton DL, Bammler TK, Kelly EJ. Interindividual differences in response to chemoprotection against aflatoxin-induced hepatocarcinogenesis: implications for human biotransformation enzyme polymorphisms. *Adv Exp Med Biol*. 2001;500:559–576.

Eaton DL, Gallagher EP. Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol*. 1994;34:135–172.

Eaton DL, Gallagher EP, Bammler TK, Kunze KL. Role of cytochrome P4501a2 in chemical carcinogenesis—implications for human variability in expression and enzyme-activity. *Pharmacogenetics*. 1995;5:259–274.

ECHA (European Chemicals Agency). Registered substances database. 2017. Available at: https://echa.europa.eu/information-on-chemicals/registered-substances. Accessed 23 January, 2017.

Eduati F, Mangravite LM, Wang T, et al. Prediction of human population responses to toxic compounds by a collaborative competition. *Nat Biotechnol*. 2015;33(9):933-40.

EGRET. *Statistics and Epidemiology Research Corporation (SERC)*. Seattle, WA: Statistics and Epidemiology Research Corporation; 1994.

Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A*. 1998;95:14863–14868.

Ershow AG, Cantor KP. Federation of American Societies for Experimental Biology. Life Sciences Research Office, and National Cancer Institute (US). Division of Cancer Etiology. Epidemiology and Biostatistics Program. *Total Water and Tapwater Intake in the United States: Population-based Estimates of Quantities and Sources*. Bethesda, MD: Life Sciences Research Office Federation of American Societies for Experimental Biology; 1989.

FAO, WHO. *Codex Alimentarius Commission—International Food Standards*. 2011. Available at: http://www.codexalimentarius.org/.

Faustman E, Omenn G. Risk assessment and the impact of ecogenetics. In: Costa L, Eaton D, eds. *Gene–Environment Interactions: Fundamentals of Ecogenetics*. Hoboken, NJ: Wiley; 2006.

Faustman EM. Short-term test for teratogens. *Mutat Res*. 1988;205:355–384.

Faustman EM, Allen BC, Kavlock RJ, Kimmel CA. Dose–response assessment for developmental toxicity: I. Characterization of data base and determination of no observed adverse effect levels. *Fundam Appl Toxicol*. 1994;23:478–486.

Faustman EM, Gohlke J, Judd NL, et al. Modeling developmental processes in animals: applications in neurodevelopmental toxicology. *Environ Toxicol Pharmacol*. 2005;19:615–624.

Faustman EM, Gohlke JM, Ponce RA, et al. Experimental approaches to evaluate mechanisms of developmental toxicity. In: Hood R, ed. *Developmental and Reproductive Toxicology: A Practical Approach*. 3rd ed. Boca Raton, FL: CRC Press; 2011.

Faustman EM, Lewandowski TA, Ponce RA, Bartell SM. Biologically based dose–response models for developmental toxicants: lessons from methylmercury. *Inhal Toxicol*. 1999;11:101–114.

Faustman EM, Ponce RA, Seeley MR, Whittaker SG. Experimental approaches to evaluate mechanisms of developmental toxicity. In: Hood R, ed. *Handbook of Developmental Toxicology*. Boca Raton, FL: CRC Press; 1997;13–41.

Faustman EM, Wellington DG, Smith WP, Kimmel CS. Characterization of a developmental toxicity dose response model. *Environ Health Perspect*. 1989;79:229–241.

Fingerman IM, McDaniel L, Zhang X, et al. NCBI epigenomics: a new public resource for exploring epigenomic data sets. *Nucleic Acids Research*. 2010;39(suppl 1):D908–D912.

Finley G, Proctor D, Scott P. Recommended distributions for exposure factors frequently used in health risk assessment. *Risk Anal*. 1994;14:533–553.

Fischhoff B. Cost–benefit analysis: an uncertain guide to public policy. *Am N Y Acad Sci*. 1981;363:173–188.

Fischhoff B, Bostrom A, Quandrel MJ. Risk perception and communication. *Annu Rev Public Health*. 1993;14:183–203.

Fischhoff B, Bostrom A, Quandrel MJ. Risk perception and communication. In: Detels R, Holland W, McEwen J, Omenn GS, eds. *Oxford Textbook of Public Health*. New York: Oxford University Press; 1996:987–1002.

Frumkin H. Introduction to environmental health. In: Frumkin H, ed. *Environmental Health: From Global to Local*. 3rd ed. San Francisco, CA: Wiley; 2016:3–25.

Gamble JF, Battigelli MC. Epidemiology. In: Clayton GD, Clayton FE, eds. *Patty's Industrial Hygiene and Toxicology*. New York: Wiley; 1978:113–127.

Gamble JF, Battigelli MC. Occupational epidemiology: some guideposts. In: Clayton GD, Clayton FE, eds. *Patty's Industrial Hygiene and Toxicology*. New York: Wiley; 1991.

Gargas ML, Finley BL, Paustenback DJ, Long TF. Environmental health risk assessment: theory and practice. In: Ballantyne B, Marrs T, Syversen T, eds. *General and Applied Toxicology*. New York: Grove's Dictionaries; 1999:1749–1809.

Gill EA, Curl CL, Adar SD, et al. Air pollution and cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis. *Prog Cardiovasc Dis*. 2011;53(5):353–360.

Gohlke JM, Griffith WC, Bartell SM, Lewandowski TA, Faustman EM. A computational model for neocortical neuronogenesis predicts ethanol-induced neocortical neuron number deficits. *Dev Neurosci*. 2002;24:467–477.

Gohlke JM, Griffith WC, Faustman EM. The role of cell death during neocortical neurogenesis and synaptogenesis: implications from a computational model for the rat and mouse. *Brain Res Dev Brain Res*. 2004;151:43–54.

Gohlke JM, Griffith WC, Faustman EM. A systems-based computational model for dose–response comparisons of two mode of action hypotheses for ethanol-induced neurodevelopmental toxicity. *Toxicol Sci*. 2005;86:470–484.

Gohlke JM, Griffith WC, Faustman EM. Computational models of neocortical neuronogenesis and programmed cell death in the developing mouse, monkey, and human. *Cereb Cortex*. 2007;17:2433–2442.

Gohlke JM, Hrynkow SH, Portier CJ. Health, economy, and environment: sustainable energy choices for a nation. *Environ Health Perspect*. 2008;116:A236–A237.

Goldman L. Environmental public health: from theory to practice. In: Frumkin H, ed. *Environmental Health: From Global to Local*. 3rd ed. San Francisco, CA: Wiley; 2016:725–746.

Gray TJB. Application of in vitro systems in male reproductive toxicology. In: Lamb JC IV, Foster PMD, eds. *Physiology and Toxicology of Male Reproduction*. San Diego, CA: Academic Press; 1988:250–253.

Great Britain, Health and Safety Executive. *Reducing Risks, Protecting People: Health & Safety Executive*. Suffolk, UK: HSE Books; 1999.

Gribble EJ, Hong SW, Faustman EM. The magnitude of methylmercury-induced cytotoxicity and cell cycle arrest is p53-dependent. *Birth Defects Res A Clin Mol Teratol*. 2005;73:29–38.

Groopman JD, Kensler TW. The light at the end of the tunnel for chemical-specific biomarkers: daylight or headlight? *Carcinogenesis*. 1999;20:1–11.

Guerrette Z, Moreira EG, Griffith WC, et al. Cytochrome P450 3A5 genotype is correlated with acetylcholinesterase inhibition levels after exposure to organophosphate pesticides. Abstract 2111. *The Toxicologist CD—An Official Journal of the Society of Toxicology*. 2011;120(March).

Guo L, Lobenhofer EK, Wang C, et al. Rat toxicogenomic study reveals analytical consistency across microarray platforms. *Nat Biotechnol*. 2006;24:1162–1169.

HapMap. *International HapMap Project*. 2009. Available at: http://snp.cshl.org/index.html.en.

Harris MW, Chapin RE, Lockhart AC. Assessment of a short-term reproductive and developmental toxicity screen. *Fundam Appl Toxicol*. 1992;19:186–196.

Hartung R. Dose–response relationships. In: Tardiff RG, Rodricks JV, eds. *Toxic Substances and Human Risk: Principles of Data Interpretation*. New York: Plenum Press; 1987:29–46.

Hartung T, Blaauboer BJ, Bosgra S, et al. An expert consortium review of the EC-commissioned report "alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010". *ALTEX*. 2011;28:183–209.

Hartung T, Bremer S, Casati S, et al. ECVAM's response to the changing political environment for alternatives: consequences of the European Union chemicals and cosmetics policies. *Altern Lab Anim*. 2003;31:473–481.

Haseman JK, Lockhart AM. Correlations between chemically related site-specific carcinogenic effects in long-term studies in rats and mice. *Environ Health Perspect*. 1993;101:50–54.

Haws LC, Su SH, Harris M, et al. Development of a refined database of mammalian relative potency estimates for dioxin-like compounds. *Toxicol Sci*. 2006;89:4–30.

Healey GF. Power calculations in toxicology. *Altern Lab Anim*. 1987;15:132–139.

Hill AB. The environment and disease: association or causation. *Proc R Soc Med*. 1965;58:295–300.

Hsieh A. A nation's genes for a cure to cancer: evolving ethical, social and legal issues regarding population genetic databases. *Columbia J Law Soc Probl*. 2004;37:359–411.

Human Genome Program. *Breaking News: GINA Becomes Law May 2008*. Genetics Privacy and Legislation. 2008. Available at: http://www.ornl.gov/sci/techresources/Human_Genome/elsi/legislat.shtml. Accessed 2011.

IARC. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. Lyon, France: World Health Organization; 2000.

IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans*. Lyon, France: International Agency for Research on Cancer; 2017.

ICCVAM. *The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds: The Results of an Independent Peer Review Evaluation*. Research Triangle Park, NC: National Institute of Environmental Health Sciences; 1999.

ICCVAM. *ICCVAM Test Method Evaluation Report on using the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products*. Research Triangle Park, NC: National Institute of Environmental Health Sciences; 2011a.

ICCVAM. *Independent Scientific Peer Review Panel Report Evaluation of the LUMI-CELL®ER (BG1Luc ER TA) Test Method*. Research Triangle Park, NC: National Institute of Environmental Health Sciences; 2011b.

ICCVAM. *Validation of the BG1Luc Estrogen Receptor Transcriptional Activation Test Method*. Research Triangle Park, NC: National Institute of Environmental Health Sciences. 2012. Available at: http://iccvam.niehs.nih.gov/methods/endocrine/end_eval.htm.

ICCVAM. Available at: https://ntp.niehs.nih.gov/iccvam/docs/about_docs/icatm-moc-mar11.pdf.

ILSI. *A Framework for Cumulative Risk Assessment*. Washington, DC: International Life Science Institute; 1999.

Institute of Medicine. *Toward Environmental Justice. Research, Education, and Health Policy Needs*. Washington, DC: National Academies Press; 1999.

Institute of Medicine. *Development of DRIs 1994–2004 Lessons Learned and New Challenges: Workshop Summary*. Washington, DC: National Academies Press; 2008.

Israeli M, Nelson CB. Distribution and expected time of residence for United States households. *Risk Anal*. 1992;12:65–72.

Johnson DE, Wolfgang GHI, Gledin MA, Braeckman RA. Toxicokinetics and toxicodynamics. In: Sipes I, McQueen C, Gandolfi A, eds. *Comprehensive Toxicology*. Oxford, UK: Pergamon; 1997:169–181.

Judd NL, Drew CH, Acharya C, et al. Framing scientific analyses for risk management of environmental hazards by communities: case studies with seafood safety issues. *Environ Health Perspect*. 2005;113:1502–1508.

Judson RS, Martin MT, Egeghy P, et al. Aggregating data for computational toxicology applications: the US Environmental Protection Agency (EPA) aggregated computational toxicology resource (ACToR) system. *Int J Mol Sci*. 2012;13(2):1805–1831.

Kavlock RJ, Allen BC, Faustman EM, Kimmel CA. Dose response assessments for developmental toxicity: IV. Benchmark doses for fetal weight changes. *Fundam Appl Toxicol*. 1995;26:211–222.

Kavlock R, Chandler K, Houck K, et al. Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. *Chem Res Toxicol*. 2012;25(7):1287–302.

Keim M. Intentional chemical disasters. In: Hogan D, Burstein J, eds. *Disaster Medicine*. Philadelphia, PA: Lippincott Williams & Wilkins; 2002:340–348.

Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci*. 2011;120(suppl 1):S28–S48.

Khoury MJ. Human genome epidemiology: translating advances in human genetics into population-based data for medicine and public health. *Genet Med*. 1999;1:71–73.

Kite-Powell HL, Fleming LE, Backer LC, et al. Linking the oceans to public health: current efforts and future directions. *Environ Health*. 2008;7:S6.

Klaunig JE, Babich M, Baetcke K, et al. PPARα agonist-induced rodent tumors: modes of action and human relevance. *Crit Rev Toxicol*. 2003;33:655–780.

Koturbash I, Scherhag A, Sorrentino J, et al. Epigenetic mechanisms of mouse interstrain variability in genotoxicity of the environmental toxicant 1,3-butadiene. *Toxicol Sci*. 2011;122:448–456.

Kramer CB, Cullen AC, Faustman EM. Policy implications of genetic information on regulation under the Clean Air Act: the case of particulate matter and asthmatics. *Environ Health Perspect*. 2006;114:313–319.

Krewski D, Van Ryzin J. Dose-response models for quantal response toxicity data. In: Csorgo M, Dawson D, Rao J, Seleh A, eds. *Statistics and Related Topics*. Amsterdam: North-Holland; 1981:201–229.

Lave LB, Ennever F, Rosenkranz HS, Omenn GS. Information value of the rodent bioassay. *Nature*. 1988;336:631–633.

Lave LB, Omenn GS. Cost-effectiveness of short-term tests for carcinogenicity. *Nature.* 1986;334:29–34.

Leiserowitz AA, Kates RW, Parris TM. Sustainability values, attitudes, and behaviors: a review of multinational and global trends. *Annu Rev Environ Resour*. 2006;31:413–444.

Leroux BG, Leisenring WM, Moolgavkar SH, Faustman EM. A biologically based dose–response model for development. *Risk Anal*. 1996;16:449–458.

Lewandowski TA, Ponce RA, Whittaker SG, Faustman EM. In vitro models for evaluating developmental toxicity. In: Gad S, ed. *Vitro Toxicology*. New York: Taylor & Francis; 2000:139–187.

Limbird LE, Taylor P. Endocrine disruptors signal the need for receptor models and mechanisms to inform policy. *Cell*. 1998;93:157–163.

Liu R, Rallo R, George S, et al. Classification NanoSAR development for cytotoxicity of metal oxide nanoparticles. *Small*. 2011;7:1118–1126.

Lowrance WW. *Of Acceptable Risk: Science and the Determination of Safety*. Los Altos, CA: William Kaufmann; 1976.

Maynard AD, Aitken RJ, Butz T, et al. Safe handling of nanotechnology. *Nature*. 2006;444:267–269.

McClain RM. Mechanistic considerations in the regulation and classification of chemical carcinogens. In: Kotsonis F, Mackey M, Hjelle J, eds. *Nutritional Toxicology*. New York: Raven Press; 1994:278–304.

McGregor DB, Rice JM, Venitt S. *The Use of Short- and Medium-Term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation*. Lyon, France: IARC; 1999.

Meek ME, Boobis AR, Crofton KM, et al. Risk assessment of combined exposure to multiple chemicals: a WHO/IPCS framework. *Regul Toxicol Pharmacol*. 2011;60(2):S1–S14.

Mendoza MA, Ponce RA, Ou YC, Faustman EM. p21(WAF1/CIP1) inhibits cell cycle progression but not G2/M-phase transition following methylmercury exposure. *Toxicol Appl Pharmacol*. 2002;178:117–125.

Moggs JG, Tinwell H, Spurway T, et al. Phenotypic anchoring of gene expression changes during estrogen-induced uterine growth. *Environ Health Perspect*. 2004;112:1589–1606.

Moolgavkar SH, Luebeck G. Two-event model for carcinogenesis: biological, mathematical, and statistical considerations. *Risk Anal.* 1990;10:323–341.

Moore JA, Daston GP, Faustman EM. An evaluative process for assessing human reproductive and developmental toxicity of agents. *Reprod Toxicol*. 1995;9:61–95.
Morgan GM. Risk analysis and management. *Sci Am*. 1993;269:32–41.
National Academy of Science. *Health Risks from Dioxin and Related Compounds: Evaluation of the EPA Reassessment*. Washington, DC: National Academies Press; 2006.
National Human Genome Research Institute. *A DNA Polymorphism Discovery Resource*. 2010. Available at: http://www.genome.gov/10001552.
Nature Biotechnology Editorial. Making the most of microarrays. *Nat Biotechnol*. 2006;24:1039–1039.
NCBI. *dbSNP Short Genetic Variations*. Available at: http://www.ncbi.nlm.nih.gov/SNP/.
NCBI. Available at: http://www.ncbi.nlm.nih.gov/. 2011b.
NCI. Available at: http://dtp.nci.nih.gov. 2017.
Neal N, Malmfors T, Slovic P. Intuitive toxicology: expert and lay judgments of chemical risks. *Toxicol Pathol*. 1994;22:198–201.
Nebert DW, Duffy JJ. How knockout mouse lines will be used to study the role of drug-metabolizing enzymes and their receptors during reproduction, development, and environmental toxicity, cancer and oxidative stress. *Biochem Pharmacol*. 1997;53:249–254.
Nel AE, Nasser E, Godwin H, et al. A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment. *ACS Nano*. 2013;7(8):6422–6433.
Neumann DA, Olin SS. Urinary bladder carcinogenesis: a working group approach to risk assessment. *Food Chem Toxicol*. 1995;33:701–704.
NHGRI. The ENCODE project: encyclopedia of DNA elements. 2017. Available at: https://www.encodeproject.org.
NIEHS. *Corrositex: An In Vitro Test Method for Assessing Dermal Corrosivity Potential of Chemicals*. Washington, DC: ICCVAM; 1999a:33109–33111.
NIEHS. *The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds*. Washington, DC: ICCVAM; 1999b:14006–14007.
NIEHS. *ICCVAM Test Method Evaluation Report: In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests*. National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Bethesda, MD: National Institutes of Health; 2006.
NLM. Available at: http://toxnet.nlm.nih.gov/. 2017.
NRC. *Risk Assessment in the Federal Government: Managing the Process*. Washington, DC: National Academies Press; 1983.
NRC. *Improving Risk Communication*. Washington, DC: National Academies Press; 1989a.
NRC. *Biological Markers in Reproductive Toxicology*. Washington, DC: National Academies Press; 1989b.
NRC. *Biological Markers in Pulmonary Toxicology*. Washington, DC: National Academies Press; 1989c.
NRC. *Biological Markers in Immunotoxicology*. Washington, DC: National Academies Press; 1992.
NRC Committee on Risk Assessment Methodology. *Issues in Risk Assessment: Use of Maximum Tolerated Dose in Animal Bioassays for Carcinogenicity*. Washington, DC: National Academies Press; 1993.
NRC. *Science and Judgement in Risk Assessment*. Washington, DC: National Academies Press; 1994
NRC. *Understanding Risk: Informing Decisions in a Democratic Society*. Stern PC, Fineberg HV, eds. Washington, DC: National Academies Press; 1996.
NRC. *Scientific Frontiers in Developmental Toxicology and Risk Assessment*. Washington, DC: National Research Council; 2000.
NRC. *Effective Risk Communication, The Nuclear Regulatory Commission's Guidelines for External Risk Communication*. Washington, DC: National Academies Press, 2004.
NRC. *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Washington, DC: National Academies Press; 2007a.
NRC. *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk*. Washington, DC: National Academies Press; 2007b.
NRC. *Science and Decisions: Advancing Risk Assessment*. Washington, DC: National Academies Press; 2009.
NRC. *Toxicity Pathway-Based Risk Assessment Preparing for Paradigm Change: A Symposium Summary*. Washington, DC: National Academies Press; 2010.
NRC. *Sustainability Concepts in Decision-making: Tools and Approaches for the US Environmental Protection Agency*. Washington, DC: National Academies Press; 2014a.
NRC. *A Framework to Guide Selection of Chemical Alternatives*. Washington, DC: National Academies Press; 2014b.
NRC. *Using 21st Century Science to Improve Risk-related Evaluations*. Washington, DC: National Academies Press; 2017.
NTP. Available at: http://ntp.niehs.nih.gov/. 2011a.
NTP, Office of Health Assessment and Translation (OHAT, Formerly CERHR). Available at: http://ntp.niehs.nih.gov/?objectid=497C419D-E834-6B35-8AF15D389859AF07. 2011b.
NTP. *14th Report on Carcinogens*; 2016.
NTP, Office of Health Assessment and Translation. *OHAT Systematic Review*. 2017a. Available at: https://ntp.niehs.nih.gov/pubhealth/hat/noms/index-2.html.
NTP, Guidance document for the developmental neurotoxicity testing arm of the modified one-generation study. 2017b. Available at https://ntp.niehs.nih.gov/ntp/test_info/mog_guidance_508.pdf
Oberdorster G. Lung particle overload: implications for occupational exposure to particles. *Regul Toxicol Pharmacol*. 1995;12:123–135.
OECD (Organisation for Economic Co-Operation and Development). *Manual for Investigation of HPV Chemicals*; 2013. Available at: www.oecd.org.
OECD. *Adverse Outcome Pathways. Molecular Screening and Toxicogenomics*; 2017. Available at: http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm.
Omenn GS. The genomic era: a crucial role for the public health sciences. *Environ Health Perspect*. 2000;108:A204–A205.
Omenn GS. Gene–environment interactions: eco-genetics and toxicogenomics. In: Ginsburg GS, Willard HF, eds. *Genomic and Personalized Medicine*. 2nd ed. Elsevier: New York; 2012.
Omenn GS, Lampen A. Scientific and cost-effectiveness criteria in selecting batteries of short-term tests. *Mutat Res*. 1988;205:41–49.
Omenn GS, Omiecinski CJ, Eaton DE. Eco-genetics of chemical carcinogens. In: Cantor C, Caskey C, Hood L, eds. *Biotechnology and Human Genetic Predisposition to Disease*. New York: Wiley-Liss; 1990:81–93.
Omenn GS, Stuebbe S, Lave LB. Predictions of rodent carcinogenicity testing results: interpretation in light of the Lave–Omenn value-of-information model. *Mol Carcinogen*. 1995;14:37–45.
Patlewicz G, Roberts DW, Aptula A, Blackburn K, Hubesch B. Workshop: use of "read-across" for chemical safety assessment under REACH. *Regul Toxicol Pharmacol*. 2013;65(2):226–228.
Patterson TA, Lobenhofer EK, Fulmer-Smentek SB, et al. Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project. *Nat Biotechnol*. 2006;24:1140–1150.
Perdue WC, Stone LA, Gostin LO. The built environment and its relationship to the public's health: the legal framework. *Am J Public Health*. 2003;93:1390–1394.
Perera FP, Weinstein IB. Molecular epidemiology: recent advances and future directions. *Carcinogenesis*. 2000;21:517–524.
REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). 2011. Available at: http://ec.europa.eu/environment/chemicals/reach/reach_en.htm.
Regalado J, Perez-Padilla R, Sansores R, et al. The effect of biomass burning on respiratory symptoms and lung function in rural Mexican women. *Am J Respir Crit Care Med*. 2006;174(8):901–905.
Renwick AG. Safety factors and the establishment of acceptable daily intakes. *Food Addit Contam*. 1991;8:135–150.
Renwick AG. Toxicokinetics. In: Ballantyne B, Marrs T, Syversen T, eds. *General and Applied Toxicology*. New York: Grove's Dictionaries; 1999:67–95.
Renwick AG, Lazarus NR. Human variability and noncancer risk assessment—an analysis of the default uncertainty factor. *Regul Toxicol Pharmacol*. 1998;27:3–20.
Rice JM, Baan RA, Blettner M. Rodent tumors of urinary bladder, renal cortex, and thyroid gland. IARC monographs: evaluations of carcinogenic risk to humans. *Toxicol Sci*. 1999;49:166–171.

Risk Commission. *The Presidential/Congressional Commission on Risk Assessment and Risk Management*. Washington, DC: Government Printing Office; 1997.

Rodericks JV, Rudenko I, Starr TB, Turnbull D. Risk assessment. In: Sipes I, McQueen C, Gandolfi A, eds. *Comprehensive Toxicology*. Oxford, UK: Pergamon; 1997.

Roe D, Pease W, Florini K, Silbergeld E. *Toxic Ignorance: The Continuing Absence of Basic Health Testing for Top-Selling Chemicals in the United States*. Washington, DC: Environmental Defense Fund; 1997.

Rooney AA, Boyles AL, Wolfe MS, Bucher JR, Thayer KA. Systematic review and evidence integration for literature-based environmental health science assessments. *Environmental Health Perspectives*. 2014;122(7):711–718.

Ryan PB. Exposure assessment, industrial hygiene, and environmental management. In: Frumkin H, ed. *Environmental Health: From Global to Local*. 2nd ed. San Francisco, CA: Jossey-Bass; 2010.

Sandman PM. *Responding to Community Outrage: Strategies for Effective Risk Communication*. Fairfax, VA: American Industrial Hygiene Association; 1993.

Sartor MA, Dolinoy DC, Rozek LS, Omenn GS. Bioinformatics for high-throughput toxico-epigenomics studies. In: Sahu SC, ed. *Toxicology and Epigenetics*. New York: Wiley; 2012.

Sartor MA, Mahavisno V, Keshamouni VG, et al. ConceptGen: a gene set enrichment and gene set relation mapping tool. *Bioinformatics*. 2010;26:456–463.

Schardein JL, Scialli AR. The legislation of toxicologic safety factors: the Food Quality Protection Act with chlorpyrifos as a test case. *Reprod Toxicol Rev*. 1999;13:1–14.

Sette WF. *Pesticide Assessment Guidelines, Subdivision D, Hazard Evaluation, Human and Domestic Animals, Addendum 10*. Neurotoxicity Series 81, 82, and 83 PB91-154617. Washington, DC: National Technical Information Services; 1991.

Sexton K, Selevan SG, Wagener DK, Lybarger JA. Estimating human exposures to environmental pollutants: availability and utility of existing databases. *Arch Environ Health*. 1992;47:398–407.

Shelby MD, Bishop JB, Mason JM, Tindall KR. Fertility, reproduction and genetic disease: studies on the mutagenic effects of environmental agents on mammalian germ cells. *Environ Health Perspect*. 1993;200:283–291.

Shuey DL, Lau C, Logsdon TR. Biologically based dose–response modeling in developmental toxicology: biochemical and cellular sequelae of 5-fluorouracil exposure in the developing rat. *Toxicol Appl Pharmacol*. 1994;126:129–144.

Silverman KC, Naumann BD, Holder DJ. Establishing data-derived adjustment factors from published pharmaceutical clinical trial data. *Hum Ecol Risk Assess*. 1999;5:1059–1089.

Simon R, Wang SJ. Use of genomic signatures in therapeutics development in oncology and other diseases. *Pharmacogenomics J*. 2006;6:166–173.

Slovic P. *The Feeling of Risk: New Perspectives on Risk Perception*. The Earthscan Risk in Society Series. Washington, DC: Earthscan, in association with the International Institute for Environment and Development; 2010.

Slovic P, Baruch F, Lichtenstein S. Rating the risks. *Environment*. 1979;21:1–20, 36–39.

Slovic P, Peters E, Finucane ML, MacGregor DG. Affect, risk, and decision making. *Health Psychol*. 2005;24:S35–S40.

Smith MN, Grice J, Cullen, Faustman EM. A toxicological framework for the prioritization of children's safe product act data. *Int J Environ Res Public Health*. 2016a;13(4):431.

Smith MT, Guyton KZ, Gibbons CF, et al. Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. *Environ Health Perspect*. 2016b;124(6):713–721.

Spielmann H, Seiler A, Bremer S, et al. The practical application of three validated in vitro embryotoxicity tests—the report and recommendations of an ECVAM/ZEBET workshop (ECVAM workshop 57). *Altern Lab Anim*. 2006;34:527–538.

Stevens JT. Risk assessment of pesticides. In: Williams P, Hottendorf G, eds. *Comprehensive Toxicology*. Oxford, UK: Pergamon; 1997:17–26.

Sudmant PH, Kitzman JO, Antonacci F, et al. Diversity of human copy number variation and multicopy genes. *Science*. 2010;330:641–646.

Teichman K, U.S. EPA. *Presentation to the Science Advisory Board and Board of Scientific Counselors (Overview)*. 2011, June. Available at: http://my.brainshark.com/Dr-Kevin-Teichman-SAB-BOSC-Presentation-720933584.

Tennant RW, Stasiewicz S, Mennear J. Genetically altered mouse models for identifying carcinogens. In: McGregor DB, Rice JM, Venitt S, eds. *The Use of Short- and Medium-Term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation*. Vol. 146. Lyon, France: IARC; 1999:123–150.

Toscano WA, Oehlke KP. Systems biology: new approaches to old environmental health problems. *Int J Environ Res Public Health*. 2005;2:4–9.

United States Act. *The Genetic Information Nondiscrimination Act of 2008 (GINA)*. Public Law 110-233; May 21, 2008.

U.S. EPA. Guidelines for mutagenicity risk assessment. *Fed Reg*. 1986;51:34006–34012.

U.S. EPA. Proposed guidelines for assessing female reproductive risk. *Fed Reg*. 1988;53:24834–24847.

U.S. EPA. *Risk Assessment Guidance for Superfund. Vol 1: Human Health Evaluation Manual, Part A*. Washington, DC: Office of Policy Analysis and Office of Emergency and Remedial Response; 1989.

U.S. EPA. Guidelines for developmental toxicity risk assessment. *Fed Reg*. 1991;56:63798–63826.

U.S. EPA. *Frameworks for Ecological Risk Assessment*. EPA\630\R-92\001; 1992a.

U.S. EPA. Guidelines for exposure assessment. *Fed Reg*. 1992b;57: 22888–22938.

U.S. EPA. *Health Assessment Document for 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) and Related Compounds*. Washington, DC: Office of Research and Development; 1994a.

U.S. EPA. *Guidelines for Carcinogen Risk Assessment (Draft Revisions)*. Washington, DC: Office of Health and Environmental Assessment; 1994b.

U.S. EPA. Guidelines for reproductive toxicity risk assessment. *Fed Reg*. 1996a;61:56278–56322.

U.S. EPA. *Food Quality Protection Act (FQPA)*. Washington, DC: Office of Pesticide Programs; 1996b.

U.S. EPA. *Chemical Hazard Availability Study*. Washington, DC: Office of Pollution Prevention and Toxics; 1998a.

U.S. EPA. Guidance for identifying pesticides that have a common mechanism of toxicity: notice of availability and solicitation of public comments. *Fed Reg*. 1998b; 63:42031–42032.

U.S. EPA. *Guidelines for Neurotoxicity Risk Assessment*. EPA Technical Report. Risk Assessment Forum. Washington, DC: U.S. Environmental Protection Agency; 1998c.

U.S. EPA. *Guidelines for Carcinogen Risk Assessment, Draft*. NCEA-F-0644. Washington, DC: EPA; 1999.

U.S. EPA. *Benchmark Dose Technical Guidance Document. External Review Draft*. Washington, DC: Risk Assessment Forum; 2000a.

U.S. EPA. *Chlorpyrifos: Re-evaluation Report of the FQPA Safety Factor Committee*. HED Doc. No. 014077. Washington, DC: U.S. Environmental Protection Agency; 2000b.

U.S. EPA. *Risk Characterization Handbook*. Washington, DC: U.S. Environmental Protection Agency Science Policy Council; 2000c.

U.S. EPA. *Generic Ecological Assessment Endpoints (GEAE) for Ecological Risk Assessment*. Washington, DC: U.S. Environmental Protection Agency; 2004.

U.S. EPA. *Guidelines for Carcinogen Risk Assessment (Cancer Guidelines)*. Federal Register Notice. Washington, DC: U.S. Environmental Protection Agency; 2005a.

U.S. EPA. *Policy on Evaluating Heath Risks to Children*. Washington, DC: U.S. Environmental Protection Agency; 2005b.

U.S. EPA. *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens*. Federal Register Notice. EPA/630/R-03/003F. Washington, DC: U.S. Environmental Protection Agency; 2005c.

U.S. EPA. *Child-Specific Exposure Factors Handbook (Final Report)*. EPA/600/R-06/096F. Washington, DC: Office of Research and Development and the National Center for Environmental Assessment; 2008.

U.S. EPA. *The Office of Chemical Safety and Pollution Prevention (OCSPP) Harmonized Test Guidelines: Series 870—Health Effects Test Guidelines*. 870 Series Final Guidelines. 2010a. Available at: http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series870.htm.

U.S. EPA. *EPA's Reanalysis of Key Issues Related to Dioxin Toxicity and Response to NAS Comments Draft*. 2010b. NAS comments are published by the National Research Council of the National Academies and available from the National Technical Information Service, Springfield, VA, and available at: http://www.epa.gov/ncea.

U.S. EPA. Integrated Exposure Uptake Biokinetic Model for Lead in Children. Windows® version (IEUBKwin v1.1 build 11). Washington, DC: U.S. Environmental Protection Agency; 2010c. Available from the National Technical Information Service. Available at: http://www.epa.gov/superfund/lead/products.htm.

U.S. EPA. *IRIS Toxicological Review of Inorganic Arsenic (Cancer) (2010 External Review Draft)*. EPA/635/R-10/001. Washington, DC: U.S. Environmental Protection Agency; 2010d.

U.S. EPA, Office of Research and Development. Presentations for the Science Advisory Board and Board of Scientific Counselors. In: Preparation for the June 29–30, 2011 Joint Meeting; 2001f. Available at: https://yosemite.epa.gov/sab/sabproduct.nsf/F53952681221F76F852578AF007EE972/$File/SABBOSC+Presentations.pdf.

U.S. EPA. *Exposure Factors Handbook: 2011 Edition. Final Report*. Washington, DC: Office of Health and Environmental Assessment; 2011a.

U.S. EPA. *High Production Volume (HPV) Challenge*. 2011b. Available at: http://www.epa.gov/hpv/.

U.S. EPA. *PMN Forms and Information. New Chemicals*. 2011c. Available at: http://www.epa.gov/oppt/newchems/pubs/pmnforms.htm.

U.S. EPA. *Integrated Risk Information System (IRIS)*. 2011d. Available at: http://www.epa.gov/IRIS/.

U.S. EPA. *Interim Genomics Policy*. 2011e. Available at: http://www.epa.gov/spc/genomics.htm.

U.S. EPA. *Benchmark Dose Technical Guidance*. EPA/100/R-12/001. 2012a. Available at: https://www.epa.gov/risk/benchmark-dose-technical-guidance.

U.S. EPA. *Integrated Risk Information System (IRIS): Glossary of IRIS Terms*. 2012b. Available at: http://www.epa.gov/iris/help_gloss.htm.

U.S. EPA. *Computational Toxicology Research*. 2012c. Available at: http://www.epa.gov/ncct/.

U.S. EPA. *Use of High-Throughput Assays and Computational Tools*. Endocrine Disruptor Screening Program. Notice of Availability and Opportunity for Comment; 2015. Available at: https://www.federalregister.gov/documents/2015/06/19/2015-15182/use-of-high-throughput-assays-and-computational-tools-endocrine-disruptor-screening-program-notice.

U.S. EPA. TRI Chemical List Changes; 2016.

U.S. EPA. Benchmark Modelling Software, Version 2.7. 2017a. Available at: https://www.epa.gov/bmds/what-benchmark-dose-software-bmds.

U.S. EPA. *ACToR*. Washington, DC: Office of Health and Environmental Assessment; 2017b. Available at: https://actor.epa.gov/actor/home.xhtml.

U.S. FDA. *Critical Path Initiative. Science & Research*. Silver Spring, MD: U.S. Food and Drug Administration; 2011. Available at: http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/default.htm.

U.S. FDA. *Pharmacogenetics*. 2017a. Available at: https://www.fds.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.html.

U.S. FDA. SNP track. 2017b. Available at: https://www.fds.gov/scienceresearch/bioinformaticstools/snptrack/default.htm.

Van den Berg M, Birnbaum L, Bosveld AT, et al. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect*. 1998;106:775–792.

Van den Berg M, Birnbaum LS, Denison M, et al. The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol Sci*. 2006;93:223–241.

Vlaanderen J, Portengen L, Rappaport SM, et al. The impact of saturable metabolism on exposure–response relations in 2 studies of benzene-induced leukemia. *Am J Epidemiol*. 2011;174:621–629.

Wambaugh JF, Setzer RW, Reif DM, et al. High-throughput models for exposure-based chemical prioritization in the ExpoCast project. *Environ Sci Technol*. 2013; 47(15):8579–8588.

Weller E, Long N, Smith A. Dose–rate effects of ethylene oxide exposure on developmental toxicity. *Toxicol Sci*. 1999;50:259–270.

Whittaker SG, Faustman EM. *In Vitro Assays for Developmental Toxicity*. New York: Raven Press; 1994.

WHO. *Principles in Governing Consumer Safety in Relation to Pesticide Residues: Report of a Meeting of a W.H.O. Expert Committee on Pesticide Residues*, held jointly with the F.A.O. Panel of Experts on the Use of Pesticides in Agriculture. World Health Organization Technical Report Series No. 240. Geneva: WHO; 1962.

WHO. *Principles for the Assessment of Risks to Human Health from Exposure to Chemicals. Environmental Health Criteria*. World Health Organization, International Programme on Chemical Safety. Geneva: World Health Organization; 1999.

WHO. *Joint IPCS/OECD Project on the Harmonization of Chemical Hazard/Risk Assessment Terminology (Harmonization Project)*. Geneva: World Health Organization; 2000.

WHO. *IPCS Risk Assessment Terminology*. Harmonization Project Document. Geneva: World Health Organization, International Program on Chemical Safety; 2004.

WHO. *Chemical-Specific Adjustment Factors for Interspecies Differences and Human Variability: Guidance Document for Use of Data in Dose/Concentration–Response Assessment*. Geneva: World Health Organization; 2005.

WHO. *Principles of Characterizing and Applying Human Exposure Models*. IPCS Harmonization Project Document No. 3. Geneva: World Health Organization; 2006a.

WHO. *Principles for Modeling Dose-response for the Risk Assessment of Chemicals*. Environmental Health Criteria Series, No. 234. Geneva: World Health Organization, International Program on Chemical Safety; 2006b.

WHO. *Uncertainty and Data Quality in Exposure Assessment*. Geneva: World Health Organization, International Program on Chemical Safety; 2008.

WHO. *WHO Human Health Risk Assessment Toolkit: Chemical Hazards*. IPCS Harmonization Project Document No. 8. International Programme on Chemical Safety, Inter-Organization Programme for the Sound Management of Chemicals. Geneva: World Health Organization; 2010.

WHO. *IPCS Harmonization Project. Programmes and Projects*. 2011a. Available at: http://www.who.int/ipcs/methods/harmonization/en/.

WHO. *The International Agency for Research on Cancer (IARC)*. 2011b. Available at: http://www.iarc.fr/.

WHO. *Concise International Chemical Assessment Documents*. International Programme on Chemical Safety (IPCS); 2011c. Available at: http://www.who.int/ipcs/publications/cicad/en/.

WHO. *Millennium Development Goals (MDGs). Health Topics*. 2011d. Available at: http://www.who.int/topics/millennium_development_goals/en/.

Yamada T, Kondo M, Miyata K, et al. An evaluation of the human relevance of the lung tumors observed in female mice treated with permethrin based on mode of action. *Toxicol Sci*. 2017;157(2):465–486.

Yu X, Griffith WC, Hanspers K, et al. A system based approach to interpret dose and time-dependent microarray data: quantitative integration of GO ontology analysis for risk assessment. *Toxicol Sci*. 2006;92:560–577.

Yu XZ, Hong S, Moreira EG, Faustman EM. Improving in vitro Sertoli cell/gonocyte co-culture model for assessing male reproductive toxicity: lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates. *Toxicol Appl Pharmacol*. 2009;239:325–336.

Zhao Q, Dourson M, Gadagbui B. A review of the reference dose for chlorpyrifos. *Regul Toxicol Pharmacol*. 2006;44:111–124.

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Unit II

Disposition of Toxicants

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chapter 5

Absorption, Distribution, and Excretion of Toxicants

Angela L. Slitt

Introduction

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- Special Transport
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  - *Active Transport*
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  - *Additional Transport Processes*

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Acknowledgments

References

INTRODUCTION

The disposition of a chemical or xenobiotic is defined as the composite actions of its absorption, distribution, biotransformation, and elimination. This chapter focuses on the contribution of absorption, distribution, and elimination to xenobiotic toxicity, whereas Chap. 6 is dedicated to biotransformation. The quantitative characterization of xenobiotic disposition is termed pharmacokinetics or toxicokinetics and is reviewed in Chap. 7.

The various factors and organs involved in affecting disposition of a toxicant are depicted in Fig. 5-1. The diagram is a pictoral overview of the processes of absorption, distribution, and excretion, and its complexity is intended to illustrate that, although they will be discussed separately, the processes that determine disposition are likely to occur simultaneously. Moreover, the disposition of any compound is a fundamental factor that contributes to its potential for toxicity. Specifically, the toxicity of a substance is usually in most circumstances directly dependent on the dose, where "dose" is defined as the amount that ultimately reaches the site or sites of action (tissue, cell, or molecular target). Therefore, the disposition of a chemical determines its concentration at the site of action such that the concerted actions of absorption, distribution, and elimination also determine the potential for adverse events to occur.

The skin, lungs, and alimentary canal are the main barriers that separate higher organisms from an environment containing a large number of chemicals (Fig. 5-1). Toxicants must cross one or several of these incomplete barriers to exert deleterious effects, and only chemicals that are caustic and corrosive (acids, bases, salts, oxidizers), which act directly at the point of contact, are exceptions to this generalization. A chemical absorbed into the bloodstream or lymphatics through any of the major barriers is distributed, at least to some extent, throughout the body, including the site where it produces damage. This site is called the target organ or target tissue. A chemical may have one or several target organs, and, in turn, several chemicals may have the same target organ or organs. For example, microcystins are an example of cyanotoxins present in harmful algal blooms that can selectively concentrate to the liver via transporter-mediated mechanisms that facilitate uptake into hepatocytes and elicit toxicity through inhibition of protein phosphatases, PP1 and PP2A (Campos and Vasconcelos, 2010). However, it cannot be assumed that the tissue of highest concentration is also the target organ where toxicity will be observed. For example, dichlorodiphenyltrichloroethane (DDT) is a chlorinated hydrocarbon insecticide that achieves high concentrations in fat depots but is not overtly toxic to that tissue.

Poor absorption of a toxicant resulting from a low amount absorbed or from a low rate of absorption will limit or prevent toxicity because a chemical may never attain a sufficiently high concentration at a potential site of action to cause toxicity. Fig. 5-2 depicts the example of microcystins, in which hepatotoxicity is attenuated when mechanisms that result in accumulation are decreased, such as targeted deletion of a key transporter, Organic

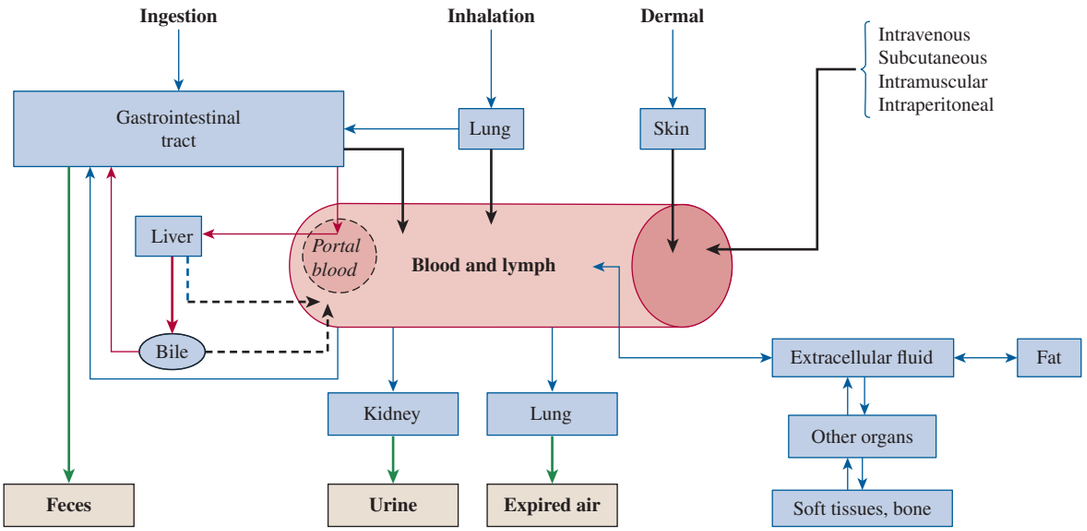

Figure 5-1. *Summary of the disposition of toxicants as determined by absorption, distribution, and excretion in the body.* Black lines represent major pathways of absorption into the body, blue designates distribution, and green lines identify pathways of final excretion (elimination) from the body, with the exception of enterohepatic circulation, which is designated in red.

anion transporting polypeptide 1b2 (Oatp1b2). In wild-type mice that expressed Oatp1b2, microcystin concentrated in liver and binding to protein phosphatase 1/2a was observed. However, in mice that lacked Oatp1b2 there was less MC in the liver, along with less binding of MC to protein phosphatase 1/2a and much less toxicity. Similarly, a chemical that is well absorbed but rapidly biotransformed or eliminated from an organism is less likely to be toxic because rapid excretion prevents it from reaching a sufficiently high concentration at a potential site of action to cause toxicity. These are examples of how the disposition of toxicants contributes to the fundamental concept that the "dose makes the poison."

The processes comprising xenobiotic disposition are interrelated and influence each other. The integrated relationship of these processes is illustrated in Fig. 5-1. In this chapter, the qualitative aspects of absorption, distribution, and excretion are presented with emphasis on the functional features and molecular determinants of these processes. As all these processes involve passage across biological membranes, we begin with a discussion of this important and ubiquitous barrier.

CELL MEMBRANES

Toxicants usually pass through a number of cells, such as the stratified epithelium of the skin, the thin cell layers of the lungs or the gastrointestinal (GI) tract, capillary endothelium, and ultimately the cells of the target organ. The plasma membranes surrounding all these cells are remarkably similar. The basic unit of the cell membrane is a lipid bilayer composed primarily of phospholipids, glycolipids, and cholesterol. Mammalian cell plasma membranes contain four major phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin) and these together comprise more than half of the lipid in most membranes. Phospholipids are amphiphilic, consisting of a hydrophilic polar head and a hydrophobic lipid tail. In membranes, the polar head groups are oriented toward the outer and inner surfaces of the membrane, whereas the hydrophobic tails are oriented inward and face each other to form a continuous hydrophobic inner space. The thickness of the cell membrane is about 7 to 9 nm. Numerous proteins are inserted or embedded in the bilayer, and some transmembrane proteins traverse the entire lipid bilayer, functioning as important biological receptors or allowing the formation of aqueous pores, ion channels, and transporters (Fig. 5-3). The fatty acids of the membrane do not have a rigid crystalline structure but are semifluid at physiological temperatures. Overall, hydrophobic interactions are the major driving force in the formation of membrane lipid bilayers, and the fluid character of membranes is determined largely by the structure and relative abundance of unsaturated fatty acids. The more unsaturated fatty acids the membranes contain, the more fluid-like they are, facilitating more rapid active or passive transport.

The membrane barrier is differentially permeable and regulates what enters into or exits from cells. Toxicants cross membranes either

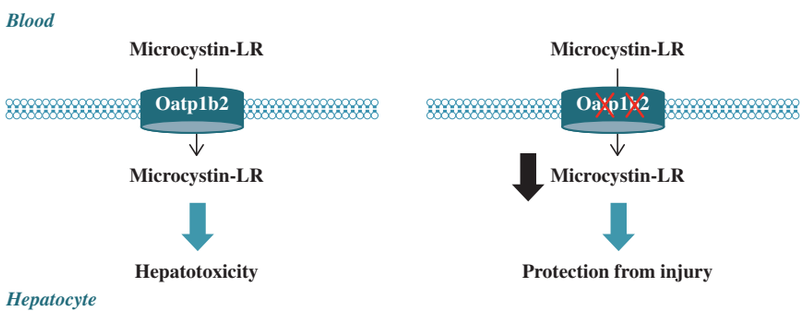

Figure 5-2. *Contribution of Oatp1b2 to microcystin-induced hepatotoxicity.*

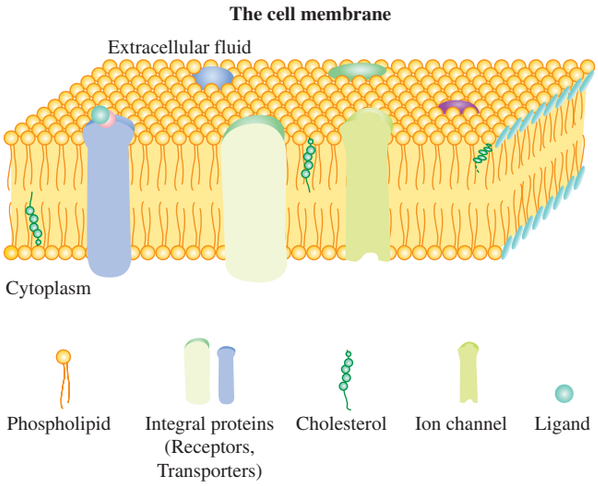

Figure 5-3. *Schematic model of a biological membrane.*

by passive processes in which the cell expends no energy or by mechanisms in which the cell provides energy to translocate the toxicant across its membrane. Physicochemical properties such as molecular weight, hydrophilicity, and polarity (total polar surface area) are determinants for the ability of a chemical to permeate the cell membrane.

Passive Transport

Simple Diffusion Many toxicants cross membranes by simple diffusion, following the principles of Fick's law, which establishes that chemicals move from regions of higher concentration to regions of lower concentration without any energy expenditure. Small hydrophilic molecules (up to about 600 Da) permeate membranes through aqueous pores (Benz et al., 1980), in a process termed paracellular diffusion, whereas hydrophobic molecules diffuse across the lipid domain of membranes (transcellular diffusion). The smaller a hydrophilic molecule is, the more readily it traverses membranes by simple diffusion through aqueous pores. Consequently, a small, water-soluble compound such as ethanol is rapidly absorbed into the blood from the GI tract and is distributed just as rapidly throughout the body by simple diffusion from blood into all tissues.

For larger organic molecules with differing degrees of lipid solubility, the rate of transfer across membranes correlates with lipophilicity. Lipid solubility is generally determined by the octanol/water partition coefficient, *P*, which is defined as the ratio of the concentration of compound in organic and aqueous phases under equilibrium conditions. log *P* is also expressed as Kow or Pow. It is usually expressed in logarithmic form as log *P*, and numerous examples are listed in Table 5-1. The log *P* is an extremely informative physicochemical parameter relative to assessing potential membrane permeability, with positive values associated with high lipid solubility. In contrast, charged molecules such as paraquat do not have a measurable log *P*. As illustrated by the compounds in Table 5-1, amino acids such as glycine are highly water-soluble and have a negative log *P*, whereas the environmental contaminants DDT and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are very lipid soluble and have a high, positive log *P*. There are a variety of experimental methods for empirical determination of log *P*, along with computational tools that can be used to predict log *P* for any chemical (Mannhold et al., 2009).

Many chemicals are weak organic acids or bases, which in solution are ionized according to Arrhenius' theory. The ionized form usually has low lipid solubility and thus does not permeate readily through the lipid domain of a membrane. There may be some transfer of organic anions and cations (depending on their molecular weight) through the aqueous pores, but this is a slow and inefficient process. In contrast, the nonionized form of weak organic acids and bases is lipid soluble to some extent, resulting in diffusion across the lipid domain of a membrane. The rate of movement of the nonionized form is proportional to its lipid solubility such that the extent to which a compound is nonionized determines its diffusion.

The molar ratio of ionized to nonionized molecules of a weak organic acid or base in solution depends on the ionization constant, which is defined as the pH at which a weak organic acid or base is 50% ionized (denoted as pK_a or pK_b for acids and bases, respectively). Like pH, pK_a and pK_b are defined as the negative logarithm of the ionization constant of a weak organic acid or base. An organic acid with a low pK_a is relatively a strong acid, and one with a high pK_a is a weak acid. The opposite is true for bases. The numerical value of pK_a does not indicate whether a chemical is an organic acid or a base. Knowledge of the chemical structure is required to distinguish between organic acids and bases.

Table 5-1

Octanol/Water Partition Coefficients (*P*) of Different Molecules Expressed as log *P*

COMPOUND	log *P*
Paraquat	Charged molecule
Cephalosporin C	−4.72
Glycine	−3.21
Glutathione	−3.05
Cysteine	−2.35
Glucose	−2.21
Ethylene glycol	−1.37
Lead acetate	−0.63
p-Aminohippuric acid	−0.25
Dimercaprol	0.18
Scopolamine	0.30
Aspirin (acetyl salicylic acid)	1.02
Colchicine	1.19
Atropine	1.32
Benzoic acid	1.88
Benzene	2.14
Salicylic acid	2.19
Methyl salicylate	2.34
2,4-D	2.73
Warfarin	2.89
Digitoxin	3.05
Parathion	3.47
DDT	6.76
TCDD	7.05

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DDT, dichlorodiphenyltrichloroethane; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

The Henderson–Hasselbalch equation describes the derivation of pH as a function of the pK_a in a biological system. Two equivalent forms of this equation are as follows:

$$pH = pK_a + \log \frac{[\text{ionized}]}{[\text{nonionized}]}$$

$$pH = pK_a - \log \frac{[\text{nonionized}]}{[\text{ionized}]}$$

Rearranging these equations enables determination of the extent to which a weak acid or base is ionized or nonionized at any pH as follows:

$$\text{For acids: } pK_a - pH = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$$

$$\text{For bases: } pK_a - pH = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$$

The effect of pH on the degree of ionization of an organic acid (benzoic acid) and an organic base (aniline) is illustrated in Fig. 5-4. According to the Brönsted–Lowry acid–base theory, an acid is a proton (H^+) donor and a base is a proton acceptor. Thus, the ionized and nonionized forms of an organic acid represent an acid–base pair, with the nonionized moiety being the acid and the ionized moiety being the base. At a low pH, a weak organic acid such as benzoic acid is largely nonionized. At pH 4, exactly 50% of benzoic acid is ionized and 50% is nonionized because this is the pK_a of the compound. As the pH increases, more and more protons are neutralized by hydroxyl groups, and benzoic acid continues to dissociate until almost all of it is in the ionized form. For an organic base such as aniline, the inverse is true. At a low pH, when protons are abundant, almost all of aniline is ionized. This form of aniline is an acid because it can donate protons. As the pH increases, ions from aniline continue to dissociate until almost all the aniline is in the nonionized form, which is the aniline base. As transmembrane passage is largely restricted to the nonionized form, benzoic acid is more readily translocated through a membrane from an acidic environment, whereas more aniline is transferred from an alkaline environment.

Lipid solubility can also be expressed as the distribution coefficient, log *D*, which is the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized) in each of the two phases, one essentially always aqueous; as such, it depends on the pH of the aqueous phase, and log D = log P for nonionizable compounds at any pH. The major difference between log *P* versus log *D* is that log *P* is a constant for the molecule under its neutral form, whereas log *D* takes into account all neutral and charged forms of the molecule. Because a charged form of the molecule might hardly enter the octanol phase, the distribution varies with pH. log D = log P in the pH region where the molecule is mostly unionized. However, a pH region where a significant fraction of the molecule is ionized, log *D* becomes a function of log *P*, pH, and pK_a. Overall, log *D* is a parameter commonly used for in silico prediction of absorption, distribution, metabolism, and excretion (ADME), as well as toxicity properties.

Filtration When water flows in bulk across a porous membrane, any solute small enough to pass through the pores flows with it. Passage through these channels is called filtration, as it involves bulk flow of water caused by hydrostatic or osmotic force. One of the main differences between various membranes is the size of these channels. In renal glomeruli, a primary site of filtration, these pores are relatively large (about 70 nm) allowing molecules smaller than albumin (approximately 60 kDa) to pass through. In contrast, there are no aqueous pores at cellular tight junctions, and channels in most cells are much smaller (3 to 6 Å), thereby only permitting substantial passage of molecules with molecular weights of no more than a few hundred daltons (Schanker, 1962; Lin, 2006).

Special Transport

There are numerous compounds whose movement across membranes cannot be explained by simple diffusion or filtration. Some compounds are too large to pass through aqueous pores or too insoluble in lipids to diffuse across the lipid domains of membranes. Nevertheless, they are often transported very rapidly across membranes, even against concentration gradients. To explain these phenomena, specialized transport systems have been identified. These systems are responsible for the transport (both influx and efflux) across cell membranes of many nutrients, such as sugars and amino and nucleic acids, along with numerous foreign compounds (Table 5-2). Based on the sequencing of the human genome, approximately 1500 genes encode for transporters or transport-related proteins (Hediger et al., 2013; Ye et al., 2014). However, not all of these genes contribute to the disposition of toxicants. Throughout this chapter, membrane-associated transporters known to contribute to the disposition and subsequent effects of xenobiotics will be emphasized. Importantly, the role of xenobiotic transporters in chemical disposition still remains an expanding research

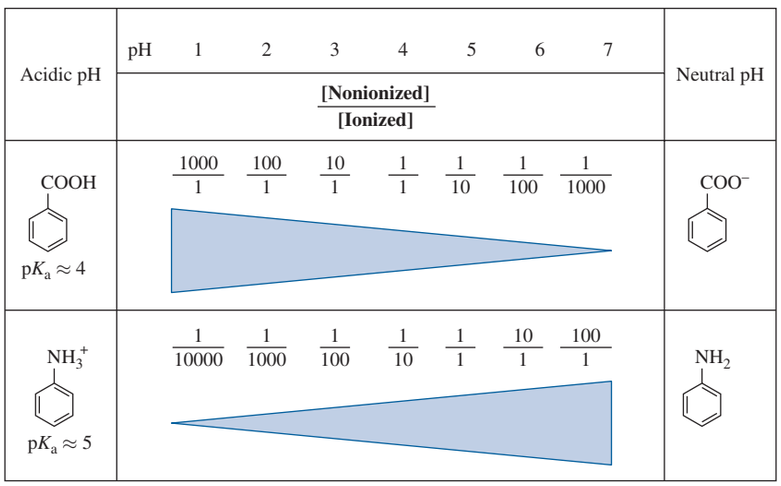

Figure 5-4. *Effect of pH on the ionization of benzoic acid (pK_a = 4) and aniline (pK_a = 5).*

Table 5-2

Distribution of Specialized Transport Systems in the Small Intestine of Man and Animals

SUBSTRATES	UPPER	MIDDLE	LOWER	COLON
Sugar (glucose, galactose, etc.)	++	+++	++	0
Neutral amino acids	++	+++	++	0
Basic amino acids	++	++	++	?
Gamma globulin (newborn animals)	+	++	+++	?
Pyrimidines (thymine and uracil)	+	+	?	?
Triglycerides	++	++	+	?
Fatty acid absorption and conversion to triglyceride	+++	++	+	0
Bile salts	0	+	+++	
Vitamin B_{12}	0	+	+++	0
Na^+	+++	++	+++	+++
H^+ (and/or HCO_3^- secretion)	0	+	++	++
Ca^{2+}	+++	++	+	?
Fe^{2+}	+++	++	+	?
Cl^-	+++	++	+	0

SOURCE: Data from Wilson (1962).

field, and new information regarding their function, molecular regulation, and genetic polymorphisms continues to inform and modify traditional concepts in toxicology.

Facilitated Transport Facilitated transport applies to carrier-mediated transport that exhibits the properties of active transport except that the substrate is not moved against an electrochemical or concentration gradient, and the transport process does not require the input of energy (e.g., ATP). For this reason, metabolic poisons do not interfere with this transport. The transport of glucose from the GI tract across the basolateral membrane of the intestinal epithelium, from plasma into red blood cells, and from blood into the central nervous system (CNS) occurs by facilitated diffusion via glucose transporters.

Active Transport Active transport is characterized by (1) movement of chemicals against electrochemical or concentration gradients, (2) saturability at high substrate concentrations, (3) selectivity for certain structural features of chemicals, (4) competitive inhibition by chemical congeners or compounds that are carried by the same transporter, and (5) requirement for expenditure of energy, so that metabolic inhibitors block the transport process. Substances actively transported across cell membranes presumably form a complex with a membrane-bound macromolecular carrier on one side of the membrane. The complex subsequently traverses to the other side of the membrane, where the substance is released. Afterward, the carrier returns to the original surface to repeat the transport cycle.

Xenobiotic Transporters Significant advances in identifying and understanding the carrier-mediated transport systems for xenobiotics have been made in the recent years. In total, it is estimated that at least 5% of all human genes are transporter related, indicative of the importance of the transport function in normal biological and toxicological outcomes (Hediger et al., 2004; Ye et al., 2014). Transporters mediate the influx (uptake) or efflux of xenobiotics and can be divided into two categories, determined by whether they mediate active or facilitated transfer of compounds. The first active, energy-dependent xenobiotic transporter identified was a phosphoglycoprotein overexpressed in tumor cells that showed resistance to anticancer drugs. The gene conferred multidrug resistance (MDR) to the cells, and was also called P-glycoprotein (P-gp or MDR1; gene name *MDR1*). This transporter functions as an efflux pump, which in cancerous cells exudes cytotoxic drugs out of the tumor cells, and thus contributes to their resistance (Ambudkar et al., 1999). MDR1 was the first member of a large superfamily of transport proteins known as ATP-binding cassette (ABC) transporters, with seven subfamilies of ABC transporters now identified (Box 5-1). These are classified as subfamilies A to G with 49 genes in humans (Ueda, 2011). Many of these transporters play key roles in the homeostasis of numerous endogenous substrates. For example, ABCA1 is a major regulator of cholesterol in cells, mediating its efflux from liver, and ABCB4 specifically transports phospholipid substrates. In this chapter, transporters that play important roles in xenobiotic disposition and toxicity are described, with emphasis on the human genes and proteins. For example, in humans, the major form of MDR1 involved in xenobiotic transport is a single protein (ABCB1; MDR1), whereas in rodents there are two drug-transporting MDR1 homologs identified as Mdr1a and Mdr1b.

The second important ABC transport subfamily is the ABCC subfamily, which is also known as the multiresistant drug protein (MRP) family (see Box 5-1). ABCC1 (MRP1) was also originally isolated from multidrug resistant cells. Members of this family excrete chemicals from cells, with MRP2 and MRP3 particularly important in the efflux of xenobiotic metabolites, especially those conjugated with UDP-glucuronic acid or glutathione. For example, the glucuronide metabolite of acetaminophen is a substrate for both MRP2 and MRP3 (Ghanem et al., 2005). The breast cancer resistance protein (BCRP) is a member of the ABCG subfamily (ABCG2) and was originally isolated from a breast cancer cell line. It is expressed in a variety of normal tissues (i.e., liver, kidney, and intestine) and contributes to the disposition of the efflux transport

Box 5-1

Human ABC Transporters: Gene Family Overview and Major Transporters Involved in Xenobiotic Disposition

ABC SUBFAMILY	GENES IN FAMILY	GENE SYMBOLS
A	12	*ABCA1-10, 12, 13*
B	**11**	***ABCB1-11***
C	**13**	***ABCC1-13****
D	4	*ABCD1-4*
E	1	ABCE1
F	3	*ABCF1-3*
G	5	***ABCG1, 2, 4, 5, 8***

Bolded subfamily designations are those with a major role in xenobiotic disposition.

**ABCC13 is reported to be a pseudogene.*

GENE SYMBOL	COMMON NAME	GENERAL FUNCTION	TOXICOLOGICAL IMPACT
ABCB1	Multidrug resistance protein/P-glycoprotein (MDR)	Efflux from gut, brain, and placenta; biliary excretion	Intestinal inhibition increases bioavailability Loss of expression increases penetration to brain and fetus
ABCB11	Bile salt export pump (BSEP)	Bile salt transport	Inhibition associated with drug-induced liver injury
ABCC1	Multidrug resistance–associated protein 1 (MRP1)	Multidrug resistance in many tissues; export pump	Inhibition may improve drug-refractory tumors
ABCC2	Multidrug resistance–associated protein 2 (MRP2)	Organic anion efflux, glucuronide and glutathione conjugates, and biliary excretion	Mediates efflux of drug-conjugates (i.e., NSAIDs) that can be deconjugated in intestine and cause intestinal injury. Loss or inhibition can cause hyperbilirubinemia
ABCC3	Multidrug resistance–associated protein 3 (MRP3)	Organic anion efflux, and glucuronide and glutathione conjugates	Some evidence for inhibition associated with drug-induced liver injury for TAK-875
ABCC4	Multidrug resistance–associated protein 4 (MRP4)	Nucleoside transport and organic anion efflux	Implicated in tenofovir disoproxil fumarate–induced renal proximal tubular toxicity, which is observed in humans
ABCG2	Breast cancer resistance protein (BCRP)	Organic anion efflux, many sulfate conjugates, and biliary excretion	Not well described Protection against protoporphyria and diet-dependent phototoxicity

of numerous endogenous and xenobiotic sulfate conjugates. It is also highly expressed in numerous stem cells, particularly the side population of human bone marrow and other organs such as placenta and mammary gland, where it is purported to provide protection from xenobiotics (van Herwaarden et al., 2007; Ni et al., 2010; Memon et al., 2014).

ABC transporters exhibit the characteristics of active transport outlined above, and most notably, they require expenditure of energy to function. For most ABC transporters, the binding and hydrolysis of ATP provide the energy required to move their substrates across membranes. The typical structure of most ABC transporters consists of two types of structural domains including hydrophobic membrane spanning domain (MSD) and the hydrophilic, intracellular nucleotide binding domain (NBD) where ATP binds and is hydrolyzed (Fig. 5-5). The typical structural organization of the ABC transporter, as exemplified by MDR1, is a tandem repeat of two domains (1 MSD followed by 1 NBD). The two repeated halves are joined by a polypeptide linker sequence. MRPs are distinguished from MDR1 in that they comprise five domains, with an extra MSD that comprises five transmembrane segments and an extracytosolic amino terminus, whereas BCRP is a "half-transporter" and requires at least two NBDs to function as a drug efflux pump (Nakanishi et al., 2003). Functional BCRP is described to be in the form of homodimers or homomultimers, with homodimer formation occurring through linkages at disulfide bridges at cysteine 603. These differences in structure account for differences in molecular weight (approximately 170, 190, and 70 kDa for MDR1, MRP1, and BCRP, respectively), but there is considerable

overlap in many of the xenobiotics that are transported by these proteins.

Members of the ABC transport family are expressed constitutively in many cells, and collectively they play important roles in absorption from the GI tract and elimination into bile or into urine for a wide range of xenobiotics. They are also critical to maintaining the barrier function of numerous tissue sites including the blood–brain barrier (BBB), the blood–testis barrier, and the maternal–fetal barrier or placenta. Hence, they play a central role in the disposition and toxicity of xenobiotics. Their function in absorption, distribution, and excretion will be discussed throughout the remaining sections of this chapter.

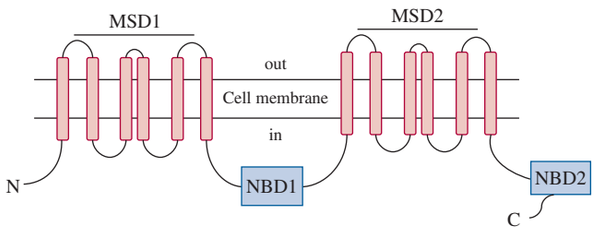

Figure 5-5. *Diagrammatic representation of the structure of MDR1, illustrating the membrane spanning domain and ATP-binding domains (NBD).*

The second major family of xenobiotic transporters is the solute carriers (SLCs), which function predominantly as facilitative transporters. There are 43 SLC gene families identified, and many of the nearly 300 genes comprising the 43 distinct SLC families play important roles in the disposition of endogenous compounds, including glucose, neurotransmitters, nucleotides, essential metals, and peptides. Additionally, there are several families that are vital to xenobiotic disposition, regulating the movement of many diverse organic anions and cations across cell membranes (Hediger et al., 2004; Klaassen and Aleksunes, 2010).

The major human solute carriers involved in xenobiotic disposition are summarized in Box 5-2. The organic anion–transporting peptides (OATPs, SLCO family) are important membrane transport

Box 5-2

Major Members of the Human Solute Carrier Transporter Families Involved in Xenobiotic Disposition

TRANSPORTER	GENE FAMILY	HUMAN PROTEINS	GENE NAME	FUNCTION	TOXICOLOGICAL IMPACT
Organic anion transporting polypeptide (OATP)	*SLCO*	OATP1A2 OATP1B1 OATP1B3 OATP1C1 OATP2A1 OATP2B1 OATP3A1 OATP4A1 OATP4C1 OATP5A1 OATP6A1	SLCO1A2 SLCO1B1 SLCO1B3 SLCO1C1 SLCO2A1 SLCO2B1 SLCO3A1 SLCO4A1 SLCO4C1 SLCO5A1 SLCO6A1	Transport of organic anions, cations, and neutral compounds	1. OATP1B1: Decreased function due to polymorphisms can decrease hepatic extraction of substrates and leading to increased extrahepatic concentrations (example: statins); transport of chemicals of environmental concern (example: perfluoroalkyl substances) and microcystins 2. Oatp2b1: Transport of microcystins in rodents 3. OATP2B1: Expression in intestine and important bioavailability of organic anions
Organic cation transporter (OCT)	*SLC22*	OCT1 OCT2 OCT3	SLC22A1 SLC22A2 SLC22A3	Transport of organic cations	1. OCT1: Important for metformin uptake into liver and efficacy 2. OCT2: Cisplatin dose-limiting nephrotoxicity
Organic cation/carnitine transporter (OCTN)	*SLC22*	OCTN1 OCTN2	SLC22A4 SLC22A5	Organic cations OCTN2 specific for carnitine	
Organic anion transporter (OAT)	*SLC22*	OAT1 OAT2 OAT3 OAT4 OAT5	SLC22A6 SLC22A7 SLC22A8 SLC22A11 SLC22A10	Transport of organic anions	OAT1: Antiviral drugs cidofovir and adefovir; methylmercury; ochratoxin A
Peptide transporter (PEPT)	*SLC15*	PEPT1 PEPT2	SLC15A1 SLC15A2	Transport of dipeptides and tripeptides, and xenobiotics	Aids in the renal excretion of beta-lactam antibiotics of the cephalosporin and penicillin classes, angiotensin-converting enzyme inhibitors (ACE inhibitors), and amino acid–conjugated antiviral drugs (e.g., valacyclovir)
Multidrug and toxin extrusion transporter (MATE)	*SLC47*	MATE1 MATE2K	SLC47A1 SLC47A2	Efflux of organic cations; MATE2K localized to kidney	Aids in metformin excretion

Gene and protein members of the solute carrier families are compiled from Mizuno et al. (2003), Hagenbuch and Meier (2004), Sahi (2005), Lin (2006), Hagenbuch and Guo (2008), Kusuhara and Sugiyama (2009), and Klaassen and Aleksunes (2010).

proteins that mediate the sodium-independent transport of a wide range of compounds, including organic acids, bases, and neutral compounds. Although they are largely regarded as influx pumps, solutes can move bidirectionally, and these proteins appear to be especially important in the hepatic uptake of xenobiotics. In human liver, OATP1B1, OATP1B3, OATP1A2, and OATP2B1 have been identified, whereas the major Oatps in rat liver are Oatp1a1, Oatp1a4, and Oatp1b2. For OATPs, the mechanism of transport is anion exchange such that the cellular influx of an organic compound is thought to be coupled to the efflux of bicarbonate, glutathione, or glutathione conjugates.

The organic-anion transporter (OAT; SLC22) family is particularly important in the renal uptake of anions, and human proteins (OAT1-5) have been identified in the kidney, whereas Oat1 and Oat3 are the major transporters identified in rodent kidneys. The organic-cation transporter (OCT; also SLC22) family is important in both the renal and hepatic uptake of xenobiotics. In this family, OCT1 is expressed and functionally important for uptake of cations into liver for human, rats, and mice (Hayer et al., 1999; Slitt et al., 2002; Wang et al., 2002; Alnouti et al., 2006). OCT2 is expressed and functionally important for organic cation uptake into kidney for humans and rodents Motohashi and Inue, 2013. Novel organic-cation transporters (OCTNs; also SLC22) have also been identified which transport exogenous cations along with important endogenous compounds such as carnitine. OATs transport substrates against an electrochemical gradient in exchange for intracellular dicarboxylates, such as α-ketoglutarate, whereas the driving force for OCT-mediated transport is the electrochemical gradient of the transported cation, typical of facilitated diffusion (described above). Peptide transporters (PEPT1 and PEPT2; SLC15) are responsible for the transport of di- and tripeptides as well as drugs and toxicants such as the β-lactam antibiotics. Finally, the multidrug and toxin extrusion transporters (MATEs; SLC47) are a unique gene family of solute carriers expressed predominantly in liver and kidney that function specifically as cation efflux pumps.

Additional Transport Processes Other forms of specialized transport have been proposed, but their overall importance is not as well established as that of active transport and facilitated diffusion. Phagocytosis and pinocytosis are proposed mechanisms for cell membranes flowing around and engulfing particles. This type of transfer has been shown to be important for the removal of particulate matter from the alveoli by phagocytes and from blood by the reticuloendothelial system of the liver and spleen.

ABSORPTION

The process by which toxicants cross body membranes to enter the bloodstream is referred to as absorption. Xenobiotics penetrate membranes during absorption by the same processes as do biologically essential substances such as oxygen, foodstuffs, and other nutrients. The main sites of absorption are the GI tract, lungs, and skin. However, absorption may also occur from other sites, such as the subcutis, peritoneum, or muscle, if a chemical is administered by special routes. Experimentalists and medical professionals often distinguish between parenteral and enteral administration of drugs and other xenobiotics. Enteral administration includes all routes pertaining to the alimentary canal (sublingual, oral, and rectal), whereas parenteral administration involves all other routes (intravenous, intraperitoneal, intramuscular, subcutaneous, etc.).

Absorption of Toxicants by the Gastrointestinal Tract

The GI tract is one of the most important sites where toxicants are absorbed. Many environmental toxicants enter the food chain and are absorbed together with food from the GI tract. This site of absorption is also particularly relevant to toxicologists because accidental ingestion is the most common route of unintentional exposure to a toxicant (especially for children) and intentional overdoses most frequently occur via the oral route.

The GI tract may be viewed as a tube traversing the body. Although it is within the body, its contents remain outside of the body. Therefore, unless a noxious chemical has direct caustic or irritating properties, toxicants remaining in the GI tract may damage the cells that make up the organ, but usually do not produce systemic injury until they are absorbed.

Absorption of toxicants can take place along the entire GI tract, even in the mouth and the rectum. Therefore, although the majority of drugs are given orally, drugs such as nitroglycerin are administered sublingually, whereas others are administered as rectal suppositories. If a toxicant is an organic acid or base, it can be absorbed by simple diffusion in the part of the GI tract where it exists in its most lipid-soluble (nonionized) form. Because gastric juice is acidic (pH about 2) and the intestinal contents are nearly neutral, the lipid solubility of weak organic acids or bases can differ markedly in these two areas of the GI tract. It should also be noted that pH along the GI differs in the fed and fasting state. The Henderson–Hasselbalch equations (discussed above) determine the fraction of a toxicant that is in the nonionized (lipid-soluble) form and estimate the rate of absorption from the stomach or intestine. As illustrated in Fig. 5-3, a weak organic acid such as benzoic acid is present mainly in the nonionized (lipid-soluble) form in the stomach and predominantly in the ionized form in the intestine. Therefore, weak organic acids are absorbed more readily from the stomach than from the intestine. In contrast, organic bases (except very weak organic bases) are likely to be ionized and not lipid soluble in the stomach, but are more likely to be in the nonionized form in the intestine, suggesting that the absorption of such compounds occurs predominantly in the intestine rather than in the stomach. However, the Henderson–Hasselbalch calculations are not an absolute determination of absorption because other factors—including the mass action law, surface area, and blood flow rate—have to be taken into consideration in examining the absorption of weak organic acids or bases. For example, only 1% of benzoic acid is present in the lipid-soluble form in the neutral pH of the intestine. Therefore, one might conclude that the intestine has little capacity to absorb this organic acid. However, absorption is a dynamic process. The blood keeps removing benzoic acid from the lamina propria of the intestine, and according to the mass action law, the equilibrium will always be maintained at 1% in the nonionized form, providing continuous availability of benzoic acid for absorption. Moreover, absorption by simple diffusion is also proportional to the surface area. The small intestine has a very large surface because the villi and microvilli increase the surface area approximately 600-fold, such that the overall capacity of the intestine for absorption of benzoic acid is quite large. Similar considerations are also valid for the absorption of all weak organic acids from the intestine.

Numerous xenobiotic transporters are expressed in the GI tract, where they function to increase or decrease absorption of xenobiotics (Fig. 5-6). There are a handful transporters in the intestine that are considered to impact absorption and for which evidence is strong that they impact bioavailability—namely, PEPT1, OATP2B1, and MDR1 (*p*-glycoprotein; PgP).

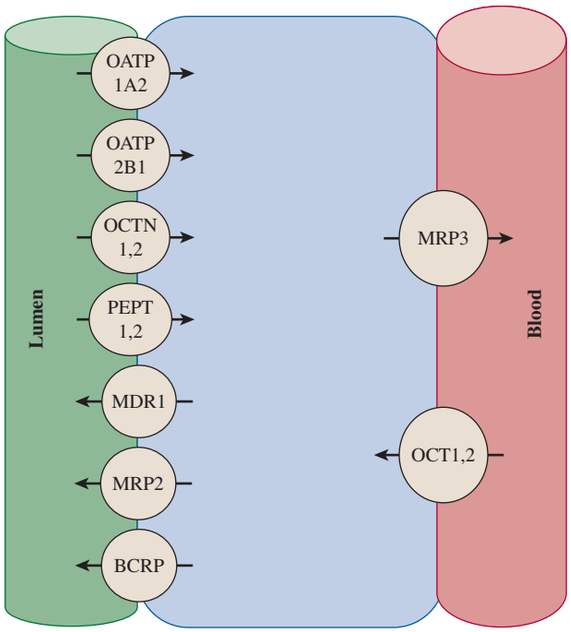

Figure 5-6. *Schematic model showing the important xenobiotic transport systems present in the human gastrointestinal tract.*

With regard to uptake, several proteins in the SLC families are expressed in the intestine where they are predominantly localized on the apical brush-border membranes of the enterocytes and increase uptake from the lumen into the enterocytes. In humans, recent proteomic analysis has characterized transporter abundance along the gastrointestinal tract in humans (Gröer et al., 2013; Drozdzik et al., 2014). Among the uptake transporters, PEPT1 relative protein abundance is higher than the other transporters for both jejunum and ileum. PEPT1 mediates the transport of peptide-like drugs such as antibiotics, particularly those containing a β-lactam structure. In the small intestine, OATP2B1 was detected but not OATP1A2. OCT1 and OCT3 proteins are detected in the intestinal tract, with OCT1 being significantly higher in expression and expressed on the basolateral membrane to contribute to xenobiotic uptake into enterocytes (Klaassen and Aleksunes, 2010; Tamai, 2012; Han et al., 2013).

MRP2 and MRP3 protein expression was higher in colon than in small intestine. OCTN1 and OCTN2 are also present in the intestine, with OCTN2 specifically involved in the uptake of carnitine. The primary active efflux transporters such as MDR1, MRP2, and BCRP are also expressed on enterocyte brush-border membranes where they function to excrete their substrates into the lumen, thereby decreasing the net absorption of xenobiotics. MRP3 is also found in the intestine, but is localized to the basolateral membrane. The expression of the intestinal transporters varies across the GI tract. For example, MDR1 expression increases from the duodenum to colon, whereas MRP2 and most of the uptake transporters are expressed most highly in the duodenum and decrease in the terminal ileum and colon. BCRP is found throughout the small intestine and colon.

The efflux transporters are particularly relevant to the disposition of toxicants, as there will be a net reduction in the absorption of chemicals that are substrates for these transporters, and this is a desirable outcome for toxic chemicals. For example, the dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine (PhIP), a heterocyclic amine produced during the cooking of meat, is a substrate for MRP2. Studies comparing the disposition of PhIP in wild-type and Mrp2-deficient rats showed that MRP is very important in limiting the oral absorption of this harmful compound (Dietrich et al., 2001). However, although limiting absorption of toxicants and carcinogens is beneficial, these transporters can also function to limit the oral absorption of drugs. For example, the immunosuppressive drug cyclosporine and the chemotherapeutic anticancer drugs paclitaxel (taxol), colchicine, and vincristine are not readily absorbed from the GI tract because they are good substrates for MDR1.

The number of toxicants actively absorbed by the GI tract is low; most enter the body by simple diffusion. Although lipid-soluble substances are absorbed by this process more rapidly and extensively than are water-soluble substances, the latter may also be absorbed to some degree. After oral ingestion, about 10% of lead, 4% of manganese, 1.5% of cadmium, and 1% of chromium salts are absorbed. If a compound is very toxic, even small amounts of absorbed material produce serious systemic effects. An organic compound that would not be expected to be absorbed on the basis of the pH-partition hypothesis is the fully ionized quaternary ammonium compound pralidoxime chloride (2-PAM; molecular weight 137 Da), yet it is absorbed almost entirely from the GI tract. The mechanism by which some lipid-insoluble compounds are absorbed is not entirely clear. It appears that organic ions of low molecular weight (<200 Da) can be transported across the mucosal barrier by paracellular transfer, that is, passive penetration through aqueous pores at the tight junctions or by active transport as discussed above.

Particles and particulate matter can also be absorbed by the GI epithelium. In this case, particle size is a major determinant of absorption, whereas factors such as the lipid solubility or ionization characteristics are less important. For particles, size is inversely related to absorption such that absorption increases with decreasing particle diameter (Florence et al., 1995). This explains why metallic mercury is relatively nontoxic when ingested orally and why powdered arsenic was found to be significantly more toxic than its coarse granular form (Schwartze, 1923). Large particles (greater than about 20 μm in diameter) enter intestinal cells by pinocytosis, a process that is much more prominent in newborns than in adults, after which they are carried through the intestinal epithelium in intact vesicles and discharged into the interstices of the lamina propria. Absorption into gut-associated lymphoid tissue (such as Peyer's patches) and the mesenteric lymph supply also plays a key role in systemic absorption of particles (Jani et al., 1990; Florence et al., 1995).There is increasing interest in particles of very small diameter that may be used in a variety of chemical and biological processes. Nanoparticles or nanomaterials are typically less than 100 nm in size, and numerous issues have been raised regarding the toxic potential of these entities (Chap. 28). Early studies that compared size demonstrated that greater absorption of smaller (50 nm) particles compared to larger (100 nm) particles was observed, and with 300-nm particles being minimally absorbed. A multistep process is required for particles to translocate through the intestinal barrier. This includes translocation through the mucus layer, contact with enterocytes and potentially M cells, and uptake, which appear to occur through endocytosis. Surface characteristics of nanoparticles also contribute to their absorption, with hydrophobic, nonionized particles being more extensively absorbed than those modified to possess an ionized surface as is the case with larger particles, the gut-associated lymphoid tissue appears to be the predominant absorption pathway for nanoparticles from the GI tract (Stern and McNeil, 2008; Fröhlich, 2012). Overall, the absorption of a toxicant from the GI tract depends on its physical properties, including lipid

solubility and its dissolution rate. Although it is often generalized that an increase in lipid solubility increases the absorption of chemicals, an extremely lipid-soluble chemical is unlikely to dissolve in the aqueous fluids within the GI tract, which limits its absorption from the GI tract. Similarly, if the toxicant is a solid and is relatively insoluble in GI fluids, it will also have limited contact with the GI mucosa and its rate of absorption will be low (Lipinski et al., 2001).

In addition to the characteristics of the compounds themselves, there are numerous additional factors relating to the GI tract itself that influence the absorption of xenobiotics. These factors include pH, the presence of food, digestive enzymes, bile acids, and bacterial microflora in the GI tract, along with the motility and permeability of the GI tract. Chemical resistance or lack of resistance to alteration by the acidic pH of the stomach, enzymes of the stomach or intestine, or the intestinal microflora is extremely important. A toxicant may be hydrolyzed by stomach acid, biotransformed by enzymes in the GI tract, or modified by the resident microflora to new compounds with a toxicity different from that of the parent compound. For example, snake venoms, which are proteinaceous moieties, are much less toxic by the oral route relative to intravenous exposure because they are degraded by digestive enzymes of the GI tract.

Intestinal microflora can also influence absorption and toxicity of compounds. For example, a variety of nitroaromatic compounds are reduced by intestinal bacteria to potentially toxic and carcinogenic aromatic amines. Nitroreduction of 2,6-dinitrotoluene to the mutagenic 2,6-diaminotoluene in the GI tract produces long-term adverse effects, including mutagenicity and carcinogenicity, which are not observed in germ-free animals (Rickert et al., 1984). Similarly, the nitroaromatic perfume, musk xylene, can inhibit the activity of cytochrome P450 enzymes in the liver only after it is reduced to an amine derivative by resident microflora in the GI tract and subsequently absorbed (Lehman-McKeeman et al., 1997). It has also been shown that ingestion of well water with a high nitrate content produces methemoglobinemia much more frequently in infants than in adults. In this case, bacteria in the GI tract convert nitrate to nitrite, increasing the likelihood of methemoglobinemia (Mensinga et al., 2003). Infants are more susceptible to methemoglobinemia because the higher pH of the neonatal GI tract is permissive for the growth of bacteria (such as *Escherichia coli*) that convert nitrate to nitrite. One example wherein intestinal microfloras reduce the potential toxicity is that of the mycotoxin, deoxynivalenol, which is found in numerous grains and foodstuffs. Strict anaerobes detoxify this compound leading to the absorption of a less toxic reductive metabolite (Awad et al., 2010). The contribution of bacteria in the GI tract to other aspects of xenobiotic disposition, particularly enterohepatic circulation, is discussed in greater detail later in this chapter.

Chemicals, such as the chelator, ethylenediaminetetraacetic acid (EDTA), increase absorption of some toxicants by increasing intestinal permeability. An increase in permeability is thought to result from the chelation of calcium that is involved in the formation and maintenance of tight intercellular junctions (Ballard et al., 1995) such that binding of calcium reduces the integrity of the cell junctions, allowing more paracellular absorption. Furthermore, simple diffusion is proportional not only to the surface area and the permeability but also to the residency time within various segments of the GI tract. Therefore, the rate of absorption of a toxicant remaining for longer periods in the intestine increases, whereas that with a shorter residency time decreases. Experiments have shown that the oral toxicity of some chemicals is increased by diluting the dose (Borowitz et al., 1971). This phenomenon may be explained by more rapid stomach emptying induced by increased dosage volume, which in turn leads to greater absorption in the duodenum because of the larger surface area there. Furthermore, some chemicals used as laxatives reduce absorption of xenobiotics by increasing intestinal motility, whereas agents used as antidiarrheals may increase absorption by slowing intestinal motility.

The amount of a chemical that enters the systemic circulation after oral administration depends on several factors. The amount absorbed into the cells of the GI tract is important, and transporters can influence this amount by affecting the uptake or efflux from the cells. Further, before a chemical enters the systemic circulation, it can be biotransformed by the cells in the GI tract or extracted by the liver and excreted into bile with or without prior biotransformation. This phenomenon of the removal of chemicals before entrance into the systemic circulation is referred to as presystemic elimination or first-pass effect. The lung can also contribute to the biotransformation or elimination of chemicals before their entrance into the systemic circulation, although its role is less well defined than that of the intestine and the liver. Chemicals that have a high first-pass effect will appear to have a lower absorption because they are eliminated as quickly as they are absorbed. For toxicants, a high first-pass effect will serve to limit exposure and typically minimizes toxic potential.

A number of other factors have been shown to alter absorption. For example, although lead and many other heavy metal ions are not absorbed readily from the GI tract, EDTA and other chelators increase the lipid solubility and thus the absorption of complexed ions. Thus, it is important not to administer a chelator orally when excess metal is still present in the GI tract after oral ingestion. Furthermore, metal ions can affect absorption of other ions. For example, cadmium decreases the absorption of zinc and copper, calcium decreases cadmium absorption, and magnesium decreases absorption of fluoride (Pfeiffer, 1977). Consumption of grapefruit juice can also influence GI absorption through the actions of naringin, a flavonoid that inhibits the function of several transporters including MDR1 (Pgp) (Bailey, 2010). By reducing MDR1-dependent efflux, grapefruit juice increases GI absorption of numerous drugs (such as calcium-channel blockers and cholesterol-lowering agents), and, in some cases, this effect leads to toxic or adverse reactions resulting from increased exposure to the drugs. Alternatively, inhibition of OATPs reduces absorption and could limit toxicity. Atenolol, celiprolol, and fexofenadine are considered to be the most sensitive substrates to evaluate intestinal DDIs related to OATP inhibition (Yu et al., 2017). OATP inhibition in the gut has been reported for common juices, such as apple juice, grapefruit juice, and orange juice. Also, interestingly, decreased intestinal BCRP function can also impact absorption. Sulfasalazine and rosuvastatin exposure was higher in polymorphic individuals that have an ABCG2 421C/A mutation (Kashihara et al., 2017).

Species differences in absorption across the GI tract are widely recognized. As one example, absorption of nadolol after intraperitoneal and oral dosing was compared across species by calculating the exposure as plasma area-under-the-curve (AUC) and was found to be essentially complete in dogs, substantially less in humans, and limited in rats. Urinary and fecal excretion of nadolol support the bioavailability data, as the low oral absorption of nadolol in rats is consistent with the large amount of drug recovered in feces (Table 5-3). However, excretory data further indicate that in addition to the nonabsorbed portion of this compound, biliary and possibly nonbiliary sources also contribute to the fecal excretion of this compound. Similar comparisons across species provide further evidence for species differences in the absorption of numerous compounds, indicating that nadolol is not an exceptional case (Calabrese, 1984).

Table 5-3

Absorption and Excretion of Radioactivity in Rats, Dogs, and Humans After Nadolol Dosages*

SPECIES	DOSE (mg/kg)	PERCENT OF DOSE EXCRETED			PERCENT OF DOSE ABSORBED
		ROUTE	URINE	FECES	
Rat	20	ip	62	31	(100)
	20	po	11	84	18
Dog	25	ip	75	12	(100)
	25	po	76	28	102
Human	2	ip	73	23	(100)
	2	po	25	77	34

po and ip denote oral and intraperitoneal administration, respectively.
**SOURCE: Data from Dreyfuss et al. (1978).*

Table 5-4

pH of the Gastrointestinal Contents of Various Species*

SPECIES	PH				
	STOMACH	JEJUNUM	CECUM	COLON	FECES
Monkey	2.8	6.0	5.0	5.1	5.5
Dog	3.4	6.6	6.4	6.5	6.2
Rat	3.8	6.8	6.8	6.6	6.9
Rabbit	1.9	7.5	6.6	7.2	7.2

**SOURCE: Data from Smith (1965).*

The factors that contribute to species differences in absorption are not fully understood. Anatomical considerations are likely to contribute to species differences in intestinal absorption, and substantial functional differences exist between species. Because most xenobiotics are transported across the GI mucosa by passive diffusion, and because this transport is surface area- and site-dependent, it is likely that these factors will also contribute to species differences. Furthermore, species differences in the pH along the GI tract exist (Table 5-4), varying by as much as two pH units. The magnitude of this diversity can translate into differences up to two orders of magnitude in the concentration of the nonionized versus ionized moiety of a weak organic acid or base available for absorption. In addition, the GI absorption is often higher in dogs compared to other species because paracellular absorption is thought to occur to a greater extent in dogs.

Another factor that may result in species-dependent absorption of xenobiotics is the presence and function of the GI microflora. Bacteria in the GI tract are highly abundant, with strict anaerobes (oxygen intolerant) being the most prevalent. Approximately 1000 bacterial species have been characterized along the gastrointestinal tract (Frank et al., 2007). The intestinal microflora impacts the bioavailability of numerous drugs, such as diclofenac, lovastatin, and metronidazole (Enright et al., 2016). Use of germ-free mice indicates that the presence of bacteria impacts the mRNA expression of biotransformation enzymes and transporters in the host intestine (Fu et al., 2017). It should be noted that species differences in gut microflora composition are appreciable. In general, mice are considered to be an excellent model for studying changes in gut microbial diversity and much knowledge has been gained through using mice as a model species. However, although some similarities exist between the intestinal microflora of humans and mice for many common disease models (Nguyen et al., 2015), rats are suggested to better reflect the human gut composition (Wos-Oxley et al., 2012). Humanized rats even more closely reflect the gut microbiota of human donors (Wos-Oxley et al., 2012). As expected, nonhuman primates, chimpanzees, have a similar gut microbial composition as found in humans (Moeller et al., 2012).

Absorption of Toxicants by the Lungs

Toxic responses to chemicals can occur from absorption following inhalation exposure. Relevant examples include carbon monoxide poisoning and silicosis, an important occupational disease. These toxicities result from absorption or deposition of airborne poisons in the lungs.

A major group of toxicants that are absorbed by the lungs are gases (e.g., carbon monoxide, nitrogen dioxide, and sulfur dioxide), vapors of volatile or volatilizable liquids (e.g., benzene and carbon tetrachloride), and aerosols. Because the absorption of inhaled gases and vapors differs from that of aerosols, aerosols are discussed separately below. However, the absorption of gases and vapors is governed by the same principles, and therefore the word gas is used to represent both in this section.

Gases and Vapors A vapor is the gas form of substance that can also exist as a liquid or solid at atmospheric pressure and normal temperature. Most organic solvents evaporate and produce vapors, and some solids can also sublimate into a gaseous form. Vapor pressure is that exerted by a vapor above its own liquid in a closed system, such that liquids that have a high vapor pressure have a higher tendency to evaporate. A toxicant with a high vapor pressure at room temperature is considered to be volatile. Examples of the vapor pressure of several toxicants are presented in Table 5-5.

The absorption of inhaled gases takes place mainly in the lungs. However, when inhaled, gases first pass through the nose, filtering through delicately scrolled, simple epithelial-lined turbinates, which serve to increase the surface area of exposure. Because the mucosa of the nose is covered by a film of fluid, gas molecules can be retained by the nose and do not reach the lungs if they are very water soluble or react with cell surface components. Therefore, the nose acts as a "scrubber" for water-soluble and highly reactive gases, partially protecting the lungs from potentially injurious insults. Although these actions may serve to reduce systemic

Table 5-5

Vapor Pressures of Common Solvents or Toxicants

SUBSTANCE	VAPOR PRESSURE (SI UNITS; KPA*)	VAPOR PRESSURE (MM HG)
Ethylene glycol	0.5	3.75
Water	2.3	17.5
Ethanol	5.83	43.7
Chloroform	30	225
Acetaldehyde	98.7	740
Formaldehyde	435.7	3268

**SI units are the International System of Units for pressure with the Pascal (Pa) as the standard unit. All values obtained at 20°C.*

exposure or to protect the lungs, they also increase the risk that the nose could be adversely affected. Such is the case with formaldehyde (Kerns et al., 1983) and vinyl acetate (Bogdanffy et al., 1999), which cause tumors of the nasal turbinates in rats.

Absorption of gases in the lungs differs from intestinal and percutaneous absorption of compounds in that the dissociation of acids and bases and the lipid solubility of molecules are less important factors in pulmonary absorption because diffusion through cell membranes is not rate-limiting in the pulmonary absorption of gases. There are at least three reasons for this. First, ionized molecules are of very low volatility, so that they do not achieve significant concentrations in normal ambient air. Second, type I pneumocytes (epithelial cells lining the alveoli; Chap. 15) are very thin and the capillaries are in close contact with the pneumocytes, so that the distance for a chemical to diffuse is very short. Third, chemicals absorbed by the lungs are removed rapidly by the blood, and blood moves very quickly through the extensive capillary network in the lungs.

When a gas is inhaled into the lungs, gas molecules diffuse from the alveolar space into the blood and then dissolve. Except for some gases with a special affinity for certain body components (e.g., the binding of carbon monoxide to hemoglobin), the uptake of a gas by a tissue usually involves the simple physical process of dissolving. The end result is that gas molecules partition between two media, namely air and blood during the absorptive phase and blood and other tissues during the distributive phase. As the inspired gas remains in contact with blood in the alveoli, more molecules dissolve in blood until gas molecules in blood are in equilibrium with gas molecules in the alveolar space. At this equilibrium, the ratio of the concentration of chemical in the blood and chemical in the gas phase is constant. This solubility ratio is called the blood-to-gas partition coefficient, and it is unique for each gas. Note that although the ratio is constant, the concentrations achieved vary in accordance with Henry's law, which dictates that the amount of gas dissolved in a liquid is proportional to the partial vapor pressure of the gas in the gas phase at any given concentration before or at saturation. Thus, the higher the inhaled concentration of a gas (i.e., the higher the partial pressure), the higher the gas concentration in blood, but the blood:gas ratio does not change unless saturation has occurred. When equilibrium is reached, the rate of transfer of gas molecules from the alveolar space to blood equals the rate of removal by blood from the alveolar space. For example, chloroform has a relatively high blood-to-gas partition coefficient (approximately 20), whereas ethylene has a low coefficient (0.14). By comparison, a smaller percentage of the total ethylene in the lungs is removed into the blood during each circulation because the low blood-to-gas partition coefficient dictates that blood is quickly saturated with this gas. Therefore, an increase in the respiratory rate or minute volume does not change the transfer of such a gas to blood. In contrast, an increase in the rate of blood flow increases the rate of uptake of a compound with a low solubility ratio because of more rapid removal from the site of equilibrium, that is, the alveolar membranes. It has been calculated that the time to equilibrate between the blood and the gas phase for a relatively insoluble gas is about 10 to 20 minutes.

A gas with a high blood-to-gas partition coefficient, such as chloroform, is readily transferred to blood during each respiratory cycle so that little if any remains in the alveoli just before the next inhalation. The more soluble a toxic chemical is in blood, the more of it will be dissolved in blood by the time equilibrium is reached. Consequently, the time required to equilibrate with blood is much longer for a gas with a high blood-to-gas partition coefficient than for a gas with a low ratio. This has been calculated to take a minimum of 1 hour for compounds with a high solubility ratio, although it may take even longer if the gas also has high tissue affinity (i.e., high fat solubility). With highly soluble gases, the principal factor limiting the rate of absorption is respiration. Because the blood is already removing virtually all gases with a high solubility ratio from the lungs, increasing the blood flow rate does not substantially increase the rate of absorption. However, the rate can be accelerated greatly by increasing the rate of respiration.

The blood carries the dissolved gas molecules to the rest of the body. In each tissue, the gas molecules are transferred from the blood to the tissue until equilibrium is reached at a tissue concentration dictated by the tissue-to-blood partition coefficient. After releasing part of the gas to tissues, blood returns to the lungs to take up more of the gas. The process continues until a gas reaches equilibrium between blood and each tissue according to the tissue-to-blood partition coefficients characteristic of each tissue. This equilibrium is referred to as steady state, and at this time, no net absorption of gas takes place as long as the exposure concentration remains constant.

AEROSOLS AND PARTICLES

Absorption of aerosols and particles is distinguished from gases and vapors by the factors that determine absorption from the inhalation route of exposure. The absorption of gases and vapors by inhalation is determined by the partitioning of the compound between the blood and the gas phase along with its solubility and tissue reactivity. In contrast, the important characteristics that affect absorption after exposure to aerosols are the aerosol size and water solubility of any chemical present in the aerosol.

The site of deposition of aerosols and particulates depends largely on the size of the particles. This relationship is discussed in detail in Chap. 15. In general, the smaller the particle, the further into the respiratory tree the particle will deposit (Fig. 5-7). Particles ranging from 5 μm or larger, described as "course particles," usually are deposited in the nasopharyngeal region. Those deposited on the unciliated anterior or rostral portion of the nose tend to remain at the site of deposition until they are removed by nose wiping, blowing, or sneezing. The mucous blanket of the ciliated nasal surface propels insoluble particles by the movement of the cilia. These particles and particles inhaled through the mouth are swallowed within minutes. Soluble particles may dissolve in the mucus and be carried to the pharynx or may be absorbed through the nasal epithelium into the blood.

Particulate matter with diameters of approximately 2.5 μm, referred to as "fine particles," are deposited mainly in the tracheobronchiolar regions of the lungs, from which they may be cleared by retrograde movement of the mucus layer in the ciliated portions of the respiratory tract (also known as the mucociliary escalator). The rate of cilia-propelled movement of mucus varies in different parts of the respiratory tract, although in general it is a rapid and efficient transport mechanism. Toxicants or viral infections that damage cilia may impair the efficiency of this process. Measurements have shown transport rates between 0.1 and 1 mm/min, resulting in removal half-lives between 30 and 300 minutes. Coughing and sneezing greatly increase the movement of mucus and particulate matter toward the mouth. Particles eventually may be swallowed and absorbed from the GI tract or expectorated.

Particles 1 μm and smaller penetrate to the alveolar sacs of the lungs. Ultrafine particles or nanoparticles, particularly those that are approximately 10 to 20 nm in size, have the greatest likelihood of depositing in the alveolar region. These extremely small particles may be absorbed into blood or cleared through

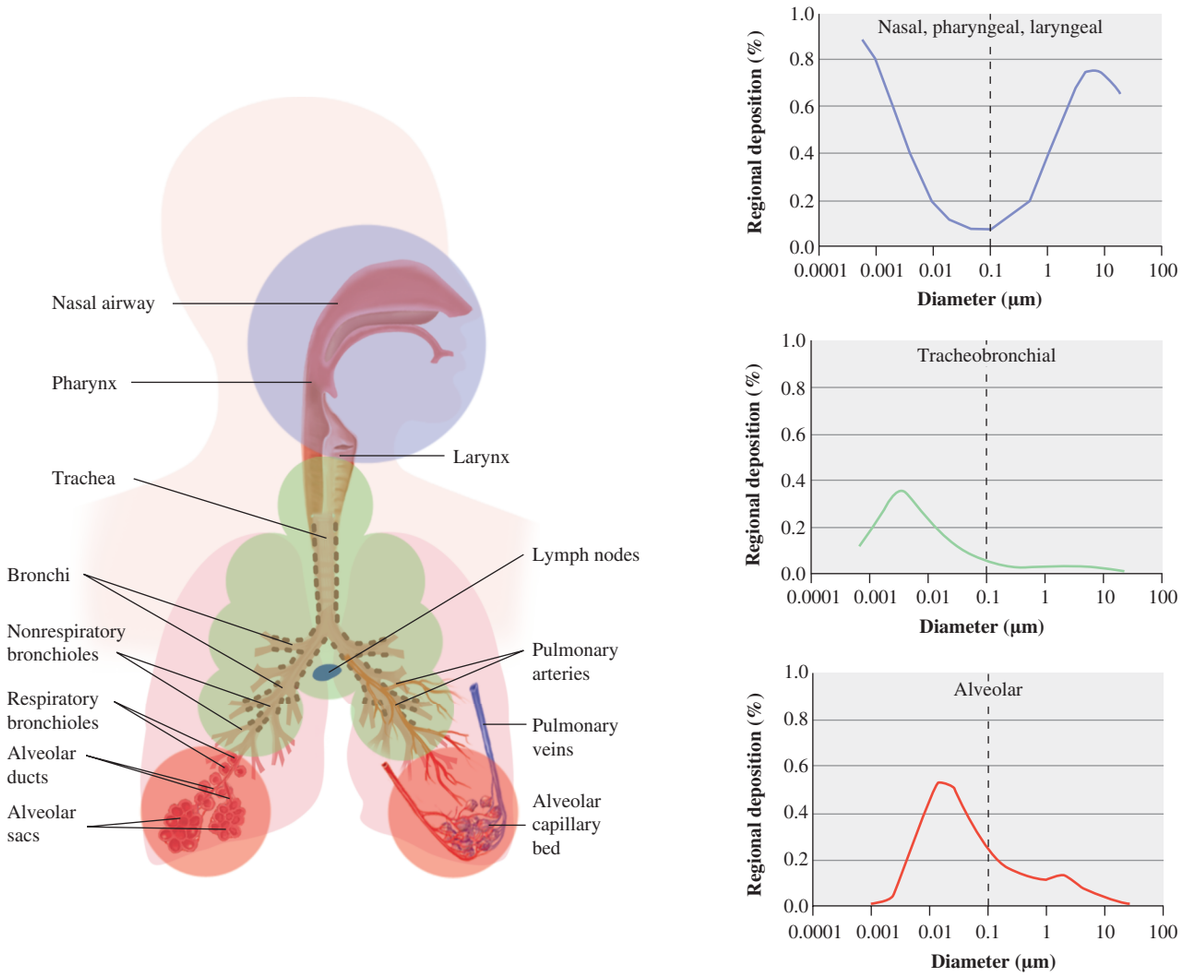

Figure 5-7. *Representation of particle distribution in the lungs relative to size.* (From Oberdorster et al., 2005, with permission.)

the lymphatics after being scavenged by alveolar macrophages (Oberdorster et al., 2005).

In addition to being a major determinant of lung deposition, as particle size decreases, the number of particles in a unit of space increases along with the total surface area of the particles. This relationship, illustrated in Table 5-6, indicates that nanoparticles have the propensity to deliver high amounts of particulates to the lung. However, it appears that the surface properties of nanoparticles may be more important determinants of toxic potential than their size or surface area. The contribution of nanoparticles to toxic responses, with special emphasis on their disposition (and factors that influence disposition including size, composition, surface structure, surface group modification, solubility, and aggregation), is a major area of toxicological and human health effects research (Nel et al., 2006; Stern and McNeil, 2008).

The mechanisms responsible for the removal or absorption of particulate matter from the alveoli are less clear than those responsible for the removal of particles deposited in the tracheobronchial tree. Removal appears to occur by three major mechanisms. First, particles may be removed from the alveoli by a physical process. As described earlier, it is thought that particles deposited on the fluid layer of the alveoli are aspirated onto the mucociliary escalator of the tracheobronchial region. From there, they are transported to the mouth and may be swallowed. The origin of the thin fluid layer in the alveoli is probably a transudation of lymph and secretions of lipids and other components by the alveolar epithelium. The alveolar fluid flows by an unknown mechanism to the terminal bronchioles. This flow seems to depend on lymph flow, capillary action, the respiratory motion of the alveolar walls, the cohesive nature of the respiratory tract's fluid blanket, the propelling power of the ciliated bronchioles, and the surface tension within the fluid

Table 5-6

Particle Number and Surface Area for 10 μg/m³ Airborne Particles

PARTICLE DIAMETER (μm)	PARTICLES/mL AIR	PARTICLE SURFACE AREA (μm²/mL AIR)
5	0.15	12
2	2	30
0.5	153	120
0.02	2,400,000	3016
0.05	153,000,000	12,000

SOURCES: Modified from Oberdorster et al. (2005) and Nel et al. (2006).

layer. Second, particles from the alveoli may be removed by phagocytosis. The principal cells responsible for engulfing alveolar debris are the resident alveolar macrophages. These phagocytic cells are found in large numbers in normal lungs and contain many phagocytized particles of both exogenous and endogenous origin. They apparently migrate to the distal end of the mucociliary escalator and are cleared and eventually swallowed. Third, removal may occur via the lymphatics. The endothelial cells lining lymphatic capillaries are permeable to very large molecules and particles (molecular weight >1000 kDa), although the rate of penetration is low when molecular weight exceeds 10 kDa. Nevertheless, the lymphatic system plays a prominent role in collecting high-molecular-weight proteins leaked from cells or blood capillaries and particulate matter from the interstitium and the alveolar spaces. Particulate matter may remain in lymphatic tissue for long periods, and this explains the phenomenon of "dust store of the lungs."

In general, the overall removal of particles from the alveoli is relatively inefficient. The rate of clearance by the lungs can be predicted by a compound's solubility in lung fluids. The lower the solubility, the lower the removal rate. Thus, it appears that removal of particles that enter the alveoli is largely due to dissolution and vascular transport. Some particles may remain in the alveoli indefinitely. This may occur when long-lived alveolar macrophages phagocytose indigestible dust particles and secrete cytokines that stimulate the development of a local network of type I and III collagen fibers to form an alveolar dust plaque or nodule.

Absorption of Toxicants Through the Skin

Skin is the largest body organ and provides a relatively good barrier for separating organisms from their environment. Overall, human skin comes into contact with many toxic chemicals, but exposure is usually limited by its relatively impermeable nature. However, some chemicals can be absorbed by the skin in sufficient quantities to produce systemic effects. For example, there are several insecticides for which fatal exposures have occurred in agricultural workers after absorption through intact skin (see Chap. 19). In addition, there are numerous chemicals that increase tumor development in other organs after dermal application.

The skin comprises two major layers, the epidermis and dermis (Fig. 5-8). The epidermis is the outermost layer and contains keratinocytes that are metabolically competent and able to divide. Proliferating keratinocytes in the stratum germinativum displace maturing keratinocyte layers upward until they reach the outermost layer, the stratum corneum. The stratum corneum contains densely packed keratinized cells that have lost their nuclei and are biologically inactive. The stratum corneum is replaced approximately every 3 to 4 weeks in adult humans. This complex process involves dehydration and polymerization of intracellular matrix forming keratin-filled dried cell layers. During the process, the cell walls apparently double in thickness and transform into a dry, keratinous semisolid state with much lower permeability for diffusion of toxicants. The stratum corneum is unique anatomically and represents the single most important barrier to preventing fluid loss from the body while also serving as the major barrier to prevent the absorption of xenobiotics into the body.

The dermis is situated beneath the epidermis and consists primarily of fibroblasts. This region also contains the vascular network that provides the dermis and epidermis with blood supply and serves to carry absorbed compounds into the body. Although the major anatomical area that controls absorption across the skin is the stratum corneum, compounds may also be absorbed through dermal appendages, including sweat and sebaceous glands and hair follicles found in the dermis. Sweat glands and hair follicles are scattered in varying densities on skin. These appendages account for no more than 1% of the total cross-sectional area of the total skin

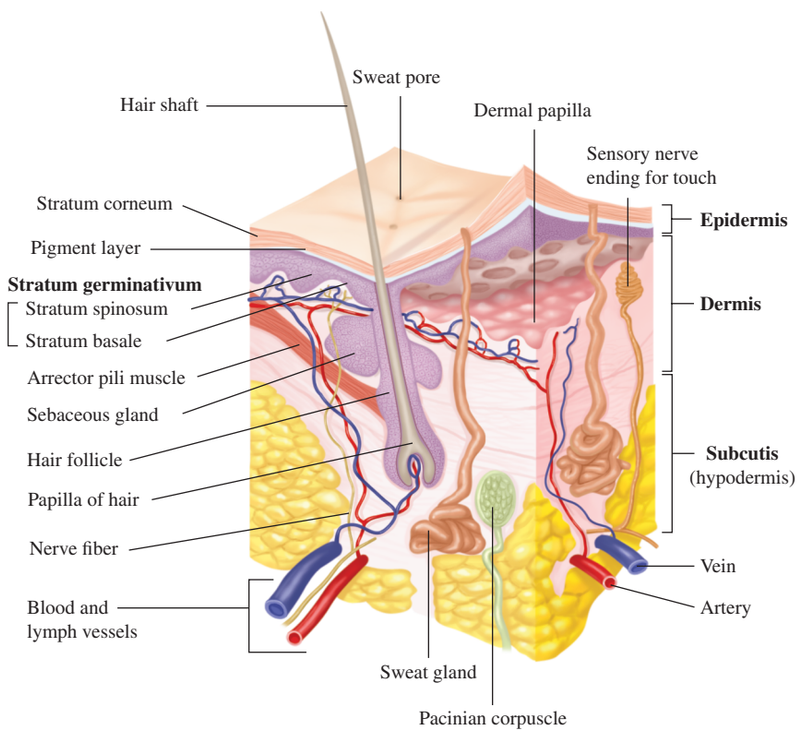

Figure 5-8. *Diagram of a cross section of human skin illustrating the various layers, cellular composition, and blood supply* (www.dentalarticles.com/visual/d/skin-layers.php).

surface, and, in general, passage through these areas is much more rapid than passage through the stratum corneum. Ultimately, to be absorbed a chemical must pass the barrier of the stratum corneum and then traverse the other six layers of the skin (Dugard, 1983; Poet and McDougal, 2002).

In contrast to the complexity of the GI tract, the skin is a simpler penetration barrier for chemicals because passage through the stratum corneum is the rate-determining step. All toxicants move across the stratum corneum by passive diffusion. In general, lipophilic (fat-soluble) compounds are absorbed more readily across the stratum corneum, whereas the penetration of hydrophilic (water-soluble) compounds is more limited. Nonpolar toxicants diffuse through the skin in a manner that is proportional to their lipid solubility and inversely related to molecular weight. However, although lipophilic compounds may pass more readily through the stratum corneum, their passage through the dermis may become rate-limiting. Hydrophilic compounds are more likely to penetrate the skin through appendages such as hair follicles.

Human stratum corneum is significantly different in structure and chemistry in various regions of the body, and these differences affect the permeability of the skin to chemicals. Skin from the palmar and plantar regions is much different from skin from other areas of the body in that the stratum corneum of the palms and soles is adapted for weight bearing and friction and there are no hair follicles present. The stratum corneum of the rest of the body surface is adapted for flexibility and fine sensory discrimination. The permeability of the skin also depends on both the diffusivity and the thickness of the stratum corneum. Although the stratum corneum is much thicker on the palms and soles (400 to 600 μm in callous areas) than on the arms, back, legs, and abdomen (8 to 15 μm), it has much higher diffusivity per unit thickness. In contrast, the skin of the scrotum is characterized by a thin stratum corneum and a high diffusivity. Consequently, as illustrated by the comparative absorption of malathion across different human skin sites (Table 5-7), toxicants are likely to readily cross scrotal skin, whereas absorption across forehead skin is less extensive, and penetration across the palm is lowest because of the thickness of the stratum corneum and the lack of dermal appendages.

The second phase of percutaneous absorption consists of diffusion of the toxicant through the lower layers of the epidermis (stratum germinativum, spinosum, and granulosum) and the dermis. Despite possessing tight intercellular junctions, these cell layers are far inferior to the stratum corneum as diffusion barriers. In contrast to the stratum corneum, they contain a porous, nonselective, aqueous diffusion medium. Toxicants pass through this area by diffusion and enter the systemic circulation through the numerous venous and lymphatic capillaries in the dermis. The rate of diffusion depends on blood flow, interstitial fluid movement, and perhaps other factors, including interactions with dermal constituents.

Table 5-7

Absorption of Malathion Across Different Human Skin Regions

ANATOMICAL REGION	ABSORPTION (%)	STRATUM CORNEUM THICKNESS (μm)	HAIR FOLLICLES/ cm^2
Scrotum	101.6	5	60
Forehead	23.2	13	770
Hand (back)	12.5	49	18
Palm	5.8	400	—

SOURCE: Adapted from Poet and McDougal (2002).

There are several factors that can influence the absorption of toxicants through the skin, including (1) the integrity of the stratum corneum, (2) the hydration state of the stratum corneum, (3) temperature, (4) solvents as carriers, and (5) molecular size. Because the stratum corneum plays a critical role in determining cutaneous permeability, removal of this layer causes a dramatic increase in permeability of the epidermis for a variety of large or small molecules, both lipid-soluble and water-soluble (Poet and McDougal, 2002). Caustic agents, such as acids and alkalis, that damage the stratum corneum increase its permeability. The most frequently encountered penetration-enhancing damage to the skin results from burns and various skin diseases. Water also plays an extremely important role in skin permeability. Under normal conditions, the stratum corneum is partially hydrated, containing about 7% water by weight. This amount of water increases the permeability of the stratum corneum approximately 10-fold over the permeability that exists when it is completely dry. On contact with water, the stratum corneum can increase its weight of tightly bound water up to fivefold, which increases permeability an additional two to threefold. In many studies, the site of application is covered with plastic wrap (occlusive application), originally described by Draize et al. (1944), to hydrate the stratum corneum and enhance the absorption of some toxicants. Similarly, an increase in temperature will increase dermal penetration by increasing dermal blood flow. This is particularly important for occupational exposures to chemicals such as insecticides in which agricultural workers are likely to be working strenuously at relatively high temperatures. Such environmental conditions increase dermal penetration and may increase the risk of systemic toxicity.

Solvents used to dissolve compounds of interest can also influence dermal penetration. In general, lower absorption will be observed if a toxicant is highly soluble in the vehicle, whereas low solubility of the toxicant in the vehicle will tend to increase dermal penetration. In addition, solvents such as dimethyl sulfoxide (DMSO) facilitate the penetration of toxicants through the skin by increasing the permeability of the stratum corneum. Although the mechanism by which DMSO enhances skin permeability is not fully understood, it has been suggested that it (1) removes much of the lipid matrix of the stratum corneum, making holes on artificial shunts in the penetration barrier; (2) alters keratin configuration to change protein structure; and (3) functions as a swelling agent (Williams and Berry, 2004).

With respect to molecular size, it is generally recognized that compounds above 400 Da exhibit poor dermal penetration. As such, nanoparticles may exhibit higher dermal absorption and systemic exposures as a result of their small size. However, although nanoparticles appear to penetrate the skin as a function of their size, their overall absorption is relatively low. For nanomaterials, hair follicles can contribute to dermal penetration, and as a general rule, particles that are approximately 300 to 600 nm penetrate deeply in to hair follicles, where the extensive capillary network around the follicles facilitates uptake (Lademann et al., 2011). Based on these features, nanoparticle formulations may facilitate absorption from the skin. In contrast, nanosized particles of titanium dioxide and zinc oxide, which are widely used in sunscreens, do not appear to penetrate beyond the stratum corneum, a feature that is consistent with their skin protective effects (Newman et al., 2009).

Dermal absorption has been studied in most laboratory animals including rats, mice, rabbits, guinea pigs, primates, and pig, and dermal absorption varies widely across these species. As a general rule, dermal absorption across rodent skin is greater than human skin, whereas the cutaneous permeability characteristics of guinea pigs, pigs, and monkeys are more similar to those observed in humans (Wester and Maibach, 1993). Species differences in percutaneous absorption account for the differential toxicity of insecticides in insects and humans. For example, the LD_{50} of injected DDT is approximately equal in insects and mammals, but DDT is much less toxic to mammals than to insects when it is applied to the skin. This appears to be due to the fact that DDT is poorly absorbed through the skin of mammals but passes readily through the chitinous exoskeleton of insects. Furthermore, insects have a much greater body surface area relative to weight than mammals.

Species differences in dermal absorption of xenobiotics result from several anatomic, physiological, and biochemical factors (Dugard, 1983; Poet and McDougal, 2002). First, the composition and thickness of the stratum corneum along with the nature of the dermal appendages are highly variable across species. The stratum corneum is much thicker in humans than in most laboratory animals, making human skin typically less permeable than animal skin. However, the thinner stratum corneum in animals is often compensated for by a relatively thick hair cover, diminishing direct contact of the skin with a xenobiotic. Sweat and pilosebaceous ducts also vary across species. For example, eccrine sweat glands are located in the pads of the extremities of all mammals. However, the general body surface of humans contains 100 to 600/m^2 of coiled tubular sweat glands, whereas rodents, rabbits, cats, and dogs have none. The number of pilosebaceous ducts in humans and pigs is similar (about 40/cm^2), but rodents may have up to 100 times more. Another important potential rate-limiting step in the dermal absorption of chemicals is the cutaneous blood flow. In humans, the skin plays an important thermoregulatory function as opposed to furred animals. Consequently, there is a much more extensive vasculature in humans than in most mammals (Calabrese, 1984). Biotransformation reactions in skin can also facilitate absorption, and the presence of metabolizing enzymes is highly variable across species. Xenobiotic transporters, including members of the ABCC family and the SLCO and SLC families, have been identified in skin with expression predominantly in keratinocytes. Differences in levels or patterns of expression of these transporters may also contribute to species differences in dermal absorption of toxicants (Kao et al., 1985; Baron et al., 2001; Hashimoto et al., 2013). Recently PgP and BCRP inhibition have been noted to increase the dermal absorption of dermal accumulation of regorafenib and its active metabolites M-2 and M-5, conceptually suggesting that inhibition of transport can impact distribution to skin (Fujita et al., 2017). It is not clear whether the impact is due to change in systemic inhibition levels or at the level of the skin, but the mechanism is potentially relevant to dermal toxicants.

Overall, commonly used laboratory animals are typically not good models of human absorption of toxicants. An example of the species differences and lack of concordance with human data for dermal absorption of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is illustrated in Table 5-8. As illustrated by these data, absorption through rat skin was higher than in other species. In general, absorption through primate or pig skin is considered to most closely predict human dermal absorption (Wester and Maibach, 1993). Additionally, in vitro methods using human skin may provide reasonable alternatives to the use of laboratory animals for evaluating dermal absorption of toxicants (Bronaugh et al., 1982).

Table 5-8

Comparison of Dermal Absorption of 2,4-D Across Species

SPECIES	WASH TIME (HOUR)	COLLECTION TIME (HOUR)	% ABSORBED
Rat	24	336	20
Rabbit	24	336	12
Monkey	24	336	6
Human	4	120	3.7

NOTE: Data from Ross et al. (2005) and represent application of the dimethylamine salt of 2,4-dichlorophenoxyacetic acid (2,4-D). In all cases, the dose applied was 4 μg/cm^2. For rats, rabbits, and monkeys, the application site was washed after 24 hours, whereas human skin was washed after 4 hours. The collection time denotes the interval over which data were collected to determine absorption.

Absorption of Toxicants After Special Routes of Administration

Toxicants usually enter the bloodstream after absorption through the skin, lungs, or GI tract. However, in studying the effects of chemicals in laboratory animals, other routes of administration may also be used. The most common routes are (1) intravenous, (2) intraperitoneal, (3) subcutaneous, and (4) intramuscular. The intravenous route introduces the toxicant directly into the bloodstream, eliminating the process of absorption. Intraperitoneal injection results in rapid absorption of xenobiotics because of the rich peritoneal and mesenteric blood supply and the relatively large surface area of the peritoneal cavity. In addition, this route of administration circumvents the delay and variability of gastric emptying. Intraperitoneally administered compounds are absorbed primarily through the portal circulation and therefore pass through the liver before reaching other organs. Subcutaneous and intramuscular injections usually result in slower absorption rates, but toxicants enter directly into the general circulation. The rate of absorption by these two routes can be altered by changing the blood flow to the injection site. For example, epinephrine causes vasoconstriction and will decrease the rate of absorption if it is coinjected intramuscularly with a toxicant. The formulation of a xenobiotic may also affect the rate of absorption, as toxicants are typically absorbed more slowly from suspensions than from solutions.

The toxicity of a chemical may or may not depend on the route of administration. If a toxicant is injected intraperitoneally, most of the chemical enters the liver via the portal circulation before reaching the general circulation. Therefore, with intraperitoneal administration, a compound may be completely extracted and biotransformed by the liver with subsequent excretion into bile without gaining access to the systemic circulation. Propranolol (Shand and Rangno, 1972) and lidocaine (Boyes et al., 1970) are classical examples of drugs with efficient extraction during the first pass through the liver. A chemical with a high first-pass effect that is toxic in an organ other than the liver and GI tract is likely to be less toxic when administered intraperitoneally than when administered by other routes (intravenously, intramuscularly, or subcutaneously) because the intraperitoneal route favors extraction in the liver to reduce what is available systemically. In contrast, compounds with no appreciable biotransformation in the liver are likely to show similar toxicity independent of the route of administration if the rates of absorption are equal. Therefore, preliminary information on

the contribution of biotransformation and excretion of xenobiotics to toxic outcome can be derived by comparing toxic responses after administration by different routes.

DISTRIBUTION

After gaining entry into the bloodstream, regardless of route of exposure, a toxicant distributes to tissues throughout the body. The rate of distribution to organs or tissues is determined primarily by blood flow and the rate of diffusion out of the capillary bed into the cells of a particular organ or tissue, and usually occurs rapidly. The final distribution depends largely on the affinity of a xenobiotic for various tissues. In general, the initial phase of distribution is dominated by blood flow, whereas the eventual distribution is determined largely by affinity. The penetration of toxicants into cells occurs by passive diffusion or special transport processes, as discussed previously. Small water-soluble molecules and ions apparently diffuse through aqueous channels or pores in the cell membrane. Lipid-soluble molecules readily permeate the membrane itself. Very polar molecules and ions of even moderate size (molecular weight of 50 or more) cannot enter cells easily except by special transport mechanisms because they are surrounded by a hydration shell, making their actual size much larger.

Volume of Distribution

A key concept in understanding the disposition of a toxicant is its volume of distribution (Vd), a primary determinant of the concentration of a toxicant in blood that is used to quantify distribution throughout the body. It is defined as the volume in which the amount of drug would need to be uniformly dissolved in order to produce the observed blood concentration. Total body water is derived from that which is either extracellular or intracellular and represents three distinct compartments: plasma water and interstitial water comprise the extracellular compartment and are distinguished from intracellular water (Box 5-3). If a chemical distributes only to the plasma compartment (no tissue distribution), it has a high plasma concentration and hence a low Vd. In contrast, if a chemical distributes throughout the body (total body water), the effective plasma concentration is low and hence a high Vd. However, the distribution of toxicants is complex and under most circumstances cannot be simply equated with distribution into one of the water compartments of the body. Binding to and/or dissolution in various storage sites of the body, such as fat, liver, and bone, are usually more important factors in determining the distribution of chemicals.

Some toxicants do not readily cross cell membranes and therefore have restricted distribution, whereas other toxicants rapidly pass through cell membranes and are distributed throughout the body. Some toxicants selectively accumulate in certain parts of the body as a result of protein binding, active transport, or high solubility in fat. The target organ for toxicity may be the site of accumulation, but this is not always the case. If a toxicant accumulates at a site other than the target organ or tissue, the accumulation is likely to be protective because plasma levels and consequently its concentration at the site of action are reduced. In this case, it is assumed that the chemical in the storage depot is toxicologically inactive. However, a chemical in a storage depot is also in equilibrium with the free fraction of the toxicant in plasma, so that it is released into the circulation as the unbound fraction of toxicant is eliminated. Examples illustrating a wide range of Vd are noted in Table 5-9, and the factors that contribute to the Vd are discussed below.

Storage of Toxicants in Tissues

Because only the free fraction of a chemical is in equilibrium throughout the body, binding to or dissolving in certain body constituents greatly alters the distribution of a xenobiotic. Some xenobiotics attain their highest concentrations at the site of toxic action, such as carbon monoxide, which has a very high affinity for hemoglobin, and paraquat, which accumulates in the lungs. Other chemicals concentrate at sites other than the target organ. For example, lead is stored in bone, but manifestations of lead poisoning appear in soft tissues. The compartment where a toxicant is concentrated, but is not the major site of toxicity for that chemical, is described as a storage depot. Toxicants in these depots are always in equilibrium with the free fraction in plasma, so that as a chemical is biotransformed or excreted from the body, more is released from the storage site. As a result, the biological half-life of stored compounds can be very long.

Table 5-9

Examples of Factors That Contribute to Volume of Distribution (Vd)

COMPOUND	VD (L/KG)	FACTORS INFLUENCING DISTRIBUTION
Warfarin	0.1	High plasma protein binding with little distribution into tissues
Ethanol	0.5	Distribution in total body water
Propranolol	4.3	Distributed to peripheral tissues
Tamoxifen	50	Extensive distribution to peripheral tissues and high protein binding
Chloroquine	100	High tissue uptake and trapping in lysosomes

Box 5-3

Estimates of the Size of the Major Compartments That Contribute to Volume of Distribution

COMPARTMENT	PERCENTAGE OF TOTAL	LITER IN 70-KG HUMAN	PLASMA CONCENTRATION AFTER 1 G OF CHEMICAL (MG/L)
Plasma water	4.5	3	333
Total extracellular water	20	14	71
Total body water	55	38	26
Tissue binding	—	—	0–25

Plasma Proteins as Storage Depot Binding of chemicals to plasma proteins is a major storage depot, and several different plasma proteins bind xenobiotics and some endogenous constituents of the body. As depicted in Fig. 5-9, albumin is the major protein in plasma and it binds many different drugs and xenobiotics. α_1-Acid glycoprotein, although present at a much lower concentration than albumin, is also an important protein in plasma, and compounds with basic characteristics tend to bind to it. Transferrin, a β-globulin, is important for the transport of iron in the body. The other major metal-binding protein in plasma is ceruloplasmin, which carries copper. The α- and β-lipoproteins are very important in the transport of lipid-soluble compounds such as vitamins, cholesterol, and steroid hormones as well as xenobiotics. Plasma γ-globulins are antibodies that function specifically in immunological reactions. Overall, plasma proteins bind acidic compounds such as phenylbutazone, basic compounds such as imipramine, and neutral compounds such as digitoxin.

Albumin, present in the plasma at a concentration of 500 to 600 μM, is the most abundant protein in plasma and serves as both a depot and multivalent transport protein for many endogenous and exogenous compounds. Protein–ligand interactions occur primarily as a result of hydrophobic forces, hydrogen bonding, and Van der Waals forces. Because of their high molecular weight, plasma proteins and the toxicants bound to them cannot cross capillary walls. Consequently, the fraction of a toxicant bound to plasma proteins is not immediately available for distribution into the extravascular space or filtration by the kidneys. However, the interaction of a chemical with plasma proteins is a reversible process, and as unbound chemical diffuses out of capillaries, bound chemical dissociates from the protein until the free fraction reaches equilibrium between the vascular space and the extravascular space. In turn, diffusion in the extravascular space to sites more distant from the capillaries continues, and the resulting concentration gradient provides the thermodynamic force for continued dissociation of the bound fraction in plasma. Active transport processes are not limited by the binding of chemicals to plasma proteins.

The binding of toxicants to plasma proteins is usually determined by equilibrium dialysis or ultrafiltration. The total amount in plasma is determined prior to these separation methods, and the fraction that passes through a dialysis membrane or appears in the ultrafiltrate is the unbound (or free) fraction. The bound fraction is determined as the difference between the total and unbound fractions. The extent of plasma protein binding varies considerably among xenobiotics. Some, such as antipyrine, are not bound; others, such as secobarbital, are bound to about 50%; and some, such as warfarin, are 99% bound. For warfarin, the high protein binding is the single most important determinant of its Vd, which is limited to the plasma compartment (Table 5-9).

The binding of chemicals to plasma proteins is an important concept in toxicology for two reasons. Toxicity is typically manifested by the amount of a xenobiotic that is unbound. Therefore, a compound with a high degree of plasma protein binding may not show toxicity when compared to one that is less extensively bound to plasma proteins. Ironically, a high degree of protein binding also tends to increase the risk of adverse effects resulting from interactions with other highly bound compounds. In particular, severe toxic reactions can occur if a toxicant with a high degree of protein binding is displaced from plasma proteins by another chemical, increasing the free fraction of the toxicant in plasma. This interaction increases the equilibrium concentration of the toxicant in a target organ, thereby increasing the potential for toxicity. For example, if a compound is 99.9% bound to plasma protein (0.1% free), then an interaction that decreases protein binding to 99.5%, which may seem to be a minor change, is effectively a fivefold increase in the free plasma concentration (0.5% free). Most research on the adverse interactions associated with binding of xenobiotics to plasma proteins have been conducted with drugs. For example, if a strongly bound sulfonamide is given

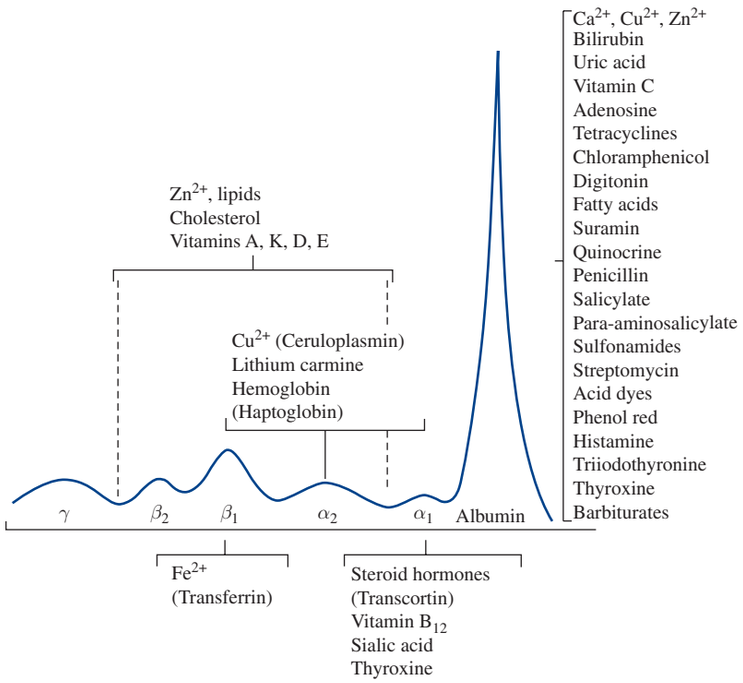

Figure 5-9. *Schematic representation of the electrophoretic separation of plasma proteins and xenobiotics that interact with these proteins.*

Table 5-10

Plasma Protein Binding and Half-life of Clofibric Acid in the Mouse, Rat, and Man

SPECIES	PLASMA PROTEIN BINDING (%)	HALF-LIFE (HOURS)
Human	97	21
Rat	75	6
Mouse	45	2

SOURCE: Data from Cayen (1980).

concurrently with an antidiabetic drug, the sulfonamide may displace the antidiabetic drug and induce a hypoglycemic coma. Similarly, interactions resulting from displacement of warfarin can lead to inappropriate blood clotting and possible deleterious effects. Xenobiotics can also compete with and displace endogenous compounds that are bound to plasma proteins. The importance of this phenomenon was demonstrated in a notable clinical trial comparing the efficacy of tetracycline with that of a penicillin–sulfonamide mixture in the management of bacterial infections in premature infants (Silverman et al., 1956). The penicillin–sulfonamide mixture led to much higher mortality than did the tetracycline because the sulfonamide displaced a considerable amount of bilirubin from albumin. Free bilirubin then diffused into the brain of the newborns (because the BBB is not fully developed), causing a severe form of brain damage termed kernicterus. In addition to drugs, some chemicals, such as the insecticide dieldrin, also bind avidly to plasma proteins (99%). Therefore, it is to be expected that chemical–chemical interactions that alter plasma protein binding occur with many different xenobiotics.

Plasma protein binding can also give rise to species differences in the disposition of xenobiotics. For example, plasma protein binding of clofibric acid is considerably different between mice, rats, and humans and correlates with the half-lives of this compound in these species (Table 5-10). Because clofibric acid is primarily eliminated in all three species by renal glomerular filtration without tubular reabsorption, differences in the free fraction of this compound in plasma across the species contribute to the observed species differences in drug half-life. Additional factors that influence plasma protein binding across species include differences in the concentration of albumin, in binding affinity, and/or in competitive binding of endogenous substances.

Liver and Kidney as Storage Depots The liver and kidney have a high capacity for acting as a storage depot for many chemicals. These two organs probably concentrate more toxicants than do all the other organs combined, and, in most cases, active transport or binding to tissue components are likely to be involved.

Hepatic uptake of lead illustrates how rapidly the liver binds foreign compounds: just 30 minutes after a single dose, the concentration of lead in liver is 50 times higher than the concentration in plasma (Klaassen and Shoeman, 1974). In humans OATP1B1 and OATP1B3 (Oatp1a1 and Oatp1b2 in rodents) largely contribute to the deposition of drugs and xenobiotics to the liver. For example, chemicals that have long half-lives, such as perfluorooctanoic acid (PFOA), preferentially deposit in the liver due to the high affinity of liver-enriched OATP transporters that preferentially transport certain perfluorinated chemicals into the liver for deposition (Weaver et al., 2010; Zhao et al., 2017).

In addition, some proteins can also sequester xenobiotics in the liver or kidney. For example, metallothionein (MT), a specialized metal-binding protein, sequesters both essential and toxic metals including zinc and cadmium (Cd) with high affinities in the kidney and liver. In the liver, Cd bound to MT serves to concentrate and sequester the heavy metal while preventing its excretion into bile. In the kidney, however, the Cd–MT complex is very toxic and is mechanistically involved in the chronic toxicity of Cd (Klaassen and Liu, 1997). Another protein that sequesters certain toxicants in the kidney is α2u-globulin. This protein, which is synthesized in large quantities only in male rats, binds to a diverse array of xenobiotics including metabolites of D-limonene (a major constituent of orange juice) and 2,4,4-trimethylpentane (found in unleaded gasoline). The chemical–α2u-globulin complex is taken up by the kidney, where it accumulates within the lysosomal compartment and damages the proximal tubule cells. Ultimately, the accumulation of this complex in the kidney is responsible for male rat–specific nephrotoxicity and carcinogenicity (Lehman-McKeeman, 2010).

As noted in Table 5-9, chloroquine has a very high Vd, which is determined largely by extensive storage in peripheral tissues. This compound is an example of a wide range of chemicals referred to as cationic amphiphiles. At physiological pH, chloroquine is nonionized, but once in cells, it is taken up into the acidic milieu of the lysosome where it becomes highly ionized (as per Henderson–Hasselbalch equations) and is subsequently trapped in this compartment. A variety of xenobiotics share these attributes, which ultimately lead to lysosomal dysfunction and the accumulation of phospholipids, referred to as phospholipidosis (Hanumegowda et al., 2010).

Fat as Storage Depot There are many organic compounds that are highly stable and lipophilic, leading to their accumulation in the environment. The lipophilic nature of these compounds also permits rapid penetration of cell membranes and uptake by tissues, and it is not surprising that highly lipophilic toxicants are distributed and concentrated in body fat where they are retained for a very long time. The environmental accumulation and potential toxicological significance of long-term storage of numerous compounds including the pesticides aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex, and toxaphene, along with polychlorinated and polybrominated biphenyls, polybrominated flame retardants, dioxins, and furans are a major issue in toxicology. There is extensive research efforts designed to assess the potential for these compounds to produce carcinogenic, developmental, and endocrine effects, which is directly related to their accumulation and storage in body fat (Jandacek and Tso, 2001).

Toxicants appear to accumulate in fat by dissolution in neutral fats, which constitute about 50% and 20% of the body weight of obese individuals and lean athletic individuals, respectively. Thus, toxicants with a high lipid/water partition coefficient may be stored in body fat, and higher amounts are likely to be retained in obese individuals. Storage lowers the concentration of the toxicant in the target organ such that toxicity is likely to be less severe in an obese person than in a lean individual. However, of more practical toxicological concern is the possibility that a sudden increase in the concentration of a chemical in blood and the target organ of toxicity may occur if rapid mobilization from fat occurs. Several studies have shown that signs of intoxication can be produced by short-term starvation of experimental animals that were previously exposed to persistent organochlorine insecticides.

There have been numerous attempts to alter storage of lipophilic toxicants in adipose tissues in animal models and humans. Changes in body fat composition appear to be the most effective in reducing body burdens, and interventions designed to reduce the absorption of these compounds from the GI tract appear to be

somewhat helpful in reducing storage depots of persistent organic pollutants in fat (Jandacek and Tso, 2001).

Bone as Storage Depot Compounds such as fluoride, lead, and strontium may be incorporated and stored in the bone matrix. For example, 90% of the lead in the body is eventually found in the skeleton. Skeletal uptake of xenobiotics is essentially a surface chemistry phenomenon, with exchange taking place between the bone surface and the fluid in contact with it. The fluid is the extracellular fluid, and the surface is that of the hydroxyapatite crystals of bone mineral. Many of those crystals are very small, resulting in a large surface area relative to the mass of the bone. The extracellular fluid brings the toxicant into contact with the hydration shell of the hydroxyapatite, allowing diffusion through it and penetration of the crystal surface. As a result of similarities in size and charge, F^- may readily displace OH^-, whereas lead or strontium may substitute for calcium in the hydroxyapatite lattice matrix through an exchange–adsorption reaction.

Foreign compounds deposited in bone are not sequestered irreversibly by that tissue. Toxicants can be released from the bone by ionic exchange at the crystal surface and dissolution of bone crystals through osteoclastic activity. An increase in osteolytic activity such as that seen after parathyroid hormone administration leads to enhanced mobilization of hydroxyapatite lattice, which can be reflected in an increased plasma concentration of toxicants. Ultimately, deposition and storage of toxicants in bone may or may not be detrimental. Lead is not toxic to bone, but the chronic effects of fluoride deposition (skeletal fluorosis) and radioactive strontium (osteosarcoma and other neoplasms) are well documented.

Blood–Brain Barrier

Access to the brain is restricted by the presence of two barriers: the blood–brain barrier (BBB) and the blood–cerebral spinal fluid barrier (BCSFB). Although neither represents an absolute barrier to the passage of toxic chemicals into the CNS, many toxicants do not enter the brain in appreciable quantities because of these barriers.

The BBB is formed primarily by the endothelial cells of blood capillaries in the brain (Fig. 5-10). Each endothelial cell forms a tight junction with adjacent cells, essentially forming a tight seal between the cells and preventing diffusion of polar compounds through paracellular pathways. Diffusion of more lipophilic compounds through endothelial cell membranes is counteracted by xenobiotic efflux transporters present in the endothelial cells. Glial cells, particularly astrocytes, contribute to the BBB by secreting chemical factors that modulate endothelial cell permeability, and astrocytes and perivascular microglial cells extend processes that support the integrity of the BBB. For small- to medium-sized water-soluble molecules, the tighter junctions of the capillary endothelium and the lipid membranes of the glial cell processes represent the major barrier. Although the absorption of lipid-soluble compounds is favored in the brain, such compounds must traverse the membranes of the endothelial cells, not be substrates for xenobiotic transporters, and then traverse the glial cell processes to enter the brain.

Although the BBB is a physical structure that limits distribution to the brain, active transport processes also play a pivotal role in determining the concentration of xenobiotics in the brain. Numerous ATP-dependent transporters have been identified as part of the BBB, comprising various members of the ABC and SLC families (Fig. 5-10) (Kusuhara and Sugiyama, 2005; Deeken and Loscher, 2007; Miller, 2010). Efflux transporters including MDR1 (PGP), BCRP, MRP1, MRP2, MRP4, and MRP5 are located on the apical (blood side) plasma membrane and function to move xenobiotics absorbed into the capillary endothelial cells out into the blood, thereby limiting distribution into the brain. Uptake transporters including OATP1A2 and OATP1C1 are found on both the basolateral and apical sides of the endothelium, and can drive a concentrative efflux if coupled energetically to the electrical potential difference across the endothelial cell membrane. OATP1C1 is suggested to specifically transport thyroid hormone into the brain. Finally, uptake transporters on the apical membranes include OAT3 and CTN2. In combination, these transporters can efficiently efflux a wide range of anionic, cationic, uncharged, and numerous drug conjugates from the brain.

PGP and BCRP are the two transporters that are known to significantly impact brain penetration and neurotoxicity. The importance of transport processes to the maintenance of the BBB has been demonstrated in genetically modified mouse models. Compounds that are substrates for MDR1 achieve much higher brain levels in P-gp null ($Mdr1a^{-/-}$/$Mdr1b^{-/-}$) mice relative to wild-type mice, and are likely to show increased toxicity as well. Seminal studies demonstrated that MDR1 null mice were far more sensitive to the insecticide, ivermectin, and the anticancer agent, vinblastine. The brain uptake of these compounds was increased 26- and 80-fold, respectively, and the sensitivity to neurotoxicity or lethality was increased up to 100-fold (Schinkel et al., 1994; Lankas et al., 1997). Because PGP can limit impact penetration, screens for whether a new chemical entity is a PGP substrate are often conducted early in the discovery/development process. BCRP also serves to pump drugs and xenobiotics from the capillary endothelial cells out into the blood. Use of knockout mice have illustrated the role of BCRP to limit brain penetration for various anticancer agents, such as sorafenib and erlotinib (Agarwal et al., 2011; de Vries et al., 2012). For some drugs, targeted deletion of both PGP and BCRP resulted in even greater brain penetration than for either deleted transporter alone (Kodaira et al., 2010).

The BCSFB is found between the circulating blood and the circulating cerebral spinal fluid in the brain (Fig. 5-11). It comprises

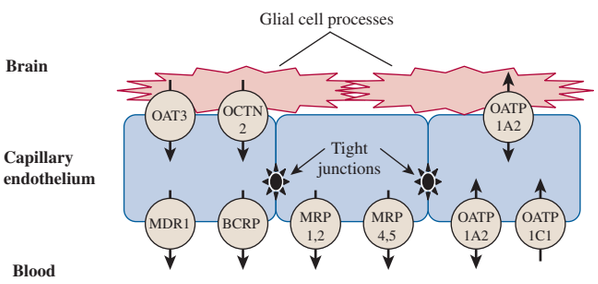

Figure 5-10. *Schematic model showing the xenobiotic transporting systems that contribute to the human blood–brain barrier.*

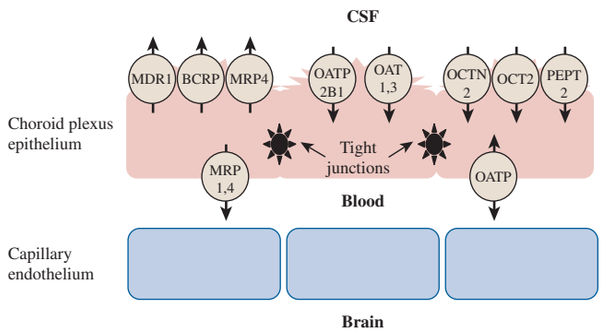

Figure 5-11. *Schematic model showing the xenobiotic transporting systems that contribute to the human blood–cerebral spinal fluid barrier.*

elements of the choroid plexus, the arachnoid membrane, and certain periventricular locations (including the area postrema). The choroid plexus has highly permeable endothelial cells on the blood side but is lined by epithelial cells on the cerebrospinal fluid (CSF) side that form the barrier. Xenobiotic transporters also contribute to removing compounds that may enter the endothelial cells. Blood flow through the choroid plexus is about 10 times higher than in the brain, a feature that provides the high turnover of biological fluids necessary to support the dynamic changes in nutrients required for neuronal function. Furthermore, the protein concentration in CSF is much lower than that in other body fluids. The low protein content of the CSF also limits the movement of water-insoluble compounds by paracellular transport, which is possible in a largely aqueous medium only when such compounds are bound to proteins. In general, toxicants achieve concentrations in the CSF that are no higher than the concentration of the unbound toxicant in the plasma. The BCSFB is more of an anatomical entity than a true barrier, but it does provide some protection against the distribution of toxicants to the CNS, thereby affording some protection against toxicity.

Xenobiotic transporters are also expressed in the epithelial cells of the choroid plexus, but within the BSCFB, MDR1, and MRP1 are thought to play the most important roles (Fig. 5-11). In this barrier, MDR1 is expressed on the luminal side, with efflux from the choroidal epithelial cells into the CSF. In contrast, MRP1 is expressed on the basolateral side of the choroidal epithelium, catalyzing the transport of xenobiotics out of the cells and into the vascular compartment, and it has been suggested that MDR1 and MRP1 may play an important function to coordinate the absorption and secretion of toxicants into the CNS at the BSCFB (Deeken and Loscher, 2007). Members of the OATP, OAT, OCT, and PEPT families are also expressed in the choroid plexus (Klaassen and Aleksunes, 2010). The function of these proteins is not well characterized, but studies in rodents suggest that Oatps, particularly Oatp1a4, play an important role in regulating xenobiotic concentrations in the CSF.

In addition to its role in regulating distribution of xenobiotics into the CNS, transporter function is also implicated in neurological diseases. MDR1 and BCRP contribute to the efflux of β-amyloid from the brain. This protein is known to accumulate in Alzheimer's disease, and there is evidence that MDR1 levels are decreased in Alzheimer's patients, with a concomitant increase in BCRP expression. There is also ongoing work to determine the potential for MDR1 to remove toxicants from the brain in a manner that may ultimately be protective against the development of Parkinson's disease (Miller, 2010). These observations require more research, with particular emphasis on how altered transporter function or expression may be affected by toxicant exposures that could contribute to human diseases.

The effectiveness of the BBB varies from one area of the brain to another. For example, the cortex, the lateral nuclei of the hypothalamus, the area postrema, the pineal body, and the posterior lobe of the hypophysis are more permeable than are other areas of the brain. It is not clear whether this is due to the increased blood supply to those areas, or because the BSCFB represents a more permeable barrier, or both. In general, the entrance of toxicants into the brain follows the same principle that applies to transfer across other cells in the body. Only the free fraction of a toxicant (i.e., not bound to plasma proteins) equilibrates rapidly with the brain. Lipid solubility plays an important role in determining the rate of entry of a compound into the CNS, as does the degree of ionization, as discussed earlier. Lipid solubility enhances the rate of penetration of toxicants into the CNS, whereas ionization greatly diminishes it. 2-PAM, a quaternary nitrogen derivative, does not readily penetrate the brain and is ineffective in reversing the inhibition of brain cholinesterase caused by organophosphate insecticides. Some very lipophilic compounds may enter the brain but are so efficiently removed by xenobiotic transporters that they do not reach appreciable concentrations. This is particularly true for agents such as cyclosporin. TCDD, which is highly lipophilic, is also not readily distributed into the brain, but it is not known whether transporters influence its distribution to the brain. It is likely that strong binding to plasma proteins or lipoproteins, as well as the composition of the brain (mainly phospholipids), limits the entry of very lipophilic compounds into the brain. Some xenobiotics, although very few, may enter the brain by carrier-mediated processes. For example, methylmercury combines with cysteine, forming a sulfhydryl complex that is similar to methionine, which can be utilized as the large neutral amino acid carrier of the capillary endothelial cells for uptake into the brain (Clarkson, 1987).

The BBB is not fully developed at birth, and this is one reason why some chemicals are more toxic in newborns than adults. The example of kernicterus resulting from increased brain levels of bilirubin in infants has been noted earlier. In addition, morphine is 3 to 10 times more toxic to newborn than to adult rats because of the higher permeability of the brain of a newborn to morphine. Similarly, lead produces encephalomyelopathy in newborn rats but not in adults, apparently because of differences in the stages of development of the BBB.

Passage of Toxicants Across the Placenta

The term placental barrier has been associated with the concept that the main function of the placenta is to protect the fetus against the passage of noxious substances from the mother. However, the placenta is a multifunctional organ that also provides nutrition for the conceptus, exchanges maternal and fetal blood gases, disposes of fetal excretory material, and maintains pregnancy through complex hormonal regulation. Placental structure and function show more species differences than any other mammalian organ. Anatomically, the placental barrier consists of several cell layers interposed between the fetal and maternal circulations, but the number of cell layers varies across species and with the stage of gestation. The maximum number of cell layers in placentas is six (three maternal and three fetal layers), and when all six layers are present, the placenta is termed epitheliochorial (Table 5-11). Those in which the maternal epithelium is absent are referred to as syndesmochorial. When only the endothelial layer of the maternal tissue remains, the tissue is termed endotheliochorial; when even the endothelium is gone (as in humans), the chorionic villi bathe in the maternal blood, and the tissue is called hemochorial. In some species, the maternal layers are absent along with the fetal endothelial and connective tissue, and these are called hemoendothelial. Within the same species, the placenta may also change its histological classification during gestation. For example, at the beginning of gestation, the placenta of a rabbit has six major layers (epitheliochorial), and at the end it has only one (hemoendothelial). Overall, there are marked species differences in placental structure. The animal models that are most similar to the organization of the human placenta based on its hemochorial histological organization are rodents and primates, but the decidual nature of the rodent placenta still differs markedly from that of humans (Leiser and Kaufmann, 1994; Myllynen et al., 2005).

Most of the vital nutrients necessary for the development of the fetus are transported by active transport systems. For example, vitamins, amino acids, essential sugars, and ions such as calcium and iron are transported from mother to fetus against a concentration

Table 5-11

Features of the Placental Barrier Based on Separation of Fetal and Maternal Blood

		MATERNAL TISSUE		
TYPE	SPECIES	ENDOTHELIUM	CONNECTIVE TISSUE	EPITHELIUM
Epitheliochorial	Pig, horse, donkey	+	+	+
Synepitheliochorial	Cow, sheep, goat	+	+	–
Endotheliochorial	Cat, dog, elephant	+	–	–
Hemochorial	Human, monkey	–	–	–
Hemoendothelial	Rat, rabbit, guinea pig	–	–	–

Fetal layers that separate maternal and fetal blood also include the endothelial, connective tissue, and epithelial components in all types of placentas. In the hemoendothelial type, only the fetal epithelium is present.
SOURCES: Modified from Morris et al. (1994) and Enders and Carter (2004).

gradient (Ganapathy et al., 2000). Like nutrients, toxic chemicals can be transported across the placental barrier or pass through the placenta via passive permeability. The only exceptions are a few antimetabolites that are structurally similar to endogenous purines and pyrimidines, which are the physiological substrates for active transport from the maternal to the fetal circulation. The placenta also has biotransformation capabilities that may prevent some toxic substances from reaching the fetus (Syme et al., 2004; Prouillac and Lecoeur, 2010). Among the substances that cross the placenta by passive diffusion, more lipid-soluble substances rapidly attain a maternal–fetal equilibrium. Under steady-state conditions, concentrations of a toxic compound in the plasma of the mother and fetus are usually the same.

Many foreign substances can cross the placenta, and the same factors that dictate the passage of xenobiotics across biological membranes are important determinants of the placental transfer. These include previously discussed attributes including degree of ionization, lipophilicity, protein binding, and molecular weight along with blood flow and the concentration gradient across the barrier. In addition to chemicals, viruses (e.g., rubella virus), cellular pathogens (e.g., syphilis spirochetes), and globulin antibodies can traverse the placenta. In this regard, the placental barrier is not as precise an anatomical unit like the BBB. The human placenta includes the syncytiotrophoblast and cytotrophoblast layers. The apical membrane of the syncytiotrophoblast, which forms a continuous epithelial layer, is bathed in maternal blood and the basolateral surface is in contact with the discontinuous cytotrophoblast layer, the stromal tissue, or the fetal vasculature. To reach the fetus, toxins must traverse the apical and basolateral membranes of the syncytiotrophoblast as well as the endothelium of the fetal capillaries. Xenobiotic transporters are differentially expressed in these various cells and contribute to the barrier function that restricts distribution of toxicants to the fetus. The transporters are also critical to the movement of nutrients from the maternal circulation to the fetus along with the transfer of toxicants or waste products from the fetus back to the maternal circulation. Of the various cells in the placenta, the syncytiotrophoblasts appear to have the most extensive cohort of xenobiotics transporters (Fig. 5-12). In particular, BCRP is highly expressed in these cells. In fact, BCRP expression is highest in the human placenta relative to any other organ. BCRP, MDR1 (PGP), and several MRPs are also expressed on the apical border of the syncytiotrophoblast where they play important roles in protecting the fetus from toxicants while regulating the movement of essential nutrients. Several uptake transporters are also expressed on the apical membrane, including OCTNs and OATPs. In contrast,

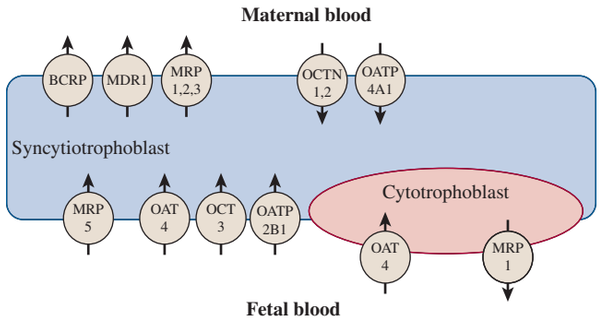

Figure 5-12. *Schematic model showing the transport systems that contribute to the barrier function of the human placenta.*

the efflux transporter MRP5 is expressed on the basolateral side of the synctiotrophoblast along with uptake transporters including OAT4, OCT3, and OATP2B1. Expression of transporters in other cells of the placenta is less characterized, but it appears that the cytotrophoblasts express MRP1 and OAT4 (Klaassen and Aleksunes, 2010; Prouillac and Lecoeur, 2010).

There are several examples illustrating that placental transporters protect the developing fetus from toxicant exposure and possible abnormal development. For example, the fetal concentration of the antibiotic nitrofurantoin is increased fivefold in Bcrp-null mice, demonstrating that it limits fetal exposure to this drug (Zhang et al., 2007). Furthermore, homozygous offspring of female MDR1 null mice ($Mdr1a^{-/-}/Mdr1b^{-/-}$) develop cleft palate when treated with a photoisomer of avermectin, whereas heterozygous littermates showed intermediate susceptibility and wild-type mice were resistant to this adverse effect (Lankas et al., 1998). Finally, it has been shown that some anticonvulsants, including known developmental toxicants such as valproic acid and phenytoin (see Chap. 10), inhibit OCTN2 function in human placental membrane vesicles (Wu et al., 2004). This transporter is critical for the uptake of carnitine, leading to the hypothesis that inhibition of carnitine uptake by valproic acid (or other drugs) with subsequent fetal deficiency of carnitine may be involved in the adverse developmental effects associated with a variety of anticonvulsant drugs.

Despite the presence of biotransformation systems and xenobiotic transporters designed to nourish and protect the fetus, the transfer of xenobiotics across the placenta can still occur. One important consequence of placental transfer is that of transplacental carcinogenesis. In this case exposing the mother during gestation increases

the likelihood of tumor development in the offspring later in life (see Chap. 8). The most well-known transplacental carcinogen in humans is diethylstilbestrol (Newbold and McLachlan, 1996), but other compounds such as the antiviral drug zidovudine (Diwan et al., 1999) and inorganic arsenic (Waalkes et al., 2003; Tokar et al., 2011) induce tumors in mice when exposed to these chemicals only during gestation.

Redistribution of Toxicants

The most critical factors that affect the distribution of xenobiotics are the organ blood flow and its affinity for a xenobiotic. The initial phase of distribution is determined primarily by blood flow to the various parts of the body. Therefore, a well-perfused organ such as the liver may attain high initial concentrations of a xenobiotic. However, chemicals may have a high affinity for a binding site (e.g., intracellular protein or bone matrix) or to a cellular constituent (e.g., fat), and with time, will redistribute to these high-affinity sites. For example, although 50% of a dose of lead is found in the liver 2 hours after administration, 1 month later, 90% of the lead remaining in the body is associated with the crystal lattice of bone. Similarly, persistent organic pollutants do not distribute rapidly to fat but accumulate selectively in adipose tissue over time.

EXCRETION

Toxicants are eliminated from the body by several routes. The kidney is perhaps the most important organ for the excretion of xenobiotics because more chemicals are eliminated from the body by this route than by any other (see Chap. 14). Biotransformation to more water-soluble products is usually a prerequisite to the excretion of xenobiotics through urine (see Chap. 6). The second important route of elimination of many xenobiotics is through feces, and the third, primarily for gases, is through the lungs. Biliary excretion of xenobiotics and/or their metabolites is most often the major source of fecal excretion, but a number of other sources can be significant for some compounds. All body secretions appear to have the ability to excrete chemicals; toxicants have been found in sweat, saliva, tears, and milk.

Urinary Excretion

The kidney is a very efficient organ for the elimination of toxicants from the body. The functional unit of the kidney is the nephron, comprising the glomerulus, a capillary tuft that initiates the process of filtration of the blood, and tubular elements within the renal cortex and medulla that function to produce and concentrate urine. Toxic compounds are excreted in urine by the same mechanisms the kidney uses to remove the end products of intermediary metabolism from the body, including glomerular filtration, tubular excretion by passive diffusion, and active tubular secretion. In general, the excretion of small molecular weight (<350 Da), water-soluble compounds is favored in urine.

The kidney receives about 25% of the cardiac output, about 20% of which is filtered at the glomeruli. The glomerular capillaries have large pores (approximately 70 nm), which filter compounds up to a molecular weight of about 60 kDa (smaller than albumin). Thus, the degree of plasma protein binding affects the rate of glomerular filtration because protein–xenobiotic complexes, particularly those bound to albumin, will not be filtered. Glomerular filtration rates vary considerably across species, ranging from a high of approximately 10 mL/min/kg in mice to about 1.8 mL/min/kg in humans. This difference appears to be determined by the relative number of nephrons per kilogram of body weight, with mice being the highest (Lin, 1995; Walton et al., 2004).

A toxicant filtered at the glomerulus may remain in the tubular lumen and be excreted in urine. Depending on the physicochemical properties of a compound, it may be reabsorbed across the tubular cells of the nephron back into the bloodstream. The principles governing the reabsorption of toxicants across the kidney tubules are the same as those discussed earlier in this chapter for passive diffusion across cell membranes. Thus, toxicants with a high lipid/water partition coefficient are reabsorbed efficiently, whereas polar compounds and ions are excreted in urine. The pH of urine may vary but it is usually slightly acidic (approximately 6 to 6.5). Just as the Henderson–Hasselbach calculations determine the absorption of nonionized compounds from the GI tract, they also determine urinary excretion. In this case, urinary excretion of the ionized moiety is favored, such that bases are excreted to a greater extent at lower pH whereas excretion of acids predominates at higher urinary pH. A practical application of this knowledge is illustrated by the treatment of phenobarbital poisoning with sodium bicarbonate. The percentage of ionization is increased markedly within physiologically attainable pH ranges for a weak organic acid such as phenobarbital ($pK_a = 7.2$) such that alkalinization of urine increases the fraction of drug in the ionized state, thereby increasing the urinary excretion of phenobarbital. Similarly, excretion of salicylate can be accelerated by administering sodium bicarbonate. In a similar manner, urinary acidification can be used to increase the excretion of a weak base such as phencyclidine (PCP) in drug abusers.

Toxic chemicals can also be excreted from plasma into urine by passive diffusion through the tubule. This process is probably of minor significance because filtration is much faster than excretion by passive diffusion through the tubules, providing a favorable concentration gradient for reabsorption rather than excretion. Exceptions to this generalization may be some organic acids ($pK_a \approx 3$–5) and bases ($pK_a \approx 7$–9) that would be largely ionized and thus trapped at the pH of urine (pH ≈ 6). For renal excretion of such compounds, the flow of urine is likely to be important for the maintenance of a concentration gradient, favoring excretion. Thus, diuretics can hasten the elimination of weak organic acids and bases because they increase urine flow.

Xenobiotics can also be excreted into urine by active secretion. This process involves the uptake of toxicants from the blood into the cells of the renal proximal tubule, with subsequent efflux from the cell into the tubular fluid from which urine is formed. Fig. 5-13 illustrates the various families of transporters expressed in the human kidney that are directly involved in xenobiotic disposition. There are numerous other transporters such as specific glucose transporters that play a role predominantly in the flux of endogenous substances, which are not presented here. Transporters may be expressed on the apical cell membrane where efflux pumps contribute to tubular secretion and influx pumps are important for reabsorption. Transporters localized to the basolateral membranes serve to transport xenobiotics to and from the systemic circulation or the renal tubular cells and also contribute to reabsorptive and excretory processes. Renal functional activity, as may be altered by toxicant exposure, can also be evaluated by determining the renal clearance of transporter substrates such as the widely used organic anion *p*-aminohippurate (PAH).

Recent proteomic analysis has shed light on the transporters expressed in human kidney cortex (Prasad et al., 2016). OAT1-4, OCT2, OCTN1 and OCTN2, PgP, MRP2, MRP4, MATE1, and SGLT2 were detected above the limit of detection, whereas BCRP and MATE2-K proteins were detectable but were below the lower limit of quantification. Transporters expressed on the basolateral side of the renal tubules in humans that contribute mainly to uptake into the kidney and subsequent excretion include OATs, OCTs,

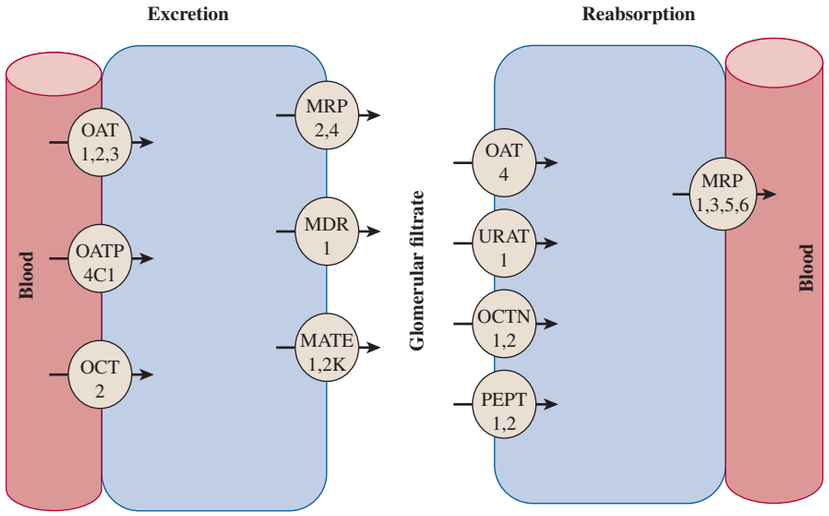

Figure 5-13. *Schematic model showing the transport systems in the human proximal tubule of the kidney.*

and OATP4C1. The OAT family is a major contributor to urinary excretion as these proteins mediate the renal uptake of organic acids such as *p*-aminohippurate (PAH) and the renal exchange of dicarboxylates. In humans, OAT1, OAT2, and OAT3 are expressed on the basolateral membrane, whereas OAT4 is localized to the brush-border membrane. Similarly, in rodents, Oat1 and Oat3 are localized to the basolateral membranes. Although the OATs play an important role in the physiological exchange of metabolites in the kidney, they can also contribute to the development of renal toxicity. In particular, the clinical use of antiviral drugs cidofovir and adefovir is limited by nephrotoxicity that is mediated by the accumulation of these drugs in the proximal tubule by OAT1 (Ho et al., 2000). Additionally, OAT1 mediates the uptake of methylmercury and ochratoxin A, and as such, may play a role in the nephrotoxicity associated with these toxicants (Rizwan and Burckhardt, 2007).

The OCT transporter family is responsible for uptake of some cations, particularly those of low molecular weight into the kidney. Substrates for OCTs include endogenous compounds such as choline, tetraethylammonium (TEA), and certain cationic drugs including cisplatin, cimetidine, metformin, and acyclovir. OCT2 is highly expressed in the human kidney, whereas Oct1 and Oct2 are expressed on the basolateral membranes of the rodent kidney (Shitara et al., 2005). The antineoplastic drug, cisplatin is a substrate and inhibitor of OCT2, and the dose-limiting nephrotoxicity associated with this drug may be directly related to uptake-mediated concentration of drug in the proximal tubule epithelium (Zolk and Fromm, 2011). A second cation transporter family is OCTN, which has two members OCTN1 and OCTN2 that are localized to the brush-border membranes in the human and rodent kidney. Substrates include cations such as TEA, and OCTN2 is particularly important in the renal reabsorption of carnitine, which is an essential cofactor for fatty acid β-oxidation.

Peptide transporters, localized to the brush-border membrane of the proximal tubule, are important for the reuptake of dipeptides and tripeptides. Two transporters PEPT1 and PEPT2 have been identified in the human and rodent kidney, with PEPT1 localized in the upper region of the proximal tubule (pars convoluta) and PEPT2 expressed in the lower region (pars recta) of the convoluted tubule. Examples of PEPT1 and PEPT2 substrates are peptide-like drugs, such as beta-lactam antibiotics of the cephalosporin and penicillin classes, angiotensin-converting enzyme inhibitors (ACE inhibitors), and amino acid–conjugated antiviral drugs (valacyclovir). In PEPT2 knockout mice urinary excretion of the antibiotic cefadroxil was markedly increased due to almost complete lack of reabsorption from urine.

MDR1, MRP2, and MRP4 are efflux transporters localized to the luminal brush border of the proximal tubule, where they pump xenobiotics out of the cells and into the tubular fluid, thereby enhancing excretion. In general, these efflux transporters work in concert with uptake transporters to accomplish the urinary excretion of xenobiotics. For example, following OAT-mediated uptake of adefovir works in concert with MRP4 to transport the potentially toxic drug out of the cell and into the tubular fluid. Similarly, MATE1 and MATE2K are expressed on the apical membrane of renal tubular cells and contribute to the functional coordination of the urinary excretion of cationic compounds. There is a recognized interplay between OCT2-mediated uptake of organic cations, such as metformin, followed by the secretion into the tubular fluid by MATE1 and MATE2K. This is particularly important in the case of metformin because the drug does not undergo extensive hepatic metabolism and is eliminated from the body almost exclusively by urinary excretion (Zolk and Fromm, 2011).

Finally, Bcrp is expressed at high levels in the apical brush border of the rat and mouse kidney and is functional in the rodent kidney and is also detected in the human kidney (Huls et al., 2008). Loss of Bcrp in the rodent kidney is associated with decreased urinary excretion of the Bcrp substrates (11)C-SC-62807 and 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole sulfate (E3040S) (Mizuno et al., 2004; Takashima et al., 2013). The contribution of BCRP with respect to renal excretion and contribution to toxic responses remains to be fully evaluated.

As in all active transport systems, there is competition for renal secretion of xenobiotics. This fact was taken advantage of during World War II, when penicillin was in short supply. Penicillin is actively secreted by the organic acid systems (OATs) of the kidney. To lengthen its half-life and duration of action, another acid was sought to compete with penicillin for renal secretion, and probenecid was successfully introduced for this purpose. Uric acid is also secreted actively by renal tubules, and a unique member of the OAT family, the urate transporter (URAT1; SLC22A12) is expressed only in the kidney where it functions specifically as a mechanism for reabsorption of urate. Various uricosuric drugs

Table 5-12

Urinary and Biliary Excretion of Griseofulvin and/or Metabolites in Rats and Rabbits

	RATS*		RABBITS*	
	URINE	BILE	URINE	BILE
Total	12	77	78	11
Phase I metabolites	ND	23	70	3
Phase II metabolites	ND	54	8	8

Abbreviation: ND, not determined.
**Expressed as percent of dose.*
SOURCE: Data from Symchowicz et al. (1967), with permission.

including probenecid and phenylbutazone inhibit URAT1 to facilitate the excretion of uric acid from the body.

Because many functions of the kidney are incompletely developed at birth, some xenobiotics are eliminated more slowly in newborns than in adults, and therefore may be more toxic to newborns. For example, the clearance of penicillin by premature infants is only about 20% of that observed in older children. In contrast, some compounds, such as cephaloridine, are nephrotoxic in adult animals but not in newborns. The reduced toxicity results directly from the lack of active uptake of cephaloridine by the kidneys in newborns, such that the chemical is not concentrated in the tubules.

The renal proximal tubule reabsorbs small plasma proteins that are filtered at the glomerulus. This largely occurs by pinocytosis at the brush-border membrane of the proximal tubule epithelium. If a toxicant binds to those small proteins, it can be carried into the proximal tubule cells and exert toxicity. As discussed previously, cadmium bound to MT is readily taken up by the kidney and is the major cause of Cd-induced nephrotoxicity (Klaassen and Liu, 1997). Similarly, chemicals such as limonene and 2,4,4-trimethyl pentane bind to the male-rat-specific protein, α_{2u}-globulin, and are taken up by the proximal tubule to produce hyaline droplet nephropathy and eventually renal tumors in male rats (Lehman-McKeeman, 2010).

Species differences in regard to the urinary excretion of weak organic acids and bases are observed frequently, as the pH of urine varies widely among species. Differences in renal clearance also can occur for compounds filtered at the glomeruli because of differences in plasma protein binding. Similarly, differences in xenobiotic transporter expression, regulation, and function can contribute to differences in the renal excretion of toxicants.

Additional factors affecting the excretion of xenobiotics are exemplified by the disposition of griseofulvin in rats and rabbits (Table 5-12). Rabbits metabolize griseofulvin to 6-demethylgriseofulvin (a phase-I metabolite), a low-molecular-weight species that is predominantly excreted in urine. In contrast, rats metabolize griseofulvin to many conjugates (phase-II metabolites) that are high-molecular-weight species (>350) that are preferentially excreted in bile. In this example, species differences in biotransformation ultimately determine the route of excretion of griseofulvin.

Fecal Excretion

Fecal excretion, the second major pathway for the elimination of xenobiotics, is a complex process that is not as well understood as urinary excretion. Excretion of toxicants via the feces can result from direct elimination of nonabsorbed compounds in the GI tract, from delivery to the GI tract via the bile, and from secretion into intestinal luminal contents from the enterocytes.

Nonabsorbed Ingesta In addition to undigested material, varying proportions of nutrients and xenobiotics that are present in food or are ingested voluntarily (drugs) pass through the alimentary canal unabsorbed, contributing to fecal excretion. The physicochemical properties of xenobiotics and the biological characteristics that facilitate absorption were discussed earlier in this chapter. Although most chemicals are lipophilic to some extent and thereby available for absorption, it is rare for 100% of a compound to be absorbed. However, some macromolecules and some high-molecular-weight compounds that are essentially completely ionized are not absorbed at all. For example, the absorption of polymers or quaternary ammonium bases is quite limited in the intestine. Consequently, most of a dose of orally administered sucrose polyester, cholestyramine, or paraquat can be found in feces. The nonabsorbed portion of xenobiotics contributes to the fecal excretion of most chemicals to some extent. One other factor contributing to fecal excretion is intestinal secretion, which likely occurs by passive diffusion out of enterocytes or via transporter-mediated processes.

Biliary Excretion The biliary route of elimination is a significant source contributing to the fecal excretion of xenobiotics and is even more important for the excretion of metabolites. The liver plays an important role in removing toxic chemicals from blood after absorption from the GI tract because blood from the GI tract passes via the portal circulation through the liver before reaching the general circulation. A compound can be extracted by the liver, thereby preventing its distribution to other parts of the body. The liver is also the main site for biotransformation of toxicants, and metabolites may be excreted directly into bile. In this manner, the liver can remove xenobiotics and their metabolites before entering the general circulation, which is referred to as the "first-pass effect." Furthermore, xenobiotics and/or their metabolites excreted into bile enter the intestine and may be excreted with feces. However, if the physicochemical properties favor reabsorption, an enterohepatic circulation may ensue (discussed below).

Toxic chemicals bound to plasma proteins are fully available for active biliary excretion. The factors that determine whether a chemical will be excreted into bile or into urine are not fully understood. However, as a general rule, low-molecular-weight compounds (<325) are poorly excreted into bile, whereas compounds or their conjugates with molecular weights exceeding approximately 325 can be excreted in appreciable quantities. Glutathione and glucuronide conjugates have a high predilection for excretion into bile, but there are marked species differences in the biliary excretion of foreign compounds with consequences for the biological half-life of a compound and its toxicity. Table 5-13 provides examples of species differences in biliary excretion, and demonstrates that species variation in biliary excretion is also compound specific. It is therefore difficult to categorize species into "good" or "poor" biliary excretors, but, in general, rats and mice tend to be better biliary excretors than are other species (Klaassen and Watkins, 1984).

Foreign compounds excreted into bile are often divided into three classes on the basis of the ratio of their concentration in bile versus that in plasma. Class A substances have a ratio of nearly 1 and include sodium, potassium, glucose, mercury, thallium, cesium, and cobalt. Class B substances have a ratio of bile to plasma greater than 1 (usually between 10 and 1000). Class B substances include bile acids, bilirubin, lead, arsenic, manganese, and many other xenobiotics. Class C substances have a ratio below 1 (e.g., inulin, albumin, zinc, iron, gold, and chromium). Compounds rapidly excreted into bile are usually class B substances. However, a compound does not have to be highly concentrated in bile for biliary

Table 5-13

Biliary Excretion of Xenobiotic Conjugates Across Species

(MOLECULAR WEIGHT)	INDOCYANINE GREEN, (775)	PHENOLPHTHALEIN GLUCURONIDE, (495)	SULFADIMETHYOXINE *n*-GLUCURONIDE, (487)	STILBESTROL GLUCURONIDE, (445)
		% EXCRETED IN BILE		
Rat	82	54	43	95
Dog	97	81	43	65
Rabbit	97	13	10	32
Guinea pig	Not done	6	12	20

SOURCE: Data modified from Lin (1995).

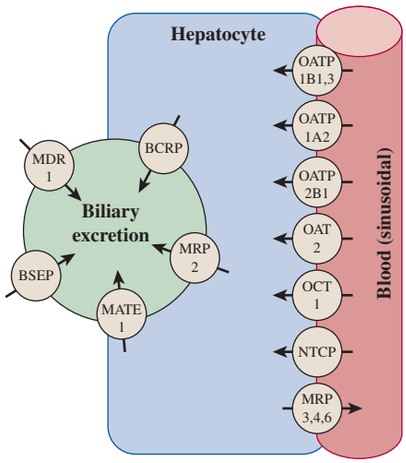

Figure 5-14. *Schematic model showing the xenobiotic transporting systems present in the human liver.*

excretion to be of quantitative importance. For example, mercury is not concentrated in bile, yet bile is the main route of excretion for this slowly eliminated substance.

Biliary excretion is regulated predominantly by xenobiotic transporters present on the canalicular membrane, which include MRP2, BCRP, MDR1 (PGP), MATE1, and BSEP (Fig. 5-14). MRP2 is extremely important in biliary excretion because it is largely responsible for the transport of organic anions including glucuronide and glutathione conjugates of many xenobiotics. BCRP has particular affinity for sulfated conjugates of toxicants, whereas MDR1 (PGP) transports a variety of substrates, but primarily organic bases into bile. MATE1 is specifically involved in biliary excretion of organic cations, and BSEP is critical for the secretion of bile salts and the regulation of bile flow.

The important contribution of MRP2 to biliary excretion of toxicants was established in part by the characterization of two naturally occurring mutant strains of rat, the Groningen/Yellow transport deficient (TR^-) rat and the Eisai hyperbilirubinemic (EHBR) rat, both of which lack functional Mrp2 protein. These rats are phenotypically similar to humans suffering from Dubin–Johnson syndrome, a rare inherited disorder associated with mutations in MRP2 and characterized by chronic conjugated hyperbilirubinemia. The mutant rats also present with conjugated hyperbilirubinemia, show reduced biliary excretion of glutathione, and are defective in the normal biliary excretion of glucuronide and glutathione conjugates of many xenobiotics. A $Mrp2^{-/-}$ null mouse has been engineered, and like its mutant rat counterparts, shows marked reductions in bile flow, biliary glutathione concentrations, and reduced ability to eliminate xenobiotics. For example, plasma levels of the food-derived carcinogen, PhIp, increased 1.9-fold in $Mrp2^{-/-}$ null mice compared to wild-type controls, demonstrating that functional Mrp2 plays a role in reducing exposure to toxic chemicals (Vlaming et al., 2006). Finally, species differences in Mrp2 function may contribute to the qualitative differences observed across species in biliary excretion. In a direct comparison of the transport of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) across canalicular membrane vesicles from various species, clearance ranged from a high rate of 64.2 μL/min/mg protein in rats to a low of 3.8 μL/min/mg protein in humans, with intermediate values for other species including mouse, guinea pig, rabbit, and dog (Ishizuka et al., 1999). Such differences in transporter function and expression are likely to contribute to species differences in biliary excretion illustrated in Table 5-13.

Although the highest levels of BCRP are found in the placenta, the transporter is expressed on the bile canalicular membrane of hepatocytes where it preferentially transports sulfate conjugates of xenobiotics (Maliepaard et al., 2001). Known substrates for BCRP include estrone 3-sulfate and 4-methylembelliferone sulfate and methotrexate. MDR1 (PGP) is important in the biliary elimination of compounds such as doxorubicin and vinblastine. Bcrp knockout along with Mrp2 and Mrp3 (triple knockout) can cause diclofenac-induced liver injury (Lagas et al., 2010).

As described earlier, MATE1 and MATE2K are involved in the urinary excretion of organic cations such as metformin. MATE1, but not MATE2K, is also expressed on the bile canaliculus, where it also functions to transport organic cations into bile. In the case of metformin, a drug that works to inhibit gluconeogenesis in the liver, biliary excretion reduces hepatic drug concentrations, and thereby limits pharmacological activity.

The biliary excretion of xenobiotics mediated by MRP2, BCRP, and MDR1 usually results in increased excretion of toxicants out of hepatocytes and into bile. In doing so, these transporters increase excretion of xenobiotics and generally limit the likelihood of toxicity in the liver. However, adverse reactions can occur if the function of these transporters is inhibited, as evidenced by the examples illustrated above for the genetic mutant or knockout models. Furthermore, although BSEP does not appear to play a major role in the excretion of xenobiotics, some compounds can inhibit the function of this transporter, thereby decreasing the biliary excretion of bile acids and leading to cholestasis (defined as a decrease in bile flow with increased serum bilirubin) and cholestatic liver injury (Arrese and Ananthanarayanan, 2004). For example, inhibition of BSEP by the pharmaceutical agent troglitazone and to a much greater extent, troglitazone sulfate competitively inhibits BSEP function, leading to

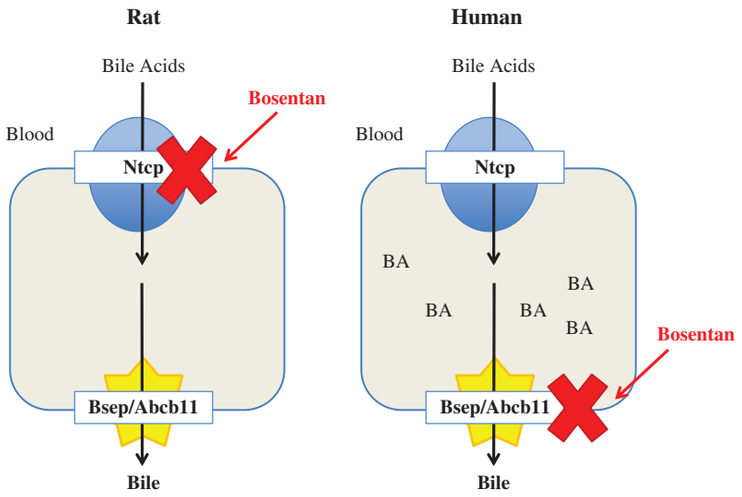

Figure 5-15. *Schematic model species difference in bosentan hepatotoxicity.*

intrahepatic cholestasis, which may contribute to the hepatotoxicity of troglitazone. The inhibitory properties of 24 Most-drug-induced liver injury (DILI)-, 28 Less-DILI-, and 20 No-DILI-concern drugs were identified using United States National Center for Toxicological Research Liver Toxicity Knowledge Base (NCTR-LTKB). Overall, drug potency for inhibiting BSEP or mitochondrial activity generally correlated across human DILI concern categories. However, drugs that caused both BSEP inhibition and mitochondrial inhibition were associated with more severe human DILI and restrictive product safety labeling (Aleo et al., 2014). Furthermore, inhibition of BSEP contributes to the cholestasis observed with steroids such as estradiol. Collectively, the ability of a chemical to interfere with the normal function of BSEP may be involved in hepatotoxicity (Morgan et al., 2010). Also species differences in BSEP inhibition must be considered for evaluating potential toxicity in preclinical models. A well-known example is bosentan-induced hepatotoxicity, which is observed in humans but not in rats. The suspected mechanism that explains the difference in toxicity is that bosentan is an inhibitor of human BSEP, but not rat Bsep (Fig. 5-15).

Although the transporters located on the canalicular membrane are directly responsible for biliary excretion of xenobiotics, other xenobiotic transporters localized to the sinusoidal membranes are also important in determining hepatic concentrations of toxicants and thereby contribute to hepatic disposition and biliary elimination. Transporters present on sinusoidal membranes include the ABC transport family members MRP3, MRP4, and MRP6, along with OATPs, OATs, and OCT. The Na^+/taurocholate cotransporting polypeptide is also found on the sinusoidal membrane where it functions specifically in the uptake of bile acids into the liver.

MRP3 transports many of the same organic anions that are substrates for MRP2 from the liver into the blood circulation. It has been shown to be important in the excretion of acetaminophen and morphine glucuronide conjugates from the liver into blood (Borst et al., 2006). In humans, it is upregulated during cholestasis and in patients with Dubin–Johnson syndrome, and in a similar manner, it is increased in TR^- and EHBR rats. The increase in MRP3 is recognized as a compensatory response that helps to protect hepatocytes from toxic chemicals for cells deficient in MRP2. MRP4 is specifically involved in the cellular efflux of purine analogs, nucleoside antiviral drugs, and cyclic nucleotides, including cAMP and cGMP. It has also been shown to be a carrier for some xenobiotics including conjugated and unconjugated compounds (Borst et al., 2006). A major role for MRP6 in xenobiotic disposition has not been established, but mutations in human MRP6 are associated with pseudoxanthoma elasticum, a hereditary disease affecting the elasticity of connective tissues (Bergen et al., 2007).

Transporters that are localized to the sinusoidal membranes of hepatocytes also contribute to hepatic uptake and efflux. OATPs contribute to the uptake of many organic anions. OATP1B1, OATP1B3, and OATP2B1 in human versus Oatp1a1 and Oatp1b2 in rodent contribute to hepatic uptake. Thus, uptake transporters also contribute to disposition to the liver and the hepatobiliary clearance of xenobiotics. In addition, the efficacy of hepatic extraction of drugs and xenobiotics from blood can impact extra-hepatic concentrations of drug and cause potential adverse drug toxicities. A well-known example of dysfunction hepatic extraction causing toxicity is statin-induced myopathy corresponding to OATP1B1 and OATP1B3. Fig. 5-16 depicts hepatic OATP–mediated uptake of statins is decreased; the

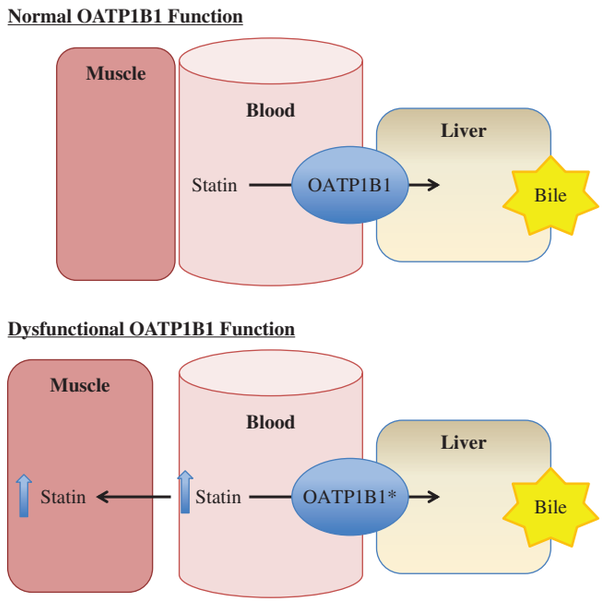

Figure 5-16. *Schematic model showing contribution of OATP1B1 in statin-induced myotoxicity.*

net result is increased blood statin levels and the potential for statins to be taken up via skeletal muscle OATP2B1 and impair mitochondrial function resulting in rhabdomyolysis (Knauer et al., 2010). Certain OATP variants that decrease OATP protein function have been associated with increased plasma exposure and statin-induced myopathy (SEARCH Collaborative Group et al., 2008; Niemi et al., 2011; Canestaro et al., 2014; Tornio et al., 2015). Several plant toxins, including phalloidin (from mushrooms) and microcystin-LR (from blue–green algae), are transported by the OATP1B proteins, and Oatp1b2-null mice are resistant to hepatotoxicity induced by both of these toxins (Lu et al., 2008). In addition to statins, Oatps exhibit broad substrate specificity and are important for the hepatic uptake of numerous drugs and xenobiotics, along with a variety of hormones, including thyroxin, and hormone conjugates. In addition to OATPs, OAT2 is expressed in liver and transports small organic anions such as indomethacin, salicylate, and prostaglandins (Anzai et al., 2006). OCT1 is also expressed in human and rodent liver, and contributes to the uptake of organic cations. This includes numerous endogenous compounds such as choline and dopamine, as well as cationic xenobiotics such as metformin. Oct1 null mice are generally resistant to lactic acidosis than is caused by metformin (Wang et al., 2003), suggesting a role for this uptake transporter (along with the biliary transporters highlighted above) in the elimination and toxic potential of this compound. In concert, the OATPs, OAT, and OCT family of transporters are important in the uptake of toxicants into the liver, a step that will also influence the contribution of hepatic metabolism, efflux into the bile, or efflux back into the circulation.

An important concept relating to biliary excretion is the phenomenon of enterohepatic circulation. After a compound is excreted into bile, it enters the intestine where it can be reabsorbed or eliminated with feces (illustrated in Fig. 5-14). Many organic compounds are conjugated with UDP-glucuronic acid, sulfate, or glutathione (see Chap. 6) before excretion into bile, and these polar metabolites are not sufficiently lipid soluble to be reabsorbed. However, enzymes found in the intestinal microflora may hydrolyze glucuronide and sulfate conjugates, liberating a more lipophilic moiety and increasing the likelihood of reabsorption. Intestinal reabsorption of the liberated xenobiotic completes a cycle in which the compound can return to the liver, where it can again be metabolized and excreted back into bile. Repeated enterohepatic cycling can lead to very long half-lives of xenobiotics in the body. Therefore, it is often desirable to interrupt this cycle to hasten the elimination of a toxicant from the body (Genius, 2011). This principle has been utilized in the treatment of dimethylmercury poisoning; ingestion of a polythiol resin binds the mercury and thus prevents its reabsorption (Magos and Clarkson, 1976).

An increase in hepatic excretory function also has been observed after pretreatment with some drugs (Klaassen and Watkins, 1984). For example, it has been demonstrated that phenobarbital increases plasma disappearance of bromosulfophthalein (BSP) by enhancing its biliary excretion. The increase in bile flow caused by phenobarbital is an important factor in increasing the biliary excretion of BSP. However, other factors including induction of phase-II enzymes (see Chap. 6) and xenobiotic transporters may also enhance biliary elimination. Induction of metabolizing enzymes and transporters works in concert to increase the clearance of a toxicant from the plasma. In particular, induction of these processes increases the capacity for a xenobiotic to be (1) taken up into the liver; (2) metabolized to conjugates that are likely to be excreted into bile; and (3) excreted into bile and removed from the general circulation. However, not all microsomal enzyme inducers increase bile flow and excretion, as agents such as 3-methylcholanthrene and benzo[*a*]pyrene are relatively ineffective in this regard.

The toxicity of some compounds can also be directly related to their biliary excretion. For example, the intestinal toxicity of several xenobiotics and drugs is increased by their excretion into bile. In the intestine deconjugation can occur, which can result in high concentrations of the chemical in the intestine. This has been observed in patients on nonsteroidal anti-inflammatory and cancer drugs. Nonsteroidal anti-inflammatory drugs cause intestinal ulcerations that can be abolished by bile-duct ligation (Duggan et al., 1975). More recently, irinotecan, an anticancer drug, has been found to induce severe GI toxicity (severe diarrhea) in humans and rats. The mechanism for the effect of this drug involved metabolism to an active metabolite that was a good substrate for MRP2. The excretion into bile mediated by MRP2 results in high concentrations of the toxic metabolite in the intestinal lumen and toxicity ensues. The toxicity of irinotecan can be modulated by inhibiting MRP2 function (Chu et al., 1997).

Many xenobiotic transporters are not expressed early in development, and the hepatic excretory system is not fully developed in newborns. Transporter expression in rodent liver is both age and isoform specific (Cui et al., 2012). As a result, there are numerous examples of compounds that are more toxic to newborns than to adults (Klaassen and Slitt, 2005). For example, ouabain is about 40 times more toxic in newborn than in adult rats. This is due to an almost complete inability of the newborn rat liver to remove ouabain from plasma by Oatp1a4. The development of hepatic excretory function can be promoted in newborns by administering microsomal enzyme inducers. In humans, less is known, but there is evidence also for ontogenic differences in transporter expression. When comparing protein expression from human liver samples, OCT1, OATP1B3, and P-gp were differentially expressed in neonates versus adults. OCT1 protein expression increased about fivefold with age (Prasad et al., 2016). Expression of MRP2, OATP1B1, and OATP1B3 in liver was lower in pediatric samples than adults. In intestine, MDR1 and MRP2 expression was similar between neonates and adults, and OATP2B1 expression in neonates was significantly higher than in adults.

Finally, the plasma clearance or biliary excretion of organic acids such as BSP, indocyanine green (ICG), or DBSP has been used to evaluate liver function. In particular, ICG is a valuable diagnostic agent in clinical practice. It is injected intravenously after which its disappearance from plasma is easily monitored. A change in plasma clearance of ICG does not distinguish between changes in hepatic blood flow or cellular function, but is highly suggestive of reduced hepatic function, including reduced biliary excretion, consistent with liver dysfunction or injury (Sakka, 2007).

Exhalation

Substances that exist predominantly in the gas phase at body temperature are eliminated mainly by the lungs. Because volatile liquids are in equilibrium with their gas phase in the alveoli, they may also be excreted via the lungs. The amount of a liquid eliminated via the lungs is proportional to its vapor pressure. A practical application of this principle is seen in the breath analyzer test for determining the amount of ethanol in the body. Highly volatile liquids such as diethyl ether and certain volatile anesthetics (nitrous oxide) are excreted almost exclusively by the lungs.

No specialized transport systems have been described for the excretion of toxic substances by the lungs. Some xenobiotic transporters, including MRP1 and MDR1, have been identified in the lung, but overall, compounds excreted via exhalation in the lung are most likely to be eliminated by simple diffusion. Elimination of gases is roughly inversely proportional to the rate of their

absorption. Therefore, gases with low solubility in blood, such as ethylene, are rapidly excreted, whereas chloroform, which has a much higher solubility in blood, is eliminated very slowly by the lungs. Trace concentrations of highly lipid-soluble anesthetic gases such as halothane and methoxyflurane may be present in expired air for as long as 2 to 3 weeks after a few hours of anesthesia. Undoubtedly, this prolonged retention is due to deposition in and slow mobilization from adipose tissue of these very lipid-soluble agents. The rate of elimination of a gas with low solubility in blood is perfusion-limited, whereas that of a gas with high solubility in blood is ventilation-limited.

Other Routes of Elimination

Cerebrospinal Fluid A specialized route of removal of toxic chemicals from a specific organ is represented by the cerebrospinal fluid (CSF). All compounds can leave the CNS with the bulk flow of CSF through the arachnoid villi. In addition, lipid-soluble toxicants also can exit at the site of the BBB. As discussed earlier, toxicants also can be removed from the CSF by active transport, using the transport systems present in the BCSFB.

Milk The secretion of toxic compounds into milk is extremely important because (1) a toxic material may be passed with milk from the mother to the nursing offspring and (2) compounds can be passed from cows to people via dairy products. Toxic agents are excreted into milk by simple diffusion. Because milk has an acidic pH (about 6.5), basic compounds may be concentrated in milk, whereas acidic compounds may attain lower concentrations in milk than in plasma (Findlay, 1983; Wilson, 1983). More important, about 3% to 4% of milk consists of lipids, and the lipid content of colostrum after parturition is even higher. Lipid-soluble xenobiotics diffuse along with fats from plasma into the mammary glands and are excreted with milk during lactation. Many of the same compounds that accumulate in fat such as aldrin, chlordane, DDT, polychlorinated and polybrominated biphenyls, dibenzo-*p*-dioxins, and furans (Van den Berg et al., 1987; Li et al., 1995) have been found in human breast milk, and milk can be a major route of their excretion. More recently, other compounds of environmental exposure concern such as nitromusk perfume ingredients, bisphenol A (BPA) and tetrabromobisphenols, polybrominated diphenyl ethers (PBDEs), and alternative halogenated flame retardants (AFRs) have been detected in milk, but it is unclear as to whether the presence of these compounds is directly responsible for possible adverse effects (Leibl et al., 2000; Nakao et al., 2015; Čechová et al., 2017). Species differences in the excretion of xenobiotics with milk are to be expected, as the proportion of milk fat derived from the circulation versus that synthesized de novo in the mammary gland differs widely among species. Metals chemically similar to calcium, such as lead, and chelating agents that form complexes with calcium also can be excreted into milk to a considerable extent.

Some transporters are detected in mammary epithelial tissue. For example, OCT1, OCT3, OCTN1, OCTN2, OATP-A, OATP-B, OATP-D, OATP-E, MRP1, MRP2, MRP5, MDR1, CNT1, CNT3, ENT1, ENT3, NCBT1, PEPT1, and PEPT2 transcripts were detected in lactating mammary epithelial cells (MEC) purified from pooled fresh breast milk samples. OCT1, OCTN1, PEPT2, CNT1, CNT3, and ENT3 were more than fourfold higher than nonlactating MEC. Little is known about whether the latter transcripts that are detected are expressed at an appreciable level that impacts function. There is evidence in Oct1/2 knockouts that milk-to-plasma concentration ratio (M/P) of terbutaline and metformin was significantly decreased compared to control mice (Ito et al., 2014). BCRP is expressed in the alveolar epithelial cells of mammary glands and actively secretes a variety of compounds into milk. Endogenous compounds such as vitamin B_{12} (riboflavin) are actively transported into breast milk by BCRP, as well as numerous toxicants including heterocyclic amines and aflatoxin. BCRP is considered to be a predominant transporter to efflux chemicals to breast milk (Ito et al., 2015). The contribution of BCRP to toxicants in the mammary gland and breast milk is paradoxical in that secretion into the milk is likely to be protective to the mother, while simultaneously increasing the potential for exposure in suckling infants or dairy consumers to a variety of toxic chemicals (Van Herwaarden and Schinkel, 2006; Vlaming et al., 2009).

Sweat and Saliva The excretion of toxic agents in sweat and saliva is quantitatively of minor importance. Again, excretion depends on the diffusion of the nonionized, lipid-soluble form of an agent. Toxic compounds excreted into sweat may produce dermatitis. Substances excreted in saliva enter the mouth, where they are usually swallowed to become available for GI absorption.

COMPUTATIONAL AND EXPERIMENTAL APPROACHES TO ASSESS XENOBIOTIC DISPOSITION

In general, the empiric determination of xenobiotic absorption, distribution, and elimination in experiments involving laboratory animals or studies in human subjects is the most reliable means by which disposition can be studied. However, such studies may require the development of sophisticated analytical methods, the use of radioactive compounds, and in the case of human studies, controlled laboratory conditions and constant monitoring. As such, these types of studies are not always practical or feasible, and a variety of computational models, nonanimal tools, or in vitro cellular systems have been developed to predict dispositional attributes of drugs and toxicants. A brief overview of several of the most widely used tools is presented here, but it is noted that this is a dynamic and rapidly changing field. In general, these models are particularly useful in studying absorption and excretion, particularly biliary excretion. Tissue distribution, with particular emphasis on target organ dosimetry, can also be assessed with pharmacokinetic models (Chap. 7).

Absorption A variety of models are used to estimate log *P* because the lipid solubility is an important, general feature of any toxicant (Mannhold et al., 2009). Additionally, based on the general principles outlined earlier, computational tools to estimate permeability have been developed. One such model predicts poor absorption as a function of the calculated log *P* (*C* log *P*), the molecular weight, and the presence of hydrogen bond donors and acceptors. The concept based on these four elements is referred to as the "rule of 5" because the determinants are based on multiples of 5 and include (1) molecular weight greater than 500; (2) *C* log *P* greater than 5; (3) more than 5 H-bond donors; and (4) 10 H-bond acceptors (Lipinski et al., 2001).

The human colon adenocarcinoma cell line, Caco-2, is widely used to evaluate xenobiotic permeability. These cells form a confluent epithelial monolayer with well-defined tight junctions and typical microvilli on the apical surface. A variety of xenobiotic transporters are expressed on Caco-2 cells. The uptake transporters OATP2B1, PEPT1, and OCTN2 are expressed on the apical membrane along with efflux transporters MDR1, MRP2, and BCRP. On the basolateral membrane, efflux transporters MRP3, MRP4, and MRP6 have been identified (Sun et al., 2008). Caco-2 cells are specifically used to estimate the fraction of xenobiotic that will be absorbed in the GI tract. Movement across the apical (A) and basolateral (B) membranes is also used to determine whether a xenobiotic

is substrate or inhibitor of xenobiotic transporters. Transport in the A→B direction is indicative of absorption, whereas movement in the B→A direction suggests that carrier-mediated efflux is favored. Madin–Darby canine kidney (MDCK) and MDCKII–low expressing (MDCKII-LE) cell lines have become preferred cell lines to assess permeability. For example, MDCKII-LE PgP protein expression is about 20% of that present in wild-type MDCKII cells, and the functional efflux capacity is less than 10% of that observed in MDCKII wild-type cells (Di et al., 2011).

Another model used to predict absorption utilizes artificial membranes such as that exemplified by the parallel artificial membrane permeability assay (PAMPA). As an artificial membrane, this system lacks transporters or paracellular pathways and is most useful for assessing the non-energy-dependent diffusion of toxicants.

Dermal exposure can be evaluated in a variety of in vitro systems, either with skin biopsy samples or with models developed to represent human skin. Mathematical models, with emphasis on the lipid matrix as the principal pathway of permeation, have also been developed and used with increasing frequency. Such methods emphasize log *P* and molecular size in model development (Lian et al., 2008).

Hepatobiliary Excretion Xenobiotic transporter function can be evaluated with membrane vesicles isolated from specific organs or with expressed cell systems. The development of a variety of transporter-deficient models, particularly in mice, has also proved to be very useful for assessing transporter contribution to toxicity as has been described throughout this chapter (Klaassen and Lu, 2008). The latter tools along with more sophisticated primary hepatocyte culture models are typically utilized to aid in the prediction of transporter-mediated effects in absorption and excretion processes. For example, the use of sandwich-cultured human hepatocytes (SCHH) have become a standard tool to aid in the prediction of biliary excretion and drug-induced liver injury. Briefly, when hepatocytes are cultured between two layers of gelled collagen (hence the sandwich configuration), they retain molecular and biochemical characteristics more consistent with their properties in the whole organ than monolayer cultures of cells. These features include the formation of canalicular networks necessary for biliary excretion. This system has been optimized to assess toxicant accumulation, estimate biliary excretion, and investigate the interplay between metabolism and transport, and has proved to be a useful in vitro system to aid in the evaluation of hepatobiliary disposition (Swift et al., 2010). When compared to in vivo models, such as monkey, rat, and dog, SCHH displayed good predictability to monkey and human for drugs with no significant active uptake and improved predictability when scaled to take extended clearance into account (Kimoto et al., 2017).

Classification Systems Classification systems have evolved to utilize physiochemical properties to predict solubility, disposition, and excretion. These systems are predominantly used in the pharmaceutical industry; however, their likely usefulness in the field of toxicology is to aid in understanding/predicting disposition for chemicals of environmental concern or predict the impact of active pharmaceutical ingredients excreted and present in sewage (Daughton, 2014). In 2005, the Biopharmaceutics Drug Disposition Classification System (BDDCS) was described with the purpose to aid in the prediction of drug disposition characteristics at early stages of drug discovery and development (Wu and Benet, 2005). The BDDCS was further updated in 2011 to include 927 drugs and aid in prediction of transporter-mediated effects (Benet et al., 2011).

The Extended Clearance Classification System (ECCS) was described in 2015 (Varma et al., 2015) and uses known physicochemical properties and in vitro/in silico data readily available in early drug discovery, namely molecular weight, ionization, and measured/calculated in vitro permeability (Fig. 5-17). It is used to predict absorption trends, impact of intestinal and hepatic first-pass effect on new molecular entities oral profiles, drug clearance rate–determining step, and extent of metabolism. The ECCS classifies molecules into six classes, which are as follows (Fig. 5-17):

1. ECCS Class 1A: These molecules are either acids or zwitterions that have high permeability and low molecular weight (<400 Da). ECCS Class 1A molecules typically have good oral absorption and intestinal or hepatic first pass does not limit their oral bioavailability. The clearance rate–determining step of this class is metabolism.
2. ECCS Class 1B: This is a class of molecules that are acids or zwitterions. They have high permeability and molecular weight (≥400 Da). The oral profile of these molecules is mainly influenced by their intestinal and hepatic first-pass effect. The rate-determining step is active hepatic uptake mediated by OATP1B1/OATP1B3. Once in the liver, they are metabolized and have a high extent of metabolism (≥70%).

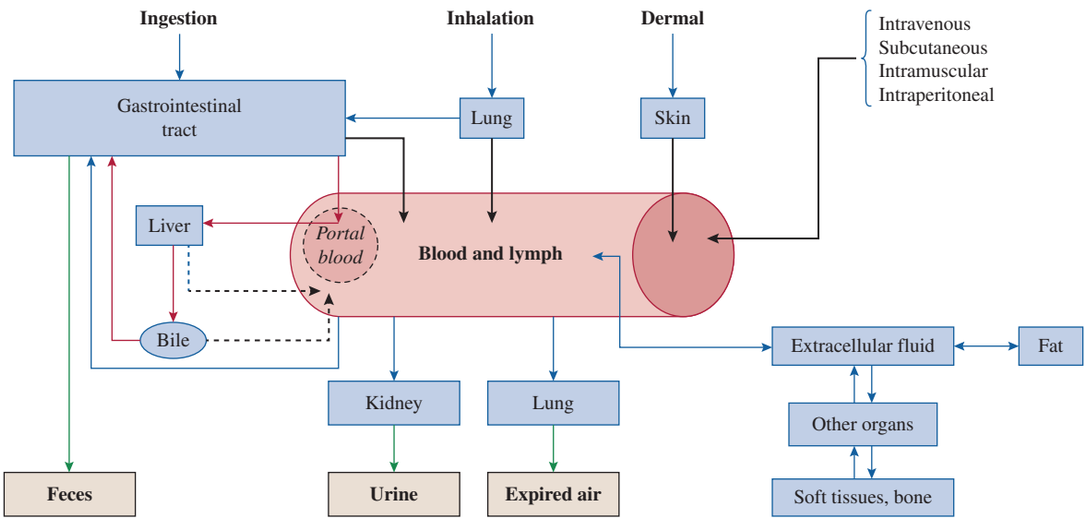

Figure 5-17. *Schematic model showing the extended clearance classification system.*

3. ECCS Class 2: These are either basic or neutral chemicals with high membrane permeability. The oral profile of ECCS Class 2 is influenced by intestinal and hepatic first-pass effect because many ECCS Class 2 molecules are metabolized by CYP3A4, which is highly expressed in human intestine and liver. Metabolism is the rate-determining step of their clearance mechanism. Similar to ECCS classes 1A and 1B, ECCS Class 2 molecules have a high extent of metabolism (≥70%).
4. ECCS Class 3A: These are acids and zwitterions with low molecular weight (<400 Da) and low permeability. The oral profile of these molecules is determined by their intestinal permeability and affinity to uptake intestinal transporters, for example, PEPT1 and OATP2B1. The rate-determining step is renal elimination.
5. ECCS Class 3B: These molecules are either acids or zwitterions with low permeability and high molecular weight (≥400 Da). Their oral absorption is governed by their intestinal permeability and affinity to uptake and efflux intestinal transporters. Their clearance rate–determining step is active hepatic uptake and/or renal elimination. The hepatic uptake is typically mediated by OATP1B1 and OATP1B3, and once in the liver they are excreted in the bile as unchanged drug. If renally eliminated, they are also excreted in the urine as unchanged drug.
6. ECCS Class 4: These molecules are either basic or neutral chemicals. They have low membrane permeability with oral absorption as a key determinant of their oral bioavailability. Renal elimination is the rate-determining step of their clearance mechanism. Similar to classes 3A and 3B, they have a low extent of metabolism (<30%).

CONCLUSION

Humans are in continuous contact with toxic chemicals. Toxicants are in the food we eat, the water we drink, and the air we breathe. Depending on their physical and chemical properties, toxic chemicals may be absorbed by the GI tract, the lungs, and/or the skin. Fortunately, the body has the ability to biotransform and excrete these compounds into urine, feces, and air. However, when the rate of absorption exceeds the rate of elimination, toxic compounds may accumulate and reach a critical concentration at a certain target site, and toxicity may ensue. Whether a chemical elicits toxicity depends not only on its inherent potency and site specificity but also on whether, and if so how, it is absorbed, distributed, and eliminated. Therefore, knowledge of the disposition of chemicals is of great importance in judging the toxicity of xenobiotics. For example, for a potent CNS suppressant that displays a strong hepatic first-pass effect, oral exposure is of less concern than is exposure by inhalation. Also, two equipotent gases, with the absorption of one being perfusion rate-limited and that of the other being ventilation rate-limited, will exhibit completely different toxicity profiles at a distant site because of differences in the concentrations attained in the target organ.

Many chemicals have very low inherent toxicity but can be metabolically activated into toxic metabolites, and toxicity may be determined by the rate of formation of toxic metabolites. Alternatively, a very potent toxicant may be detoxified rapidly by biotransformation. The fundamental and overarching concept is that adverse effects are related to the unbound concentration of the "toxic chemical" at the site of action (in the target organ), whether a chemical is administered or generated by biotransformation in the target tissue or at a distant site. Accordingly, the toxic response exerted by chemicals is critically influenced by the rates of absorption, distribution, biotransformation, and excretion.

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REFERENCES

Agarwal S, Sane R, Ohlfest JR, Elmquist WF. The role of the breast cancer resistance protein (ABCG2) in the distribution of sorafenib to the brain. *J Pharmacol Exp Ther*. 2011;336:223–233.

Aleo MD, Luo Y, Swiss R, et al. Human drug-induced liver injury severity is highly associated with dual inhibition of liver mitochondrial function and bile salt export pump. *Hepatology*. 2014;60:1015–1022.

Alnouti Y, Petrick JS, Klaassen CD. Tissue distribution and ontogeny of organic cation transporters in mice. *Drug Metab Dispos*. 2006;34:477–482.

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol*. 1999;39:361–398.

Anzai N, Kanai Y, Endou H. Organic anion transporter family: current knowledge. *J Pharmacol Sci*. 2006;100:411–426.

Arrese M, Ananthanarayanan M. The bile salt export pump: molecular properties, function and regulation. *Pflugers Arch*. 2004;449:123–131.

Awad Wa, Ghareeb K, Bohm J, Zentek J. Decontamination and detoxification strategies for the Fusarium mycotoxin deoxynivalenol in animal feed and the effectives of microbial biodegradation. *Food Add Contam*. 2010;27:510–520.

Bailey DG. Fruit juice inhibition of uptake transport: a new type of food-drug interaction. *Br J Clin Pharmacol*. 2010;70:645–655.

Ballard ST, Hunter JH, Taylor AE. Regulation of tight-junction permeability during nutrient absorption across the intestinal epithelium. *Annu Rev Nutr*. 1995;15:35–55.

Baron JM, Holler D, Schiffer R, et al. Expression of multiple cytochrome P450 enzymes and multidrug resistance-associated transport proteins in human skin keratinocytes. *J Invest Dermatol*. 2001;116:541–548.

Benet LZ, Broccatelli F, Oprea TI. BDDCS applied to over 900 drugs. *AAPS J*. 2011;13(4):519–547.

Benz R, Janko K, Länger P. Pore formation by the matrix protein (porin) to *Escherichia coli* in planar bilayer membranes. *Ann N Y Acad Sci*. 1980;358:13–24.

Bergen AA, Plomp AS, Hu X, deJong PT, Gorgels TG. ABCC6 and pseudoxanthoma elasticum. *Pflugers Arch*. 2007;453:685–691.

Bogdanffy MS, Manning LA, Sarangapani R. High-affinity nasal extraction of vinyl acetate vapor is carboxylesterase dependent. *Inhal Toxicol*. 1999;11:927–941.

Borowitz JL, Moore PF, Him GKW, Miya TS. Mechanism of enhanced drug effects produced by dilution of the oral dose. *Toxicol Appl Pharmacol*. 1971;19:164–168.

Borst P, Zelcer N, van de Wetering K. MRP2 and 3 in health and disease. *Cancer Lett*. 2006;234:51–61.

Boyes RN, Adams HJ, Duce BR. Oral absorption and disposition kinetics of lidocaine hydrochloride in dogs. *J Pharmacol Exp Ther*. 1970;174:1–8.

Bronaugh RL, Stewart RF, Congdon ER, Giles ALJr. Methods for in vitro percutaneous absorption studies. I. Comparison with in vivo results. *Toxicol Appl Pharmacol*. 1982;62:474–480.

Campos A, Vasconcelos V. Molecular mechanisms of microcystin toxicity in animal cells. *Int J Mol Sci*. 2010;11:268–287.

Calabrese EJ. Gastrointestinal and dermal absorption: interspecies differences. *Drug Metab Rev*. 1984;15:1013–1032.

Canestaro WJ, Austin MA, Thummel KE. Genetic factors affecting statin concentrations and subsequent myopathy: a HuGENet systematic review. *Genet Med*. 2014, 16:810–819.

Cayen MN. Metabolic disposition of antihyperlipidemic agents in man and laboratory animals. *Drug Metab Rev*. 1980;11:291–323.

Čechová E, Vojta Š, KukuČka P, et al. Legacy and alternative halogenated flame retardants in human milk in Europe: implications for children's health. *Environ Int*. 2017;108:137–145.

Chu XY, Kato Y, Niinuma K, et al. Multi-specific organic anion transporter (cMOAT) is responsible for the biliary excretion of the camptothecin derivative irinotecan, CPT-11 and its metabolites in rats. *J Pharmacol Exp Ther*. 1997;281:304–314.

Clarkson TW. Metal toxicity in the central nervous system. *Environ Health Perspect*. 1987;75:59–64.

Cui JY, Gunewardena SS, Yoo B, et al. RNA-Seq reveals different mRNA abundance of transporters and their alternative transcript isoforms during liver development. *Toxicol Sci*. 2012;127:592–608.

Daughton CG. Eco-directed sustainable prescribing: feasibility for reducing water contamination by drugs. *Sci Total Environ*. 2014; 493:392–404.

Deeken JG, Loscher W. The blood-brain barrier and cancer: transporters, treatment and Trojan horses. *Clin Cancer Res*. 2007;13:1663–1674.

de Vries NA, Buckle T, Zhao J, et al. Restricted brain penetration of the tyrosine kinase inhibitor erlotinib due to the drug transporters P-gp and BCRP. *Invest New Drugs*. 2012;30:443–449.

Di L, Whitney-Pickett C, Umland JP, et al. Development of a new permeability assay using low-efflux MDCKII cells. *J Pharm Sci*. 2011;100:4974–4985.

Dietrich CG, Rudi de Waart D, Ottenhoff R, Schoots IG, Oude Elferink RPJ. Increased bioavailability of the food-derived carcinoge 2-amino-1-methyl-6-phenylimidazo[4,5]pyridine in MRP2-deficient rats. *Mol Pharmacol*. 2001;59:974–980.

Diwan BA, Riggs CW, Logsdon D, et al. Multiorgan transplacental and neonatal carcinogenicity of 3′-axido-3′-deoxythymidine in mice. *Toxicol Appl Pharmacol*. 1999;161:82–99.

Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther*. 1944;82:377–390.

Dreyfuss J, Shaw JM, Ross JJ. Absorption of the adrenergic-blocking agent, nadol, by mice, rats, hamsters, rabbits, dogs, monkeys and man: unusual species differences. *Xenobiotica*. 1978;8:503–510.

Drozdzik M, Gröer C, Penski J, et al. Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine. *Mol Pharm*. 2014;11:3547–3555.

Dugard PH. Skin permeability theory in relation to measurements of percutaneous absorption in toxicology. In: Marzulli FN, Maibach HI, eds. *Dermatotoxicology*. 2nd ed. New York: Hemisphere; 1983:91–116.

Duggan DE, Hooke KF, Noll RM, Kwan KC. Enterohepatic circulation of indomethacin and its role in intestinal irritation. *Biochem Pharmacol*. 1975;24:1749–1754.

Enders AC, Carter AM. What can comparative studies of placental structure tell us?—a review. *Placenta*. 2004;25(suppl A): S3–S9.

Enright EF, Gahan CG, Joyce SA, Griffin BT. The impact of the gut microbiota on drug metabolism and clinical outcome. *Yale J Biol Med*. 2016;89:375–382.

Findlay JWA. The distribution of some commonly used drugs in human breast milk. *Drug Metab Rev*. 1983;14:653–686.

Florence AT, Hillery AM, Hussain N, Jani PU. Factors affecting the oral uptake and translocation of polystyrene nanoparticles: histological and analytical evidence. *J Drug Target*. 1995;3:65–70.

Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A*. 2007;104:13780–13785.

Fröhlich E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int J Nanomedicine*. 2012;7:5577–5591.

Fu ZD, Selwyn FP, Cui JY, Klaassen CD. RNA-Seq profiling of intestinal expression of xenobiotic processing genes in germ-free mice. *Drug Metab Dispos*. 2017;45(12):1225–1238.

Fujita KI, Masuo Y, Yamazaki E, et al. Involvement of the transporters P-glycoprotein and breast cancer resistance protein in dermal distribution of the multikinase inhibitor regorafenib and its active metabolites. *J Pharm Sci*. 2017;106: 2632–2641.

Fullmer CS. Intestinal interactions of lead and calcium. *Neurotoxicology*. 1992;13:799–807.

Ganapathy V, Prasad PD, Ganapathy ME, Leibach FH. Placental transporters relevant to drug disposition across the maternal-fetal interface. *J Pharmacol Exp Ther*. 2000;294:413–420.

Genius SJ. Elimination of persistent toxicants from the human body. *Human Exp Toxicol*. 2011;30:3–18.

Ghanem CI, Ruiz ML, Villanueva SS, et al. Shift from biliary to urinary elimination of acetaminophen-glucuronide in acetaminophen-pretreated rats. *J Pharmacol Exp Ther*. 2005;315:987–995.

Gröer C, Brück S, Lai Y, et al. LC-MS/MS-based quantification of clinically relevant intestinal uptake and efflux transporter proteins. *J Pharm Biomed Anal*. 2013;85:253–261.

Hagenbuch B, Guo C. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica*. 2008;38:778–801.

Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch*. 2004;447:653–665.

Han TK, Everett RS, Proctor WR, et al. Organic cation transporter 1 (OCT1/mOct1) is localized in the apical membrane of Caco-2 cell monolayers and enterocytes. *Mol Pharmacol*. 2013;84:182–189.

Hanumegowda UM, Wenke G, Regueiro-Ren A, et al. Phospholipidosis as a function of basicity, lipophilicity and volume of distribution of compounds. *Chem Res Toxicol*. 2010;23:749–755.

Hashimoto N, Nakamichi N, Uwafuji S, et al. ATP binding cassette transporters in two distinct compartments of the skin contribute to transdermal absorption of a typical substrate. *J Control Release*. 2013;165:54–61.

Hayer M, Bönisch H, Brüss M. Molecular cloning, functional characterization and genomic organization of four alternatively spliced isoforms of the human organic cation transporter 1 (hOCT1/SLC22A1). *Ann Hum Genet*. 1999;63:473–482.

Hediger MA, Clémençon B, Burrier RE, Bruford EA. The ABCs of membrane transporters in health and disease (SLC series): introduction. *Mol Aspects Med*. 2013;34:95–107.

Hediger MA, Romero MF, Peng J-B, et al. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. *Pflugers Arch*. 2004;447:465–468.

Ho ES, Lin DC, Mendel DB, Cihlar T. Cytotoxicity of antiviral nucleotides adefovir and cidofovir induced by the expression of the human renal organic anion transporter I. *J Am Soc Nephrol*. 2000;11:383–393.

Huls M, Brown CD, Windass AS, et al. The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. *Kidney Int*. 2008;73:220–225.

Ishizuka H, Donno K, Shiina T, et al. Species differences in the transport activity for organic anions across the bile canalicular membrane. *J Pharmacol Exp Ther*. 1999;290:1324–1330.

Ito N, Ito K, Ikebuchi Y, et al. Organic cation transporter/solute carrier family 22a is involved in drug transfer into milk in mice. *J Pharm Sci*. 2014, 103(10):3342–3348.

Ito N, Ito K, Ikebuchi Y, et al. Prediction of drug transfer into milk considering breast cancer resistance protein (BCRP)-mediated transport. *Pharm Res*. 2015;32(8):2527–2537.

Jandacek RJ, Tso P. Factors affecting the storage and excretion of toxic lipophilic xenobiotics. *Lipids*. 2001;36:1289–1305.

Jani P, Halbert GW, Langridge J, Florence AT. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. *J Pharm Pharmacol*. 1990;42:821–826.

Kao J, Patterson FU, Hall J. Skin penetration and metabolism of topically applied chemicals in six mammalian species, including man: an in vitro study with benzo(a)pyrene and testosterone. *Toxicol Appl Pharmacol*. 1985;81:502–516.

Kashihara Y, Ieiri I, Yoshikado T, et al. Small-dosing clinical study: pharmacokinetic, pharmacogenomic (SLCO2B1 and ABCG2), and interaction (atorvastatin and grapefruit juice) profiles of 5 probes for OATP2B1 and BCRP. *J Pharm Sci*. 2017;106:2688–2694.

Kerns WD, Pavkov KL, Donofrio DJ, Gralla EJ, Swenberg JA. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. *Cancer Res*. 1983;43:4382–4392.

Kimoto E, Bi YA, Kosa RE, Tremaine LM, Varma MVS. Hepatobiliary clearance prediction: species scaling from monkey, dog, and rat, and in vitro-in vivo extrapolation of sandwich-cultured human hepatocytes using 17 drugs. *J Pharm Sci*. 2017;106:2795–2804.

Klaassen CD, Aleksunes LM. Xenobiotic, bile acid and cholesterol transporters: function and regulation. *Pharmacol Rev*. 2010;62:1–96.

Klaassen CD, Liu J. Role of metallothionein in cadmium-induced hepatotoxicity and nephrotoxicity. *Drug Metab Rev*. 1997;29:79–102.

Klaassen CD, Lu H. Xenobiotic transporters: ascribing function from gene knockout and mutation studies. *Toxicol Sci*. 2008;101:186–196.

Klaassen CD, Shoeman DW. Biliary excretion of lead in rats, rabbits and dogs. *Toxicol Appl Pharmacol*. 1974;29:434–446.

Klaassen CD, Slitt AL. Regulation of hepatic transporters by xenobiotic receptors. *Curr Drug Metab*. 2005;6:309–328.

Klaassen CD, Watkins JB. Mechanisms of bile formation, hepatic uptake, and biliary excretion. *Pharmacol Rev*. 1984;36:1–67.

Knauer MJ, Urquhart BL, Meyer zu Schwabedissen HE, et al. Human skeletal muscle drug transporters determine local exposure and toxicity of statins. *Circ Res*. 2010, 106:297–306.

Kodaira H, Kusuhara H, Ushiki J, Fuse E, Sugiyama Y. Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. *J Pharmacol Exp Ther*. 2010;333:788–796.

Kusuhara H, Sugiyama Y. Active efflux across the blood-brain barrier: role of the solute carrier family. *NeuroRx*. 2005;2:73–85.

Kusuhara H, Sugiyama Y. In vitro-in vivo extrapolation of transporter-mediated clearance in the liver and kidney. *Drug Metab Pharmacokinet*. 2009;24:37–52.

Lademann J, Richter H, Schanzer S, et al. Penetration and storage of particles in human skin: perspectives and safety aspects. *Eur J Pharm Biopharm*. 2011;77:465–468.

Lagas JS, Sparidans RW, Wagenaar E, Beijnen JH, Schinkel AH. Hepatic clearance of reactive glucuronide metabolites of diclofenac in the mouse is dependent on multiple ATP-binding cassette efflux transporters. *Mol Pharmacol*. 2010;77:687–694.

Lankas GR, Cartwright ME, Umbenhauer D. P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicol Appl Pharmacol*. 1997;143:357–365.

Lankas GR, Wise LD, Cartwright ME, et al. Placental p-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod Toxicol*. 1998;12:457–463.

Lehman-McKeeman LD. α2u-Globulin nephropathy. In: Schnellman RG, ed. *Comprehensive Toxicology*. 2nd ed. New York: Elsevier; 2010:507–521.

Lehman-McKeeman LD, Johnson DR, Caudill D. Induction and inhibition of mouse cytochrome P450 2B enzymes by musk xylene. *Toxicol Appl Pharmacol*. 1997;142:169–177.

Leibl B, Mayer R, Ommer S, Sonnichsen C, Koletzko B. Transition of nitro musks and polycyclic musks into human milk. *Adv Exp Med Biol*. 2000;478:289–305.

Leiser P, Kaufmann R. Placental structure: in a comparative aspect. *Exp Clin Endocrinol*. 1994;102:122–134.

Li X, Weber LWD, Rozman KK. Toxicokinetics of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in female Sprague–Dawley rats including placental and lactational transfer to fetuses and neonates. *Fundam Appl Toxicol*. 1995;27:70–76.

Lian G, Chen L, Han L. An evaluation of mathematical models for predicting skin permeability. *J Pharm Sci*. 2008;97:585–598.

Lin JH. Species similarities and differences in pharmacokinetics. *Drug Metab Dispos*. 1995;23:1008–1021.

Lin JH. Tissue distribution and pharmacodynamics: a complicated relationship. *Curr Drug Metab*. 2006;7:39–65.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 2001; 46:3–26.

Lu H, Choudhuri S, Ogura K, et al. Characterization of organic anion transporting polypeptide 1b2-null mice: essential role in hepatic uptake/toxicity ophalloidin and microcystin-LR. *Toxicol Sci*. 2008;103:35–45.

Magos L, Clarkson TW. The effect of oral doses of a polythiol resin on the excretion of methylmercury in mice treated with cystein, d-penicillamine or phenobarbitone. *Chem Biol Interact*. 1976;14:325–335.

Maliepaard M, Scheffer GL, Faneyte IF, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res*. 2001;6:3458–3464.

Mannhold R, Poda GI, Ostermann C, Tetko IV. Calculation of molecular lipophilicity: state-of-the-art and comparison of log *P* methods on more than 96,000 compounds. *J Pharm Sci*. 2009;98:861–893.

Memon N, Bircsak KM, Archer F, et al. Regional expression of the BCRP/ABCG2 transporter in term human placentas. *Reprod Toxicol*. 2014;43:72–77.

Mensinga TT, Speijers GJ, Meulenbelt J. Health implications of exposure to environmental nitrogenous compounds. *Toxicol Rev*. 2003;22:41–51.

Miller DS. Regulation of P-glycoprotein and other ABC drug transporters at the blood-brain barrier. *Trends Pharm Sci*. 2010;31:246–254.

Mizuno N, Niwa T, Yotsumoto Y, Sugiyama Y. Impact of drug transporter studies on drug discovery and development. *Pharmacol Rev*. 2003;55:425–461.

Mizuno N, Suzuki M, Kusuhara H, et al. Impaired renal excretion of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) sulfate in breast cancer resistance protein (BCRP1/ABCG2) knockout mice. *Drug Metab Dispos*. 2004;32:898–901.

Moeller AH, Degnan PH, Pusey AE, Wilson ML, Hahn BH, Ochman H. Chimpanzees and humans harbour compositionally similar gut enterotypes. *Nat Commun*. 2012;3:1179.

Morgan RE, Trauner M, van Staden CJ, et al. Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. *Toxicol Sci*. 2010;118:485–500.

Morris FH Jr, Boyd DH, Mahendran D. Placental transport. In: Knobel E, Neill JD, eds. *The Physiology of Reproduction*. New York: Raven Press; 1994:813–861.

Motohashi H, Inui K. Organic cation transporter OCTs (SLC22) and MATEs (SLC47) in the human kidney. *AAPS J*. 2013 Apr;15:581–588 doi: 10.1208/s12248-013-9465-7.

Myllynen P, Pasanen M, Pelkonen O. Human placenta: a human organ for developmental toxicology research and biomonitoring. *Placenta*. 2005;26:361–371.

Nakanishi T, Doyle LA, Hassel B, et al. Functional characterization of human breast cancer resistance protein (BCRP, ABCG2) expressed in the oocytes of *Xenopus laevis*. *Mol Pharmacol*. 2003;64:1452–1462.

Nakao T, Akiyama E, Kakutani H, Mizuno A, Aozasa O, Akai Y, Ohta S. Levels of tetrabromobisphenol A, tribromobisphenol A, dibromobisphenol A, monobromobisphenol A, and bisphenol a in Japanese breast milk. *Chem Res Toxicol*. 2015;28(4):722–728.

Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science*. 2006;311:622–627.

Newbold RR, McLachlan JA. Transplacental hormonal carcinogenesis: diethylstilbestrol as an example. *Prog Clin Biol Res*. 1996;394:131–147.

Newman MD, Stotland M, Ellis JI. The safety of nanosized particles in titanium dioxide and zinc oxide-based sunscreens. *J Am Acad Dermatol*. 2009;61:685–692.

Nguyen TL, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? *Dis Model Mech*. 2015;8:1–16.

Ni Z, Bikadi Z, Rosenberg MF, Mao Q. Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). *Curr Drug Metab*. 2010;11:603–617.

Niemi M, Pasanen MK, Neuvonen PJ. Organic anion transporting polypeptide 1B1: a genetically polymorphic transporter of major importance for hepatic drug uptake. *Pharmacol Rev*. 2011;63:157–181.

Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect*. 2005;113:823–839.

Pfeiffer CJ. Gastroenterologic response to environmental agents—absorption and interactions. In: Lee DHK, ed. *Handbook of Physiology. Section 9: Reactions to Environmental Agents*. Bethesda, MD: American Physiological Society; 1977:349–374.

Poet TS, McDougal JN. Skin absorption and human risk assessment. *Chem Biol Interact*. 2002;140:19–34.

Prasad B, Johnson K, Billington S, et al. Abundance of drug transporters in the human kidney cortex as quantified by quantitative targeted proteomics. *Drug Metab Dispos*. 2016;44:1920–1924.

Prouillac C, Lecoeur S. The role of the placenta in fetal exposure to xenobiotics: importance of membrane transporters and human models for transport studies. *Drug Metab Dispos*. 2010;38:1623–1635.

Rickert DE, Butterworth BE, Popp JA. Dinitrotoluene: acute toxicity, oncogenicity, genotoxicity and metabolism. *CRC Crit Rev Toxicol*. 1984;13:217–234.

Rizwan AN, Burckhardt G. Organic anion transporters of the SLC22 family: biopharmaceutical, physiological and pathological roles. *Pharm Res*. 2007;24:9181–2004.

Ross JH, Driver JH, Harris SA, Maibach HI. Dermal absorption of 2,4-D: a review of species differences. *Regul Toxicol Pharmacol*. 2005;41:82–91.

Rowland IR, Mallet AK, Wise A. The effect of diet on mammalian gut flora and its metabolic activities. *CRC Crit Rev Toxicol*. 1985;16:31–103.

Sahi J. Use of in vitro transporter assays to understand hepatic and renal disposition of new drug candidates. *Expert Opin Drug Metab Toxicol*. 2005;1:409–427.

Sakka SG. Assessing liver function. *Curr Opin Crit Care*. 2007;13:207–214.

Schanker LS. Passage of drugs across body membranes. *Pharmacol Rev*. 1962;74:501–530.

Schinkel A, Smith JJM, van Tellingen O, et al. Disruption of the mouse mdr1a p-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. *Cell*. 1994;47:491–502.

Schwartze EW. The so-called habituation to arsenic: variation in the toxicity of arsenious oxide. *J Pharmacol Exp Ther*. 1923;20:181–203.

SEARCH Collaborative Group, Link E, Parish S, et al. SLCO1B1 variants and statin-induced myopathy—a genomewide study. *N Engl J Med*. 2008;359:789–799.

Shand DG, Rangno RE. The deposition of propranolol: I. Elimination during oral absorption in man. *Pharmacology*. 1972;7:159–168.

Shitara Y, Sato H, Sugiyama Y. Evaluation of drug–drug interaction in the hepatobiliary and renal transport of drugs. *Annu Rev Pharmacol Toxicol*. 2005;45:689–723.

Silverman WA, Andersen DH, Blanc WA, Crozier DN. A difference in mortality rate and incidence of kernicterus among premature infants allotted to two prophylactic antibacterial regimens. *Pediatrics*. 1956;18:614–625.

Slitt AL, Cherrington NJ, Hartley DP, Leazer TM, Klaassen CD. Tissue distribution and renal developmental changes in rat organic cation transporter mRNA levels. *Drug Metab Dispos*. 2002;30:212–219.

Smith HW. Observations on the flora of the alimentary tract of animals and factors affecting its composition. *J Pathol Bacteriol*. 1965;89:95–107.

Stern ST, McNeil SE. Nanotechnology safety concerns revisited. *Toxicol Sci*. 2008;101:4–21.

Sun H, Chow ECY, Liu S, Du Y, Pang KS. The Caco-2 cell monolayer: usefulness and limitations. *Expert Opin Drug Metab Toxicol*. 2008;4:395–411.

Swift B, Pfeifer ND, Brouwer KLR. Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. *Drug Metab Rev*. 2010;42:446–471.

Symchowicz S, Staub MS, Wong KKA. Comparative study of griseofulvin-^{14}C metabolism in the rat and rabbit. *Biochem Pharmacol*. 1967;16:2405–2411.

Syme MR, Paxton JW, Keelan JA. Drug transfer and metabolism by the human placenta. *Clin Pharmacokinet*. 2004;43:487–514.

Takashima T, Wu C, Takashima-Hirano M, et al. Evaluation of breast cancer resistance protein function in hepatobiliary and renal excretion using PET with 11C-SC-62807. *J Nucl Med*. 2013, 54:267–276.

Tamai I. Oral drug delivery using intestinal OATP transporters. *Adv Drug Deliv Rev*. 2012;64:508–514.

Tokar EJ, Qu W, Waalkes MP.Arsenic, stem cells and the developmental basis of adult cancer. *Toxicol Sci*. 2011;120(suppl 1):S192–S203.

Tornio A, Vakkilainen J, Neuvonen M, et al. SLCO1B1 polymorphism markedly affects the pharmacokinetics of lovastatin acid. *Pharmacogenet Genomics*. 2015, 25:382–387.

Ueda K. ABC proteins protect the human body and maintain optimal health. *Biosci Biotechnol Biochem*. 2011;75:401–409.

Van den Berg M, Heeremans C, Veerhoven E, Olie K. Transfer of polychlorinated dibenzo-p-dioxins and dibenzofurans to fetal and neonatal rats. *Fundam Appl Toxicol*. 1987;9:635–644.

Van Herwaarden AE, Schinkel AH. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. *Trends Pharmacol Sci*. 2006;27:10–16.

van Herwaarden AE, Wagenaar E, Merino G, et al. Multidrug transporter ABCG2/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk. *Mol Cell Biol*. 2007;27:1247–1253.

Varma MV, Steyn SJ, Allerton C, El-Kattan AF. Predicting clearance mechanism in drug discovery: Extended Clearance Classification System (ECCS). *Pharm Res*. 2015;32:3785–3802.

Vlaming ML, Lagas JS, Schinkel AH. Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice. *Adv Drug Deliv Rev*. 2009;61:14–25.

Vlaming MLH, Morhmann K, Wagenaar E, et al. Carcinogen and anticancer drug transport by Mrp2 in vivo: studies using Mrp2 (Abcc2) knockout mice. *J Pharmacol Exp Ther*. 2006;318:319–327.

Waalkes MP, Ward JM, Liu J, Diwan BA. Transplacental carcinogenesis of inorganic arsenic in drinking water: induction of hepatic, ovarian, pulmonary and adrenal tumors in mice. *Toxicol Appl Pharmacol*. 2003;186:7–17.

Walton K, Dorne JLCM, Renwick AG. Species-specific uncertainty factors for compounds eliminated principally by renal excretion in humans. *Food Chem Toxicol*. 2004;42:261–274.

Wang DS, Jonker JW, Kato Y, Kusuhara H, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *J Pharmacol Exp Ther*. 2002;302:510–515.

Wang DS, Kusuhara H, Kato Y, Jonker JW, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in the lactic acidosis caused by metformin. *Mol Pharmacol*. 2003;63:844–848.

Weaver YM, Ehresman DJ, Butenhoff JL, Hagenbuch B. Roles of rat renal organic anion transporters in transporting perfluorinated carboxylates with different chain lengths. *Toxicol Sci*. 2010;113:305–314.

Wester RC, Maibach HI. Animal models for percutaneous absorption. In: Wang RGM, Knaak JB, Maibach HI, eds. *Health Risk Assessment—Dermal and Inhalation Exposure and Absorption of Toxicants*. Boca Raton, FL: CRC Press; 1993:89–103.

Williams AC, Berry BW. Penetration enhancers. *Adv Drug Deliv Rev*. 2004;56:603–618.

Wilson JT. Determinants and consequences of drug excretion in breast milk. *Drug Metab Rev*. 1983;14:619–652.

Wilson TH. *Mechanisms of Absorption*. Philadelphia, PA: WB Saunders; 1962:40–68.

Wos-Oxley M1, Bleich A, Oxley AP, et al. Comparative evaluation of establishing a human gut microbial community within rodent models. *Gut Microbes*. 2012;3(3):234–249.

Wu CY, Benet LZ. Predicting drug disposition via application of BCS: transport/absorption/ elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm Res*. 2005;22:11–23.

Wu SP, Shyu MK, Liou HH, Gau CS, Lin CJ. Interaction between anticonvulsants and human placental carnitine transporter. *Epilepsia*. 2004;45:204–210.

Xu J, Liu Y, Yang Y, Bates S, Zhang J. Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. *J Biol Chem*. 2004;279:19781–19789.

Ye AY, Liu Q-R, Li C-Y, Zhao M, Qu H. Human transporter database: comprehensive knowledge and discovery tools in the human transporter genes. *PLoS ONE*. 2014;9:e88883.

Yu J, Zhou Z, Tay-Sontheimer J, Levy RH, Ragueneau-Majlessi I. Intestinal drug interactions mediated by OATPs: a systematic review of preclinical and clinical findings. *J Pharm Sci*. 2017;106:2312–2325.

Zhang Y, Wang H, Unadkat JD, Mao Q. Breast cancer resistance protein limits fetal distribution of nitrofurantoin in the pregnant mouse. *Drug Metab Dispos*. 2007;35:2154–2158.

Zhao W, Zitzow JD, Weaver Y, et al. Organic anion transporting polypeptides contribute to the disposition of perfluoroalkyl acids in humans and rats. *Toxicol Sci*. 2017;156:84–95.

Zolk O, Fromm MF. Transporter-mediated drug uptake and efflux: important determinants of adverse drug reactions. *Clin Pharmacol Ther*. 2011;89:798–805.

chapter 6

Biotransformation of Xenobiotics

Andrew Parkinson, Brian W. Ogilvie, David B. Buckley, Faraz Kazmi and Oliver Parkinson

Introduction

Principles of Xenobiotic Biotransformation

Hydrolysis, Reduction, and Oxidation

Hydrolysis
- *Carboxylesterases*
- *Cholinesterases (AChE and BChE)*
- *Paraoxonases (Lactonases)*
- *Prodrugs and Alkaline Phosphatase*
- *Peptidases*
- *β-Glucuronidase*
- *Epoxide Hydrolases*

Reduction
- *Azo- and Nitro-Reduction*
- *Carbonyl Reduction: AKRs, SDRs, and Others*
- *Disulfide Reduction*
- *Sulfoxide and N-Oxide Reduction*
- *Quinone Reduction: NQO1 and NQO2*
- *Dihydropyrimidine Dehydrogenase*
- *Dehalogenation*
- *Dehydroxylation: mARC, Cytochrome b_5, b_5 Reductase, and Aldehyde Oxidase*
- *Aldehyde Oxidase—Reduction Reactions*

Oxidation
- *Alcohol, Aldehyde, and Ketone Oxidation–Reduction Systems*
- *Alcohol Dehydrogenase*
- *Aldehyde Dehydrogenase*
- *Dimeric Dihydrodiol Dehydrogenase*
- *Molybdenum Hydroxylases (Molybdoenzymes)*
- *Xanthine Oxidoreductase*
- *Aldehyde Oxidase*
- *Amine Oxidases*
- *Aromatization*
- *Peroxidase-Dependent Cooxidation*
- *Flavin Monooxygenases*
- *Cytochrome P450*
- *Endogenous Biomarkers of Cytochrome P450*
- *Activation of Xenobiotics by Cytochrome P450*
- *Inhibition of Cytochrome P450*
- *Induction of Cytochrome P450: Xenosensors*

Conjugation
- Glucuronidation and Formation of Acyl-CoA Thioesters
- Sulfonation
- Methylation
- Acetylation
- Amino Acid Conjugation
- Glutathione Conjugation
- Thiosulfate Sulfurtransferase (Rhodanese)
- 3-Mercaptopyruvate Sulfurtransferase
- Unusual Conjugation Reactions
- Phosphorylation

Acknowledgments

References

INTRODUCTION

The overall fate or disposition of a xenobiotic, which encompasses its absorption, distribution, metabolism, and elimination or ADME (an acronym commonly used by the pharmaceutical industry), is determined by three major factors, namely passive diffusion across biological membranes or between cells (i.e., transcellular and paracellular passive diffusion, respectively), facilitated transport by uptake and/or efflux transporters, and biotransformation (invariably called metabolism in the case of drugs). In the case of xenobiotics that permeate cells with a high rate of passive diffusion, which enables them to be absorbed from the gastrointestinal tract, lung, or skin (depending on the route of exposure), biotransformation plays a key role in their elimination. Without biotransformation, a highly permeable xenobiotic excreted in urine or bile will be reabsorbed in the kidney or intestine rather than eliminated in urine or feces. If a xenobiotic can permeate cells with a high rate of passive diffusion but it cannot be biotransformed at an appreciable rate then the xenobiotic can have a long residency time in the body. Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD) illustrates this principle. Dioxin permeates cells with a high rate of passive diffusion, but it is largely resistant to biotransformation. For this reason, dioxin and numerous other polyhalogenated aromatic hydrocarbons (PCDFs, PCBs, PBBs, etc.), industrial chemicals present in the environment, are renowned for their persistence in the body and for their bioaccumulation in the food chain. The lesson from dioxin and related compounds, namely that halogenation is not an obstacle to passive permeation but is an obstacle to biotransformation, is not lost on the pharmaceutical industry. Many drugs are fluorinated (and some are chlorinated, brominated or iodinated) at metabolically labile sites to slow their rate of biotransformation and thereby prolong their residency time in the body in order to extend their therapeutic effect, which may make once-a-day dosing at a low dose feasible (Obach et al., 2016).

In all previous versions of this chapter, biotransformation has been described as an enzymatic process of chemical modification that changes the physicochemical properties of a xenobiotic from those that favor absorption and distribution (i.e., high passive permeability associated with high lipophilicity) to those that favor elimination (i.e., low passive permeability associated with high hydrophilicity). This rationalization of biotransformation is still

valid but it overlooks an important principle, namely that facilitated transport also plays a key role in the disposition of xenobiotics, including those whose elimination is determined by their rate of biotransformation. Facilitated transport resolves a conundrum that has long been overlooked in drug metabolism circles: If biotransformation converts a lipophilic xenobiotic to hydrophilic metabolites, how do the hydrophilic metabolites get out of the cell that forms them? The short answer is: They are transported out of the cell. In many cases transporters play an important role in the elimination of drugs whose systemic clearance is primarily determined by their rate of biotransformation in the liver. The opposite is also true: In many cases biotransformation plays an important role in the elimination of drugs whose systemic clearance is primarily determined by their rate of transporter-mediated uptake into liver. Whether biotransformation (with support from transporters) or facilitated transport (with support from biotransformation) plays the key role in the elimination of a drug depends on the physicochemical properties of the xenobiotic. The following information on the importance of physicochemical properties comes largely from studies of drug disposition, but the principles apply to all xenobiotics.

Charge is an obstacle to membrane permeation by passive diffusion. There are several drugs that resemble the neurotransmitter acetylcholine, $CH_3CO–O–C_2H_4–N^+(CH_3)_3$, inasmuch as they contain one or more quaternary ammonium ions (R_1,R_2,R_3,R_4N^+) and are used to block neurotransmission by acetylcholine (nicotinic or muscarinic acetylcholine receptor antagonists such as alcuronium, atracurium, cisatracurium, doxacurium, metocurine, mivacurium, pancuronium, rocuronium, tubocurarine, and vecuronium) or inhibit the hydrolysis of acetylcholine (acetylcholinesterase inhibitors such as edrophonium, neostigmine, and pyridostigmine). All of these drugs have extracellular targets (acetylcholinesterase in the neuromuscular gap junction or acetylcholine receptors on synaptic cell surfaces) and they are all administered by intramuscular (IM) or intravenous (IV) injection because their permanent positive charge (due to the quaternary ammonium ion) is an obstacle to oral absorption from the gastrointestinal tract. (Pyridostigmine is administered orally but at 30 times the IV dose.) Xenobiotics with a permanent positive charge do not permeate membranes with a high rate of passive diffusion but they can enter cells if they are substrates for an uptake transporter. Because of its permanent positive charge, tetramethylammonium ion, $(CH_3)_4N^+$, does not permeate membranes with a high rate of passive diffusion; however, it is transported into hepatocytes and is used in vitro as a probe/index substrate for hepatic organic cation transporter-1 (OCT1).[1] Metformin, a natural product used to treat type 2 diabetes, is widely used as an in vitro probe/index substrate for OCT2, a renal organic cation transporter. Metformin does not have a single permanent positive charge, but it does have a pair of basic nitrogen atoms with high pK_a(B) values (10.3 and 12.3), each of which is >99.9% ionized at pH 7.4, which results in a miniscule percentage of drug in the nonionized state in blood.

Drugs with a permanent charge are the minority. The majority of drugs contain one or more ionizable groups, such as a carboxylic acid moiety ($R–COOH \leftrightharpoons R–COO^- + H^+$) or a basic amine ($R–NH_3^+ \leftrightharpoons R–NH_2 + H^+$). Only the nonionized form of these drugs permeates cell membranes with a high rate of passive diffusion. The ionized and nonionized (neutral) forms are in pH-dependent equilibrium. The pH producing equal amounts of the ionized and nonionized form of an ionizable group is defined as pK_a(A) in the case of acids and pK_a(B) in the case of bases. Physiological pH values range widely: pH 1.5 to 3 in the fasted stomach, pH 3 to 5 in the fed stomach, pH 5 to 8 in the small intestine, pH 4 to 5 in lysosomes, pH 5 to 6 in endosomes, pH 7.2 in cytosol, and pH 7.4 in blood (Newton and Kluza, 1978). Consequently, for compounds with ionizable groups, the ratio of ionized-to-neutral drug varies throughout the body. Studies of drug permeation across artificial phospholipid membranes (the parallel artificial membrane permeation assay or PAMPA), which are devoid of transporters, reaffirm the lesson from permanently charged drugs, namely, that permeation of membranes with a high rate of passive diffusion is a property of the nonionized form of a drug, which is favored by low pH in the case of acidic drugs and high pH in the case of basic drugs (Ruell et al., 2003).

In addition to charge, two other physicochemical properties impact the permeation of drugs by passive diffusion, namely log *P* and topological polar surface area (TPSA). log *P* is a measure of the lipophilicity of the nonionized form of a drug. log *P* is the octanol–water partition ratio (the ratio of the concentration of nonionized drug in octanol vs. the concentration of nonionized drug in water) expressed on a $\log_{10}$ scale. Incidentally, log *D*, the distribution coefficient, takes into account the concentration of ionized drug in water, which is pH dependent; hence, log *D* values for compounds with ionizable groups vary with pH, whereas log *P*, which applies only to the nonionized drug, is a constant. Permeation of membranes by passive diffusion increases with increasing log *P*. However, increasing log *P* can come at the expense of aqueous solubility; hence, drugs with a log $P > 5$ often show poor oral bioavailability because they do not dissolve in gastrointestinal fluid. This consideration generally has limited applicability to xenobiotics like dioxin where prolonged exposure to *low* concentrations, rather than once-a-day dosing to a drug at *high* concentrations, is the norm.

TPSA is an overall measure of the number of oxygen and nitrogen atoms that bind water by functioning as hydrogen donors or hydrogen acceptors. For example, dimethylamine, $(CH_3)_2NH$, has one hydrogen donor that can form a hydrogen bond with the oxygen atom of water, $(CH_3)_2NH$ ||||| OH_2, and one hydrogen acceptor (the lone pair of electrons on nitrogen) that can form a hydrogen bond with a hydrogen atom in water, $(CH_3)_2HN$: ||||| H–O–H. Trimethylamine, dimethylamine, and methylamine each has one hydrogen acceptor (the lone pair of electrons on nitrogen) and zero, one, and two hydrogen donors, respectively; their respective TPSA values are 3, 12, and 26 Å^2. Hydrogen bonding to water affects membrane permeation by passive diffusion because only the desolvated (water-free) form on the nonionized drug can permeate membranes with a high rate of passive diffusion. Accordingly, the rate of membrane permeation by passive diffusion (and the extent of oral absorption of nontransported drugs) decreases with increasing TPSA (Palm et al., 1997; Price et al., 2009; Smith and Dalvie, 2012). As a general rule, a TPSA > 75 Å^2 restricts membrane permeation by passive diffusion of acidic drugs, whereas a TPSA > 100 Å^2 restricts membrane permeation by passive diffusion of basic drugs. The difference reflects the property of phospholipid membranes, which are negatively charged. As such, basic drugs are attracted electrostatically to membranes, whereas acidic drugs are repelled. In effect, the equilibrium between the ionized and nonionized form of a drug occurs close to the membrane in the case of basic drugs and away from the membrane in the case of acidic drugs. Transporters play a key role in the disposition of drugs with a high TPSA. If any drug with a TPSA greater than 140 Å^2 is absorbed from the intestine

[1]Alternative names for the transporters mentioned throughout this chapter are as follows: ASBT (SLC10A2), BCRP (ABCG2), BSEP (ABCB11), MATE1 (SLC47A1), MATE2-K (SLC47A2), MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), MRP6 (ABCC6), MRP7 (ABCC10), MRP8 (ABCC11), NTCP (SLC10A1), OAT1 (SLC22A6), OAT3 (SLC22A8), OATP1A2 (SLCO1A2), OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OATP2B1 (SLCO2B1), OCT1 (SLC22A1), OCT2 (SLC22A2), *P*-gp (ABCB1 and MDR1), and URAT1 (SLC22A12).

and distributes into tissues, it is probably a substrate for one or more transporters (Palm et al., 1997; Ertl et al., 2000; Shitara et al., 2013).

The roles of passive diffusion, transport, and biotransformation in the disposition of acidic and basic drugs in humans (based on studies of drug pharmacokinetics) are compared and contrasted in Figs. 6-1 and 6-2, respectively. Nonsteroidal anti-inflammatory drugs (NSAIDs) are carboxylic acid-containing drugs with physicochemical properties that generally favor membrane permeation by passive diffusion (they have pK_a(A) values ranging from 2.5 to 5.0 with log *P* values >1 and, in most cases, TPSA values <75 Å^2). Likewise, antidepressant drugs are aliphatic amine–containing drugs with physicochemical properties that favor membrane permeation by passive diffusion (in most cases they have pK_a(B) values in the 8.5 to 10.5 range with log *P* values >1 and, with few exceptions, TPSA values <100 Å^2). As shown in Table 6-1, all NSAIDs have a small volume of distribution (0.1 to 0.3 L/kg, which is less than the volume of body water) and they have a short T_{max} (the time to reach maximum plasma levels after oral dosing). The low volume of distribution of NSAIDs is attributable partly to their extensive binding to albumin and other plasma proteins (>99% in the majority of cases) and partly to their extensive ionization (>99%) at pH 7.4, both of which disfavor passive diffusion into tissues. The short T_{max} of some NSAIDs is consistent with their absorption from the stomach (pH 1.5 to 3 in the fasted state) and duodenum (pH 5 to 6), two sites with low pH, which favors the nonionized form of NSAIDs and, hence, their absorption by passive diffusion. Oral absorption of NSAIDs is delayed in the fed state due, in part, to the increase in gastric pH (to pH 3 to 5) and the delay in gastric emptying time.

In contrast, as shown in Table 6-2, antidepressant drugs have a large volume of distribution (>10 L/kg, which is more than total body water) and, in most cases, a relatively long T_{max}. The large volume of distribution of antidepressants is attributable to their rapid permeation of tissues by passive diffusion and their extensive partitioning into phospholipid membranes and ion partitioning of the charged (cationic) species into acidic organelles like lysosomes (pH 4 to 5) and, to a lesser extent, endosomes (pH 5 to 6). Following their absorption from the small intestine, antidepressants and many other classes of cationic amphiphilic drugs (CADs) reach high organ-to-blood levels (>10-fold) in the liver, lung, and kidney, the organs with the highest concentration of phospholipids and lysosomes. Ion partitioning of such drugs into lysosomes (and their direct complexing with phospholipids) often impairs the lysosomal metabolism of phospholipids and mucopolysaccharides, which is why CADs are renowned for causing phospholipidosis and/or mucopolysaccharidosis. CADs (positively charged) are also renowned for adsorbing to melanin, a negatively charged pigment in the uveal gland of the eye and other pigmented cells.

Transporters, such as OATP2B1 and OATP1A2, play an important role in the intestinal absorption of acidic drugs with a high TPSA, such as statins, many of which permeate membranes with a low rate of passive diffusion (Shirasaka et al., 2010, 2011; Varma et al., 2011). They also play a key role in the intestinal absorption of certain basic drugs, such as aliskerin, atenolol, and celiprolol, and zwitterionic drugs, such as ciprofloxacin and fexofenadine—drugs whose intestinal absorption by OATPs is partially inhibited by large volumes of orange and apple

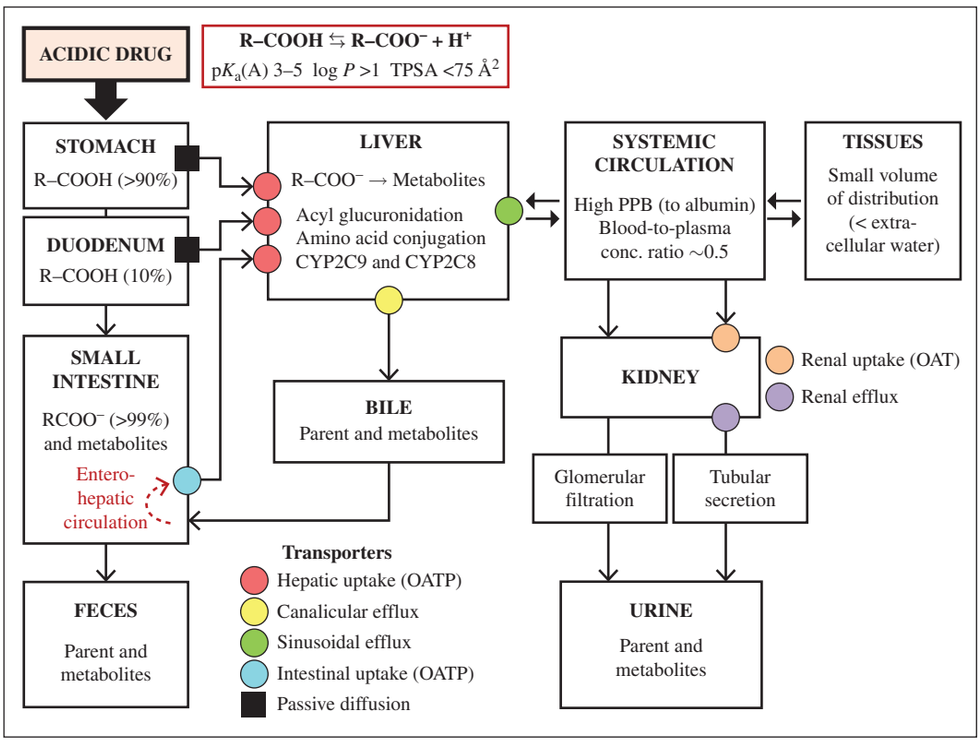


Figure 6-1. *Role of passive diffusion, transport, and biotransformation in the disposition of a lipophilic acidic xenobiotic.* OAT, organic anion transporter; OATP, organic anion transporting polypeptide; PPB, plasma protein binding; TPSA, topological polar surface area.

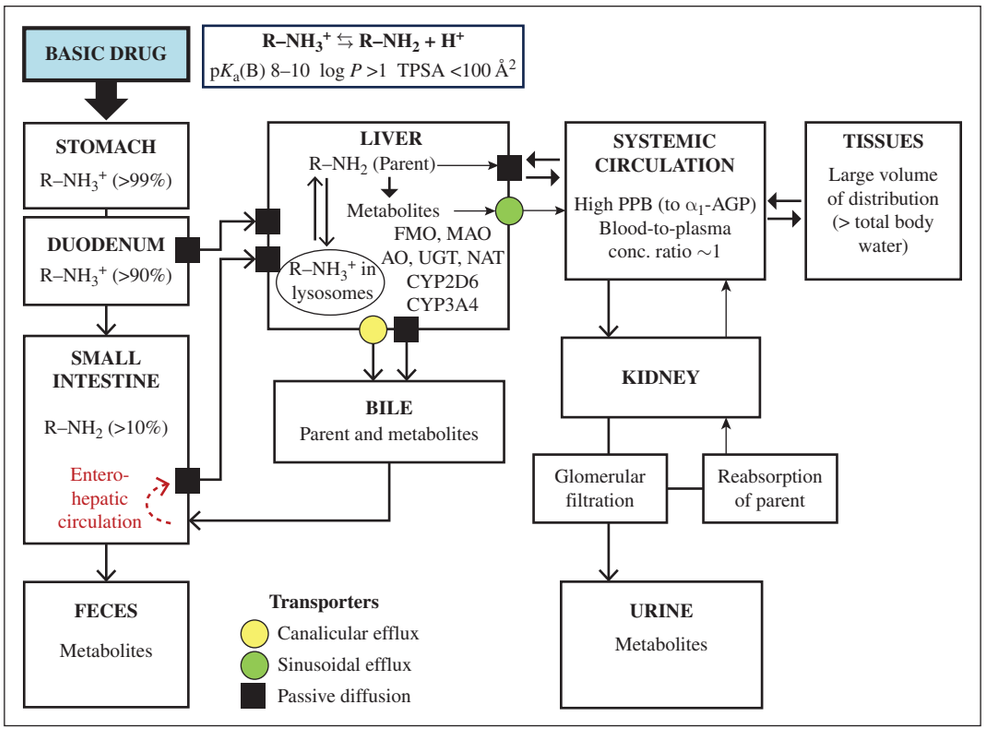


Figure 6-2. *Role of passive diffusion, transport, and biotransformation in the disposition of a lipophilic basic xenobiotic.* α1-AGP, α1-acid glycoprotein; AO, aldehyde oxidase; CYP, cytochrome P450; MAO, monoamine oxidase; NAT, *N*-acetyltransferase; PPB, plasma protein binding; TPSA, topological polar surface area; UGT, UDP-glucuronosyltransferase.

Table 6-1

Volume of Distribution (V_d) of Carboxylic Acid–Containing Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) and Plasma T_{max} in the Fasted and Fed State in Human Subjects

	PHYSICOCHEMICAL PROPERTIES*				PLASMA T_{max} (h)‡	
NSAID (R–COOH)	pK_a(A)	TPSA (Å²)	log *P*	V_d (L/kg)†	FASTED§	FED§
Diclofenac	4.0	49	4.3	0.12	0.5	1.5
Dexketoprofen	3.9	55	3.6	0.24	0.6	1.5
Ibuprofen fast-acting	4.9	40	3.8	0.15	0.7	1.4
Bromfenac	3.8	80	3.7	0.26	0.8	2.4
Suprofen	4.0	54	3.5	0.17	0.9	1.6
Naproxen sodium	4.2	47	3.0	0.10	1.3	3.2
Ibuprofen	4.9	37	3.8	0.15	1.3	2.0
Aspirin (salicylic acid)	3.0	58	2.0	0.15	1.7	2.7
Ketoprofen (racemic)	3.9	54	3.6	0.11	1.9	4.8
Naproxen	4.2	47	3.0	0.10	1.9	1.9
Diflunisal	2.7	58	3.9	0.10	2.4	3.1

**Physicochemical properties are from https://chemicalize.com. pK_a(A) is the pKa of the acidic group (the pH at which the drug is 50% ionized and 50% nonionized). TPSA stands for topological polar surface area, which is an overall measure of the number of oxygen and nitrogen atoms that bind water by functioning as hydrogen donors or hydrogen acceptors. log P is the octanol-water partition coefficient, which is $\log_{10}$ of the ratio of the concentration of nonionized drug in octanol versus the concentration of nonionized drug in water.*

†Values for the volume of distribution (V_d) are from Davies and Skjodt (2000).

‡Plasma T_{max} is the time to reach the maximum observed plasma level of drug following oral dosing. Values are from Moore et al. (2015).

§In the fasted and fed state, gastric pH is 1.5 to 3 and 3 to 5, respectively.

Table 6-2

Volume of Distribution (V_d) and Plasma T_{max} of Aliphatic Amine–Containing Antidepressant Drugs (Amphiphilic Cationic Amines or CADs) in Humans

	PHYSICOCHEMICAL PROPERTIES*				
ANTIDEPRESSANT	pK_a(B)	TPSA (Å^2)	log *P*	V_d (L/kg)	T_{max} (h)
Amitriptyline	8.7	3.2	2.9	15†	2–4†
Clomipramine	9.2	6.5	4.9	12–17†	2–4†
Citalopram	9.8	36	3.8	12–16‡	2–4§
Imipramine	9.2	6.5	4.3	10–20†	3–4†
Desipramine	10.0	15	3.9	22†	3–6†
Maprotiline	10.5	12	4.4	23–70†	3–8†
Nortriptyline	9.8	21	4.2	14–40†	3–24†
Sertraline	9.9	12	5.2	20‡	4.5–8.4§
Fluoxetine	9.8	21	4.2	14–100‡	6–8§
Paroxetine	9.8	40	3.2	13‡	6–10§

**Physicochemical properties are from https://chemicalize.com. pK_a(B) is the pK_a of the basic group (the pH at which the drug is 50% ionized and 50% nonionized). TPSA stands for topological polar surface area, which is an overall measure of the number of oxygen and nitrogen atoms that bind water by functioning as hydrogen donors or hydrogen acceptors. log P is the octanol-water partition coefficient, which is $\log_{10}$ of the ratio of the concentration of nonionized drug in octanol versus the concentration of nonionized drug in water.*

†Values from Jack (1992).

‡Values from Catterson and Preskorn (1996).

§Values from Ciraulo et al. (2011).

juice (Dolton et al., 2012). The systemic clearance of statins and other drugs with a high TPSA (including certain basic and zwitterionic drugs) is largely determined by transporter (OATP1B1 and OATP1B3)-mediated uptake into liver, followed by hepatic biotransformation or transporter-mediated efflux into bile (by P-glycoprotein [P-gp], BCRP, MRP2, BSEP, and/or MATE1) (Shitara et al., 2013). Acidic drugs that undergo biliary excretion (i.e., transporter-mediated uptake from blood coupled with transporter-mediated efflux into bile) can potentially be reabsorbed by intestinal uptake transporters, which gives rise to enterohepatic circulation. Such recycling of acidic drugs is analogous to the enterohepatic circulation of bile acids (lipophilic carboxylic acids), which are synthesized in the liver, effluxed into bile by BSEP, reabsorbed from the small intestine by ASBT (in conjunction with OSTα/β), and actively transported back into the liver by NTCP and OATP1B1 to begin the process again (Kuipers et al., 2014). Transporter (ASBT)-mediated reabsorption in the kidney similarly prevents the loss of bile acids in urine due to glomerular filtration.

Because carboxylic acid–containing drugs are extensively ionized at pH 7.4, they are predicted NOT to enter tissues with a high rate of passive diffusion, which is consistent with their low volume of distribution. However, various acidic drugs such as NSAIDs, statins, and the anticonvulsant valproic acid enter the brain due to transport by OATPs (including OATP2B2 and OATP1A2, which are also expressed in the small intestine) and/or one or more members of the family of monocarboxylate transporters (MCTs) (Sanchez-Covarrubias et al., 2014; Vijay and Morris, 2014). Uptake transporters (OATs) also play a role in the tubular secretion of NSAIDs and other acid drugs. Biotransformation might be assumed to play a negligible role in the disposition of acidic drugs but, in many cases, biotransformation plays a key role in the disposition of acidic drugs and the metabolites of acidic drugs (particularly acyl glucuronides) play a key role in the toxicity and drug interaction potential of acidic drugs (discussed in the sections "Glucuronidation and Formation of Acyl-CoA Thioesters" and "CYP2C8"). Transporters also play a key role in the disposition of zwitterionic drugs. Zwitterionic drugs are predominantly cations in the stomach, which does not favor their absorption by passive diffusion, and are both negatively and positively charged in the small intestine and blood, which disfavors intestinal absorption and tissue distribution by passive permeation. Certain zwitterionic drugs target the brain, such as the anticonvulsants gabapentin and pregabalin, which are transported across the blood–brain barrier by members of the family of large amino acid transporters (LATs).

Interestingly, many antihistamines are also lipophilic aliphatic amines (pK_a(B) > 8) that, like antidepressants, have physicochemical properties that favor membrane permeation with a high rate of passive diffusion (i.e., log *P* >1 and TPSA <100 Å^2). Some antihistamines, such as chlorpheniramine and diphenhydramine, readily enter the CNS and cause sedation, but others are prevented from entering the CNS because they are substrates for the efflux transporter P-gp (a component of the blood–brain barrier). Desloratadine and terfenadine are examples of lipophilic amines that, because they are P-gp substrates, are nonsedating antihistamines (Raeissi et al., 1999; Chen et al., 2003). Many drugs classified as neutrals contain an amine group with a pK_a(B) < 7; they are predominantly nonionized at pH 7.4. Aromatic amines and amides fall into this class. Many of them permeate membranes with a high rate of passive diffusion and biotransformation plays a key role in their elimination. However, in contrast to amine-containing drugs with a pK_a(B) > 8, amine-containing drugs with a pK_a(B) < 7 do not partition

extensively into phospholipid membranes or lysosomes; hence, they have moderate volumes of distribution (usually <5 L/kg) in contrast to the aliphatic amines classified as CADs (with volumes of distribution >10 L/kg).

These considerations apply to all xenobiotics, not just drugs. For example, dioxin permeates membranes with a high rate of passive diffusion, which is consistent with its lack of ionizable groups at physiologic pH values, and its high log *P* (>5) and low TPSA (18 Å^2). But there are exceptions (or *apparent* exceptions) especially among certain natural products. For example, the aminoglycoside antibiotics gentamicin, tobramycin, and amikacin contain four or five cationic sites, and have strongly *negative* log *P* values and very high TPSA values (200 to 330 Å^2). They are administered intravenously or intramuscularly due to their low oral bioavailability. Aminoglycosides do not diffuse through membranes but they get into various cells by a passive process, namely endocytosis: an invagination of the plasma membrane that occurs when the four or five positively charged sites on the aminoglycoside form a complex with a corresponding number of negatively charged sites on the phospholipid membrane. Some drugs, such as sildenafil (Viagra), permeate membranes with a higher rate of passive diffusion than would be predicted from their TPSA value, but this is due to *internal* hydrogen bonding, which effectively reduces TPSA, a measure of *external* hydrogen bonding to water. In the case of sildenafil, internal hydrogen bonding reduces its predicted TPSA by 20 Å^2 (from 118 to 98 Å^2), which enables sildenafil to be completely absorbed from the intestine. However, oral bioavailability of *parent* drug is less than 100% due to presystemic biotransformation of sildenafil in the small intestine and liver.

The final point in the preceding paragraph warrants further consideration because several factors, including biotransformation, can contribute to an absolute oral bioavailability of less than 100%. Absolute oral bioavailability is usually determined by comparing systemic exposure to parent drug following oral administration (AUC_{po}) and intravenous (IV) administration (AUC_{iv}) with adjustment for dose (if necessary):

$$\text{Absolute oral bioavailability}(\%) = 100 \cdot \frac{AUC_{po} \cdot Dose_{iv}}{AUC_{iv} \cdot Dose_{po}}$$

where AUC is the area under the plasma concentration versus time curve, a measure of systemic exposure. Absolute oral bioavailability is a pharmacokinetic term that applies only to the *parent* drug. There are several reasons why absolute oral bioavailability may be less than 100%:

1. The drug may be partially or completely insoluble in the gastrointestinal tract at the dose administered orally. In this case, absorption of both parent drug and drug-derived material (parent + metabolites) is less than 100%. In this case, oral absorption may decrease with increasing dose (due to precipitation of the drug at high doses), which gives rise to an *infra*-proportional dose–exposure relationship.
2. The drug may permeate membranes or pass between cells with a low rate of passive diffusion and the drug is not efficiently transported by an intestinal uptake transporter. Once again, oral absorption of both parent drug and drug-derived material is less than 100%. The intestine is the therapeutic target for some drugs, such as linaclotide, an agonist of gluanylate cyclase 2 (expressed on the cell surface of intestinal epithelial cells) and is used to treat constipation and irritable bowel syndrome. Linaclotide has a very low log *P* (close to −6) and very high TPSA (726 Å^2)—properties that strongly disfavor passive permeation of membranes—and is not absorbed from the gastrointestinal tract.
3. The drug may permeate membranes with a high rate of passive diffusion or be efficiently transported by an intestinal uptake transporter, but it is biotransformed in the small intestine, liver or, in principle, the lung (three presystemic organs). In this case, oral absorption of parent drug is less than 100%, but oral absorption of drug-derived material (parent + metabolites) is 100% (all of the drug is absorbed but the parent drug undergoes presystemic biotransformation). Sildenafil is one of a large number of drugs that are completely absorbed from the intestine but undergoes significant presystemic (or first-pass) metabolism.
4. The drug may permeate membranes with a high rate of passive diffusion or is a substrate for an intestinal uptake transporter, but its intestinal absorption is restricted by one or more intestinal efflux transporters, such as P-gp. For highly soluble, highly permeable drugs, efflux by intestinal transporters can be saturated at high doses, which gives rise to a *supra*-proportional dose–exposure relationship.

In the case of drugs that are substrates for both the intestinal efflux transporter P-gp and the drug-metabolizing enzyme CYP3A (the major cytochrome P450 enzyme in the intestine and a major enzyme in the liver), oral absorption of *parent* drug can be especially reduced because efflux by P-gp slows the rate of intestinal absorption, which increases the extent of metabolism by intestinal CYP3A (Raeissi et al., 1999; Benet, 2009). This transporter–enzyme interplay underscores the importance of BOTH transporters and drug-metabolizing enzymes to the overall disposition of certain drugs and other xenobiotics.

Extrinsic factors that impact the oral bioavailability of drugs include (1) aqueous solubility, which is affected by pH, food, bile acids, and drug formulation); (2) uptake by intestinal transporters, which are inhibited by compounds in apple and orange juice, and uptake by hepatic transporters, which are inhibited by drugs such as rifampin); (3) efflux by intestinal transporters such as P-gp, which can be inhibited by some drugs and induced by others; and (4) biotransformation by intestinal enzymes such as CYP3A, which is inhibited by compounds in grapefruit juice, and hepatic enzymes, many of which are inhibited by some drugs and induced by others. In addition to drugs that cause inhibition or induction, genetic polymorphism (an intrinsic factor) can also affect the activity of transporters and drug-metabolizing enzymes. For a growing number of transporters and drug-metabolizing enzymes, genetic polymorphisms have been identified that decrease their activity (loss-of-function allelic variants or gene deletion) or increase it (gain-of-function allelic variants or gene duplication).

Biotransformation has traditionally been divided into two phases. Phase 1 biotransformation involves oxidation, reduction, and hydrolysis, which result in the introduction or exposure of functional groups such as –OH, –NH_2, –SH, or –COOH. Phase 2 biotransformation involves conjugation of the xenobiotic or its Phase 1 metabolite(s) with an acid (glucuronic acid or sulfonic acid) or zwitterion (glutathione or amino acids such as glycine, taurine, and glutamine). Phase 1 biotransformation facilitates the elimination of xenobiotics by increasing their TPSA and lowering their log *P*, both of which decrease membrane permeation by passive diffusion and decrease the likelihood of the excreted metabolites being

Table 6-3

Effects of Common Phase 1 and Phase 2 Biotransformation Reactions on log *P* and Topological Polar Surface Area (TPSA) of Xenobiotics

REACTION	EXAMPLE	CHANGE in log *P**	CHANGE in TPSA (Å²)*
Hydroxylation—aliphatic	$R–CH_3 \rightarrow R–CH_2OH$	−1.5	20
Hydroxylation—aromatic	Benzene (Ø) → phenol (Ø-OH)	−0.3	20
Alcohol dehydrogenation	$R–CH_2OH \rightarrow R–CHO$	−0.2	−3
Aldehyde oxidation	$R–CHO \rightarrow R–COOH$	+0.2	20
Epoxidation	$R–HC{=}CH_2 \rightarrow R–HC(O)CH_2$	−1.1	13
O-demethylation	$R–CH_2O–CH_3 \rightarrow R–CH_2OH$	−0.6	11
N-demethylation	$R–CH_2NH–CH_3 \rightarrow R–CH_2NH_2$	−0.4	14
N-oxygenation	$R_1,R_2,R_3N \rightarrow R_1,R_2,R_3N–O$	−1.1	24
N-hydroxylation	$R_1,R_2NH \rightarrow R_1,R_2N–OH$	−0.4	11
Hydrolysis	$R_1CO–OR_2 \rightarrow R_1COOH + R_2OH$	−0.1 −0.4	11 2
O-methylation	Catechol → *O*-methylcatechol	+0.6	−6
N-acetylation	$R–NH_2 \rightarrow R–NH–COCH_3$	−0.2	3
O-sulfonation	Phenol → phenol sulfate	−0.5	43
O-glucuronidation	R–OH → R–O–glucuronide	−1.5	96
N-glucuronidation	$R–NH_2$ → R–NH–glucuronide	−1.8	93
Acyl-glucuronidation	R–COOH → R–COO–glucuronide	−1.8	96
Glutathionylation (addition of glutathione)	Diethyl maleate → DEA-glutathione	−3.5	159
Glycine conjugation	Benzoic acid → hippuric acid Cholic acid → glycocholic acid	−1.1	29
Taurine conjugation	Cholic acid → taurocholic acid	−1.6	46

**Changes in log P and TPSA were determined from https://chemicalize.com.*

reabsorbed from the small intestine or kidney. Phase 1 biotransformation also produces metabolites that potentially undergo Phase 2 biotransformation. Phase 2 biotransformation facilitates the elimination of xenobiotics by adding an ionizable group (often an acidic moiety that is predominantly [>99%] negatively charged above pH 7) that is associated with relatively large increases in TPSA and decreases in log *P*. Phase 1 biotransformation increases TPSA by up to 24 Å^2 and decreases log *P* by up to 1.5 units. Phase 2 biotransformation increases TPSA by as much as about 160 Å^2 and decreases log *P* by as much as 3.5 units. The effects of common pathways of Phase 1 and Phase 2 biotransformation on TPSA and log *P* are summarized in Table 6-3.

In general, the physicochemical properties of metabolites formed by Phase 1 biotransformation are similar to those of the parent compound because Phase 1 biotransformation results in relatively modest changes in TPSA and log *P* (Table 6-3). In the case of drugs with high passive permeability, their Phase 1 metabolites (formed in the small intestine or liver) are often present in the systemic circulation (Smith and Dalvie, 2012). In fact several drugs on the market today are pharmacologically active Phase 1 metabolites of previously approved drugs (such as desloratadine, the *N*-dealkylated metabolite of loratadine). In contrast, conjugated metabolites formed by Phase 2 biotransformation do not permeate membranes with a high rate of passive diffusion. Their elimination from the site of formation is mediated by transporters. In the case of Phase 2 metabolites formed in the liver, the conjugates can be effluxed into bile by transporters on the canalicular membrane (such as MRP2 and BCRP) or effluxed into blood (with subsequent elimination in urine) by transporters on the sinusoidal membrane (such as MRP1, MRP3, MRP4, MRP5, and MRP6) (Zamek-Gliszczynski et al., 2006). The efflux of Phase 2 metabolites into bile versus blood (the so-called vectorial route of elimination) can occur in a species-dependent manner. In the same species, treatment with drugs that induce the expression of transporters can affect the vectorial route of elimination of Phase 2 metabolites (Slitt et al., 2003). Accordingly, Phase 2 metabolites of drugs can sometimes be observed as circulating metabolites in humans but not rodents (or vice versa) even though humans and rodents form the same Phase 2 metabolite.

Conjugations with glucuronic acid, sulfonic acid, glutathione, and various amino acids are not the only Phase 2 biotransformation reactions. Acetylation and methylation are Phase 2 biotransformation reactions but with a notable difference: they do not add an ionizable group to the xenobiotic. In contrast to all other pathways of Phase 2 biotransformation, methylation actually decreases TPSA and increases log *P* (Table 6-3), which can slow the overall rate of xenobiotic elimination from the body. Acetylation of a basic amine ($R–NH_2 \rightarrow R–NH–CO–CH_3$) has a modest effect on TPSA and log *P* (Table 6-3), but it can greatly lower the $pK_a(B)$ of the nitrogen atom from one that favors partitioning into phospholipid membranes and lysosomes ($pK_a(B) > 8$) to one that does not support such partitioning ($pK_a(B) << 7$). In this case, acetylation can decrease the volume of distribution and thereby promote xenobiotic elimination.

By facilitating the elimination of Phase 2 metabolites in bile or urine (via blood), transporters play an important role in the elimination of xenobiotics that permeate membranes with a high rate of passive diffusion, although the rate of metabolism generally determines the overall rate of elimination of the xenobiotic (i.e., metabolism represents the rate-limiting step in elimination). As noted above, the interplay between intestinal efflux transporters like P-gp and intestinal drug-metabolizing enzymes like CYP3A can increase the extent of metabolism and decrease oral absorption of highly permeable drugs and other xenobiotics. What about xenobiotics that permeate membranes with a low rate of passive diffusion, such as drugs with a high TPSA, but which are absorbed, distributed, and eliminated because they are substrates for transporters? It might be assumed that drugs whose systemic clearance depends on facilitated transport (i.e., drugs for which transporter-mediated uptake into liver or kidney represents the rate-limiting step in systemic clearance) are slowly biotransformed if, in fact, they are biotransformed at all. It seems reasonable to assume that drug-metabolizing enzymes would have evolved NOT to metabolize low permeability xenobiotics because such compounds already have a high TPSA and/or low log *P*, so there would be little advantage to increasing it further. It is perhaps surprising, therefore, that several drugs whose permeation into liver is dependent on uptake transporters (namely, OATP1B1 and OATP1B3) are among the most rapidly metabolized drugs by the human liver microsomal cytochrome P450 (Phase 1 biotransformation), as shown in Fig. 6-3.

This feature may have evolved for the following reason. In the case of xenobiotics that permeate membranes with a high rate of passive diffusion, the *unbound* concentration of xenobiotic inside the cell cannot be greater than the *unbound* concentration outside the cells because they are in rapid equilibrium. However, in the case of xenobiotics that do not permeate cells rapidly by passive diffusion but are actively transported into cells (such as cells in the small intestine and liver), the *unbound* intracellular concentration can be much greater than the *unbound* extracellular concentration. (Emphasis is placed on the *unbound* concentration because the *total* intracellular concentration of a xenobiotic, including those that permeate membranes with a high rate of passive diffusion, is usually much higher than its total extracellular concentration because xenobiotics inside the cell partition into phospholipid membranes and organelles and/or bind extensively to various proteins and other cellular constituents.) A xenobiotic that is transported into the small intestine or liver can be eliminated either by transporter-mediated efflux or by biotransformation. Many of the drugs that are transported into the liver, such as the statins, are indeed effluxed into bile by canalicular transporters. But high rates of biotransformation of xenobiotics that are substrates for uptake transporters may have evolved as a second mechanism to protect cells from high and potentially toxic intracellular levels of xenobiotics that are transported into the liver. When xenobiotics with low passive permeability are transported into hepatocytes, it seems reasonable to assume that their unbound intracellular concentration would be *higher* than its unbound extracellular concentration. But several drugs with these properties are metabolized so quickly (in addition to being effluxed into bile) that their intracellular unbound concentration is actually *lower* than their unbound extracellular concentration.

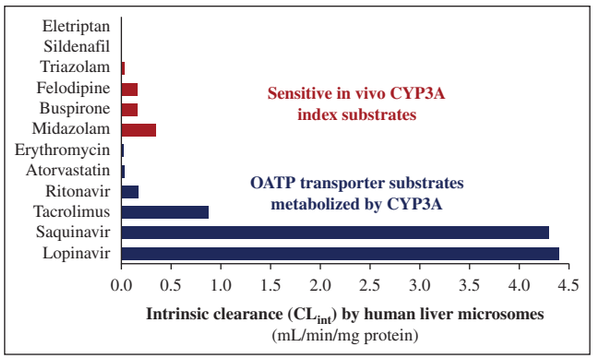


Figure 6-3. *Intrinsic metabolic clearance by human liver microsomes of drugs that are sensitive in vivo CYP3A probe substrates and OATP substrates (drugs whose uptake into hepatocytes is largely dependent on facilitated transport, not passive diffusion).* Intrinsic clearance values (determined with human liver microsomes) are from Evans et al. (2003) for eletriptan, from Hyland et al. (2001) for sildenafil, from De Bruyn et al. (2016) for lopinavir, saquinavir, and ritonavir, from Park et al. (2008) for atorvastatin, and from Tseng et al. (2014) for all other substrates. Sensitive in vivo CYP3A probes are drugs whose plasma AUC increase fivefold or more when coadministered with a strong CYP3A inhibitor. A complete list of sensitive CYP3A probe substrates (and strong CYP3A inhibitors and inducers) can be found at the University of Washington Drug Interaction Database (https://www.druginteractioninfo.org).

CYP3A enzymes are often involved in the biotransformation of drugs that are substrates for the efflux transporter P-gp or are substrates for the hepatic OATP uptake transporters. As noted above, the interplay between intestinal P-gp and intestinal CYP3A increases the extent of biotransformation of various drugs during their intestinal absorption and, as shown in Fig. 6-3, some of the drugs transported into the liver by OAPT1B1/3 are rapidly metabolized by hepatic CYP3A enzymes. CYP3A is unusual for its ability to metabolize high-molecular-weight xenobiotics with a high TPSA, but it is not alone. Many acidic drugs with a high molecular weight and high TPSA (including glucuronide conjugates of drugs) are substrates for CYP2C8. CYP2C9 also metabolizes numerous acidic drugs but with a preference for low-molecular-weight compounds. Humans and cynomolgus monkeys evolved CYP2C enzymes capable of biotransforming acidic drugs. In contrast, dogs are renowned for expressing CYP enzymes that, in general, do not biotransform acidic drugs. Rats and mice (the other two non-clinical species widely used in drug safety and toxicological testing) are intermediate in their ability to biotransform acidic drugs by CYP enzymes. It is difficult to know what evolutionary pressures led to these species differences in CYP-dependent biotransformation of acidic compounds, but gastric pH offers a potential insight. Humans and cynomolgus monkeys have low gastric pH (pH 1.5 and 2.1, respectively) compared with mice, rats, and dogs (pH 3.8, 4.4, and 4.5, respectively); hence, humans and cynomolgus monkeys are more likely to absorb acidic xenobiotics by passive diffusion

from the stomach than many other species (Beasley et al., 2015). It is conceivable that species differences in gastric pH provided an evolutionary pressure for the selection of acid-metabolizing CYP enzymes in humans and cynomolgus monkeys. Species differences in gastric pH are partly responsible for species differences in the composition of the intestinal microbiome (Beasley et al., 2015), which in turn influences the biotransformation of certain xenobiotics and bile acids (Klaassen and Cui, 2015).

In summary, the overall fate or disposition of a xenobiotic, which encompasses its absorption, distribution, metabolism, and elimination (ADME), is determined by three major factors, namely passive diffusion, facilitated transport, and biotransformation (metabolism). In most cases, all three processes are involved. Xenobiotics that rapidly permeate membranes by passive diffusion are biotransformed inside cells. Such xenobiotics do not require uptake transporters to enter cells, but they require efflux transporters to facilitate the elimination of certain metabolites, notably those formed by Phase 2 biotransformation reactions (except methylation and acetylation). Xenobiotics with low passive permeability that are transported into cells, resulting in potentially high intracellular concentrations, can be eliminated by efflux transporters, but in several cases they are rapidly biotransformed to lower their intracellular concentration and further facilitate their elimination by efflux transporters. A few xenobiotics permeate membranes with a high rate of passive diffusion but they are neither substrates for Phase 1 or Phase 2 biotransforming enzymes nor substrates for uptake and efflux transporters. Such xenobiotics will be absorbed quickly but eliminated slowly, as is the case with dioxin and other persistent polyhalogenated aromatic hydrocarbons.

This chapter describes some fundamental principles of xenobiotic biotransformation, and describes the major enzyme systems involved in the biotransformation (or metabolism) of drugs and other xenobiotics. The examples given are biased toward drugs and human enzyme systems for two reasons. First, many of the fundamental principles of xenobiotic biotransformation stem from such studies. This is especially true of drugs with a narrow therapeutic index (where the toxic dose is not much greater than the therapeutic dose), which have revealed a large number of genetic and environmental factors that affect xenobiotic biotransformation and, hence, drug toxicity. Second, adverse drug events (ADEs) are one of the leading causes of death in the United States. Lazarou et al. (1998) estimated that in 1994 over 2 million hospitalized patients had serious ADEs (also known as adverse drug reactions or ADRs) and 106,000 had fatal outcomes, placing ADEs only behind heart disease, cancer, stroke, and pulmonary disease as a leading cause of death. Of the 548 new chemical entities approved by the Food and Drug Administration (FDA) between 1974 and 1999, about 10% (56 drugs) required black box warnings or were withdrawn from the market because of ADEs that included hepatotoxicity, cardiotoxicity (several as a result of drug–drug interactions), myelotoxicity, immunotoxicity, and warnings to pregnant women (Lasser et al., 2002; Thomas, 2002). Between 1998 and 2005 the number of serious ADEs reported voluntarily to the FDA through the Adverse Events Reporting System (AERS), known as MedWatch reports, increased 2.6-fold (from 34,966 in 1998 to 89,842 in 2005) and the number of ADE-related fatalities increased 2.7-fold (from 5519 in 1998 to 15,107 in 2005) (Moore et al., 2007). These increases in ADE incidence were four times greater than the increase in the total number of outpatient prescriptions written during the same period. In many cases, xenobiotic biotransformation is central to understanding these ADEs.

PRINCIPLES OF XENOBIOTIC BIOTRANSFORMATION

It is difficult to make categorical statements about xenobiotic biotransformation because there is an exception to every rule. Nevertheless, the following points, which might be considered principles or rules, apply in the majority of cases:

Point 1 Xenobiotic biotransformation or drug metabolism is the process of converting lipophilic (fat-soluble) chemicals, which are readily absorbed from the gastrointestinal tract and other sites, into hydrophilic (water-soluble) chemicals, which are readily excreted in urine or bile. There are exceptions even to this most basic rule. For example, acetylation and methylation are biotransformation reactions that result in a nominal increase or a small decrease in the water solubility of xenobiotics, as shown in Table 6-3. As a general rule, xenobiotics with a log $D_{7.4} > 0$ require biotransformation to facilitate their elimination (Williams et al., 2003a).

Point 2 The biotransformation of xenobiotics is catalyzed by various enzyme systems that can be divided into four categories based on the reaction they catalyze:

1. Hydrolysis (e.g., carboxylesterase)
2. Reduction (e.g., carbonyl reductase)
3. Oxidation (e.g., cytochrome P450 [CYP])
4. Conjugation (e.g., UDP-glucuronosyltransferase [UGT])

The mammalian enzymes involved in the hydrolysis, reduction, oxidation, and conjugation of xenobiotics are listed in Table 6-4, together with their principal subcellular location. The conjugation reactions include glucuronidation, sulfonation (often called sulfation), acetylation, methylation, conjugation with glutathione (GSH; mercapturic acid synthesis), and conjugation with amino acids (such as glycine, taurine, and glutamine). Examples of the major chemical groups that undergo biotransformation together with the enzymes that commonly mediate their biotransformation are given in Table 6-5 (Williams et al., 2003a; Testa and Krämer, 2008, 2010).

Xenobiotic biotransformation is generally catalyzed by enzymes, but there are exceptions. For example, hydrolysis of certain carboxylic and phosphoric acid esters, reduction of sulfoxides to sulfides (e.g., rabeprazole), isomerization involving enol–keto tautomerization (e.g., thalidomide), and the conjugation of certain xenobiotics with GSH can occur nonenzymatically at an appreciable rate. Certain reactions are catalyzed by gastric acid, such as the hydrolysis of esters and the conversion of indole-3-carbinol to a dimer that is a potent agonist of the aryl hydrocarbon receptor (AhR) and, consequently, an inducer of various enzymes including certain CYP enzymes (CYP1A1, CYP1A2, CYP1B1, and CYP2S1) (Grose and Bjeldanes, 1992).

Point 3 In general, individual xenobiotic-biotransforming enzymes are located in a single organelle. In Table 6-4, some enzymes are listed with two or more subcellular locations. However, in such cases, the enzyme name generally refers to two or more enzymes, each with its own distinct subcellular location. For example, the epoxide hydrolase located in microsomes is the same enzyme located in the plasma membrane but is a different enzyme from the epoxide hydrolase located in cytosol (i.e., they are distinct gene products). From a practical perspective, it is noteworthy that during the homogenization of tissue and the preparation of subcellular fractions, a certain degree of cross-contamination of organelles occurs. For example, microsomes contain detectable levels of monoamine oxidase (MAO) and other outer mitochondrial enzymes, such as the mitochondrial amidoxime-reducing component (mARC) due

Table 6-4

General Pathways of Xenobiotic Biotransformation and Their Major Subcellular Location

REACTION	ENZYME OR SPECIFIC REACTION	LOCALIZATION
Hydrolysis	Carboxylesterase	Microsomes, cytosol, lysosomes, blood
	Butyrylcholinesterase	Plasma, most tissues
	Acetylcholinesterase	Erythrocytes, most tissues
	Paraoxonases	Plasma, microsomes, inner mitochondrial membrane
	Alkaline phosphatase	Plasma membrane
	Peptidase	Blood, lysosomes
	β-Glucuronidase	Microsomes, lysosomes, microflora
	Epoxide hydrolase	Microsomes, plasma membrane, cytosol
Reduction	Azo- and nitro-reduction	Microflora
	Carbonyl (aldo-keto) reduction	Cytosol, microsomes, blood
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Quinone reduction	Cytosol, microsomes
	Dihydropyrimidine dehydrogenase	Cytosol
	Reductive dehalogenation	Microsomes
	Dehydroxylation (mARC)	Mitochondria
	Dehydroxylation (aldehyde oxidase)	Cytosol
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidoreductase	Cytosol
	Class I Amine Oxidases	
	MAO-A and B	Outer mitochondrial membrane, platelets
	PAO	Cytosol, peroxisomes, plasma
	SMOX	Cytosol, nucleus
	Class II Amine Oxidases (CuAOs)	
	SSAOs (e.g., AOC3)	Cytosolic, membrane-associated forms
	DAOs	Microsomes, extracellular matrix
	LOs	Extracellular matrix
	Peroxidases	Microsomes, lysosomes, saliva
	Flavin-monooxygenases	Microsomes
	Cytochrome P450	Microsomes, mitochondria
Conjugation	UDP-glucuronosyltransferase	Microsomes
	Acyl-CoA synthetase	Mitochondria
	Sulfotransferase	Cytosol
	Glutathione transferase	Cytosol, microsomes, mitochondria
	Amino acid transferase	Mitochondria, microsomes
	N-acetyltransferase	Mitochondria, cytosol
	Methyltransferase	Cytosol, microsomes, blood

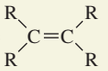
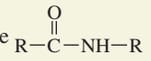
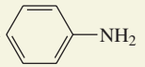
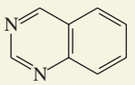
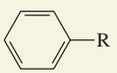
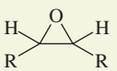
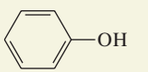
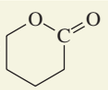
Abbreviation: mARC, mitochondrial amidoxime-reducing complex.

to their contamination with the outer mitochondrial membrane. In contrast, some xenobiotic-biotransforming enzymes are present in two or more subcellular locations. Some CYP enzymes, such as CYP2E1, are present in microsomes and the inner mitochondrial membrane, which has implications for the site of xenobiotic toxicity, as discussed in the section "CYP2E1."

Point 4 In general, xenobiotic biotransformation is accomplished by a limited number of enzymes with broad substrate specificities. In humans, for example, two CYP enzymes—namely, CYP2D6 and CYP3A4—metabolize 40% of drugs ($n = 860$) and 21% of natural products/endobiotics ($n = 331$ compounds) (Rendic and Guengerich, 2015). The broad and sometimes overlapping substrate specificities of xenobiotic-biotransforming enzymes preclude the possibility of naming the individual enzymes after the reactions they catalyze (which is how most other enzymes are named). Many of the enzymes involved in xenobiotic biotransformation are arranged in families and subfamilies and named according to nomenclature systems based on the primary amino acid sequence of the individual enzymes. CYP2D6, for example, is in CYP family 2, subfamily D, and gene number 6. Some enzymes are given the same name across all mammalian species, whereas others are named in a species-specific manner. For example, some CYP enzymes, such as CYP1A1, CYP1A2, and CYP1B1, are so named in all mammalian species, whereas CYP enzymes in the CYP2, CYP3, and CYP4 families (with certain exceptions such as CYP2E1) are named in a species-specific manner. The convention of using italic and regular

Table 6-5

Common Chemical Groups and Enzymes Possibly Involved in Their Metabolism

CHEMICAL GROUP	ENZYME(S)	REACTION(S)	CHEMICAL GROUP	ENZYME(S)	REACTION(S)
Alkane $R-CH_2-R$	CYP	Hydroxylation, dehydrogenation	Aldehyde	CYP, ALDH, aldehyde oxidase	Oxidative de-formylation, oxidation to carboxylic acid
Alkene 	CYP, GST	Epoxidation, glutathionylation	Aliphatic amide 	Amidase (esterase)	Hydrolysis
Alkyne $R-C\equiv C-R$	CYP	Oxidation to ketocarbenes and carboxylic acid	Aniline 	CYP, NAT, UGT, peroxidase, SULT	*N*-hydroxylation, *N*-acetylation, *N*-glucuronidation, *N*-oxidation, *N*-sulfonation
Aliphatic alcohol $R-CH_2-OH$	CYP, ADH, catalase, UGT, SULT	Oxidation, glucuronidation and sulfonation	Aromatic azaheterocycles 	UGT, CYP, aldehyde oxidase	*N*-glucuronidation, hydroxylation, *N*-oxidation, ring cleavage, oxidation
Aliphatic amine $R-NH_2$	CYP, FMO, MAO, UGT, SULT, MT, NAT, peroxidase	*N*-dealkylation, *N*-oxidation, deamination, *N*-glucuronidation, *N*-carbamoyl glucuronidation, *N*-sulfonation, *N*-methylation, *N*-acetylation	Carbamate $R\text{-}NH\text{-}C(=O)\text{-}O\text{-}R$	CYP, esterase	Oxidative cleavage, hydrolysis
Amidine $HN{=}CR{-}NH_2$	CYP	*N*-oxidation	Ester $R-CH_2\text{-}O\text{-}C(=O)\text{-}R$	CYP, esterase	Oxidative cleavage, hydrolysis
Arene 	CYP	Hydroxylation and epoxidation	Ether $R-CH_2\text{-}O\text{-}CH_2\text{-}R$	CYP	*O*-dealkylation
Carboxylic acid $R-COOH$	UGT, amino acid transferases, acyl-CoA synthetase	Glucuronidation, amino acylation, Coenzyme A thioesterification	Ketone $R-CH_2-C(=O)-R$	CYP, FMO, SDR, AKR	Baeyer–Villiger oxidation, reduction
Epoxide 	Epoxide hydrolase, GST	Hydrolysis, glutathionylation	Phenol 	CYP, UGT, SULT, MT	Ipso-substitution, glucuronidation, sulfonation, methylation
Lactone 	Lactonase (paraoxonase)	Hydrolysis (ring opening)	Thioether $R-CH_2\text{-}S-CH_2\text{-}R$	CYP, FMO	*S*-dealkylation, *S*-oxidation

Abbreviations: ADH, alcohol dehydrogenase; AKR, aldo-keto reductases; ALDH, aldehyde dehydrogenase; FMO, flavin monooxygenase; GST, glutathione S-transferase; MT, methyltransferase; SDR, short-chain dehydrogenases/reductases; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

SOURCE: Data from Williams et al. (2003a).

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letters to distinguish between the gene and gene products (mRNA and protein), respectively, and the convention of using lower case letters to designate mouse genes and gene products will not be followed in this chapter.

The structure (i.e., amino acid sequence) of a given xenobiotic-biotransforming enzyme may differ among individuals, which can give rise to differences in rates of drug metabolism. In general, a variant form of a xenobiotic-biotransforming enzyme (known as an *allelic variant* or *allelozyme*) has diminished enzymatic activity or level of expression compared with that of the wild-type enzyme, although this is not always the case (see the sections "Alcohol Dehydrogenase," "Aldehyde Oxidase," "Cholinesterases (AChE and BChE)," "Carboxylesterases," "Epoxide Hydrolases," "Flavin Monooxygenases," "Glutathione Conjugation," "Paraoxonases (Lactonases)," "Sulfonation," "CYP1A1, CYP1A2, and CYP1B1," "CYP2C8," "CYP2C19," and "CYP3A5"). The impact of amino acid substitution(s) on the catalytic activity of a xenobiotic-biotransforming enzyme may be substrate-dependent, such that an allelic variant may interact normally with some substrates (and inhibitors) but interact atypically with others.

The broad substrate specificity of xenobiotic-biotransforming enzymes makes them catalytically versatile but slow compared with most other enzymes (with the exception of hydrolytic reactions). The sequential oxidation, conjugation, and transport of a xenobiotic tend to proceed quicker at each subsequent step, which prevents the accumulation of intracellular metabolites.

Point 5 Hydrolysis, reduction, and oxidation expose or introduce a functional group (such as −OH, $-NH_2$, −SH, or −COOH) that can be converted to a water-soluble conjugate (see Table 6-5). For subsequent conjugation, the functional group introduced or exposed by hydrolysis, reduction, or oxidation must be nucleophilic (in the case of glucuronidation, sulfonation, methylation, acetylation, and conjugation with glycine or taurine) or electrophilic (in the case of glutathionylation). The first three reactions (hydrolysis, reduction, and oxidation) are called Phase 1 reactions, and the conjugation reactions are called Phase 2 reactions. These terms were originally coined to distinguish biotransformation reactions that could potentially increase the toxicity of a xenobiotic (by forming a reactive metabolite), which was attributed to Phase 1 biotransformation, from those that resulted in only detoxication, which was attributed to Phase 2 biotransformation (Williams, 1959). However, on a case-by-case basis, ALL biotransformation reactions are capable of increasing the toxicity of xenobiotics. Formation of hepatotoxic acyl glucuronides of carboxylic acid–containing drugs such as NSAIDs involves conjugation with glucuronic acid (a Phase 2 biotransformation reaction). In the United States, NSAIDs and other carboxylic acid–containing drugs represent the largest category of drugs whose clinical development was halted or whose regulatory approval was subsequently withdrawn because of liver toxicity in humans (Fung et al., 2001). Although Phase 1 biotransformation often precedes Phase 2 metabolism, some drugs, such as acetaminophen (Tylenol), are conjugated directly (by glucuronidation and sulfonation), whereas other drugs, such as the lipid-lowering drug gemfibrozil and the NSAID diclofenac, undergo Phase 1 metabolism (by CYP [CYP2C8]) *after* Phase 2 metabolism (glucuronidation) (Ogilvie et al., 2006; Tornio et al., 2014).

Point 6 Oxidation, reduction, hydrolysis, methylation, and acetylation generally cause a modest increase (or a small decrease in the case of methylation) in the water solubility of a xenobiotic, whereas glucuronidation, sulfonation, glutathionylation, and amino acid conjugation generally cause a marked increase in hydrophilicity, as shown in Table 6-3. The impact of biotransformation on the physiochemical properties and disposition of xenobiotics and their metabolites has been discussed in the section "Introduction." For a drug to be absorbed by passive diffusion in the gut (so that it can be orally effective and distribute throughout the body) it must generally have a log *P* of 0 to 5 and a calculated total polar surface area (TPSA) less than 100 Å^2 (Smith and Dalvie, 2012). Water-soluble drugs (those with a negative log *P*) might be orally active if their absorption is mediated by an intestinal drug transporter. Extremely lipophilic drugs (log $P > 5$) tend to be too insoluble or they dissolve too slowly to be well absorbed from the intestine unless the dose is particularly low. Drugs must undergo desolvation (i.e., they must dissociate from water molecules) to penetrate the lipid bilayer of cells. Drugs begin to lose their lipoidal permeability when their TPSA increases above about 75 Å^2 and lose it entirely at a TPSA of about 140 Å^2. When drugs meet the aforementioned criteria (log $P = 0-5$, TPSA < 100 Å^2), their metabolites formed by oxidation, reduction, hydrolysis, methylation, or acetylation also tend to meet these same criteria; hence, the metabolites formed by these reactions, such as the parent drug, readily diffuse across biological membranes down a concentration gradient (unless their diffusion into tissues such as the brain is restricted by efflux transporters). Metabolites formed by conjugation reactions (other than methylation and acetylation) contain a negatively charged glucuronide, sulfate, or amino acid (glycine or taurine) moiety (or a zwitterionic GSH moiety) and consequently have a much lower log *P* and a much greater TPSA than the parent drug, so much so that they cannot readily diffuse passively across biological membranes. The elimination of such conjugates from the liver (where they are largely formed) requires active transport across the canalicular (apical) membrane so that they can be eliminated in bile or across the sinusoidal (basolateral) membrane, which enables their transport into blood (where they are generally highly bound to albumin given their anionic nature [negative charge]) and then to the kidney for elimination in urine (by either glomerular filtration or possibly active transport [tubular secretion]). Drug conjugates are actively transported into bile primarily by MRP2 on the canalicular membrane and actively transported into blood primarily by MRP1 (in the case of GSH conjugates) and MRP3 (in the case of glucuronide and sulfate conjugates) on the sinusoidal membrane, although on a case-by-case basis other transporters may be involved (Borst et al., 2006).

Point 7 Not all biotransformation reactions are catalyzed by the mammalian enzymes listed in Table 6-4. Some biotransformation reactions are catalyzed by enzymes that participate in intermediary (endobiotic) metabolism, whereas others are catalyzed by enzymes in the gut microbiota (largely anaerobic bacteria in the colon) (Klaassen and Cui, 2015). This principle is illustrated in Fig. 6-4 for three xenobiotics that are all converted to benzoic acid and then hippuric acid. The conversion of toluene to hippuric acid is catalyzed by some of the xenobiotic-biotransforming enzymes listed in Table 6-4: microsomal CYP converts toluene to benzoic acid in three oxidative steps ($R–CH_3 \rightarrow R–CH_2OH \rightarrow RCHO \rightarrow RCOOH$), which introduces a functional group (namely, –COOH) that is conjugated with glycine to produce hippuric acid, which is excreted in urine. Cinnamic acid is also converted to benzoic acid but, in this case, the reaction is catalyzed by the mitochondrial enzymes involved in the β-oxidation of fatty acids. Quinic acid is also converted to benzoic acid, but this reductive, multistep reaction is catalyzed by gut microbiota. Incidentally, the conversion of benzoic acid to hippuric acid is of historical interest because it is generally recognized as the first xenobiotic biotransformation reaction to be discovered (in dogs by Woehler in 1828, and in humans by Ure in 1841).

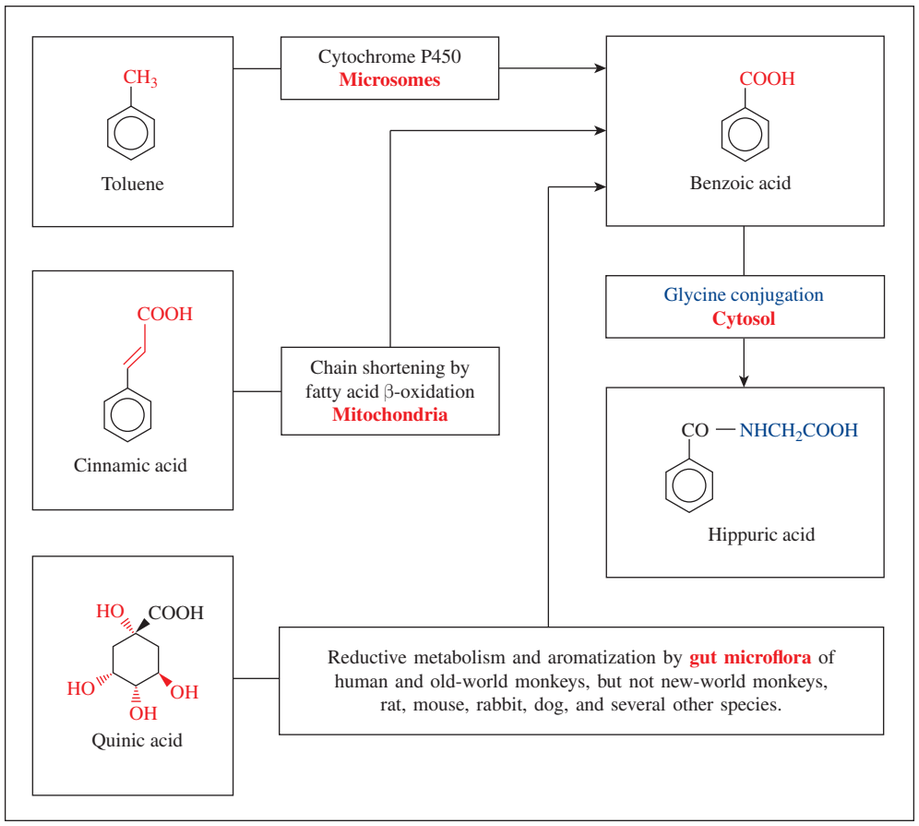


Figure 6-4. *Examples of xenobiotic biotransformation by different enzyme systems: a xenobiotic biotransforming enzyme (cytochrome P450), an endobiotics metabolizing enzyme, and gut microbiota.*

Some drugs are intentionally designed to be biotransformed by endobiotic-metabolizing enzymes. For example, the anti-HIV drug zidovudine (AZT) is converted to a triphosphate nucleoside by enzymes in the salvage pathway (nucleoside kinase, nucleoside monophosphate kinase [NMK], and nucleoside diphosphate kinase [NDK]). HIV reverse transcriptase inhibitors, such as zidovudine, are administered as nonphosphorylated analogs to facilitate their absorption and cellular uptake. Conversely, some drugs are administered as a phosphorylated prodrug (e.g., fosamprenavir) to promote their water solubility and rate of dissolution. The luminal surface of the small intestine contains high levels of alkaline phosphatase, which hydrolyzes prodrugs such as fosamprenavir and thereby releases the active drug at the surface of the enterocyte, where it is readily absorbed. Generally speaking, kinase and alkaline phosphatase are not usually considered to be xenobiotic-biotransforming enzymes.

Point 8 Just as some xenobiotics are biotransformed by the so-called endobiotic-metabolizing enzymes (Point 7), certain endobiotics are biotransformed by the so-called xenobiotic-metabolizing enzymes. For example, the same CYP enzymes implicated in xenobiotic biotransformation also contribute to the hepatic catabolism of steroid hormones, and the same UGTs that conjugate xenobiotics also glucuronidate bilirubin, thyroid hormones, and steroid hormones. Interestingly, the CYP and UGT enzymes that play a major role in steroid metabolism (namely, CYP3A4 and UGT1A1) display positive homotropic cooperativity (i.e., the enzymes have Hill coefficients >1) such that the rate of metabolism of steroid hormones by these enzymes is slow at low substrate concentrations but increases dramatically as the concentration of steroid hormone increases. Benzoic acid, a xenobiotic, is conjugated with glycine (as shown in Fig. 6-4), and so are bile acids (endobiotics). Certain leukotrienes are GSH conjugates. Conjugates of endogenous substrates, such as the glucuronide and sulfate conjugates of steroid and thyroid hormones, tend to be transported out of the hepatocyte by MRP2, the same canalicular transporter that transports drug conjugates into bile. From the few examples in Points 7 and 8, it is apparent that, on a case-by-case basis, there is often no clear-cut distinction between endobiotic- and xenobiotic-biotransforming enzymes.

The human genome project has helped to establish that what were once thought to be two distinct enzymes, one involved in the metabolism of endobiotics and one involved in the metabolism of xenobiotics, are in fact one and the same enzyme. For example, the microsomal enzyme known as 11β-hydroxysteroid dehydrogenase is identical to the xenobiotic-metabolizing enzyme known as microsomal carbonyl reductase (gene symbol HSD11B1; aka SDR26C1). Likewise, hepoxilin A_3 hydrolase was thought to represent a fifth class of epoxide hydrolases, but was subsequently identified as the same enzyme as soluble epoxide hydrolase (sEH) (Cronin et al., 2011).

Point 9 Several xenobiotic-biotransforming enzymes are inducible, meaning their expression can be increased (upregulated) usually in response to exposure to high concentrations of xenobiotics. Induction is mediated by ligand-activated receptors (so-called xenosensors) that are activated by xenobiotics (ligands) to DNA-binding proteins that upregulate the transcription of various genes encoding xenobiotic-biotransforming enzymes, especially CYP enzymes, which are usually induced to the greatest extent (in terms of fold increase). The major xenosensors are the aryl hydrocarbon receptor

(AhR), which induces CYP1 enzymes, the constitutive androstane receptor (CAR), which induces CYP2B, CYP2C, and CYP3A enzymes, the pregnane X receptor (PXR), which also induces CYP2B, CYP2C, and CYP3A enzymes, and the peroxisome proliferator–activated receptor α (PPARα), which induces CYP4 enzymes in rodents. CAR and PXR are closely related nuclear receptors, and they tend to be activated by the same ligands and bind to the same DNA-response elements (i.e., the same discrete regions of DNA that control gene expression), for which reason there is said to be considerable *crosstalk* between these two xenosensors. Certain xenosensors are activated by endogenous ligands (e.g., bilirubin, bile acids, and fatty acids activate CAR, PXR, and PPARα, respectively), and certain nuclear receptors, such as the vitamin D receptor (VDR), can mimic PXR and induce CYP3A4, which inactivates the active metabolite of vitamin D. These examples illustrate how xenosensors are not just involved in xenobiotic disposition but also play a role in endobiotic homeostasis. Two proteins called Kelch-like ECH-related protein 1 (KEAP-1) and nuclear factor E2 p45-related factor-2 (Nrf2) function as xenosensors inasmuch as they induce enzymes in response to oxidative stress, which is often associated with xenobiotic biotransformation. Binding of the transcription factor Nrf2 to the region of DNA known as the antioxidant response element (also known as the electrophilic response element [EpRE]) induces enzymes that detoxify electrophiles and metabolites that generate reactive oxygen species, including glutathione transferase (GST; GSTA1), microsomal epoxide hydrolase (mEH), aldo-keto reductase (AKRs; AKR7A2 and AKR7A3, also known as aflatoxin aldehyde B1 reductases 1 and 2), NAD(P)H-quinone oxidoreductase (NQO1, also known as DT-diaphorase), microsomal heme oxygenase, and glutamate–cysteine ligase (GCL), which catalyzes the rate-limiting step in GSH synthesis. Induction of GCL increases the rate of GSH synthesis under conditions of oxidative stress and a decrease in GSH concentration (Lee and Johnson, 2004). Experiments in wild type and Nrf2 knockout mice revealed that activation of AhR leads to activation of Nrf2, which contains an AhR-response element in its promoter region; hence, AhR agonists such as dioxin induce enzymes under the direct control of AhR (such as CYP1A1) as well as enzymes under the control of Nrf2 (such as NQO1 and the other enzymes mentioned above) (Yeager et al., 2009). Induction of Nrf2 via activation of AhR protects mice from the hepatotoxic effects of dioxin (Lu et al., 2011).

Induction is a reversible, adaptive response to xenobiotic exposure. The induced enzymes (and transporters) usually accelerate the elimination of the xenobiotic that triggered the induction process, in which case the xenobiotic is said to be an autoinducer (one that induces its own metabolism). However, xenobiotics often induce enzymes that are not capable of metabolizing them, in which case the induction is said to be gratuitous. Induction is a pleiotropic response: activation of AhR, CAR, PXR, PPARα, and Nrf2 all results in alterations in the expression of numerous genes.

Suppression (downregulation) of drug-metabolizing enzymes is often associated with inflammatory diseases (such as arthritis), cancer, infectious diseases (both bacterial and viral), vaccination, and treatment with certain proinflammatory biologics (therapeutic proteins). These disease processes activate nuclear factor kappa-B (NF-κB) (and other nuclear receptors), which suppresses the expression and induction of CYP and other xenobiotic-metabolizing enzymes because activated NF-κB suppresses all four xenosensors (AhR, CAR, PXR, and PPARα), as well as several other nuclear receptors. By reversing the disease process—such as lessening the inflammation associated with rheumatoid arthritis—some biologics (large drug molecules such as monoclonal antibodies and other types of therapeutic proteins) can reverse the suppression of drug-metabolizing enzymes and restore their activity to normal (predisease) levels (Morgan, 2009; Huang et al., 2010).

Point 10 The ability of certain xenobiotic-biotransforming enzymes to metabolize hormones and other endobiotics (Point 8) and the ability of certain xenobiotics to induce xenobiotic-biotransforming enzymes (Point 9) have implications for understanding an important mechanism by which certain xenobiotics can alter homeostasis or cause toxicity. Persistent exposure to xenobiotics that are enzyme inducers (especially CAR and PPARα activators) can increase the rate of steroid hormone oxidation by CYP and increase the rate of thyroid hormone glucuronidation and sulfonation that, in rodents, can lead to the development of Leydig cell tumors (due to elevated levels of LH and FSH) and thyroid follicular tumors (due to elevated levels of thyroid-stimulating hormone [TSH]), respectively (Grasso et al., 1991). Persistent exposure to enzyme inducers can also cause liver tumors, although the mechanism is not fully understood. Activation of certain xenosensors (CAR or PPARα) is critical to liver tumor development in mice, although upregulation of xenobiotic-biotransforming enzymes is less important than other xenosensor-dependent events, such as, in the case of the CAR activator phenobarbital, altered DNA methylation of numerous genes, upregulation of GADD45B (an antiapoptotic factor) and MDM2 (a negative regulator of the tumor suppressor p53), and the downregulation of gap junctional proteins (connexin 32), which diminishes cell–cell communication (Omiecinski et al., 2011b; Elcombe et al., 2014; Lake et al., 2015).

Phenobarbital, Wy-14643, methapyrilene, and Ponceau S are representatives of four classes of nongenotoxic rodent tumorigens (tumor promoters) that cause hepatocellular hyperplasia and hypertrophy in association with proliferation of the endoplasmic reticulum, peroxisomes, mitochondria, and lysosomes, respectively (Grasso et al., 1991). In the first two cases, the hepatocellular changes depend on activation of CAR (in the case of phenobarbital) and PPARα (in the case of Wy-14643). Prolonged activation of these receptors in rodents results in the development of liver and/or thyroid tumors. However, this is thought to be a rodent-specific phenomenon because, in the case of phenobarbital and other enzyme-inducing antiepileptic drugs (EIAEDs), there is compelling epidemiological evidence that these chemicals do not cause liver tumors in humans even after >30 years of treatment (Parkinson et al., 2006; Elcombe et al., 2014; Lake et al., 2015).

The major UGT responsible for conjugating bilirubin is UGT1A1, which is inducible by CAR activators such as phenobarbital and scoparone (6,7-dimethylesculetin). At one time, the management of neonatal jaundice included treatment with phenobarbital to induce bilirubin conjugation, but this practice has been discontinued. However, the Chinese herbal Yin Zhi Huang (active ingredient scoparone) is still used to treat neonatal jaundice. Bilirubin is also a CAR agonist, and high levels of bilirubin induce UGT1A1 and bilirubin/bilirubin glucuronide transporters (MRP2 and MRP3) to increase the rate of bilirubin elimination when bilirubin levels are elevated (e.g., during hemolytic anemia).

Although prolonged activation of PXR in rats is associated with thyroid tumor promotion due to induction of triiodothyronine (T3) glucuronidation (Richardson and Klaassen, 2010), this effect is thought not to occur in humans. However, prolonged activation of PXR by rifampin in humans (which induces hepatic and intestinal CYP3A4) is associated with osteomalacia caused by an enhanced rate of conversion of the active metabolite of vitamin D to inactive metabolites by CYP3A4 (Xu et al., 2006; Zhou et al., 2006a).

Point 11 Xenobiotic biotransformation can alter the biological properties of a xenobiotic. It can make the xenobiotic less toxic (detoxication), but in some cases it can make it more toxic (activation). The oxidation of ethanol (alcohol) to acetaldehyde is an example of xenobiotic activation, and the subsequent oxidation of acetaldehyde to acetic acid is an example of detoxication. The biotransformation of drugs can result in (1) a loss of pharmacological

activity (e.g., the conversion of acetaminophen to acetaminophen glucuronide and the conversion of morphine to morphine-3-glucuronide), (2) no change in pharmacological activity (e.g., the conversion of fluoxetine to its *N*-demethylated metabolite norfluoxetine), or (3) an increase in pharmacological activity (e.g., the conversion of codeine to morphine, as well as the conversion of morphine to morphine-6-glucuronide). When the parent drug is pharmacologically active, diminished metabolism (due to a genetic deficiency or enzyme inhibition) can result in an exaggerated pharmacological response (Type A toxicity) (Aronson and Ferner, 2003),[2] such as the prolonged or increased hypotension caused by debrisoquine, alprenolol, bufuralol, metoprolol, propranolol, and timolol in individuals lacking CYP2D6 (Turner et al., 2001; Ingelman-Sundberg, 2005). When a metabolite is the pharmacologically active component, diminished formation (due to a genetic deficiency or enzyme inhibition) can result in little or no pharmacological response (Type F toxicity) (Aronson and Ferner, 2003), such as the diminished effects of codeine, an analgesic, and tamoxifen, an antiestrogen drug, in individuals lacking CYP2D6 and the diminished effects of clopidogrel, an antiplatelet drug, in individuals lacking CYP2C19 (discussed in the sections "CYP2D6" and "CYP2C19").

Point 12 In many cases, the toxicity of a xenobiotic is due to the parent compound (the compound that was absorbed), in which case xenobiotic biotransformation serves as a detoxication mechanism. This principle is underscored by the clinical observation that the incidence of ADEs is often higher in individuals with a poor metabolizer (PM) phenotype (discussed in Point 24). However, xenobiotic-biotransforming enzymes can convert certain xenobiotics to reactive metabolites (e.g., electrophiles and radicals), and this activation process plays an important role in chemical toxicity and chemical mutagenicity/carcinogenicity; the former often involves the covalent binding of reactive metabolites to critical cellular nucleophiles such as proteins, whereas the latter involves covalent binding to one or more purine or pyrimidine bases in DNA. Formation of reactive metabolites can also lead to irreversible CYP inhibition (a process known as metabolism-dependent inhibition [MDI] or time-dependent inhibition [TDI]), which is an important cause of drug–drug interactions. As illustrated in Fig. 6-5, carboxylic acid–containing drugs (especially the acetic and propionic acid–containing NSAIDs that cause liver toxicity) can be converted to reactive acyl glucuronides and/or acyl-CoA thioesters, whereas many neutral and basic drugs are converted by CYP to electrophilic metabolites or carbon-centered radicals that may cause liver toxicity (or damage to the cells harboring the activating CYP). Several nitrogen- and sulfur-containing drugs can be activated by peroxidases to heteroatom-centered radicals that can cause blood dyscrasias such as agranulocytosis/neutropenia, aplastic anemia, and thrombocytopenia (Tang and Lu, 2010; Walsh and Miwa, 2011). In Fig. 6-5, the scheme depicting the formation and toxicity of reactive metabolites is an oversimplification. Furthermore, some drugs can be converted to two or more reactive metabolites. The diuretic tienilic acid, for example, contains a carboxylic acid group that is converted to a reactive acyl glucuronide and acyl-CoA thioester, and a furan (a neutral group) that is converted by CYP to an epoxide and other reactive metabolites (such as an enedial). Tienilic was withdrawn from the market due to hepatotoxicity.

CYP is particularly effective at converting proximate carcinogens to ultimate carcinogens by converting the former to electrophilic metabolites that bind to and mutate DNA, thereby leading to mutations and tumor initiation. The Ames bacterial mutagenicity assay, which is representative of several mutagenicity tests, is performed in the absence and presence of CYP (i.e., liver microsomes) to evaluate the mutagenic potential of both the parent compound and any electrophilic metabolites, respectively. Although such in vitro assays help to identify compounds that can be metabolized to reactive metabolites, they can potentially provide misleading information on the identity of the enzymes responsible for activating

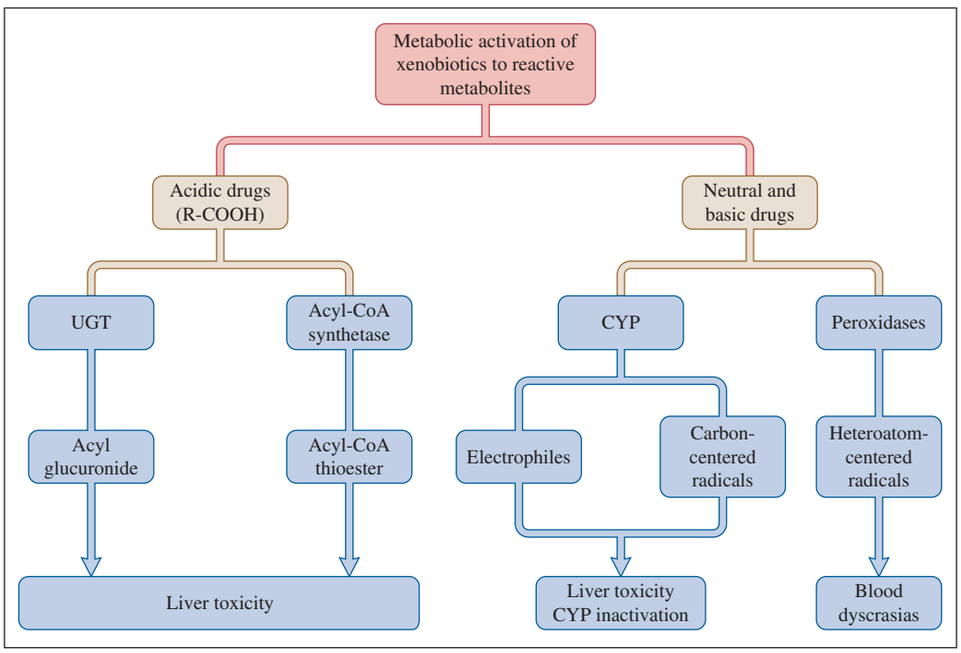


Figure 6-5. *Some major pathways of metabolic activation of xenobiotics to reactive metabolites that cause toxicity.*

[2]Aronson and Ferner classified adverse drug effects into types A to F as follows: **Type A**, exaggerated pharmacological effects involving the intended pharmacological target (Type A1) or an unintended (off-target) pharmacological target (Type A2); **Type B**, idiosyncratic reaction (rare, not closely related to dose or time); **Type C**, chemical reactions with macromolecules (e.g., alkylating agents); **Type D**, delayed effect (e.g., carcinogenicity); **Type E**, discontinuation symptoms; and **Type F**, failure of therapy (Aronson and Ferner 2003).

compounds in vivo because they detect activation but not deactivation and they do not measure the overall balance between activation and deactivation. For example, CYP1A1 is more active than other CYP enzymes at activating benzo[*a*]pyrene to reactive metabolites, but in vitro mutagenicity assays conceal the fact that CYP1A1 is even more active than other CYP enzymes at converting this carcinogen to inactive metabolites that can be eliminated from the body. Consequently, metabolism of benzo[*a*]pyrene by CYP1A1 would seem to be a liability based on in vitro mutagenicity assays but induction of CYP1A1 in vivo protects against benzo[*a*]pyrene toxicity and carcinogenicity, whereas deletion of CYP1A1 (i.e., CYP1A1 knockout mice) has a potentiating effect (discussed in the sections on "CYP1A1, CYP1A2, and CYP1B1" and "Enzyme Induction and Chemical Carcinogenesis"). Polycyclic aromatic hydrocarbons (PAHs; combustion pollutants and components of tobacco smoke), aromatic amines (industrial chemicals), aflatoxin (a mycotoxin), cooked food pyrolysis products, and tobacco-specific nitrosamines are all examples of proximate carcinogens that require metabolic activation to form electrophilic, DNA-binding metabolites (ultimate carcinogens).

Certain anticancer drugs require activation by xenobiotic-metabolizing enzymes in order to exert their antineoplastic effects. For example, cyclophosphamide is activated by CYP (CYP2B6 and CYP3A4), and canfosfamide is activated by GST, namely, GSTP1-1, which is often overexpressed in tumors thereby conferring resistance against certain anticancer drugs (see the section "Glutathione Conjugation") (Gonzalez and Tukey, 2006)

Liver microsomes are widely used in three in vitro systems to investigate the potential of drugs or drug candidates to be converted to reactive metabolites. First, liver microsomes from Aroclor 1254–treated (induced) rats are used in the Ames bacterial mutagenicity assay. A limitation of this assay is that it is well suited to identify reactive metabolites formed by CYP but not by other xenobiotic-biotransforming enzymes, examples of which are given in the section "Conjugation." This limitation has been addressed through genetic engineering of *Salmonella typhimurium* so that test bacteria express one or more human xenobiotic-metabolizing enzymes (Iwata et al., 1998; Glatt and Meinl, 2004b). Second, human and rat liver microsomes are widely used to assess the formation of reactive metabolites that bind covalently to microsomal protein or GSH (or a related nucleophile such as *N*-acetylcysteine [NAC]). This system does a poor job of distinguishing hepatotoxic drugs from nonhepatotoxic drugs (Obach et al., 2008), but the distinction is improved when covalent binding is assessed in human hepatocytes and dose is taken into account (Bauman et al., 2009; Nakayama et al., 2009). Third, human liver microsomes are widely used to evaluate a drug candidate's potential to cause irreversible inhibition of the major drug-metabolizing CYP enzymes. False-negative results can occasionally occur when metabolism by a non-CYP enzyme is instrumental to CYP inactivation, such as the need to glucuronidate gemfibrozil and clopidogrel in order to inactivate CYP2C8. In such cases of so-called system-dependent inhibition the use of human hepatocytes offers advantages over human liver microsomes (Parkinson et al., 2010).

Structural alerts (functional groups) for the bioactivation of xenobiotics to reactive metabolites that cause toxicity and/or CYP inactivation include the following (which is not an exhaustive list): (1) acetic and propionic acids (activated by UGT and/or acyl-CoA synthetase), (2) nitro aromatics (activated by reductases and/or CYP), and (3) a large number of functional groups that are activated by CYP and, in some cases, peroxidases: acetylenes, activated aromatics, 3-alkly indoles, anilines, cyclopropylamines, formamides, furans, hydrazines, pyrroles, sulfonylureas, thiazolidinones, thioamides, thiophenes, and thioureas (Walsh and Miwa, 2011).

Point 13 The toxicity and potential carcinogenicity of electrophilic metabolites produced by CYP and other xenobiotic-biotransforming enzymes are reduced and often altogether eliminated by their conjugation with GSH, which is often described as a noncritical cellular nucleophile. Conjugation with GSH can occur both enzymatically (by GST) and nonenzymatically. In the majority of cases, conjugation with GSH represents a detoxication reaction, one that protects critical cellular nucleophiles, such as protein and DNA, from covalent modification. However, there are cases (e.g., the fumigants methylbromide and dibromoethane) where conjugation with GSH actually produces a DNA-reactive (mutagenic) metabolite. Activation of haloalkanes and dihaloalkanes (which are used industrially as fumigants) is usually catalyzed by the theta class of GSTs (Josephy, 2010). Depletion of GSH or genetic deficiency of a particular GST can predispose to certain drug toxicities. For example, the genetic loss of glutathione transferase mu 1 (GSTM1) predisposes to the hepatotoxic effects of carbamazepine and valproic acid, two antiepileptic drugs (Saruwatari et al., 2010), whereas the combined loss of GSTM1 and glutathione transferase theta 1 (GSTT1) is a risk factor for hepatotoxicity from troglitazone, an antidiabetic drug withdrawn from the market (Ikeda, 2011).

As mentioned in Point 9, several Phase 2 enzymes including GSTs and enzymes involved in GSH synthesis are under the control of Nrf2, which allows cells to respond to oxidative stress and reactive metabolites by increasing the cytoprotective effects of GSH-dependent processes. Such induction is a possible mechanism by which administration of low doses of acetaminophen to mice for 8 days, which causes Nrf2 activation (Goldring et al., 2004), protects them from a subsequent high hepatotoxic dose of acetaminophen (Shayiq et al., 1999). This finding may explain why some individuals who misuse opioid/acetaminophen drugs (such as Percocet [oxycodone/acetaminophen] and Vicodin [hydrocodone/acetaminophen]) can consume 3 to 19 g of acetaminophen per day for 1 to 14 years (and in one case as much as 50-g acetaminophen per day) — doses well in excess of the maximum daily recommended dose of 4 g/day—without developing liver damage (Shayiq et al., 1999).

Point 14 The biotransformation of some xenobiotics results in the production of reactive oxygen species, which can cause cell toxicity (including DNA damage) through oxidative stress and lipid peroxidation. GSH, GSTs, and glutathione peroxidases (GPXs) all limit the toxic effects of reactive oxygen species just as they limit the toxicity of reactive metabolites formed directly from xenobiotics. Oxidative stress and the formation of electrophilic metabolites reduce GSH levels and thus result in the concurrent oxidation of KEAP-1, which then releases Nrf2, which in turn upregulates the expression of enzymes that detoxify electrophilic metabolites (e.g., epoxides) and those metabolites that generate reactive oxygen species (e.g., quinones) (see Point 9). Certain xenobiotics and ischemic reperfusion injury cause an accumulation of unfolded proteins in the endoplasmic reticulum (where proteins are folded and post-translationally modified), which gives rise to the unfolded protein response (UPR; also known as endoplasmic reticulum stress [ER stress]), which activates Nrf2 and Bax-inhibitor-1 (BI-1). The heme oxygenase induced by Nrf2 as well as BI-1 (which complexes with NADPH–cytochrome P450 reductase) protects cells from oxidative stress by inhibiting the production of reactive oxygen species by CYP2E1 and other CYP enzymes (discussed in the section "CYP2E1").

Point 15 The balance between activation and detoxication by xenobiotic-biotransforming enzymes is often a key determinant of chemical toxicity, and is often the basis for organ or species differences in toxicity. For example, aflatoxin is converted by liver microsomal CYP to a reactive epoxide that is thought to

be responsible for the hepatotoxic and hepatocarcinogenic effect of this mycotoxin. The fact that this reaction occurs in the liver explains why aflatoxin causes liver toxicity and liver tumors. On this basis, mice would be expected to be more sensitive than rats to the hepatotoxic effects of aflatoxin because mice catalyze the epoxidation of aflatoxin faster than rats. However, through GSH conjugation, mice also detoxify aflatoxin epoxide faster than rats. Consequently, despite their slower rate of activation, rats are more susceptible than mice to the toxic effects of aflatoxin. Coumarin is hepatotoxic to rats, but not to humans. This species difference is attributable to differences in the metabolites formed by CYP. Rats convert coumarin to a reactive epoxide that rearranges to a reactive aldehyde, whereas humans convert coumarin to the relatively nontoxic metabolite 7-hydroxycoumarin (umbelliferone).

Point 16 Exposure to xenobiotics (especially drugs) is largely through oral ingestion, and the small intestine and liver are highly developed to limit systemic exposure to orally ingested xenobiotics, a process known as *first-pass elimination* (*or presystemic elimination*). The enterocytes at the tips of the small intestinal villi express various efflux transporters, such as P-gp, BCRP, and MRP2, which restrict intestinal absorption and limit systemic exposure to various xenobiotics including many drugs. The enterocytes express high levels of certain CYP and UGT enzymes, which biotransform a wide variety of xenobiotics. The liver expresses a number of uptake transporters that actively remove xenobiotics from the blood. They also express a number of efflux transporters that actively discharge xenobiotics or their metabolites (especially conjugates) into the bile canaliculus for biliary excretion, or that actively discharge xenobiotic metabolites (especially conjugates) across the sinusoidal membrane back into the blood for urinary excretion. The liver expresses the largest number and, with few exceptions, the highest concentrations of xenobiotic-biotransforming enzymes.

Although the liver contains higher concentrations of most xenobiotic-biotransforming enzymes, and because the number of hepatocytes in the liver exceeds the number of enterocytes in the small intestine, it might be assumed that, compared with the liver, the small intestine would make only a small contribution to first-pass metabolism, but this is not the case in part because in the small intestine xenobiotics are recycled by P-gp, BCRP, and MRP2 on the luminal surface of enterocytes, which delays absorption and increases the opportunity for xenobiotic biotransformation by intestinal enzymes such as CYP (especially CYP3A4), UGT, and sulfotransferase (SULT) (Benet, 2009). Furanocoumarins in grapefruit juice inhibit intestinal but not hepatic CYP3A4 and yet grapefruit juice increases systemic exposure to felodipine (a calcium channel blocker used to treat hypertension) and other drugs that undergo first-pass metabolism by CYP3A4 (Paine et al., 2006). The impact of the CYP3A4 inhibitor ketoconazole (an antifungal drug) on the disposition of the CYP3A4 substrate midazolam (an antianxiety/sedative drug) depends on whether midazolam is given orally or intravenously. When midazolam is given intravenously, such that its clearance is dependent only on hepatic metabolism, ketoconazole causes a three- to fivefold increase in the area under the plasma concentration–time curve (AUC). However, when midazolam is given orally, ketoconazole causes a 10- to 15-fold increase in AUC, the difference reflecting the significant role of intestinal metabolism to the presystemic clearance of midazolam. In rats, administration of the general CYP inhibitor 1-aminobenzotriazole (ABT) by the intravenous route (which inhibits only hepatic CYP enzymes) and the oral route (which inhibits both intestinal and hepatic CYP enzymes) can be used to assess the relative role of the intestine and liver to the first-pass metabolism of a drug or other xenobiotic (Strelevitz et al., 2006; Boily et al., 2015).

The small intestine and liver are exposed to high concentrations of xenobiotics, and they possess high levels of the enzymes that potentially convert xenobiotics to reactive or toxic metabolites. It is perhaps not surprising, therefore, that both tissues possess protective mechanisms to minimize the risk of xenobiotic toxicity and carcinogenicity. As already mentioned, both tissues have enzymes and transporters that facilitate the elimination of xenobiotics and their metabolites. In both tissues, several of the xenobiotic-biotransforming enzymes and transporters are inducible, enabling the liver and the small intestine to respond to high levels of xenobiotics by enhancing the rate of xenobiotic biotransformation and elimination. In the small intestine, the enterocytes at the villus tips undergo extensive turnover (half-life of about 2 days), such that the mature cells that are exposed to high levels of xenobiotics and/or reactive metabolites are quickly lost (exfoliated) and replaced in a matter of days. In liver, high levels of GSH (5 to 10 mM), a large proportion of diploid (binucleated) cells, and a high regenerative capacity all protect the liver from xenobiotic toxicity or help the liver to repair chemical-mediated toxicity. In addition, severely damaged hepatocytes can undergo apoptosis (cell-programmed death) to eliminate precancerous cells (i.e., cells with extensive DNA damage).

Point 17 Some of the same mechanisms that protect the small intestine and liver from xenobiotic toxicity also protect certain organs such as the brain and reproductive organs. Xenobiotics that enter the systemic circulation are often excluded from the brain by the blood–brain barrier, which poses a physical barrier to xenobiotic transport (in the form of tight junctions) and a biochemical barrier notably in the form of the efflux transporters P-gp, BCRP, and MRP2. Germ cells in the testis are protected in part by a blood–testis barrier, by high levels of GSH (and GST and GPX), and by high cell turnover (as in the case of enterocytes). The ovum, with virtually no cell turnover, possesses high levels of GSH (8 mM or more), which protects it from DNA-reactive electrophiles and from the oxidative stress that accompanies both xenobiotic exposure and fertilization. Efflux transporters and GSTs are often overexpressed in tumor cells as a result of chromosomal rearrangements that place the genes encoding these proteins under the control of a strong promoter. The overexpression of a transporter and/or GST can confer resistance to certain cancer chemotherapeutic drugs (many of which are intended to be converted to reactive metabolites that damage DNA and kill tumor cells).

Point 18 In view of the important role of CYP in the metabolic activation of proximate carcinogens to ultimate carcinogens, it may seem paradoxical to list CYP induction among the defense mechanisms that protect organisms from the carcinogenic effects of xenobiotics. Activation by CYP is definitely required for certain xenobiotics to exert their carcinogenic effects, and induction of CYP is associated with an increase in the toxicity of certain xenobiotics. However, contrary to expectation, treatment of rodents with a CYP inducer prior to treatment with a known proximate carcinogen (such as aflatoxin, various nitrosamines, or PAHs) is generally associated with a decrease, not an increase, in tumor incidence (Parkinson and Hurwitz, 1991). The route of exposure to the carcinogen can affect the impact of enzyme induction; it protects against orally administered drugs but may increase the tumorigenicity of carcinogens applied directly to their site of action (Nebert et al., 2004), and prolonged treatment of rodents with an enzyme inducer *after* application of the initiating carcinogen can lead to an increase in tumor incidence because many enzyme inducers act as tumor promoters (see Point 10). Nevertheless, for the most part, enzyme induction provides protection against chemical toxicity and carcinogenesis (Nebert and Dalton, 2006; Shimada, 2006; Ma and Lu, 2007; Nebert et al., 2013).

Point 19 Although the small intestine and liver contain the highest concentrations, xenobiotic-biotransforming enzymes are nevertheless widely distributed throughout the body. In terms of specific content (i.e., the amount of enzyme on a per-milligram-protein basis), some of the highest concentrations of xenobiotic-metabolizing enzymes are to be found in nasal epithelium, a portal of entry for many volatile xenobiotics. The presence of high levels of xenobiotic-biotransforming enzymes in nasal epithelium is of interest because numerous xenobiotics cause nasal cytotoxicity and cancer in rodents (Jeffrey et al., 2006). Xenobiotic-biotransforming enzymes in the lung, eye, and skin can be especially important for the metabolism of drugs delivered by inhalation, eye drop, or topical application, respectively. The kidney expresses several xenobiotic-biotransforming enzymes, in addition to numerous transporters that actively secrete xenobiotics (especially acidic metabolites) into urine. The kidney also metabolizes GSH conjugates formed in the liver and converts them to mercapturic acids (*N*-acetylcysteine conjugates), which is discussed in the section "Glutathione Conjugation."

Point 20 Species differences in xenobiotic-biotransforming enzymes are often the basis for species differences in both the qualitative and quantitative aspects of xenobiotic biotransformation and toxicity. As mentioned in Point 15, species difference in coumarin metabolism and toxicity (activation by epoxidation in rats, detoxication by aromatic hydroxylation in humans) reflects a species difference in hepatic microsomal CYP, whereas species differences in GST activity account for the difference between rats and mice in terms of their susceptibility to aflatoxin-induced liver toxicity. Species differences in the fetal expression of xenobiotic-biotransforming enzymes have raised questions about the suitability of laboratory animals to assess the risk of human teratogenicity because human fetal liver expresses CYP3A7, an enzyme capable of activating aflatoxin to reactive metabolites, whereas no such enzyme is expressed in the fetus of rodents (Li et al., 1997; Pang et al., 2012). Biotransformation, inhibition, and induction can occur in a species-specific manner. For example, furafylline, an antiasthma drug that was not approved, and omeprazole, a proton pump inhibitor (antacid), are inhibitors and inducers of human CYP1A2, respectively, but they do not inhibit or induce CYP1A2 in rodents. Such species differences are the impetus for the development of chimeric mice (in which the mouse hepatocytes are replaced with human hepatocytes) (Grompe and Strom, 2013) and so-called humanized mice, which involves substituting the murine genes that encode xenobiotic-metabolizing enzymes (and the receptors that regulate their expression) with their human counterparts (Gonzalez, 2003; Gonzalez and Yu, 2006).

In some species, entire pathways of xenobiotic biotransformation are low or absent from a particular species. For example, cats and dogs are considered poor glucuronidators and poor acetylators of xenobiotics, respectively. Because of their inability to glucuronidate drugs, cats are sensitive to acetaminophen hepatotoxicity (Baillie and Rettie, 2011). Dogs are poor acid secretors, which disfavors the gastric absorption of acidic drugs (see the section "Introduction"). Interestingly, although dogs can conjugate acidic drugs, they are poor metabolizers of acidic drugs in terms of oxidative biotransformation by CYP enzymes (Baillie and Rettie, 2011). Metabolism by gut microbiota can occur in a species-dependent manner. For example, as shown in Fig. 6-4, quinic acid (the perceived acid in coffee) is converted to benzoic acid in humans and old-world monkeys but not in new-world monkeys, mice, rats, and most other laboratory animals (Adamson et al., 1970).

Point 21 In sexually mature rats and, to a lesser extent, mice there are marked gender differences in the expression of certain xenobiotic-biotransforming enzymes (both oxidative and conjugating enzymes) (Waxman and Holloway, 2009). In other species, including humans, gender differences either do not exist or generally represent less than a twofold difference, whereas the differences in rodents can be an order of magnitude or greater (Parkinson et al., 2004; Soldin et al., 2011). Against a large background of interindividual variation in each gender, it is often difficult to discern gender differences in xenobiotic biotransformation in humans, although, in general, women appear to have lower CYP1A2 and higher CYP3A4 activity than men (Parkinson et al., 2004). Few drugs are dosed on the basis of gender, but in 2014 the FDA recommended that, due to their slower rate of metabolism, women should be administered half the dose administered to men for zolpidem (Intermezzo, a sedative) and zolpidem-containing drugs like Ambien, Edluar, and Zolpimist (FDA, 2018). It is particularly interesting that, compared with men, women appear to have a higher incidence of idiosyncratic drug toxicity (Walgren et al., 2005), which has been the cause of a number of drug withdrawals and black box warnings (see Point 22).

Point 22 Idiosyncratic drug reactions (IDRs) are rare adverse events (generally $<0.1\%$) that do not involve an exaggerated pharmacological response, do not occur in most patients at any dose, and typically do not occur immediately after exposure but do so after weeks or months of repeated administration (Walgren et al., 2005). IDRs are also known as Type B or hypersensitivity reactions and they can be divided into allergic and nonallergic IDRs (Aronson and Ferner, 2003). Allergic IDRs tend to develop relatively quickly (in days or weeks) and, after the drug is discontinued, patients respond robustly when rechallenged with the same or a closely related drug, whereas the latter tend to develop relatively slowly (with symptoms sometimes appearing after 6 months or more of drug treatment) and patients may or may not respond to rechallenge with the drug. Clinical characteristics suggest that IDRs are immune mediated, and there is substantive evidence that most, but not all, IDRs are caused by chemically reactive species (Uetrecht and Naisbitt, 2013). The major targets are skin, liver, and bone marrow. Drugs that were withdrawn from the U.S. market or carry a black box warning for idiosyncratic hepatotoxicity are listed in Table 6-6. The drugs are structurally diverse and fall into numerous therapeutic classes.

Several factors predispose to some, but not all, IDRs including gender (women are often more prone than men), age (the elderly are more prone than infants although infants are more prone to IDRs involving valproic acid), ethnicity, viral infection (a particularly strong predisposing factor), and other disease states (such as diabetes and obesity), possibly alcohol consumption, comedications that cause pharmacokinetic drug–drug interactions, genetic polymorphisms in xenobiotic-biotransforming enzymes and transporters (discussed in Point 23), and genetic polymorphisms that affect immune function, especially allelic variants of human leukocyte antigens (HLA haplotypes), part of the major histocompatibility complex (MHC) (Alfirevic and Pirmohamed, 2010; Chalasani and Bjornsson, 2010; Uetrecht and Naisbitt, 2013; Roth and Lee, 2017; Roth et al., 2017).

One of the best understood IDR is the skin hypersensitivity reaction to the anti-HIV drug abacavir, which is determined in a highly predictable manner by an individual's HLA subtype (Alfirevic and Pirmohamed, 2010). Abacavir binds to the antigen-presenting groove of MHC I molecule in patients carrying the B*5701 allele (in patients of all ethnic groups). In vitro studies have demonstrated that treatment of antigen-presenting cells positive for B*5701, but not for the related alleles B*5702 or B*5801, with abacavir can stimulate a CD8+ T-cell response. Topical exposure to abacavir (patch test) reveals activated CD8+ T cells in skin biopsies. Genetic testing for B*5701 is highly predictive; abacavir hypersensitivity is almost never seen in individuals without the B*5701 allele and

Table 6-6

Drugs That Have Been Withdrawn or Carry a Black Box Warning for Idiosyncratic Hepatotoxicity*

DRUG NAME	TYPICAL DAILY DOSE	INDICATION	REACTIVE METABOLITE AND EVIDENCE OF METABOLISM-DEPENDENT ENZYME INHIBITION	INDUCTION†, IMMUNOLOGICAL AND OTHER MECHANISTIC ASPECTS
Drugs Withdrawn				
Benoxaprofen	300–600 mg	NSAID	Acyl glucuronide	Inducer (rat AhR and PPARα agonist)
Bromfenac	25–50 mg	NSAID	Acyl glucuronide	
Iproniazid	25–150 mg	Depression	Multiple. MDI of MAO-B	Anti-MAO-B and antimitochondria
Nefazodone	200 mg	Depression	Quinoneimine. MDI of CYP3A4	
Tienilic acid	250–500 mg	Diuretic	Thiophene epoxide, sulfoxide. MDI of CYP2C9	Anti-LKM_2 (anti-CYP2C9)
Troglitazone	400 mg	Diabetes	Quinone methide, sulfenic acid, α-ketoisocyanate	Inducer (CAR/PXR agonist)
Black Box Warnings				
Acitretin	25–50 mg	Psoriasis	No. Acitretin, a retinoid, is esterified to etretinate	Etretinate was withdrawn in 2002
Bosentan	125–250 mg	PAH	No	Inhibits BSEP. Inducer (PXR agonist)
Dacarbazine	140–315 mg	Melanoma, lymphoma	Methyl diazohydroxide and methyl cation (CH_3^+)	Hepatotoxic to laboratory animals
Dantrolene	300–400 mg	Muscle relaxant	Nitro-reduction intermediates bind to GSH	Inducer (AhR agonist)
Felbamate	1200 mg	Epilepsy	2-Phenylpropenal (atropaldehyde)	Inducer (PXR agonist)
Flutamide	750 mg	Prostate cancer	Nitro-reduced, nitrogen radicals and ortho-quinoneimines bind to protein and GSH‡	Inhibits mitochondrial respiration
Gemtuzumab	9 mg/m^2	AML	Reductive activation to a diradical	Designed to intercalate and bind DNA
Isoniazid	300 mg	Tuberculosis	Multiple. MDI of CYP1A1, 2A6, 2C19, and 3A4	Radicals formed by myeloperoxidase
Ketoconazole	200 mg	Fungal infections	Dialdehyde	Auto-inhibitor (inhibits CYP3A4)
Naltrexone	50 mg	Alcoholism, addiction	No	
Nevirapine	200–400 mg	AIDS	Quinonemethide. MDI of CYP3A4§	Inducer (PXR agonist)
Pemoline	37.5–112.5 mg	Hyperactivity	No	
Tolcapone	300 mg	Parkinsonism	Nitro reduction and then quinoneimine formation	Uncouples mitochondrial respiration
Trovafloxacin	100–500 mg	Antibiotic	No, but it has a lipophilic difluorinated side chain present in a toxic fluoroquinolone—now withdrawn	Eosinophilia suggests an immune-mediated hepatitis
Valproic acid (VPA)	1000–4200 mg	Epilepsy	CoA thioesterification and epoxidation of 4-ene-VPA	Disrupts mitochondrial β-oxidation

Abbreviations: AhR, aryl hydrocarbon receptor; AIDS, acquired immunodeficiency syndrome; AML, acute myeloid leukemia; anti-LKM_2, auto-antibodies against liver and kidney microsomes (anti-LKM_1 contains antibodies against CYP2D6, whereas anti-LKM_2 contains antibodies against CYP2C9); BSEP, bile salt export pump (a bile canalicular transporter); CYP, cytochrome P450; GSH, glutathione; MAO, monoamine oxidase; MBI, mechanism-based inhibitor (aka suicide inactivator); NSAID, nonsteroidal anti-inflammatory drug; PAH, pulmonary arterial hypertension; PPARα, peroxisome proliferator–activated receptor alpha; PXR, pregnane X receptor; VPA, valproic acid.

*Data adapted from Walgren et al. (2005).

†The receptor agonist assignment is based on the CYP-induced enzyme (CYP1A for AhR; CYP2B, CYP2C, and/or CYP3A for CAR/PXR; CYP4A for PPARα).

‡Wen et al. (2008).

§Chen et al. (2008).

it is seen in about half of B*5701-positive carriers. Genetic testing for B*5701 prior to abacavir treatment is required by the FDA and is cost effective (the cost of treating the severe skin lesions that occur in 5% to 9% of abacavir-treated patients outweighs the costs of genetic testing). Another strong association (odds ratio >1000) between HLA alleles (HAL B*1502) and IDR is seen with carbamazepine-induced skin lesions (Stevens–Johnson syndrome [SJS] and toxic epidermal necrolysis [TEN]) in Han Chinese, Thai, Malay, and Indian populations. However, B*1502 is neither predictive of SJS/TEN in Japanese or Caucasian populations (in whom HLA-A*3101 is a moderate risk factor) (McCormack et al., 2011) nor predictive of other carbamazepine-induced IDRs such as maculopapular exanthema or drug reaction with systemic symptoms (DRESS) in Asian populations (Alfirevic and Pirmohamed, 2010).

Hypersensitivity to abacavir is unusual for two reasons (Uetrecht and Naisbitt, 2013). First, an individual's HLA haplotype

(the expression of B*5701) is the dominant determining factor. In other words, sensitivity is observed in all subjects carrying the B*5701 allele. Second, the parent drug, which binds to the antigen-binding grove of B*5701, rather than a (reactive) metabolite, appears to be the precipitating factor. In other IDRs, especially drug-induced liver injury (DILI), there is evidence that a reactive metabolite triggers the idiosyncratic response by forming a protein adduct (hapten) that triggers an acquired immune response (activation of killer T cells and/or antibody-producing B lymphocytes). There is strong evidence, such as the production of antibodies against drug-modified proteins, that the idiosyncratic DILI caused by halothane (an anesthetic) and tienilic acid (a diuretic) is due to the formation of reactive metabolites, which is why, during the process of drug development, considerable attention is devoted to avoiding drug candidates that are converted to reactive metabolites that bind covalently to protein (see Point 12) (Obach et al., 2008; Bauman et al., 2009; Nakayama et al., 2009; Tang and Lu, 2010; Ikeda, 2011; Park et al., 2011; Walsh and Miwa, 2011). However, formation of reactive metabolites (which occurs in all individuals) is insufficient to account for the rarity of idiosyncratic DILI. For many drugs that cause idiosyncratic DILI, HLA haplotype is a factor but not to the extent seen with abacavir (Alfirevic and Pirmohamed, 2010; Uetrecht and Naisbitt, 2013).

In several cases, the drugs that cause idiosyncratic liver injury (those listed in Table 6-6) have closely related structures that do not cause idiosyncratic DILI. For example, rosiglitazone and pioglitazone contain the same thiazolidinedione ring that in troglitazone is converted to a reactive metabolite, and yet, of these three antidiabetic drugs, only troglitazone has been withdrawn from the market because of idiosyncratic hepatotoxicity. This difference may be due to the much larger daily dose of troglitazone (300 mg) compared with rosiglitazone (4 to 8 mg) and pioglitazone (15 to 45 mg). Uetrecht (2001) has proposed the general rule that drugs administered at a daily dose of 10 mg or less do not cause idiosyncratic toxicity, and this rule is consistent with the dosing information shown in Table 6-6. Both dose (more than 50 mg/day) and extent of hepatic metabolism (greater than 50%) are now recognized as risk factors for idiosyncratic drug-induced liver injury (Lammert et al., 2008; Chalasani and Bjornsson, 2010; Lammert et al., 2010). Other examples of closely related drugs where one does and one does not cause hepatotoxicity include tolcapone/entacapone, ticlopidine/clopidogrel, amineptine/tianeptine, tamoxifen/toremifene, ibufenac/ibuprofen, ebrotidine/famotidine, and niperotidine/ranitidine (Walgren et al., 2005). Labetalol (an antihypertensive drug) has two chiral centers and is a racemic mixture of four enantiomers, one of which is dilevalol. Labetrol carries a black box warming for hepatotoxicity; dilevalol was not approved by the FDA because it caused a high incidence of liver toxicity during clinical trials. In some cases, the difference is thought to be due to differences in dose (which is invariably higher for the drug partner that causes hepatotoxicity), but in other cases the basis for the difference is not known. Furthermore, more than 20 drugs (many of which do not cause idiosyncratic toxicity) bind covalently to their pharmacological target, including aspirin, omeprazole (and other proton pump inhibitors), clopidogrel, and second-generation inhibitors of EGFR-linked tyrosine kinase (such as afatinib, dacomitinib, and neratinib) (Singh et al., 2011).

Just as the acquired immune system can be activated by drugs that cause Type B toxicity (idiosyncratic toxicity), so the innate immune system can be activated by drugs that cause Type C (off-target) toxicity (Aronson and Ferner, 2003). For example, acetaminophen (Tylenol), which is a leading cause of acute liver failure in humans (Kaplowitz, 2005), is activated to a reactive metabolite (*N*-acetylbenzo-*p*-quinoneimine) that binds covalently to mitochondrial proteins, which leads to cell death. The cell damage (necrosis) caused by acetaminophen results in the release of cellular constituents such as fragmented DNA (and other molecules that make up the so-called danger-associated molecular pattern [DAMP]) that bind to toll-like receptors and other receptors that activate macrophages, which in turn recruit neutrophils and monocytes to the site of injury (Jaeschke et al., 2012). Whether activation of the innate immune system in this way is detrimental (by leading to a second round of hepatocellular damage) or beneficial (by limiting damage and promoting tissue repair) is controversial. In fact, the acute liver toxicity caused by acetaminophen is not an IDR (Uetrecht and Naisbitt, 2013). However, it is noteworthy that, in the case of Type C toxicity, the innate immune system is activated by cellular components released from damaged cells (DAMP) and not by the drug or its metabolites (although in the example given it is recognized that the reactive metabolite of acetaminophen plays a critical role in causing the cell damage that subsequently activates the innate immune system). This contrasts with the direct role of the drug and/or its metabolites (including reactive metabolites) in Type B toxicity. In the case of idiosyncratic toxicity, the drug/metabolite plays a key role in activating the acquired immune system.

Point 23 The same dose of drug (or any other xenobiotic) administered to humans often results in large interindividual differences in exposure to the parent compound (typically measured as the plasma AUC). This variation can reflect genetically determined differences in the activity of xenobiotic-biotransforming enzymes or transporters (genetic polymorphisms) or environmental factors, such as drug–drug interactions.

The study of the causes, prevalence, and impact of heritable differences in xenobiotic-biotransforming enzymes is known as *pharmacogenetics*. It is the basis for the concept of individualized or personalized medicine, whereby the dosage of drugs with a narrow therapeutic index (such as anticancer drugs) is adjusted in accordance with an individual's genotype (i.e., drug-metabolizing potential).

Genetic variation in a xenobiotic-metabolizing enzyme produces four discernible phenotypes: poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs), which arise from the expression of no functional alleles (−/−) for PMs, one nonfunctional allele and one partially functional allele or two partially active alleles (−/* or */*) for IMs, one or two functional alleles (+/−, +/*, or +/+) for EMs, and gene duplication ([+/+]*n*) for UMs. This traditional classification scheme has been revised on the basis of an activity score, which assigns to each allelic variant a functional activity value from one (for the wild-type or *1 allele) to zero (for any completely nonfunctional allele), as shown in Table 6-7 (Zineh et al., 2004; Gaedigk et al., 2008).

For those enzymes that are polymorphically expressed, such as CYP2D6, the incidence of the PM (and UM) genotype can vary widely from one ethnic group to the next. For example, the CYP2D6 PM genotype is common among Caucasians but not Asians, whereas the converse is true of the CYP2C19 PM genotype.

When the clearance of a drug is largely determined by a polymorphically expressed enzyme, such as CYP2D6, exposure to the parent drug follows the rank order: PMs > IMs > EMs > UMs. Exposure to metabolites (especially the maximum concentration, C_{max}) follows the opposite rank order. The difference in exposure between PMs and EMs (i.e., the fold increase in C_{max} or AUC associated with the PM phenotype vs. the EM phenotype) depends on fractional metabolism (fm), a measure of how much of the drug's clearance is determined by CYP2D6 (or whatever enzyme

Table 6-7

The Relationship between Genotype and Phenotype for the Polymorphically Expressed Enzyme CYP2D6 with Active (wt), Partially Active (*x), and Inactive (*y) Alleles

GENOTYPE	ALLELES	CONVENTIONAL	ACTIVITY SCORE*	ACTIVITY SCORE PHENOTYPE†
Duplication of active alleles ($n = 2$ or more)	(wt/wt)n	UM‡	$2 \times n$	UM
Two fully active wild-type (wt) alleles	wt/wt	EM	$1 + 1 = 2$	High EM
One fully active + one partially active allele	wt/*x	EM	$1 + 0.5 = 1.5$	Medium EM
One fully active + one inactive allele	wt/*y	EM	$1 + 0 = 1$	Low EM
Two partially active alleles	*x/*x	EM or IM	$0.5 + 0.5 = 1$	Low EM
One partially active + one inactive allele	*x/*y	IM	$0.5 + 0 = 0.5$	IM
Two inactive alleles	*y/*y	PM	$0 + 0 =$ zero	PM

**In the case of CYP2D6, various activity scores have been assigned as follows:*
*Activity score = 1.0 for each *1 (wt), *2, *35, and *41 [2988G].*
*Activity score = 0.75 for each *9, *29, *45, and *46.*
*Activity score = 0.5 for each *10, *17, and *41 [2988A].*
*Activity score = 0 for each *3, *4, *5, *6, *7, *8, *11, *12, *13, *14, *15, *19, *20, *21, *30, *36, *38, *40, *42, *68, *92, *100, and *101.*

†Activity scores are classified as follows:
*UM activity score = > 2.0 (e.g., [*1/*1]n where n is 2 or more gene duplications).*
*High EM activity score = 1.75 to 2.0 (e.g., *1/*1).*
*Medium EM activity score = 1.5 (e.g., *1/*17 or *9/*9).*
*Low EM activity score = 1.0 to 1.25 (e.g., *1/*4, *17/*17, or *9/*17).*
*IM activity score = 0.5 to 0.75 (e.g., *4/*9 or *4/*17).*
*PM activity score = 0 (e.g., *4/*4).*

*‡The phenotypes are ultrarapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM), and poor metabolizer (PM), based on the particular combination of alleles that are fully active (wt), partially active (*x), or inactive (*y).*

SOURCES: Data adapted from Gaedigk et al. (2008) and Zineh et al. (2004).

is responsible for metabolizing the drug in question) and the hepatic extraction ratio (E_H, the ratio of blood clearance to hepatic blood flow) (Kirby and Unadkat, 2010). For low-extraction drugs ($E_H < 0.3$), the fold increase in AUC (AUC_{PM}/AUC_{EM}) approximates to $1/(1 - fm)$; hence, the impact of the polymorphism increases dramatically as fm approaches unity. If CYP2D6 accounted for half of the clearance of a low-extraction drug (fm = 0.5), exposure to that drug would increase twofold in CYP2D6 PMs. However, if CYP2D6 accounted for 90% of a drug's clearance (fm = 0.9), then exposure to that drug would increase 10-fold in CYP2D6 PMs. This is why PMs are often at increased risk of ADEs either due to an exaggerated pharmacological response to the drug or due to its toxic side effects. An exception is omeprazole and other proton pump inhibitors, which actually provide improved control over gastroesophageal reflux disease (GERD) in CYP2C19 PMs compared with EMs (Furuta et al., 2005).

There are a minority of cases where the UMs, not the PMs, are at increased risk, especially in cases where the polymorphic enzyme catalyzes the formation of a pharmacologically active or toxic metabolite. For example, CYP2D6 converts the prodrug codeine to the active metabolite morphine, and there are reports of morphine intoxication in CYP2D6 UMs, one case being the death of a baby being breastfed by a CYP2D6 UM mother who was prescribed codeine (Gasche et al., 2004; Koren et al., 2006).

Many cases have now been described where the PM phenotype requires dosage adjustment to prevent off-target (Type C) toxicity or an exaggerated pharmacological response (Type A toxicity) (Aronson and Ferner, 2003). For example, CYP2D6 PMs are at increased risk for perhexiline hepatotoxicity and at increased risk for an exaggerated pharmacological response to debrisoquine and sparteine, three drugs that were not approved in the United States because of the high incidence of adverse events in the PMs. In contrast, when drugs are converted to active metabolites by CYP2D6, PMs derive inadequate therapeutic effect (Type F toxicity). For example, CYP2D6 converts codeine to morphine; hence, codeine is a less effective analgesic in PMs. Similarly, CYP2D6 converts the breast cancer drug tamoxifen to endoxifen (which is 30- to 100-fold more potent than tamoxifen in suppressing estrogen-dependent cell proliferation); hence, CYP2D6 PMs are at increased risk for breast cancer recurrence following tamoxifen adjuvant therapy (Goetz et al., 2005). Other examples of genetic polymorphisms that affect drug disposition in humans include CYP2C8 (repaglinide and paclitaxel), CYP2C9 (warfarin), CYP2C19 (clopidogrel, omeprazole, lansoprazole, and pantoprazole), CYP3A5 (tacrolimus), UGT1A1 (irinotecan), UGT2B7 (morphine), thiopurine methyltransferase (TPMT) (6-mercaptopurine), *N*-acetyltransferase 2 (NAT2) (isoniazid), catechol-*O*-methyltransferase (COMT) (levodopa), dihydropyrimidine dehydrogenase (DPD) (5-fluorouracil), the efflux transporter P-gp (digoxin), and the hepatic uptake transporters OATP1B1/3 (various statins) (Robert et al., 2005; Giacomini and Sugiyama, 2006; Relling and Giacomini, 2006). Details of these genetic polymorphisms are given later in the chapter. Many of the drugs identified within parentheses have a narrow therapeutic index. Some victim drugs have a high therapeutic index; hence, they are therapeutically effective in PMs to UMs and cause roughly the same incidence of adverse effects regardless of genotype. However,

in the case of victim drugs with a narrow therapeutic index, dosage must be increased in UMs to achieve a therapeutic effect and/or it must be decreased in PMs to prevent ADEs.

Genetic polymorphisms in xenobiotic-biotransforming enzymes have an impact on the incidence of certain environmental diseases. For example, ethnic differences in the incidence of polymorphisms in alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) impact the incidence of alcoholism (Li, 2000). Genetic polymorphisms in CYP2A6 impact the incidence of cigarette-smoking-induced lung cancer. Individuals lacking CYP2A6 are PMs of nicotine (so they tend to smoke less than CYP2A6 EMs) and are poor activators of tobacco-specific mutagens (so they form fewer DNA-reactive metabolites than do CYP2A6 EMs) (Kamataki et al., 2005).

Genetic polymorphisms can be the underlying cause of a disease. For example, the severe and mild hyperbilirubinemias associated with Crigler–Najjar syndrome and Gilbert syndrome, respectively, reflect a complete and partial loss of the UGT responsible for conjugating bilirubin, namely, UGT1A1. The hyperbilirubinemic Gunn rat is the rodent equivalent of Crigler–Najjar syndrome. Genetic polymorphisms in CYP1B1 are associated with primary congenital glaucoma.

Genetic polymorphisms have been described in laboratory animals. For example, laboratory-bred rabbits have about 50:50 chance of being a poor or rapid acetylator of certain drugs (such as isoniazid) because NAT2 is polymorphically expressed in rabbits. CYP1A2 is polymorphically expressed in dogs.

Point 24 Environmental factors can introduce as much variation in drug metabolism as can genetic factors, and this is especially true of drug–drug interactions. To take CYP3A4 as an example, PMs can be created pharmacologically with inhibitors (such as ketoconazole, erythromycin, and ritonavir), whereas UMs can be created pharmacologically with inducers (such as rifampin and the herbal agent St. John's wort). While the impact of genetic polymorphisms on drug disposition and safety is often identified during clinical trials, the impact of drug–drug interactions may not be identified until after the drug has been approved and is under postmarketing surveillance.

Drugs (and other xenobiotics) can be viewed from a *victim* and *perpetrator* perspective. A drug whose clearance is largely determined by a single route of elimination, such as metabolism by a single CYP enzyme, is considered a victim drug (also known as a sensitive or object drug). Such drugs have a high victim potential because a diminution or loss of that elimination pathway, either due to a genetic deficiency in the relevant CYP enzyme or due to its inhibition by another, concomitantly administered drug, will result in a large decrease in victim drug clearance and a correspondingly large increase in exposure (the magnitude of which will depend on fm, as indicated in Point 23). Perpetrators are those drugs (or other environmental factors) that inhibit or induce the enzyme that is otherwise responsible for clearing a victim drug. In other words, perpetrators are drugs that cause drug–drug interactions. Perpetrators are also known as precipitants. Genetic polymorphisms that result in the partial or complete loss of enzymatic activity can also be viewed as perpetrators inasmuch they cause a decrease in the clearance of—and an increase in exposure to—victim drugs. A combination of two perpetrators (such as two drugs that inhibit different enzymes or an inhibitory drug plus a genetic polymorphism) can have a dramatic effect on the disposition of a victim drug if they impair *parallel* pathways of clearance, a phenomenon called *maximum exposure* (Collins et al., 2006; Ogilvie and Parkinson, 2014). This is illustrated by voriconazole, whose metabolic clearance is determined by CYP2C19 and CYP3A4 (which represent two parallel pathways of clearance). Based on plasma AUC, CYP2C19 PMs (individuals lacking CYP2C19) are exposed to roughly twice as much voriconazole compared with CYP2C19 EMs. Loss of CYP3A4 (and only CYP3A4) by administering ritonavir to CYP2C19 EMs also causes about a doubling in systemic exposure to voriconazole. However, when ritonavir is administered to CYP2C19 PMs (such that *both* CYP2C19 and CYP3A4 are inactive), exposure to voriconazole increases more than 25-fold. This example illustrates how two drugs (ritonavir and voriconazole) can interact dramatically in some individuals (CYP2C19 PMs) but not others (CYP2C19 EMs). Conversely, a perpetrator drug that inhibited only CYP2C19 would cause such inhibition only in CYP2C19 EMs (the PMs having no enzyme to inhibit). Many other examples of perpetrator–perpetrator–victim interactions (maximum exposure) are reviewed in Ogilvie and Parkinson (2014).

Terfenadine (Seldane), cisapride (Propulsid), astemizole (Hismanal), and cerivastatin (Baycol) are all victim drugs, so much so that they have all been withdrawn from the market (Fung et al., 2001). The first three are all victim drugs because they are extensively metabolized by CYP3A4. Inhibition of CYP3A4 by various antimycotic drugs, such as ketoconazole, and antibiotic drugs, such as erythromycin, decreases the clearance of terfenadine, cisapride, and astemizole and increases their plasma concentrations to levels that, in some individuals, cause ventricular arrhythmias (QT prolongation) that can result in fatal heart attacks. Cerivastatin is extensively metabolized by CYP2C8. Its metabolism is inhibited by the glucuronide conjugates of gemfibrozil and clopidogrel (Ogilvie et al., 2006; Tornio et al., 2014). The coadministration of cerivastatin (Baycol) with either gemfibrozil (Lopid) or clopidogrel (Plavix) was associated with a high incidence of fatal, cerivastatin-induced rhabdomyolysis which resulted in the withdrawal of cerivastatin.

Mibefradil (Posicor) is the only drug withdrawn from the U.S. market largely because of its perpetrator potential. This calcium channel blocker not only causes extensive inhibition of CYP3A4 but also causes prolonged inhibition of the enzyme by virtue of being a metabolism-dependent inhibitor of CYP3A4. By inactivating CYP3A4 in an irreversible manner—such that restoration of normal CYP3A4 activity requires the synthesis of new enzyme—mibefradil inhibits CYP3A4 long after treatment with the drug is discontinued.

The in vitro technique of reaction phenotyping (also known as enzyme mapping) is the process of identifying which enzyme or enzymes are largely responsible for metabolizing a drug candidate (Williams et al., 2003a; Ogilvie et al., 2008; Zientek and Youdim, 2015; Bohnert et al., 2016). Reaction phenotyping allows an assessment of the victim potential of a drug candidate or other xenobiotic. Drug candidates can also be evaluated in vitro for their potential to inhibit or induce CYP enzymes (and selected UGT enzymes and transporters), which allows an assessment of their perpetrator potential (Tucker et al., 2001; Bjornsson et al., 2003; Huang et al., 2008; EMA, 2012; FDA, 2017).

Herbal products can also interact with drugs. For example, St. John's wort contains hyperforin, which is a potent PXR agonist and, as such, is an inducer of CYP3A4 (along with several other xenobiotic-metabolizing enzymes) and P-gp. To prevent a loss of therapeutic efficacy, the use of St. John's wort is not recommended for patients on antirejection drugs (such as cyclosporine and tacrolimus), anti-HIV drugs (such as indinavir and nevirapine), anticoagulants (such as warfarin and phenprocoumon), or oral contraceptive steroids (Pal and Mitra, 2006). Food can affect drug disposition in a number of ways. It can affect absorption, which is why some drugs are taken with meals and others are taken between meals. Large quantities of cruciferous vegetables (e.g., broccoli and Brussels

sprouts) can induce hepatic CYP1A2 (Conney, 1982), whereas grapefruit juice can inhibit intestinal CYP3A4 (Paine et al., 2006) and large quantities of orange and apple juice can inhibit the intestinal absorption of drugs by OATP2B1 and/or OATP1A2 (Dolton et al., 2012).

Point 25 Although drug–drug interactions can cause an increase in the incidence of adverse events or, in the case of induction, a loss of therapeutic efficacy, not all drug–drug interactions are undesirable. For example, some anti-HIV drugs, such as ritonavir, inhibit CYP3A4 and P-gp and thereby improve the pharmacokinetic profile of other anti-HIV drugs, such as saquinavir, lopinavir, and elitegravir. Cobicistat, an analog of ritonavir with no antiviral activity, is used with several anti-HIV drugs (atazanavir, darunavir, and tenofovir alafenamide) to enhance their systemic exposure by improving their oral absorption and decreasing their rate of elimination. Tumors often overexpress various transporters and GSTs, which limit the effectiveness of several anticancer drugs. Drugs that inhibit transporters and GST are being developed to enhance the efficacy of anticancer drugs.

Point 26 Although the amount of functional enzyme determines whether an individual is a PM, IM, EM, or UM, disease states and cofactor levels can impact the rate of xenobiotic metabolism. Not surprisingly, severe liver disease decreases the rate of metabolism of a large number of drugs whose clearance is determined by hepatic metabolism, as can kidney disease, which decreases the hepatic expression of CYP2D6 (Rostami-Hodjegan et al., 1999). For certain xenobiotic-metabolizing enzymes, particularly the conjugating enzymes, cofactor availability can impact the rate of xenobiotic biotransformation. The liver usually has sufficient NADPH to saturate reactions catalyzed by CYP, but it does not have sufficient levels of UDP-glucuronic acid (UDPGA) or 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to saturate the enzymes responsible for glucuronidating or sulfonating xenobiotics. Consequently, alterations in the levels of these cofactors can impact the rate or extent of glucuronidation and sulfonation. For example, fasting lowers hepatic levels of these cofactors, and fasting decreases the extent to which acetaminophen is conjugated. Consequently, fasting increases the extent to which acetaminophen is metabolized to a toxic metabolite by CYP (including CYP2E1, which is induced by fasting), for which reason fasting is suspected of being one of the risk factors for acetaminophen hepatotoxicity (Whitcomb and Block, 1994).

Point 27 Stereochemical aspects can play an important role in the interaction between a xenobiotic and its biotransforming enzyme (from both a substrate and an inhibitor perspective), and xenobiotic-biotransforming enzymes can play a key role in converting one stereoisomer to another, a process known as *mutarotation* or *inversion of configuration*.

A xenobiotic that contains a chiral center can exist in two mirror-image forms called stereoisomers or enantiomers. The biotransformation of some chiral xenobiotics occurs stereoselectively, meaning that one enantiomer (stereoisomer) is biotransformed faster than its antipode. For example, the antiepileptic drug Mesantoin®, which is a racemic mixture of *R*- and *S*-mephenytoin, is biotransformed stereoselectively in humans, such that the *S*-enantiomer is rapidly hydroxylated (by CYP2C19) and eliminated faster than the *R*-enantiomer. The ability of some chiral xenobiotics to inhibit xenobiotic-biotransforming enzymes can also occur stereoselectively. For example, quinidine is a potent inhibitor of CYP2D6, whereas quinine, its antipode, has relatively little inhibitory effect on this enzyme. In some cases, achiral molecules (or achiral centers) are converted to a mixture of enantiomeric metabolites, and this conversion may proceed stereoselectively such that one enantiomer is formed preferentially over its antipode. Inversion of configuration is the process whereby one enantiomer is converted to its antipode via an achiral intermediate. This interconversion can occur nonenzymatically, as in the case of thalidomide, or it can be catalyzed by a xenobiotic-metabolizing enzyme, as in the case of lisofylline, or by an endobiotic-metabolizing enzyme, as in the case of simvastatin (Fig. 6-6).

In the case of thalidomide, inversion of configuration (as shown in Fig. 6-6) occurs spontaneously, although the process is facilitated by albumin (Eriksson et al., 1998). In the mid-to-late 1950s, mainly in Europe, thalidomide was prescribed to pregnant women in the first trimester to treat morning sickness. Unfortunately, while (*R*)-thalidomide is an effective sedative, the (*S*)-enantiomer is a teratogen that produces phocomelia (limb shortening) and other congenital defects in the offspring largely as a result of its ability to inhibit angiogenesis (vasculogenesis). The teratogenic effect of thalidomide cannot be circumvented by administering only the (*R*)-enantiomer because spontaneous or albumin-facilitated racemization quickly produces the teratogenic (*S*)-enantiomer. Thalidomide blocks the release of tumor necrosis factor-α (TNF-α) and it has been approved for the treatment of erythema nodosum leprosum (an acute inflammatory reaction associated with lepromatous leprosy), and is useful in the treatment of several other inflammatory conditions and immune-mediated diseases. Because of its ability to inhibit angiogenesis (the process that supplies tumors with new blood vessels), thalidomide is also under investigation as an anticancer drug. It is ironic that a drug with antivascular side effects was originally prescribed as a sedative and was subsequently approved for the treatment of a vascular disease (leprosy) with sedation as a side effect (Franks et al., 2004). Thalidomide (Thalomid) plus dexamethasone is also approved for the treatment of multiple myeloma.

Ketones can be reduced by carbonyl reductases to a mixture of enantiomeric secondary alcohols, and this can occur with a high degree of stereoselectivity. For example, pentoxifylline is reduced by carbonyl reductases in blood and liver to a mixture of secondary alcohols with the major metabolite having an *S*-configuration, as shown in Fig. 6-6. Interestingly, the minor metabolite, a secondary alcohol with the *R*-configuration, has pharmacological properties distinct from those of its *S*-antipode and its ketone precursor pentoxifylline. This minor metabolite is known as lisofylline, which was once under clinical investigation as a possible treatment for various inflammatory diseases and type 1 diabetes. Through the action of carbonyl reductase, lisofylline is oxidized to pentoxifylline and then reduced to its antipode (i.e., *R*-alcohol → ketone → *S*-alcohol), the net result being an inversion of configuration (Lillibridge et al., 1996a). The same type of interconversion explains why the administration of pure *R*-albuterol to human volunteers results in the formation of *S*-albuterol, just as the administration of pure *S*-albuterol leads to the formation of *R*-albuterol (Boulton and Fawcett, 1997).

In the case of simvastatin (which contains a β-hydroxylactone), the interconversion of two secondary alcohols (β-hydroxylactone ↔ α-hydroxy-lactone) involves hydrolysis of the lactone ring (by paraoxonase 3 [PON3] in humans) followed by formation of an acyl-CoA thioester with CoA, the first step in the β-oxidation of fatty acids, followed by dehydration to an achiral intermediate. Reversal of the steps by a combination of hydrolysis and lactonization (condensation) restores the lactone ring with the hydroxyl group in the original β-configuration or in the opposing α-configuration. In humans, both the initial hydrolysis of the lactone ring and the final lactonization (condensation) reaction are catalyzed by PON3 (Draganov and La Du, 2004). Conversion to an acyl-CoA thioester also plays a role in the chiral inversion of ketoprofen. In this

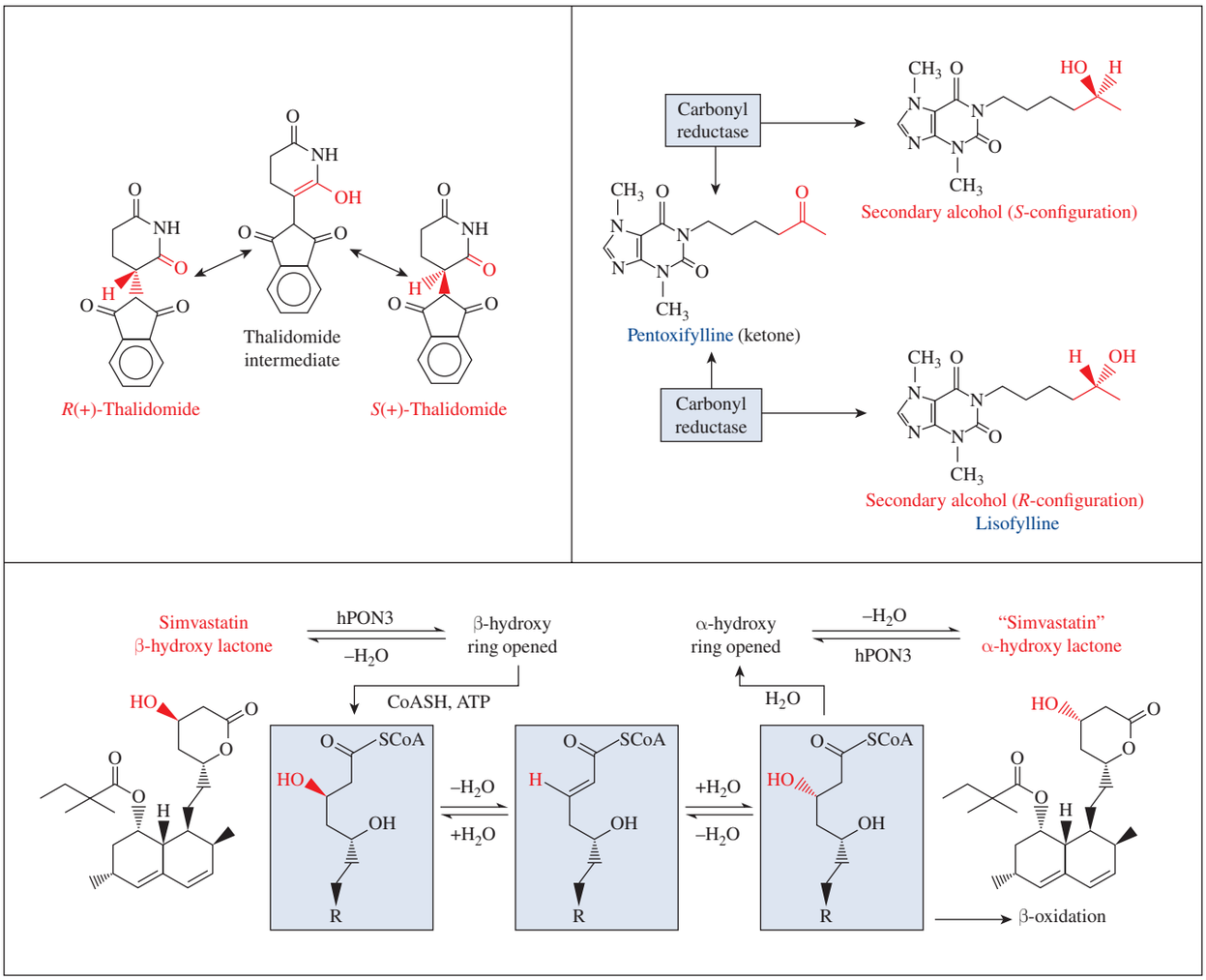


Figure 6-6. *Stereochemical aspects of xenobiotic biotransformation: inversion of configuration by nonenzymatic means (thalidomide), by carbonyl reductase (lisofylline), and by hydrolysis and condensation (lactonization) of a lactone ring (simvastatin).*

case, only the *R*-enantiomer is converted to an acyl-CoA thioester, which isomerizes and undergoes hydrolysis to the *S*-enantiomer. Although both *R*- and *S*-ketoprofen are converted to acyl glucuronides, the former is hydrolyzed twice as quickly as the latter, which further contributes to the accumulation of the *S*-enantiomer (Regan et al., 2010).

Atropisomers are stereoisomers arising because of hindered rotation about a single bond. Polyhalogenated biphenyls (PCBs and PBBs) with two or more halogens in the *ortho* position (adjacent to the bond connecting the two phenyl rings) are atropisomers. Telenzepine is an example of a drug with atropisomers (Clayden et al., 2009).

Point 28 Mass spectrometry is widely used to characterize the structure of metabolites, and many instruments now come equipped with software to assist in this process, based on the fact that certain xenobiotic reactions are associated with discrete changes in mass (Holcapek et al., 2008). For example, the loss of 2 mass/charge (m/z) units signifies dehydrogenation, whereas the loss of 14 m/z units usually signifies demethylation ($-CH_2$). Several reactions result in an increase in mass, including reduction (+2 m/z units = 2H), methylation (+14 m/z units = CH_2), oxidation (+16 m/z units = O), hydration (+18 m/z units = H_2O), acetylation (+42 m/z units = C_2H_2O), glucosidation (+162 m/z units = $C_6H_{10}O_5$), sulfonation (+80 m/z units = SO_3), glucuronidation (+176 m/z units = $C_6H_8O_6$), carbamoyl glucuronidation (+220 m/z units = $C_7H_8O_8$), and conjugation with GSH (+305 m/z units = $C_{10}H_{15}N_3O_6S$), glycine (+57 m/z units = C_2H_3NO), taurine (+107 m/z units = $C_2H_5NO_2S$), and glutamine (+107 m/z units = $C_5H_7NO_3$) (Holcapek et al., 2008). Conjugation of acidic drugs with CoA (to form acyl-CoA thioesters) increases mass by 749 m/z units, but these conjugates are not transported out of cells and, hence, are not detected in blood, bile, or urine.

Occasionally, routine changes in mass can arise from unexpected reactions. For example, ziprasidone is converted to two metabolites, each of which involves an increase of 16 m/z units, which normally indicates addition of oxygen (e.g., hydroxylation, sulfoxidation, *N*-oxygenation). One of the metabolites is indeed formed by addition of oxygen to ziprasidone (sulfoxidation), as shown in Fig. 6-7 (Beedham et al., 2003). However, the other metabolite is formed by a combination of reduction (+2 m/z units) and methylation (+14 m/z units). Therefore, nominal changes in mass can sometimes leave in doubt the biotransformation event that led to metabolite formation, which accounts for the popularity of accurate or high-resolution mass spectrometry, a technique that can

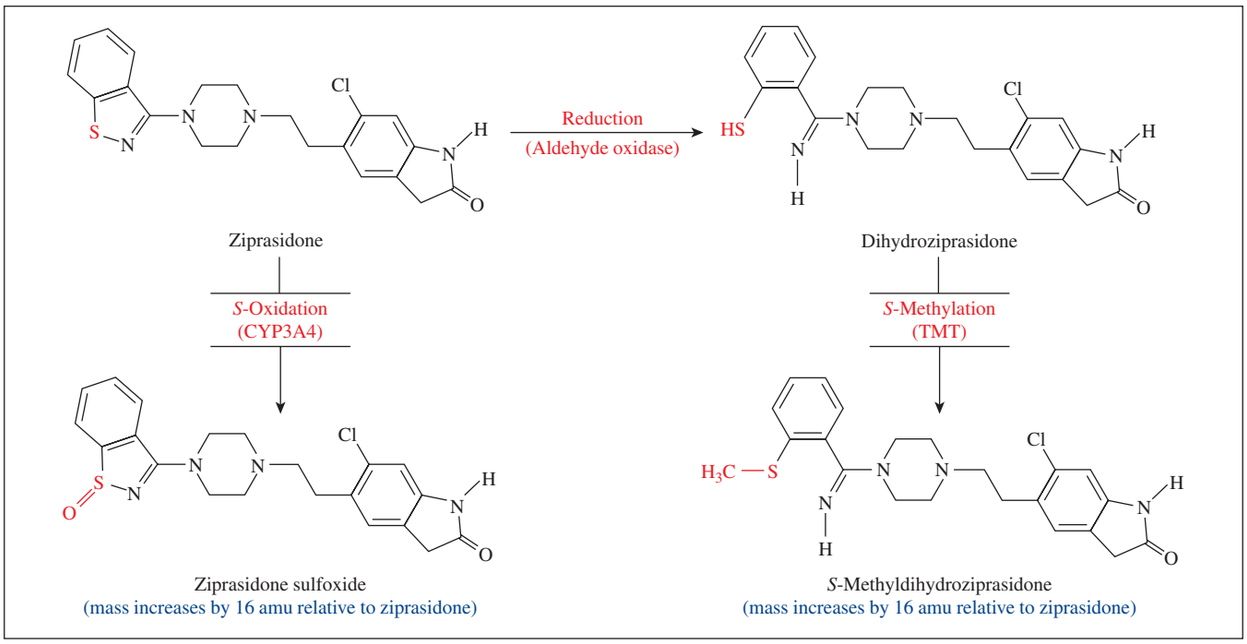


Figure 6-7. *Conversion of ziprasidone to two different metabolites both involving a mass increase of 16 m/z (relative to ziprasidone).*

distinguish, in the case of ziprasidone, for example, oxidation from a combination of reduction and methylation.

Mass spectrometry can typically provide information on which region of a molecule has undergone biotransformation, but in some cases it cannot distinguish between several closely related possibilities. For example, based on mass spectrometry alone, it might be possible to ascertain that a certain phenyl group has been hydroxylated. However, analysis by nuclear magnetic resonance (NMR) may be required to ascertain whether the hydroxylation occurred at the *ortho*, *meta*, or *para* position.

Point 29 As mentioned in Point 19, xenobiotic-biotransforming enzymes are widely distributed throughout the body. The advent of high-throughput assays based on quantitative real-time reverse-transcription polymerase chain reaction (QPCR) has facilitated comprehensive measurements of the levels of mRNA encoding numerous xenobiotic-biotransforming enzymes, transporters, and xenosensors in a wide range of human tissues (Nishimura et al., 2003; Furukawa et al., 2004; Nishimura et al., 2004; Nishimura and Naito, 2005, 2006). These data are very informative as far as mRNA expression is concerned, but it can be difficult to draw conclusions about the activity of a given enzyme in a given tissue based on mRNA levels alone. For instance, based on mRNA levels in the human liver, the levels of the major xenobiotic-biotransforming CYP enzymes follow the rank order: CYP2E1 > 2A6 > 2C8 > 2C18 ≈ 1A2 ≈ 4A11 ≈ 2C9 ≈ 3A4 (Nishimura et al., 2003). This contrasts substantially from the levels of CYP protein (as determined by LC–MS/MS), which follow the rank order: CYP2C9 > 3A4 ≈ 2E1 ≈ 2A6 > 2C8 > 1A2 > 2D6 > 2B6 ≈ 2C19 > 3A5 ≈ 3A43 ≈ 3A7 (Kawakami et al., 2011; Ohtsuki et al., 2012). In the case of CYP2E1, hepatic mRNA levels are more than 17 times higher than the levels of CYP3A4 mRNA, but CYP2E1 protein levels in microsomes are comparable to those of CYP3A4. This discrepancy is due to the fact that, under normal conditions, most of the mRNA encoding CYP2E1 is sequestered in the cytoplasm and is not available for translation (Gonzalez, 2007). The mRNA for FMO2 is present at very high levels in the human lung, but it is not translated into functional enzyme due to the presence of a truncation mutation in Caucasians and Asians. However, about 33% of sub-Saharan Africans, 26% of African Americans, 7% of Puerto Ricans, and 2% of Mexicans have one normal allele and express a functional protein (Cashman and Zhang, 2006; Veeramah et al., 2008). Human CYP2A7 and CYP4B1 are full-length genes that encode enzymes incapable of incorporating heme and are therefore nonfunctional.

Mass spectrophotometric techniques (LC–MS/MS) for the quantitative analysis of proteins have been applied to drug-metabolizing enzymes and transporters (Kawakami et al., 2011; Sakamoto et al., 2011; Ohtsuki et al., 2012; Shrivas et al., 2013). They will undoubtedly play an important role in examining the tissue distribution and subcellular location of the large diversity of variant forms of drug-metabolizing enzymes and transporters that arise from alternative splicing of DNA transcripts and pre-mRNA, which can produce both functional variants and catalytically inactive proteins of unknown function (Annalora et al., 2017). Such diversification is not unique to drug-metabolizing enzymes and transporters, as evidenced by the observation that about 22,000 genes in the human genome produce a proteome with over 200,000 distinct proteins (Annalora et al., 2017).

HYDROLYSIS, REDUCTION, AND OXIDATION

Hydrolysis

Mammals contain a variety of enzymes that hydrolyze xenobiotics containing such functional groups as a carboxylic acid ester (delapril and procaine), amide (procainamide), thioester (spironolactone), carbamate (irinotecan), phosphoric acid ester (paraoxon), acid anhydride (diisopropylfluorophosphate [DFP]), lactone (lovastatin and simvastatin), and thiolactone (erdosteine), most of which are shown in Figs. 6-8 and 6-9. The major hydrolytic

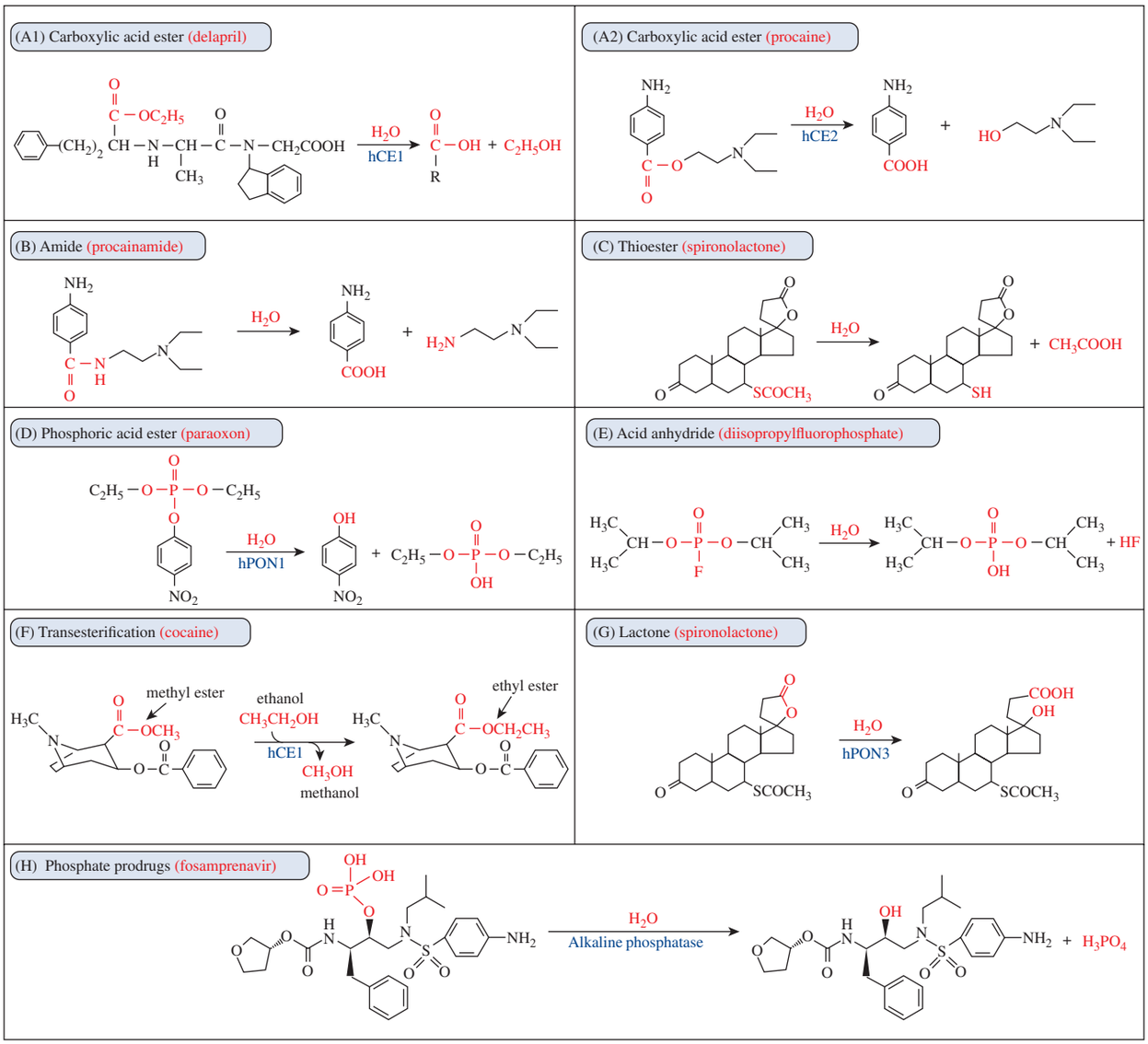


Figure 6-8. *Examples of reactions catalyzed by carboxylesterases, cholinesterases, organophosphatases, and alkaline phosphatase. CES1 and CES2, human carboxylesterases 1 and 2; PON1 and PON3, human paraoxonase 1 and 3.*

enzymes are the carboxylesterases, cholinesterases, and paraoxonases (for which lactonase is a more encompassing name), but they are by no means the only hydrolytic enzymes involved in xenobiotic biotransformation. The first two classes of hydrolytic enzymes, the carboxylesterases and cholinesterases, are known as serine esterases because their catalytic site contains a nucleophilic serine residue that participates in the hydrolysis of various xenobiotic and endobiotic substrates and the stoichiometric (one-to-one) binding of organophosphorus (OP) compounds and other cholinergic neurotoxins.

Approximately 100 human genes encode serine hydrolases that are classified as esterases, amidases, thioesterases, lipases, peptidases, or proteases (Evans and Cravatt, 2006; Ross and Crow, 2007; Testa and Krämer, 2008, 2010). The active-site serine residue of carboxylesterases, cholinesterases, and other serine hydrolases becomes phosphorylated (or phosphonylated) by OP compounds, such as those used as insecticides, herbicides, fungicides, nematicides, and plant growth regulators. Binding of OP compounds to carboxylesterases, cholinesterases, and other targets, some of which have been identified as receptors and enzymes involved in the hydrolysis of endobiotics (Casida and Quistad, 2005), plays a key role in limiting the binding of OP compounds to acetylcholinesterase (AChE). Phosphorylation of AChE, which hydrolyzes acetylcholine and thereby terminates its neurotransmitter activity, is the principal mechanism of OP toxicity in mammals, insects, and nematodes, with 70% to 90% inhibition usually proving lethal. Reversal of this phosphorylation event with pyridinium oximes such as pralidoxime (2-PAM) and obidoxime (toxogonin) is one of the strategies to treat OP poisoning (Casida and Quistad, 2005).

In the presence of an alcohol, carboxylesterases and certain other hydrolytic enzymes can catalyze the transesterification of xenobiotics, which accounts for the conversion of cocaine (a methyl ester) to ethylcocaine (the corresponding ethyl ester)

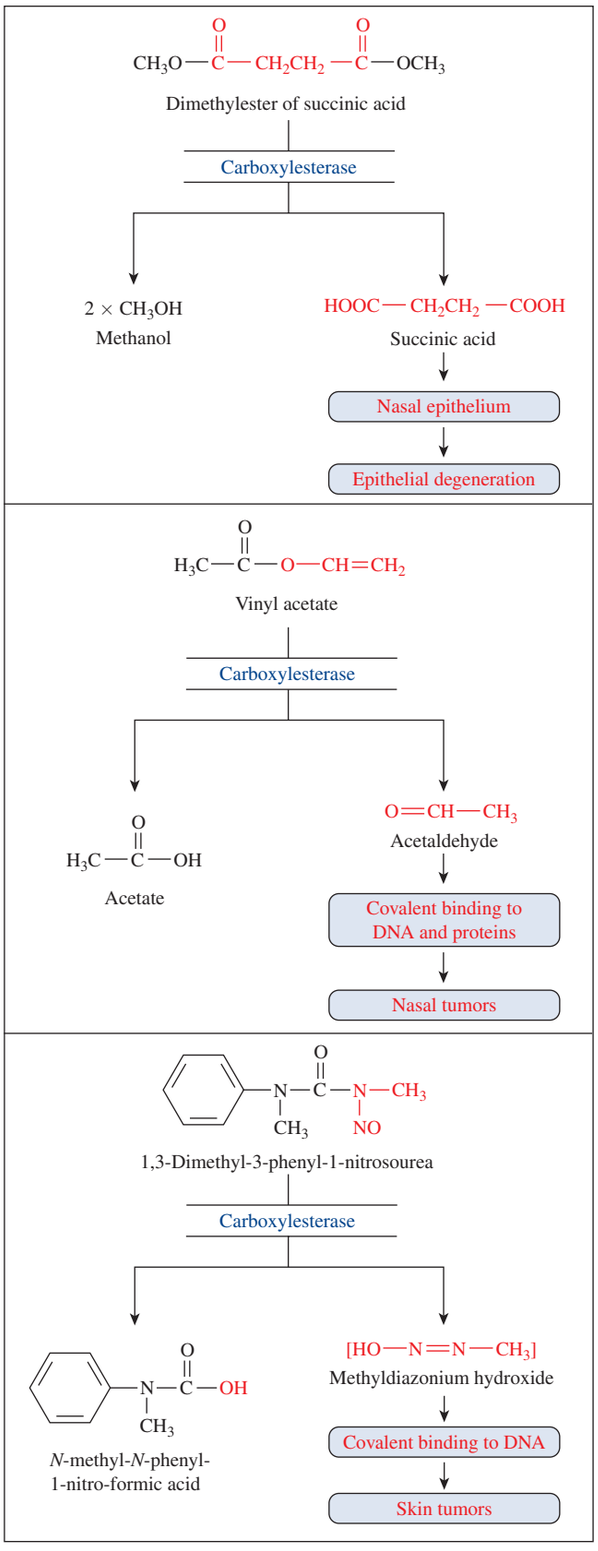


Figure 6-9. *Activation of xenobiotics to toxic and tumorigenic metabolites by carboxylesterases.*

(Fig. 6-8). The same transesterification occurs with clopidogrel, which is converted from a methyl to an ethyl ester (Tang et al., 2006). Transesterification occurs when ethanol, not water, cleaves the catalytic transition state, that is, the esteratic bond between the active serine residue on the enzyme and the carbonyl group on the xenobiotic:

$$\text{Enzyme}-\text{O}-\text{CO}-\text{R} + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{Enzyme} - \text{OH} + \text{CH}_3\text{CH}_2 - \text{O} - \text{CO} - \text{R}$$

In humans, the hydrolysis of xenobiotics (including many prodrugs) is largely catalyzed by microsomal carboxylesterases in liver (CES1 and CES2) and intestine (CES2), and by cholinesterases, paraoxonases, and albumin in blood (some of these enzymes are present in plasma; others are bound to erythrocytes in a species-dependent manner) (Li et al., 2005). Compared with many other mammalian species humans and monkeys are unusual because they lack a plasma carboxylesterase (Li et al., 2005; Oda et al., 2015). However, human erythrocytes contain esterase D, a carboxylesterase used as a genetic marker for retinoblastoma (Wu et al., 2009). In humans, enzymes other than those mentioned above can be involved in xenobiotic hydrolysis (Oda et al., 2015). These so-called orphan esterases include:

1. α/β hydrolase domain containing 10 (ABHD10), which hydrolyzes the acyl glucuronides of mycophenolic acid and probenecid;
2. Acylpeptide hydrolase (APEH), which hydrolyzes the acyl glucuronide of valproic acid;
3. Biphenyl hydrolase-like protein (BPHL or valacyclovirase), which hydrolyzes the valine ester prodrugs of acyclovir (valacyclovir) and gangciclovir (valgangciclovir) as well as nucleoside drugs such as zidovudine, floxuridine, and gemcitabine;
4. Carboxymethylenebutenolidase homolog (CMBL), which hydrolyzes olmesartan, medoxomil, faropenen, and lenampicillin;
5. Sialic acid 9-*O*-acetylesterase (SIAE), which hydrolyzes alacepril;
6. Platelet-activating factor acetylhydrolase (PAFAH), which hydrolyzes aspirin;
7. Aldehyde dehydrogenase (ALDH), which hydrolyzes nitroglycerin.

Albumin, lipases, peptidases, proteases, and carbonic anhydrases have also been shown to have hydrolytic (esteratic) activity toward various xenobiotics. CYP can catalyze the cleavage of certain xenobiotics containing a carboxylic acid ester, phosphoric acid ester, or carbamate (see the section "Cytochrome P450" for examples).

In general, esters are hydrolyzed more rapidly than amides, which can impact the duration and site of action of drugs. For example, procaine, a carboxylic acid ester, is rapidly hydrolyzed, which is why this drug is used mainly as a local anesthetic. In contrast, procainamide, the amide analog of procaine, is hydrolyzed much more slowly; hence, this drug reaches the systematic circulation, where it is useful in the treatment of cardiac arrhythmia. In general, enzymatic hydrolysis of amides occurs more slowly than esters, although electronic factors can influence the rate of hydrolysis. The presence of electron-withdrawing substituents weakens an amide bond, making it more susceptible to enzymatic hydrolysis.

The hydrolysis of xenobiotics by carboxylesterases and other hydrolytic enzymes is not always a detoxication process. Fig. 6-9 shows some examples in which carboxylesterases convert xenobiotics to toxic and tumorigenic metabolites.

In 1953, Aldridge classified hydrolytic enzymes on the basis of their interaction with OP compounds, classifying those that hydrolyze OP compounds as A-esterases, those that are inhibited

by OP compounds as B-esterases, and those that do not interact with OP compounds as C-esterases (Aldridge, 1953). Although the terms are still used, the classification system of Aldridge can be somewhat confusing because it divides the paraoxonases into the A- and C-esterase classes: the human paraoxonase PON1 hydrolyzes OP compounds and so can be classified as an A-esterase, whereas PON2 and PON3 can be classified as C-esterases because they neither hydrolyze OP compounds nor, in most cases, are inhibited by them. Furthermore, carboxylesterases and cholinesterases, two distinct classes of hydrolytic enzymes, are both B-esterases according to Aldridge because both are inhibited by OP compounds.

Carboxylesterases Carboxylesterases are predominantly microsomal enzymes (about 60 kDa glycoproteins) that are present in liver, intestine, kidney, and a wide variety of other tissues. As shown in Table 6-8, humans, monkeys, and dogs express two carboxylesterases (CES1 and CES2) in a tissue-specific manner, whereas rats and mice express 10 and 11 carboxylesterase (Ces) enzymes, respectively, and only rodents express a carboxylesterase (Ces1c) in plasma (Oda et al., 2015). Based on LC–MS/MS (proteomic) analysis, the levels of CES1 in 16 samples of human liver microsomes (171 to 801 pmol/mg) were much greater than those of CES2 (16.3 to 57.2 pmol/mg) (Sato et al., 2012). Interindividual variation of both enzymes was low (less than fivefold) and the levels of CES1 were negatively correlated with the levels of CES2. Both enzymes were also detected in human liver cytosol at roughly one-seventh the concentration in microsomes (Sato et al., 2012).

Human CES1 prefers to hydrolyze xenobiotics with a small alcohol leaving group (large acid–small alcohol), whereas CES2 prefers to hydrolyze xenobiotics with a large alcohol leaving group (small acid–large alcohol). This is illustrated in Fig. 6-8 for the hydrolysis of delapril by CES1 (which releases ethanol, a small alcohol) and the hydrolysis of procaine by CES2 (which releases 2-hydroxytriethylamine, a large alcohol). In the case of cocaine (the structure of which is shown in Fig. 6-8), the methyl ester is hydrolyzed by CES1 (to release a small alcohol), whereas the benzoic ester is hydrolyzed by CES2 (to release a large alcohol). CES1 also catalyzes the transesterification of the methyl ester of cocaine, as shown in Fig. 6-8. CES1 is more active than CES2 at catalyzing the hydrolysis of oseltamivir, benazepril, cilazepril, quinapril, temocapril, imidapril, meperidine, delapril, capecitabine, methylphenidate, and clopidogrel, whereas CES2 is more active than CES1 at hydrolyzing aspirin, flutamide, prasugrel, heroin, cocaine benzoyl ester, 6-acetylmorphine, oxybutynin, and the anticancer drug irinotecan (also known as CPT-11) (Satoh and Hosokawa, 2006; Shi et al., 2006; Tang et al., 2006; Oda et al., 2015). Individual pyrethroids such as *trans*-permethrin represent an example of xenobiotics that are hydrolyzed by both CES1 and CES2 (Ross and Crow, 2007).

In addition to hydrolyzing xenobiotics, carboxylesterases hydrolyze numerous endogenous compounds, such as long- and short-chain acyl-glycerols (both monoacylglycerols and diacylglycerols), long-chain acyl-carnitine, long-chain acyl-CoA thioesters (e.g., palmitoyl-CoA), retinyl ester, platelet-activating factor, and other esterified lipids. Carboxylesterases can also catalyze the synthesis of fatty acid ethyl esters, which represents a nonoxidative pathway of ethanol metabolism in adipose and certain other tissues. In the case of platelet-activating factor, carboxylesterases catalyze both the deacetylation of PAF and its subsequent esterification with fatty acids to form phosphatidylcholine (Satoh and Hosokawa, 1998).

In addition to CES1 and/or CES2, human liver and intestinal microsomes contain another serine hydrolase known as AADAC, which stands for arylacetamide deacetylase. This enzyme catalyzes the hydrolysis of phenacetin (a discontinued drug), 2-acetylaminofluorene (2-AAF, a carcinogen), and flutamide (an antiandrogen drug) (Watanabe et al., 2010). AADAC deacetylates phenacetin and 2-aminofluorene (2-AF) to aromatic amines that can be *N*-hydroxylated by CYP and then conjugated to form reactive metabolites. Examples of this type of metabolic activation are given later in the sections "Azo- and Nitro-Reduction" and "Conjugation"). Due to risk of hepatotoxicity, the antifungal drug ketoconazole is no longer recommended for use as a CYP3A inhibitor in clinical studies designed to assess the role of CYP3A in the disposition of a drug. AADAC catalyzes the first step in the metabolic activation of ketoconazole (Fukami et al., 2016). In subsequent steps, desacetylated ketoconazole is oxidized by FMO to an hepatotoxic nitrone and/or dialdehyde, as shown in Fig. 6-10 (Foti and Dalvie, 2016).

CES1 (the major liver form, which is also expressed in lung and other tissues) and CES2 (the major intestinal form, which is also expressed in liver, kidney, and brain) represent two of the six families of human carboxylesterases (Satoh and Hosokawa, 2006; Holmes et al., 2010; Merali et al., 2014). The other enzymes are CES3 (expressed in brain, liver, and colon), CES4A (previously called CES6 or CES8, which is expressed in brain, lung, and kidney), CES5A (previously called CES7, which is expressed in brain, lung, and testis), and CES6 (which is expressed in brain cerebellum).

Table 6-8

Tissue Distribution of Carboxylesterase (CES) Enzymes in Various Species

SPECIES (NUMBER OF FORMS)	TISSUE DISTRIBUTION OF CARBOXYLESTERASES (CES)				
	LIVER	SMALL INTESTINE	KIDNEY	LUNG	PLASMA
Human ($n = 2$)	CES1, CES2	CES2	CES2	CES1	–
Cynomolgus monkey ($n = 2$)	CES1, CES2	CES1, CES2	CES1, CES2	?	–
Dog ($n = 2$)	CES1, CES2	–	CES1, CES2	CES1, CES2	–
Rat ($n = 10$)	Ces1c, 1d, 1e, and 1f	Ces1e and 1f	Ces1e and 1f	Ces1d	Ces1c
	Ces2a, 2c, 2e, & 2g	Ces2a, 2c, and 2h	Ces2g		
Mouse ($n = 11$)	Ces1c, 1d, 1e, 1f, and 1g	Ces1d, 1e, 1f, and 1g	Ces1d, 1e, 1f, and 1g	Ces1d	Ces1c
	Ces2a, 2c, and 2e	Ces2a, 2b, 2c, 2e, and 2g	Ces2c		

SOURCE: Table adapted from Table 6-9 in Oda et al. (2015).

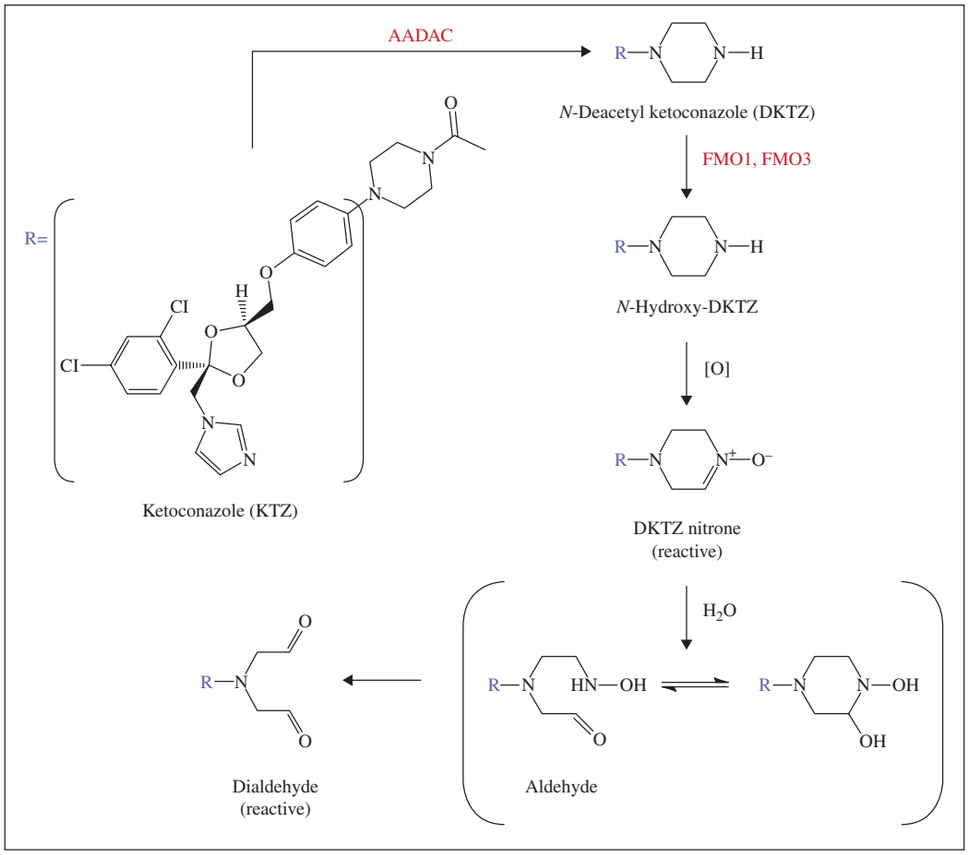


Figure 6-10. *Role of arylacetamide deacetylase (AADAC) and flavin monooxygenase (FMO) in the activation of ketoconazole (KTZ) to hepatotoxic metabolites.*

The human gene encoding CES1 (also known as CES1A1) is adjacent to a duplicated segment of CES1 called CES1P1. Despite a high degree of sequence homology, this pseudogene does not encode a functional carboxylesterase due to the presence of stop codon at exon 3. In some individuals, the nonfunctional CES1P1 gene is replaced with hybrid gene composed partly of CES1P1 (the 5′ end and exon 1) and mostly of CES1A1, which eliminates the premature stop codon at exon 3 and encodes a functional enzyme. This hybrid gene is variously called CES1P1 variant, CES1P1VAR or CES1A2, to distinguish it from the original copy of CES1, namely CES1A1 (Rasmussen et al., 2017). This variant form of CES1 (in addition to CES1A1) is expressed in 4.4% of Caucasians, 5.1% of African Americans, and 31.3% of Japanese subjects (Oda et al., 2015). Two variants of CES1A1 have been described in which exon 1 of CES1A1 is replaced by exon 1 from CES1P1 (with or without its flanking sequences). These variants are called CES1A1b and CES1A1c to distinguish them from the "original" form designated CES1A1a (Rasmussen et al., 2017).

Genetic polymorphisms (single-nucleotide polymorphisms or SNPs) that affect carboxylesterase activity or expression levels underscore the importance of CES1 and CES2 in drug metabolism (Merali et al., 2014). Genetic polymorphisms of CES1 (rs71647871, rs71647872, and rs1219127770) affect the disposition of methylphenidate (Ritalin, a methyl ester) and the antiviral prodrug oseltamivir (an ethyl ester) (Zhu and Markowitz, 2009). Dabigatran etexilate, an anticoagulant with a narrow therapeutic index, is a substrate for CES1. A genome-wide association study (GWAS) identified an allelic variant of CES1 (rs2244613) in roughly one-third of patients on long-term anticoagulation therapy which was associated with lower exposure to dabigatran (the active metabolite formed by CES1) and a lower risk of bleeding (a consequence of excessive anticoagulation) (Pare et al., 2013). A phenotype for CES1 that might be classified as high EM has been described. It arises from a SNP (rs3785161) in the promoter region of CES1A2 (but not CES1A1) that increases the expression of CES1 and thereby increases the rate of hydrolysis of imidapril to its active metabolite imidaprilat, an angiotensin-converting enzyme (ACE) inhibitor, which increases its antihypertensive effect (Geshi et al., 2005). Genetic polymorphisms of CES2 (rs72547531 and rs72547532) affect the disposition of the anticancer drug irinotecan (CPT-11, a carbamate), which is converted by CES2 to the active metabolite SN-38, a topoisomerase inhibitor (Kubo et al., 2005). However, genetic polymorphisms of UGT1A have a greater impact on the disposition and toxicity of irinotecan, as detailed in the section "Glucuronidation and Formation of Acyl-CoA Thioesters."

Certain carboxylesterases also have a physiological function in anchoring other proteins to the endoplasmic reticulum. For example, the lysosomal enzyme β-glucuronidase is also present in the endoplasmic reticulum, where it is anchored in the lumen by egasyn, a microsomal carboxylesterase designated Ces1e in mouse and rat (Holmes et al., 2010). In rodents, egasyn binds to β-glucuronidase at its active-site serine residue, which effectively abolishes the carboxylesterase activity of egasyn, although there is no corresponding loss of β-glucuronidase activity. In humans, β-glucuronidase binds to egasyn but at a site distinct from its active site (Islam et al., 1999). Binding of OP compounds to egasyn causes

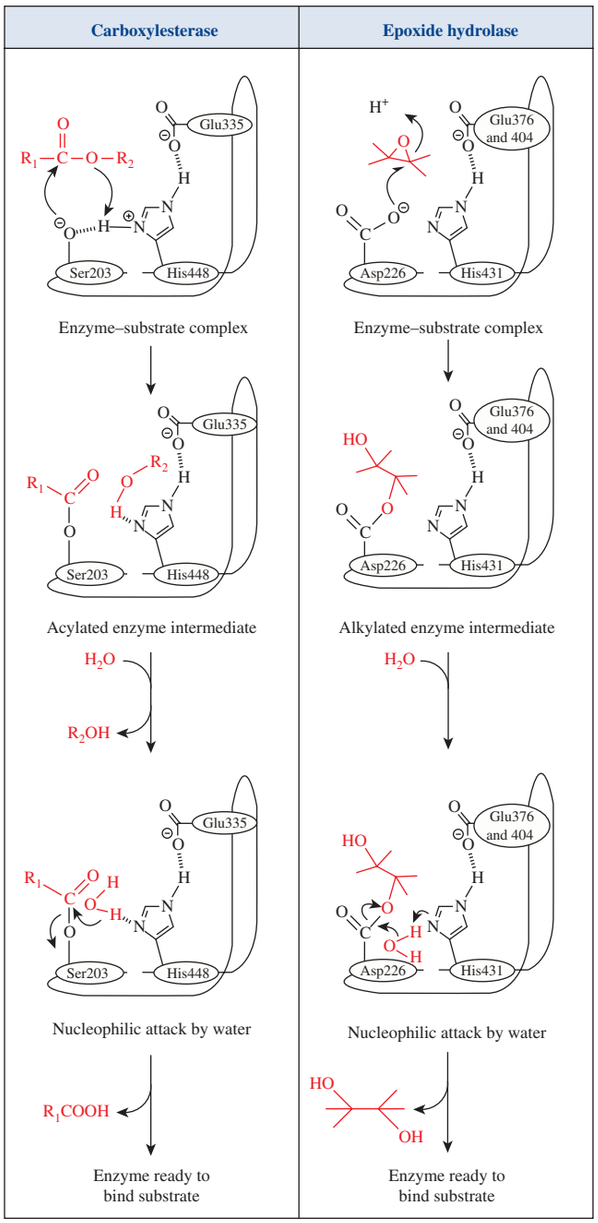


Figure 6-11. *Catalytic cycle of microsomal carboxylesterase (left) and microsomal epoxide hydrolase (right), both of which are α/β-hydrolase fold enzymes.*

the release of β-glucuronidase into plasma, which serves as the basis for a test for OP exposure (Fujikawa et al., 2005). The retention of β-glucuronidase in the lumen of the endoplasmic reticulum is thought to be physiologically significant. Glucuronidation by microsomal UGTs is a major pathway in the clearance of many of the endogenous aglycones (such as bilirubin) and xenobiotics (such as drugs). However, hydrolysis of glucuronides by β-glucuronidase complexed with egasyn in the lumen of the endoplasmic reticulum appears to be an important mechanism for recycling endogenous compounds, such as steroid hormones (Dwivedi et al., 1987). The acute-phase response protein, C-reactive protein, is similarly anchored in the endoplasmic reticulum by egasyn.

The mechanism of catalysis by carboxylesterases is analogous to the mechanism of catalysis by serine proteases. In the case of carboxylesterases, it involves charge relay among a catalytic triad comprising an acidic amino acid residue (glutamate [Glu_{335}]), a basic residue (histidine [His_{448}]), and a nucleophilic residue (serine [Ser_{203}]) (Yan et al., 1994; Satoh and Hosokawa, 1998). (These amino acid residues, numbered for a rat carboxylesterase, differ slightly in other species, but the overall location and function of these residues are the same in all mammalian carboxylesterases.) The mechanism of catalysis of carboxylesterases is shown in Fig. 6-11, and is discussed in more detail in the section "Epoxide Hydrolases." OP compounds bind to the nucleophilic OH group on the active-site serine residue to form a phosphorus–oxygen bond, which is not readily cleaved by water. Therefore, OP compounds bind stoichiometrically to carboxylesterases and inhibit their enzymatic activity, for which reason they are also classified as B-esterases (Aldridge, 1953). Surprisingly, the stoichiometric binding of OP compounds to carboxylesterases and cholinesterases is an important determinant of OP toxicity, as outlined in the section "Cholinesterases (AChE and BChE)."

As mentioned previously in this section, a plasma carboxylesterase, namely, Ces1c (previously known as Es1), is present in mouse plasma but not human or monkey plasma and accounts, at least in part, for the relative resistance of mice to the OP nerve agent soman. Duysen et al. (2011) demonstrated that plasma carboxylesterase knockout mice are considerably more susceptible to soman toxicity than wild-type mice.

Given the lack of plasma carboxylesterase in humans, the hydrolysis of xenobiotics in human blood is catalyzed by cholinesterases and paraoxonases (with a significant contribution from albumin on a case-by-case basis), as described in the following sections.

Cholinesterases (AChE and BChE) Humans have two cholinesterases, namely, acetylcholinesterase (AChE; gene name ACHE) and butyrylcholinesterase (BChE, also known as pseudocholinesterase; gene name BCHE), which are related enzymes (about 54% identical). They are present in most tissues. The levels of BChE are higher than those of AChE except in brain and muscle, tissues where AChE terminates the action of the neurotransmitter acetylcholine. In human plasma, the levels of BChE are 100- to 1000-fold greater than those of AChE, although the latter enzyme is present in erythrocytes (Li et al., 2005). As the names imply, AChE and BChE have high activity toward acetylcholine and butyrylcholine, respectively. AChE is highly selective for acetylcholine and plays little or no significant role in the hydrolysis of xenobiotics, whereas BChE hydrolyzes numerous drugs (and other xenobiotics) including aspirin, bambuterol, chlorpropaine, cocaine, flestolol, heroin, irinotecan, isosorbide diaspirinate, methylprednisolone acetate, mivacurium, moxisylyte, *n*-octanoyl ghrelin, procaine, succinylcholine (suxamethonium), and tetracaine (Li et al., 2005). Eserine (physostigmine) is an inhibitor of both enzymes, whereas BW284C51 is a selective inhibitor of AChE, and iso-OMPA, bambuterol, tolserine, and bis-norcymserine are selective inhibitors of BChE (Liederer and Borchardt, 2006; Masson and Lockridge, 2010). Drugs that selectively inhibit AChE, such as tacrine (Cognex®), gelantamine (Reminyl®), and donepezil (Aricept®), or inhibit both AChE and BChE, namely rivastigmine (Exelon®), have been approved for the treatment of Alzheimer disease, which is characterized by a loss of cholinergic neurons that project from the basal forebrain to the cortex and hippocampus (Wang et al., 2016b).

Both enzymes exist in six different forms with differing solubility: monomer (G1), dimer (G2), tetramer (G4), tailed tetramers (A4), double tetramers (A8), and triple tetramers (A12). G1, G2, and G4 contain 1, 2, and 4 subunits, each with a catalytic site. These various forms can each exist in three states: soluble (hydrophilic), immobilized (asymmetric), and amphiphilic globular (membrane-bound

through attachment to the phospholipid bilayer) (Nigg and Knaak, 2000). All forms are expressed in muscle. In the case of AChE, the major form in brain is the tetramer G4 (anchored with a 20-kDa side chain containing fatty acids), but the major form in erythrocytes is the dimer G2 (anchored with a glycolipid-phosphatidylinositol side chain). In the case of BChE, the major form in plasma is the tetramer G4 (a glycoprotein with M_r 342 kDa). In both AChE and BChE, the esteratic site (containing the same catalytic triad—Ser-His-Glu—present in carboxylesterases [Fig. 6-11]) is adjacent to an anionic (negatively charged) site that interacts with the positively charged nitrogen on acetylcholine and butyrylcholine.

Genetic variants of AChE that eliminate its activity have not been described, which is not surprising given the key role that AChE plays in terminating neurotransmission by acetylcholine, although AChE knockout mice ($AChE^{-/-}$) are born alive and, despite developmental abnormalities, survive up to 21 days (Xie et al., 2000). Based on measurements of erythrocyte AChE activity, familial reductions of 30% have been reported, and a reduction of 50% has been linked to paroxysmal nocturnal hemoglobinuria (Nigg and Knaak, 2000).

More than 70 genetic variants of BChE have been described following the discovery of poor metabolizers (PMs) of succinylcholine (suxamethonium) and, later, mivacurium. Succinylcholine and mivacurium are muscle relaxants whose duration of action is determined by plasma BChE. Succinylcholine (1.5 to 2.0 mg/kg) is a rapidly acting muscle relaxant (the onset of paralysis takes 30 to 60 seconds) with a short duration of action (8 to 15 minutes) making it well suited for intubating patients. In some individuals, succinylcholine causes prolonged (60 to 120 minutes) paralysis (muscular relaxation and apnea), which led to the discovery of two BChE genetic polymorphisms (now known as BCHE*A and BCHE*K) (Cerf et al., 2002). The so-called A variant of BChE ($Asp_{70}Gly$) has markedly reduced enzymatic activity (less than 10% of the wild-type enzyme) but is relatively rare; about 1 in 300 Caucasians are homozygous for the A variant. The K variant ($Ala_{539}Thr$) is considerably more common (with 1 in 63 individuals being homozygous), but the K variant still retains approximately two-thirds of its enzymatic activity. Consequently, the A variant causes a greater impairment of succinylcholine (and mivacurium) metabolism than does the K variant (La Du, 1992; Lockridge, 1992; Levano et al., 2008). Although the A variant has markedly diminished activity toward succinylcholine (due to about 100-fold increase in K_m), it nevertheless has appreciable activity toward other substrates, such as acetylcholine and benzoylcholine. Wild-type BChE and the A variant are equally sensitive to the inhibitory effect of OP compounds, but the allelic variant is relatively resistant to the inhibitory effect of dibucaine, a local anesthetic, which forms the basis of a diagnostic test for its presence (frequently called a test for atypical pseudocholinesterase). The percent inhibition of hydrolysis of benzoylcholine by dibucaine (with both the substrate and inhibitor at 10 μM) is called the dibucaine number; it is 80% or more with wild-type BChE and about 40% with the A variant in most but not all cases (Cerf et al., 2002). The discovery of the A variant of BChE (the so-called atypical pseudocholinesterase) is of historical interest because it ushered in the new field of pharmacogenetics, a field pioneered by Dr Werner Kalow, after whom the K variant of BChE is named.

Carboxylesterases and cholinesterases in the blood and tissues play an important role in limiting the amount of cholinergic neurotoxins that reach AChE in the brain, marked inhibition (70% to 90%) of which is lethal to mammals, insects, and nematodes. The cholinergic neurotoxins that are bound covalently to—or are hydrolyzed by—these enzymes include OP nerve agents (such as soman and sarin), OP pesticides (such as parathion, malathion, and chlorpyros, which are converted to oxons by CYP), carbamate pesticides (such as aldicarb, carbaryl, carbofuran), the naturally occurring OP compound anatoxin-a(*S*) from blue-green algae, physostigmine (eserine) from the Calabar bean, huperzine A from the club moss *Huperzia serrata*, solandine from green potatoes, and cocaine from the *Erythroxylum coca* plant. The covalent interaction between OP compounds and brain AChE is analogous to their binding to the active-site serine residue in all serine esterases (B-esterases). As previously mentioned, certain OP compounds are hydrolyzed by A-esterases (the paraoxonase PON1) but bind stoichiometrically and, for the most part, irreversibly to B-esterases (carboxylesterases and cholinesterases). Surprisingly, stoichiometric binding of OP compounds to carboxylesterase and cholinesterase (and perhaps to numerous other enzymes and receptors that have structural features common to serine esterases) plays an important role in limiting the toxicity of OP compounds. Numerous studies have shown an inverse relationship between serine esterase activity and susceptibility to the toxic effect of OP compounds. Factors that decrease serine esterase activity potentiate the toxic effects of OP compounds, whereas factors that increase serine esterase activity have a protective effect. For example, the susceptibility of animals to the toxicity of parathion, malathion, and diisopropylfluorophosphate (DFP) is inversely related to the level of plasma esterase activity (which reflects BChE activity and, in some species, carboxylesterase activity). Differences in the susceptibility of several mammalian species to OP toxicity can be abolished by pretreatment with selective serine esterase inhibitors such as cresylbenzodioxaphosphorin oxide, the active metabolite of tri-*ortho*-tolylphosphate (which is also known as tri-*ortho*-cresylphosphate [TOCP]). Knockout mice that lack plasma carboxylesterase (Ces1c, previously known as Es1) or AChE are more susceptible to the toxic effects of OP compounds, as are PON1 knockout mice in some but not all cases (discussed in the section "Paraoxonases (Lactonases)") (Xie et al., 2000; Duysen et al., 2011). Somewhat surprisingly, BChE knockout mice are not more sensitive to OP toxicity than wild-type mice (Duysen et al., 2007), but this may not reflect the situation in humans because mouse plasma contains a carboxylesterase, whereas humans do not (see Table 6-8 and the section "Carboxylesterases"). In humans, BChE plays an important role in preventing OP compounds from reaching AChE in the brain. Carboxylesterases, cholinesterases, and paraoxonases are not the only enzymes involved in the detoxication of OP pesticides. Certain OP compounds are detoxified by CYP, flavin monooxygenases, and GSTs. However, paraoxonases, enzymes that catalyze the hydrolysis of certain OP compounds, appear to play a limited role in determining susceptibility to OP toxicity, as outlined in the following section.

Once bound to the active site of cholinesterase or carboxylesterase, OP compounds can undergo dealkylation reactions that further retard their release from the active-site serine residue of these enzymes. This process is called "aging." In many cases the phosphorylated or phosphonylated enzyme can be reactivated by displacement of the OP adduct with nucleophilic compounds such as fluoride, hydroxamates, and oximates. The pyridinium oximes pralidoxime (2-PAM) and obidoxime (toxogonin) are used therapeutically as antidotes to OP poisoning. More potent oximes, namely HI-6 (asoxime) and MMB-4 (ICD-039, methoxime), have been developed for the treatment of poisoning from OPs including chemical warfare agents (Masson and Lockridge, 2010; Lundy et al., 2011; Kovalevsky et al., 2016).

Cocaine is hydrolyzed by CES1 and CES2 in the liver and intestine, and by BChE in plasma. Genetic polymorphisms that reduce BChE activity toward succinylcholine (such as the A variant

described earlier in this section) also reduce its activity toward cocaine (again by increasing K_m), which exacerbates cocaine toxicity (Masson and Lockridge, 2010). Despite its important role in cocaine toxicity, BChE hydrolyzes cocaine slowly. Masson and Lockridge (2010) reviewed efforts based on molecular dynamics simulation to improve the hydrolytic function of BChE through site-directed mutagenesis. A BChE variant with four mutations hydrolyzes cocaine with a catalytic efficiency 1500- to 5000-fold greater than that of the wild-type enzyme, whereas a variant with five mutations is 6500 times more active. Cocaine hydrolase (CocH), a modified form of BChE with high activity toward cocaine, coupled with albumin or a modified Fc region of immunoglobulin G1 (IgG1) to prolong its plasma half-life, is under development for the treatment of cocaine overdose and addition (Chen et al., 2016). Based on its ability to hydrolyze and inactivate ghrelin, a gastric peptide hormone acylated with a fatty acid that stimulates hyperphagia and adiposity (earning ghrelin the title of "hunger hormone"), CocH also has potential as an antiobesity drug (Zheng et al., 2016).

Paraoxonases (Lactonases) Paraoxonases catalyze the hydrolysis of a broad range of organophosphates, organophosphinites, aromatic carboxylic acid esters (such as phenylacetate), cyclic carbonates, lactones, and oxidized phospholipids. They are calcium-dependent enzymes containing a critical sulfhydryl (−SH) group; as such they are inhibited by EDTA, metal ions (Cu and Ba), and various mercurials such as phenylmercuric acetate (PMA), *para*-chloromercuribenzoate (PCMB), and the PCMB hydrolysis product, *para*-hydroxymercuribenzoate. (*Note*: Calcium must be added to measure paraoxonase activity in plasma prepared from EDTA-anticoagulated blood.) Based on the observation that A-esterases are inhibited by PCMB but not OP compounds, Augustinsson (1966) postulated that, in the case of paraoxonases, OP compounds bind to a nucleophilic SH group on an active-site cysteine residue and form a phosphorus–sulfur bond, which is readily cleaved by water. A strong argument against this postulate is the fact that there is no loss of enzymatic activity when the only potential active-site cysteine residue in human paraoxonase (Cys_{283}) is substituted with serine or alanine (Sorenson et al., 1995). However, substitution with serine or alanine renders paraoxonase resistant to inhibition by PCMB, placing Cys_{283} near but not in the active site of paraoxonase. Paraoxonase requires Ca^{2+} for both stability and catalytic activity, which raises the possibility that the hydrolysis of OP compounds by paraoxonase involves metal-catalyzed hydrolysis, analogous to that proposed for calcium-dependent phospholipase A2 or zinc-dependent phosphotriesterase activity (Sorenson et al., 1995). A structurally modified but catalytically active form of recombinant PON1 has been crystallized and shown by x-ray analysis to contain two calcium ions in a sixfold β-propeller protein similar to that found in squid diisopropylfluorophosphatase (DFPase), a paraoxonase-like enzyme (Otto et al., 2009).

Humans express three paraoxonases designated PON1, PON2, and PON3. PON1 is present in liver microsomes and plasma, where it is associated exclusively with high-density lipoprotein (HDL). PON2 is not present in plasma but it is expressed in the inner mitochondrial membrane of vascular cells and many tissues (Devarajan et al., 2011). PON3 is expressed in liver and kidney microsomes and plasma. Only PON1 has appreciable arylesterase activity and the ability to hydrolyze the toxic oxon metabolites of OP insecticides such as parathion (paraoxon), diazinon (diazoxon), and chlorpyrifos (chlorpyrifos oxon) (Gupta and DuBois, 1998; Draganov and La Du, 2004). However, all three enzymes can catalyze the hydrolysis of various lactones, for which reason the name "lactonase" is more encompassing. A lactone derived from arachidonic acid (5-hydroxy-cicosate traeomic acid-1,5-lactone) is one of the few substrates hydrolyzed by all three paraoxonases (Gupta et al., 2009). Lactone hydrolysis of the statins lovastatin and simvastatin is catalyzed only by PON3. Reports of the same reaction being catalyzed by PON1 appear to be attributable to trace contamination with PON3 (Draganov and La Du, 2004). However, both PON1 and PON3 hydrolyze the lactone form of atorvastatin, which reverses the lactonization of atorvastatin, a reaction that involves formation of an acyl glucuronide of atorvastatin by UGT1A3 (Riedmaier et al., 2011). PON1 hydrolyzes thiolactones such as homocysteine thiolactone (an endobiotic substrate) and a thiolactone metabolite of clopidogrel (discussed later in the section "CYP2C19"), whereas PON3 hydrolyzes the lactone spironolactone (a diuretic drug) (Gupta et al., 2009). PON1 is the major plasma enzyme responsible for hydrolyzing the prodrug olmesartan medoxomil to its active metabolite (Ishizuka et al., 2012) and is one of the plasma enzymes responsible for hydrolyzing the structurally related prodrugs prulifloxacin and ceftobiprole medocaril to their active metabolite (Eichenbaum et al., 2012).

PON2, located on the inner mitochondrial membrane, appears to play no significant role in xenobiotic biotransformation, although it can hydrolyze the lactone dihydrocoumarin. In the inner mitochondrial membrane, PON2 is bound to coenzyme Q_{10} and complexed with respiratory complex III where it protects the mitochondrion from oxidative damage from superoxide anion and its derivative reactive oxygen species (hydrogen peroxide, peroxynitrate, and hydroxy radicals) (Devarajan et al., 2011).

PON1 has two prominent polymorphisms in the coding region, namely, $Q_{192}R$ ($Glu_{192}Arg$) and $L_{55}M$ ($Leu_{55}Met$), and several polymorphisms in the promoter region. Polymorphisms in the promoter region and the $L_{55}M$ polymorphism in the coding region do not affect PON1 activity but they do affect expression levels (Gupta et al., 2009; Furlong et al., 2010). The commonest genetic polymorphism, $Q_{192}R$, affects PON1 activity in a substrate-dependent manner. The glutamine (Q_{192}) and arginine (R_{192}) allelozymes have the same hydrolytic activity toward *para*-nitrophenylacetate (a measure of arylesterase activity) and diazoxon (the oxon of diazinon) but R_{192} is more active toward paraoxon (the oxon of parathion), chlorpyrifos oxon, the prodrugs olmesartan medoxomil and prulifloxacin, and the lactone pilocarpine, whereas Q_{192} is more active toward soman, sarin, the thiolactone metabolite of clopidogrel (Bouman et al., 2011; discussed later in the section "CYP2C19"), and lipid peroxides, the significance of which is discussed later in this section (Gupta et al., 2009; Furlong et al., 2010; Eichenbaum et al., 2012; Ishizuka et al., 2012).

There is evidence to suggest that PON1 protects against atherosclerosis by hydrolyzing specific derivatives of oxidized cholesterol and/or phospholipids in atherosclerotic lesions and in oxidized low-density lipoprotein (LDL). Low-serum PON-1 activity is associated with a higher risk of cardiovascular disease (CVD), which is partly dependent on the serum levels of high-density lipoprotein cholesterol (HDL-C), which binds PON-1 and is a recognized risk factor for CVD (Kunutsor et al., 2016). Mice lacking PON1 (knockout mice or PON1 null mice) are predisposed to atherogenesis, whereas mice overexpressing PON1 are protected (Draganov and La Du, 2004; Gupta et al., 2009). Some studies show that individuals who are homozygous for the R_{192} allele are at increased risk of atherosclerosis and ischemic stroke compared with individuals who are homozygous for the Q_{192} allele, suggesting that the absence of the latter enzyme, the PON1 allelozyme with high activity toward lipid peroxides and offering more protection against LDL oxidation, is a risk factor for CVD (Gupta et al., 2009; Dahabreh et al., 2010). However, the association is controversial. A complicating factor is that the R_{192} allelozyme is expressed at higher levels than

the Q_{192} enzyme, which may partially offset its lower ability to hydrolyze lipid peroxides. Bayrak et al. (2012) proposed that, in terms of assessing the risk of atherosclerosis, an assessment of both plasma PON1 activity and genotype is more reliable than genotype alone. This seems appropriate in view of the finding that several environmental factors affect PON1 levels, which are upregulated by hypolipidemic drugs (fenofibrate and statins) and cardioprotective dietary components such as polyphenols (such as resveratrol), oleic acid, and olive oil, and downregulated by diabetes and a high-fat (proatherogenic) diet (Gupta et al., 2009).

It seems reasonable to assume that PON1 would play an important role in determining the susceptibility of humans to OP toxicity based on three considerations: first, PON1 hydrolyzes OP compounds (rather than simply bind them stoichiometrically like BChE). Second, the concentration of PON1/PON3 in human plasma is 10 times greater than that of BChE (50 ng/mL vs.5 ng/mL) (Li et al., 2005). Third, human plasma does not contain carboxylesterase (Table 6-8). PON1/PON3 may protect against OP toxicity in some but not all cases. PON1 knockout mice are no more susceptible than wild-type mice to the toxic effects of paraoxon (the active metabolite of parathion), and administration of human PON1 (either the R_{192} or the Q_{192} allelozyme) to PON1 knockout mice does not confer protection against paraoxon. However, administration of either the R_{192} or Q_{192} human allelozymes to PON1 knockout mice does protect against diazoxon toxicity, and administration of the R_{192} allelozyme (but not the Q_{192} allelozyme) protects against chlorpyrifos oxon (Cole et al., 2010). The allelozyme-dependent pattern of protection corresponds with the rate of hydrolysis of these OP oxons by R_{192} and Q_{192} (high and equal for diazoxon, high but greater with R_{192} for chlorpyrifos oxon, and low but greater with R_{192} for paraoxon). Although the concentration of BChE in human plasma is only one-tenth that of PON1/PON3 (about 50 nM vs. 500 nM), its apparent second-order rate constant with OP compounds is very high (10^7 to 10^9 M^{-1} min^{-1}) compared with the catalytic efficiency of PON1 (10^5 M^{-1} min^{-1}) (Masson and Lockridge, 2010).

Diisopropylfluorophosphatase (DFPase), a squid enzyme that catalyzes the release of fluoride from DFP (Fig. 6-9), is a hydrolytic enzyme related to the paraoxonases. It hydrolyzes the nerve gas agents sarin and soman (Liederer and Borchardt, 2006). Human paraoxonases do not hydrolyze DFP but are inhibited by this OP compound.

Prodrugs and Alkaline Phosphatase Many prodrugs are designed to be hydrolyzed by hydrolytic enzymes (Liederer and Borchardt, 2006; Yang et al., 2011). Some prodrugs, such as propranolol ester, are hydrolyzed by both carboxylesterases and cholinesterases (both AChE and BChE), whereas others are preferentially or specifically hydrolyzed by carboxylesterases (capecitabine and irinotecan), BChE (bambuterol and methylprednisolone acetate), hPON1 (prulifloxacin and olmesartan medoxomil), or PON3 (lovastatin and simvastatin). On a case-by-case basis, specific enzymes other than those mentioned above can be involved in xenobiotic hydrolysis. For example, the valine ester prodrugs of acyclovir (valacyclovir) and gangciclovir (valgangciclovir) are hydrolyzed by valacyclovirase (gene code BPHL), a serine- and α/β-fold-hydrolase that hydrolyzes other antiviral and anticancer nucleoside drugs such as zidovudine, floxuridine, and gemcitabine (Lai et al., 2008). As a means of targeting the release of cytotoxic anticancer drugs toward cancer cells, amino acid or peptide conjugates of drugs such as doxorubicin have been designed for hydrolysis (activation) by various matrix metalloproteinases, which play a key role in tumor angiogenesis, invasion, and metastasis (Yang et al., 2011). Several anticancer drugs have been conjugated with peptides that are specifically cleaved by prostate-specific antigen (PSA) to target PSA-expressing prostate cells (Yang et al., 2011). Some prodrugs are hydrolyzed with a high degree of stereoselectivity. For example, in the case of prodrugs of ibuprofen and flurbiprofen, the *R*-enantiomer is hydrolyzed about 50 times faster than the *S*-enantiomer. The acyl glucuronides of ibuprofen and other NSAIDs are also hydrolyzed stereoselectively (see the section "Glucuronidation and Formation of Acyl-CoA Thioesters").

Some prodrugs, such as fosphenytoin (Cerebyx®) and fosamprenavir (Lexiva®), are designed to be hydrolyzed by alkaline phosphatase, high concentrations of which are present on the luminal surface of the enterocytes lining the wall of the small intestine. Hydrolysis of these prodrugs by alkaline phosphatase releases the active drug at the surface of the enterocytes, where it can be readily absorbed. Soluble epoxide hydrolase (sEH) is a bifunctional enzyme; its C-terminus contains an epoxide hydrolase domain, whereas its N-terminus contains a phosphatase domain, as described in the section "Epoxide Hydrolases." Although it may play a role in the hydrolysis of endogenous phosphates, such as polyisoprenyl phosphates and lysophosphatidic acids (Oguro and Imaoka, 2012), sEH is an intracellular enzyme and, as such, can play no significant role in the hydrolysis of phosphate/phosphonate prodrugs.

Peptidases Peptidases are proteolytic enzymes that hydrolyze the amide (peptide) bond connecting adjacent amino acids in peptides and proteins (R_1–NH–CO–R_2 + H_2O → R_1–NH_2 + R_2–COOH). Traditionally they have not been viewed as drug-metabolizing enzymes but that has changed with the emerging field of therapeutic peptides and proteins.

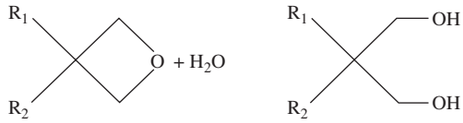
On January 16, 2017, Botox made the cover of *Time Magazine* under the title: *How Botox Became the Drug That's Treating Everything—Depression, Heart Trouble, Migraines, Erectile Dysfunction, Back Pain, Sweaty Palms, Drooling and 793 Other Problems*. Botox is a large therapeutic protein (about 1300 amino acids) derived from botulinum neurotoxin type A (BoNT/A), which ranks as one of the most toxic substances known and is the cause of botulism following infection with the bacterium *Botulinum clostridium*. The manufacturer of Botox, Allergan, holds over 800 patents on potential therapeutic applications for Botox. Today, Botox is probably the best known therapeutic protein, although insulin held this position in the past. Therapeutic proteins (9%), therapeutic peptides (8%), and monoclonal antibodies (8%) collectively accounted for 25% of drugs approved by the FDA between 2009 and 2011, compared with 34% for small molecule drugs (Albericio and Kruger, 2012). Therapeutic peptides/proteins are used or can potentially be used to treat infection, cancer, diabetes, cardiovascular diseases and, even with Botox alone, too many other conditions to list.

Proteolysis is one of the major factors limiting the usefulness of therapeutic peptides and proteins. Proteolysis in the intestine restricts oral bioavailability and proteolysis in the blood/tissues leads to the rapid systemic elimination of therapeutic peptides/proteins and loss of therapeutic efficacy. Parenteral administration overcomes the former problem. Overcoming proteolysis in blood and tissues is more problematic. A larger number of proteolytic enzymes are expressed in blood, on cell surfaces, or in tissues, including serine proteases ($n = 9$), cysteine proteases ($n = 3$), aspartate proteases ($n = 2$), metalloproteinases ($n = 2$), aminopeptidases ($n = 3$), dipeptidyl- and/or tripeptidyl-peptidases ($n = 4$), and metallocarboxypeptidases ($n = 6$) (Vlieghe et al., 2010). The stability of therapeutic peptides can be enhanced by various ways, including incorporation of D-amino acids (to render them protease resistant), cyclization (to prevent hydrolysis by exopeptidases [the last four classes of proteases listed in the preceding sentence]), and incorporation of α-aminoxy amino acids, which greatly slow

proteolysis (Gentilucci et al., 2010; Chen et al., 2012a). Another strategy to overcome rapid elimination of therapeutic peptides by proteolysis is the use of modified peptides, which are known as peptidomimetics or quasi-peptides. When intracellular targeting is desirable, therapeutic peptides can be linked to one of a variety of cell-penetrating peptides, which are generally peptides containing multiple amino acids with cationic (basic) side chains (such as lysine and arginine) that can bind to phospholipid membranes and enter cells by endocytosis, much the same way as aminoglycosides permeate cell membranes (see the section "Introduction") (Zheng et al., 2016; Guidotti et al., 2017).

β-Glucuronidase β-Glucuronidase is present in liver lysosomes and microsomes (where it is bound to the lumen of the endoplasmic reticulum by egasyn [Ces1e], as mentioned in the section "Carboxylesterases") and in gut microbiota. The enzyme hydrolyzes xenobiotic glucuronides (in which the glucuronide is in the β-configuration). When a drug is glucuronidated directly and excreted in bile, hydrolysis by β-glucuronidase in the gut can release the aglycone (the parent drug) and result in a second phase of drug absorption, a process called enterohepatic circulation. In the case of the anticancer drug irinotecan, which is hydrolyzed to SN-11 (the pharmacologically active and toxic metabolite) and then glucuronidated, hydrolysis of SN-11-glucuronide by microbiota β-glucuronidase is undesirable because it releases SN-11 in the colon and causes dose-limiting diarrhea. To reduce the risk of such an adverse event, inhibitors of gut β-glucuronidase have been developed (Wallace et al., 2010; Roberts et al., 2013) which is described in more detail in the section "Glucuronidation and Formation of Acyl-CoA Thioesters."

Epoxide Hydrolases Epoxide hydrolases catalyze the *trans*-addition of water to alkene epoxides and arene oxides (oxiranes), which can form during the CYP-dependent oxidation of aliphatic alkenes and aromatic hydrocarbons, respectively. As shown in Fig. 6-12, the products of this hydrolysis are vicinal diols with a *trans*-configuration (i.e., *trans*-1,2-dihydrodiols). There are two notable exceptions to this general rule. Epoxide hydrolase catalyzes the hydration of the epoxide leukotriene A_4 (LTA_4) to leukotriene B_4 (LTB_4) and the hydration of AZD1979, an investigational drug containing an oxetane moiety (a four-membered ring composed of three carbons and one oxygen atom) to a ring-opened diol, and in both cases the two hydroxyl groups appear on nonadjacent carbon atoms (Li et al., 2016; Toselli et al., 2017). Hydration of a generic oxetane by epoxide hydrolase is shown below:



Epoxide hydrolases play an important role in detoxifying electrophilic epoxides that might otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations. In the case of PAHs, however, microsomal epoxide hydrolase plays a critical role in forming diol epoxides, the ultimate carcinogenic metabolites of benzo[*a*]pyrene (B[*a*]P), 7,12-dimethylbenz[*a*]anthracene (DMBA), and many other PAHs (discussed later in this section).

There are four distinct forms of epoxide hydrolase in mammals: microsomal epoxide hydrolase (mEH; gene name EPHX1), soluble epoxide hydrolase (sEH; gene name EPHX2), cholesterol-5,6-epoxide hydrolase (ChEH; EC 3.3.2.11), and leukotriene A_4 hydrolase (LTA_4 hydrolase; gene name LTA4H) (Fretland and Omiecinski, 2000; Morisseau and Hammock, 2005; Kodani and Hammock, 2015). Hepoxilin A_3 hydrolase was thought to represent a fifth class of epoxide hydrolase but it was subsequently identified as being the same enzyme as sEH (Cronin et al., 2011). As their names imply, cholesterol-5,6-epoxide hydrolase (ChEH) and LTA_4 hydrolase hydrolyze endogenous epoxides specifically, and have virtually no capacity to detoxify xenobiotic oxides. LTA_4 hydrolase is distinct from the other epoxide hydrolases because it is a bifunctional zinc metalloenzyme that has both epoxide hydrolase and peptidase activity, and because the two hydroxyl groups introduced during the conversion of LTA_4 to LTB_4 are eight carbon atoms apart. sEH is a bifunctional enzyme; its C-terminus contains an epoxide hydrolase domain, whereas its N-terminus contains a phosphatase domain. The latter domain is structurally related to members of the haloacid dehalogenase (HAD) superfamily, which includes dehalogenases, phosphonatases, phosphomutases, phosphatases, and ATPases. The phosphatase domain of sEH is thought to play a role in the hydrolysis of endogenous phosphates, such as polyisoprenyl phosphates (which regulate cholesterol levels) and lysophosphatidic acids (Imig and Hammock, 2009; Oguro and Imaoka, 2012).

mEH hydrolyzes a wide variety of xenobiotics with an alkene epoxide or arene oxide. sEH hydrolyzes some xenobiotic epoxides and oxides, such as *trans*-stilbene oxide, but it also plays an important role in the hydrolysis of endogenous fatty acid epoxides, such as the epoxyeicosatrienoic acids (EETs) that are formed by epoxidation of arachidonic acid by CYP (particularly CYP2C8, CYP2C9, and CYP2J2) and the leukotoxins that are formed by the epoxidation of linoleic acid by leukocytes (Fretland and Omiecinski, 2000; Morisseau and Hammock, 2005; Imig and Hammock, 2009; Fleming, 2014). EETs are endothelin-derived hyperpolarizing factors (EDHFs) (i.e., vasodilators) that possess anti-inflammatory properties and protect tissues from ischemic injury. Hydrolysis of EETs by sEH terminates their vasodilatory and anti-inflammatory effects. Accordingly, sEH is a potential therapeutic target for the treatment of various cardiovascular diseases such as hypertension and atherosclerosis (Imig and Hammock, 2009; Wang et al., 2010c; Oguro and Imaoka, 2012; Fleming, 2014; Kodani and Hammock, 2015; He et al., 2016). Several disubstituted ureas (including the anticancer drug sorafenib) have been identified as competitive inhibitors of sEH that are both potent (K_i values in the nanomolar range) and specific (they do not inhibit the phosphatase domain of sEH and they do not potently inhibit mEH). Despite promising efficacy in nonclinical species, a clinical trial of a sHE inhibitor

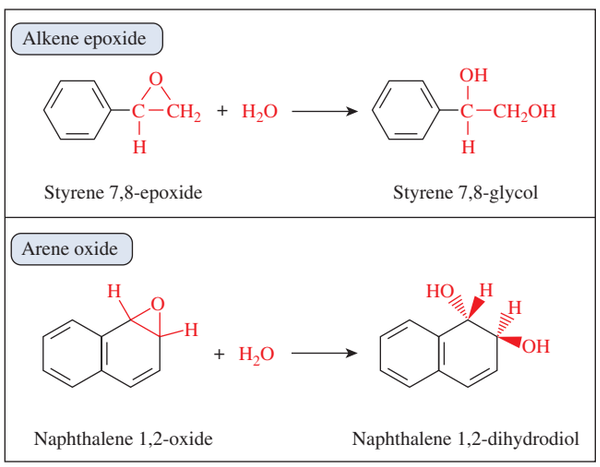


Figure 6-12. *Examples of the hydration of an alkene epoxide (top) and an arene oxide (bottom) by epoxide hydrolase.*

(1-(1-acetyl-piperidin-4-yl)-3-adamantan-1-yl-urea) was terminated for lack of efficacy in Phase 2 trials (He et al., 2014). In addition to their vasodilatory and anti-inflammatory effects, EETs promote endothelial cell proliferation and migration; hence, they are angiogenic (i.e., they stimulate blood vessel supply). A potential side effect of sEH inhibitors, therefore, may be potentiation of EET-mediated angiogenesis resulting in accelerated tumorigenesis (Imig and Hammock, 2009; Wang et al., 2010c; Fleming, 2014).

Although the levels vary from one tissue to the next, mEH has been found in the microsomal fraction (and in some cases the plasma membrane) of virtually all tissues, including the liver, testis, ovary, lung, kidney, skin, intestine, colon, spleen, thymus, brain, and heart. sEH is also widely distributed in tissues; high levels of sEH are present in the cytosol (and in some cases the lysosomes) of liver, kidney, brain, and vasculature and lower levels are present in lung, spleen, and testis. In general, mEH prefers monosubstituted epoxides and disubstituted epoxides with a *cis* configuration, such as *cis*-stilbene oxide, whereas sEH prefers tetrasubstituted and trisubstituted epoxides and disubstituted epoxides with a *gem* configuration (both substituents on the same carbon atom) or the *trans* configuration, such as *trans*-stilbene oxide, as shown in Fig. 6-13. In rodents, both sEH and mEH are inducible enzymes; sEH is under the control of PPARα, so it is induced following treatment of rats and mice with peroxisome proliferators, whereas mEH is under the control of Nrf2, so it is induced in response to oxidative stress or exposure to electrophiles and GSH depletors (see the section "Quinone Reduction: NQO1 and NQO2"). Treatment of mice with the CAR agonist phenobarbital induces mEH about two- to threefold, whereas treatment with Nrf2 activators such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ethoxyquin induces mEH by an order of magnitude or more. mEH is one of several proteins (so-called preneoplastic antigens) that are overexpressed in chemically induced foci and nodules that eventually develop into liver tumors.

Many epoxides and oxides are intermediary metabolites formed during the CYP-dependent oxidation of unsaturated aliphatic and aromatic xenobiotics. These electrophilic metabolites might otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations. In general, sEH and mEH are found in the same tissues and cell types that contain CYP enzymes.

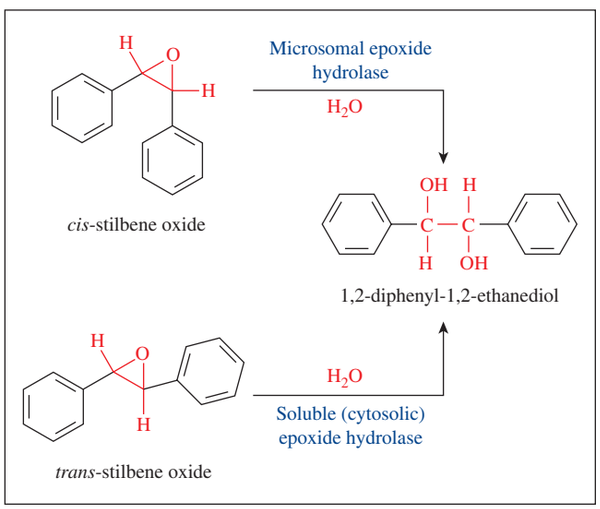


Figure 6-13. *Stereoselective hydration of stilbene oxide by microsomal and soluble epoxide hydrolase.*

For example, the distribution of epoxide hydrolase parallels that of CYP enzymes in the liver, lung, and testis. In other words, both enzymes are located in the centrilobular region of the liver (zone 3), in Clara and type II cells in the lung, and in Leydig cells in the testis. The colocalization of epoxide hydrolase and CYP presumably ensures the rapid detoxication of alkene epoxides and arene oxides generated during the oxidative metabolism of unsaturated xenobiotics.

Electrophilic epoxides and arene oxides are constantly produced during the CYP-dependent oxidation of unsaturated aliphatic and aromatic xenobiotics, and are potentially reactive to cellular macromolecules such as DNA and protein. Epoxide hydrolase can rapidly convert these potentially toxic metabolites to the corresponding dihydrodiols, which are less reactive and easier to excrete. Thus, epoxide hydrolases are widely considered as a group of detoxication enzymes. In some cases, however, further oxidation of a dihydrodiol can lead to the formation of diol epoxide derivatives that are no longer substrates for epoxide hydrolase because the oxirane ring is protected by bulky substituents that sterically hinder interaction with the enzyme. This point proved to be extremely important in elucidating the mechanism by which PAHs cause tumors in laboratory animals (Conney, 1982). Tumorigenic PAHs, such as B[*a*]P, are converted by CYP (particularly by CYP1B1 and CYP1A1) to a variety of arene oxides that bind covalently to DNA, making them highly mutagenic to bacteria. One of the major arene oxides formed from B[*a*]P, namely, the 4,5-oxide, is highly mutagenic to bacteria but weakly mutagenic to mammalian cells. This discrepancy reflects the rapid inactivation of B[*a*]P 4,5-oxide by epoxide hydrolase in mammalian cells. However, one of the arene oxides formed from B[*a*]P, namely, B[*a*]P 7,8-dihydrodiol-9,10-oxide, is not a substrate for epoxide hydrolase and is highly mutagenic to mammalian cells and considerably more potent than B[*a*]P as a lung tumorigen in mice.

B[*a*]P 7,8-dihydrodiol-9,10-oxide is known as a bay-region diol epoxide, and analogous bay-region diol epoxides are now recognized as tumorigenic metabolites of numerous PAHs. A feature common to all bay-region epoxides is their resistance to hydration by mEH, which results from steric hindrance from the nearby dihydrodiol group. As shown in Fig. 6-14, B[*a*]P 7,8-dihydrodiol-9,10-oxide is formed in three steps: B[*a*]P is converted to the 7,8-oxide, which is converted to the 7,8-dihydrodiol, which is converted to the corresponding 9,10-epoxide (which can exist in four diastereomeric forms). The first and third steps are epoxidation reactions catalyzed by CYP (especially CYP1B1 and CYP1A1) or prostaglandin H synthase, but the second step is catalyzed by mEH. Consequently, even though mEH plays a major role in detoxifying several B[*a*]P, such as the 4,5-oxide, it nevertheless plays a role in converting B[*a*]P to its ultimate tumorigenic metabolite B[*a*]P 7,8-dihydrodiol-9,10-oxide. Diol epoxides are also the carcinogenic metabolites of DMBA and many other PAHs (Shimada, 2006).

The importance of mEH in the conversion of PAHs to their ultimate carcinogenic metabolites, namely, diol epoxides, is illustrated by the observation that mEH knockout mice (mEH-null mice) are completely resistant to the tumorigenic effects of DMBA (Shimada, 2006). Genetic polymorphisms of human mEH also impact cigarette-smoking-induced cancer of the lung and upper aerodigestive tract (UADT) in a manner consistent with the protective effect conferred by a lack of mEH in DMBA-treated mice (Li et al., 2011b). More than 110 SNPs have been identified in the human EPHX1 gene, 2 of which have been studied in detail: one of them ($Tyr_{113}His$) decreases mEH activity (by about 40%) and the other ($His_{139}Arg$) increases mEH activity (by about 25%).

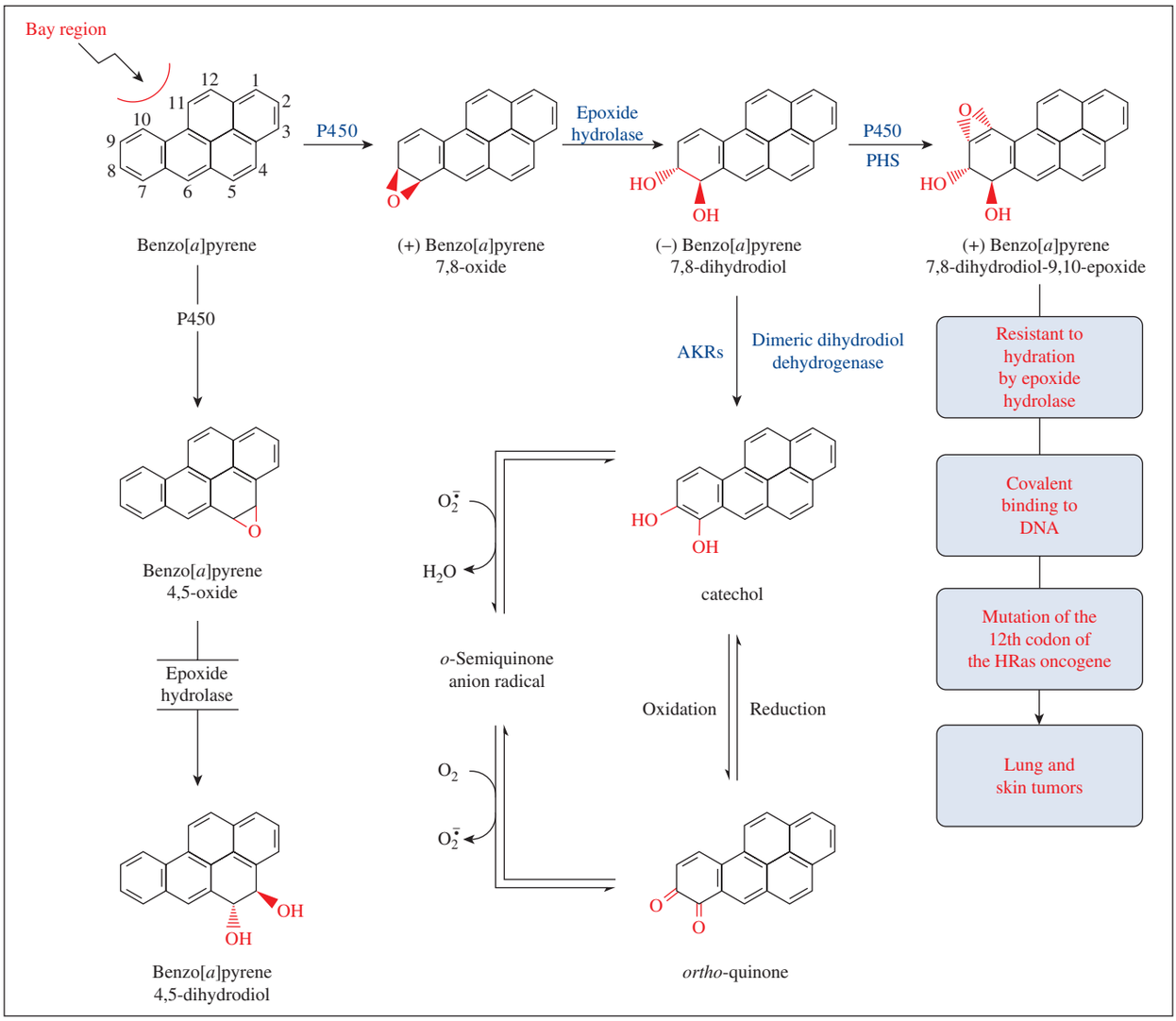


Figure 6-14. *Role of epoxide hydrolase in the inactivation of benzo[a]pyrene 4,5-oxide and in the conversion of benzo[a]pyrene to its tumorigenic bay-region diol epoxide.* Also shown is the role of dimeric dihydrodiol dehydrogenase, and the aldo-keto reductase (AKR) superfamily, in the formation of reactive catechol and ortho-quinone metabolites of benzo[*a*]pyrene.

The low-activity mEH (observed in individuals who are homozygous or heterozygous for the His_{133} allelozyme) protects from tobacco-induced cancer of the lung and UADT, whereas the high-activity variant (observed in individuals who are homozygous or heterozygous for the Arg_{139} allelozyme) predisposes to these cancers (Li et al., 2011b). These findings do not translate to the risk of other cancers. Chen et al. (2015b) found that the low activity variant of mEH was associated with an increased risk of head and neck (odds ratio of ~ 1.3), in contrast to the decreased risk of lung and UADT cancer, and found no association between head and neck cancer and the high activity variant of mEH.

Not all epoxides are highly reactive and toxic to the cells that produce them. Some drugs actually contain an epoxide, such as scopolamine, tiotropium, troleandomycin, carfilzomib, and oprozomib. Vitamin K epoxide is also a nontoxic epoxide, which is formed and consumed during the vitamin K–dependent γ-carboxylation of prothrombin and other clotting factors in the liver. Vitamin K epoxide is not hydrated by mEH but is reduced by vitamin K epoxide reductase. This enzyme is inhibited by warfarin and related coumarin anticoagulants, which interrupts the synthesis of several clotting factors. The major metabolite of carbamazepine is an epoxide, which is so stable that carbamazepine 10,11-epoxide is a major circulating metabolite in patients treated with this antiepileptic drug. (Carbamazepine is converted to a second epoxide, which is less stable and more cytotoxic, as shown in the section "Cytochrome P450.") Circulating levels of carbamazepine 10,11-epoxide are elevated in individuals expressing the low-activity variant of mEH (the His_{133} allelozyme) but, with one possible exception, this genetic polymorphism is not associated with an increase in the adverse effects of this or other anticonvulsant drugs (Daly, 1999). The exception is a case report of a man who had a defect in mEH expression and suffered acute and severe phenytoin toxicity (Morisseau and Hammock, 2005). Certain drugs such as valpromide (the amide analog of valproic acid) and progabide (a γ-aminobutyric acid [GABA] agonist) cause clinically significant inhibition of mEH and may impair epoxide hydrolase activity more

than genetic polymorphisms. These two drugs potentiate the neurotoxicity of carbamazepine by inhibiting mEH, leading to increased plasma levels of carbamazepine 10,11-epoxide and presumably its more toxic 2,3-epoxide (Kroetz et al., 1993). mEH can be inhibited in vitro by certain epoxides, such as 1,1,1-trichloropropene oxide and cyclohexene oxide, and stimulated by several alcohols, ketones, and imidazoles.

The microsomal and soluble forms of epoxide hydrolase show no evident sequence identity and, accordingly, are immunochemically distinct proteins (Beetham et al., 1995). Nevertheless, mEH and sEH catalyze reactions by the same mechanism, and similar amino acids are involved in catalysis, namely, a nucleophilic acid (Asp_{226} in mEH and Asp_{334} in sEH), a basic histidine (His_{431} in mEH and His_{523} in sEH), an orienting acid (Glu_{404} in mEH and Asp_{495} in sEH), and polarizing tyrosine residues (Tyr_{299} and Tyr_{374} in mEH and Tyr_{382} and Tyr_{465} in mEH) (Morisseau and Hammock, 2005). The mechanism of catalysis by epoxide hydrolase is similar to that of carboxylesterase, in that the catalytic site comprises three amino acid residues that form a catalytic triad, as shown in Fig. 6-11. The attack of the nucleophile Asp_{226} on the carbon of the oxirane ring initiates enzymatic activity, leading to the formation of an α-hydroxyester-enzyme intermediate, with the negative charge developing on the oxygen atom stabilized by a putative oxyanion hole. The His_{431} residue (which is activated by Glu_{376} and Glu_{404}) activates a water molecule by abstracting a proton (H^+). The activated (nucleophilic) water then attacks the Cγ atom of Asp_{226}, resulting in the hydrolysis of the ester bond in the acyl–enzyme intermediate, which restores the active enzyme and results in the formation of a vicinal diol with a *trans*-configuration (Armstrong, 1999). The second step, namely, cleavage of the ester bond in the acyl–enzyme intermediate, resembles the cleavage of the ester or amide bond in substrates for serine esterases and proteases.

Although both epoxide hydrolase and carboxylesterase have a catalytic triad comprising a nucleophilic, basic, and acidic amino acid residue, there are striking differences in their catalytic machinery, which account for the fact that carboxylesterases primarily hydrolyze esters and amides, whereas epoxide hydrolases primarily hydrolyze epoxides and oxides. In the triad, both enzymes have histidine as the base and either glutamate or aspartate as the acid, but they differ in the type of amino acids for the nucleophile. Even during catalysis, there is a major difference. In carboxylesterases, the same carbonyl carbon atom of the substrate is attacked initially by the nucleophile Ser_{203} to form an α-hydroxyester-enzyme ester that is subsequently attacked by the activated water to release the alcohol product. In contrast, two different atoms in epoxide hydrolase are targets of nucleophilic attacks. First the less hindered carbon atom of the oxirane ring is attacked by the nucleophile Asp_{226} to form a covalently bound ester, and next this ester is hydrolyzed by an activated water molecule that attacks the Cγ atom of the Asp_{226} residue, as illustrated in Fig. 6-11. Therefore, in carboxylesterase, the oxygen incorporated into the product is derived from the activated water molecule. In contrast, in epoxide hydrolase, the oxygen incorporated into the product is derived from the nucleophile Asp_{226} (Fig. 6-11).

Carboxylesterases and epoxide hydrolases exhibit no primary sequence identity, but they share surprising similarities in the topology of the structure and sequential arrangement of the catalytic triad. Both are members of the α/β-hydrolase fold enzymes, a superfamily of proteins that includes lipases, esterases, and haloalkane dehydrogenases (Beetham et al., 1995). Functionally, proteins in this superfamily all catalyze hydrolytic reactions; structurally, they all contain a similar core segment that is composed of eight β-sheets connected by α-helices. They all have a catalytic triad and the arrangement of the amino acid residues in the triad (i.e., the order of the nucleophile, the acid, and the base in the primary sequence) is the mirror image of the arrangement in other hydrolytic enzymes such as trypsin. All three active-site residues are located on loops that are the best conserved structural features in the fold, which likely provides catalysis with certain flexibility to hydrolyze numerous structurally distinct substrates.

Reduction

Certain metals (e.g., pentavalent arsenic) and xenobiotics containing an aldehyde, ketone, alkene, disulfide, sulfoxide, quinone, *N*-oxide, hydroxamic acid, amidoxime, isoxazole, isothiazole, azo, or nitro group are often reduced in vivo, although it is sometimes difficult to ascertain whether the reaction proceeds enzymatically or nonenzymatically by interaction with reducing agents (such as the reduced forms of glutathione, FAD, FMN, and NAD[P]). Some of these functional groups can be either reduced or oxidized. For example, aldehydes (R–CHO) can be reduced to an alcohol ($R–CH_2OH$) or oxidized to a carboxylic acid (R–COOH), whereas sulfoxides ($R_1–SO–R_2$) can be reduced to a sulfide ($R_1–S–R_2$) or oxidized to a sulfone ($R_1–SO_2–R_2$). Likewise, some enzymes, such as alcohol dehydrogenase (ADH), aldehyde oxidase (AO), and CYP, can catalyze both reductive and oxidative reactions depending on the substrate or conditions (e.g., aerobic vs. anaerobic). In the case of halogenated hydrocarbons, such as halothane, dehalogenation can proceed by an oxidative or reductive pathway, both of which are catalyzed by the same enzyme (namely, CYP). In some cases, such as azo-reduction, nitro-reduction, and the reduction of certain alkenes, the reaction is largely catalyzed by intestinal microbiota.

Azo- and Nitro-Reduction Prontosil and chloramphenicol are examples of drugs that undergo azo- and nitro-reduction, respectively, as shown in Fig. 6-15. Reduction of prontosil is of historical interest. Treatment of streptococcal and pneumococcal infections with prontosil marked the beginning of specific antibacterial chemotherapy. Subsequently, it was discovered that the active drug was not prontosil but its metabolite sulfanilamide (*para*-aminobenzene sulfonamide), a product of azo-reduction. During azo-reduction, the nitrogen–nitrogen double bond is sequentially reduced and cleaved to produce two primary amines, a reaction requiring four reducing equivalents. RT Williams subsequently provided additional evidence that intestinal microbiota were involved in prontosil and neoprontosil azo-reduction with the finding that total sulfanilamide excretion was significantly reduced in rats pretreated with antibiotics (Gingell et al., 1971). Nitro-reduction requires six reducing equivalents, which are consumed in three sequential reactions, as shown in Fig. 6-15, for the conversion of nitrobenzene to aniline.

Azo- and nitro-reduction reactions are generally catalyzed by intestinal microbiota. However, under certain conditions, such as low oxygen tension, the reactions can be catalyzed by liver microsomal CYP and NAD(P)H-quinone oxidoreductase (NQO1, a cytosolic flavoprotein that is also known as DT-diaphorase) and, in the case of nitroaromatics, by cytosolic AO. The anaerobic environment of the lower gastrointestinal tract is well suited for azo- and nitro-reduction, which is why intestinal microbiota contribute significantly to these reactions. The reduction of quinic acid to benzoic acid is another example of a reductive reaction catalyzed by gut microbiota, as shown in Fig. 6-4.

Nitro-reduction by intestinal microbiota is thought to play an important role in the toxicity of several nitroaromatic compounds including 2,6-dinitrotoluene, which is hepatotumorigenic to male rats. The role of nitro-reduction in the metabolic activation of 2,6-dinitrotoluene is shown in Fig. 6-16 (Long and Rickert,

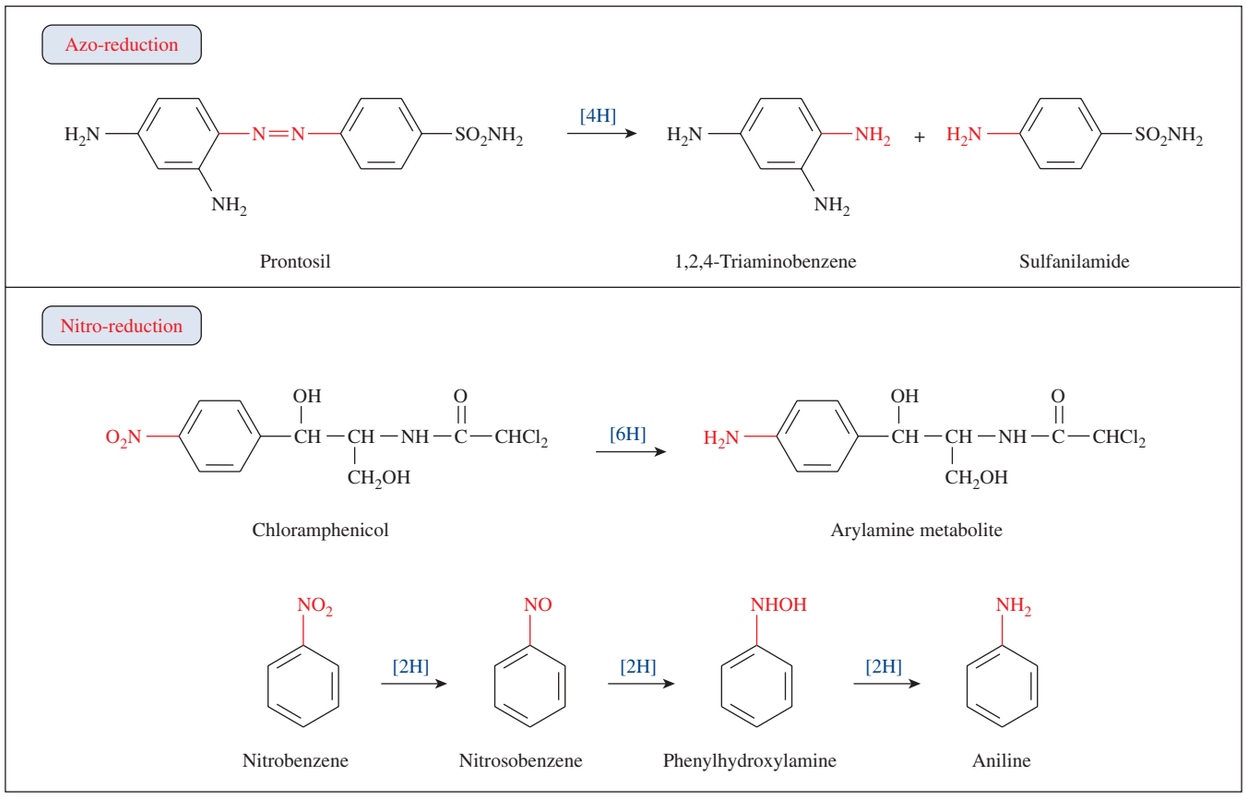


Figure 6-15. *Examples of drugs that undergo azo reduction (prontosil) and nitro-reduction (chloramphenicol and nitrobenzene).*

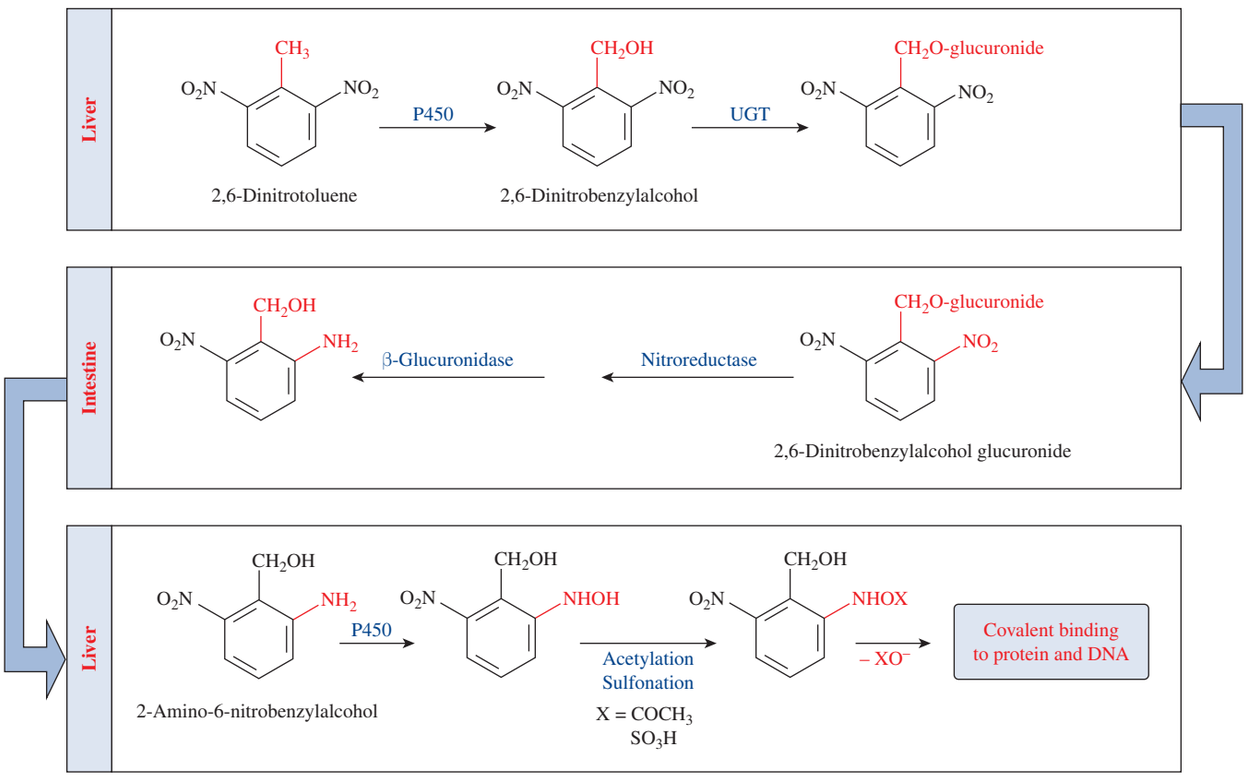


Figure 6-16. *Role of nitro reduction by intestinal microbiota in the activation of the rat liver tumorigen, 2,6-dinitrotoluene.*

1982; Mirsalis and Butterworth, 1982). The biotransformation of 2,6-dinitrotoluene begins in the liver, where it is oxidized by CYP and conjugated with glucuronic acid. This glucuronide is excreted in bile and undergoes biotransformation by intestinal microbiota. One or both of the nitro groups are reduced to amines by nitroreductase, and the glucuronide is hydrolyzed by β-glucuronidase. The reduced/deconjugated metabolites are absorbed and transported to the liver, where the newly formed amine group is *N*-hydroxylated by CYP and conjugated with acetate or sulfonate. These conjugates form good leaving groups, which renders the nitrogen highly susceptible to nucleophilic attack from proteins and DNA; this ostensibly leads to mutations and the formation of liver tumors. Compared with females, male rats are more susceptible to hepatotumorigenicity of 2,6-dinitrotoluene due to their higher rate of biliary excretion of 2,6-dinitrobenzylalcohol glucuronide. The complexity of the metabolic scheme shown in Fig. 6-16 underscores an important principle that the activation of some chemical tumorigens to DNA-reactive metabolites involves several different biotransforming enzymes and may take place in more than one tissue. Consequently, the ability of 2,6-dinitrotoluene to bind to DNA and cause mutations is not revealed in most of the short-term assays for assessing the genotoxic potential of chemicals. These in vitro assays for genotoxicity do not make allowance for biotransformation by intestinal microbiota or, in some cases, the conjugating enzymes.

Nitro-reduction by intestinal microbiota plays an important role in the biotransformation of musk xylene (1-*tert*-butyl-3,5-dim ethyl-2,4,6-trinitrobenzene). Reduction of one or both of the nitro groups is required for musk xylene to induce (as well as markedly inhibit) liver microsomal CYP (namely, CYP2B) in rodents (Lehman-McKeeman et al., 1999). Azo- and nitro-reduction catalyzed by intestinal microbiota also play a role in the toxicity and disposition of several drugs. For instance, the teratogenicity (e.g., exencephaly, cleft palate, and limb reductions) observed with nitrazepam (approved in the United Kingdom and several other countries) appears to be due to this drug's conversion to 7-aminonitrazepam and 7-acetylaminonitrazepam in normal pregnant rats, but not in those pretreated with antibiotics to deplete intestinal microbiota (Takeno and Sakai, 1991; Klaassen and Cui, 2015; Enright et al., 2016). Clonazepam and misonidazole also undergo nitro-reduction catalyzed by the intestinal microbiota (Klaassen and Cui, 2015; Jourova et al., 2016). In addition, metronidazole bears a black box warning for carcinogenicity in mice and rats, which may be in part due to nitro-reduction and further conversion of this drug to acetamide and *N*-(2-hydroxyethyl)-oxamic acid by the intestinal microbiota (Koch and Goldman, 1979; Enright et al., 2016). Sulfasalazine is a poorly absorbed prodrug indicated for ulcerative colitis that links 5-aminosalicylic acid (5-ASA) with sulfapyridine with an azo bond. In the colon, it undergoes azo-reduction by the microbiota to release both drugs, with the anti-inflammatory action of 5-ASA attributable to suppression of cytokines within the mucosa (Rachmilewitz et al., 1992; Klaassen and Cui, 2015). Other drugs that undergo azo-reduction by the intestinal microbiota include balsalazide and olsalazine (Klaassen and Cui, 2015; Jourova et al., 2016).

Azo-reduction by the intestinal microbiota is also implicated in the activation of azo dyes (Chung, 2013; Claus et al., 2016). More than 3000 azo dyes are used in various industries, notably in the production of processed foods and pharmaceuticals where small amounts of the dyes are directly ingested and can theoretically be acted upon by the intestinal microbiota. For instance, at least five azo dyes are used as food colorants, including Sunset Yellow (FD&C Yellow 6), Orange B, Allura Red (FD&C Red 40), Citrus Red No. 2, and tartrazine (FD&C Yellow 5). These dyes have no carcinogenic, mutagenic, or cytotoxic effects, but some studies provide evidence that azo-reductases from the intestinal microbiota can cleave the azo bond to form potentially carcinogenic aromatic amines (Claus et al., 2016).

Carbonyl Reduction: AKRs, SDRs, and Others A variety of xenobiotics contain a carbonyl function (R–CHO and R_1–CO–R_2) that undergoes reduction in vivo. The reduction of aldehydes to primary alcohols and of ketones to secondary alcohols is generally catalyzed in mammals by NAD(P)H-dependent reductases belonging to one of several superfamilies, the aldo–keto reductases (AKRs), the short-chain dehydrogenases/reductases (SDRs), the medium-chain dehydrogenases/reductases (MDR), aldehyde dehydrogenases (ALDH), and NAD(P)H-quinone oxidoreductases (NQO), as shown in Table 6-9 (Jez and Penning, 2001; Oppermann et al., 2001; Matsunaga et al., 2006; Malatkova et al., 2010; Skarydova and Wsol, 2012).

The 15 human AKRs are members of a superfamily of cytosolic, generally monomeric, enzymes that reduce both xenobiotic and endobiotic compounds, as their alternative names imply (Table 6-9). Although AKRs can catalyze oxidations in vitro, it is likely that they function nearly exclusively as reductases in vivo (with the notable exception of oxidation of several *trans*-dihydrodiols of polycyclic aromatic hydrocarbon oxiranes—covered below) (Penning, 2015). The propensity for AKRs to catalyze reductions rather than oxidations is mainly due to their affinity for NADP(H) (10 to 120 nM) versus NAD(H) (100 to 250 μM), which also ensures potent product inhibition for any NAD+-dependent oxidations that may occur (Penning, 2015).

Dimeric dihydrodiol dehydrogenase and various members of the AKR superfamily (particularly AKR1A1, AKR1C1, AKR1C2, AKR1C3, and AKR1C4), functioning as dihydrodiol dehydrogenases, can oxidize the *trans*-dihydrodiols of various polycyclic aromatic hydrocarbon oxiranes (formed by epoxide hydrolase) to the corresponding *ortho*-quinones, as shown previously in Fig. 6-14 (Penning, 2014). The role of AKRs as oxidizing enzymes is discussed in the section on oxidative enzymes (see the section "Dimeric Dihydrodiol Dehydrogenase"). As mentioned earlier (see Point 9 in the "Principles of Xenobiotic Biotransformation"), AKR7A2 and AKR7A3 (also known as aflatoxin aldehyde B_1 reductases 1 and 2) are two of the many enzymes induced following activation of Nrf2 by oxidative stress, exposure to electrophiles, or depletion of glutathione (Ahmed et al., 2011; Li et al., 2015). The dimeric AKR7As also rapidly reduce the *ortho*-quinones once formed (but cannot oxidize the *trans*-dihydrodiols), increasing the likelihood of redox cycling in cells where both sets of enzymes are expressed (Penning, 2014).

Various members of the AKR superfamily have been implicated in the reduction of carbonyl-containing xenobiotics as the tobacco-specific nitrosamine NNK, acetohexamide, daunorubicin, naloxone, naltrexone, befunolol, ethacrynic acid, ketoprofen, ketotifen, haloperidol, loxoprofen, metyrapone, oxonortriptyline, and numerous aromatic and aliphatic aldehydes (Rosemond and Walsh, 2004). Putative AKR1A, AKR1B, AKR1C1–4, or AKR1D1-selective inhibitors, statil, flufenamic acid, phenolphthalein, and finasteride, respectively, can aid in the evaluation of which reductive enzyme(s) is(are) involved in the formation of specific metabolites. Many of the xenobiotics reduced by AKRs are also reduced by SDRs, and in most cases the relative contribution of individual carbonyl-reducing enzymes is not known. Genetic polymorphisms of human AKR1C3, AKR1C4, AKR7A2, and cytosolic carbonyl reductase (CBR1) have been associated with decreased metabolism of carbonyl-containing xenobiotics such as doxorubicin and daunorubicin in vitro

Table 6-9

Human Aldo-Keto Reductases (AKRs), Short-Chain Dehydrogenases/Reductases (SDRs), Medium-Chain Dehydrogenases/Reductases (MDRs), and Quinone Reductases (NQOs)

ENZYME SUPERFAMILY	EXAMPLE ENZYMES	ALTERNATIVE NAMES	SUBCELLULAR LOCALIZATION
Aldo-keto reductase (AKR), 15 enzymes	AKR1A1	Aldehyde reductase	Cytosol
	AKR1B1	Aldose reductase	Cytosol
	AKR1B10	Small intestine reductase (small intestine and liver)	Cytosol
	AKR1B15	Putative aldo-keto reductase family 1 member B15	Membrane-associated
	AKR1C1	20α-Hydroxysteroid dehydrogenase, dihydrodiol dehydrogenase (DD1)	Cytosol
	AKR1C2	3α-Hydroxysteroid dehydrogenase, dihydrodiol dehydrogenase (DD2), bile acid binding protein	Cytosol
	AKR1C3	3α-Hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase type V, dihydrodiol dehydrogenase (DD3)	Cytosol
	AKR1C4	3α-Hydroxysteroid dehydrogenase, chlordecone reductase, dihydrodiol dehydrogenase (DD4)	Cytosol
	AKR1D1	Δ^4-3-Ketosteroid-5β-reductase	Cytosol
	AKR1E2	AKRDC1; AKR1CL2; LoopADR	Unknown
	AKR6A3, 6A5, 6A9	Shaker-channel subunit Kvb1, Kvb2, Kvb3 (KCNAB1-3)	Cytosol
	AKR7A2	Aflatoxin B_1 aldehyde reductase 2	Golgi
	AKR7A3	Aflatoxin B_1 aldehyde reductase 3	Cytosol
Short-chain dehydrogenase/ reductases (SDR), 80 enzymes	Cytosolic carbonyl reductases (gene symbols CBR1 and CBR3)	Xenobiotic ketone reductase with pH 6.0 activity; prostaglandin 9-ketoreductase; human placental NADP-linked 15-hydroprostaglandin dehydrogenase, SDR21C1, and SDR21C2	Cytosol
	Microsomal carbonyl reductase (gene symbol HSD11B1)	11β-Hydroxysteroid dehydrogenase; 11β-HSD1; 11β-reductase, 11-oxidoreductase, SDR26C1	Microsomes
Medium-chain dehydrogenase/ reductases (MDR), 7 ADH enzymes	ADH1A	Class I ADH; ADH1, α, β, γ; hADH1,2,3	Cytosol
	ADH1B	Class I ADH; ADH2, β, γ; ADHII; hADH4	Cytosol
	ADH1C	Class I ADH; ADH3, γ	Cytosol
	ADH4	Class II ADH; π, hADH7	Cytosol
	ADH5	Class III ADH; χ, ADH3	Cytosol
	ADH6	Class V ADH	Cytosol
	ADH7	Class IV ADH; μ (σ)	Cytosol
NQO, 2 enzymes	NQO1	DT diaphorase, menadione reductase	Cytosol
	NQO2	*N*-Ribosyldihydronicotinamide dehydrogenase	Cytosol

Abbreviation: NQO, NADPH-quinone oxidoreductase.

Members of the aldo-keto reductase superfamily can be found at http://www.med.upenn.edu/akr/members.shtml. Information on human SDR membranes can be found at http://www.sdr-enzymes.org/resources.htm.

(Gonzalez-Covarrubias et al., 2007; Bains et al., 2010). Allelic variants of human cytosolic AKR1C2 with reduced enzymatic activity toward the androgen 5α-dihydrotestosterone (DHT) have also been identified (Takahashi et al., 2009).

Humans contain at least 80 SDR members, three of which, namely, cytosolic carbonyl reductases (CBR1 and CBR3) and a microsomal carbonyl reductase (HSD11B1), play a role in the reduction of a wide variety of carbonyl-containing xenobiotics (other species express more than two carbonyl reductases) (see SDR [2018]). Erythrocytes also contain carbonyl reductase, which contributes significantly to the reduction of haloperidol, as shown in Fig. 6-17. From the alternative names given in Table 6-9, it is apparent that both cytosolic and microsomal carbonyl reductases have been studied for their role in endobiotic metabolism, namely, the reduction of prostaglandin derivatives and 11β-hydroxysteroids, respectively (Kavanagh et al., 2008). There appear to be no reports of clinically significant drug–drug interactions involving the inhibition or induction of AKRs or SDRs (Rosemond and Walsh, 2004).

In certain cases, the reduction of aldehydes to alcohols can be catalyzed by alcohol dehydrogenase, as shown in Fig. 6-17, for the conversion of the sedative-hypnotic, chloral hydrate, to trichloroethanol. As shown in Table 6-9, alcohol dehydrogenases belong to the medium-chain dehydrogenases/reductases (MDRs). They typically convert alcohols to aldehydes, for which reason they are discussed later in the section on oxidative reactions (see section "Alcohol Dehydrogenase"). In the case of chloral hydrate, the reverse reaction is favored by the presence of the trichloromethyl group, which is a strong electron-withdrawing group.

The SDR carbonyl reductases are monomeric, NADPH-dependent enzymes present in erythrocytes and both the cytosolic and microsomal fractions of the liver, kidney, brain, and many other tissues. The major circulating metabolite of the antipsychotic drug

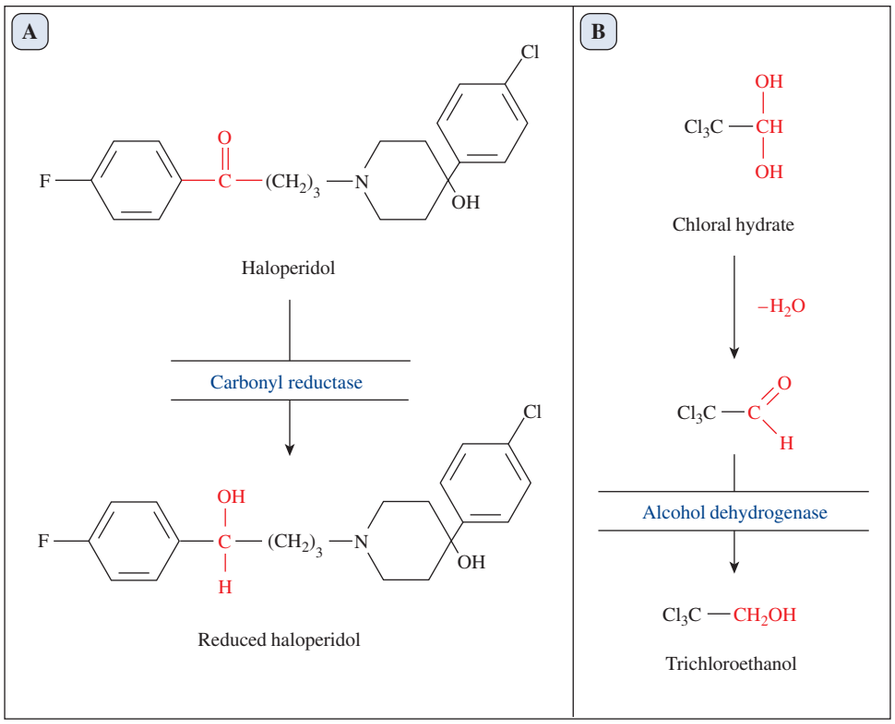


Figure 6-17. *Reduction of xenobiotics by carbonyl reductase (**A**) and alcohol dehydrogenase (**B**).*

haloperidol is a secondary alcohol formed by carbonyl reductases in the blood and liver, as shown in Fig. 6-17. Other xenobiotics that are reduced by carbonyl reductases include pentoxifylline (see Fig. 6-6), acetohexamide, daunorubicin, doxorubicin, loxoprofen, menadione, 4-nitroacetophenone, timiperone, and *R*-warfarin (Rosemond and Walsh, 2004). As shown in Fig. 6-6, the reduction of ketones to secondary alcohols by carbonyl reductases may proceed with a high degree of stereoselectivity, as in the case of pentoxifylline (Lillibridge et al., 1996a).

Liver cytosol contains at least two carbonyl reductases (CBR1 and CBR3), and microsomes contain at least one other form of carbonyl reductase (HSD11B1), and these can differ in the degree to which they stereoselectively reduce ketones to secondary alcohols. For example, keto-reduction of pentoxifylline produces two enantiomeric secondary alcohols: one with the *R*-configuration (which is known as lisofylline) and one with the *S*-configuration, as shown in Fig. 6-6. Reduction of pentoxifylline by cytosolic carbonyl reductases results in the stereospecific formation of the optical antipode of lisofylline, whereas the same reaction catalyzed by microsomal carbonyl reductase produces both lisofylline and its optical antipode in a ratio of about 1 to 5 (Lillibridge et al., 1996a).

The well-known microsomal carbonyl reductase is 11β-hydroxysteroid dehydrogenase (gene symbol HSD11B1, aka SDR26C1) (Skarydova and Wsol, 2012). However, there are several other microsomal carbonyl-reducing enzymes, such as the six human retinol dehydrogenases (e.g., RDHs) and the six human 17β-hydroxysteroid dehydrogenases. Although these enzymes have not been well-characterized for their ability to reduce carbonyl-containing xenobiotics, there is indirect evidence from the ratio of enantiomers of metabolites that multiple microsomal enzymes may play a role in the metabolism of some xenobiotics such as 7α-methyl-19-nortestotsterone (Skarydova and Wsol, 2012). There are at least two monomeric cytosolic carbonyl reductases (gene symbols CBR1 [aka SDR21C1] and CBR3 [aka SDR21C2]) that have broad and somewhat overlapping substrate specificities toward many endobiotics and xenobiotics such as menadione, isatin, NNK, daunorubicin, and doxorubicin (Malatkova et al., 2010). A human tetrameric mitochondrial carbonyl reductase, namely CBR4 (aka SDR45C1), has also been identified with activity toward 9,10-phenanthrenequinone and 1,4-benzoquinone, and therefore acts as a mitochondrial quinone reductase (Malatkova et al., 2010).

In rat liver cytosol, the reduction of quinones is primarily catalyzed by NQO1 and NQO2 (see the section "Quinone Reduction: NQO1 and NQO2"), whereas in human liver cytosol, quinone reduction is catalyzed by both NQO and carbonyl reductases.

Disulfide Reduction Some disulfides are reduced and cleaved to their sulfhydryl components, as shown in Fig. 6-18, for the alcohol deterrent disulfiram (Antabuse). As shown in Fig. 6-18, disulfide reduction by glutathione is a three-step process, the last of which is catalyzed by glutathione reductase. The first step can be catalyzed by glutathione transferase, or it can occur nonenzymatically. The disulfide, thioctic acid (α-lipoic acid), is a prescription drug in Germany and some other countries that is indicated mainly for diabetic polyneuropathy. It can be reduced in NADPH-dependent reactions catalyzed by cytosolic glutathione reductase (GSR) and thioredoxin reductases (cytosolic TXNRD1, and mitochondrial TXNRD2), as well as by the NADH-dependent mitochondrial enzyme (Arner et al., 1996; Haramaki et al., 1997; Testa and Kramer, 2008).

Sulfoxide and *N*-Oxide Reduction Thioredoxin-dependent enzymes in liver and kidney cytosol have been reported to reduce sulfoxides, which themselves may be formed by CYP or flavin monooxygenases (Anders et al., 1981). Recycling through these counteracting enzyme systems, a process known as retro-reduction or futile cycling, may prolong the half-life of certain xenobiotics (Hinrichs et al., 2011). As shown in Fig. 6-19, sulindac is a sulfoxide that undergoes reduction to a sulfide, which is excreted in bile and reabsorbed from the intestine (Ratnayake et al., 1981). This

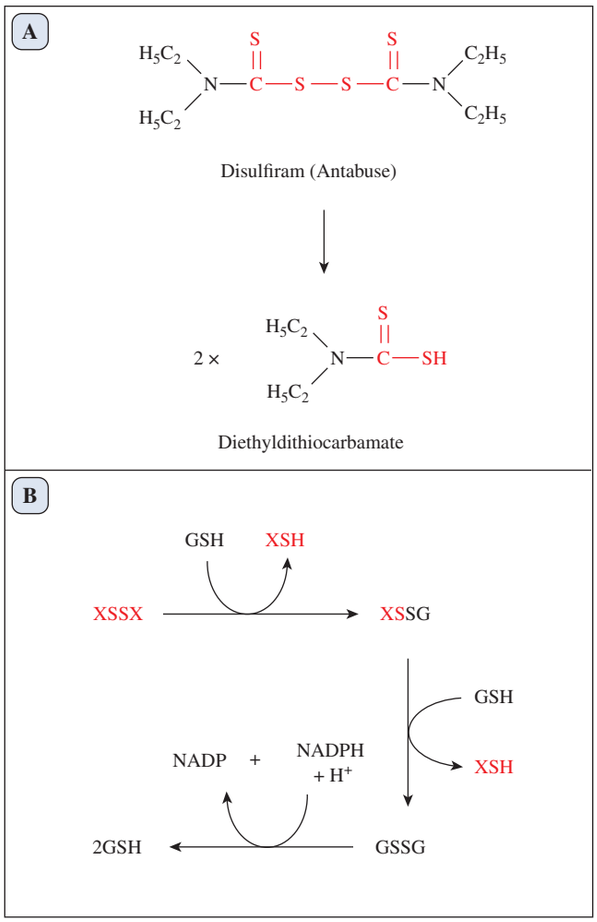


Figure 6-18. *Biotransformation of disulfiram by disulfide reduction (**A**) and the general mechanism of glutathione-dependent disulfide reduction of xenobiotics (**B**). GSH, glutathione; GSSG, reduced glutathione; XSSX, xenobiotic disulfide*. The last reaction in (**B**) is catalyzed by glutathione reductase.

enterohepatic cycling prolongs the duration of action of the drug such that this NSAID need only be taken twice daily. In the human liver, glutaredoxin and thioredoxin may also be involved in reducing the mixed disulfide formed between xenobiotics and glutathione, as is the case in the formation of the pharmacologically active metabolite of prasugrel, an inhibitor of $P2Y_{12}$, the platelet ADP-binding receptor (Hagihara et al., 2011).

Sulfoxide reduction may also occur nonenzymatically at an appreciable rate, as in the case of the proton pump inhibitor rabeprazole (Miura et al., 2006). Diethyldithiocarbamate methyl ester, a metabolite of disulfiram, is oxidized to a sulfine, which is reduced to the parent methyl ester by glutathione. In the latter reaction, two molecules of glutathione (GSH) are oxidized with reduction of the sulfine oxygen to water (Madan et al., 1994) as shown below:

$$R_1R_2C = S^+ - O^- + 2GSH \rightarrow R_1R_2C = S + GSSG + H_2O$$

Just as sulfoxide reduction can reverse the effect of sulfoxidation, so the reduction of *N*-oxides can reverse the *N*-oxygenation of amines, which is catalyzed by FMO and CYP enzymes. Under reduced oxygen tension, reduction of the *N*-oxides of imipramine, tiaramide, indicine, and *N*, *N*-dimethylaniline can be catalyzed by mitochondrial and/or microsomal enzymes in the presence of NADH or NADPH (Sugiura and Kato, 1977). The NADPH-dependent reduction of *N*-oxides in liver microsomes appears to be catalyzed by CYP (Sugiura et al., 1976) although in some cases NADPH-cytochrome P450 reductase may play an important role.

As a class, *N*-oxides are not inherently toxic compounds. However, certain aromatic and aliphatic *N*-oxides have been exploited as bioreductive drugs (also known as DNA-affinic drugs) for the treatment of certain cancers and infectious diseases (Wardman et al., 1995). In these cases, *N*-oxides have been used as prodrugs that are converted to cytotoxic or DNA-binding drugs under hypoxic conditions. The fact that *N*-oxides of certain drugs are converted to toxic metabolites under hypoxic conditions is the basis for their selective toxicity to certain solid tumors (namely, those that are hypoxic and, hence, resistant to radiotherapy) and anaerobic bacteria. For example, tirapazamine (SR 4233) is a benzotriazine di-*N*-oxide that is preferentially toxic to hypoxic cells, such as those present in solid tumors, apparently due to its rapid activation by one-electron reduction of the *N*-oxide to an oxidizing nitroxide radical, as shown in Fig. 6-19 (Walton et al., 1992). This reaction is catalyzed by CYP and NADPH-CYP reductase (Saunders et al., 2000). Two-electron reduction of the di-*N*-oxide, SR 4233, produces a mono-*N*-oxide, SR 4317, which undergoes a second *N*-oxide reduction to SR 4330. Like SR 4233, the antibacterial drug quindoxin is a di-*N*-oxide whose cytotoxicity is dependent on reductive activation, which is favored by anaerobic conditions. AQ4N is a di-*N*-oxide prodrug that is converted by *N*-oxide reduction to the potent topoisomerase II inhibitor 4QA (Nishida et al., 2010). The reductive reaction is catalyzed by CYP2S1 and CYP2W1, two hypoxia-inducible CYP enzymes expressed in many solid tumors. The induction of CYP2S1 and CYP2W1 in hypoxic tumor cells provides a basis for their application in the selective bioreductive activation of antineoplastic drugs (Nishida et al., 2010).

Bioreductive alkylating agents, which include such drugs as mitomycins, anthracyclins, and aziridinylbenzoquinones, represent another class of anticancer drugs that require activation by reduction. However, for this class of drugs, bioactivation also involves a two-electron reduction reaction, which is largely catalyzed by NQO, which is described in the next section.

Quinone Reduction: NQO1 and NQO2 Quinones can be reduced to hydroquinones by two closely related, cytosolic flavoproteins, namely, NQO1 and NQO2. The former enzyme, NAD(P)H-quinone oxidoreductase-1, is also known as DT-diaphorase. The latter enzyme, NAD(P)H-quinone oxidoreductase-2, is also known as NRH-quinone oxidoreductase because it prefers the unusual electron donor dihydronicotinamide riboside (NMR) over NAD(P)H. Although they are closely related enzymes (both contain two 27-kDa subunits each with an FAD prosthetic group), NQO1 and NQO2 have different substrate specificities, and they can be distinguished on the basis of their differential inhibition by dicoumarol and quercetin (which are selective inhibitors of NQO1 and NQO2, respectively). NQO2 may have a physiological role in the metabolism of vitamin K hydroquinone (Chen et al., 2000).

An example of the type of reaction catalyzed by NQO is shown in Fig. 6-20. Formation of the hydroquinone involves a two-electron reduction of the quinone with stoichiometric oxidation of NAD[P]H *without* oxygen consumption. (The two-electron reduction of certain quinones can also be catalyzed by carbonyl reductase, especially in humans.) In contrast, NADPH-cytochrome P450 reductase, a microsomal flavoprotein, catalyzes the one-electron reduction of quinones to semiquinone radicals that, in addition to being reactive metabolites themselves, cause oxidative stress by reacting with oxygen to form reactive oxygen species, which leads to nonstoichiometric oxidation of NADPH and oxygen

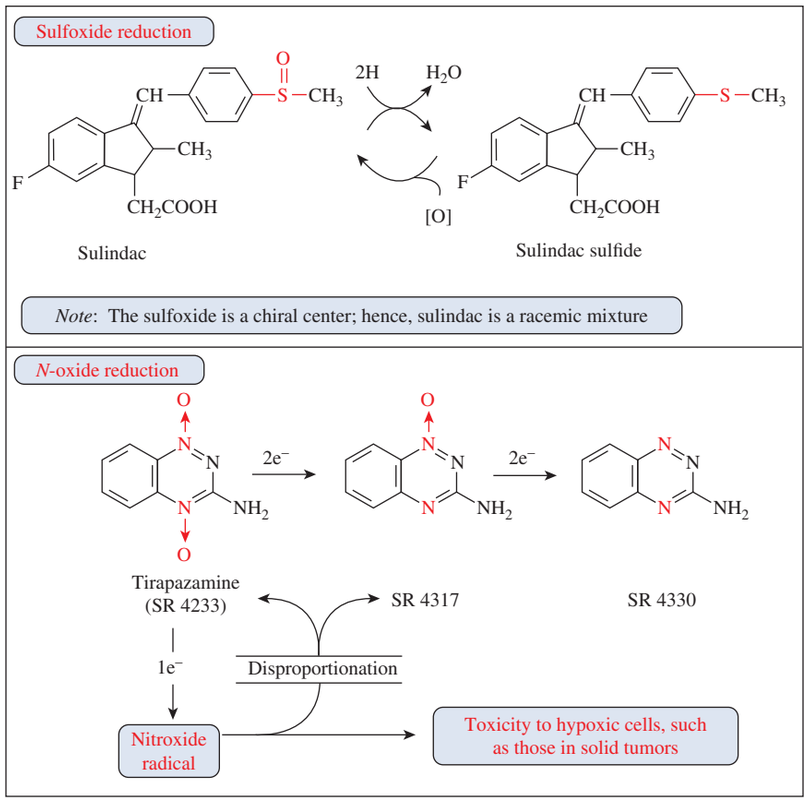


Figure 6-19. *Examples of sulfoxide and N-oxide reduction.* Note that tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide) is a representative of a class of agents that are activated by reduction, which may be clinically useful in the treatment of certain tumors.

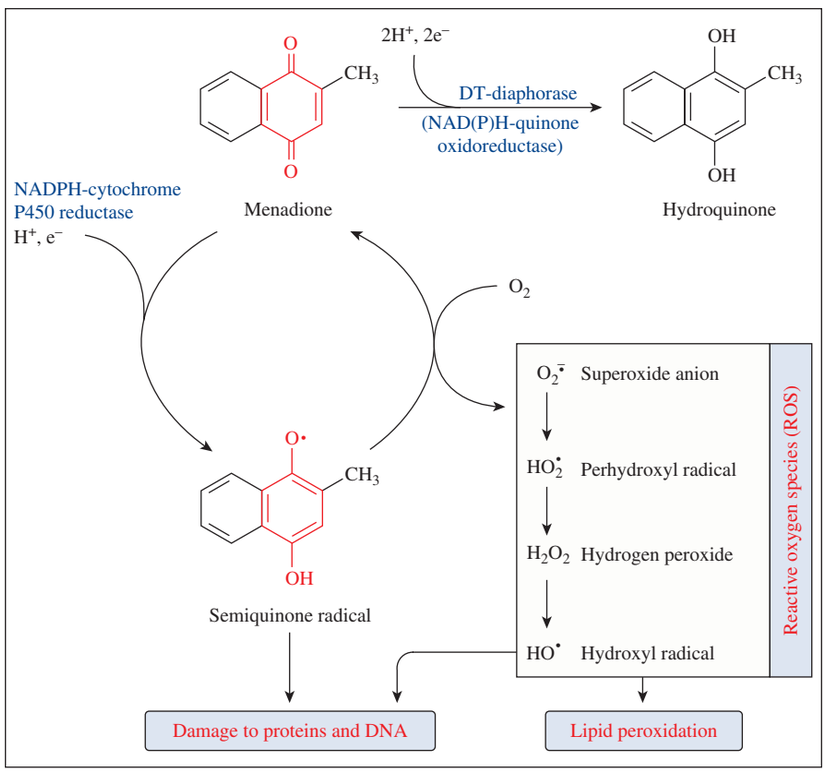


Figure 6-20. *Two-electron reduction of menadione to a hydroquinone, and production of reactive oxygen species during its one-electron reduction to a semiquinone radical.*

consumption, as shown in Fig. 6-20. The two-electron reduction of quinones is a nontoxic reaction—one that is not associated with semiquinone formation and oxidative stress—provided the resultant hydroquinone is sufficiently stable to undergo glucuronidation or sulfonation. However, there are quinone-containing xenobiotics that, despite undergoing two-electron reduction by NQO, produce semiquinone free radicals, oxidative stress, DNA damage, and cytotoxicity. Many of these xenobiotics are being developed as anticancer drugs because NQO1 is often overexpressed in tumor cells (Oh and Park, 2015). The impact of the null allele NQO1*2 (discussed below in this section) on the sensitivity and resistance to antitumor quinones remains to be established (Siegel et al., 2012). The properties of the hydroquinone determine whether, during the metabolism of quinone-containing xenobiotics, NQO functions as a protective antioxidant or a pro-oxidant activator leading to the formation of reactive oxygen species and reactive semiquinone free radicals. The latter are thought to form not from the one-electron reduction of the quinone but from the two-electron reduction of the quinone (Q) to the hydroquinone (QH_2), which then undergoes one-electron oxidation or perhaps disproportionation to form the reactive semiquinone (QH):

$$QH_2 + Q \leftrightarrow 2QH$$

Drugs or drug candidates that are activated by NQO to anticancer agents include the aziridinylbenzoquinone diaziquone, the anthraquinone mitoxantrone, the indolquinones mitomycin C and EO9 (an analog of mitomycin C that is more rapidly reduced by NQO1), and the anthracycline antibiotics daunorubicin and doxorubicin (Gutierrez, 2000). These so-called bioreductive alkylating agents are reduced by NQO1 to generate semiquinone free radicals and other reactive intermediates that undergo nucleophilic additions with DNA, resulting in single-strand DNA breaks. The reason such drugs are preferentially toxic to tumor cells is that tumor cells, especially those in solid tumors, are hypoxic, and hypoxia induces the synthesis of NQO1 (by a mechanism that involves the activator protein 1 [AP-1] and nuclear factor-κB [NF-κB] response elements in the 5′-promoter region of the NQO1 gene). Therefore, tumor cells often express high levels of NQO1, which predisposes them to the toxic effects of quinone-reductive anticancer drugs like mitomycin C. Interestingly, mitomycin C also upregulates the expression of NQO1, which may enable this anticancer drug to stimulate its own metabolic activation in tumor cells (Yao et al., 1997). Some cancer chemotherapeutic drugs, such as the *N*-oxide SR 4233 (tirapazamine), are inactivated, not activated, by NQO, as shown in Fig. 6-19.

NQO can activate certain nitroaromatic compounds ($R–NO_2$) to the corresponding hydroxylamine (R–NHOH), which can be activated by acetylation or sulfonation (by pathways analogous to those shown in Fig. 6-16). Dinitropyrenes and the nitroaromatic compound CB 1954 are activated by NQO. The latter compound was under consideration as an anticancer agent. However, although it is activated by reduction by rat NQO, the nitroaromatic compound CB 1954 is not activated by human NQO.

Oxidative stress is an important component to the mechanism of toxicity of several xenobiotics that either contain a quinone or can be biotransformed to a quinone (Anders, 1985). The production of superoxide anion radicals and oxidative stress are responsible, at least in part, for the cardiotoxic effects of doxorubicin (adriamycin) and daunorubicin (daunomycin), the pulmonary toxicity of paraquat and nitrofurantoin, and the neurotoxic effects of 6-hydroxydopamine. Oxidative stress also plays an important role in the destruction of pancreatic beta cells by alloxan and dialuric acid. Tissues low in superoxide dismutase activity, such as the heart, are especially susceptible to the oxidative stress associated with the redox cycling of quinones. This accounts, at least in part, for the cardiotoxic effects of adriamycin and related anticancer drugs, although other susceptibility factors have been proposed (Mordente et al., 2001).

As already mentioned in this section, it is now apparent that the structure of the hydroquinones produced by NQO determines whether the two-electron reduction of quinones results in xenobiotic detoxication or activation. Hydroquinones formed by two-electron reduction of unsubstituted or methyl-substituted 1,4-naphthoquinones (such as menadione) or the corresponding quinone epoxides are relatively stable to autoxidation, whereas the methoxyl, glutathionyl, and hydroxyl derivatives of these compounds undergo autoxidation with production of semiquinones and reactive oxygen species. The ability of glutathionyl derivatives to undergo redox cycling indicates that conjugation with glutathione does not prevent quinones from serving as substrates for NQO. The glutathione conjugates of quinones can also be reduced to hydroquinones by carbonyl reductases, which actually have a binding site for glutathione. In human carbonyl reductase, this binding site is Cys_{227}, which is involved in binding both substrate and glutathione (Tinguely and Wermuth, 1999). Although oxidative stress is an important mechanism by which quinones cause cellular damage (through the intermediacy of semiquinone radicals and the generation of reactive oxygen species), it should be noted that quinones are Michael acceptors, and cellular damage can occur through direct alkylation of critical cellular proteins and/or DNA (Bolton et al., 2000).

NQO1 is inducible up to 10-fold by two classes of inducers, which have been categorized as *bifunctional* and *monofunctional* inducers (Prochaska and Talalay, 1988). The bifunctional inducers include compounds like β-naphthoflavone, benzo[*a*]pyrene, 3-methylcholanthrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin or TCDD), which are AhR (aryl hydrocarbon receptor) agonists that induce both oxidative enzymes (such as the CYP enzyme CYP1A1) and conjugating enzymes (such as glutathione transferase and UDP-glucuronosyltransferase). The monofunctional inducers are Nrf2 activators that tend to induce conjugating and other non-CYP enzymes (although in mice, monofunctional inducers can induce CYP2C55 and CYP2U1, as well as aldehyde oxidase). The AhR agonists (the so-called bifunctional inducers) signal through XRE (the xenobiotic-response element), whereas Nrf2 activators (the so-called monofunctional inducers) signal through ARE (the antioxidant response element), which is also known as the EpRE (the electrophilic response element). (Response elements are short sequences of DNA, often located in the 5′-promoter region of a gene, that bind the transcription factors that control gene expression.) The monofunctional inducers can be subdivided into two chemical classes: those that activate Nrf2 by causing oxidative stress through redox cycling (e.g., the quinone, menadione, and the phenolic antioxidants *tert*-butylhydroquinone and 3,5-di-*tert*-butylcatechol), and those that activate Nrf2 by causing oxidative stress by depleting glutathione (e.g., fumarates, maleates, acrylates, isothiocyanates, and other Michael acceptors that react with glutathione). Experiments in wild-type and Nrf2 knockout mice revealed that activation of AhR leads to activation of Nrf2, which contains an AhR-response element in its promoter region; hence, AhR agonists such as dioxin induce enzymes under the direct control of AhR (such as CYP1A1) as well as enzymes under the control of Nrf2 (such as NQO1 and the other enzymes mentioned above) (Yeager et al., 2009). Induction of Nrf2 via activation of AhR protects mice from the hepatotoxic effects of dioxin (Lu et al., 2011).

The bifunctional inducers are direct activators of AhR and indirect activators of Nrf2 due to the upregulation of Nrf2 by activated AhR. However, several bifunctional inducers also activate Nrf2 directly due to their ability to form electrophilic metabolites or to cause oxidative stress. The flavonoid β-naphthoflavone and the polycyclic aromatic hydrocarbon benzo[*a*]pyrene induce NQO1 (and other enzymes) by both mechanisms; the parent compound activates AhR, whereas electrophilic and/or redox active metabolites activate Nrf2. The situation with benzo[*a*]pyrene is quite intriguing. This polycyclic aromatic hydrocarbon binds directly to AhR, which binds to XRE and induces the synthesis of CYP1A1 and CYP1B1, which in turn convert benzo[*a*]pyrene to electrophilic metabolites (such as arene oxides and diol-epoxides) and redox active metabolites (such as catechols), as shown in Fig. 6-14. These electrophilic and redox active metabolites activate Nrf2 and induce various enzymes that protect against oxidative stress. However, the catechol metabolites of benzo[*a*]pyrene are further converted by AKRs and/or dimeric dihydrodiol dehydrogenase to *ortho*-quinones (Fig. 6-14), and are thereby converted back into planar, hydrophobic compounds that are highly effective ligands for the AhR (Burczynski and Penning, 2000). This may be toxicologically important, because the AhR may translocate *ortho*-quinone metabolites of benzo[*a*]pyrene into the nucleus, where they might damage DNA (Bolton et al., 2000).

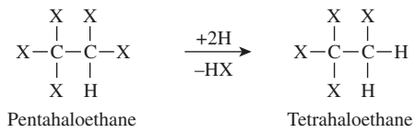
Sulforaphane and various isothiocyanates are Nrf2 activators that are present in broccoli and are thought responsible for the anticarcinogenic effects of this cruciferous vegetable (Zhang et al., 1992). These Nrf2 activators induce glutathione transferase (GSTA1), microsomal epoxide hydrolase, aldo–keto reductase (AKR7A, also known as aflatoxin aldehyde reductase), NAD(P)H-quinone oxidoreductase (NQO1, also known as DT-diaphorase), glutamate-cysteine ligase (GCL), as well as genes involved in apoptosis. One isothiocyanate in particular, phenethylisothiocyanate, has been found to activate Nrf2 and activate numerous genes in addition to those encoding xenobiotic-biotransforming enzymes and oxidant defense systems. Microarray studies carried out in wild-type and Nrf2 knockout mice treated with phenethylisothiocyanate showed that the most highly inducible genes include the very low density lipoprotein (VLDL) receptor, G-protein signaling modulator 2, early growth response 1, pancreatic lipase–related protein 2, histocompatibility 2 (K region), general transcription factor IIB, myoglobin, potassium voltage–gated channel Q2, and SLC39A10 (Hu et al., 2006). As with other xenosensors, activation of Nrf2 results in a pleiotypic response in which a large number of genes are activated (or repressed) (Klaassen and Reisman, 2010; Wu et al., 2012). As mentioned in this section, hypoxia and the anticancer drug mitomycin C are also inducers of NQO1, which has implications for cancer chemotherapy.

NQO1 and NQO2 are polymorphically expressed enzymes, and several lines of evidence suggest that NQO1 and/or NQO2 plays a key role in protecting bone marrow from the hematotoxic effects of benzene or other environmental factors (Iskander and Jaiswal, 2005). In humans, a high percentage of individuals with myeloid and other types of leukemia are homo- or heterozygous for a null mutant allele of NQO1. This polymorphism, NQO1*2, is a SNP (C609T) that changes Pro187 to Ser187, which destabilizes the protein and targets it for rapid degradation by the ubiquitin proteasomal pathway; this polymorphism is also associated with increased risk of colorectal and esophageal cancers (Ross, 2005; Chen et al., 2012b). Mice lacking NQO1 or NQO2 (knockout or null mice) have no developmental abnormalities but have increased granulocytes in the blood and myelogenous hyperplasia of the bone marrow (due to decreased apoptosis). Mice lacking NQO1 are substantially more susceptible than wild-type mice to benzene-induced hematotoxicity (Iskander and Jaiswal, 2005; Ross, 2005). The hematotoxicity of benzene is thought to involve its conversion to hydroquinone in the liver and its subsequent oxidation to benzoquinone by myeloperoxidase in the bone marrow (discussed later in the section "Peroxidase-Dependent Cooxidation"). NQO would be expected to play a role in detoxifying benzoquinone, and the loss of this protective mechanism may be the mechanism by which loss of NQO potentiates benzene hematotoxicity. However, loss of NQO also impairs apoptosis, which also represents a plausible explanation for the association between loss of NQO and increased susceptibility to benzene hematotoxicity. This latter mechanism (i.e., impaired apoptosis) likely accounts for the observation that NQO1 and NQO2 null mice are more susceptible than wild-type mice to skin carcinogenesis by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene, an effect attributable to the diol-epoxides, not the quinone metabolites, of these polycyclic aromatic hydrocarbons (Iskander and Jaiswal, 2005).

Dihydropyrimidine Dehydrogenase In 1993, 18 Japanese patients died as a result of an interaction between two oral medications: sorivudine—a new antiviral drug for herpes zoster—and tegafur—a prodrug that is converted in the liver to the anticancer agent 5-fluorouracil. The deaths occurred within 40 days of the Japanese government's approval of sorivudine for clinical use. The mechanism of the lethal interaction between sorivudine and tegafur is illustrated in Fig. 6-21, and involves inhibition of dihydropyrimidine dehydrogenase (DPD), an NADPH-requiring, homodimeric protein (M_r about 210 kDa) containing FMN/FAD and an iron–sulfur cluster in each subunit. The enzyme is located mainly in liver cytosol, where it catalyzes the reduction of 5-fluorouracil and related pyrimidines. Sorivudine is converted in part by gut microbiota to (*E*)-5-(2-bromovinyl) uracil (BVU), which lacks antiviral activity but which is converted by DPD to a metabolite that binds covalently to the enzyme. The irreversible inactivation (aka suicidal inactivation) of DPD by sorivudine causes a marked inhibition of 5-fluorouracil metabolism, which increases blood levels of 5-fluorouracil to toxic and, in some cases, lethal levels (Ogura et al., 1998; Kanamitsu et al., 2000).

Several genetic polymorphisms that result in a partial or complete loss of DPD activity, affecting about 8% of the population, have been described (van Kuilenburg et al., 2004; Robert et al., 2005). Severe 5-fluorouracil toxicity has also been documented in individuals who are heterozygous for allelic variants of DPD, and 5-fluorouracil lethality has been documented in rare individuals who are completely deficient in DPD (one individual in about 10,000) (Caudle et al., 2013; Meulendijks et al., 2015). 5-Fluorouracil is one of the most frequently prescribed anticancer drugs, for which reason assessing an individual's DPD genotype (by analyzing DNA for allelic variants) or phenotyping (by measuring DPD activity in peripheral blood mononuclear cells or PBMCs) is advocated prior to 5-fluorouracil or capecitabine (a 5-flurouracil prodrug) therapy so that the dosage of these anticancer drugs can be adjusted on an individual basis.

Dehalogenation There are three major mechanisms for removing halogens (F, Cl, Br, and I) from aliphatic xenobiotics (Anders, 1985). The first, known as *reductive dehalogenation*, involves replacement of a halogen with hydrogen, as shown below:



Pentahaloethane Tetrahaloethane

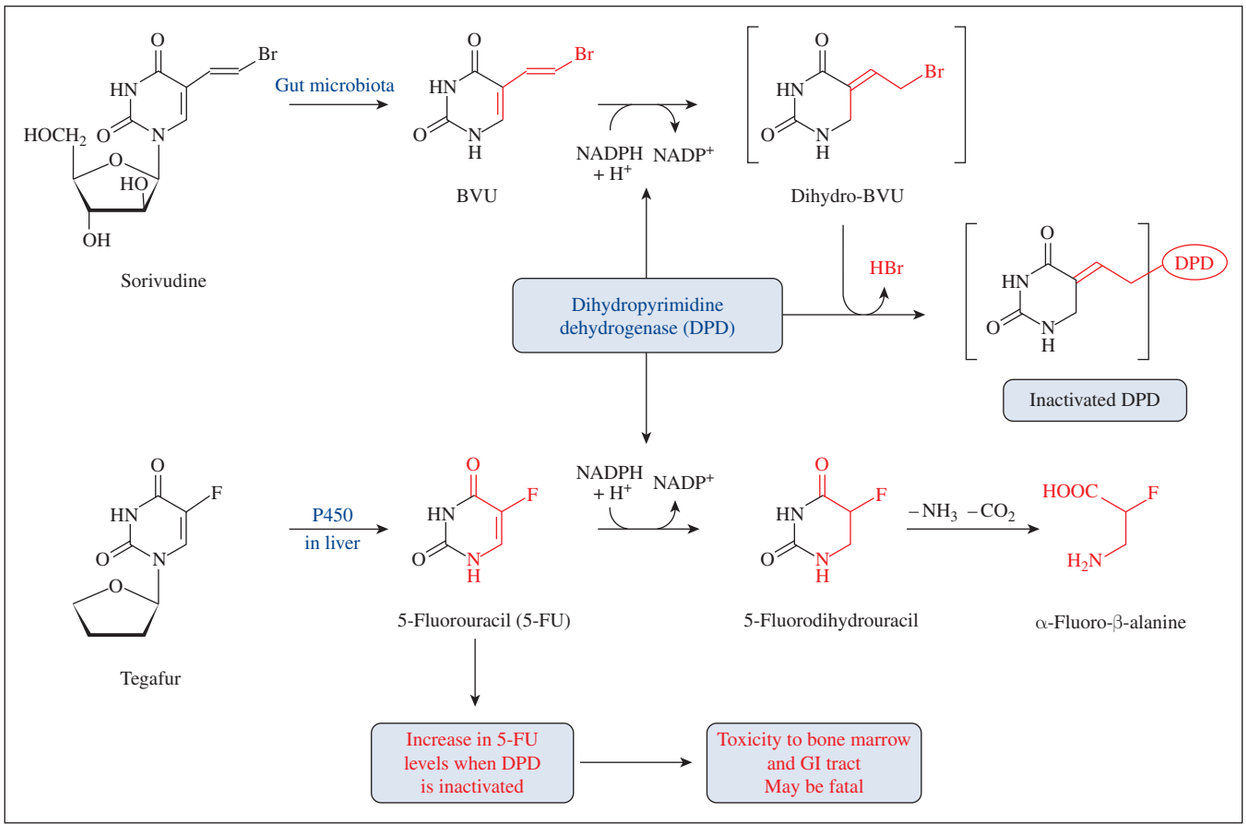
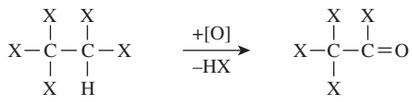
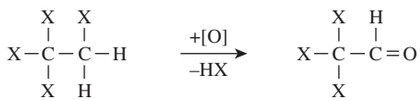


Figure 6-21. *Reduction of 5-fluorouracil by dihydropyrimidine dehydrogenase and its inhibition (suicide inactivation) by sorivudine.* Note that inhibition of dihydropyrimidine dehydrogenase is the mechanism of fatal interactions between sorivudine and the 5-fluorouracil prodrug, tegafur.

In the second mechanism, known as *oxidative dehalogenation*, a halogen and hydrogen on the same carbon atom are replaced with oxygen. Depending on the structure of the haloalkane, oxidative dehalogenation leads to the formation of an acylhalide or aldehyde, as shown below:

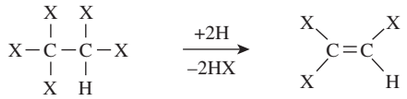


Pentahaloethane Tetrahaloacetylhalide



Tetrahaloethane Trihaloacetaldehyde

A third mechanism of dehalogenation involves the elimination of two halogens on adjacent carbon atoms to form a carbon–carbon double bond, as shown below:



Pentahaloethane Trihaloethylene

A variation on this third mechanism is *dehydrohalogenation*, in which a halogen and hydrogen on adjacent carbon atoms are eliminated to form a carbon–carbon double bond.

Reductive and oxidative dehalogenations are both catalyzed by CYP. (The ability of CYP to catalyze both reductive and oxidative reactions is explained later in the section "Cytochrome P450".) Dehalogenation reactions leading to double bond formation are catalyzed by CYP and glutathione transferase. These reactions play an important role in the biotransformation and metabolic activation of several halogenated alkanes, as the following examples illustrate.

The hepatotoxicity of carbon tetrachloride (CCl_4) and several related halogenated alkanes is dependent on their biotransformation by reductive dehalogenation (Plaa, 2000). The first step in reductive dehalogenation is a one-electron reduction catalyzed by CYP, which produces a potentially toxic, carbon-centered radical and inorganic halide. In the case of CCl_4, reductive dechlorination produces a trichloromethyl radical (•CCl3), which initiates lipid peroxidation and produces a variety of other metabolites, as shown in Fig. 6-22.

Halothane can also be converted by reductive dehalogenation to a carbon-centered radical, as shown in Fig. 6-23. The mechanism is identical to that described for carbon tetrachloride, although in the case of halothane the radical is generated through loss of bromine, which is a better leaving group than chlorine. Fig. 6-23 also shows that halothane can undergo oxidative dehalogenation, which involves oxygen insertion at the C–H bond to generate an unstable halohydrin ($CF_3COHClBr$) that decomposes to a reactive acylhalide (CF_3COCl), which can bind to cellular proteins (particularly to amine groups) or further decompose to trifluoroacetic acid (CF_3COOH).

Both the oxidative and reductive pathways of halothane metabolism generate reactive intermediates capable of binding to proteins and other cellular macromolecules. The relative importance of these two pathways to halothane-induced hepatotoxicity is species dependent. In rats, halothane-induced hepatotoxicity is promoted by those conditions favoring the reductive dehalogenation of halothane, such

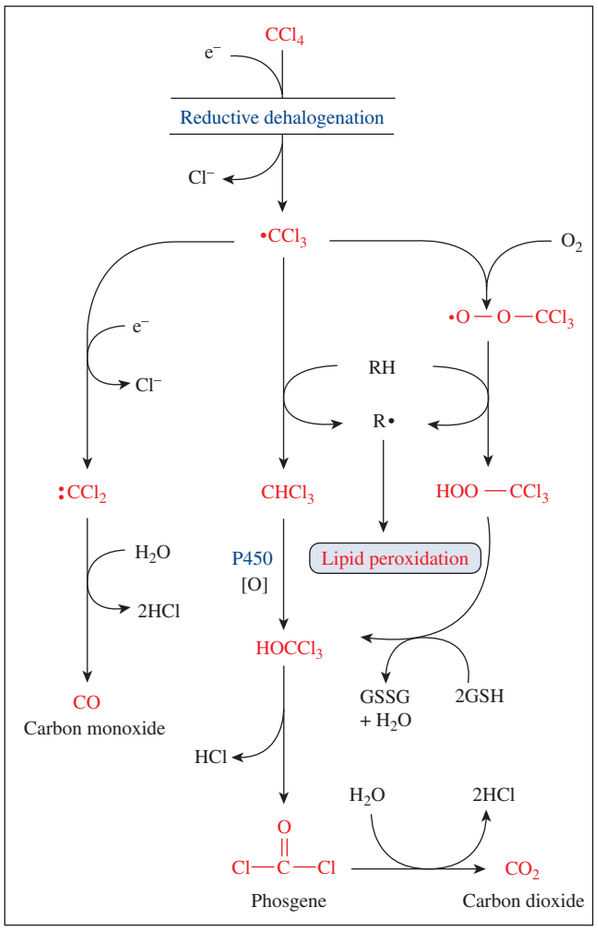


Figure 6-22. *Reductive dehalogenation of carbon tetrachloride to a trichloromethyl free radical that initiates lipid peroxidation. RH, unsaturated lipid; R•, lipid dienyl radical; GSH, reduced glutathione; GSSG, oxidized glutathione.*

as moderate hypoxia (10% to 14% oxygen) plus treatment with the CYP inducers, phenobarbital, and pregnenolone-16α-carbonitrile. In contrast to the situation in rats, halothane-induced hepatotoxicity in guinea pigs is largely the result of oxidative dehalogenation of halothane (Lunam et al., 1989). In guinea pigs, halothane hepatotoxicity is not enhanced by moderate hypoxia and is diminished by the use of deuterated halothane, which impedes the oxidative dehalogenation of halothane because the CYP-dependent insertion of oxygen into a carbon–deuterium bond is energetically less favorable (and therefore slower) than inserting oxygen into a carbon–hydrogen bond.

Halothane hepatitis in humans is a rare but severe form of liver necrosis associated with repeated exposure to this volatile anesthetic. In humans, as in guinea pigs, halothane hepatotoxicity results from the oxidative dehalogenation of halothane, as shown in Fig. 6-23. Serum samples from patients suffering from halothane hepatitis contain antibodies directed against neoantigens formed by the trifluoroacetylation of proteins. These antibodies have been used to identify which specific proteins in the endoplasmic reticulum are targets for trifluoroacetylation during the oxidative dehalogenation of halothane (Pohl et al., 1989).

The concept that halothane is activated by CYP to trifluoroacetylhalide, which binds covalently to proteins and elicits an immune response, has been extended to other volatile anesthetics, such as enflurane, methoxyflurane, and isoflurane. In other words, these halogenated aliphatic hydrocarbons, such as halothane, may be converted to acylhalides that form immunogens by binding covalently to proteins. In addition to accounting for rare instances of enflurane hepatitis, this mechanism of hepatotoxicity can also account for reports of a *cross-sensitization* between enflurane and halothane, in which enflurane causes liver damage in patients previously exposed to halothane.

One of the metabolites generated from the reductive dehalogenation of halothane is 2-chloro-1,1-difluoroethylene (Fig. 6-23). The formation of this metabolite involves the loss of two halogens from adjacent carbon atoms with formation of a carbon–carbon double bond. This type of dehalogenation reaction can also be catalyzed by glutathione transferases. Glutathione initiates the reaction with a nucleophilic attack either on the electrophilic carbon to which the halogen is attached (mechanism A) or on the halide itself (mechanism B), as shown in Fig. 6-24, for the dehalogenation of 1,2-dihaloethane to ethylene. The insecticide DDT is detoxified by dehydrochlorination to DDE by a lyase (i.e., DDT-dehydrochlorinase), as shown in Fig. 6-25. The activity of this glutathione-dependent reaction correlates well with resistance to DDT in houseflies.

Dehydroxylation: mARC, Cytochrome b_5, b_5 Reductase, and Aldehyde Oxidase Mitochondrial amidoxime-reducing complex (mARC) is a molybdenum-containing enzyme that, in the presence of NADH, cytochrome b_5 and NADH-cytochrome b_5 reductase, can catalyze the *N*-dehydroxylation of various amidoximes and related *N*-hydroxy compounds, as shown in Fig. 6-26 (Havemeyer et al., 2010). Reactions catalyzed by mARC/cytochrome b_5/NADH-cytochrome b_5 reductase include the *N*-dehydroxylation of amidoximes formed during the activation of the antiparasitic prodrug pafuramidine (DB-289), the *N*-dehydroxylation of *N*-hydroxysulfonamides, and the *N*-dehydroxylation of *N*-hydroxy-valdecoxib to its active principal, a COX-2 inhibitor (Saulter et al., 2005; Havemeyer et al., 2010). The mARC complex can also *N*-dehydroxylate the aryl hydroxylamine metabolites of carcinogenic arylamines such as 4-aminobiphenyl and PhIP; *N*-hydroxy metabolites that are formed by CYP1A1, CYP1A2, CYP1B1, lactoperoxidase, and myeloperoxidase (see the sections "Cytochrome P450" and "Peroxidase-Dependent Cooxidation") (Kurian et al., 2006). Because these carcinogenic aryl hydroxylamines can be further activated by glucuronidation, sulfonation, or acetylation in various tissues (see the sections "Glucuronidation," "Sulfonation," and "Acetylation"), reduction by mARC/cytochrome b_5/NADH-cytochrome b_5 reductase represents a competing detoxication pathway. Gut microbiota can also catalyze dehydroxylation reactions as shown in Fig. 6-4 for quinic acid. Gut microbiota can also reduce *N*- and *S*-oxides formed by FMO and/or CYP, as described for trimethylamine *N*-oxide in the section "Flavin Monooxygenases."

Aldehyde Oxidase—Reduction Reactions Aldehyde oxidase is a cytosolic molybdoenzyme that catalyzes the oxidation of some xenobiotics and the reduction of others. The types of oxidative and reductive reactions catalyzed by aldehyde oxidase are shown in Fig. 6-27. In contrast to the large number of drugs that are known to be (or suspected of being) oxidized by aldehyde oxidase in vivo, only a few drugs are known to be (or suspected of being) reduced by aldehyde oxidase in vivo, including nitrofurazone, zonisamide, and ziprasidone. The reductive metabolism of ziprasidone by aldehyde oxidase is shown in Fig. 6-7. The features of aldehyde oxidase and the oxidative reactions it catalyzes are discussed later in the section "Aldehyde Oxidase."

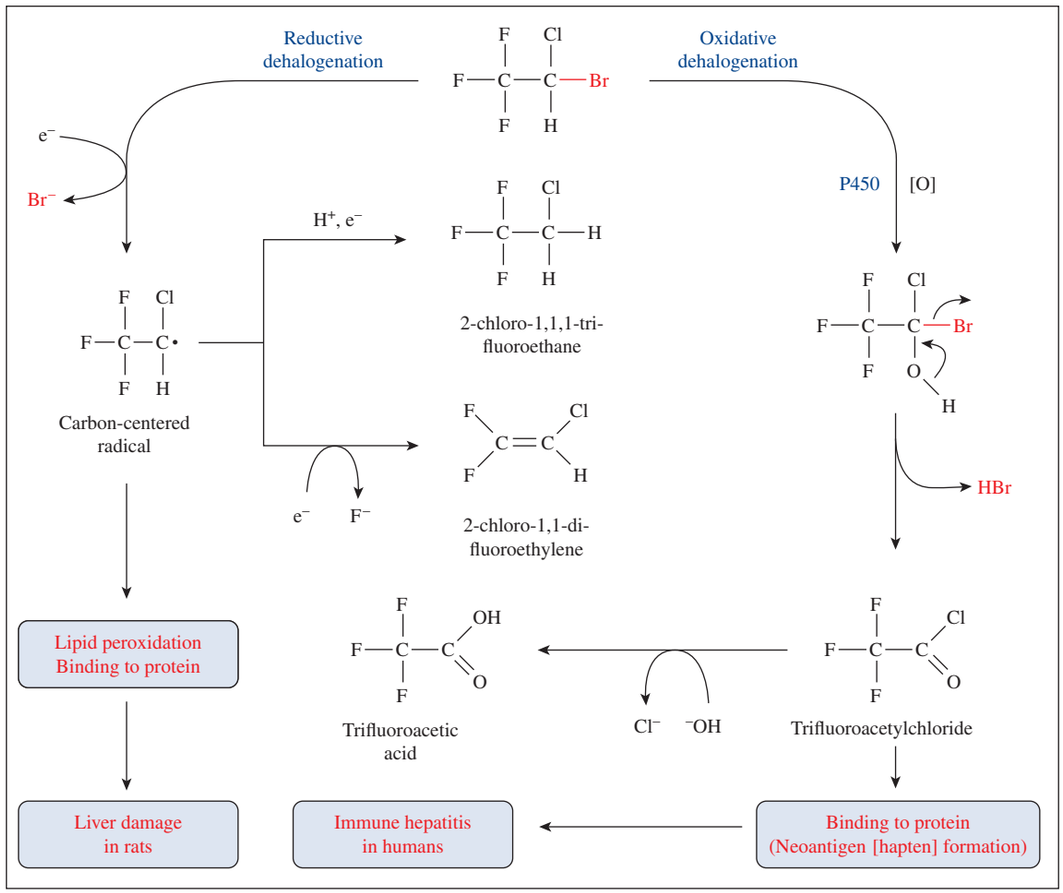


Figure 6-23. *Activation of halothane by reductive and oxidative dehalogenation and their role in liver toxicity in rats and humans.*

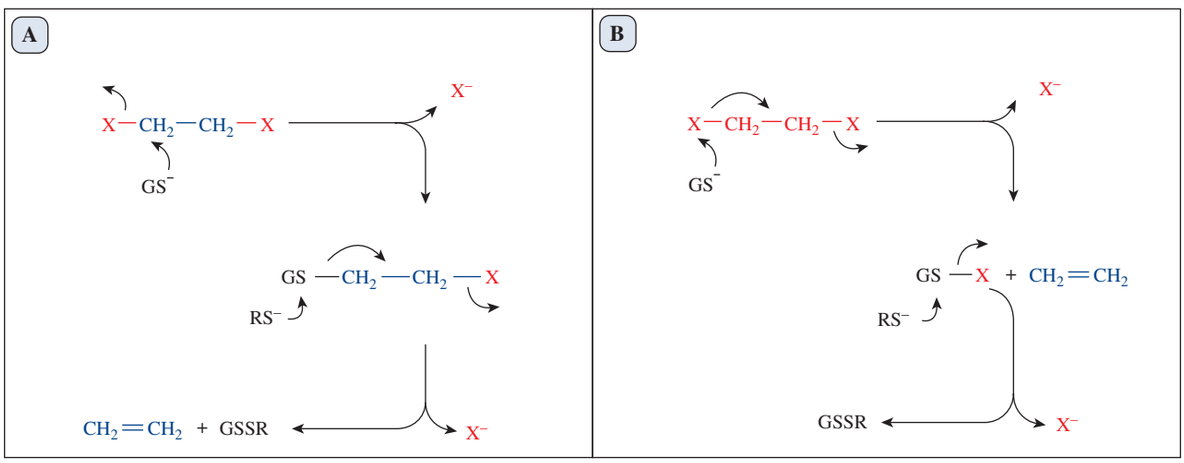


Figure 6-24. *Glutathione-dependent dehalogenation of 1,2-dihaloethane to ethylene. (**A**) Nucleophilic attack on carbon and (**B**) nucleophilic attack on halide.*

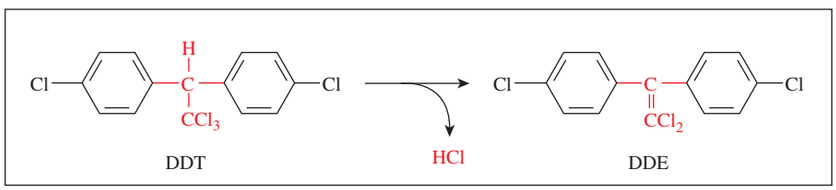


Figure 6-25. *Dehydrochlorination of the pesticide DDT to DDE, a glutathione-dependent reaction.*

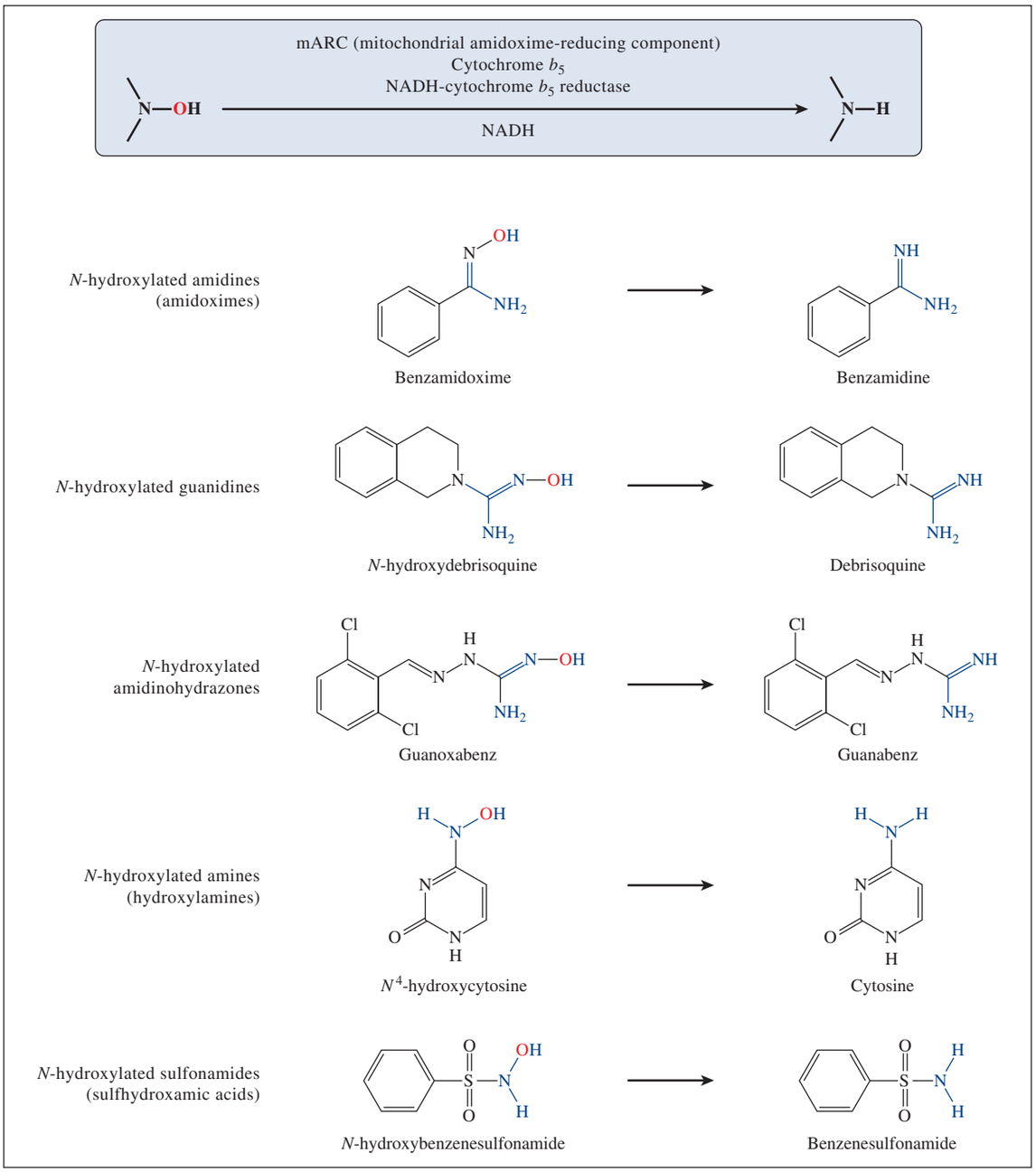


Figure 6-26. *Dehydroxylation reactions catalyzed by the molybdoenzyme mitochondrial amidoxime-reducing component (mARC), cytochrome b5, and NADH-cytochrome b5 reductase.* Note that aldehyde oxidase can also catalyze dehydroxylation reactions as shown in Fig. 6-27.

Oxidation

Alcohol, Aldehyde, and Ketone Oxidation–Reduction Systems Alcohols, aldehydes, and ketones are oxidized by a number of enzymes, including alcohol dehydrogenases, aldehyde dehydrogenases, AKR1A1, AKR1C1–1C4 (which have dihydrodiol dehydrogenase activity toward *trans*-dihydrodiols of various polycyclic aromatic hydrocarbon oxiranes), the molybdenum-containing enzymes (namely aldehyde oxidase and xanthine dehydrogenase/oxidase), and CYP. For example, simple alcohols (such as methanol and ethanol) are oxidized to aldehydes (namely, formaldehyde and acetaldehyde) by alcohol dehydrogenase. These aldehydes are further oxidized to carboxylic acids (formic acid and acetic acid) by aldehyde dehydrogenase, as shown in Fig. 6-28. Many of the aforementioned enzymes can also catalyze the reduction of xenobiotics, as discussed in the previous section "Reduction."

Alcohol Dehydrogenase Alcohol dehydrogenases (ADHs) belong to the medium-chain dehydrogenases/reductases (MDRs), as shown in Table 6-9. ADHs are zinc-containing, cytosolic enzymes present in several tissues including liver (which has the

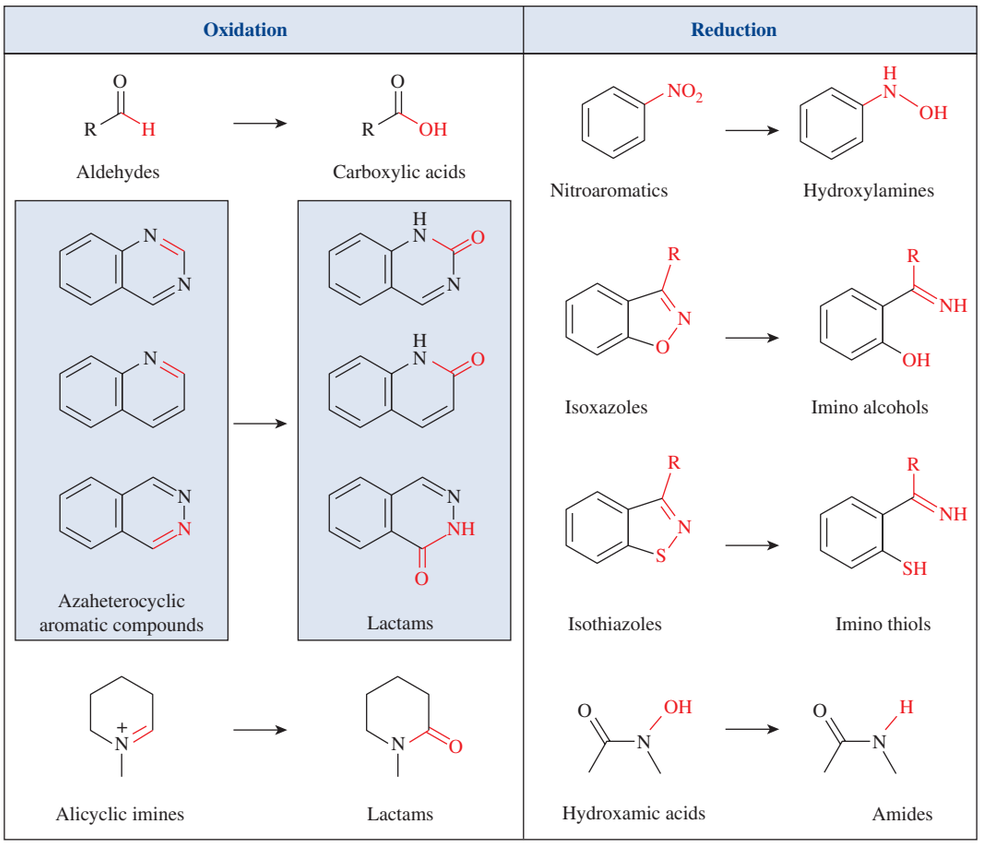


Figure 6-27. *Examples of oxidation and reduction catalyzed by aldehyde oxidase.*

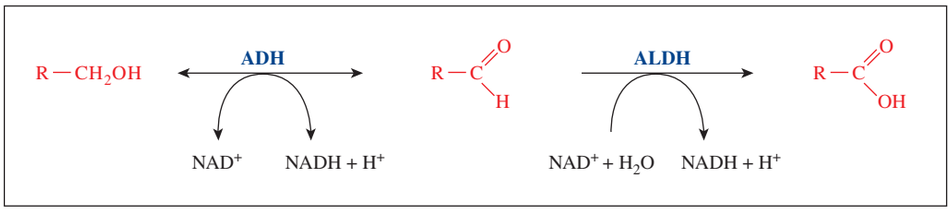


Figure 6-28. *Oxidation of alcohols to aldehydes and carboxylic acids by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH).*

highest levels), kidney, lung, and gastric mucosa (Agarwal, 1992; Ramchandani, 2004). These enzymes oxidize several types of alcohols including hydroxysteroids, retinol, ethanol, lipid peroxidation products, and other simple and complex (e.g., ring-containing) alcohols. Human ADHs are dimeric proteins consisting of two about 40-kDa subunits designated α, β, γ, π, χ, σ (previously known as μ), or ADH6 (the latter having no subunit designation). As shown in Table 6-9, there are seven human ADHs, and these are categorized into five classes (I to V) based on patterns of tissue-specific expression, catalytic properties, and amino acid sequence. Class I comprises three hepatically expressed genes: ADH1A, ADH1B, and ADH1C, which were formerly known as ADH1, ADH2, and ADH3, respectively. The class I isozymes consist of homo- and hetero-dimeric forms of the three subunits (e.g., αα, αβ, ββ, βγ, and γγ). Class II contains ADH4, which is composed of two pi subunits (ππ). Class III contains ADH5, which is composed of two chi subunits (χχ). Class IV contains ADH7, which is composed of two sigma subunits (σσ). Class V contains ADH6 (for which there is no subunit designation) (Brennan et al., 2004; Ramchandani, 2004). Therefore, there are over 20 human ADH isozymes and these differ in their substrate specificity and catalytic efficiency toward ethanol (Ramchandani, 2004). The human ADH genes have similar sequences (i.e., 60% to 70% identical coding regions), and all have nine exons and eight introns with the exception of ADH6, which lacks the last exon (Han et al., 2005). In addition, several hundred genetic variants have been described across the human ADH cluster which lies on chromosome 4q (Li et al., 2008). Alcohols can be oxidized to aldehydes by non-ADH enzymes in microsomes and peroxisomes, although these are quantitatively less important than ADH for ethanol oxidation (Lieber, 2004). The microsomal ethanol oxidizing system (formerly known as MEOS) is the CYP enzyme, CYP2E1. The corresponding peroxisomal enzyme is catalase. The

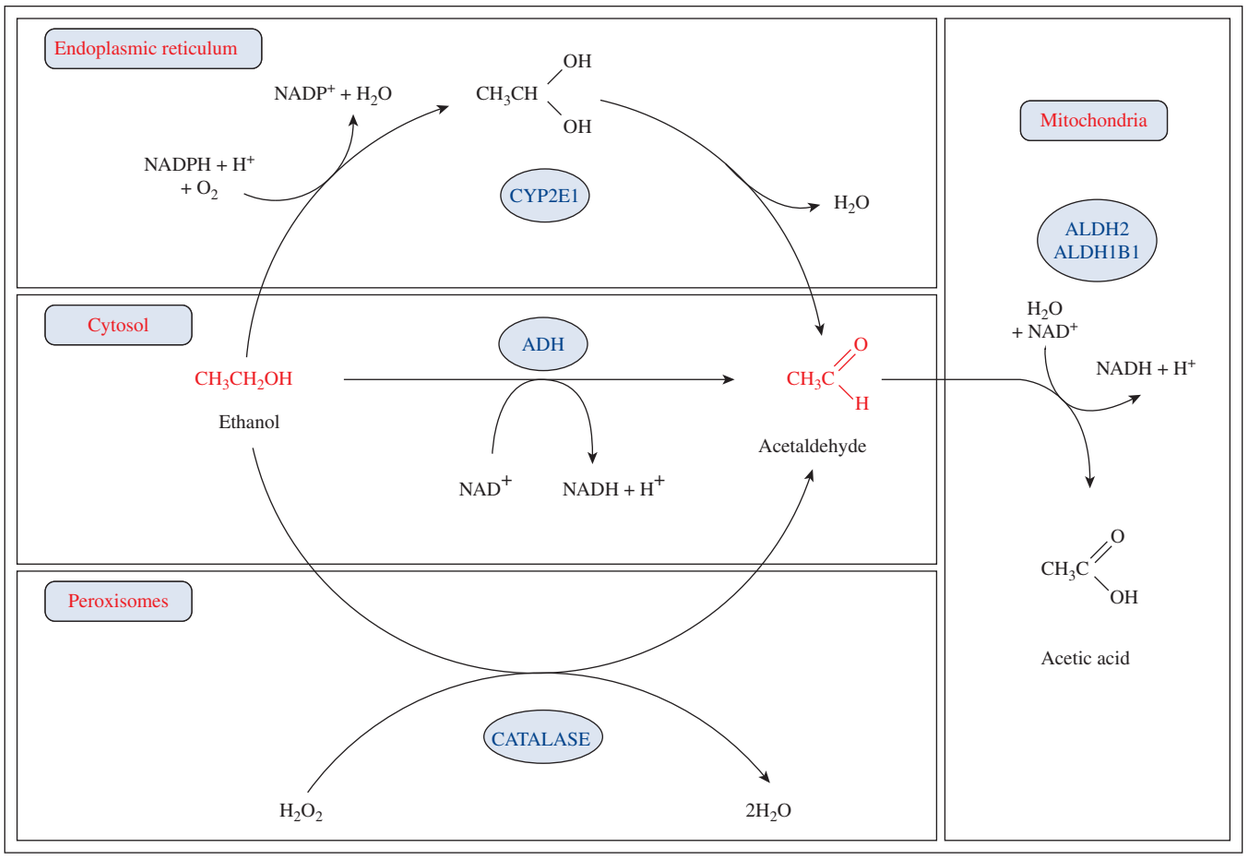


Figure 6-29. *Oxidation of alcohol to acetaldehyde by alcohol dehydrogenase (ADH), cytochrome P450 (CYP2E1), and catalase.* Note that the oxidation of alcohol to acetic acid involves multiple organelles.

oxidation of ethanol to acetaldehyde by these three enzyme systems is shown in Fig. 6-29.

Class I (ADH1A, ADH1B, ADH1C) The class I ADH isozymes (ADH1A or α-ADH, ADH1B or β-ADH, and ADH1C or γ-ADH) are responsible for the oxidation of ethanol and other small, aliphatic alcohols, and they are strongly inhibited by pyrazole and its 4-alkyl derivatives (e.g., 4-methylpyrazole). High levels of class I ADH isozymes are expressed in liver and adrenals, with lower levels in kidney, lung, blood vessels (in the case of ADH1B), gastric mucosa (in the case of ADH1C), and other tissues, but not brain. It is noteworthy that the liver expresses a very large amount of ADHs (approximately 3% of all soluble protein) and also expresses the widest variety of isozymes (Ramchandani, 2004).

The class I ADH isozymes differ in their capacity to oxidize ethanol. Polymorphisms have been well described for the class I ADH isozymes. Even the allelozymes, which differ in a single amino acid, differ markedly in their affinity (K_m) and/or capacity (V_{max}) for oxidizing ethanol to acetaldehyde. There are at least three allelic variants of ADH1B (i.e., *1, *2, and *3), with a single amino acid change at position 48. The homodimer, $\beta_2\,\beta_2$ and heterodimers containing at least one β_2 subunit (i.e., the ADH1B*2 allelozymes) are especially active in oxidizing ethanol at physiological pH. ADH1B*2 (formerly known as ADH2*2) is known as *atypical* ADH, and is responsible for the unusually rapid conversion of ethanol to acetaldehyde in up to 90% of the Pacific Rim Asian population (e.g., Japanese, Chinese, Korean), whereas only about 10% of Caucasians express this allele. The ADH1B*3 is relatively common in individuals of African descent. The latter two alleles have greater activity toward ethanol than the ADH1B1*1 allele (Kimura and Higuchi, 2011). These population differences in ADH1B allelozyme expression contribute to ethnic differences in alcohol consumption and toxicity, as discussed in the next section "Aldehyde Dehydrogenase."

Unlike the allelic variants of ADH1B, the allelic variants of ADH1C do not differ markedly in their ability to oxidize ethanol. However, as in the case of the ADH1B allelozymes, the expression of the ADH1C allelozymes also varies from one ethnic group to the next. The two allelozymes of ADH1C, namely, ADH1C*1 (γ_1-ADH) and ADH1C*2 (γ_2-ADH), are respectively expressed ~50:50 in Caucasians, but 90:10 in Pacific Rim Asians, with the *2 allele having somewhat higher activity toward ethanol than the *1 allele (Li, 2000; Kimura and Higuchi, 2011). An additional SNP in ADH1C has been found in up to 20% of some Native American populations (Ramchandani, 2004).

Class II (ADH4) The class II enzyme ADH4 (π-ADH) is mainly expressed in liver (and to some extent in other gastrointestinal tissues), where it preferentially oxidizes larger alcohols (Ramchandani, 2004). ADH4 differs from the ADH1 isozymes in that it is less sensitive to pyrazole inhibition, but may play some role in ethanol oxidation, especially at high concentrations (Lockley et al., 2005;

Edenberg, 2007; Kimura and Higuchi, 2011). Some studies support a role for polymorphisms of ADH4 in the susceptibility to alcoholism (Kimura and Higuchi, 2011).

Class III (ADH5) The class III enzyme ADH5 (a homodimer of the χ-subunit) preferentially oxidizes long-chain alcohols (pentanol and larger), omega-hydroxy-fatty acids, and other alcohols (such as cinnamyl alcohol). Like ADH4, ADH5 is less sensitive to pyrazole inhibition than ADH1 enzymes. However, in contrast to ADH4, which is largely confined to the liver, ADH5 is ubiquitous, being present in virtually all tissues (including brain), where it catalyzes the rate-limiting step in detoxifying formaldehyde through oxidation of *S*-hydroxymethylglutathione (which is formed spontaneously from formaldehyde and glutathione) to *S*-formylglutathione. In fact, ADH5 and the glutathione-dependent formaldehyde dehydrogenase (also referred to in literature as FDH, ADH3, or *S*-nitrosoglutathione reductase [GSNOR]) are identical enzymes (Koivusalo et al., 1989; Edenberg, 2007; Just et al., 2011). This enzyme appears to be the ancestral form of ADH from which all other vertebrate ADHs have evolved, and so far functional polymorphisms have not been identified in ADH5 (Just et al., 2011).

Class IV (ADH7) The class IV enzyme ADH7 (σ-ADH; also referred to as the μ subunit) is a low-affinity (high K_m), high-capacity (high V_{max}) enzyme, and is the most active of the medium-chain ADHs in oxidizing retinol (a member of the vitamin A family). It is the major ADH expressed in human stomach and other areas of the upper aero-digestive tract (e.g., stomach, esophagus, pharynx, gingiva, mouth, and tongue), as well as the eyes (Han et al., 2005). In contrast to the other ADHs, ADH7 is not expressed in the adult human liver (Ramchandani, 2004). Among the human ADH forms, ADH7 has the highest activity toward ethanol (Han et al., 2005). Inasmuch as ADH7 is expressed in the upper gastrointestinal tract, where chronic alcohol consumption leads to cancer development, there is considerable interest in the role of ADH7 in the preabsorptive conversion of ethanol to acetaldehyde (a suspected upper gastrointestinal tract carcinogen or co-carcinogen) and in its role in the metabolism of retinol (a vitamin required for epithelial cell growth and differentiation), which might be inhibited by alcohol consumption (Seitz and Oneta, 1998). The role for a protective effect of high-activity polymorphisms against alcoholism remains to be fully elucidated, with some studies showing a positive association (Han et al., 2005), and others no association (Duell et al., 2012). However, the A92G SNP (rs1573496; C → G) in ADH7 has been found to confer a reduced risk of squamous cell carcinoma of the head and neck in Caucasians (Wei et al., 2010).

Compared with hepatic ADH, gastric ADH has a lower affinity (higher K_m) but higher capacity (larger V_{max}) for oxidizing ethanol; the former being dominated by the class I ADH isozymes, the latter by the class IV enzyme ADH7. Although ethanol is largely biotransformed by hepatic ADH1, gastric ADH7 nevertheless can limit the systemic bioavailability of alcohol. This first-pass elimination of alcohol by gastric ADH7 can be significant depending on the manner in which the alcohol is consumed; large doses over a short time produce high ethanol concentrations in the stomach, which compensate for the low affinity (high K_m) of gastric ADH7. Young women have lower gastric ADH7 activity than do men, and gastric ADH7 activity tends to be lower in alcoholics (Frezza et al., 1990). Some alcoholic women have no detectable gastric ADH7, and blood levels of ethanol after oral consumption of alcohol are the same as those that are obtained after intravenous administration. Gastric ADH7 activity decreases during fasting, which is one reason alcohol is more intoxicating when consumed on an empty stomach. Several commonly used drugs (e.g., cimetidine, ranitidine, and aspirin) are noncompetitive inhibitors of gastric ADH7. Under certain circumstances these drugs increase the systemic availability of alcohol, although the effect is too small to have serious medical, social, or legal consequences (Levitt, 1993). About 30% of Asians appear to be genetically deficient in ADH7, the main gastric ADH. In addition to biotransforming ethanol and retinol, ADH7 also detoxifies the dietary carcinogen nitrobenzaldehyde. It has been suggested that a lack of ADH7 in some Japanese subjects may impair their ability to detoxify nitrobenzaldehyde and may possibly be linked to the high rate of gastric cancer observed in the Japanese population (Seitz and Oneta, 1998).

Class V (ADH6) The mRNA for class V ADH (namely ADH6, for which there is no subunit designation) (Brennan et al., 2004; Ramchandani, 2004) has been found in fetal and adult liver (Edenberg, 2007). However, the protein has not yet been isolated from human tissue so little is known about its in vivo function. ADH6 has been expressed in vitro and metabolizes ethanol with a K_m of approximately 28 mM and has higher affinity for benzyl alcohol (K_m 0.12 mM) and propanol (K_m 3.2 mM) (Zhi et al., 2000).

Aldehyde Dehydrogenase Aldehyde dehydrogenases (ALDHs) oxidize four major types of aldehydes: (1) saturated alkanals (e.g., formaldehyde and acetaldehyde), (2) unsaturated alkenals (e.g., acrolein), (3) aromatic aldehydes (e.g., benzaldehyde), and (4) dicarbonyls (e.g., glyoxal and malondialdehyde) to their corresponding carboxylic acids, generally with NAD^+ as the cofactor. However, it should be noted that ALDH1L1 prefers $NADP^+$ over NAD^+, and ALDH3B1 can use either cofactor in a substrate-dependent manner. In addition, ALDH6A1 requires acetyl- or propionyl-CoA (Marchitti et al., 2007, 2008). Most of the enzymes also have esterase activity (Yoshida et al., 1998; Marchitti et al., 2008). Several ALDH enzymes are involved in the oxidation of xenobiotic aldehydes, such as those formed from ethanol, allyl alcohol, carbon tetrachloride, cyclophosphamide, and ifosfamide (Marchitti et al., 2007). Formaldehyde dehydrogenase, which specifically oxidizes formaldehyde that is complexed with glutathione, is not a member of the ALDH family but is a class III ADH (ADH5) (Koivusalo et al., 1989; Edenberg, 2007; Just et al., 2011). At least 19 ALDH genes have been identified in humans, and a correspondingly large number of ALDH genes appear to be present in other mammalian species (Sládek, 2003; Vasiliou et al., 2004; Marchitti et al., 2007; Marchitti et al., 2008). The name, tissue distribution, subcellular location, and major substrate for the human ALDHs are summarized in Table 6-10. The ALDHs differ in their primary amino acid sequences. They may also differ in the quaternary structure. For example, ALDH3A1 is a dimer of two 85-kDa subunits, whereas ALDH1A1 and ALDH2 are homotetramers of 54-kDa subunits (Goedde and Agarwal, 1992; Marchitti et al., 2008).

As shown in Fig. 6-29, ALDH2 is a mitochondrial enzyme that, by virtue of its high affinity, is primarily responsible for oxidizing simple aldehydes, such as acetaldehyde (K_m for acetaldehyde <1 μM), and by acting as a nitrate reductase, ALDH2 is the principal enzyme necessary for the activation of nitroglycerin (Marchitti et al., 2008). In fact, the genetic polymorphism ALDH2*2 is associated with decreased nitroglycerin efficacy in certain Chinese populations (Marchitti et al., 2008). Several genetic polymorphisms in human ALDH2 have been described, including the well-described ALDH2*2 point mutation ($Glu_{487} \rightarrow Lys_{487}$) which results in a loss of activity due to a greatly diminished affinity for NAD^+ (Marchitti et al., 2008). The mutant ALDH2*2 allele is dominant, such that heterotetrameric ALDH2 proteins that contain even a single ALDH2*2 subunit are inactive (Marchitti et al., 2008). A high percentage (40% to 50%) of individuals of Asian descent are deficient in ALDH2

Table 6-10

Properties of the Human Aldehyde Dehydrogenases (ALDHs)

ALDH	ALTERNATIVE NAMES	FORM	TISSUE	SUBCELLULAR LOCALIZATION	MAJOR SUBSTRATE
1A1	ALDH1, ALDH11, RALDH1	Homotetramer	Liver, kidney, lung, stomach, testis, lens, retina, CNS, etc.	Cytosol	Retinal
1A2	RALDH2	Homotetramer	Intestine, testis, lung, liver, kidney, retina, CNS, etc.	Cytosol	Retinal, nitroglycerin
1A3	ALDH6, RALDH3	Homodimer	Prostate, salivary gland, stomach, kidney, breast, CNS, etc.	Cytosol	Retinal, aliphatic aldehydes
1B1	ALDH5	Homotetramer	Muscle, liver, kidney, lung, testis, muscle, heart, CNS, etc.	Mitochondria	Acetaldehyde, aliphatic aldehydes
1L1	FDH	Homotetramer	Liver, kidney, pancreas, lung, prostate, heart, muscle, testis, ovary, CNS, etc.	Cytosol, mitochondria	10-Formyltetrahydrofolate, propanal, and acetaldehyde
1L2	mtFDH	Homotetramer	Spleen, cervix, uterus, corpus collusum	Mitochondria	10-Formyltetrahydrofolate
2	ALDM, ALDHI	Homotetramer	Liver, kidney, lung, heart, stomach, muscle, CNS, etc.	Mitochondria	Acetaldehyde
3A1	ALDH3	Homodimer	Cornea, esophagus, trachea, stomach, lung, bladder, etc.	Cytosol, nucleus	Aromatic and medium-chain aliphatic aldehydes
3A2	ALDH10, FALDH	Homodimer	Adrenal gland, kidney, liver, lung, intestine, pancreas, stomach, heart, muscle, CNS, etc.	Microsomes, peroxisomes	Fatty and aromatic aldehydes
3B1	ALDH4, ALDH7	Unknown	Kidney, lung, liver, cerebral astrocytes, testis, macrophages, pancreas, spleen	Cytosol (membrane-bound in some tissues)	Medium- and long-chain aliphatic and aromatic aldehydes
3B2	ALDH8	Unknown	Parotid, adrenal gland, prostate, lung, kidney, liver, CNS	Microsomes	Unknown*
4A1	ALDH4, P5CDH	Homodimer	Liver, muscle, kidney, CNS	Mitochondria	Glutamate γ-semialdehyde, short- and medium-chain aliphatic aldehydes
5A1	SSDH, SSADH	Homotetramer	Liver, kidney, muscle, heart, CNS	Mitochondria	Succinate semialdehyde
6A1	MMSDH	Homotetramer	Kidney, liver, heart, muscle, CNS	Mitochondria	Malonate semialdehyde
7A1	ATQ1, EPD	Homotetramer	Cochlea, eye, ovary, heart, kidney, liver, CNS, etc.	Cytosol, nucleus, mitochondria	α-Aminoadipic semialdehyde
8A1	ALDH12	Unknown	Liver, kidney, CNS, breast, testis, prostate, etc.	Cytosol	9-*cis*-Retinal
9A1	ALDH4, ALDH7, ALDH9, γABHD	Homotetramer	Liver, kidney, muscle, CNS	Cytosol	γ-Aminobutyraldehyde
16A1	MGC10204	Unknown	CNS, uterus, placenta, bone marrow, heart, kidney, lung	Cytosol	Unknown
18A1	GSAS, Δ^1-P5CS†	Unknown	Pancreas, ovary, testis, kidney, heart, colon, muscle, CNS, etc.	Mitochondria	Glutamic γ-semialdehyde

Abbreviations: γABHD, 4-aminobutyraldehyde; FALDH, fatty aldehyde dehydrogenase; MMSDH, methylmalonate semialdehyde dehydrogenase; SSDH, succinic dehydrogenase.

SOURCE: Data adapted from Marchitti et al. (2007, 2008, 2010), Sládek (2003), Strickland et al. (2011), and Vasiliou et al. (2004).

**ALDH3B2 contains an in-frame stop codon, suggesting the possibility that it is a pseudogene, although transcripts have been found in various tissues.*

† Δ^1-Pyrroline-5-carboxylate synthase, P5CS.

activity due to the presence of the ALDH2*2 allele. This same population also has a high incidence of the atypical form of ADH1B (i.e., ADH1B*2), which means that they rapidly convert ethanol to acetaldehyde but only slowly convert acetaldehyde to acetic acid. (They also have a relatively high prevalence of a deficiency of ADH7 activity, which impairs gastric metabolism of ethanol.) As a result, many Asian subjects experience a flushing syndrome after consuming alcohol due to a rapid buildup of acetaldehyde, which triggers the dilation of facial blood vessels through the release of catecholamines. Some Native American populations also experience a flushing syndrome after consuming alcohol, possibly because they express ADH1B*2 (Ramchandani, 2004) or a different allelic variant of ALDH2 and/or because acetaldehyde oxidation in blood erythrocytes is impaired in these individuals, possibly due to the expression of a variant form of ALDH1A1. The functional genetic variants of ADH that rapidly convert ethanol to acetaldehyde (i.e., ADH1B*2) and the genetic variants of ALDH that slowly detoxify acetaldehyde (i.e., ALDH2*2) both protect against heavy drinking and alcoholism. Inhibition of ALDH by disulfiram (Antabuse) causes an accumulation of acetaldehyde in alcoholics. The nauseating effect of acetaldehyde serves to deter continued ethanol consumption (Goedde and Agarwal, 1992). However, it is important to note that a predisposition toward alcoholism is not simply determined by factors that affect the pharmacokinetics of ethanol and its metabolites. Studies in humans and rodents implicate 5-HT1B, 5-HT2A, 5-HT3A, and 5-HT3B receptors, dopamine-related genes, GABA receptors, cholinergic muscarinic receptor 2, the endogenous opioid system, tryptophan hydroxylase, and neuropeptide Y as candidate targets of genetic susceptibility in the pharmacodynamic actions of ethanol (Li, 2000; Kimura and Higuchi, 2011). Monoamine oxidase may also be a risk factor for alcoholism, as discussed later (see the section "Amine Oxidases").

Genetic deficiencies in other ALDHs impair the metabolism of other aldehydes, which is the underlying basis of certain diseases. For example polymorphisms in certain ALDH genes may alter the risk for, or cause, the following: spina bifida (ALDH1A2); Sjögren–Larsson syndrome (ALDH3A2); paranoid schizophrenia (ALDH3B1); type II hyperprolinemia (ALDH4A1); γ-hydroxybutyric aciduria (ALDH5A1); methylmalonic aciduria (ALDH6A1); pyroxidine-dependent seizures (ALDH7A1); nonalcoholic steatohepatitis or NASH (ALDH9A1); and hyperammonemia and/or hypoprolinemia (ALDH18A1) (Marchitti et al., 2008).

The toxicological consequences of an inherited (i.e., genetic) or acquired (e.g., drug-induced) deficiency of ALDH illustrate that aldehydes are more cytotoxic than the corresponding alcohol. This is especially true of allyl alcohol (CH_2=$CHCH_2OH$), which is converted by ADH to the highly hepatotoxic aldehyde acrolein (CH_2=CHCHO) (Marchitti et al., 2007, 2008).

The oxidation of ethanol by ADH and ALDH leads to the formation of acetic acid, which is rapidly oxidized to carbon dioxide and water. However, in certain cases, alcohols are converted to toxic carboxylic acids, as in the case of methanol and ethylene glycol, which are converted via aldehyde intermediates to formic acid and oxalic acid, respectively. Formic and oxalic acids are considerably more toxic than acetic acid. For this reason, methanol and ethylene glycol poisoning are commonly treated with ethanol, which competitively inhibits the oxidation of methanol and ethylene glycol by ADH and ALDH. The potent inhibitor of ADH, 4-methylpyrazole (fomepizole) (Lockley et al., 2005), is also used to treat methanol and ethylene glycol poisoning.

The reduction of aldehydes and ketones to primary and secondary alcohols by carbonyl reductases has already been discussed (see the section "Carbonyl Reduction: AKRs, SDRs, and Others"). In contrast to ADH and ALDH, carbonyl reductases typically use NADPH as the source of reducing equivalents. Aldehydes, especially aromatic aldehydes, can also be oxidized by aldehyde oxidase and xanthine oxidoreductase, which are discussed in the section "Molybdenum Hydroxylases (Molybdoenzymes)."

Dimeric Dihydrodiol Dehydrogenase As mentioned in the section "Carbonyl Reduction: AKRs, SDRs, and Others," several members of the aldo–keto reductase (AKR) superfamily (particularly AKR1A1, 1C1, 1C2, 1C3, and 1C4) are dihydrodiol dehydrogenases that oxidize the *trans*-dihydrodiols of various polycyclic aromatic hydrocarbons to the corresponding *ortho*-quinones, as shown in Fig. 6-14 (Penning, 1997; Burczynski and Penning, 2000). There is also a dihydrodiol dehydrogenase (gene symbol DHDH; completely unrelated to AKRs or SDRs) that encodes a dimeric enzyme that oxidizes *trans*-dihydrodiols of aromatic hydrocarbons to the corresponding catechols in a NADP+-dependent manner (Carbone et al., 2008). The overall reaction catalyzed by dimeric dihydrodiol dehydrogenase is a two-electron oxidation of one of the hydroxyl groups of a *trans*-dihydrodiol to an intermediate ketol, which rapidly enolizes to the catechol (Carbone et al., 2008). It also activates naphthalene *trans*-dihydrodiol to 1,2-dihydroxynaphthalene, which rapidly auto-oxidizes to 1,2-naphthoquinone, a cytotoxic *ortho*-quinone that may be responsible for naphthalene-induced cataracts (Carbone et al., 2008). Dimeric dihydrodiol dehydrogenase is not active toward hydroxysteroids, but it does oxidize several endogenous sugars, including D-glucose and D-xylose, and may therefore play a role in the metabolism of dietary sugars with subsequent generation of NADPH. Dimeric dihydrodiol dehydrogenase can also catalyze reductions in the presence of NADPH, such as the carbonyl reduction of methylglyoxal and 3-deoxyglucusone, nitrobenzaldehydes, and camphoroquine (Carbone et al., 2008). Dimeric dihydrodiol dehydrogenase can be inhibited by L-ascorbic and isoascorbic acids and also by 4-hydroxyphenylketones and 2,6-dihydroxyanthraquinone, and is inactivated by magnesium chloride and some phosphate salts (Carbone et al., 2008). This enzyme was first characterized in rabbit liver, where it oxidizes benzene dihydrodiol to the genotoxic and immunotoxic catechol. It has also been isolated from pig, dog, and cynomolgus monkey (*Macaca fascicularis*). The purified enzyme is composed of identical subunits with molecular weights of 32 to 36 kDa, and amino acid sequence in humans indicates that each subunit is composed of 335 amino acids (Carbone et al., 2008). Dimeric dihydrodiol dehydrogenase is ubiquitously expressed in dogs and pigs, but only in the kidney of monkey, lens and intestine of rabbit, and apparently only the intestine of humans (Carbone et al., 2008).

It should be noted that polycyclic aromatic hydrocarbons can be oxidized by CYP to an arene oxide, an electrophilic and potentially toxic metabolite that is detoxified by its conversion to a *trans*-dihydrodiol by epoxide hydrolase. By oxidizing the *trans*-dihydrodiol to an *ortho*-quinone, AKR1A1, 1C1-1C4 can generate yet another potentially toxic metabolite. The roles of these AKRs (which can act as monomeric dihydrodiol dehydrogenases) in the metabolism of PAHs are well known but the activity of dimeric dihydrodiol dehydrogenase toward the trans-dihydrodiols of PAHs has not yet been reported (Carbone et al., 2008). In terms of their ability to cause oxidative stress and cellular toxicity, *ortho*-quinones can be considered equivalent to *para*-quinones, which were discussed earlier in the section "Quinone Reduction: NQO1 and NQO2."

Molybdenum Hydroxylases (Molybdoenzymes) There are five human molybdenum-containing enzymes (molybdoenzymes), namely aldehyde oxidase (AO; gene symbol AOX1), xanthine

oxidoreductase (XOR; also known as xanthine oxidase or xanthine dehydrogenase, gene symbol XDH), sulfite oxidase (gene symbol SUOX), and the more recently discovered "mitochondrial amidoxime reducing component" (mARC-1 and -2; gene symbols MARC1 and MARC2) (Havemeyer et al., 2011; Neve et al., 2015). Aldehyde oxidase and xanthine oxidoreductase are the two major molybdoenzymes that participate in the biotransformation of xenobiotics. The human mitochondrial amidoxime-reducing component (composed of mARC 1 and 2) is involved in the activation of amidoxime prodrugs and detoxication of some *N*-hydroxylated xenobiotics, as described in the section "Dehydroxylation." Sulfite oxidase will not be described in detail here except that, as its name implies, the enzyme oxidizes sulfite, an irritating air pollutant, to sulfate, which is relatively innocuous, and is also required to metabolize the sulfur-containing amino acids cysteine and methionine in foods.

Genetic polymorphisms can affect all molybdoenzymes or individual enzymes. Homozygosity for nonfunctional forms of some of the genes involved in the synthesis of the active molybdenum cofactor (MoCo), namely MOCS1, MOCS2, MOCOS, or GPHN, result in a complete lack of activity of all molybdoenzymes, and is clinically indistinguishable from isolated sulfite oxidase deficiency (Reiss and Hahnewald, 2010), a rare condition that gives rise to progressive neurological damage and death in early childhood (Reiss and Johnson, 2003; Feng et al., 2007). Isolated xanthine oxidoreductase deficiency results in the classical type I xanthinuria which can lead to xanthine stones (calculi) because of the inability to oxidize xanthine and hypoxanthine to uric acid, but it is not life-threatening (Reiss and Hahnewald, 2010). Although no isolated forms of AO deficiency have been described, type II xanthinuria results from a combined deficiency of xanthine oxidoreductase and aldehyde oxidase as evidenced by both the inability to oxidize xanthine and hypoxanthine as well as allopurinol to oxypurinol. Certain polymorphisms in aldehyde oxidase do appear to cause a decrease or complete loss of activity toward certain substrates, as discussed in the section "Aldehyde Oxidase" (Hartmann et al., 2012). Not surprisingly, given its recent discovery, isolated mARC deficiency has not yet been described.

Xanthine oxidoreductase and aldehyde oxidase comprise the human members of the "xanthine oxidase family" of molybdoenzymes. Both enzymes are only active as homodimers, each with about 145-kDa monomer consisting of an N-terminal region that binds two nonidentical iron–sulfur clusters of the [2Fe-2S]-type, followed by an FAD-binding region and C-terminal domains that bind the molybdenum center (Wahl et al., 2010). In xanthine oxidoreductase, however, an additional NAD^+ binding site is present. In xanthine oxidoreductase and aldehyde oxidase, the molybdenum is ligated to an oxo ligand, a hydroxyl group, two dithiolene sulfurs, and a sulfido group (as shown in the upper left panel of Fig. 6-31). The molybdenum-sulfur bond is inserted through a posttranslational reaction catalyzed by MoCo sulfurase (gene symbol MOCOS) which substitutes one of the oxo groups of the molybdenum cofactor with a sulfo double bond and is essential for the activity of these enzymes (Garattini et al., 2008). In contrast, in sulfite oxidase (the only fully accepted human member of the "sulfite oxidase family" of molybdoenzymes), the molybdenum is coordinated by the two sulfurs on the pterin, two oxo ligands, and a protein-derived cysteinyl sulfur (Wahl et al., 2010). Sulfite oxidase does not require posttranslational sulfuration of the molybdenum cofactor to become active. The two 35-kDa human mARC enzymes may represent a separate family of molybdoenzymes in that they do not contain an FAD domain and may bind molybdenum in a manner different than either the xanthine oxidase or sulfite oxidase families. However, there is some evidence to suggest that the mARCs are in fact members of the human sulfite oxidase family of molybdoenzymes (Havemeyer et al., 2011). In addition, the mARCs share significant sequence similarity with the C-terminal domain of MOCOS and appear to require cytochrome b_5, cytochrome b_5 reductase, and NADH for catalytic activity (Wahl et al., 2010). Furthermore, while xanthine oxidoreductase, aldehyde oxidase, and sulfite oxidase are localized in the cytosol, the two mARC proteins appear to be localized to the outer mitochondrial membrane (Havemeyer et al., 2011).

The catalytic cycle for xanthine oxidoreductase and aldehyde oxidase involves an interaction between the molybdenum center with a reducing substrate, which results in the reduction of the molybdenum cofactor, after which reducing equivalents are transferred intramolecularly to the flavin cofactors (which act as electron sinks), with reoxidation occurring via the flavin moiety by molecular oxygen (in the case of aldehyde oxidase and the xanthine oxidase form of xanthine oxidoreductase) or NAD^+ (in the case of the xanthine dehydrogenase form of xanthine oxidoreductase). Electrons are transferred from the molybdenum cofactor to the flavin cofactor through the intermediacy of the iron–sulfur centers. During substrate oxidation, aldehyde oxidase and xanthine oxidoreductase are reduced and then reoxidized by molecular oxygen; hence, they function as true oxidases. The oxygen incorporated into the xenobiotic is derived from water rather than oxygen, which distinguishes these oxidases from oxygenases. The main functional difference between aldehyde oxidase and xanthine oxidoreductase is the ability of the latter to use both NAD^+ (in its XD form) *and* molecular oxygen (in its XO form), as the final acceptors of the reducing equivalents (Garattini and Terao, 2011). Reduction of oxygen by the XO form of xanthine oxidoreductase leads to the formation of hydrogen peroxide or superoxide anion (in a substrate-dependent manner) as follows:

$$RH + H_2O + O_2 \rightarrow ROH + H_2O_2$$

$$RH + H_2O + 2O_2 \rightarrow ROH + 2O_2^- + 2H^+$$

Additional details of the catalytic cycle are described in the next section "Xanthine Oxidoreductase." Xanthine oxidoreductase and aldehyde oxidase catalyze the oxidation of carbon atoms with a low electron density such as the electron-deficient sp^2-hybridized carbon atom, double-bonded to nitrogen in aromatic azaheterocycles (R_1–CH=N–R_2) or oxygen in various aldehydes (R_1–CH=O). In aromatic azaheterocycles (such as purines, pyrimidines, pteridines, quinolines, iminium ions, and diazanaphthalenes), oxidation by aldehyde oxidase (and in most cases by xanthine oxidoreductase) occurs at the carbon with the lowest electron density, which is typically the α-carbon (next to the nitrogen), but in some cases the γ-carbon (two carbons removed) (Strolin-Benedetti, 2011). This contrasts with oxidation by CYP, which generally catalyzes the oxidation of carbon atoms with a high electron density. For this reason, xenobiotics that are good substrates for molybdoenzymes (nucleophilic oxidizing enzymes) tend to be poor substrates for CYP (an electrophilic oxidizing enzyme), and vice versa (Pryde et al., 2010). Aldehyde oxidase and, to a lesser extent, xanthine oxidoreductase hydroxylate the carbonyl carbon in certain aldehydes and convert them to carboxylic acids (R–CHO → R–COOH). Both molybdoenzymes hydroxylate the α-carbon in aromatic azaheterocycles (R_1–CH=N–R_2) to form α-hydroxyimines (R_1–COH=N–R_2) that rapidly tautomerizes to the corresponding lactam (R_1–CO–NH–R_2) (α-aminoketone). Certain aromatic aldehydes, such as tamoxifen aldehyde and benzaldehyde, are good substrates for aldehyde oxidase and xanthine oxidoreductase, whereas aliphatic aldehydes tend to be poor substrates. Consequently, aldehyde oxidase and xanthine

oxidoreductase contribute negligibly to the metabolism of acetaldehyde under normal conditions. Some reactions catalyzed by aldehyde oxidase and xanthine oxidoreductase are shown in Fig. 6-30. It should be noted that although aldehyde oxidase and xanthine oxidoreductase tend to oxidize similar substrates, there are some differences in substrate specificity (e.g., vanillin is rapidly oxidized by aldehyde oxidase, but there is little contribution of xanthine oxidoreductase) (Strolin-Benedetti, 2011). An interesting difference between xanthine oxidoreductase and aldehyde oxidase is their regioselective oxidation of the purine prodrug 6-deoxyacyclovir, which is oxidized by xanthine oxidoreductase in the six-membered ring to form the active drug acyclovir (discussed below) but is oxidized by aldehyde oxidase in the five-membered ring to form the pharmacologically inactive metabolite 6-deoxy-8-hydroxy-acyclovir (Testa and Krämer, 2008, 2010). In the case of nonaromatic azaheterocycles, the preferred substrate is the protonated form (–CH=NH$^+$–) because this form lowers the electron density at the α-carbon. For this same reason, *N*-alkylation of aromatic azaheterocycles to form a quaternary (positively charged) nitrogen next to the α-carbon improves oxidation by aldehyde oxidase (more so than by xanthine oxidoreductase), as in the case of *N*-methylnicotinamide. Under certain conditions, both enzymes can also catalyze the reduction of xenobiotics (Testa and Krämer, 2008, 2010). Examples of reductive reactions catalyzed by aldehyde oxidase are shown in Fig. 6-27.

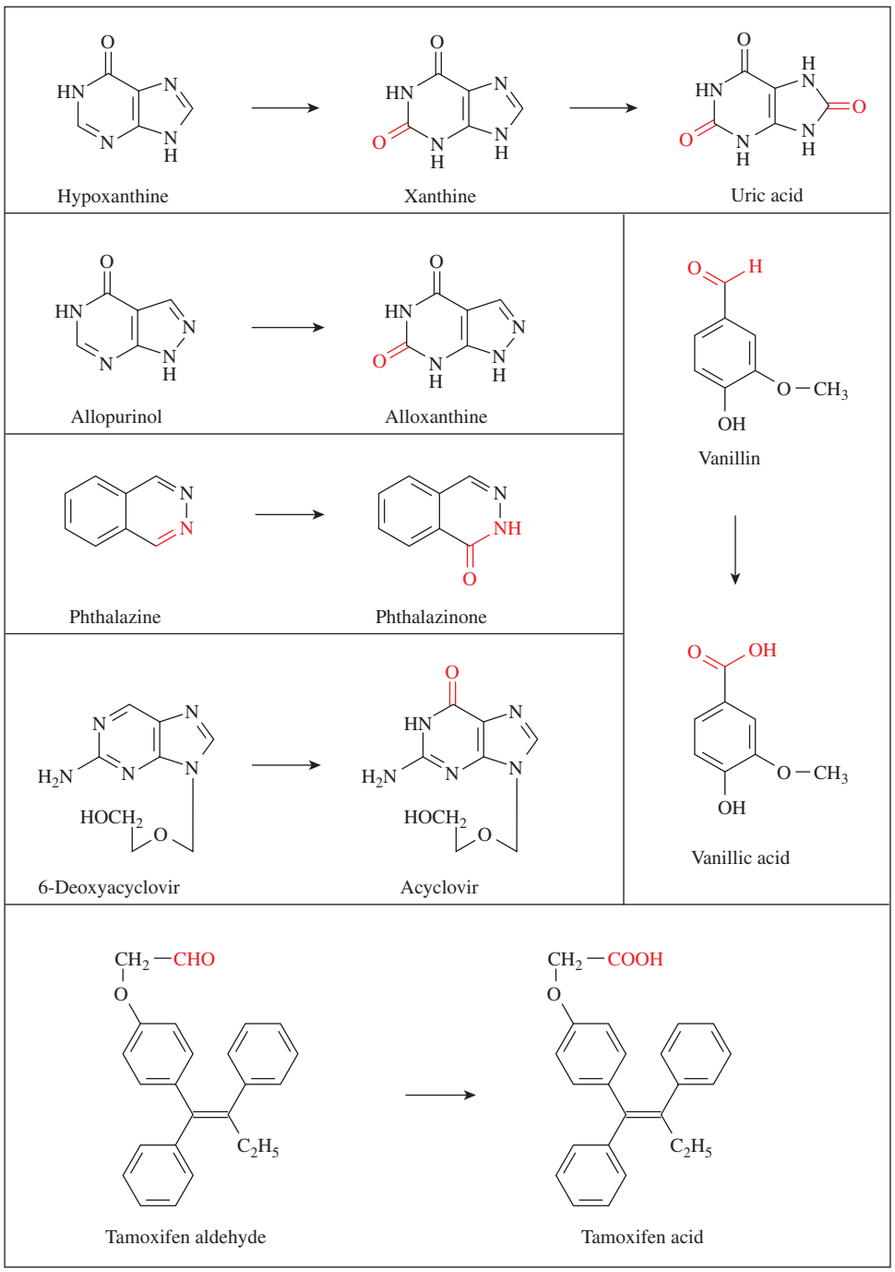


Figure 6-30. *Examples of reactions catalyzed by the molybdoenzymes, xanthine oxidoreductase, and aldehyde oxidase.*

Xanthine Oxidoreductase Xanthine dehydrogenase (XD) and xanthine oxidase (XO) are two forms of the same cytosolic enzyme (xanthine oxidoreductase or XOR, gene symbol XDH) that differ in the electron acceptor used in the final step of catalysis. In the case of XD, the final electron acceptor is NAD^+ (dehydrogenase activity), whereas in the case of XO the final electron acceptor is oxygen (oxidase activity). Although both xanthine oxidoreductase and aldehyde oxidase are expressed in many tissues, the highest xanthine oxidoreductase expression in humans is found in the proximal intestine, liver, and lactating mammary glands (Strolin-Benedetti, 2011). XD is converted reversibly to XO by oxidation of sulfhydryl groups or irreversibly by proteolytic cleavage (Testa and Krämer, 2008, 2010). In humans, xanthine oxidoreductase is encoded by a single gene (i.e., XDH). Under normal physiologic conditions, XD is the predominant form of the enzyme found in vivo. However, during tissue processing, the dehydrogenase form tends to be converted to the oxidase form; hence, most in vitro studies are conducted with XO or a combination of XO and XD. The induction (upregulation) of XD and/or the conversion of XD to XO in vivo is thought to play an important role in ischemia-reperfusion injury, lipopolysaccharide (LPS)-mediated tissue injury, and alcohol-induced hepatotoxicity (Pacher et al., 2006). During ischemia, XO levels increase because hypoxia induces xanthine oxidoreductase gene transcription, and because XD is converted to XO. During reperfusion, XO contributes to oxidative stress and lipid peroxidation because the oxidase activity of XO involves the reduction of molecular oxygen, which can lead to the formation of reactive oxygen species of the type shown in Fig. 6-20. Similarly, treatment with LPS, a bacterial endotoxin that triggers an acute inflammatory response, increases XO activity both by inducing xanthine oxidoreductase transcription and by converting XD to XO. The associated increase in oxidative stress has been implicated in LPS-induced cytotoxicity. Ethanol facilitates the conversion of XD to XO, and the conversion of ethanol to acetaldehyde provides a substrate and, hence, a source of electrons for the reduction of oxygen. Hereafter, the two forms of the enzyme will be referred to as xanthine oxidoreductase.

Typical reactions catalyzed by xanthine oxidoreductase are shown in Fig. 6-30. Xanthine oxidoreductase contributes significantly to the first-pass elimination of several purine derivatives (e.g., 6-mercaptopurine and 2,6-dithiopurine), and limits the therapeutic effects of these cancer chemotherapeutic drugs. In contrast, certain prodrugs are activated by xanthine oxidoreductase. For example, the antiviral prodrugs 6-deoxyacyclovir and 2′-fluoroarabino-dideoxypurine, which are relatively well absorbed after oral dosing, are oxidized by xanthine oxidoreductase to their respective active forms acyclovir and 2′-fluoroarabino-dideoxyinosine, which are otherwise poorly absorbed (see Fig. 6-30). Furthermore, xanthine oxidoreductase has been implicated in the bioactivation of mitomycin C and related antineoplastic drugs, although this bioactivation reaction is thought to be largely catalyzed by NQO1 (DT-diaphorase), as discussed previously in the section "Quinone Reduction: NQO1 and NQO2." Other xenobiotic substrates of xanthine oxidoreductase include 6-thioxanthine, methotrexate, and the benzylic aldehyde metabolite of tolbutamide (Chladek et al., 1997; Testa and Krämer, 2008, 2010; Kudo et al., 2010).

Xanthine oxidoreductase catalyzes an important physiological reaction, namely, the sequential oxidation of hypoxanthine to xanthine and uric acid, as shown in Fig. 6-30 (Rajagopalan, 1980). By competing with hypoxanthine and xanthine for oxidation by xanthine oxidoreductase, allopurinol inhibits the formation of uric acid, making allopurinol a useful drug in the treatment of gout (a complication of hyperuricemia). Allopurinol can also be used to evaluate the contribution of xanthine oxidoreductase to xenobiotic biotransformation in vivo. Allopurinol is often added to fluids used to perfuse human livers intended for transplantation or research purposes. Cytosol prepared from such human livers lacks xanthine oxidoreductase activity but still retains aldehyde oxidase activity (Barr et al., 2014). Like allopurinol, hydroxylated coumarin derivatives, such as umbelliferone (7-hydroxycoumarin) and esculetin (7,8-dihydroxycoumarin), are potent inhibitors of xanthine oxidoreductase. Allopurinol and other xanthine oxidoreductase inhibitors are being evaluated for the treatment of various types of ischemia reperfusion and vascular injury that appear to be mediated, at least in part, by xanthine oxidoreductase (Pacher et al., 2006).

Monomethylated xanthines are preferentially oxidized to the corresponding uric acid derivatives by xanthine oxidoreductase. In contrast, dimethylated and trimethylated xanthines, such as theophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine), are oxidized to the corresponding uric acid derivatives primarily by CYP. Through two sequential *N*-demethylation reactions, CYP converts caffeine to 1-methylxanthine, which is converted by xanthine oxidoreductase to 1-methyluric acid. The urinary ratio of 1-methylxanthine to 1-methyluric acid provides an in vivo marker of xanthine oxidoreductase activity.

The mechanism of catalysis by molybdoenzymes depicted in Fig. 6-31 is based on studies of xanthine oxidoreductase by Okamoto et al. (2004), Amano et al. (2007), and Alfaro and Jones (2008). The molybdopterin active center of the enzyme consists of molybdenum (Mo) bound to the following groups: pterin cofactor, thioxo (=S), oxo (=O), and hydroxyl (–OH). For simplicity, this cofactor will be written as S=Mo–OH. The hydroxyl group (S=Mo–OH) is activated by a nearby glutamic acid residue in the enzyme to S=Mo–O^-, which initiates a concerted nucleophilic attack on the substrate at the electron-deficient carbon atom double-bonded to the nitrogen ($H–CR_1=N–R_2$) with hydride transfer from the α-carbon to the thioxo group to form the intermediate ($HS–Mo–O–CR_1=N–R_2$). The oxygen bound to the α-carbon in the substrate (e.g., xanthine) is replaced by oxygen from water to complete formation of product (e.g., uric acid). The resting enzyme is restored by the removal of two reducing equivalents, which may be transferred to NAD^+ (in the case of XD) or oxygen (in the case of XO), as shown in Fig. 6-31. In the XO mode, xanthine oxidoreductase (and aldehyde oxidase, which exists only in the XO mode) can transfer both reducing equivalents to a single molecule of oxygen to produce hydrogen peroxide or it can transfer the two electrons to two molecules of oxygen to produce superoxide anion. In addition to the concerted mechanism described above, a stepwise mechanism involving a tetrahedral intermediate is also possible (Alfaro and Jones, 2008).

Aldehyde Oxidase The term aldehyde oxidase is something of a misnomer, in that this enzyme does not solely act on aldehyde-containing substrates (see Table 6-11). Aldehyde oxidase is the second of two molybdoenzymes that play an important role in xenobiotic biotransformation; the other being xanthine oxidoreductase (discussed in the preceding section). While xanthine oxidoreductase exists in two forms, a dehydrogenase form (XD) that relays electrons to NAD^+ and an oxidase form (XO) that relays electrons to molecular oxygen, aldehyde oxidase exists only in the oxidase form because it lacks an NAD^+ binding site (Garattini and Terao, 2011). Another significant difference between these two molybdoenzymes is that high levels of xanthine oxidoreductase appear to be widely distributed throughout the body, whereas high levels of aldehyde oxidase (or at least its mRNA) are found in the liver and adrenal gland, with somewhat less expression in the small and large

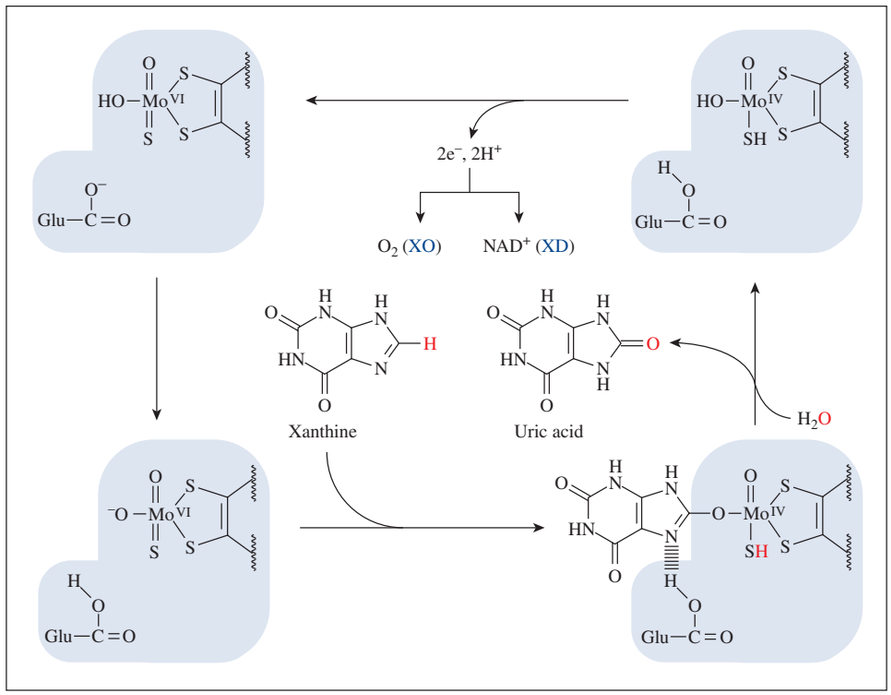


Figure 6-31. *Catalytic cycle of the molybdoenzyme xanthine oxidoreductase. Xanthine oxidoreductase is a molybdoenzyme with xanthine dehydrogenase (XD) and xanthine oxidase (XO) activities*. During the final step of the catalytic cycle, the enzyme is reoxidized by transferring electrons to NAD^+ (in the case of XD) or oxygen (in the case of XO). The same mechanism of catalysis likely applies to aldehyde oxidase, although there is no dehydrogenase form of this molybdoenzyme.

Table 6-11

Substrates Associated with Oxidation by Aldehyde Oxidase

SUBSTRATE GROUPS	EXAMPLE SUBSTRATES
Aldehydes	Acetaldehyde, benzaldehyde, citalopram aldehyde, phenylacetaldehyde, pyridoxal, retinal, tamoxifen aldehyde, tolbutamide aldehyde, vanillin
Aromatic azaheterocycles	Phthalazines, cinchona alkaloids, quinazolines, carbazeran, chloroquinazolinone, famciclovir, 6-mercaptopurine, *N*-methyl-nicotinamide, *N*-methyl-phthalazinium, phenanthridine, 3-descladinosyl-11,12-dideoxy-6-O-methyl-3-oxo-12,11-(oxycarbonyl-(1-(1R-([1,8]naphthyridin-4-yl)-ethyl)azetidin-3-yl) imino)erythromycin A, 2,4-diaminopteridine, *N*-{trans-4-[2-(6-cyano-3,4-dihydroisoquinolin-2(1H)-yl) ethyl]cyclohexyl}quinoline-4-carboxamide (SB-277011), methotrexate, 6-thioxanthine, zebularine, 5-fluoro-pyrimidinone, 5-ethinylpyrimidinone, caffeine, 6-deoxyacyclovir, 8-hydroxy-6-deoxyacyclovir, allopurinol, pyrazinamide, *N*-[(20-dimethylamino) ethyl]acridine-4-carboxamide (DACA), 2-[4-(7-chloro-2-quinoxalinyloxyphenoxy]-propionic acid (XK-469), dibenzo[*b,f*]-1,4-oxazepine, briminodine, 3-*tert*-butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-*d*][1,2,4]triazine (MRK-016), 6-(2,4-difluoro-phenoxy)-2-((R)-2-hydroxy-1-methyl-ethylamino)-8-((S)-2-hydroxy-propyl)-8H-pyrido[2,3-d] pyrimidin-7-one (RO1), zaleplon
Iminium ions	Nicotine iminium ion, *N*-methyl-nicotinamide iminium ion, iminium ion of 3-methyl-5-[(2*S*)-1-methyl-2-pyrrolidinyl]isoxazole (ABT-418), prolintane iminium ion, azapetine iminium ion

SOURCES: Data from Pryde et al. (2010) and Hartmann et al. (2012).

intestine, ovary, prostate, proximal, distal, and collecting tubules of the kidney, the epithelia of trachea and bronchium, and the alveolar cells of the lung, with only detectable levels of transcript in the endocrine tissues, esophagus, pancreas, brain, and few other tissues, at least in humans (Pryde et al., 2010; Garattini and Terao, 2011). The human AOX1 gene is complex with 35 exons, and exists as an active homodimer. There is also considerable interindividual variability in the activity of aldehyde oxidase in humans, which may be due to genetic (as discussed further below) as well as environmental factors such as age, disease state (e.g., cancer and inflammatory conditions leading to altered levels of various cytokines), smoking, heavy ethanol consumption, and drug use (Pryde et al., 2010; Fu et al., 2013). Apart from these differences, many of the features of xanthine oxidoreductase apply to aldehyde oxidase, including subcellular location (cytosol), enzyme structure and cofactor composition, typical mechanism of catalysis, preference for oxidizing sp^2-hybridized carbon atoms adjacent to the nitrogen atoms in nitrogen heterocycles, and its preference for oxidizing aromatic

aldehydes over aliphatic aldehydes. Other aldehyde oxidase substrate types include nitro- or nitroso-containing compounds as well as aldehyde or iminium ion intermediates formed by CYP, alcohol dehydrogenases, or MAOs (Pryde et al., 2010). Furthermore, aldehyde oxidase also transfers electrons to molecular oxygen, which can generate reactive oxygen species (e.g., superoxide anion which dismutates to hydrogen peroxide) and lead to oxidative stress and lipid peroxidation (Garattini and Terao, 2011). However, in contrast to xanthine oxidoreductase, the physiological functions of aldehyde oxidase remain largely unknown, although endogenous compounds such as indole-3-acetate, retinaldehyde, retinoic acid, nicotinamide, and pyridoxal are substrates for aldehyde oxidase (Garattini and Terao, 2011).

In vitro human aldehyde oxidase activity varies significantly in human liver cytosols from one individual donor to another, and appears to be substrate-dependent (e.g., 90-fold differences in carbazeran oxidation vs. 19-fold for phthalazine oxidation across 20 donors) (Fu et al., 2013). Many single-nucleotide polymorphisms in the human AOX1 gene have been described in the NCBI dbSNP database (NCBI, 2018). In a population of 180 Italian individuals, relatively frequent nonsense, synonymous, or missense SNPs in aldehyde oxidase have been described, with a total of 51 SNPs identified, with 10 individuals being homozygous for a SNP (Hartmann et al., 2012). Two of the SNPs resulted in a fast metabolizer phenotype (FMs; N1135S, frequency = 2.9%, and H1297R, frequency = 5.3%) and one in a poor metabolizer phenotype (PMs, i.e., R921H, frequency = 0.3%). In addition, a relatively frequent nonsense mutation in exon 5 (Y126stop, frequency = 0.026) was identified that results in a very short and nonfunctional 126–amino acid protein, but no subjects were homozygous for this loss-of-function mutation. An important point about these polymorphisms described so far is that the effects appear to be somewhat substrate-dependent based on the functional characterization of purified, heterologously expressed variants described above with the aldehyde oxidase substrates benzaldehyde, phthalazine, phenathridine, and chloroquinazolinone (Hartmann et al., 2012). Somewhat equivocal evidence for the clinical relevance of AOX1 SNPs has been published. The selective topoisomerase II β inhibitor and former drug candidate 2-(4-((7-chloro-2-quinoxalinyl)oxy)phenoxy)propanoic acid (XK469) is metabolized mainly by aldehyde oxidase and showed high intersubject variability in drug clearance during Phase 1 clinical trials (Ramirez et al., 2014). Although the authors found associations between certain aldehyde oxidase SNPs and decreased clearance of XK469, they concluded that the variability in clearance could not be explained by these SNPs. However, the University of Washington Metabolism and Transport Drug Interaction Database indicates that two SNPs (AOX1 IVS27-152A/IVS27-152G and IVS27-152G/IVS27-152G) are associated with significant reductions in the clearance of XK469 (–29% and –40%, respectively) (University of Washington DIDB, 2018). It is likely that genotype is only one of several factors that play a role in the relatively high variability in aldehyde oxidase activity.

Substrates for aldehyde oxidase do not appear to correlate with any specific area of "chemical space" based on physicochemical properties, which have a wide variety of lipophilicity, polar surface area, polarity, and structure, in contrast to P450 substrates, for which there is a high correlation between higher log *P* and P450-mediated metabolism as measured by microsomal stability in the presence of NADPH (see Table 6-11 for example substrates) (Pryde et al., 2010). Therefore, although it has been assumed that, in general, xenobiotics that are good substrates for aldehyde oxidase are poor substrates for CYP, and vice versa (Rettie and Fisher, 1999), there is some overlap, and structural motifs alone may be a better predictor of aldehyde oxidase substrates (Pryde et al., 2010). Naphthalene (with no nitrogen atoms) is oxidized by CYP, but not by aldehyde oxidase, whereas the opposite is true of pteridine (1,3,5,8-tetraazanaphthalene), which contains four nitrogen atoms. The intermediate structure quinazolone (1,3-diazanaphthalene) is a substrate for both enzymes. This complementarity in substrate specificity reflects the opposing preference of the two enzymes for oxidizing carbon atoms; CYP prefers to oxidize carbon atoms with high electron density, whereas aldehyde oxidase (and xanthine oxidoreductase) prefers to oxidize carbon atoms with low electron density. Because of their opposing mechanisms of action (electrophilic for CYP and nucleophilic for aldehyde oxidase), the common practice in drug development of adding a fluorine atom to block a site of oxidation by CYP (to improve metabolic stability) can potentially increase the rate of metabolism by aldehyde oxidase. In nearly all cases, oxidation of aromatic heterocycles by aldehyde oxidase occurs at the α-carbon but occasionally, depending on ring structure, the γ-carbon (or even the β-carbon) has the lowest electron density and is the site of oxidation. Examples of γ-carbon (or β-carbon) oxidation by aldehyde oxidase include *N*-methylnicotinamide, quinolone, cinnoline, and DACA (*N*-[(2′-dimethylamino)ethyl]acridine-4-carboxamide) (Schofield et al., 2000; Testa and Krämer, 2008, 2010). In terms of its role in drug metabolism, it is reasonable to conclude that human aldehyde oxidase does play—and will continue to play—a significant role in the oxidation of a wide range of structurally diverse nitrogen heterocyclic substrates (Pryde et al., 2010).

As shown in Figs. 6-27 and 6-30, aldehyde oxidase can oxidize a number of substituted pyrroles, pyridines, pyrimidines, purines, pteridines, and iminium ions (see Table 6-11 for additional examples) by the mechanism described for xanthine oxidoreductase in the previous section (Alfaro and Jones, 2008). Aldehyde oxidase can oxidize aldehydes to their corresponding carboxylic acids, but the enzyme shows a marked preference for aromatic aldehydes (e.g., benzaldehyde and tamoxifen aldehyde). Consequently, aldehyde oxidase contributes negligibly to the oxidation of aliphatic aldehydes, such as acetaldehyde. Rodrigues (1994) found that, in a bank of human liver samples, aldehyde oxidase activity toward N^1-methylnicotinamide varied more than 40-fold, whereas activity toward 6-methylpurine varied less than threefold. Although this suggests human liver cytosol contains two or more forms of aldehyde oxidase, subsequent Southern blot analysis has provided evidence for only a single copy of the aldehyde oxidase gene in humans (Terao et al., 1998), although genomic analysis of chromosome 2q has revealed the presence of two nearby pseudogenes (Garattini and Terao, 2011). Species differences in the number of functional AOX genes are discussed further below.

The substrate specificity of aldehyde oxidase differs among mammalian species, often leading to erroneous conclusions about the potential metabolic stability of certain drugs in humans, as detailed below. Aldehyde oxidase activity among animal species can vary depending on the particular substrate, but in general it is high in monkeys and humans, low in rats, and essentially absent in dogs (because the latter only express AOX3L1 and AOX4) (Garattini and Terao, 2011). These differences have been ascribed to the size of the active site (Pryde et al., 2010). However, large differences in activity have also been found among individual strains of rats and mice (e.g., high activity in Sea:SD rats and low in WKA/Sea rats; large differences between Sprague–Dawley and Wistar as well as between C129/C57 and CB57B1/6J mice) (Pryde et al., 2010). Gender differences between rats and mice due to hormonal regulation further complicate attempts to extrapolate stability of aldehyde oxidase substrates from toxicologically relevant species

to humans (e.g., two- to fourfold higher activity in male mice than in female) (Pryde et al., 2010). Finally, whereas humans encode only a single functional aldehyde oxidase (namely AOX1; the others being transcribed pseudogenes, i.e., AOX3P and 3L1P), mice and rats possess four functional AOX genes (namely, AOX1, AOX3, AOX4, and AOX3L1) (Garattini and Terao, 2011). Rhesus monkeys possess two functional AOs (AOX3 and AOX3L1). In the mouse and monkey, AOX3L1 is expressed only in nasal mucosa (Garattini and Terao, 2011). A further complication is the observation of species differences in the relative roles of aldehyde oxidase and xanthine oxidoreductase in xenobiotic biotransformation. For example, the 6-oxidation of antiviral deoxyguanine prodrugs is catalyzed exclusively in rats by xanthine oxidoreductase, but by aldehyde oxidase in humans (Rettie and Fisher, 1999). Similarly, only human aldehyde oxidase catalyzes the oxidation of 6-deoxypenciclovir to penciclovir (Strolin-Benedetti, 2011).

Aldehyde oxidase in the human liver has proved to be rather unstable, which complicates an in vitro assessment of species differences in aldehyde oxidase activity in frozen stocks of human liver cytosol (Rodrigues, 1994; Rettie and Fisher, 1999; Garattini and Terao, 2011). In addition, few human cell lines express functional aldehyde oxidase (e.g., HepG2 cells express the AOX1 transcript, but lack the functional enzyme) (Garattini and Terao, 2011). Fresh or cryopreserved human hepatocytes are therefore most likely to have relevant levels of aldehyde oxidase activity.

Aldehyde oxidase is the second of two enzymes involved in the formation of cotinine, a major metabolite of nicotine excreted in the urine of cigarette smokers. The initial step in this reaction is the formation of a double bond (C=N) in the pyrrole ring, which produces nicotine $\Delta^{1',5'}$ -iminium ion. Like nicotine, several other drugs are oxidized either sequentially or concomitantly by CYP and aldehyde oxidase, including quinidine, azapetine, cyclophosphamide, carbazeran, and prolintane. Other drugs that are oxidized by aldehyde oxidase include bromonidine, citalopram, propionaldehyde, O^6-benzylguanine, 6-mercaptopurine, metyrapone, quinine, pyrazinamide, methotrexate, vanillin, isovanillin, zaleplon, and famciclovir (an antiviral prodrug that is converted by aldehyde oxidase to penciclovir) (for additional examples, see Table 6-11). Several pyrimidine derivatives are oxidized by aldehyde oxidase, including 5-ethyl-2(1*H*)-pyrimidone, which is converted by aldehyde oxidase to 5-ethinyluracil. Like sorivudine, 5-ethinyluracil is a metabolism-dependent (suicide) inactivator of dihydropyrimidine dehydrogenase (see Fig. 6-21).

Raloxifene is an extraordinarily potent inhibitor of human aldehyde oxidase (K_i values as low as 0.9 nM), whereas it is at least three orders of magnitude less potent in other species (Obach, 2004; Pryde et al., 2010). Perphenazine and menadione are also potent inhibitors of aldehyde oxidase (IC_{50} about 0.03 and about 0.2 μM) and are often used together with allopurinol to discriminate between aldehyde oxidase–catalyzed and xanthine oxidoreductase–catalyzed reactions. The ability of proadifen to inhibit aldehyde oxidase is noteworthy because this methadone analog, commonly known as SKF 525A, is widely used as a CYP inhibitor. Hydralazine is an irreversible (time-dependent) inhibitor of aldehyde oxidase. Nitroso-imidacloprid has been characterized as a mechanism-based inhibitor of rabbit aldehyde oxidase with a K_i value of 1.3 mM and k_{inact} of 0.35 min^{-1} (Dick et al., 2007). Several other inhibitors of aldehyde oxidase have also been described, but raloxifene is probably the most useful for in vitro studies to evaluate the contribution of aldehyde oxidase to the metabolism of a substrate (Pryde et al., 2010). For instance, if a substrate that undergoes oxidative metabolism is found (1) to have higher in vitro turnover in human hepatocytes than in human liver microsomes, (2) is oxidized in human liver S9 or cytosol in the absence of NADPH, and (3) contains a structure amenable to oxidation by aldehyde oxidase, then raloxifene can be used to confirm involvement of aldehyde oxidase in the metabolism of the substrate (Pryde et al., 2010).

Under certain conditions, aldehyde oxidase and xanthine oxidoreductase can also catalyze the reduction of xenobiotics, including azo-reduction (e.g., 4-dimethylaminoazobenzene), nitro-reduction (e.g., 1-nitropyrene, imidacloprid and other nitroguanidines, and nitromethylenes), *N*-oxide reduction (e.g., *S*-(-)-nicotine-1′-*N*-oxide, imipramine *N*-oxide, and cyclobenzaprine *N*-oxide), nitrosamine reduction (e.g., *N*-nitrosodiphenylamine), hydroxamic acid reduction (e.g., *N*-hydroxy-2-acetylaminofluorene), sulfoxide reduction (e.g., sulindac; see Fig. 6-19, fenthion sulfoxide), quinone reduction (e.g., diethylstilbestrol quinone), epoxide reduction (e.g., benzo[*a*]pyrene 4,5-oxide), and heterocycle reduction (e.g., ziprasidone and zonisamide) (Testa and Krämer, 2008, 2010; Pryde et al., 2010). Oximes (C=NOH) can also be reduced by aldehyde oxidase to the corresponding ketimines (C=NH), which may react nonenzymatically with water to produce the corresponding ketone or aldehyde (C=O) and ammonia (Testa and Krämer, 2008, 2010). The reduction of ziprasidone by aldehyde oxidase is shown in Fig. 6-7, and examples of other reductive reactions catalyzed by aldehyde oxidase are shown in Fig. 6-27. Xenobiotic reduction by aldehyde oxidase in vitro requires anaerobic conditions or the presence of a reducing substrate, such as N^1-methylnicotinamide, 2-hydroxypyrimidine, or benzaldehyde. These "co-substrates" reduce the enzyme, which in turn catalyzes azo-reduction, nitro-reduction, etc. by relaying electrons to xenobiotics (rather than molecular oxygen). These unusual requirements make it difficult to assess the degree to which aldehyde oxidase functions as a reductive enzyme in vivo.

Amine Oxidases Human amine oxidases can be divided into two classes: (1) the familiar FAD-containing mitochondrial monoamine oxidases, MAO-A and MAO-B (gene symbols MAOA and MAOB), and polyamine oxidase (gene symbol PAOX) and spermine oxidase (gene symbol SMOX); and (2) the copper-containing amine oxidases (CuAOs) that contain a tightly bound Cu^{II} and a quinone residue (typically 2,4,5-trihydroxyphenylalanine quinone [TPQ]) as the redox cofactor (Largeron, 2011). The latter class of human amine oxidases belongs to the larger class of so-called quinoproteins class, which are present in plants, animals, fungi, yeast, and bacteria. In humans the CuAOs include (1) the intracellular lysyl oxidase (gene symbol LOX), (2) diamine oxidase (also known as AOC1 [from "amine-oxidase, copper-containing] and DAO [gene symbol ABP1, from "amiloride binding protein"]), and (3) the two known so-called semicarbazide-sensitive copper-containing amine oxidases (or SSAOs), which are located in plasma and the plasma membranes of various tissues (gene symbols AOC2 for the retina-specific amine oxidase, and AOC3 for the gene encoding what is probably the originally identified SSAO, which is the same as vascular adhesion protein-1 (VAP-1)) (Largeron, 2011).

Class I amine oxidases differ markedly from class II amine oxidases in their substrate specificity, but there is some overlap. For instance, dopamine and tyramine are good substrates for MAOs, SSAOs, and DAO, whereas benzylamine is a good substrate for both MAOs and the CuAOs (Largeron, 2011). Features of some of these enzymes are discussed in the following section, with examples of reactions catalyzed by MAO, DAO, and PAO, as shown in Fig. 6-32.

Class I Amine Oxidases: MAO-A and MAO-B, PAO, and SMOX Monoamine oxidase (MAO; gene symbols MAOA and MAOB), spermine oxidase (gene symbol SMOX), and polyamine oxidase (PAO; gene symbol PAOX) are involved in the oxidative deamination of primary, secondary, and tertiary amines (Benedetti, 2001; Agostinelli et al., 2004; Edmondson et al., 2004). Substrates

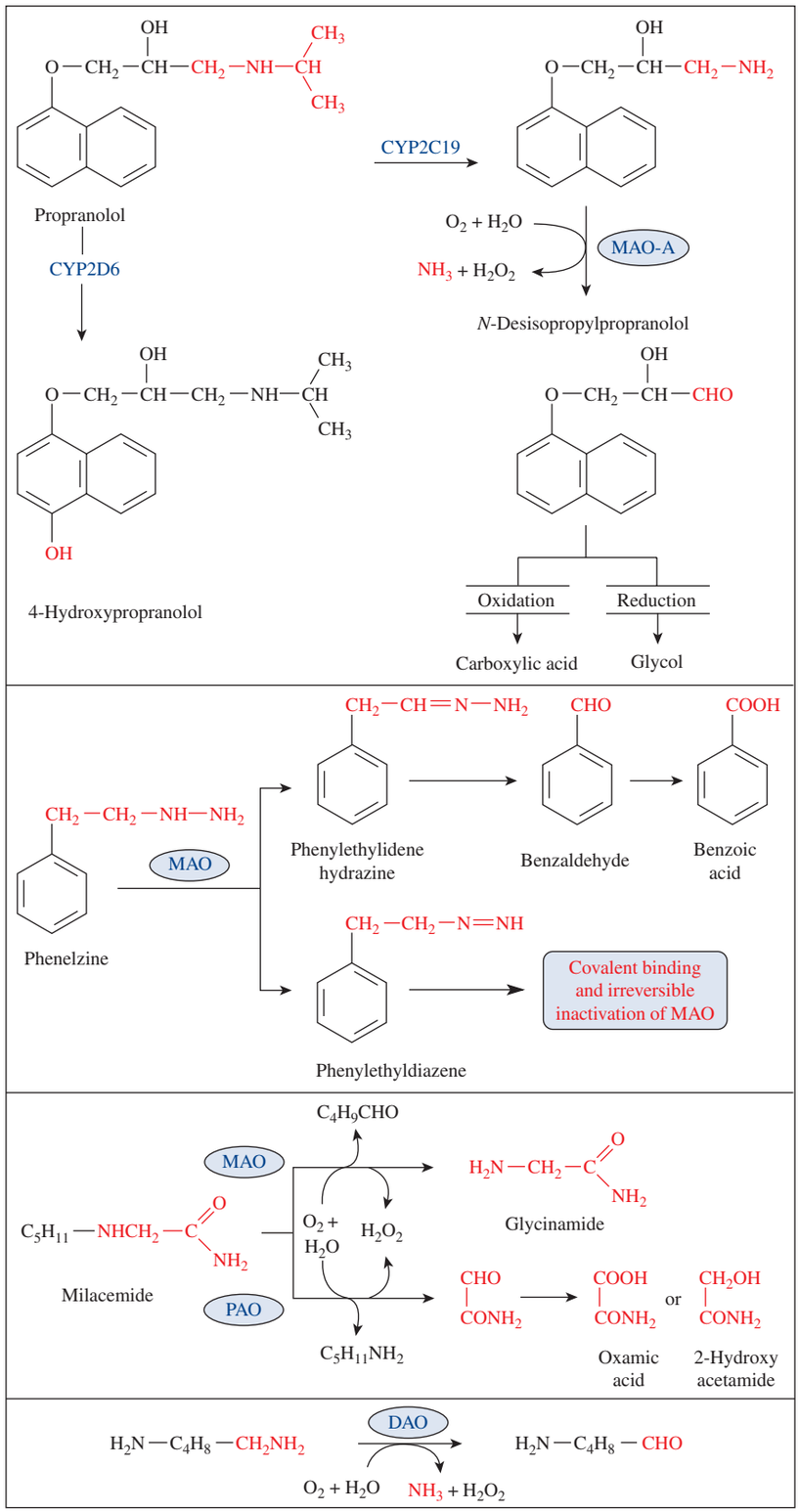


Figure 6-32. *Examples of reactions catalyzed by selected amine oxidases.* Note that phenelzine is a metabolism-dependent (mechanism-based) inhibitor of MAO-A and MAO-B.

for these enzymes include several naturally occurring amines, such as the monoamine serotonin (5-hydroxytryptamine), and monoacetylated derivatives of the polyamines spermine and spermidine.

A number of xenobiotics are substrates for these enzymes, particularly MAOs. Oxidative deamination of a primary amine produces ammonia and an aldehyde, whereas oxidative deamination

of a secondary amine produces a primary amine and an aldehyde. (The products of the former reaction—i.e., an aldehyde and ammonia—are those produced during the reductive biotransformation of certain oximes by aldehyde oxidase, as described in the preceding section "Aldehyde Oxidase".) The aldehydes formed by MAO are usually oxidized further by other enzymes to the corresponding carboxylic acids, although in some cases they are reduced to alcohols.

Monoamine oxidase is located throughout the brain, and is present at high levels in the liver, kidney, intestine, heart, blood platelets, and blood vessels (but absent from erythrocytes) in the outer membrane of mitochondria, although some activity has been found in other cellular compartments, possibly because human liver microsomes prepared from frozen tissue are contaminated with the outer mitochondrial membrane (Pearce et al., 1996a; Testa and Krämer, 2008, 2010). Although MAOs are expressed in most tissues, there are some that express predominantly one form over the other. For example, MAO-A is the predominant form in human placenta, whereas MAO-B is the predominant form in human platelets, lymphocytes, and chromaffin cells (Strolin Benedetti et al., 2007). MAO substrates include milacemide (Fig. 6-32), a dealkylated metabolite of propranolol (Fig. 6-32), primaquine, haloperidol, citalopram, sertraline, doxylamine, MPTP, β-phenylethylamine, tyramine, catecholamines (e.g., dopamine, norepinephrine, and epinephrine), tryptophan derivatives (tryptamine and serotonin), and tryptophan analogs known as triptans, which include the antimigraine drugs almotriptan, sumatriptan, zolmitriptan, and rizatriptan (Strolin Benedetti et al., 2007; Testa and Krämer, 2008, 2010). In the case of the triptans, when a nitrogen exists in either a pyrrolidine or a piperidine ring, MAOs are not involved in their metabolism. Also, as a general rule, the introduction of a methyl group in the α-position relative to the nitrogen atom in what would otherwise be an MAO substrate renders it resistant to oxidative deamination by MAO (Strolin Benedetti et al., 2007).

MAO-A preferentially oxidizes serotonin (5-hydroxytryptamine), norepinephrine, and the dealkylated metabolite of propranolol, and is preferentially inhibited by clorgyline, whereas MAO-B preferentially oxidizes β-phenylethylamine, benzylamine, and citalopram, and is preferentially inhibited by L-deprenyl (selegiline) (Strolin Benedetti et al., 2007). Species differences in the substrate specificity of MAO have been documented. For example, dopamine is oxidized by MAO-B in humans, but by MAO-A in rats, and by both enzymes in several other mammalian species. The distribution of MAO in the brain shows little species variation, with the highest concentration of MAO-A in the locus coeruleus, and the highest concentration of MAO-B in the raphe nuclei. MAO-A is expressed predominantly in catecholaminergic neurons, whereas MAO-B is expressed largely in serotonergic and histaminergic neurons and glial cells. The distribution of MAO throughout the brain does not always parallel that of its substrates. For example, serotonin is preferentially oxidized by MAO-A, but MAO-A is not found in serotonergic neurons.

The mechanism of catalysis by monoamine oxidase is illustrated below:

$$RCH_2NH_2 + FAD \rightarrow RCH{=}NH + FADH_2$$

$$RCH{=}NH + H_2O \rightarrow RCHO + NH_3$$

$$FADH_2 + O_2 \rightarrow FAD + H_2O_2$$

The substrate is oxidized by the enzyme, which itself is reduced (FAD → $FADH_2$). The oxygen incorporated into the substrate is derived from water, not molecular oxygen; hence, the enzyme functions as a true oxidase. The catalytic cycle is completed by reoxidation of the reduced enzyme ($FADH_2$ → FAD) by oxygen, which generates hydrogen peroxide (which may be a cause of oxidative stress). The initial step in the catalytic cycle is abstraction of hydrogen from the α-carbon adjacent to the nitrogen atom, hence the oxidative deamination of xenobiotics by MAO is generally blocked by substitution of the α-carbon. For example, amphetamine and other phenylethylamine derivatives carrying a methyl group on the α-carbon atom are not oxidized well by MAO. (Amphetamines can undergo oxidative deamination, but the reaction is catalyzed by CYP.) The abstraction of hydrogen from the α-carbon adjacent to the nitrogen atom can occur stereospecifically; therefore, only one enantiomer of an α-substituted compound may be oxidized by MAO. For example, while MAO-B catalyzes the oxidative deamination of both *R*- and *S*-β-phenylethylamine, only the *R*-enantiomer is a substrate for MAO-A. The oxidative deamination of the dealkylated metabolite of propranolol is catalyzed stereoselectively by MAO-A, although in this case the preferred substrate is the *S*-enantiomer (which has the same absolute configuration as the *R*-enantiomer of β-phenylethylamine) (Benedetti and Dostert, 1994).

Clorgyline and L-deprenyl (selegiline) are metabolism-dependent inhibitors (i.e., mechanism-based or suicide inactivators) of MAO-A and MAO-B, respectively. Both enzymes are irreversibly inhibited by phenelzine, a hydrazine that can be oxidized either by abstraction of hydrogen from the α-carbon atom, which leads to oxidative deamination with formation of benzaldehyde and benzoic acid, or by abstraction of hydrogen from the terminal nitrogen atom, which leads to formation of phenylethyldiazene and covalent modification of the enzyme, as shown in Fig. 6-32.

Monoamine oxidase has received considerable attention for its role in the activation of MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine) to a neurotoxin that causes symptoms characteristic of Parkinson disease in humans and monkeys but not rodents (Gerlach et al., 1991). In 1983, Parkinsonism was observed in young individuals who, in attempting to synthesize and use a narcotic drug related to meperidine (Demerol®), instead synthesized and self-administered MPTP, which causes selective destruction of dopaminergic neurons in the substantia nigra. MPTP crosses the blood–brain barrier, where it is oxidized by MAO in the astrocytes (a type of glial cell) to 1-methyl-4-phenyl-2,3-dihydropyridine ($MPDP^+$), which in turn autoxidizes to the neurotoxic metabolite, 1-methyl-4-phenylpyridine MPP^+, as shown in Fig. 6-33. Because it is transported by the dopamine transporter, MPP^+ concentrates in dopaminergic neurons, where it impairs mitochondrial respiration. The neurotoxic effects of MPTP can be blocked with pargyline (an inhibitor of both MAO-A and MAO-B), by L-deprenyl (a selective inhibitor of MAO-B) but not by clorgyline (a selective inhibitor of MAO-A). This suggests that the activation of MPTP to its neurotoxic metabolite is catalyzed predominantly by MAO-B. This interpretation is consistent with the finding that MAO-B knockout mice (i.e., transgenic mice that lack MAO-B) do not sustain damage to the dopaminergic terminals of nigrostriatal neurons after MPTP treatment (Shih et al., 1999; Quinn et al., 2007).

Genetic and environmental factors both appear to play important roles in the etiology of Parkinson disease. Apart from MPTP, Parkinsongenic neurotoxins to which humans are exposed have not been identified unequivocally; hence, the environmental factors that cause Parkinson disease remain to be identified. It is interesting that the bipyridyl herbicide, paraquat, is similar in structure to the toxic metabolite of MPTP, as shown in Fig. 6-33. Some epidemiological studies have shown a positive correlation between herbicide exposure and the incidence of Parkinsonism in some but not all rural

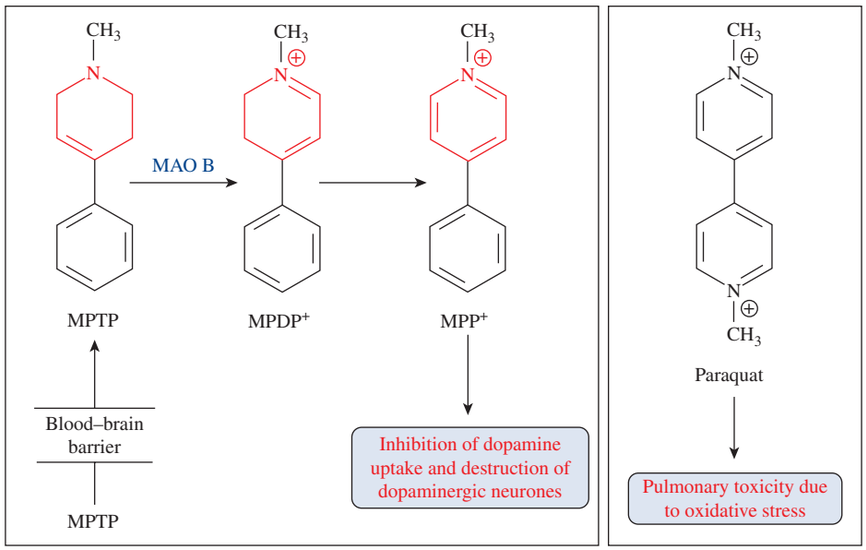


Figure 6-33. *Activation of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) to the neurotoxic metabolite, 1-methyl-4-phenylpyridine (MPP^+), by monoamine oxidase B.* The toxic pyridinium metabolite, MPP^+, is structurally similar to the herbicide paraquat $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridine.

communities. Haloperidol can also be converted to a potentially neurotoxic pyridinium metabolite (Subramanyam et al., 1991).

MAO-B may be among the genetic factors that affect susceptibility to Parkinson disease. MAO-B activity in the human brain increases with aging, perhaps due to a proliferation of glial cells. It has been proposed that increased oxidation of dopamine by MAO-B in the elderly may lead to a loss of dopaminergic neurons in the substantia nigra, which underlies Parkinson disease. Such damage may be caused by the oxidative stress associated with the oxidative deamination of dopamine by MAO-B. In support of this proposal, patients with Parkinson disease have elevated MAO-B activity in the substantia nigra, and the MAO-B inhibitors L-deprenyl (selegiline), lazabemide, and rasagiline provide some symptomatic relief and can delay the progression of symptoms and need for levodopa (Sano et al., 1997; Lohle and Reichmann, 2011). Furthermore, there are allelic variants of MAO-B, some of which (such as alleles 1, A, B4, and G) appear to be associated with an increased risk of developing Parkinson disease, especially in women (Shih et al., 1999; Kang et al., 2006). No such association has been found between Parkinson disease and MAO-A gene polymorphisms (Williams-Gray et al., 2009). Cigarette smoking, which carries a number of health risks, nevertheless provides some protection against Parkinson disease (Gorell et al., 1999; Gu et al., 2010). Although the mechanism of protection remains to be determined, it is interesting to note that cigarette smokers are known to have decreased levels of MAO-B (and MAO-A) (Shih et al., 1999), the degree of which is proportional to cigarette usage (i.e., it is dose related) (Whitfield et al., 2000).

MAO-A knockout mice have elevated brain levels of serotonin and a distinct behavioral syndrome, including enhanced aggression in adult males. The enhanced aggressive behavior exhibited by MAO-A knockout mice is consistent with the abnormal aggressive behavior in individuals who lack MAO-A activity due to a point mutation in the MAO-A gene (Shih et al., 1999). Other polymorphisms in the MAO-A gene appear to be risk factors for alcoholism among Euro-Americans and Han Chinese (Shih et al., 1999). MAO-B may also be a factor in alcoholism inasmuch as alcoholics (especially male Type 2 alcoholics) tend to have lower MAO activity in platelets, which only contain MAO-B. However, MAO-B activity is not lower in alcoholics when cigarette smoking status is taken into account, which suggests that MAO-B activity tends to be lower in alcoholics because smoking and alcohol dependence are strongly associated with each other (Whitfield et al., 2000).

Although not present in mitochondria, the polyamine oxidase, PAOX (approved name: "polyamine oxidase [exo-N4-amino]"), and SMOX (approved name: "spermine oxidase") resemble MAO in their cofactor requirement and basic mechanism of action. These enzymes use oxygen as an electron acceptor, which results in the production of hydrogen peroxide. However, spermine and spermidine are first acetylated by spermidine/spermine N^1-acetyltransferases (gene symbols SAT1 and SAT2), and subsequently oxidized by PAOX, which produces not only hydrogen peroxide but also 3-acetamidopropanal (Hakkinen et al., 2010). SMOX has very different substrate specificity, preferring nonacetylated polyamines (but not spermidine). The polyamines, spermine, spermidine, and the diamine precursor putrescine (discussed below) are ubiquitous in mammalian cells. The intracellular levels of these amines are strictly controlled by several enzymes (including PAOX and SMOX) as well as various transporters (Hakkinen et al., 2010). Dysregulation of polyamine metabolism is associated with cancer and other diseases. Because of differences in polyamine regulation in various parasites, several xenobiotic polyamine synthesis inhibitors or substrates have been investigated as antiparasitic, chemopreventive, or chemotherapeutic drugs, including *N*-alkylated polyamine analogs such as diethylnorspermine, as well as some *N*-benzyl-substituted polyamines (Hakkinen et al., 2010).

The MAO inhibitor pargyline also inhibits PAOX. The anticonvulsant milacemide is one of the few xenobiotic substrates for PAOX (the structure of which is unrelated to polyamines), although it is also a substrate for MAO (Fig. 6-32) (Strolin Benedetti et al., 1992). By converting milacemide to glycine (via glycinamide), MAO plays an important role in the anticonvulsant therapy with milacemide (Benedetti and Dostert, 1994).

Class II Amine Oxidases (CuAOs): SSAOs, DAO, and LO The term semicarbazide-sensitive amine oxidase (SSAO) is confusing because the term has been previously used to refer only to amine oxidases that are active only toward primary amines, although this has not been proved, and several publications that mention SSAOs

actually describe DAO. There are at least three types of SSAOs (Largeron, 2011). The first (which probably includes the original SSAO) has the recommended name PrAOs, for "primary amine oxidases," and includes gene symbols AOC2 and AOC3, the latter of which is probably the originally identified SSAO. The second semicarbazide-sensitive amine oxidase is diamine oxidase (DAO) represented by AOC1; gene symbol ABP1, for "amiloride-binding protein"). A third type of SSAO is lysyl oxidase (LOX) (Largeron, 2011). All of these enzymes are copper-containing amine oxidases. In general, the SSAOs (i.e., PrAOs, for "primary amine oxidases") do oxidize primary monoamines and have little or no activity toward diamines. DAO (ABP1) oxidizes both diamines such as histamine and some primary amines. Neither DAO nor SSAOs have activity toward secondary and tertiary amines (Largeron, 2011). These primary amine oxidases can be either soluble or membrane associated and are expressed in the liver, lung, lymph nodes, small intestine, and blood plasma (Testa and Krämer, 2008, 2010). The PrAO/SSAO known as AOC3 is a 180-kDa dimeric endothelial transmembrane glycoprotein that mediates leukocyte extravasation, for which reason it is widely known as the "vascular adhesion protein 1" (Kaitaniemi et al., 2009).

SSAOs/PrAOs catalyze fundamentally the same reaction catalyzed by monoamine oxidase (Kaitaniemi et al., 2009):

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + H_2O_2 + NH_3$$

The endogenous roles of the copper-containing amine oxidases (CuAOs) include regulating the levels of endogenous and exogenous primary monoamines and polyamines (e.g., methylamine, putrescine, and cadaverine, as well as specific lysine residues of extracellular matrix proteins in the case of LOX) by catalyzing their oxidative deamination, which produces an aldehyde, hydrogen peroxide, and ammonia (Yraola et al., 2009; Largeron, 2011). Because all of the latter chemicals are potentially cytotoxic, these metabolic pathways can activate endo- and xenobiotics. The activity of plasma CuAOs is generally increased in various disease states, including Alzheimer disease, congestive heart failure, cirrhosis, type 1 and 2 diabetes, atherosclerosis, and other inflammatory conditions, which leads to the overproduction of toxic species, especially aldehydes and hydrogen peroxide, and may further contribute to the disease process (Largeron, 2011).

Although CuAOs do not play a large role in xenobiotic oxidation, there is some evidence for the involvement of SSAOs in the oxidative deamination of a few xenobiotics, including allylamine, primaquine, mescaline, amlodipine, and amifostine (Testa and Krämer, 2008, 2010; Largeron, 2011). The calcium channel blocker amlodipine is first oxidized (mainly by CYP3A4) to dehydroamlodipine, with subsequent deamination to an aldehyde (consistent with the action of an SSAO) and finally oxidation to the corresponding carboxylic acid by an ALDH and/or aldehyde oxidase (Largeron, 2011). Amifostine is a thiophosphate prodrug used as a cytoprotective adjuvant used in cancer chemo- and radiotherapy; it is rapidly converted to 2-[3-aminopropylamino] ethanethiol by alkaline phosphatase followed by oxidative deamination catalyzed by one or more CuAOs to an aldehyde that spontaneously decomposes to acrolein and cysteamine (Largeron, 2011). Benzylamine (and several analogs) may be used in vitro as a substrate for SSAOs (Yraola et al., 2009). MAO-B can also catalyze the deamination of benzylamine, but MAO-B acts by removal of the *pro-R* H-atom, whereas SSAOs act by removal of the *pro-S* H-atom (Testa and Krämer, 2008, 2010). There also appear to be some substrate selectivity between the individual SSAOs in that the preferred in vitro substrates of AOC2 (highly expressed in the retina) include 2-phenylethylamine, tryptamine, and tyramine, rather than the benzylamines and methylamine, which appear to be the preferred substrates for AOC3 (Kaitaniemi et al., 2009).

Diamine oxidase (DAO) is a cytosolic, copper-containing, pyridoxal phosphate-dependent enzyme that is highly expressed in the lung, liver, kidney, intestine, and placenta, among other tissues, for which two crystal structures have been described (McGrath et al., 2010). It is located in the extracellular space (secreted form) or in the endoplasmic reticulum (Testa and Krämer, 2008, 2010). Its preferred substrates include histamine and simple alkyl diamines with a four- or five-carbon chain length such as putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) (McGrath et al., 2010). Diamines with carbon chains longer than nine are not substrates for DAO, although they can be oxidized by MAO. DAO or a similar enzyme is present in cardiovascular tissue and appears to be responsible for the cardiotoxic effects of allylamine, which is converted by oxidative deamination to acrolein. Although histamine is a substrate for DAO (which catalyzes oxidative deamination of the primary amine to imidazole acetaldehyde by the secreted diamine oxidase) (McGrath et al., 2010), there is little DAO in brain (nor is there a receptor-mediated uptake system for histamine, in contrast to other neurotransmitters). For this reason, the major pathway of histamine metabolism in the brain is by methylation (see the section "Methylation").

Aromatization The conversion of MPTP to MPP^+ (Fig. 6-33) is an example of a xenobiotic whose oxidation involves the introduction of multiple double bonds to achieve some semblance of aromaticity (in this case, formation of a pyridinium ion). Aromatization of xenobiotics is an unusual reaction, but some examples have been documented. A mitochondrial enzyme in the guinea pig and rabbit liver can oxidize several cyclohexane derivatives to the corresponding aromatic hydrocarbon, as shown in Fig. 6-34, for the aromatization of cyclohexane carboxylic acid (hexahydrobenzoic acid) to benzoic acid. Mitochondria from rat liver are less active, and those from cat, mouse, dog, monkey, and human are inactive (Mitoma et al., 1958). The reaction requires magnesium, coenzyme

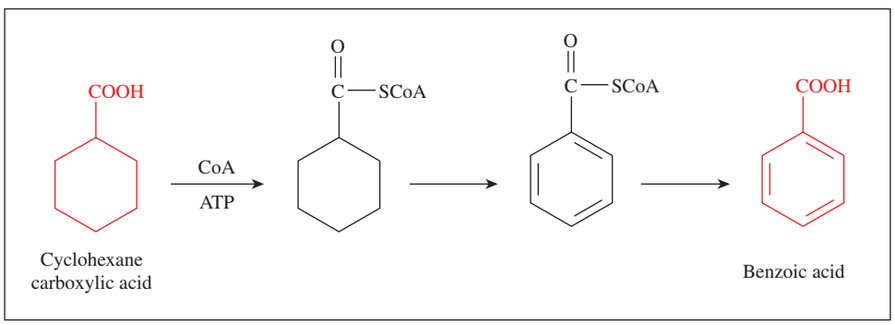


Figure 6-34. *Aromatization of cyclohexane carboxylic acid, a reaction catalyzed by rabbit and guinea pig liver mitochondria.*

A, oxygen, and ATP. The first step is the formation of hexahydrobenzoyl-CoA, which is then dehydrogenated to the aromatic product. Glycine stimulates the reaction, probably by removing benzoic acid through conjugation to form hippuric acid. The conversion of androgens to estrogens involves aromatization of the A-ring of the steroid nucleus. This reaction is catalyzed by CYP19A1, one of the CYP enzymes involved in steroidogenesis. The major substrate of CYP19A1 is androstenedione, which is converted to estrone by three successive oxidation steps (Kuhl and Wiegratz, 2007). Gut microbiota can catalyze aromatization reactions, as shown in Fig. 6-4, for the conversion of quinic acid to benzoic acid.

Evidence for the apparent aromatization of a few xenobiotics by other CYP enzymes has also been described. For instance, 19-nortestosterone derivatives can be converted to aromatic metabolites, namely, ethinylestradiol (from norethisterone and norethynodrel) and 7α-methylethinylestradiol (from tibolone) (Kuhl and Wiegratz, 2007). Formation of these A-ring aromatized steroids occurs in the liver, which does not express the steroidogenic enzyme CYP19A1 (aromatase). In addition, indoline (and some derivatives) is aromatized to indole in human liver microsomes, and mainly by recombinant human CYP3A4 (as well as CYP1A2, CYP2B6, CYP2C19, CYP2D6, and CYP2E1) by a dehydrogenation pathway (Sun et al., 2007). The aromatization mechanism involves two one-electron oxidations, which is different from that catalyzed by CYP19A1, which catalyzes two sequential carbon oxidations followed by cleavage of a carbon–carbon bond (Sun et al., 2007). The indoline-containing diuretic, indapamide, is also dehydrogenated to the aromatic indole by CYP3A4 (Sun et al., 2009). This type of dehydrogenation can lead to P450 inactivation, as exemplified by the mechanism-based inhibition of CYP3A4 by the 3-alkylindole-containing TNF-α inhibitor, SPD-304, which forms an electrophilic 3-methylenindolenine (Sun and Yost, 2008).

Peroxidase-Dependent Cooxidation The oxidative biotransformation of xenobiotics generally requires the reduced pyridine nucleotide cofactors NADPH and NADH, although oxidation by AO and MAO is a notable exception. Another exception is xenobiotic biotransformation by peroxidases, which are heme-containing enzymes that couple the reduction of hydrogen peroxide (or a lipid hydroperoxide) to the one-electron oxidation of other substrates (O'Brien, 2000; Tafazoli and O'Brien, 2005). Several different peroxidases catalyze the biotransformation of xenobiotics (in addition to performing important physiological functions), and these enzymes occur in a variety of tissues and cell types. Peroxidases do not play an important role in the first-pass metabolism or clearance of drugs as most other xenobiotics because their contribution is usually negligible compared with CYP and other oxidative enzymes. However, peroxidases do play an important role in xenobiotic toxicity, especially the activation of drugs associated with idiosyncratic hepatotoxicity, blood dyscrasias (e.g., agranulocytosis, neutropenia, aplastic anemia, and thrombocytopenia), and skin rashes, and the activation of xenobiotics (including the activation of proximate carcinogens to ultimate carcinogens) in skin, bladder, bone marrow, and various other extrahepatic tissues. As shown in Fig. 6-5, peroxidases form reactive intermediates by converting nitrogen- and sulfur-containing xenobiotics to heteroatom-centered radicals (Tang and Lu, 2010; Walsh and Miwa, 2011).

In humans, the family of peroxidases includes myeloperoxidase (gene symbol MPO), eosinophil peroxidase (EPO; gene symbol EPX), lactoperoxidase (gene symbol LPO), thyroid peroxidase (gene symbol TPO), catalase, two forms of prostaglandin H synthase (also known as PHS1 and PHS2 or cyclooxygenase-1 [COX-1] and COX-2; gene symbols PTGS1 and PTGS2), as well as the selenium-containing glutathione peroxidases (gene symbol GPX1-8). Some basic features of these peroxidases are summarized in Table 6-12. These peroxidases all have physiological functions: MPO, EPO, and LPO are lysosomal enzymes present in neutrophils, eosinophils, and secretory cells of exocrine glands, respectively. During infection, MPO and EPO are released into phagocytic vacuoles (granules) and into the plasma, whereas LPO is released into saliva and tears, where they kill microorganisms and thereby provide protection against infectious agents such as bacteria and parasites. (Peroxidases are also present in milk: LPO is the predominant peroxidase in cow's milk, whereas MPO is the predominant peroxidase in human milk.) The hydrogen peroxide required by MPO and EPO is produced by a membrane-bound NADPH oxidase that is activated by the presence of infectious agents. Unlike MPO, EPO, and LPO, which are soluble enzymes, TPO is a membrane-bound peroxidase located on the apical membrane of thyroid follicular cells; it catalyzes the iodination of tyrosine residues and the oxidative coupling of di-iodinated and monoiodinated tyrosine residues in thyroglobulin to form T3- and thyroxine (T4)-bound thyroglobulin, from which thyroid hormones are released. An NAD(P)H-oxidase known as p138 TOX (gene symbol DUOX2), which is also localized on the apical plasma membrane, produces the hydrogen peroxide required by TPO to synthesize thyroid hormones. PHS1 and PHS2 are the peroxidase component of COX-1 and COX-2; they are enzymes that convert arachidonic acid (and closely related fatty acids) to a variety of eicosanoids (prostaglandins, leukotrienes, thromboxane, and prostacyclin). In contrast to the other peroxidases, PHS1 and PHS2 do not require a source of hydrogen peroxide (although they can use it); hence,

Table 6-12

Characteristics of Some Human Peroxidases

PEROXIDASES	CELLULAR LOCATION	SUBCELLULAR LOCATION	HOMOLOGY WITH MPO
Myeloperoxidase (MPO)	Neutrophils, leukocytes	Lysosomes (human milk)	100%
Eosinophil peroxidase (EPO)	Eosinophils	Lysosomes	70%
Lactoperoxidase (LPO)	Mammary duct epithelial cells	Saliva, tears (bovine milk)	61%
Thyroid peroxidase (TPO)	Thyroid follicular cells	Microsomes, Golgi	47%
Prostaglandin-H synthase			
PHS1 (COX1)*	Numerous extrahepatic tissues	Microsomes	
PHS2 (COX2)*	Inflammatory cells	Microsomes	

**There are two forms of prostaglandin H synthase, PHS1 and PHS2; they are the peroxidase component of the bifunctional enzymes known as COX1 and COX2 (gene symbols PTGS1 and PTGS2), respectively.*

SOURCE: Data adapted from O'Brien (2000).

their activity is not dependent on an H_2O_2-generating NAD(P)H-oxidase. Catalase is also a peroxidase. This peroxisomal enzyme catalyzes the disproportionation of hydrogen peroxide to water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$). At low concentrations of hydrogen peroxide, catalase can catalyze the oxidation of ethanol (see Fig. 6-29) and various other small molecules. GPXs are a family of selenium-containing enzymes that also detoxify hydrogen peroxide (and lipid hydroperoxides) by reducing it to water, which is associated with the formation of oxidized GSH as follows:

$$2GSH + HOOH \rightarrow GS\text{-}SG + 2H_2O$$

In mammalian peroxidases (in contrast to plant peroxidases), the heme prosthetic group is covalently attached to the enzyme. Iron is bound to four pyrrole nitrogen atoms with the nitrogen on the imidazole ring of histidine serving as the usual fifth ligand. The sixth coordination position is vacant so that peroxidases can interact with hydrogen peroxide (or other hydroperoxides), just as the sixth coordinate position of hemoglobin and myoglobin (both of which have low peroxidase activity) is available to bind molecular oxygen (Tafazoli and O'Brien, 2005). The oxidation of a xenobiotic ($X \rightarrow XO$ or $2XH \rightarrow 2X^{\bullet} + H_2O$) by peroxidase involves the conversion of hydrogen peroxide to water or the conversion of a hydroperoxide to the corresponding alcohol ($ROOH \rightarrow ROH$), during which the peroxidase (Fe^{III}) is converted to spectrophotometrically distinct states known as compound I and compound II, shown as follows for the conversion of a xenobiotic phenol (XOH) to the corresponding phenoxyl radical ($XO^{\bullet}$) (Tafazoli and O'Brien, 2005):

$$\text{Peroxidase} + ROOH \rightarrow \text{Compound I} + ROH$$

$$\text{Compound I} + XOH \rightarrow \text{Compound II} + XO^{\bullet} + H^+$$

$$\text{Compound II} + XOH \rightarrow \text{Peroxidase} + XO^{\bullet} + H_2O$$

In this example, the hydroperoxide is converted to the corresponding alcohol ($ROOH \rightarrow ROH$), and the peroxyl oxygen atom is reduced to water (H_2O) by the one-electron oxidation of two molecules of xenobiotic (XOH) to produce two xenobiotic radicals ($XO^{\bullet}$). In some cases, the oxygen from the hydroperoxide is incorporated into the xenobiotic itself ($XH \rightarrow XOH$). Examples of each reaction are given later in this section. The conversion of ROOH to ROH involves the release of an oxygen atom that coordinates with the heme iron (initially in the ferric or Fe^{III} state). This iron-bound oxygen formally contains only six (instead of eight) valence electrons, making it a powerful oxidizing species. One electron is removed from the iron to produce Fe^{IV}=O, and a second electron is removed from the tetrapyrrole ring to produce a π-porphyrin cation radical ($Por^{\bullet+}$), which corresponds to compound I (Fe^{IV}=O–$Por^{\bullet+}$). The transfer of an electron from a xenobiotic ($XOH \rightarrow XO^{\bullet} + H^+$) to the porphyrin cation radical produces compound II (Fe^{IV}=O–Por). In some cases, such as PHS, the electron donated to the initial Fe=O complex comes directly or indirectly from an amino acid rather than the tetrapyrrole ring, and this accounts in part for some of the differences among the various peroxidases (such as the ability of TPO to catalyze the iodination of tyrosine residues and the subsequent formation of thyroid hormones).

Although peroxidases are renowned for catalyzing one-electron oxidation reactions, they can catalyze the two-electron oxidation of iodide (I^-) to hypoiodous acid (HOI) and iodine which, in the case of TPO, is important for the synthesis of thyroid hormones:

$$\text{Peroxidase compound I(O)} + I^- + H^+ \rightarrow HOI$$

$$\text{Peroxidase compound I(O)} + 2 \times I^- + H^+ \rightarrow I_2\,(\text{iodine}) + OH^-$$

MPO, EPO, and LPO can all oxidize the pseudohalide thiocyanate (SCN^-) faster than iodide, which in turn is oxidized faster than bromide, which is oxidized much faster than chloride (Bafort et al., 2014). In fact, MPO is the only peroxidase with appreciable ability to oxidize chloride to hypochlorous acid (HOCl). There is an inverse relationship between the rate of conversion of halides to hypohalous acids and their physiological plasma concentrations: chloride = 100 to 140 mM, bromide = 20 to 100 μM, and iodide = 0.1 to 0.5 μM. Consequently, the relatively low rate of chloride oxidation is offset by the high levels of chloride such that about half (20% to 70%) of the hydrogen peroxide produced by activated neutrophils is converted to HOCl, which reacts with GSH, proteins, thiols, amines, unsaturated fatty acids, and cholesterol, all of which disrupt cell membranes and lead to cell lysis and death (of both infectious organisms and host cells). Formation of HOCl by MPO is important not only from a physiological perspective but also from a drug metabolism and toxicity perspective, as discussed later in this section. The preferred (pseudo) halide substrate for MPO is thiocyanate, which is present in plasma at concentrations ranging from 20 to 120 μM—which is sufficiently high that oxidation of thiocyanate consumes the other half of the hydrogen peroxide produced by activated neutrophils. While MPO oxidizes chloride to HOCl, which is cytotoxic, it oxidizes thiocyanate to thiocyanogen (SCN_2), which rapidly hydrolyzes to hypothiocyanic acid (HOSCN). HOSCN is much less reactive and cytotoxic than HOCl, such that the oxidation of thiocyanate by peroxidases is a mechanism to remove hydrogen peroxide without forming HOCl. This represents an important mechanism of hydrogen peroxide detoxication in saliva, which contains low levels of catalase but high levels (1 to 5 mM) of thiocyanate, and in the stomach, where the levels of thiocyanate in parietal cells are three times greater than plasma levels, which allows gastric peroxidase to inactivate hydrogen peroxide that otherwise stimulates gastric acid secretion by stimulating histamine release from mast cells. Inactivation of gastric peroxidase by aspirin, dexamethasone, indomethacin, or methimazole can result in gastric ulceration due to impaired detoxication of hydrogen peroxide and impaired synthesis of cytoprotective prostaglandins (O'Brien, 2000).

MPO and EPO can be distinguished by (1) their cellular distribution (neutrophils/leukocytes vs. eosinophils); (2) their ability to oxidize halides (MPO is better than EPO at converting chloride to HOCl, whereas EPO is better than MPO at converting bromide to hypobromous acid); and (3) their sensitivity to inhibitors (MPO is more sensitive to the inhibitory effect of cyanide over azide, whereas the opposite is true of EPO).

As noted earlier in this section, peroxidases convert chloride to HOCl, which is cytotoxic, and they convert thiocyanate to thiocyanogen (SCN_2), which rapidly hydrolyzes to hypothiocyanic acid (HOSCN), which is much less reactive and cytotoxic than HOCl. However, EPO can oxidize thiocyanate to a mixture of cyanate (OCN^-) and hypothiocyanous acid (HOSCN). Cyanate is a minor product but it can react with lysine residues, which results in the formation of carbamylated proteins ($\text{Protein–}NH_2 + OCN^- + H^+$) $\rightarrow \text{Protein–NH–CO–}NH_2$). EPO-mediated carbamylation of proteins occurs during allergen-induced asthma and can modulate the immune response and trigger a cascade of inflammatory signals associated with asthma (Wang et al., 2016b).

The level of EPO (or a related peroxidase) in uterine epithelial cells is regulated by estrogen; uterine peroxidase activity is inducible several hundred-fold by estrogenic steroids, including the synthetic estrogens diethylstilbestrol and tamoxifen. The level of TPO in thyroid follicular cells is regulated by TSH. The enzyme is inactivated by a variety of ethylenethiourea drugs, such as propylthiouracil and methimazole (which is used as an antithyroid drug in patients with Graves disease), as well as a number of naturally

occurring flavonoid/resorcinol compounds that also have antithyroid effects. By inactivating TPO and impairing thyroid hormone synthesis, these chemicals trigger a large and prolonged increase in TSH that in rodents (but apparently not in humans) can result in thyroid follicular cell tumor formation.

MPO is an abundant enzyme in granulocytes, where it accounts for 5% of the dry weight. It can be used as a diagnostic marker to differentiate myeloid leukemia from lymphoid leukemia. MPO is one of the peroxidases implicated in the formation of reactive metabolites of drugs that cause idiosyncratic agranulocytosis (or other blood dyscrasias), including clozapine, aminopyrine, vesnarinone, propylthiouracil, dapsone, remoxipride, sulfonamides, procainamide, amodiaquine, and ticlopidine (Liu and Uetrecht, 2000; O'Brien, 2000; Tafazoli and O'Brien, 2005; Testa and Krämer, 2008, 2010). The nitro-reduced metabolite of tolcapone can be converted to a reactive *ortho*-quinoneimine by MPO and potentially lead to hepatotoxicity (Testa and Krämer, 2008, 2010). Many of these drugs are aromatic amines, and both MPO and PHS have also been implicated in the activation of several carcinogenic aromatic amines, such as benzidine, methylaminoazobenzene, and aminofluorene. Furthermore, inactivating polymorphisms in MPO (such as MPO-463A) appear to afford protection against both the activation of aromatic amines and PAH in tobacco smoke and the tumor-promoting effects of hydrogen peroxide formed by tobacco smoke–activated neutrophils (O'Brien, 2000).

In the presence of hydrogen peroxide and chloride (which are converted to HOCl by MPO), activated neutrophils and monocytes (including Kupffer cells) oxidize the aforementioned drugs to reactive intermediates (such as nitrogen-centered radicals, hydroxylamines, *N*-chloramines, and, in the case of aromatic amines with a hydroxyl group in the *para*-position, quinoneimines or the corresponding semiquinoneimine radicals). In the case of ticlopidine, MPO converts the thiophene ring of this antiplatelet drug to a thiophene-*S*-chloride, a reactive metabolite that rearranges to 2-chloroticlopidine (minor) and dehydro-ticlopidine (major), or reacts with GSH, as shown in Fig. 6-35. When catalyzed by activated

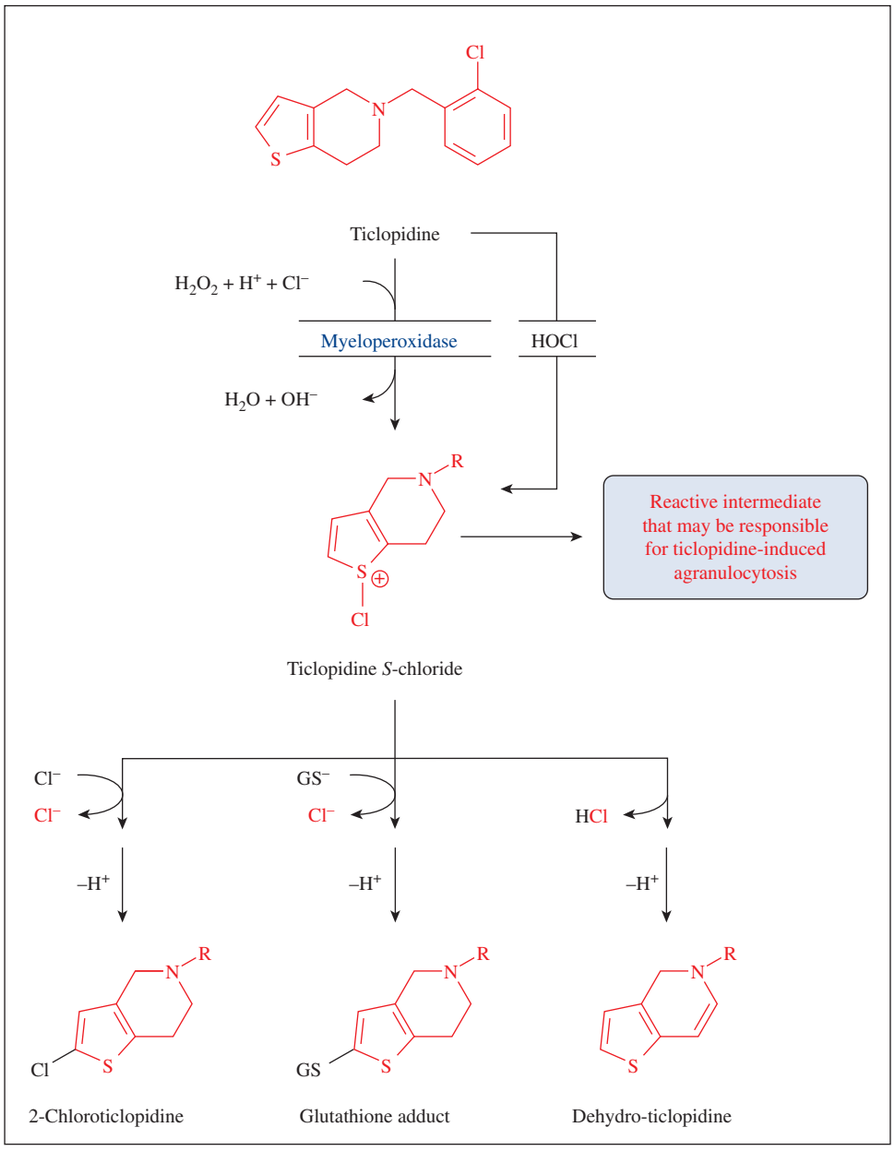


Figure 6-35. *Activation of ticlopidine to a reactive thiophene S-chloride by myeloperoxidase.*

neutrophils, ticlopidine oxidation is inhibited by low concentrations of azide and catalase. When catalyzed by purified myeloperoxidase, ticlopidine oxidation requires hydrogen peroxide and chloride, although all components of this purified system can be replaced with HOCl. It is not known whether drugs that cause agranulocytosis are activated in the bone marrow by neutrophils or their precursors that contain myeloperoxidase, or are activated in neutrophils in the general circulation. In the latter case, agranulocytosis would presumably involve an immune response triggered by neoantigens formed in neutrophils by the covalent modification of cellular component by one or more of the reactive metabolites formed by MPO. MPO is also present in Kupffer cells, which release the enzyme in the liver upon activation by hydrogen peroxide. It is possible to increase the toxicity of hydralazine (which is associated with the development of hepatitis and centrilobular necrosis) toward cultured hepatocytes by the inclusion of a hydrogen peroxide–generating system (Tafazoli and O'Brien, 2008).

MPO has also been implicated in the formation of pro-oxidant phenoxyl radicals of etoposide, a topoisomerase inhibitor that can cause myelogenous leukemia, and both MPO and PHS have been implicated in the formation of phenoxyl radicals of benzene, an industrial solvent that causes bone marrow suppression and leukemia. MPO and PHS cannot oxidize benzene itself. Liver CYP converts benzene to phenol, which in turn is oxidized to hydroquinone, which can be converted to DNA-reactive metabolites by MPO in bone marrow leukocytes and by PHS in bone marrow. The myelosuppressive effect of benzene can be blocked by the PHS inhibitor indomethacin, which suggests an important role for PHS-dependent activation in the myelotoxicity of benzene. The formation of phenol and hydroquinone in the liver is also important for myelosuppression by benzene. However, such bone marrow suppression cannot be achieved simply by administering phenol or hydroquinone to mice, although it can be achieved by coadministering hydroquinone with phenol. Phenol stimulates the MPO- and PHS-dependent activation of hydroquinone. Therefore, bone marrow suppression by benzene involves the CYP-dependent oxidation of benzene to phenol and hydroquinone in the liver, followed by the phenol-enhanced, MPO- and PHS-catalyzed peroxidative oxidation of hydroquinone to reactive intermediates that bind to protein and DNA in the bone marrow (Fig. 6-36). It is noteworthy that the CYP enzyme responsible for hydroxylating benzene has been identified as CYP2E1 (see the section "Cytochrome P450"). Although CYP2E1 was first identified in the liver, this same enzyme has been identified in bone marrow where it can presumably convert benzene to phenol and possibly hydroquinone. The importance of CYP2E1 in the metabolic activation of benzene was confirmed by the demonstration that CYP2E1 knockout mice are relatively resistant to the myelosuppressive effects of benzene (Gonzalez, 2003; Gonzalez and Yu, 2006). MPO has also been shown to convert phenol to diols. Benzene-diols are good substrates of EPO, LPO, and MPO and their oxidation by peroxidases leads to oxidative stress (Testa and Krämer, 2008, 2010).

Prostaglandin H synthase (formally known as prostaglandin-endoperoxide synthase 1 and 2, and also widely known as cyclooxygenase 1 and 2; gene symbols PTGS1 and PTGS2 and collectively referred to below as "PHS") is one of the most extensively studied peroxidases involved in xenobiotic biotransformation. As shown in Fig. 6-37, PHS is a dual-function enzyme composed of a *cyclooxygenase*, which converts arachidonic acid to PGG_2, a 15-hydroperoxide-endoperoxide (which involves the addition of two molecules of oxygen to each molecule of arachidonic acid), and a *peroxidase* that converts the 15-hydroperoxide to the corresponding 15-alcohol PGH_2, which can be accompanied by the oxidation of xenobiotics (a "cosubstrate") and formation of reactive oxygen species (Ramkissoon and Wells, 2011). PHS1 is localized in the endoplasmic reticulum and nuclear envelope and is constitutively expressed in a wide variety of tissues that synthesize and release eicosanoids (prostaglandins, leukotrienes, thromboxane, and prostacyclin), which generally bind to G-coupled cell surface receptors and regulate cellular function, largely in a paracrine (local) fashion (Ramkissoon and Wells, 2011). In contrast, PHS2 is expressed in cells that respond to inflammatory cytokines, mitogens, tumor promoters, and AhR agonists (i.e., agents such as TNF-α, LPS, phorbol esters, TPA, and dioxin) such as the macula densa of the kidney, the vas deferens, and certain regions of the brain (Ramkissoon and Wells, 2011). PGH2 produces prostaglandins that activate receptors on the nuclear membrane, and high levels of the enzyme are expressed in colorectal and other tumors, which appear to be important for tumor promotion and angiogenesis (the process of stimulating the blood vessel supply). Both enzymes play an important role in the activation of xenobiotics to toxic or tumorigenic metabolites,

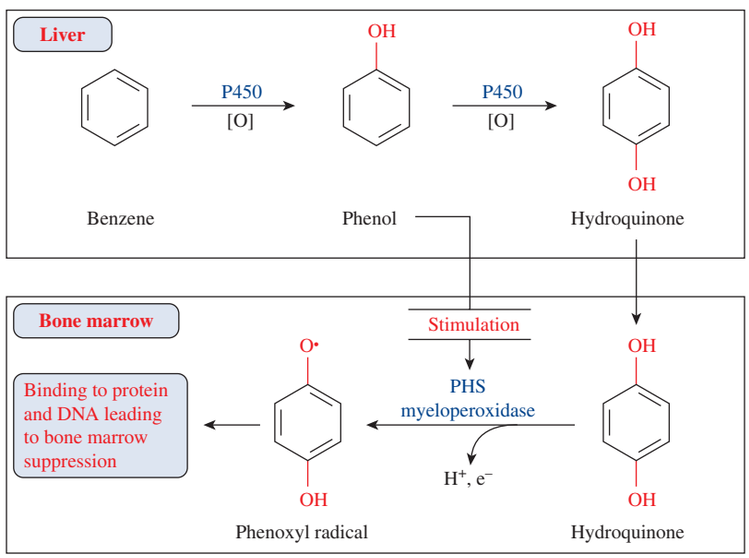


Figure 6-36. *Role of cytochrome P450 and peroxidases in the activation of benzene to myelotoxic metabolites.*

particularly in extrahepatic tissues that contain low levels of CYP. PHS2 (COX-2) also plays an important role in the subsequent response of tissues to cell damage and tumor initiation, and is a possible target for the treatment or prevention of certain types of cancer. Increasing evidence suggests that inhibition of COX-2 by NSAIDs may be part of the mechanism whereby these drugs protect against colon and other gastrointestinal cancers (Dubé et al., 2007; Wu et al., 2010). PHS has been evaluated for its contribution to the metabolism of phenytoin and other antiepileptic drugs B[*a*]P, thalidomide, methamphetamine, and 3,4-methylenedioxymethamphetamine (Ramkissoon and Wells, 2011).

In certain cases, the oxidation of xenobiotics by peroxidases involves direct transfer of the peroxide oxygen to the xenobiotic, as shown in Fig. 6-37 for the conversion of substrate X to product XO. An example of this type of reaction is the PHS-catalyzed epoxidation of B[*a*]P 7,8-dihydrodiol to the corresponding 9,10-epoxide (see Fig. 6-14). Although PHS can catalyze the final step (i.e., 9,10-epoxidation) in the formation of this tumorigenic metabolite of B[*a*]P, it cannot catalyze the initial step (i.e., 7,8-epoxidation), which is catalyzed by CYP. Several lines of evidence suggest that both PHS1 and PHS2 play an important role in PAH-induced skin carcinogenesis. First, both PHS1 knockout and PHS2 knockout mice are resistant to PAH-induced skin cancer. (It is noteworthy that PHS1 knockout mice appear normal despite having only 1% of the prostaglandin levels of wild-type mice, whereas only 60% of PHS2 knockout mice survive until weaning, and the 40% that survive past weaning usually die within a year of kidney disease.) Both resveratrol (an inhibitor of PHS1 and CYP1A1) and SC-58125 (an inhibitor of PHS2) block PAH-induced skin cancer in mice.

PHS can also catalyze the 8,9-epoxidation of aflatoxin B_1, which is one of the most potent hepatotumorigens known. Epoxidation by CYP is thought to be primarily responsible for the hepatotumorigenic effects of aflatoxin B_1. However, aflatoxin B_1 also causes neoplasia of rat renal papilla. This tissue has very low levels of CYP, but contains relatively high levels of PHS, which is suspected, therefore, of mediating the nephrotumorigenic effects of aflatoxin (Fig. 6-38).

The direct transfer of the peroxide oxygen from a hydroperoxide to a xenobiotic is neither the only mechanism of xenobiotic oxidation by peroxidases nor the most common. Xenobiotics that

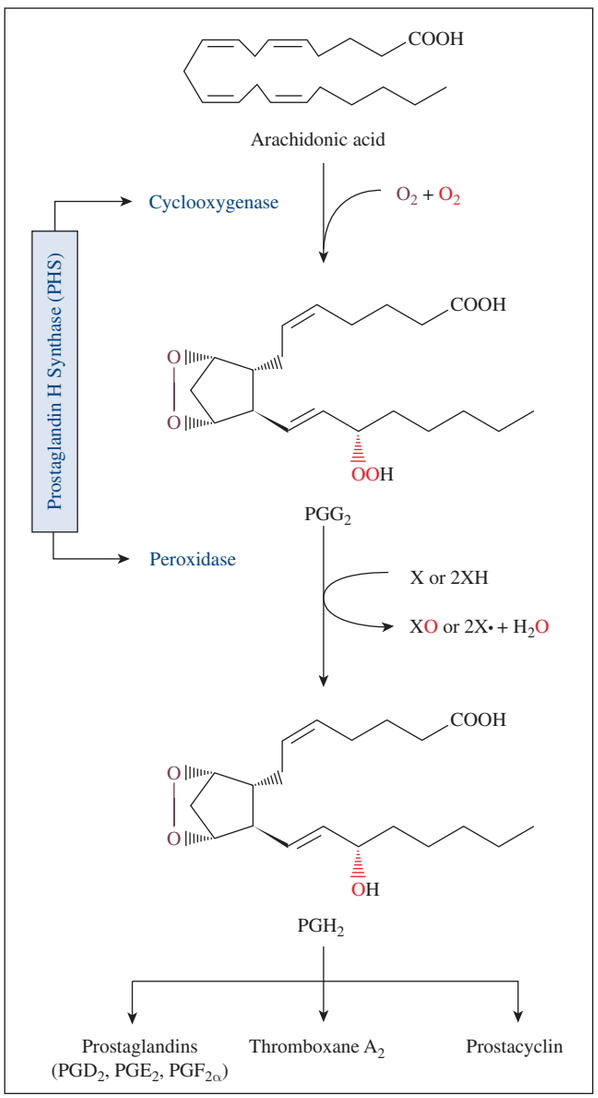


Figure 6-37. *Cooxidation of xenobiotics (X) during the conversion of arachidonic acid to PGH2 by prostaglandin H synthase.*

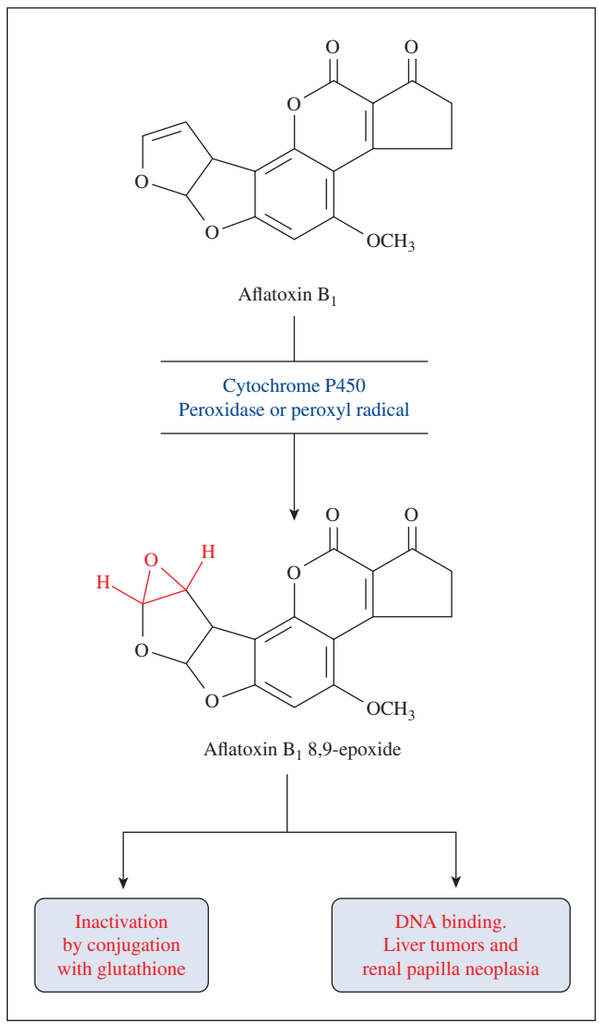


Figure 6-38. *Activation of aflatoxin B1 by cytochrome P450, leading to liver tumor formation, and by peroxidases, leading to renal papilla neoplasia.*

can serve as electron donors, such as amines and phenols, can be oxidized to free radicals during the reduction of a hydroperoxide. In this case, the hydroperoxide is still converted to the corresponding alcohol, but the peroxide oxygen is reduced to water instead of being incorporated into the xenobiotic. For each molecule of hydroperoxide reduced (which is a two-electron process), two molecules of xenobiotic can be oxidized (each by a one-electron process). Important classes of compounds that undergo one-electron oxidation reactions by peroxidase include aromatic amines, phenols, hydroquinones, and polycyclic hydrocarbons. Many of the metabolites produced are reactive electrophiles. For example, PAHs, phenols, and hydroquinones are oxidized to electrophilic quinones. Acetaminophen is similarly converted to a quinoneimine, namely, *N*-acetyl-benzoquinoneimine, a cytotoxic electrophile that binds to cellular proteins, as shown in Fig. 6-39. The formation of this toxic metabolite by CYP causes centrilobular necrosis of the liver. However, acetaminophen can also damage the kidney medulla, which contains low levels of CYP but relatively high levels of PHS; hence, PHS may play a significant role in the nephrotoxicity of acetaminophen. The two-electron oxidation of acetaminophen to *N*-acetyl-benzoquinoneimine by PHS likely involves the formation of a one-electron oxidation product, namely, *N*-acetyl-benzosemiquinoneimine radical. Formation of this semiquinoneimine radical by PHS likely contributes to the nephrotoxicity of acetaminophen and related compounds, such as phenacetin and 4-aminophenol. Metabolites of the NSAID diclofenac (namely, 4′-hydroxydiclofenac and 5-hydroxydiclofenac) can also be converted to benzoquinoneimines by peroxidases (Testa and Krämer, 2008, 2010).

Like the kidney medulla, urinary bladder epithelium also contains low levels of CYP but relatively high levels of PHS. Just as PHS in kidney medulla can ostensibly activate aflatoxin and acetaminophen to nephrotoxic metabolites, PHS in urinary bladder epithelium can potentially activate certain aromatic amines, such as benzidine, 4-aminobiphenyl, and 2-aminonaphthalene, to DNA-reactive metabolites that cause bladder cancer in certain species,

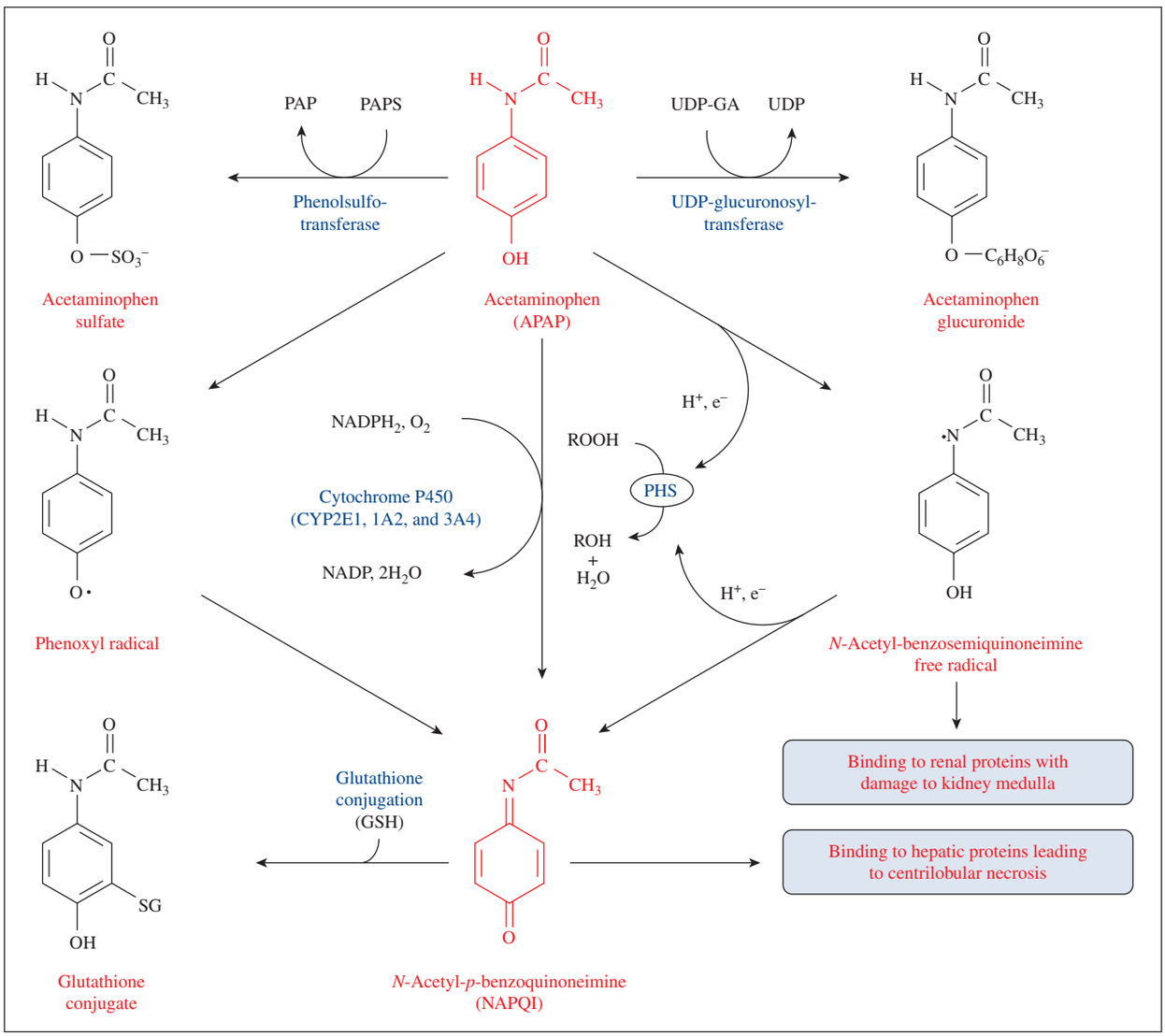


Figure 6-39. *Activation of acetaminophen by cytochrome P450, leading to hepatotoxicity, and by prostaglandin H synthase (PHS), leading to nephrotoxicity.* Conjugation with sulfonate, glucuronic acid, or glutathione represents detoxication reactions.

including humans and dogs. PHS can convert aromatic amines to reactive radicals, which can undergo nitrogen–nitrogen or nitrogen–carbon coupling reactions, or they can undergo a second one-electron oxidation to reactive diimines. Binding of these reactive metabolites to DNA is presumed to be one of the underlying mechanisms by which several aromatic amines cause bladder cancer in humans and dogs. In some cases the one-electron oxidation of an amine leads to *N*-dealkylation. For example, PHS catalyzes the *N*-demethylation of aminopyrine, although in vivo this reaction is mainly catalyzed by CYP. In contrast to CYP, PHS does not appear to catalyze the *N*-hydroxylation of carcinogenic aromatic amines (an important step in their metabolic activation), although MPO and LPO have been shown to catalyze this reaction. In liver, activation of aromatic amines by *N*-hydroxylation is catalyzed predominantly by CYP1A2, whereas this same reaction in the bladder epithelium appears to be catalyzed by another enzyme, possibly a CYP enzyme other than CYP1A2, such as CYP2A13, CYP4B1, or CYP2S1 (Nakajima et al., 2006), or a peroxidase other than PHS.

Many of the aromatic amines known or suspected of causing bladder cancer in humans have been shown to cause bladder tumors in dogs. In rats, however, aromatic amines cause liver tumors by a process that is thought to involve *N*-hydroxylation by CYP, followed by conjugation with acetate or sulfonate, as shown in Fig. 6-16. This species difference has complicated an assessment of the role of PHS in aromatic amine–induced bladder cancer, because such experiments must be carried out in dogs. However, another class of compounds, the 5-nitrofurans, such as *N*-[4-(5-nitro-2-furyl)-2-thiazole]formamide (FANFT) and its deformylated analog 2-amino-4-(5-nitro-2-furyl)thiazole (ANFT), are substrates for PHS and are potent bladder tumorigens in rats. The tumorigenicity of FANFT is thought to involve deformylation to ANFT, which is oxidized to DNA-reactive metabolites by PHS. The ability of FANFT to cause bladder tumors in rats is blocked by the COX inhibitor, aspirin, which suggests that PHS plays an important role in the metabolic activation and tumorigenicity of this nitrofuran. Unexpectedly, combined treatment of rats with FANFT and aspirin causes forestomach tumors, which are not observed when either compound is administered alone.

Increased expression of PHS2 (COX-2) has been documented in a number of tumors, including human colorectal, gastric, esophageal, pulmonary, and pancreatic carcinomas (Gupta and DuBois, 1998; Molina et al., 1999; Dubé et al., 2007; Wu et al., 2010). Aspirin and other NSAIDs block the formation of colon cancer in experimental animals, and there is epidemiological evidence that long-term use of certain NSAIDs (aspirin and sulindac), but not acetaminophen (which does not inhibit COX-2), decreases the incidence of colorectal polyps and cancer in humans, and also decreases the number of deaths from esophageal, gastric, and rectal cancers. The incidence of intestinal neoplasms in $Apc^{\Delta 716}$ knockout mice is dramatically suppressed by crossing these transgenic animals with PHS2 (COX-2) knockout mice (Oshima et al., 1996). From these few examples it is apparent that PHS2 may play at least two distinct roles in tumor formation; it may convert certain xenobiotics to DNA-reactive metabolites (and thereby *initiate* tumor formation), and it may *promote* subsequent tumor growth, perhaps through formation of growth-promoting eicosanoids, such as PGE2, which can activate EGFR, ERKs, etc. (Wu et al., 2010).

Many phenolic compounds can serve as reducing substrates for PHS peroxidase. The phenoxyl radicals produced by one-electron oxidation reactions can undergo a variety of reactions, including binding to critical nucleophiles such as protein and DNA, reduction by antioxidants such as GSH, and self-coupling. The reactions of phenoxyl radicals are analogous to those of the nitrogen-centered free radicals produced during the one-electron oxidation of aromatic amines by PHS.

It was previously mentioned in this section that phenol can enhance the peroxidative metabolism of hydroquinone, which is an important component to benzene myelotoxicity. An analogous interaction has been described between the phenolic antioxidants, BHT, and BHA. In mice, the pulmonary toxicity of BHT, which is a relatively poor substrate for PHS, is enhanced by BHA, which is a relatively good substrate for PHS. The mechanism by which BHA enhances the pulmonary toxicity of BHT involves the peroxidase-dependent conversion of BHA to a phenoxyl radical that interacts with BHT, converting it to a phenoxyl radical (by one-electron oxidation) or a quinone methide (by two-electron oxidation), as shown in Fig. 6-40. Formation of the toxic quinone methide of BHT can also be catalyzed by CYP, which is largely responsible for activating BHT in the absence of BHA. A similar outcome occurs with quercetin, which is oxidized to a quinonemethide in the presence of peroxidases (Testa and Krämer, 2008, 2010).

Several reducing substrates, such as phenylbutazone, retinoic acid, 3-methylindole, sulfite, and bisulfite, are oxidized by PHS to carbon- or sulfur-centered free radicals that can trap oxygen to form a peroxyl radical, as shown in Fig. 6-41 for phenylbutazone. The peroxyl radical can oxidize xenobiotics in a peroxidative manner. For example, the peroxyl radical of phenylbutazone can convert B[*a*]P 7,8-dihydrodiol to the corresponding 9,10-epoxide.

PHS is unique among peroxidases because it can both generate hydroperoxides and catalyze peroxidase-dependent reactions, as shown in Fig. 6-37. Xenobiotic biotransformation by PHS is controlled by the availability of arachidonic acid. The biotransformation of xenobiotics by other peroxidases is controlled by the availability of hydroperoxide substrates. Hydrogen peroxide is a normal product of cellular respiration, and lipid peroxides can form during lipid peroxidation. The level of these peroxides and their availability for peroxidase reactions depend on the efficiency of hydroperoxide scavenging by GPX and catalase.

Flavin Monooxygenases The liver, kidney, intestine, brain, and lung (among other tissues) contain one or more FAD-containing monooxygenases (FMO) that oxidize soft nucleophilic nitrogen, sulfur, selenium, phosphorus, and rarely—iodine, carbon, and boron—atoms of xenobiotics (Ziegler, 1993; Lawton et al., 1994; Cashman, 1995, 1999; Rettie and Fisher, 1999; Cashman and Zhang, 2006; Cashman, 2008; Mitchell, 2008; Testa and Krämer, 2008, 2010). Among these types of substrates, the best ones tend to be those with lone pairs of electrons available to form a polar, coordinate covalent (dative) bond (e.g., hydroxylamines and piperidines) (Cashman, 2008; Testa and Krämer, 2008, 2010). The mammalian FMO gene family comprises five enzymes (designated FMO1 to FMO5) that contain 532 to 558 amino acid residues each and are 48% to 60% identical in amino acid sequence within a species, whereas orthologous forms are 82% to 86% identical across species (Hines et al., 2002). The human enzymes range in apparent molecular weight from 52 to 64 kDa (Testa and Krämer, 2008, 2010). Six human FMO pseudogenes have also been described, including one that shares 71% sequence identity with FMO3, namely, FMO6, for which nine distinct transcripts were identified in the liver, but not the kidney. All of these variant FMO6 transcripts arise by alternative splicing and lead to truncated, and therefore nonfunctional, enzymes. Each FMO enzyme contains a highly conserved glycine-rich region (residues 4 to 32) that binds 1 mol of FAD (noncovalently) near the active site, which is adjacent to a second highly conserved glycine-rich region (residues 186 to 213) that binds NADPH (or NADH). Other structural motifs have been reviewed in detail by Ziegler (2002).

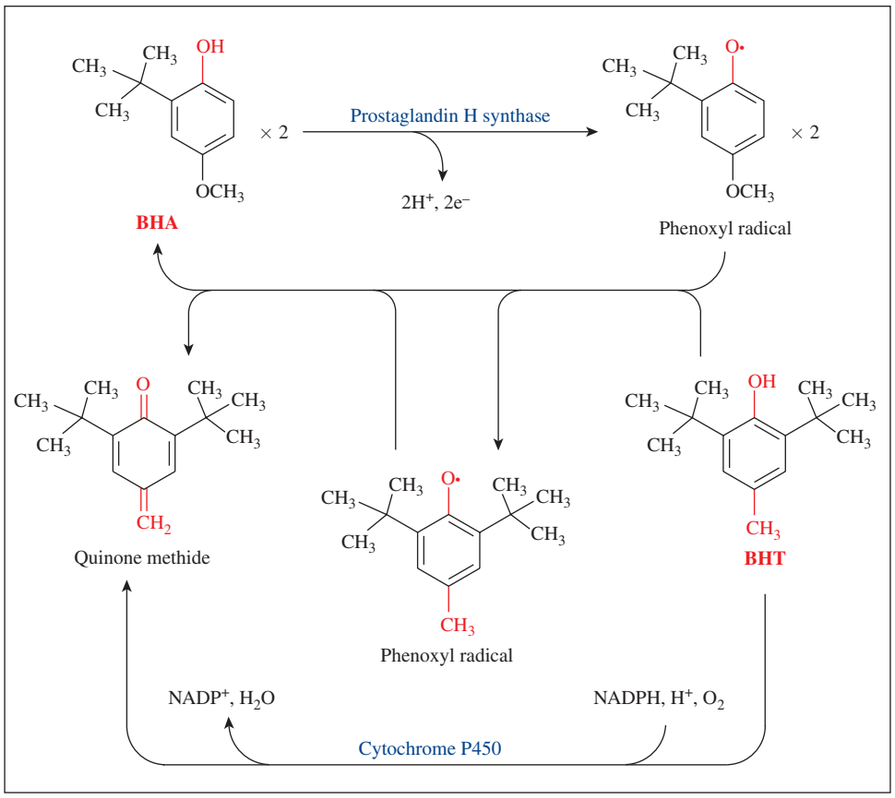


Figure 6-40. *Metabolite interaction between the phenolic antioxidants, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA).* Note that activation of BHT to a toxic quinone methide can be catalyzed by cytochrome P450 or, in the presence of BHA, by prostaglandin H synthase (PHS).

Like CYP, the FMOs are microsomal enzymes that require O_2 and NADPH (or NADH in some cases) (Lai et al., 2011), and many of the reactions catalyzed by FMO also can be catalyzed by CYP. However, while CYP catalyzes two sequential one-electron oxidations, FMO catalyzes a single two-electron oxidation (Cashman, 2008). In contrast to CYP enzymes, FMOs are not readily induced or inhibited in humans, such that metabolism of a drug by FMO tends to decrease its victim potential (i.e., its potential to be the object of drug–drug interactions) (Cashman, 2008; Phillips and Shephard, 2017). However, basal expression levels appear to vary as much as 20-fold between individuals, with the differences appearing to be caused by mutations in the promoter regions of at least FMO1 and FMO3 (Testa and Krämer, 2008, 2010). Consequently, the disposition of a drug primarily metabolized by FMO would likely show considerable interindividual variation and, for reasons outlined later in this section, infants and young children would likely be PMs.

Several in vitro techniques have been developed to distinguish reactions catalyzed by FMO from those catalyzed by CYP. In general, and in contrast to CYP, FMO is heat labile and can be inactivated in the absence of NADPH by warming microsomes to 50°C for one minute (Ogilvie et al., 2008). However, even the often-used procedure of preincubating human liver microsomes at 37°C prior to addition of NADPH can cause significant inactivation of FMO, possibly leading to an underestimation of the role of FMO in the metabolism of xenobiotics (Cashman, 2008). By comparison, CYP can be inactivated with nonionic detergent, such as 1% Emulgen 911, which has a minimal effect on FMO activity. The pH optimum for FMO-catalyzed reactions (pH 8 to 10) tends to be higher than that for most (but not all) CYP reactions (pH 7 to 8). Based on the latter approach, a higher rate of formation of *N*-oxides from tertiary amines in microsomal incubations at pH 10 relative to pH 7.4 provides strong evidence for the role of FMO in *N*-oxide formation (Cashman, 2008). Antibodies raised against purified CYP enzymes can be used not only to establish the role of CYP in a microsomal reaction but also to identify which particular CYP enzyme catalyzes the reaction. In contrast, antibodies raised against purified FMO do not inhibit the enzyme. The use of chemical inhibitors to ascertain the relative contribution of FMO and CYP to microsomal reactions is often complicated by a lack of specificity. For example, both cimetidine and SKF 525A, which are well-recognized CYP inhibitors, are substrates for FMO. Conversely, the FMO inhibitor methimazole is known to cause direct inhibition of CYP2B6 and CYP2C9, and irreversible inhibition (MDI) of CYP3A4 (Parkinson et al., 2011). Excellent FMO substrates, such as the strong nucleophile mercaptoimidazole, can potentially be used as competitive inhibitors of FMO-mediated *N*-oxidation of tertiary amines (Cashman, 2008). Zwitterions, multivalent cations such as cadaverine (a diamine), are generally not FMO substrates (Krueger et al., 2006). The situation is further complicated by the observation that the various forms of FMO differ in their thermal stability and sensitivity to detergents and other chemical modulators (examples of which are described later in this section).

FMO catalyzes the oxidation of nucleophilic tertiary amines to *N*-oxides, secondary amines to hydroxylamines and nitrones, and primary amines to hydroxylamines and oximes. Amphetamine, benzydamine, chlorpromazine, clozapine, guanethidine, imipramine, methamphetamine, olanzapine, and tamoxifen are examples of nitrogen-containing drugs that are *N*-oxygenated by FMO (and by CYP in most cases). Only sp^3-hybridized electron-rich (nucleophilic)

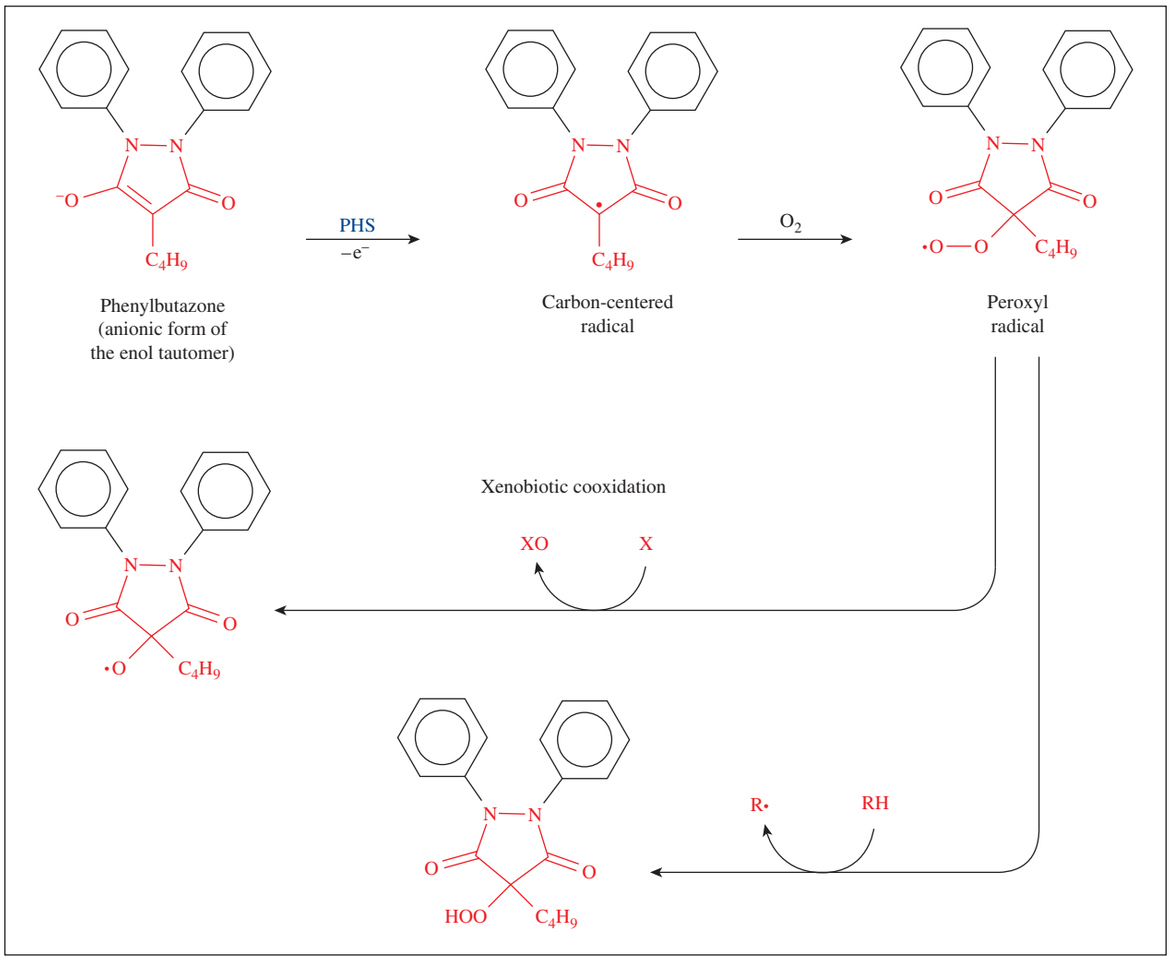


Figure 6-41. *Oxidation of phenylbutazone by prostaglandin H synthase (PHS) to a carbon-centered radical and peroxyl radical.* Note that the peroxyl radical can oxidize xenobiotics (X) in a peroxidative manner.

nitrogen atoms—those with pK_a(B) values 5 to 10—are substrates for *N*-oxygenation by FMO, hence the preference of FMO for tertiary and cyclic amines. Therefore, in general, FMO *N*-oxygenates tertiary aliphatic amines and aliphatic cyclic amines (CYP can also), but FMO does not usually *N*-oxygenate aromatic amines (pyridines and anilines such as clozapine) (Testa and Krämer, 2008, 2010). Heteroaromatics (with an sp^2-hybridized nitrogen) and amides (which have low basicity [low pK_a(B)]) are not *N*-oxygenated by FMO but they may be *N*-oxygenated by CYP. Thus, CYP can *N*-oxygenate both tertiary/cyclic amines and pyridines. In the case of anilines (arylamines), however, CYP (as will peroxidases, but rarely FMO) will *N*-hydroxylate them (Testa and Krämer, 2008, 2010). As shown in Fig. 6-42, the *N*-oxygenation of primary and secondary aliphatic amines by FMO is more complex. For instance, the aliphatic *secondary* amine methamphetamine ($R-NH-CH_3$) is *N*-oxygenated by FMO3 first to a hydroxylamine ($R-NOH-CH_3$) and then to a dihydroxylated intermediate ($R-N^+(OH)_2-CH_3$) that loses water to form a nitrone ($R-N^+O-CH_3$). The resulting nitrone can spontaneously hydrolyze to a ketone (phenylacetone in this case), such that this overall reaction represents an alternative deamination pathway (Testa and Krämer, 2008, 2010). In the case of the aliphatic *primary* amine, amphetamine ($R-CH_2-NH_2$), FMO again catalyzes two sequential *N*-hydroxylation reactions but in this case the *N*-dihydroxylated metabolite $R-CH_2-N(OH)_2$ loses water to form an oxime ($R-CH=NOH$). Primary aliphatic amines tend to be poor substrates for CYP; in fact, primary alkylamines, such as the *N*-demethylated metabolite of fluoxetine, are metabolically stable and potent inhibitors of CYP (discussed later in the section "Inhibition of Cytochrome P450").

FMO also oxygenates several sulfur-containing xenobiotics (such as thiols, thioethers, thiones, and thiocarbamates) and phosphines to *S*- and *P*-oxides, respectively. Cimetidine and sulindac sulfide are examples of sulfur-containing drugs that are converted to sulfoxides by FMO. As in the case of *N*-oxygenation, *S*-oxygenation requires a soft nucleophilic heteroatom, such as that found in thioethers. Both FMO and CYP can convert thioethers to sulfoxides ($R_1-S-R_2 \rightarrow R_1-SO-R_2$) but only CYP can convert sulfoxides to sulfones ($R_1-SO-R_2 \rightarrow R_1-SO_2-R_2$). Electron-withdrawing groups next to the sulfur (as in the case of sulfoxides and many heteroaromatics) decrease the nucleophilicity of the sulfur atom and decrease or abolish its oxygenation by FMO, whereas electronic-donating groups have the opposite effect, which is why thioamides are excellent substrates for *S*-oxygenation by FMO. Fig. 6-19 shows how sulindac, a sulfoxide, is reduced to sulindac sulfide, only to be oxidized by FMO back to the parent drug in what is often called a futile cycle as discussed further below in this section and earlier in the section "Sulfoxide and *N*-Oxide Reduction." Hydrazines, iodides, selenides, and boron-containing compounds

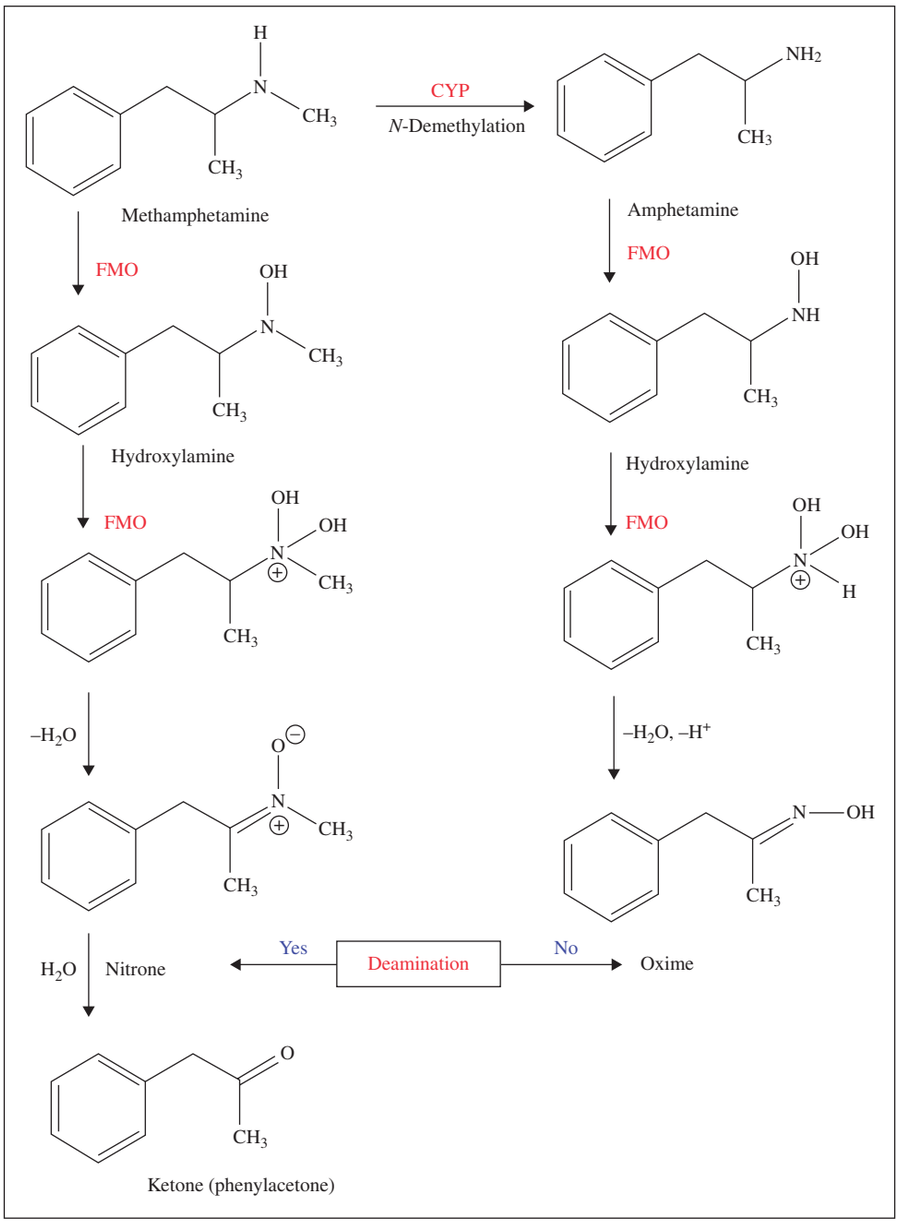


Figure 6-42. *Oxidation of methamphetamine (a secondary amine) and amphetamine (a primary amine) by flavin monooxygenase (FMO).* After an initial *N*-hydroxylation of both compounds by FMO, methamphetamine is further converted to a nitrone that undergoes deamination whereas amphetamine is converted to an oxime that does not undergo deamination.

are also substrates for FMO. Examples of FMO-catalyzed reactions are shown in Fig. 6-43A and B.

In general it is safe to assume that biotransformation reactions involving C-oxidation of a xenobiotic by NADPH-fortified liver microsomes can be ascribed to CYP, but there are a few cases where the reaction is catalyzed by FMO (Fan et al., 2016). For example, FMO1 catalyzes the oxidative defluorination of 4-fluoro-*N*-methylaniline to 4-hydroxy-*N*-methylaniline (i.e., 4-*N*-methylaminophenol) through the intermediacy of a reactive quinoneimine, as shown in Fig. 6-43A (Driscoll et al., 2010; Fan et al., 2016). In the case of 4-fluoro-*N*-methylaniline, this oxidative defluorination reaction is facilitated by delocalization of the lone pair of electrons on the aniline nitrogen. A second example of C-oxidation by FMO involves the ability of the reactive C(4a)-hydroperoxyflavin to function as an "enzymatic peroxide" to catalyze a Baeyer–Villiger oxidation, which involves the insertion of an oxygen atom into a carbon–carbon bond next to a carbonyl group (e.g., aldehyde or ketone) to form an ester (e.g., oxidation of salicylaldehyde by pig FMO1) (Lai et al., 2011). However, this reaction has not been described in humans with the notable exception of FMO5 (and only FMO5), which mediates the Baeyer–Villiger lactonization of ER-879819, the ketone metabolite of the poly(ADP-ribose) polymerase inhibitor E7016 (Lai et al., 2011) and another 4-piperidone-containing compound, namely MRX-1 (Fan et al., 2016). By inserting oxygen next to a carbonyl group FMO forms a lactone that can be hydrolyzed to a ring-open carboxylic acid and alcohol.

In general, the metabolites produced by FMO are the products of a chemical reaction between a xenobiotic and a peracid

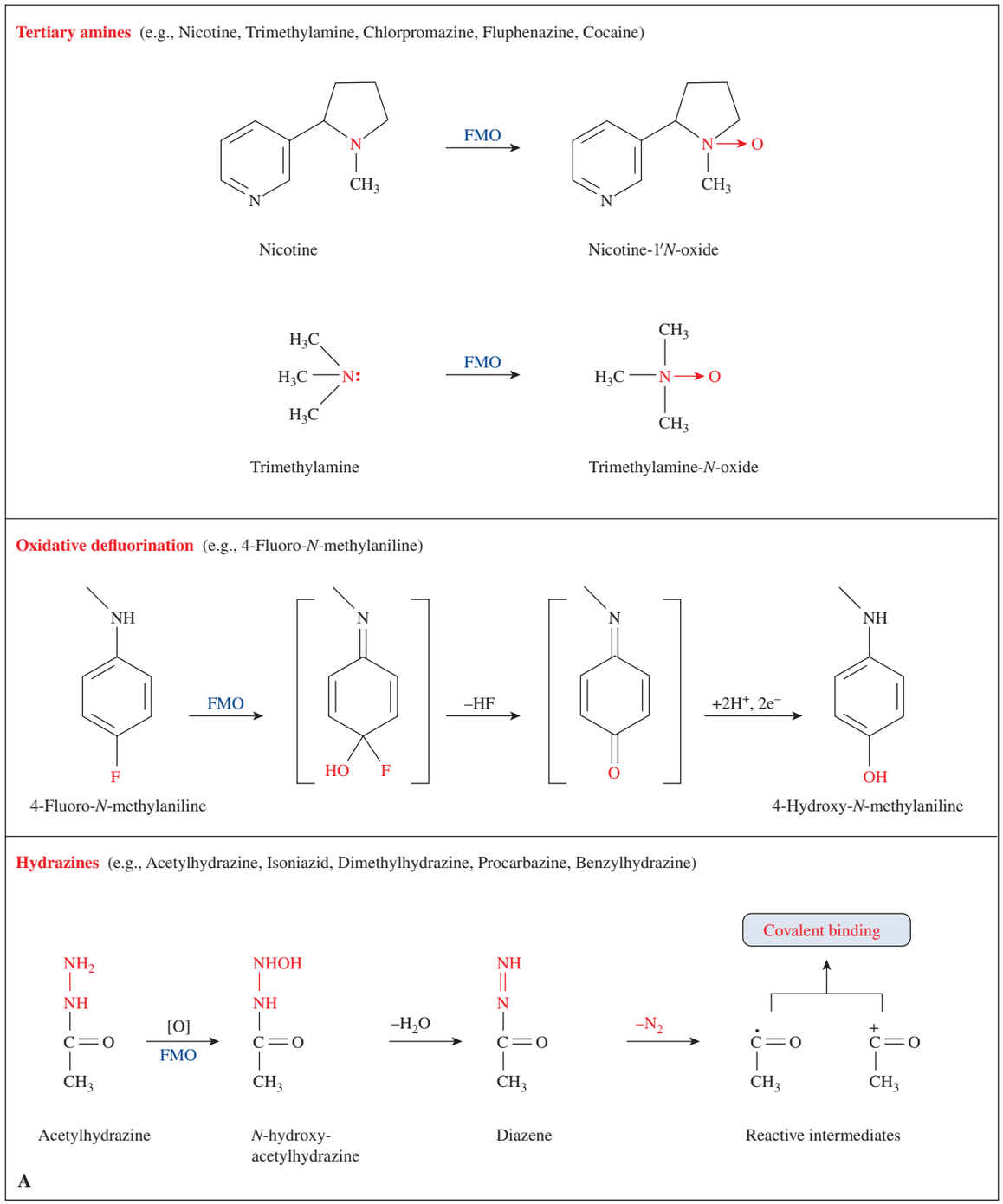


Figure 6-43. *Examples of reactions catalyzed by flavin monooxygenases (FMO).* (**A**) Nitrogen-containing xenobiotics.

or peroxide, which is consistent with the mechanism of catalysis of FMO (discussed later in this section). The reactions catalyzed by FMO are generally detoxication reactions, although there are exceptions to this rule, which are described later in this section. Inasmuch as FMO attacks nucleophilic heteroatoms, it might be assumed that substrates for FMO could be predicted from their pK_a(B) values (i.e., from a measure of their basicity). Although there is some truth to this—for example, xenobiotics containing an sp^3-hybridized nitrogen atom with a pK_a(B) of 5 to 10 are generally good substrates for FMO—predictions of substrate specificity based on pK_a(B) values alone are not very reliable presumably because steric effects influence access of substrates to the FMO active site, which is consistent with the reported lack of rabbit FMO2 activity toward imipramine and chlorpromazine (Rettie and Fisher, 1999; Krueger et al., 2006).

The mechanism of catalysis by FMO is depicted in Fig. 6-44. After the FAD moiety is reduced to $FADH_2$ by NADPH, the oxidized cofactor, $NADP^+$, remains bound to the enzyme. $FADH_2$ then binds

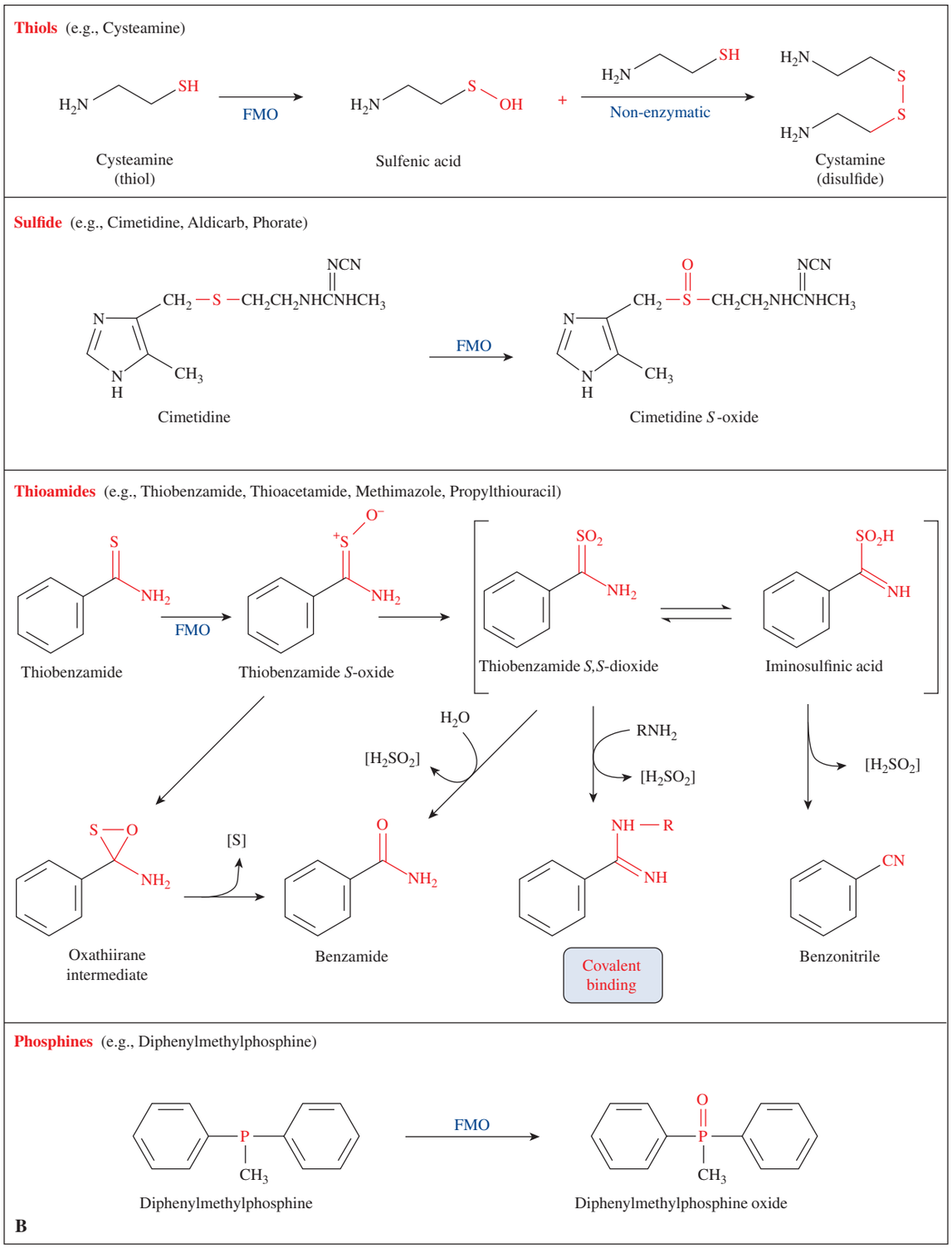


Figure 6-43. *(Continued)* (**B**) Sulfur- and phosphorus-containing xenobiotics.

oxygen to produce a peroxide (i.e., the C(4a)-hydroperoxyflavin of FAD). The FAD hydroperoxide is relatively stable, probably because the active site of FMO comprises non-nucleophilic, lipophilic amino acid residues, and this is thought to be the enzyme's resting state (i.e., the FAD hydroperoxide is the form in which FMO exists prior to substrate binding). During the oxygenation of xenobiotics (depicted as X → XO in Fig. 6-44), the nucleophilic heteroatom (N or S) attacks the terminal oxygen of the C(4a)-hydroperoxyflavin resulting in oxygen transfer to the xenobiotic (to form an *N*-oxide or sulfoxide) and formation of C(4a)-hydroxyflavin. From the latter

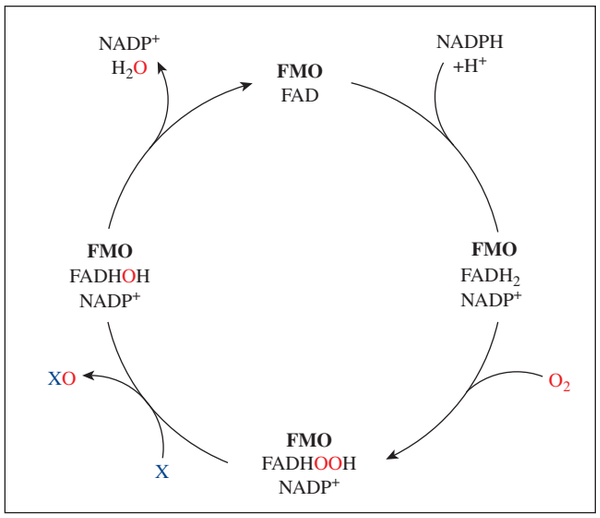


Figure 6-44. *Catalytic cycle of flavin monooxygenase (FMO). X and XO are the xenobiotic substrate and oxygenated product, respectively.* The C(4a)-hydroperoxyflavin and C(4a)-hydroxyflavin of FAD are depicted as FADHOOH and FADHOH, respectively.

step, it is understandable why the metabolites produced by FMO are generally the products of a chemical reaction between a xenobiotic and a peroxide or peracid. The final step in the catalytic cycle involves dehydration of C(4a)-hydroxyflavin (which restores FAD to the ground state) and release of $NADP^+$. This final step is important because it is rate-limiting, and it occurs after substrate oxygenation. Consequently, this step determines the upper limit of the rate of substrate oxidation. Therefore, all good substrates for FMO are converted to products at roughly the same maximum rate (i.e., V_{max} is determined by the final step in the catalytic cycle).

Binding of $NADP^+$ to FMO during catalysis is important because it prevents the reduction of oxygen to H_2O_2. In the absence of bound $NADP^+$, FMO functions as an NADPH oxidase that consumes NADPH and oxygen to produce H_2O_2. In the presence of substrate, NADPH and oxygen, recombinant human FMO1, FMO2, and FMO3 release 30% to 50% of the oxygen consumed as H_2O_2 (Siddens et al., 2014). Functional FMO2 (FMO2*1) is expressed in the lung of individuals of African (up to 49%) and Hispanic (2% to 7%) decent but is not expressed in Caucasians or Asians due to a mutation that introduces a premature stop codon and results in the synthesis of a truncated and inactive enzyme, namely FMO2*2 (discussed later in this section). A SNP in the wild-type (active) form of FMO2*1 resulting in a single amino acid substitution, namely $Ser_{195} \rightarrow Leu_{195}$, increases H_2O_2 production by an order of magnitude (Siddens et al., 2014). Whether hydrogen peroxide produced by FMO, especially by the FMO2*1 variant S195L expressed in the lung of certain ethnic groups, has toxicological consequences is not known.

The oxygenation of substrates by FMO does not lead to inactivation of the enzyme, even though some of the products are strong electrophiles capable of binding covalently to critical and noncritical nucleophiles such as protein and GSH, respectively. The products of the oxygenation reactions catalyzed by FMO and/or the oxygenation of the same substrates by CYP can inactivate CYP. For example, the FMO-dependent *S*-oxygenation of spironolactone thiol (which is formed by the deacetylation of spironolactone by carboxylesterases, as shown in Fig. 6-8) leads to the formation of an electrophilic sulfenic acid ($R-SH \rightarrow R-SOH$) that can undergo redox cycling, or be further converted to the reactive sulfinic acid ($R-SO_2H$) or sulfanyl radical ($HS^{\bullet}$) that inactivates CYP and binds covalently to other proteins (Decker et al., 1991; Krueger et al., 2006; Testa and Krämer, 2008, 2010).

In humans, FMO plays a role in the biotransformation of several drugs (e.g., albendazole, amphetamine, benzydamine, chlorpheniramine, cimetidine, clindamycin, clozapine, codeine, dasatinib, deprenyl, ethionamide, fenbendazole, guanethidine, itopride, methimazole, olanzapine, olopatadine, pargyline, ranitidine, sulindac sulfide, tamoxifen, tazarotene, thiacetazone, thioridazine, tozasertib, vandetanib, voriconazole, xanomeline, zimeldine, hydrazines such as procarbazine, and various dimethylaminoalkyl phenothiazine derivatives such as chlorpromazine and imipramine) and other xenobiotics (e.g., aldicarb, cocaine, diallyl disulfide, *N*,*N*-dimethylaniline, disulfoton, fenthion, methyl-*p*-tolyl sulfide, methamphetamine, nicotine, phorate, thioacetamide, thiobenzamide, and tyramine), and in the activation of 3,3′-iminodipropionitrile to the neurotoxic *N*-hydroxy-3,3′-iminodipropionitrile (Ballard et al., 2007; Testa and Krämer, 2008, 2010; Wang and Tompkins, 2008; Francois et al., 2009; Weil et al., 2010; Strolin-Benedetti, 2011). As discussed later in this section, FMO also plays a role in the metabolism of some endogenous substrates such as trimethylamine (TMA), cysteamine, cysteine conjugates, methionine, and lipoic acid (6,8-dithiooctanoic acid).

The major FMO in human liver microsomes, FMO3, is predominantly if not solely responsible for converting (*S*)-nicotine to (*S*)-nicotine *N*-1′-oxide (which is one of the reactions shown in Fig. 6-43A). The reaction proceeds stereospecifically; only the *trans* isomer is produced by FMO3, and this is the only isomer of (*S*)-nicotine *N*-1′-oxide excreted in the urine of cigarette smokers or individuals wearing a nicotine patch (Cashman and Zhang, 2006). Therefore, the urinary excretion of *trans*-(*S*)-nicotine *N*-1′-oxide can be used as an in vivo probe/index of FMO3 activity in humans. However, common polymorphisms in FMO1 (e.g., FMO1*6) have been shown to be associated with nicotine dependence in European patients (Hinrichs et al., 2011). Furthermore, recombinant human FMO1 catalyzes the *N*-oxidation of (*S*)-nicotine approximately fourfold more efficiently than recombinant human FMO3, and does so nonstereospecifically (i.e., 55:45 *cis*:*trans* ratio of (*S*)-nicotine *N*-1′-oxide, similar to the situation with pig, rat, and rabbit FMO1), whereas FMO3 forms predominantly the *trans*-(*S*)-nicotine *N*-1′-oxide (as described above) (Hinrichs et al., 2011). FMO1 is extrahepatic, with high expression levels in the kidney, and some expression in the intestine and brain. If FMO1 contributes significantly to the metabolism of nicotine in the brains of smokers, it is possible that the *N*-oxide formed by FMO1 could be reduced back to nicotine in the brain by AO, a process known as retro-reduction or futile cycling (Hinrichs et al., 2011).

FMO3 is also the principal enzyme involved in the *S*-oxygenation of cimetidine, an H_2-antagonist that was once widely used in the treatment of gastric ulcers and other acid-related disorders (this reaction is shown in Fig. 6-43B). Cimetidine is stereoselectively sulfoxidated by FMO3 to an 84:16 mixture of (+) and (−) enantiomers, which closely matches the 75:25 enantiomeric composition of cimetidine *S*-oxide in human urine. Therefore, the urinary excretion of cimetidine *S*-oxide, like that of (*S*)-nicotine *N*-1′-oxide, is an in vivo indicator of FMO3 activity in humans.

Sulindac is a sulfoxide that exists in two stereochemical forms (as do most sulfoxides), and a racemic mixture of *R*- and *S*-sulindac is used therapeutically as an NSAID. As shown in Fig. 6-19, the sulfoxide group in sulindac is reduced to the corresponding sulfide (which is achiral), which is then oxidized back to sulindac by retroreduction (futile cycling), as discussed in the section "Sulfoxide and *N*-Oxide Reduction." In the human liver, the sulfoxidation

of sulindac sulfide is catalyzed by FMO3 with little or no contribution from CYP. At relatively low substrate concentrations (30 μM), FMO3 converts sulindac sulfide to *R*- and *S*-sulindac in an 87:13 ratio (Hamman et al., 2000). Consequently, although sulindac is administered as a racemic mixture (i.e., a 1:1 mixture of *R*- and *S*-enantiomers), the reduction of this drug to the corresponding sulfide and its preferential sulfoxidation by FMO3 to *R*-sulindac results in stereoselective enrichment of *R*-sulindac in plasma and urine.

This retro-reduction of sulfide *S*-oxides or amine *N*-oxides formed by FMO (or CYP) back to the parent may occur more often than is currently recognized, but it can be observed in vitro when the levels of an *N*- or *S*-oxide metabolite increase rapidly at first but then quickly reach a plateau as a balance between oxidation and reduction is reached (Cashman, 2008). There are at least seven systems that can catalyze the retro-reduction of FMO metabolites: (1) AO, (2) quinone reductase, (3) hemoglobin itself or other heme-containing proteins such as CYP (which may or may not be enzymatically catalyzed), (4) xanthine oxidoreductase, (5) NADPH–cytochrome P450 reductase, (6) the mARC with NADH cytochrome b_5/cytochrome b_5, and (7) gut microbiota (Cashman, 2008; Testa and Krämer, 2008, 2010). For instance, amitriptyline *N*-oxide, imipramine *N*-oxide, cyclobenzaprine *N*-oxide, and brucine *N*-oxide are reduced back to the parent tertiary amine by quinone reductase, heme, and/or gut microbiota (Cashman, 2008; Testa and Krämer, 2008, 2010). Nicotine *N*-oxide is retro-reduced to nicotine by AO (Cashman, 2008). Recombinant human CYP1A1, CYP2A6, CYP2D6, and CYP3A4 can also retro-reduce tamoxifen *N*-oxide to the tertiary amine (Cashman, 2008). The retro-reduction of TMA *N*-oxide to TMA by gut microbiota is discussed later in this section (Cashman, 2008; Testa and Krämer, 2008, 2010).

In the case of sulindac sulfide, stereoselective sulfoxidation occurs not only with human FMO3 (the major drug-metabolizing FMO in the human liver) but also with porcine FMO1 (the major form expressed in pig liver) and rabbit FMO2 (the major form expressed in rabbit lung) (Hamman et al., 2000). However, this conformity is the exception, rather than the rule. For example, in contrast to the stereoselective oxygenation of (*S*)-nicotine and cimetidine by human FMO3 (see above), FMO1 (which is the major FMO expressed in pig, rat, and rabbit liver) converts (*S*)-nicotine to a 1:1 mixture of *cis*- and *trans*-(*S*)-nicotine *N*-1′-oxide, and similarly converts cimetidine to a 1:1 mixture of (+) and (−) cimetidine *S*-oxide, respectively. Therefore, statements concerning the role of FMO in the disposition of xenobiotics in humans may not apply to other species, or vice versa.

Several sulfur-containing xenobiotics are oxygenated by FMO to electrophilic reactive intermediates. Such xenobiotics include various thiols, thioamides, 2-mercaptoimidazoles, thiocarbamates, and thiocarbamides. The electrophilic metabolites of these xenobiotics do not inactivate FMO, but they can covalently modify and inactivate neighboring proteins, including CYP. Some of these same xenobiotics are substrates for CYP, and their oxygenation to electrophilic metabolites leads to inactivation of CYP, a process known variously as metabolism-dependent inhibition, mechanism-based inhibition, and suicide inactivation. 2-Mercaptoimidazoles undergo sequential *S*-oxygenation reactions by FMO, first to sulfenic acids and then to sulfinic acids (R-SH → R-SOH → R-SO_2H). These electrophilic metabolites, such as the sulfenic acid metabolite produced from spironolactone thiol (see above), bind to critical nucleophiles (such as proteins) or interact with GSH to form disulfides. The thiocarbamate functionality present in numerous agricultural chemicals is converted by FMO to *S*-oxides (sulfoxides), which can be further oxygenated to sulfones. These reactions involve *S*-oxygenation adjacent to a ketone, which produces strong electrophilic acylating agents, which may be responsible for the toxicity of many thiocarbamate herbicides and fungicides. The hepatotoxicity of thiobenzamide is dependent on *S*-oxidation by FMO and/or CYP. As shown in Fig. 6-43B, the *S*-oxidation of thiobenzamide produces an *S*-oxide, which can rearrange to an oxathiirane (a three-membered ring of carbon, sulfur, and oxygen) on photolysis or thermolysis. However, such oxathiiranes are readily reduced back to the *S*-oxide. In vivo, the *S*,*S*-dioxide is more likely to form, which readily tautomerizes to iminosulfinic acid, binds covalently to protein (which leads to hepatocellular necrosis), or rearranges to benzamide, a reaction known as *oxidative group transfer*. In general, thiols with a negative charge on the S atom (e.g., dithioacids) tend to be good substrates of FMOs, whereas less nucleophilic S atoms tend to be oxidized by CYP (Testa and Krämer, 2008, 2010).

Endogenous FMO substrates include cysteamine, which is oxidized to the disulfide, cystamine, and TMA, which is converted to TMA *N*-oxide (Fig. 6-43). By converting cysteamine to cystamine, FMO may serve to produce a low-molecular weight-disulfide exchange agent, which may participate in the formation of disulfide bridges during peptide synthesis or the renaturation of proteins. By converting TMA to TMA *N*-oxide, FMO3 (but neither FMO1 nor FMO5) converts a malodorous and volatile dietary product derived from the reduction of dietary TMA *N*-oxide by gut microbiota (e.g., from choline, lecithin, carnitine, and especially fish, which contain large amounts of TMA *N*-oxide) to an inoffensive metabolite. TMA smells of rotting fish (because bacteria in dead fish rapidly convert the high levels of TMA *N*-oxide to TMA), and people who are genetically deficient in FMO3 suffer from trimethylaminuria or *fish odor syndrome*, which is caused by the excretion of TMA in urine, sweat, and breath (Ayesh and Smith, 1992; Phillips and Shephard, 2008). Although the lack of functional FMO3 (and hence trimethylaminuria) occurs only rarely (e.g., about 1% of British Caucasians), it is now known to be caused by one of at least 30 mutations that decrease or abolish FMO3 activity (Cashman et al., 2000; Cashman and Zhang, 2006; Phillips and Shephard, 2008). As might be expected, trimethylaminuria is associated with an impairment of nicotine *N*-oxidation and other pathways of drug biotransformation that are primarily catalyzed by FMO3 (Rettie and Fisher, 1999). For instance, benzydamine *N*-oxygenation and oxidation of sulindac sulfide has been correlated with FMO3 genotype in trimethylaminurics (Cashman and Zhang, 2006; Testa and Krämer, 2008, 2010; Francois et al., 2009). Some polymorphisms decrease while others increase the activity of FMO3 toward certain substrates, while others affect the promoter region and alter the amount of enzyme expressed (Phillips and Shephard, 2008).

Humans and other mammals express five different flavin monooxygenases (FMO1, FMO2, FMO3, FMO4, and FMO5) in a species- and tissue-specific manner, as shown in Table 6-13, based on mRNA levels, which may not reflect protein levels (Cashman, 1995; Cashman and Zhang, 2006; Zhang and Cashman, 2006). The major hepatic FMOs expressed in humans are FMO3 and FMO5, whereas FMO1 is the major FMO expressed in the rat, rabbit, and pig liver. In humans FMO1 is expressed in the fetal liver and is very rapidly downregulated after birth (although not in kidney) while hepatic FMO3 is gradually upregulated over months to years, in contrast to other mammals (Shephard et al., 2007; Phillips and Shephard, 2008; Testa and Krämer, 2008, 2010). This delay between the hepatic silencing of FMO1 and expression of FMO3 means that most infants probably have very little drug-metabolizing FMO present in the liver for several months (Shimizu et al., 2011). In addition, given the fact that apparently only FMO3 catalyzes the *N*-oxidation of TMA produced from gut microbiota, this begs the

Table 6-13

Putative Tissue Levels of Flavin Monooxygenase (FMO) Enzymes Present in Animals and Humans

	FMO1	FMO2	FMO3	FMO4	FMO5
Liver					
Mouse	Low	None	High	Unknown	Low
Rat	High	Unknown	Low	Unknown	Low
Rabbit	High	None	Low	Unknown	Low
Human*	Trace	Low	Very high	High	Very high
Kidney					
Mouse	High	Unknown	High	Unknown	Low
Rat	High	Unknown	High	High	Low
Rabbit	Low	Low	Trace	High	Low
Human*	High	High	Low	Medium	Medium
Lung					
Mouse	Unknown	High	Trace	None	Low
Rat	Unknown	Unknown	Unknown	None	Low
Rabbit	Unknown	Very high	Unknown	None	None
Human*	Low	Variable†	Medium	Low	Medium
Small intestine					
Human*	Low	Low	Trace	Trace	High

**Human tissue levels are based on FMO mRNA levels.*

† FMO2 mRNA levels in human lung are high, but this mRNA is not translated into functional enzyme due to the presence of a truncation mutation in Caucasians and Asians. Approximately 33% of sub-Saharan Africans, 26% of African Americans, 7% of Puerto Ricans, and 2% of Mexicans have one normal allele and express a functional protein (Veeramah et al., 2008).

SOURCES: Data adapted from Cashman and Zhang (2006) and Zhang and Cashman (2006).

question of why all infants do *not* have *fish odor syndrome*. In a cohort of six patients with genetically confirmed trimethylaminuria, the average age of presentation was 6 (±3.6) years (Chalmers et al., 2006). As a genetic deficiency of FMO3, trimethylaminuria is obviously present from birth. However, the phenotype only becomes apparent when an affected child is weaned, and foods with high amounts of choline (e.g., eggs and liver) or TMA *N*-oxide (e.g., seafood) are introduced (Chalmers et al., 2006). Diagnostic delay also presumably leads to the higher than expected average age of presentation. Transient trimethylaminuria can however occur in neonates and infants if their diet sporadically contains higher than usual levels of choline (Testa and Krämer, 2008, 2010).

An epidemiological link between circulating levels of TMA *N*-oxide and the risk of cardiovascular disease (CVD) has raised the possibility that TMA *N*-oxide is proatherogenic (Fennema et al., 2016; Velasquez et al., 2016; Cho and Caudill, 2017). However, the circulating levels of TMA *N*-oxide are influenced by diet, kidney function, the composition of intestinal microbiota, and the activity of FMO3; hence, it is not known whether TMA *N*-oxide is a cause or simply a biomarker for CVD. Following up on the interesting link between FMO3/TMA *N*-oxide and CVD, Miao et al. (2015) discovered that selective loss of hepatic insulin receptors (in liver insulin receptor knockout or LIRKO mice) results in more than 1000-fold increase in FMO3 mRNA levels and a large increase in FMO3 protein. With livers unresponsive to insulin, LIRKO mice are glucose intolerant and have elevated hepatic levels of several gluconeogenic enzymes, all of which are reversed by antisense oligonucleotide knockdown of FMO3. Knockdown of FMO3 in LIRKO mice results in a decrease in Foxo1 levels, a key regulator of gluconeogenic enzymes. Knockdown of FMO3 also attenuates atherosclerosis in LDL knockout mice (Shih et al., 2015). These results suggest that, by regulating Foxo1 signaling, FMO3 plays a role in the development of insulin resistance and atherosclerosis in mice (Schugar and Brown, 2015). However, it is not clear why the marked gender difference in FMO3 levels in sexually mature mice, where FMO3 is a female-predominant enzyme, has no impact on glucose tolerance or CVD in mice. Furthermore, in humans who are homozygous for a genetic polymorphism ($Glu_{158} \rightarrow Lys_{158}$) that reduces FMO3 activity, which would be expected to protect against atherogenesis, the risk of chronic CVD was unchanged in men and increased (not decreased) in women (odds ratio 1.7) (Bushueva et al., 2015). FMO5 knockout mice do not exhibit the age-dependent increases in glucose and insulin levels characteristic of wild-type mice; that is, FMO5 knockout mice remain glucose tolerant and insulin sensitive as they age and even when fed a high-fat diet (Scott et al., 2017). FMO5 is expressed in the small intestine and the resistance of FMO5 knockout mice to age-dependent and diet-induced weight gain, obesity, and glucose intolerance resembles that observed in germ-free mice (mice lacking intestinal microbiota), leading Scott et al. (2017) to propose that FMO5 plays a role in sensing or responding to gut microbiota.

Previously, FMO3 has been considered to be the dominant FMO in the human liver, and although FMO5 expression is higher, it has activity toward fewer xenobiotic substrates, and likely plays only a minor role in xenobiotic metabolism, with the exception of E7016, the poly(ADP-ribose) polymerase inhibitor noted above (Cashman and Zhang, 2006; Lai et al., 2011). FMO5 is also the most highly expressed FMO in the small intestine, in terms of mRNA. In humans, high levels of FMO1 are expressed in the kidney, and low levels of FMO2 are expressed in the lungs of Caucasians and Asians.

Lung microsomes from most mammals, particularly rabbit, mouse, monkey, and other nonhuman primates, contain high levels of functional FMO2, and of the FMO transcripts detected in the human lung, the FMO2 transcript is found at the highest levels by far (Zhang and Cashman, 2006). The uncharacteristically low level of active FMO2 in human lung is caused by a mutation (a C → T transition at codon 472 in exon 9 [rs6661174]) in the major human

FMO2*2A allele found in essentially all Caucasian and Asian populations, which results in a premature stop codon and the synthesis of a nonfunctional, truncated protein (one lacking the last 64 amino acid residues from the C-terminus) (Dolphin et al., 1998; Veeramah et al., 2008; Francois et al., 2009). In contrast, 26% of African Americans, 7% of Puerto Ricans, and 2% of Mexicans have one normal allele and express a functional protein (Cashman and Zhang, 2006). In sub-Saharan Africa, approximately 33% of individuals possess at least one functional FMO2*1 allele, and in some subpopulations the incidence approaches 50% (Veeramah et al., 2008). In pulmonary microsomes from individuals who express one or more functional FMO2 alleles, the protein level is equal to or greater than that of CYP enzymes. Functional FMO2 has high activity toward thioureas and thioamides, which implies that individuals who express a functional FMO2 may be more susceptible to the toxic effects of sulfenic or sulfinic acids formed from *S*-oxygenation of phenylthiourea, α-naphthylthiourea, and ethylenethiourea, whereas Caucasians and Asians may be more susceptible to thioether-containing organophosphate pesticides such as phorate and disulfoton, for which the parent compounds are more toxic than products of the FMO2-catalyzed reaction (Cashman and Zhang, 2006; Veeramah et al., 2008). FMO2 has also been shown to catalyze *S*-oxygenation of the second-line antitubercular drugs ethionamide and thiacetazone, leading to the formation of reactive metabolites such as the sulfenic and sulfinic acids, carbodiimide derivatives, and *S*-oxides (Francois et al., 2009). Because these second-line antitubercular drugs are prodrugs that must be converted by the mycobacteria in FMO-like reactions to the reactive products, it is possible that administration to individuals with at least one FMO2*1 allele could decrease their efficacy and increase pulmonary toxicity. Finally, as noted earlier in this section, an allelic variant of FMO2*1 functions as an efficient NADPH oxidase and produces H_2O_2 at much higher rates than the wild-type enzyme. The toxicological consequences of this genetic polymorphism, which would be expected to increase oxidative stress in the lung, are not known.

FMO4 is expressed at low levels in the brain of several mammalian species, where it might terminate the action of several centrally active drugs and other xenobiotics. It is unstable, however, which makes its characterization somewhat difficult (Cashman and Zhang, 2006).

The various forms of FMO are distinct gene products with different physical properties and substrate specificities. For example, FMO2 *N*-oxygenates *n*-octylamine, whereas such long aliphatic primary amines are not substrates for FMO1, although they stimulate its activity toward other substrates (in some cases causing a change in stereospecificity). Conversely, short-chain tertiary amines, such as chlorpromazine, orphenadrine, and imipramine, are substrates for FMO1 but not FMO2. FMO2 exhibits no activity toward phenothiazine derivatives with only a three-carbon side chain or 1,3-diphenylthiourea (Krueger et al., 2006). FMO3 is highly selective in the *N*-oxygenation of TMA, whereas FMO5 is selective for the *N*-oxygenation of short-chain aliphatic primary amines such as *N*-octylamine, but has little activity toward other typical FMO substrates, with the exception of E7016, the poly(ADP-ribose) polymerase inhibitor noted previously (Krueger et al., 2006; Lai et al., 2011). The substrate specificity of FMO4 also is somewhat restricted. Certain substrates are oxygenated stereospecifically by one FMO enzyme but not another. For example, FMO2 and FMO3 convert (*S*)-nicotine exclusively to *trans*-(*S*)-nicotine *N*-1′-oxide, whereas the *N*-oxides of (*S*)-nicotine produced by FMO1 are a 1:1 mixture of *cis* and *trans* isomers. FMO2 is heat stable under conditions that completely inactivate FMO1, and FMO2 is resistant to anionic detergents that inactivate FMO1. Low concentrations of bile acids, such as cholate, stimulate FMO activity in rat and mouse liver microsomes but inhibit FMO activity in the rabbit and pig liver.

The FMO enzymes expressed in liver microsomes are not under the same regulatory control as CYP enzymes. In rats, the expression of FMO1 is suppressed rather than induced by treatment with phenobarbital or 3-methylcholanthrene (although some studies point to a modest [about threefold] induction of rat FMO1 by 3-methylcholanthrene). Indole-3-carbinol, which induces the same CYP enzymes as 3-methylcholanthrene, causes a marked decrease in FMO activity in the rat liver and intestine. A similar decrease in FMO3 activity occurs in human volunteers following the consumption of large amounts of Brussels sprouts, which contain high levels of indole-3-carbinol and related indoles. The decrease in FMO3 activity may result from direct inhibition of FMO3 by indole-3-carbinol and its derivatives rather than from an actual decrease in enzyme levels (Cashman, 1999). Increased levels of nitric oxide (NO), as occurs during acute inflammation (e.g., sepsis or endotoxemia), which activates nitric oxide synthase-2 (NOS-2), can inhibit FMO (as well as CYP) (Mitchell, 2008). However, AhR agonists, such as dioxin, PCB 77 (3,3′,4,4′-tetrachlorobiphenyl), and PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl), induce FMO2 and FMO3 mRNA levels in male mice by 30- and 80-fold, respectively, with much smaller increases in FMO activity or protein levels (Tijet et al., 2006; Petriello et al., 2016).

The levels of FMO3 and, to a lesser extent, the levels of FMO1 in mouse liver microsomes are sexually differentiated (female > male) due to suppression of expression by testosterone. The opposite is true of FMO1 levels in rat liver microsomes, the expression of which is positively regulated by testosterone and negatively regulated by estradiol. In pregnant rabbits, lung FMO2 is positively regulated by progesterone and/or corticosteroids.

Species differences in the relative expression of FMO and CYP appear to determine differences in the toxicity of the pyrrolizidine alkaloids, senecionine, retrorsine, and monocrotaline. These compounds are detoxified by FMO, which catalyzes the formation of tertiary amine *N*-oxides, but are activated by CYP, which oxidizes these alkaloids to pyrroles that generate toxic electrophiles through the loss of substituents on the pyrrolizidine nucleus (details of which appear in the section "Cytochrome P450"). Rats have a high pyrrole-forming CYP activity and a low *N*-oxide-forming FMO activity, whereas the opposite is true of guinea pigs. This likely explains why pyrrolizidine alkaloids are highly toxic to rats but not to guinea pigs. Many of the reactions catalyzed by FMO are also catalyzed by CYP, but differences in the oxidation of pyrrolizidine alkaloids by FMO and CYP illustrate that this is not always the case. Species differences in the levels of FMO can also complicate the findings of preclinical toxicology and metabolism studies because most toxicologically useful small animal species express FMO1 as the dominant hepatic FMO in contrast to human, in which FMO3 is the dominant form (Cashman, 2008). However, one animal model that may be more similar to the human hepatic expression of FMO is the female mouse, which has relatively high levels of FMO3 and FMO5 (Cashman, 2008).

Cytochrome P450

General Features, Reactions, and Catalytic Cycle Of all the xenobiotic-biotransforming enzymes, the CYP enzyme system ranks first in terms of catalytic versatility and the sheer number of xenobiotics it detoxifies or activates to reactive intermediates. The highest levels of CYP enzymes involved in xenobiotic biotransformation are found in liver endoplasmic reticulum (microsomes), but CYP enzymes are present in virtually all tissues. Some of the so-called microsomal CYP enzymes are also located on the inner membrane of mitochondria, the importance of which is discussed

in the section "CYP2E1." CYP enzymes play a very important role in determining the intensity and duration of action of drugs, and they also play a key role in the detoxication of xenobiotics. CYP enzymes in the liver and extrahepatic tissues play important roles in the activation of xenobiotics to toxic and/or tumorigenic metabolites. The catalytic versatility of CYP enzymes is apparent from Table 6-5, which shows some of the many chemical groups that can be metabolized by CYP.

Microsomal and mitochondrial CYP enzymes play key roles in the biosynthesis or catabolism of steroid hormones, bile acids, fat-soluble vitamins such as vitamins A and D, fatty acids, and eicosanoids such as prostaglandins, thromboxane, prostacyclin, and leukotrienes, which underscores the catalytic versatility of CYP.

The human CYP superfamily contains 55 functional genes and 60 pseudogenes (Zhou et al., 2009a; Nelson, 2009a; Guengerich et al., 2010). Many sources place the number of functional genes at 57; however, the two additional genes, namely, CYP2A7 (originally named IIA4) and CYP4B1, encode enzymes incapable of incorporating heme, rendering them catalytically inactive (note, however, that functional CYP4B1 is expressed in other mammalian species) (Yamano et al., 1990; Ding et al., 1995; Baer and Rettie, 2006). As shown in Table 6-14, the 55 human CYP enzymes can be broadly categorized on the basis of their role in (1) xenobiotic biotransformation, (2) fatty acid/eicosanoid hydroxylation/epoxidation, (3) steroidogenesis, (4) bile acid synthesis, vitamin D activation/inactivation, (5) retinoic acid metabolism, and (6) unknown function (a diminishing group of so-called orphan enzymes). In several cases, there is no clear functional distinction in terms of endobiotic and xenobiotic metabolism because, as noted in Table 6-14, there are many examples of CYP enzymes playing an important role in the metabolism of both an endobiotic and a drug or other xenobiotic. CYP2J2 and, in animals, CYP4B1 are examples of enzymes that ride the xenobiotic–endobiotic fence. In terms of endobiotic metabolism, CYP enzymes play a role in both catabolism and anabolism (several different CYP enzymes play a role in steroid hormone and bile acid synthesis). For example, CYP enzymes both activate vitamin D_3 to 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3) (CYP2R1, CYP2J2, CYP3A4, CYP27A1, and CYP27B1) and inactivate the active metabolite (CYP24A1 and CYP3A4). Arachidonic acid is epoxidated by CYP2C8, CYP2C9, and especially CYP2J2 to vasodilatory epoxyeicosatrienoic acids (EETs) but is converted by ω-hydroxylation to the vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE) by various CYP4A and CYP4F enzymes. The major xenobiotic-biotransforming CYP enzymes in human liver microsomes belong to families 1, 2, and 3, which are shown in Table 6-15 along with their homologs in the nonclinical species widely used in drug safety testing.

All CYP enzymes are heme-containing proteins. In some cases, such as many members of the CYP4 family, the heme moiety is covalently attached to the protein but in most other cases it is attached noncovalently (Ortiz de Montellano, 2008). The heme iron in CYP is usually in the ferric (Fe^{III}) state. When reduced to the ferrous (Fe^{II}) state, CYP can bind ligands such as O_2 and carbon monoxide (CO). The complex between ferrous CYP and CO absorbs light maximally at 450 nm, from which CYP derives its name (*P*igment *450*). The absorbance maximum of the CO complex differs slightly among different CYP enzymes and ranges from 447 to 452 nm. All other hemoproteins that bind CO absorb light maximally at about 420 nm. The unusual absorbance maximum of CYP is due to an unusual fifth ligand to the heme (a cysteine

Table 6-14

Classification of the 55 Functional Human CYP Enzymes

XENOBIOTICS		FATTY ACIDS/ EICOSANOIDS	STEROIDOGENIC	BILE ACIDS	VITAMIN D	RETINOIC ACID	UNKNOWN
CYP1A1	CYP2F1	CYP2U1	CYP11A1	CYP7A1	CYP2R1	CYP26A1	CYP4A22
CYP1A2	CYP2J2*,†	CYP4A11	CYP11B1	CYP7B1	CYP24A1	CYP26B1	CYP4X1
CYP1B1	CYP2S1	CYP4F2‡,§	CYP11B2	CYP8B1	CYP26C1**		CYP20A1
CYP2A6	CYP2W1	CYP4F3§	CYP17A1	CYP27A1†	CYP27B1		CYP27C1
CYP2A13	CYP3A4†,††	CYP4F8	CYP19A1	CYP39A1			
CYP2B6	CYP3A5	CYP4F11	CYP21A2	CYP46A1			
CYP2C8*	CYP3A7	CYP4F12§		CYP51A1‡‡			
CYP2C9	CYP3A43	CYP4F22					
CYP2C18		CYP4V2					
CYP2C19		CYP4Z1					
CYP2D6		CYP5A1§§					
CYP2E1		CYP8A1***					

**Also involved in fatty acid and eicosanoid metabolism.*
†*Also involved in vitamin D metabolism.*
‡*Also involved in vitamin E and vitamin K metabolism.*
§*Also involved in xenobiotic metabolism.*
***Also involved in retinoic acid metabolism.*
††*Also involved in bile acid synthesis.*
‡‡*Also involved in cholesterol biosynthesis.*
§§*Thromboxane A synthase (TBXAS1).*
***$Prostaglandin\ I_2$ (prostacyclin) synthase (PTGIS).*
Note: CYP2A7 and CYP4B1 are full length genes that probably encode inactive enzymes due to lack of heme incorporation.

Table 6-15

Homologs of the Major Xenobiotic-Metabolizing CYP Enzymes in Liver Microsomes from Humans and Nonclinical Species

SUBFAMILY	HUMAN	MONKEY	DOG	RAT	MOUSE
CYP1A	1A2 (1A1, IB1)	1A2 (1A1, IB1)	1A2 (1A1, IB1)	1A2 (1A1, IB1)	1A2 (1A1, IB1)
CYP2A	2A6, 2A13	2A23, 2A24	2A13, 2A25	2A1, 2A2, 2A3	2A4, 2A5, 2A12, 2A22
CYP2B	2B6	2B6, 2B17	2B11	2B1, 2B2, 2B3	2B9, 2B10
CYP2C	2C8, 2C9, 2C18, 2C19	2C20, 2C43, 2C76	2C21, 2C41	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23, 2C24, 2C46, 2C77, 2C79, 2C80, 2C81	2C29, 2C37, 2C38, 2C39, 2C40, 2C44, 2C50, 2C54, 2C55, 2C65, 2C66, 2C67, 2C68, 2C69, 2C70
CYP2D	2D6	2D17, 2D19, 2D29, 2D30	2D15	2D1, 2D2, 2D3, 2D4, 2D5, 2D18	2D9, 2D10, 2D11, 2D12, 2D13, 2D22, 2D26, 2D34, 2D40
CYP2E	2E1	2E1	2E1	2E1	2E1
CYP2J	2J2	2J2	2J2	2J3, 2J4, 2J10, 2J13, 2J16	2J5, 2J6, 2J7, 2J8, 2J9, 2J11, 2J12, 2J13
CYP3A	3A4, 3A5, 3A7, 3A43	3A8	3A12, 3A26	3A1/23, 3A2, 3A9, 3A18, 3A23, 3A62, 3A73	3A11, 3A13, 3A16, 3A25, 3A41, 3A44, 3A57, 3A59

Note: CYP1A1 and CYP1B1 are extrahepatic enzymes, although CYP1A1 is an AhR-inducible hepatic enzyme.
SOURCE: Data adapted from Baillie and Rettie (2011) and Nelson (2009b).

thiolate). The amino acid sequence around the cysteine residue that forms the thiolate bond with the heme moiety is highly conserved in all CYP enzymes. When this thiolate bond is disrupted, CYP is converted to a catalytically inactive form called cytochrome P420. By competing with oxygen, CO inhibits CYP. The inhibitory effect of CO can be reversed by irradiation with light at 450 nm, which photodissociates the CYP–CO complex. These properties of CYP are of historical importance (Omura, 2011). The observation that treatment of rats with certain chemicals, such as 3-methylcholanthrene, causes a shift in the peak absorbance of CYP (from 450 to 448 nm) provided some of the earliest evidence for the existence of multiple forms of CYP in liver microsomes. The conversion of CYP to cytochrome P420 by detergents and phospholipases helped to establish the hemoprotein nature of CYP. The inhibition of CYP by CO and the reversal of this inhibition by photodissociation of the CYP–CO complex established CYP as the microsomal and mitochondrial enzyme involved in drug biotransformation and steroid biosynthesis (Omura, 2011).

The basic reaction catalyzed by CYP enzymes is monooxygenation in which one atom of oxygen from O_2 is incorporated into a substrate, designated RH, and the other is reduced to water with reducing equivalents derived from NADPH, as follows:

$$\text{Substrate(RH)} + O_2 + \text{NADPH} + H^+ \rightarrow \text{Product(ROH)} + H_2O + \text{NADP}^+$$

Although CYP functions as a monooxygenase, the products are not limited to alcohols and phenols due to rearrangement reactions (Guengerich and Shimada, 1991; Guengerich, 2001a, 2007; Isin and Guengerich, 2007). During catalysis, CYP binds directly to the substrate and molecular oxygen, but it does not interact directly with NADPH or NADH. The mechanism by which CYP receives electrons from NAD(P)H depends on the subcellular localization of CYP. In the endoplasmic reticulum, which is where most of the CYP enzymes involved in xenobiotic biotransformation are localized, electrons are relayed from NADPH to CYP via a flavoprotein called NADPH–cytochrome P450 reductase or POR (also known as an oxidoreductase; gene symbol POR). Within this flavoprotein, electrons are transferred from NADPH to CYP via FMN and FAD. In mitochondria, which house many of the CYP enzymes involved in steroid hormone biosynthesis and vitamin D metabolism, electrons are transferred from NAD(P)H to CYP via two proteins: an iron–sulfur protein called ferredoxin (gene symbol FDX1) and an FMN-containing flavoprotein called ferredoxin reductase (gene symbol FDXR). These proteins are also known as adrenodoxin and adrenodoxin reductase. In bacteria such as *Pseudomonas putida*, which express P450cam (CYP101A1), electron flow is similar to that in mitochondria (NADH → flavoprotein → putidaredoxin → P450).

There are some notable exceptions to the general rule that CYP requires a second enzyme (i.e., a flavoprotein) for catalytic activity. One exception applies to two CYP enzymes involved in the conversion of arachidonic acid to eicosanoids, namely, thromboxane A synthase (CYP5A1; gene symbol TBXAS1) and prostaglandin I_2 synthase, which is also known as prostacyclin synthase (CYP8A1; gene symbol PTGIS). These two CYP enzymes convert the endoperoxide, PGH_2, to thromboxane (TXA_2) and prostacyclin (PGI_2) in platelets and the endothelial lining of blood vessels, respectively. In both cases, CYP functions as an isomerase and catalyzes a rearrangement of the oxygen atoms introduced into arachidonic acid by cyclooxygenase (see Fig. 6-37). The plant CYP, allene oxide synthase (CYP74A1), and certain invertebrate CYP enzymes also catalyze the rearrangement of oxidized chemicals (Guengerich, 2001a).

The second exception is represented by CYP102 (P450BM3) from the bacterium *Bacillus megaterium*, which produces a CYP enzyme linked directly to a flavoprotein to form a single, self-sufficient fusion protein. Some bacterial CYP enzymes are thermophilic (such as CYP119, CYP174A1, and CYP231A2), which has attracted attention for their potential industrial applications (Nishida and Ortiz de Montellano, 2005). The thermophilic P450 enzyme from *Sulfolobus acidocaldarius*, namely, CYP119, was used to confirm (and settle a long-standing debate) that the final step in the

catalytic cycle of CYP is formation of compound I ($por^{\bullet+}Fe^{IV}=O$), as in the case of peroxidases, which is discussed later in this section (Rittle and Green, 2010). Most mammalian CYP enzymes are not synthesized as a single enzyme containing both the hemoprotein and flavoprotein moieties, but this arrangement is found in nitric oxide (NO) synthases. In addition to its atypical structure, the P450 enzyme expressed in *B. megaterium*, CYP102, is unusual for another reason: it is inducible by phenobarbital, which provided insight into the mechanism of CYP induction.

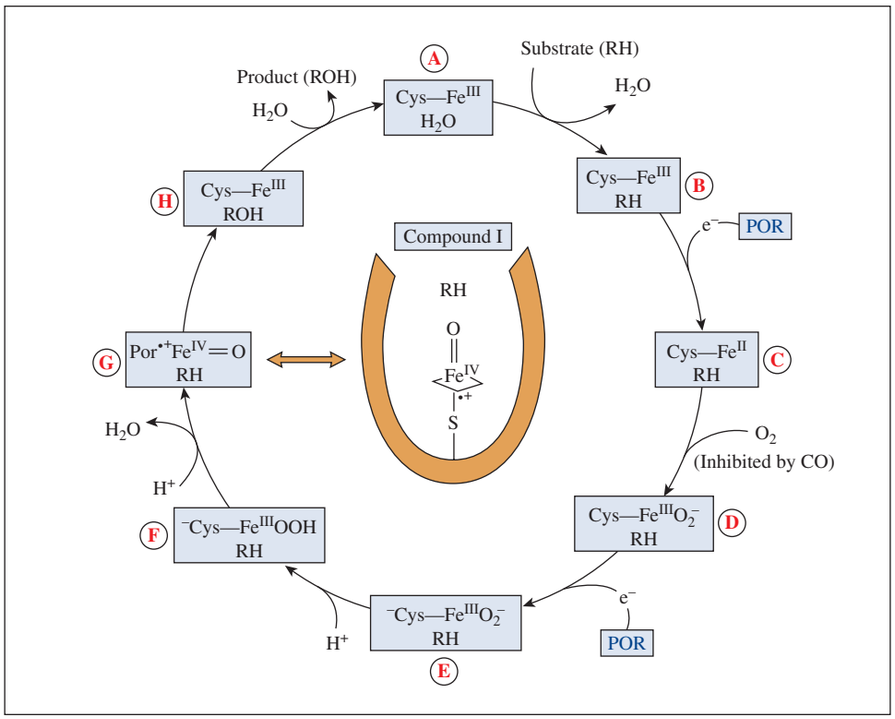
Phospholipids and cytochrome b_5 also play an important role in CYP reactions (McLaughlin et al., 2010). CYP and POR are embedded in the phospholipid bilayer of the endoplasmic reticulum, which facilitates their interaction. With the notable exception of CYP2W1 (discussed later in this section), CYP and POR face the cytoplasmic side of the endoplasmic reticulum. When the C-terminal region that anchors POR in the membrane is cleaved with trypsin, the truncated flavoprotein can no longer support CYP reactions, although it is still capable of reducing cytochrome *c* and other soluble electron acceptors. The ability of phospholipids to facilitate the interaction between POR and CYP does not appear to depend on the nature of the polar head group (serine, choline, inositol, and ethanolamine), although certain CYP enzymes (those in the CYP3A subfamily) have a requirement for phospholipids containing unsaturated fatty acids. In vitro experiments established that cytochrome b_5 can stimulate various CYP reactions by either increasing V_{max} or decreasing K_m, which was initially interpreted as evidence that cytochrome b_5 can donate the second of two electrons required by CYP. However, the same stimulation occurs with heme-depleted cytochrome b_5 (Yamazaki et al., 1996, 2001). The stimulatory effect of cytochrome b_5 is now attributed to its effect on CYP conformation/oligomerization and/or its ability to facilitate the interaction between CYP and POR. Experiments in conditional knockout mice establish that cytochrome b_5 has an important stimulatory effect on CYP in vivo (McLaughlin et al., 2010). Liver microsomes contain numerous forms of CYP but contain a single form of POR and cytochrome b_5 (CYB5A).

For each molecule of POR in rat liver microsomes, there are 5 to 10 molecules of cytochrome b_5 and 10 to 20 molecules of CYP. In human liver microsomes the ratio of CYP to POR is slightly lower (closer to 5:1). POR will reduce electron acceptors other than CYP, which enables this enzyme to be quantified based on its ability to reduce cytochrome *c* (which is why POR is often called NADPH–cytochrome *c* reductase). POR can transfer electrons much faster than CYP can use them, which more than likely accounts for the low ratio of POR to CYP in liver microsomes. Low levels of POR may also be a safeguard to protect cells from the often deleterious one-electron reduction reactions catalyzed by this flavoprotein (see Fig. 6-20). POR also supports heme oxygenase, a Nrf2-inducible enzyme, the significance of which is discussed in the section "CYP2E1."

While microsomal cytochrome b_5 is encoded by CYB5A, CYB5B encodes the cytochrome b_5 found in the outer mitochondrial membrane where it supports the molybdoenzyme mARC in the dehydroxylation of certain amidoximes and related compounds (see the section "Dehydroxylation: mARC, Cytochrome b_5, b_5 Reductase, and Aldehyde Oxidase" and Fig. 6-26).

The catalytic cycle of CYP involves eight steps (A → H), as shown in Fig. 6-45, for the oxidation of a substrate (RH) to its hydroxylated metabolite (ROH) (Dawson, 1988; Guengerich, 2007; Rittle and Green, 2010; Johnston et al., 2011; Hrycay and Bandiera, 2012). In this scheme, iron is shown bound to its fifth ligand, a heme thiolate provided by a highly conserved cysteine (Cys) residue. The first steps of the cycle (A → G) involve the activation of oxygen to compound I, and the final steps involve substrate oxidation by compound I (G → H) followed by release of the metabolite (ROH) to restore the enzyme to its resting (ferric) state (H → A). Following the binding of substrate (RH) to CYP (A → B), the heme iron is reduced from the ferric (Fe^{III}) to the ferrous (Fe^{II}) state by the introduction of a single electron from POR (B → C). In many cases the reduction of CYP is facilitated by substrate binding because binding of the substrate in the vicinity of the heme moiety converts the heme ferric iron from a low-spin to a high-spin state, although some enzymes, such as CYP1A2 and CYP2E1, are naturally in the high-spin state and can be reduced in the absence of substrate. It is at stage C, when the iron is in the ferrous state, that CYP can bind oxygen and CO. It is at this stage of the cycle that, under reduced oxygen tension, CYP can reduce certain substrates (see "Other reactions" at the bottom of the catalytic cycle shown in Fig. 6-45). In the third step (C → D) oxygen binds to the ferrous iron, which transfers an electron to oxygen to form ferrisuperoxo anion (i.e., ferric-bound superoxide anion), designated $Cys-Fe^{III}O_2^-$. At this stage, the cycle can be interrupted (uncoupled) to release superoxide anion and restore the enzyme to its resting (ferric) state (see "Other reactions" in Fig. 6-45). In the fourth step (D → E), a second electron is introduced from POR, which is delocalized over the thiolate bond to form the supernucleophilic ferriperoxo intermediate $^-Cys-Fe^{III}O_2^-$. Uncoupling of the cycle at this stage releases hydrogen peroxide and restores the enzyme to its resting (ferric) state (see "Other reactions" in Fig. 6-45). In the fifth step (E → F), addition of a proton (H^+) converts the supernucleophilic ferriperoxo intermediate to its corresponding hydroperoxide, the ferrihydroperoxy intermediate $^-Cys-Fe^{III}OOH$. In the sixth step (F → G), addition of a second proton followed by release of water converts the ferrihydroperoxy intermediate to compound I, an $iron^{IV}$-oxo porphyrin radical cation species (an oxidizing species previously described in the section "Peroxidase-Dependent Cooxidation"). The formation of compound I ($por^{\bullet+}Fe^{IV}=O$) by protonation of the ferrihydroperoxy intermediate involves the heterolytic cleavage of oxygen with the two-electron oxygen atom going to water, a reaction facilitated by the strong electron-donating effects of the heme thiolate anion. Heterolytic (two-electron) cleavage of oxygen to produce compound I places the iron in the perferryl (Fe^{V}) oxidation state, which is a considerably stronger oxidant than that formed by homolytic cleavage of oxygen, which produces the less reactive Por Fe^{IV}–OH with iron in oxidation state IV. (*Note*: It is somewhat confusing that compound I is written as $por^{\bullet+}Fe^{IV}=O$ because, without taking the porphyrin ring into account, the formula gives the erroneous impression that the iron is in the Fe^{IV} [ferryl] state, whereas it is actually in the Fe^{V} [perferryl] state.) In the seventh step (G → H), the highly electrophilic oxygen from compound I is transferred to the substrate (RH) to produce metabolite (ROH). In the final step (H → A), the metabolite is released, which restores the enzyme to its initial resting (ferric) state.

Compound I is the electrophilic oxo species responsible for the vast majority of CYP-catalyzed reactions (Hrycay and Bandiera, 2012). It can be formed as described above with NADPH and oxygen, or it can be formed by oxygen transfer from various organic hydroperoxides and peracids (X–OOH, such as cumene hydroperoxide, *tert*-butylhydroperoxide, and *meta*-chloroperbenzoic acid) in a one-step reaction called the "peroxide shunt," as shown in "Other reactions" in Fig. 6-45. Formation of CYP compound I from peroxy compounds (P450 + X–OOH → P450 compound I + X-OH) is identical to the formation of compound I in peroxidases (see the section "Peroxidase-Dependent Cooxidation"). However, by virtue of its unusual fifth ligand (the heme thiolate from cysteine) and, perhaps more importantly, its active site topology, CYP can catalyze a far greater array of oxidative reactions (or catalyze them much



Other reactions

One-electron reduction	**C** (Cys—Fe^{II} RH)	⟶ **A** (Cys—Fe^{III} + $RH^{\bullet}$)
Superoxide anion production	**D** (Cys—$Fe^{III}O_2^-$ RH)	⟶ **B** (Cys—Fe^{III} RH) + $O_2^{\bullet}$
Hydrogen peroxide production	**E** ($^-$Cys—$Fe^{III}O_2^-$ RH) + $2H^+$	⟶ **B** (Cys—Fe^{III} RH) + H_2O_2
Hydrogen peroxide shunt	**B** (Cys—Fe^{III} RH) + H_2O_2	⟶ **F** ($^-$Cys—Fe^{III}OOH RH) + H^+
Peroxide shunt to form Compound I	**B** (Cys—Fe^{III} RH) + XOOH	⟶ **G** ($Por^{\bullet+}Fe^{IV}$=O RH) + XOH

Figure 6-45. *Catalytic cycle of cytochrome P450.* Cytochrome P450 is represented as Cys-FeIII, where Cys represents the fifth ligand (a cysteine thiolate) to the ferric heme iron. RH and ROH represent the substrate and product (hydroxylated metabolite), respectively. The intermediates in the catalytic cycle are as follows: A, ferric resting state; B, substrate bound; C, ferrous intermediate; D, ferrisuperoxo anion intermediate; E, ferriperoxo intermediate with an electron delocalized over the Cys thiolate bond; F, ferrihydroperoxy intermediate (with a negative charge on the Cys thiolate bond); G, compound I, an ironIV-oxo porphyrin cation, which is responsible for most substrate oxidation reactions; H, enzyme in its resting state prior to the release of product formed by hydrogen abstraction followed by oxygen rebound (see text for details). FeII, FeIII, FeIV, and FeV refer to iron in the ferrous, ferric, ferryl, and perferryl state, respectively. It should be noted that although it is written as por•+FeIV=O, compound I is in the highly oxidized perferryl (FeV) state when the oxidation state of the porphyrin ring is also taken into account.

faster) than those typically seen with peroxidases (even chloroperoxidase, which is unusual among peroxidases for containing a Cys thiolate as the fifth ligand to the heme like that in CYP). CYP reactions supported by organic peroxides and peracids are not affected by CO, an inhibitor of reactions supported by NADPH and O_2.

Although P450 compound I generated by the peroxide shunt is as catalytically versatile as that generated by NADPH and O_2, differences in the ratio of metabolites formed from a single substrate, differences in the relative rates of oxidation of two or more substrates, and differences in kinetic isotope effects (the influence of deuterium substitution at the site of oxidation) have led to the "multiple oxidants" hypothesis. The concept is supported by the finding that site-directed mutagenesis of a highly conserved threonine residue in bacterial and mammalian CYP enzymes can differentially affect reaction rates. For example, site-directed mutagenesis (Thr268Ala) of the bacterial enzyme CYP102A1 (P450BM3) increases the rate of sulfoxidation relative to *N*-demethylation of a single substrate, namely, dimethyl-(4-methylsulfanylphenyl)amine, whereas another mutation (Phe87Ala) has the opposite effect. Although the concept that CYP forms two or more oxidizing species is well accepted, the basis for the multiplicity is a source of considerable debate (Chandrasena et al., 2004; Jin et al., 2004; Newcomb and Chandrasena, 2005; Sheng et al., 2009; Hrycay and Bandiera, 2012). The two major competing theories are the two-state and the two-oxidant hypotheses, both of which have supporting evidence. The two-state hypothesis posits that compound I is the only oxidizing species but it exists in two states, a low-spin state (that favors *N*-demethylation) and a high-spin state (that favors sulfoxidation)

(Newcomb and Chandrasena, 2005; Hrycay and Bandiera, 2012). The two-oxidant hypothesis posits that compound I is the major electrophilic oxidizing species but that its precursor, the ferrihydroperoxo intermediate ($^{-}$Cys−Fe^{III}OOH), can function as a relatively strong nucleophilic but weak electrophilic oxidizing species (Chandrasena et al., 2004; Jin et al., 2004; Sheng et al., 2009). This is supported by the observation that hydrogen peroxide (which does not form compound I but instead forms the preceding ferrihydroperoxo intermediate or its equivalent, namely, ferric iron–bound hydrogen peroxide) can support certain reactions that are not supported by organic hydroperoxides and peracids. The two mechanisms of generating multiple oxidizing species are not mutually exclusive and both may be needed to explain all the catalytic properties of CYP enzymes. There are also competing theories concerning the mechanism of substrate oxidation by P450 compound I (Hrycay and Bandiera, 2012). Hereafter, the mechanism of carbon hydroxylation is discussed in terms of just one possibility, namely, hydrogen atom transfer (HAT) from the site of substrate hydroxylation by compound I to form a carbon radical followed by oxygen rebound in a nonconcerted, stepwise manner even though experiments with so-called ultrarapid radical clocks support a nonradical mechanism (Newcomb and Chandrasena, 2005; Hrycay and Bandiera, 2012). In reactions involving heteroatoms (N and S) the initial step involves (among other possibilities) single electron transfer (SET) leading to *S*- and *N*-oxygenation or HAT leading to *S*- and *N*-dealkylation, as discussed later in this section (Li et al., 2009a; Roberts and Jones, 2010).

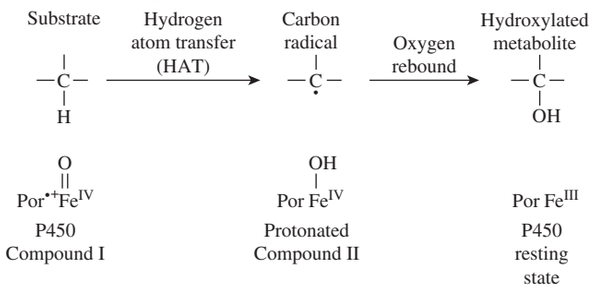
CYP catalyzes several types of oxidation reactions, including the following:

1. Hydroxylation of an aliphatic or aromatic carbon
2. Epoxidation of a double bond
3. Heteroatom (*S*-, *N*-, and *I*-) oxygenation and *N*-hydroxylation
4. Heteroatom (*O*-, *S*-, and *N*-) dealkylation
5. Oxidative group transfer
6. Cleavage of esters and carbamates
7. Dehydrogenation

In the first three cases, oxygen from P450 compound I is incorporated into the substrate, which otherwise remains intact. In the fourth case, oxygenation of the substrate is followed by a rearrangement reaction leading to cleavage of an amine (*N*-dealkylation) or an ether (*O*- and *S*-dealkylation). Oxygen from P450 compound I is incorporated into the alkyl-leaving group, producing an aldehyde or ketone. In the fifth case, oxygenation of the substrate is followed by a rearrangement reaction leading to loss of a heteroatom (oxidative group transfer). The sixth case, the cleavage of esters and carbamates, resembles heteroatom dealkylation in that the functional group is cleaved with incorporation of oxygen from P450 compound I into the leaving group, producing an aldehyde. In the seventh case, two hydrogens are abstracted from the substrate with the formation of a double bond (C=C, C=O, or C=N), with the reduction of oxygen from P450 compound I to water. It should be noted that this long list of reactions does not encompass all the reactions catalyzed by CYP (Guengerich, 2001a, 2007). CYP can catalyze reductive reactions (such as azo-reduction, nitro-reduction, *N*-oxide reduction, sulfoxide reduction, and reductive dehalogenation), ring expansion or ring formation, dearylation, dearomatization, isomerization (such as the conversion of PGH_2 to thromboxane and prostacyclin), and oxidative dehalogenation (as described previously for FMO; see Fig. 6-43). During the synthesis of steroid hormones, CYP catalyzes the cleavage of carbon–carbon bonds, which occurs during the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage enzyme (CYP11A1, which is also known as $P450_{scc}$) and the aromatization of a substituted cyclohexane, which occurs during the conversion of androgens to estrogens by aromatase (CYP19A1, also known as CYP19 and $P450_{arom}$). CYP-dependent cleavage of carbon–carbon bonds in xenobiotics is rare but it does occur. For example, Takahashi et al. (2017) reported that a minor pathway of metabolism of the investigational drug GDC-0425 involves CYP-dependent oxidative decyanation—replacement of a nitrile group (R–CN) with a hydroxyl group (R–OH) with release of the nitrile group as cyanide.

Examples of aliphatic and aromatic hydroxylation reactions catalyzed by CYP are shown in Figs. 6-46 and 6-47, respectively. The hydroxylation of aromatic hydrocarbons may proceed via an oxirane intermediate (i.e., an arene oxide) that isomerizes to the corresponding phenol. Alternatively, aromatic hydroxylation can proceed by a mechanism known as direct insertion. The *ortho*- and *para*-hydroxylation of chlorobenzene proceeds via 2,3- and 3,4-epoxidation, whereas *meta*-hydroxylation proceeds by direct insertion, as shown in Fig. 6-48. When aromatic hydroxylation involves direct insertion, hydrogen abstraction (i.e., cleavage of the C–H bond) is the rate-limiting step, so that substitution of hydrogen with deuterium or tritium considerably slows the hydroxylation reaction. This *isotope effect* is less marked when aromatic hydroxylation proceeds via an arene oxide intermediate. Arene oxides are electrophilic and, therefore, potentially toxic metabolites that are detoxified by such enzymes as epoxide hydrolase (see Figs. 6-12 to 6-14) and GST (see the section "Glutathione Conjugation"). Depending on the ring substituents, the rearrangement of arene oxides to the corresponding phenol can lead to an intramolecular migration of a substituent (such as hydrogen or a halogen) from one carbon to the next. This intramolecular migration occurs at the site of oxidation and is known as the NIH shift, so named for its discovery at the National Institutes of Health.

Aliphatic hydroxylation involves insertion of oxygen into a C–H bond. The initial step involves HAT to form a carbon radical followed by oxygen rebound, shown as follows:



In the case of simple, straight-chain hydrocarbons, such as *n*-hexane, aliphatic hydroxylation occurs at both the terminal methyl groups and the internal methylene groups. In the case of fatty acids (both saturated and unsaturated) and their derivatives (i.e., retinoic acid and eicosanoids such as prostaglandins and leukotrienes), aliphatic hydroxylation occurs at the ω-carbon (terminal methyl group) and the ω-1 carbon (penultimate carbon), as shown for lauric acid in Fig. 6-46. For thermodynamic reasons, most CYP enzymes preferentially catalyze the ω-1 hydroxylation of fatty acids and their derivatives, but one group of CYP enzymes (those encoded by the CYP4 gene family) preferentially catalyzes the less energetically favorable ω-hydroxylation of fatty acids, which can be further oxidized to dicarboxylic acids and undergo chain shortening by β-oxidation (Baer and Rettie, 2006; Johnston et al., 2011, 2015; Edson and Rettie, 2013).

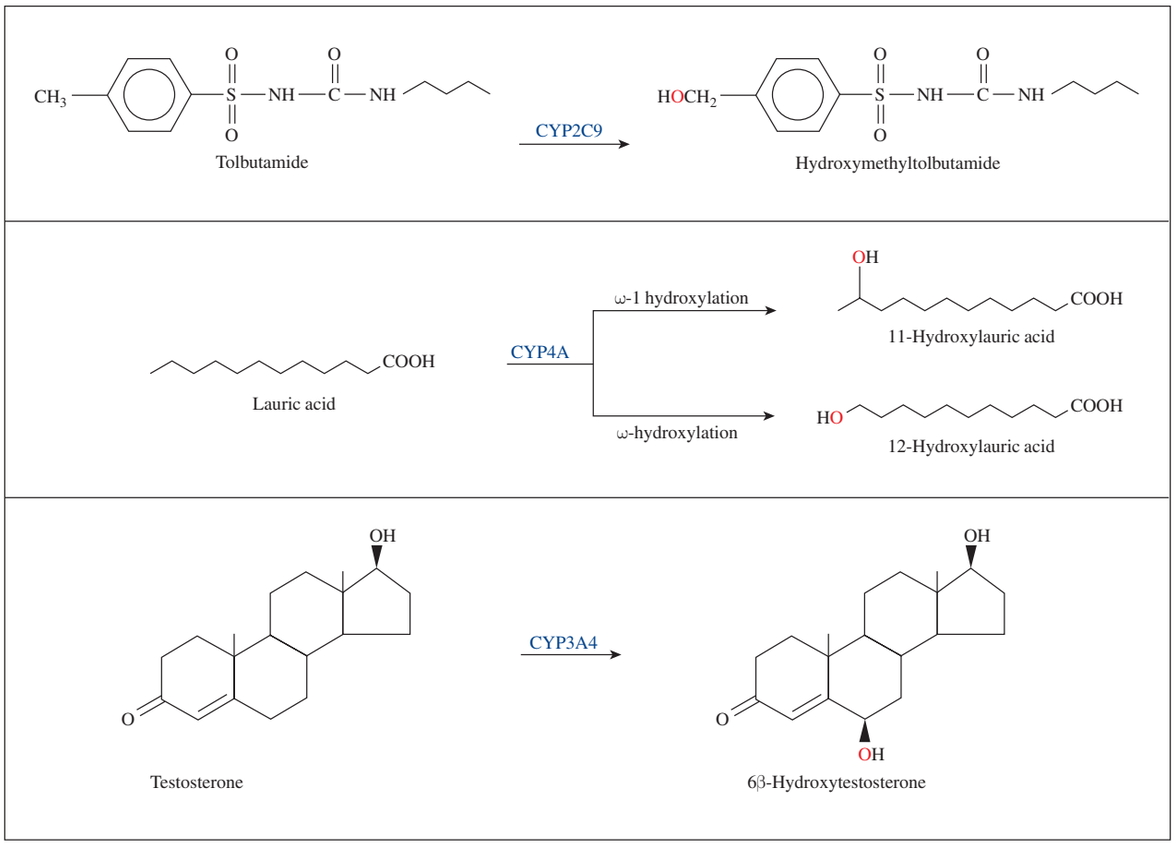


Figure 6-46. *Examples of reactions catalyzed by cytochrome P450: hydroxylation of aliphatic carbon.*

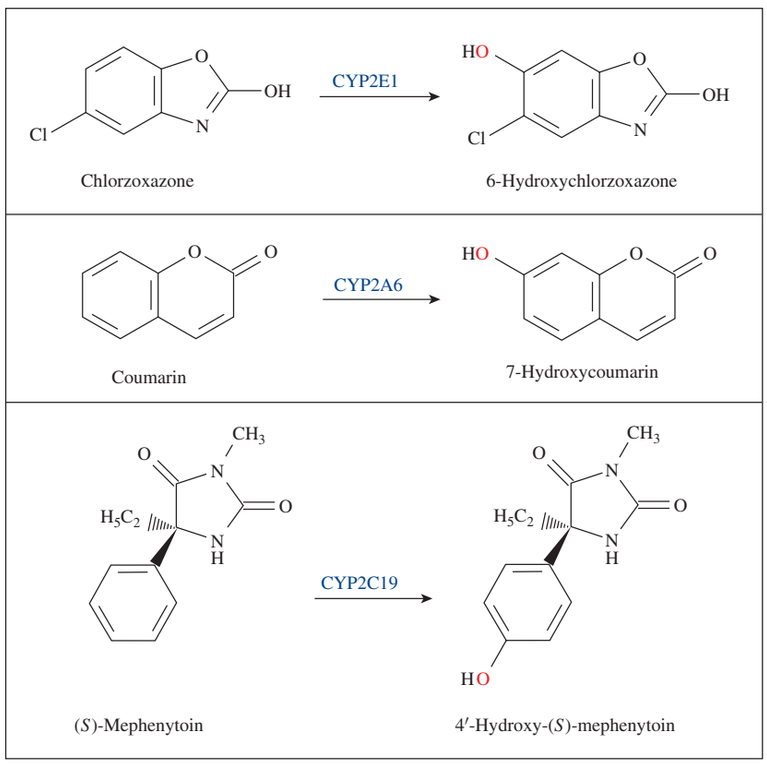


Figure 6-47. *Examples of reactions catalyzed by cytochrome P450: hydroxylation of aromatic carbon.*

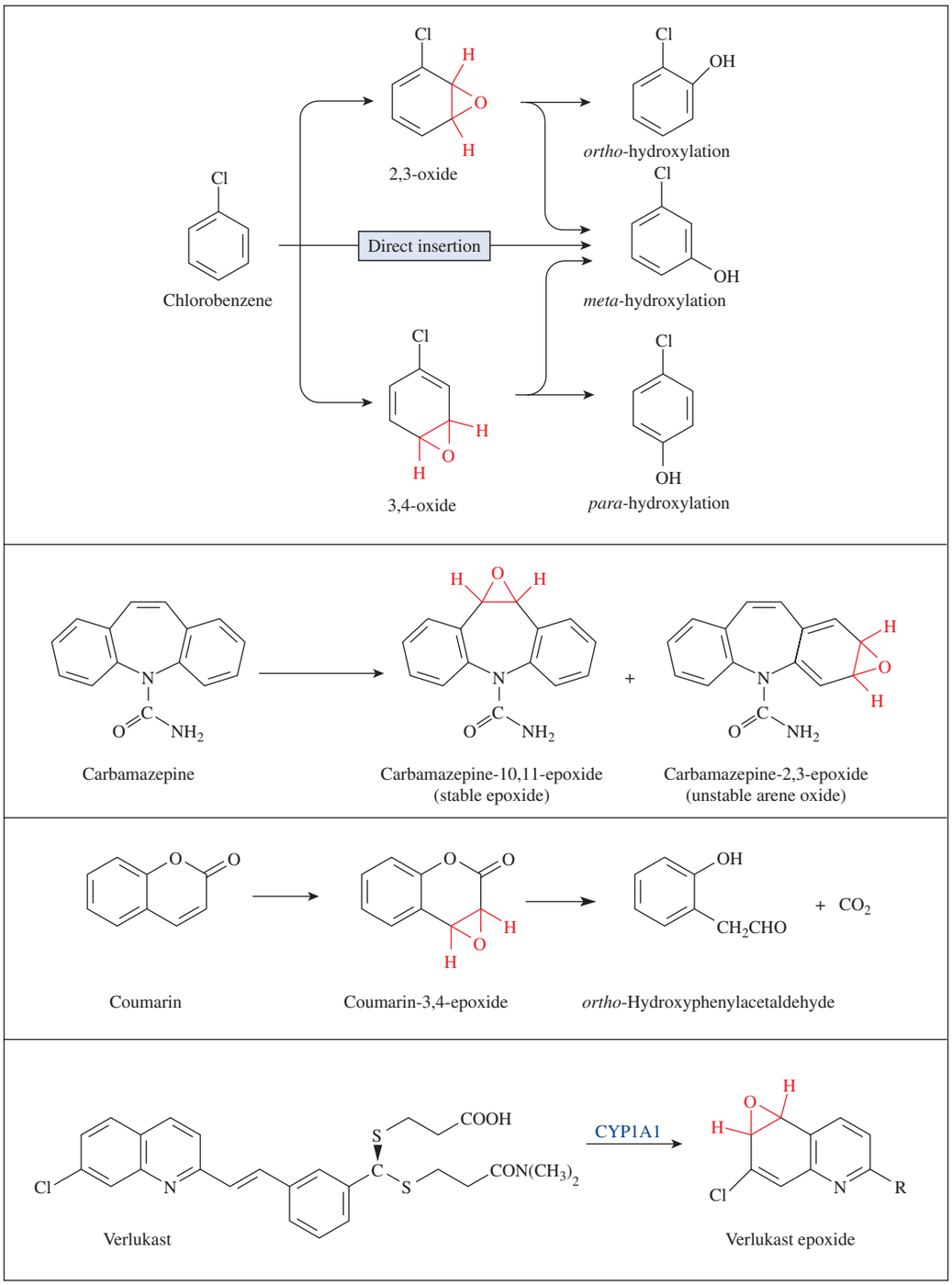
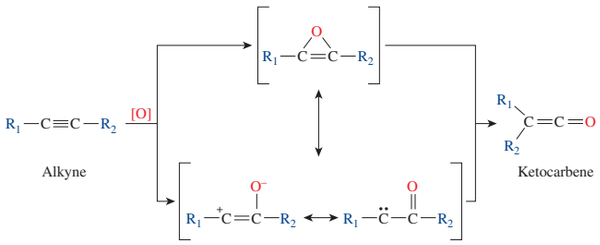


Figure 6-48. *Examples of reactions catalyzed by cytochrome P450: epoxidation.*

Like CYP, the molybdoenzymes AO and XO can also catalyze the carbon oxidation of xenobiotics as outlined in the section "Molybdenum Hydroxylases (Molybdoenzymes)." As an electrophilic oxidizing enzyme, CYP generally prefers to catalyze the oxidation of carbon atoms with a high electron density, whereas the nucleophilic oxidizing enzymes AO and XO preferentially catalyze the oxidation of carbon atoms with a low electron density (such as the sp^2 carbon atom double bonded to a nitrogen atom in various nitrogen heterocycles). For this reason, xenobiotics that are good substrates for CYP enzymes tend to be poor substrates for AO, and vice versa (Pryde et al., 2010).

Xenobiotics containing a carbon–carbon double bond (i.e., alkenes) can be epoxidated (i.e., converted to an oxirane) in an analogous manner to the oxidation of aromatic compounds to arene oxides. Just as arene oxides can isomerize to phenols, so aliphatic epoxides can isomerize to the corresponding ene-ol, the

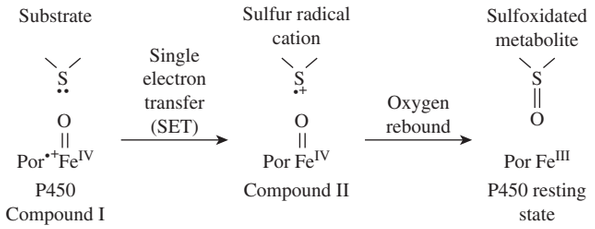
formation of which may involve an intramolecular migration (NIH shift) of a substituent at the site of oxidation (examples of intramolecular shifts accompanying epoxidation are given in the section "Activation of Xenobiotics by Cytochrome P450"). Like arene oxides, aliphatic epoxides are also potentially toxic metabolites that are inactivated by other xenobiotic-metabolizing enzymes such as epoxide hydrolase and GST. Alkynes can be epoxidated by CYP to ketocarbenes (which can be further oxidized to carboxylic acids). The conversion of an alkyne to a ketocarbene via epoxidation or other possible oxidation intermediates is shown in the following scheme:



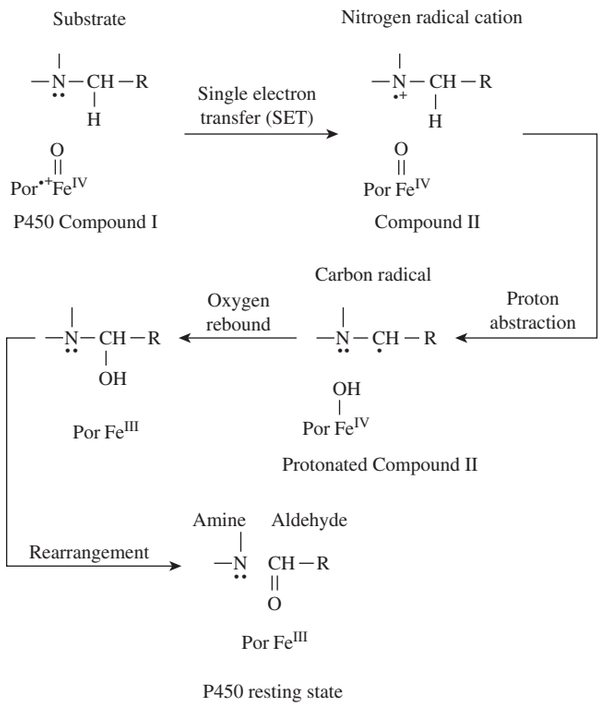
Oxidation of some aliphatic alkenes and alkynes produces metabolites that are sufficiently reactive to bind covalently to the heme moiety of CYP, a process known as suicide inactivation or metabolism-dependent inhibition (discussed later in the section "Inhibition of Cytochrome P450"). As previously discussed in the section "Epoxide Hydrolases," not all epoxides are highly reactive electrophiles. Although the 3,4-epoxidation of coumarin produces a hepatotoxic metabolite, the 10,11-epoxidation of carbamazepine produces a stable, relatively nontoxic metabolite (Fig. 6-48). Epoxyeicosatrienoic acids (EETs) are endogenous epoxides with vasodilatory, anti-inflammatory, and angiogenic properties (Imig and Hammock, 2009; Wang et al., 2010c). They are formed by CYP2C8, CYP2C9, and CYP2J2 (discussed later in this section) and inactivated by sEH (see the section "Epoxide Hydrolases").

In the presence of NADPH and O_2, liver microsomes catalyze the oxygenation of several sulfur-containing xenobiotics, including chlorpromazine, cimetidine, lansoprazole, pantoprazole, and omeprazole. Sulfur-containing xenobiotics can potentially undergo two consecutive sulfoxidation reactions: one that converts the sulfide (S) to the sulfoxide (SO), which occurs during the sulfoxidation of chlorpromazine and cimetidine, and one that converts the sulfoxide (SO) to the sulfone (SO_2), which occurs during the sulfoxidation of omeprazole and lansoprazole, as shown in Fig. 6-49. Albendazole is converted first to a sulfoxide and then to a sulfone. Both CYP and FMO can sulfoxidate sulfides to sulfoxides (S → SO) but only CYP can covert sulfoxides to sulfones (SO → SO_2) (see the section "Flavin Monooxygenases") (Testa and Krämer, 2008, 2010). Accordingly, the sulfoxidation of the proton pump inhibitors omeprazole, lansoprazole, and pantoprazole to sulfones is catalyzed by CYP (CYP3A4) and not by FMO. Examples of sulfoxidation reactions catalyzed by FMO and/or CYP are shown in Figs. 6-43B and 6-49. In the presence of NADPH and O_2, liver microsomes catalyze the oxygenation of several nitrogen-containing xenobiotics, including chlorpromazine, doxylamine, oflaxacin, morphine, nicotine, MPTP, methapyrilene, methaqualone, metronidazole, pargyline, pyridine, senecionine, strychnine, TMA, trimipramine, and verapamil, all of which are converted to stable *N*-oxides. While *S*-oxygenation might be catalyzed by both CYP and FMO, *N*-oxygenation is more likely to be catalyzed by just one of these enzymes. FMO prefers to *N*-oxygenate aliphatic amines (particularly tertiary and cyclic amines) with a highly nucleophilic/basic nitrogen atom (pK_a(B) 5 to 10) but, in contrast to CYP, FMO cannot *N*-oxygenate aromatic amines, for which reason the *N*-oxygenation of pyridines and (iso)quinolones is usually catalyzed only by CYP, as is the case for pyridine-containing xenobiotics such as the tobacco-specific nitrosamine NNK and the antihistamine temelastine, and the isoquinoline-containing muscle relaxant 6,7-dimethoxy-4-(4′-chlorobenzyl)isoquinoline. Methods to distinguish the role of CYP versus FMO in microsomal *N*- and *S*-oxygenation reactions are described in the section "Flavin Monooxygenases."

Although direct oxygen transfer is possible (Li et al., 2009a), the initial step in heteroatom oxygenation by CYP could involve SET from the heteroatom (*N*, *S*, or *I*) to P450 compound I, shown as follows for sulfoxidation:



SET from *N*, *O*, or *S* to P450 compound I may also be the initial step in heteroatom dealkylation, but in this case abstraction of the electron from the heteroatom is quickly followed by abstraction of a proton (H^+) from the α-carbon atom (the carbon atom attached to the heteroatom) to form a carbon radical. Oxygen rebound leads to hydroxylation of the α-carbon, which then rearranges to form the corresponding aldehyde or ketone with cleavage of the α-carbon from the heteroatom, as shown in the following scheme for the *N*-dealkylation of an *N*-alkylamine:



Alternatively, it seems more likely that the initial step in heteroatom dealkylation may be HAT from the α-carbon atom to compound I to produce a carbon radical that undergoes oxygen rebound

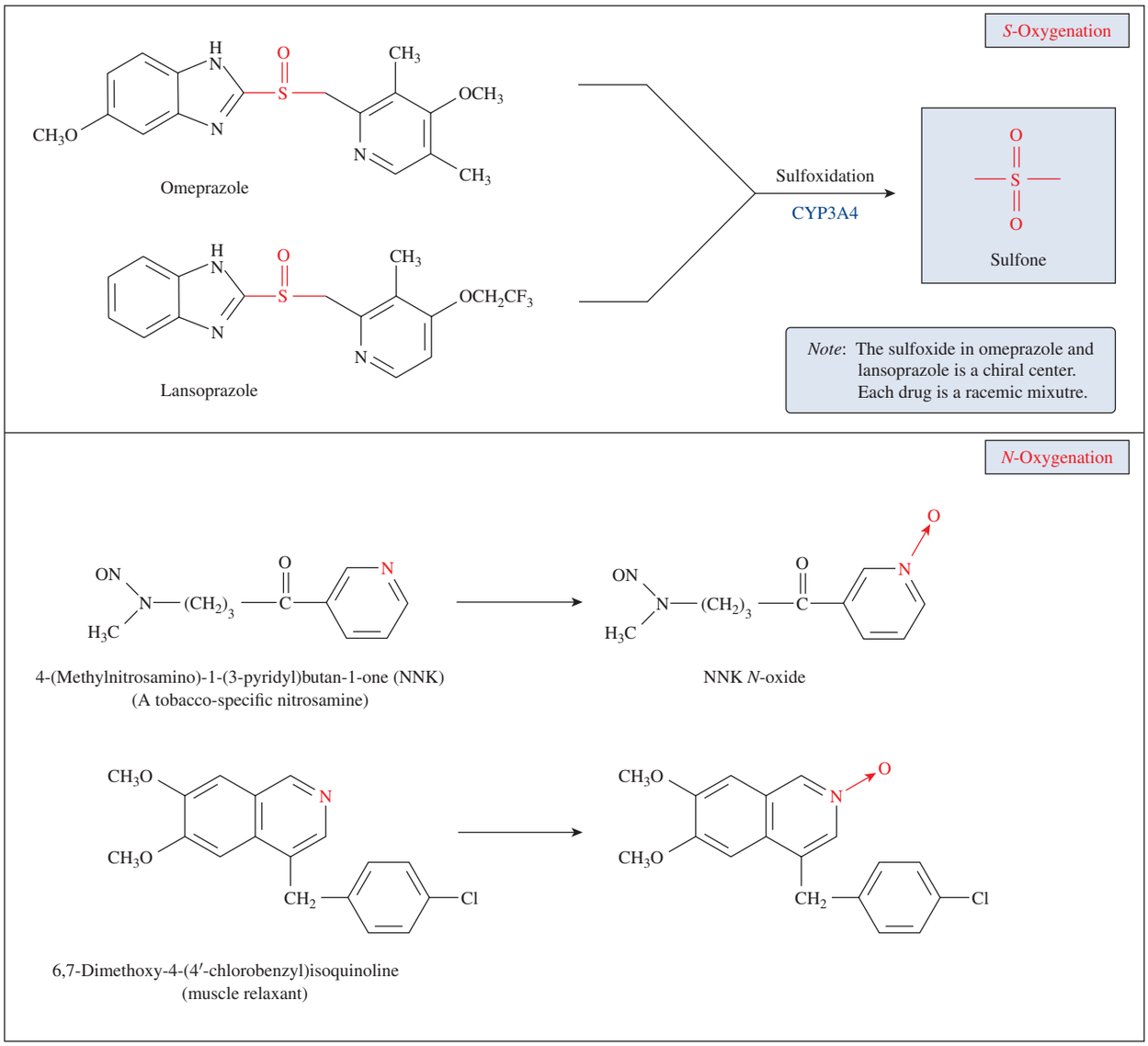
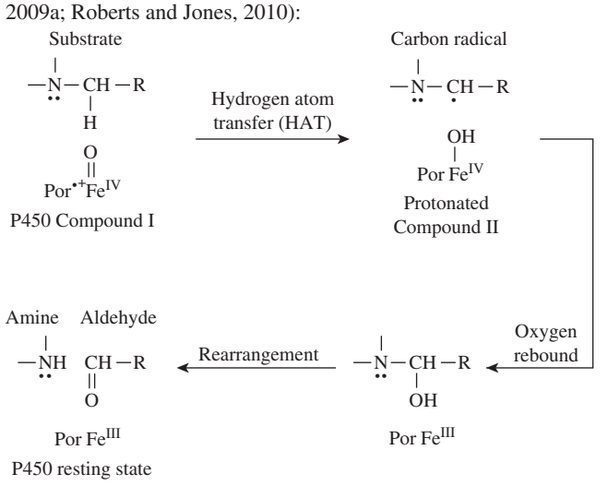


Figure 6-49. *Examples of reactions catalyzed by cytochrome P450: heteroatom oxygenation.*

and rearrangement as shown in the following scheme (Li et al., 2009a; Roberts and Jones, 2010):

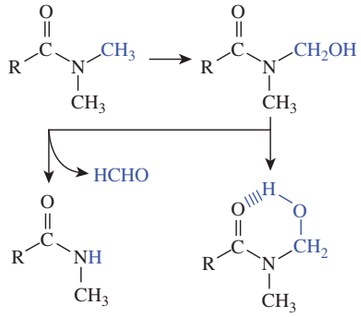


In general, CYP catalyzes the *N*-dealkylation, not the *N*-oxygenation, of amines. *N*-Oxygenation by CYP can occur if the nitrogen is next to an electron-donating group (making the nitrogen electron rich) or if α-protons are either absent (e.g., aromatic amines) or inaccessible (e.g., quinidine). In the case of aromatic amines (anilines; *aryl*-NH_2), *N*-oxidation by CYP usually results in the formation of hydroxylamines (*aryl*-NHOH). Some hydroxylamines are further oxidized to the corresponding nitroso metabolite, as observed with sulfamethoxazole (*aryl*-NH_2 → *aryl*-NOH → *aryl*-NO). Primary aromatic amides (*aryl*-$CONH_2$) are not substrates for *N*-oxidation, but secondary aromatic amides (*aryl*-NHCOR) are often *N*-hydroxylated by CYP (often by CYP1A2) to produce *N*-hydroxyamides (also known as hydroxamic acids). *N*-Hydroxylamines and hydroxyamides are of toxicological interest because under acidic conditions they can dissociate to form reactive nitrenium ions. Phenacetin, an aromatic amide (*aryl*-NH−CO−CH_3), causes kidney toxicity because it is converted by *N*-hydroxylation to a hydroxamic acid (*aryl*-NOH−CO−CH_3) that in the low pH of urine undergoes acid-catalyzed conversion to a

reactive nitrenium ion (*aryl*-NOH−CO−CH_3 + H^+ → *aryl*-N^+−CO−CH_3 + H_2O) (Testa and Krämer, 2008, 2010). *N*-Hydroxylation of aromatic amines with subsequent *O*-acetylation or *O*-sulfonation is a mechanism by which tumorigenic aromatic amines, such as 2-AAF and 4-aminobiphenyl, are converted to electrophilic reactive intermediates that bind covalently to DNA (discussed later in the sections "Glucuronidation and Formation of Acyl-CoA Thioesters" and "Acetylation").

Numerous xenobiotics are *N*-, *O*-, or *S*-dealkylated by CYP (but not by FMO), and some examples of these heteroatom dealkylation reactions are shown in Fig. 6-50. The dealkylation of xenobiotics containing an *N*-, *O*-, or *S*-methyl group results in the formation of formaldehyde, which can easily be measured by a simple colorimetric assay to monitor the demethylation of substrates in vitro. The expiration of ^{13}C- or ^{14}C-labeled carbon dioxide following the demethylation of drugs containing a ^{13}C- or ^{14}C-labeled methyl group has been used to probe CYP activity in vivo (Watkins, 1994). The activity of the human CYP enzymes involved in the *N*-demethylation of aminopyrine, erythromycin, and caffeine can be assessed by this technique. Although caffeine has three *N*-methyl groups, all of which can be removed by CYP, the major pathway in humans involves N_3-demethylation of caffeine to paraxanthine (see Fig. 6-50). Fig. 6-50 also presents an example of a xenobiotic (octamethylcyclotetrasiloxane, a component of cosmetics and deodorants) that undergoes silicone demethylation by CYP (Varaprath et al., 1999).

While the metabolism of *N*-methyl amines by CYP generally results in *N*-demethylation, with the hydroxylation of the methyl group leading to the release of formaldehyde, the metabolism of *N*-methyl amides and carbamates by CYP can result in the formation of a stable methyl-hydroxylated metabolite, one that does not release formaldehyde (which would otherwise complete an *N*-demethylation reaction) possibly because of the hydroxymethyl metabolite formation of a six-membered configuration that is stabilized by hydrogen bonding, illustrated as follows:



Zolpidem and camazepam are amide- and carbamate-containing drugs, respectively, that undergo such *N*-methyl-hydroxylation reactions.

Seto and Guengerich have shown that the *N*-demethylation and *N*-deethylation of *N*-ethyl-*N*-methylaniline not only proceed at different rates (with *N*-demethylation proceeding up to 20 times faster than *N*-deethylation) but also proceed by different mechanisms (Seto and Guengerich, 1993; Guengerich, 2001b). The initial step in the *N*-deethylation reaction involves HAT from the α-carbon atom (i.e., the carbon atom attached to the nitrogen), whereas the initial step in the *N*-demethylation reaction involves a much faster reaction, namely, a SET from the nitrogen atom, which is transferred to P450 compound I. Although the *N*-demethylation of *N*,*N*-substituted *amines* proceeds by the relatively rapid process of SET,

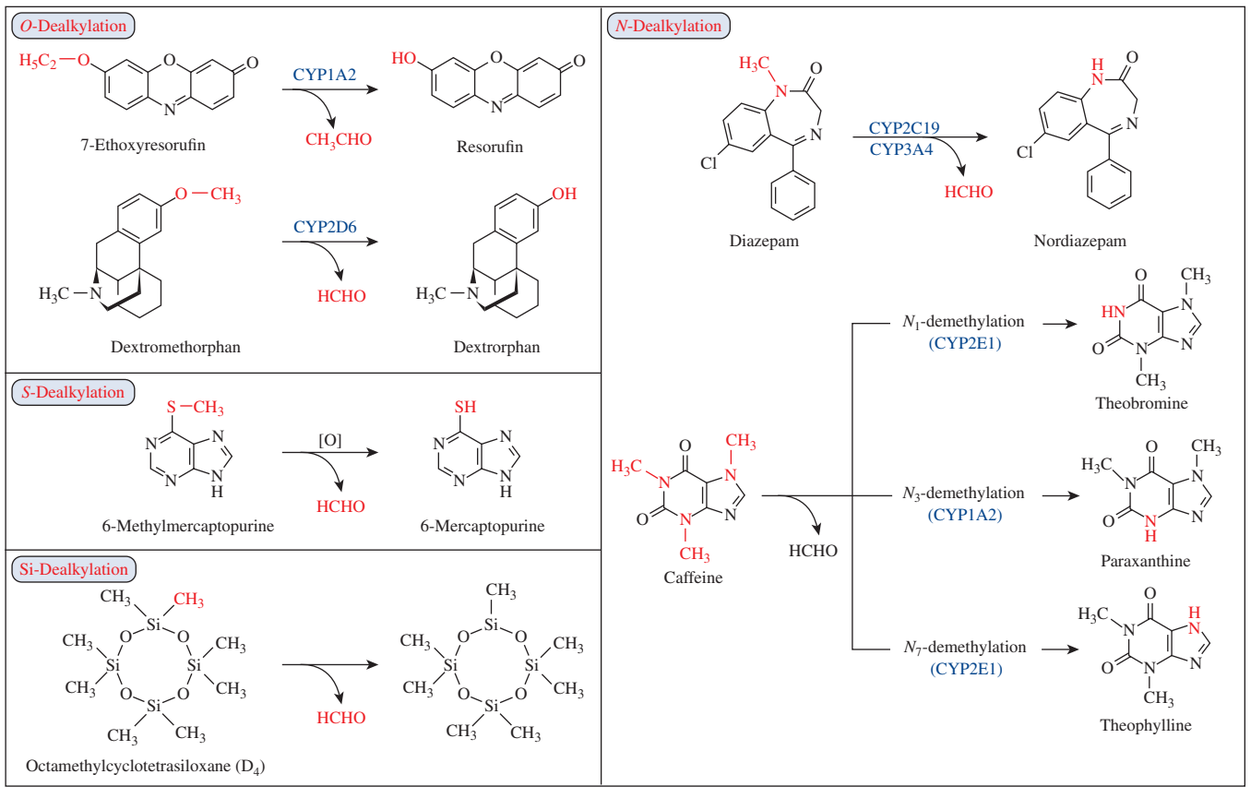


Figure 6-50. *Examples of reactions catalyzed by cytochrome P450: heteroatom dealkylation.*

the *N*-demethylation of *N*,*N*-substituted *amides*, where the adjacent carbonyl causes electrons to be withdrawn from the nitrogen atom, proceeds by the relatively slow process of HAT, for which reason the latter reactions, in contrast to the former, show a large intrinsic isotope effect when the hydrogen atoms are replaced with deuterium (because it requires more energy to break a C–D bond than a C–H bond).

In addition to *N*-dealkylation, some primary amines can also undergo oxidative deamination by CYP, which is an example of oxidative group transfer. The mechanism of oxidative deamination is similar to that of *N*-dealkylation: the α-carbon adjacent to the primary amine is hydroxylated, which produces an unstable intermediate that rearranges to eliminate ammonia with the formation of an aldehyde or ketone. The conversion of amphetamine to phenylacetone is an example of CYP-catalyzed oxidative deamination, as shown in Fig. 6-51. However, primary aliphatic amines tend to be poor substrates for CYP; in fact, primary alkylamines, such as the *N*-demethylated metabolite of fluoxetine, are metabolically stable and potent inhibitors of CYP inhibitors (discussed later in the section "Inhibition of Cytochrome P450"). Oxidative deamination is also catalyzed by MAO and FMO (Testa and Krämer, 2008, 2010). In the example given above, however, the substrate, amphetamine, contains an α-methyl group that renders it a poor substrate for MAO (as described in the section "Amine Oxidases").

In addition to oxidative deamination, CYP catalyzes two other types of oxidative group transfer, namely, oxidative desulfuration and oxidative dehalogenation. In all cases the heteroatom (*N*, *S*, or halogen) is replaced with oxygen. As shown in Fig. 6-52, oxidative desulfuration converts parathion, which has little insecticidal activity, to paraoxon, which is a potent insecticide. The same reaction converts thiopental to pentobarbital. Diethyldithiocarbamate methyl ester, a metabolite of disulfiram, also undergoes oxidative desulfuration. The initial reaction involves *S*-oxidation by CYP or FMO to a sulfine ($R_1R_2C{=}S \rightarrow R_1R_2C{=}S^+{-}O^-$). In the presence of GSH and GST, this sulfine either is converted back to the parent compound ($R_1R_2C{=}S^+{-}O^- + 2GSH \rightarrow R_1R_2C{=}S + H_2O$) or undergoes desulfuration ($R_1R_2C{=}S + 2GSH \rightarrow R_1R_2C{=}O + GSSG + H_2S$) (Madan et al., 1994). CYP catalyzes both reductive and oxidative dehalogenation reactions (Guengerich, 1991). During oxidative dehalogenation, a halogen and hydrogen from the same carbon atom are replaced with oxygen ($R_1R_2CHX \rightarrow R_1R_2CO$) to produce an aldehyde or acylhalide, as shown in Fig. 6-23, for the conversion of halothane ($CF_3CHClBr$) to trifluoroacetylchloride (CF_3COCl). Oxidative dehalogenation does not involve a direct attack on the carbon–halogen bond, but it involves the formation of an unstable halohydrin by oxidation of the carbon atom bearing the halogen substituent. The carbon–halogen bond is broken during the rearrangement of the unstable halohydrin. When the carbon atom contains a single halogen, the resulting product is an aldehyde, which can be further oxidized to a carboxylic acid or reduced to a primary alcohol. When the carbon atom contains two halogens, the dihalohydrin intermediate rearranges to an acylhalide, which can be converted to the corresponding carboxylic acid (see Fig. 6-23). Aldehydes and, in particular, acylhalides are reactive compounds that can bind covalently to protein and other critical cellular molecules. The immune hepatitis caused by repeated exposure of humans to halothane and related volatile anesthetics is dependent on oxidative dehalogenation by CYP, with neoantigens produced by the trifluoroacetylation of proteins, as shown in Fig. 6-23. Another example of oxidative dehalogenation by CYP is provided by studies of dasatinib metabolism by Li and colleagues who, in order to prevent the formation of a reactive quinoneimine metabolite that bound to GSH, used fluoro and chloro substitutions in an attempt to block *para*-hydroxylation of the substituted aniline in dasatinib, which is a metabolism-dependent inhibitor of CYP3A4 (Li et al., 2009b). The halogen substitutions did not block the formation of GSH adducts of the *para*-hydroxylated metabolite. The mechanism of oxidative dehalogenation of the substituted aniline in dasatinib (followed by glutathionylation) was presumed to arise from an epoxide intermediate

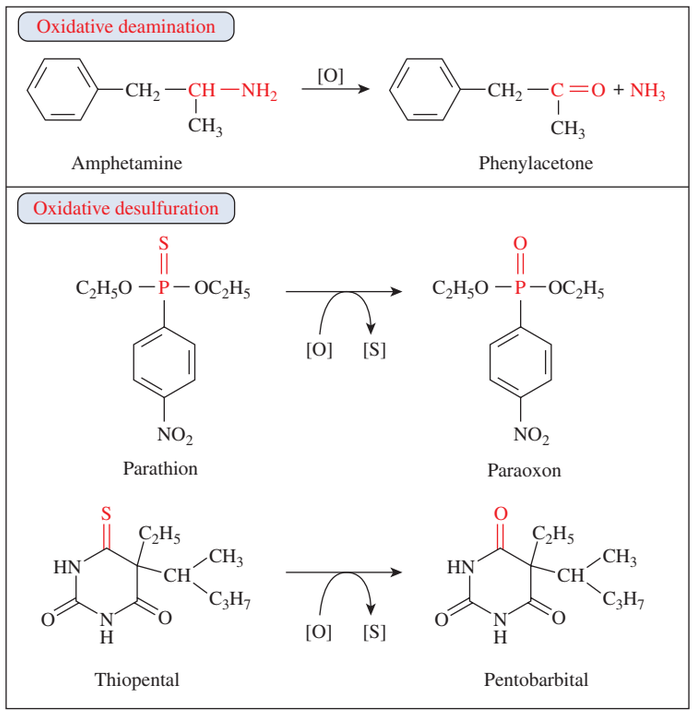


Figure 6-51. *Examples of reactions catalyzed by cytochrome P450: oxidative group transfer.*

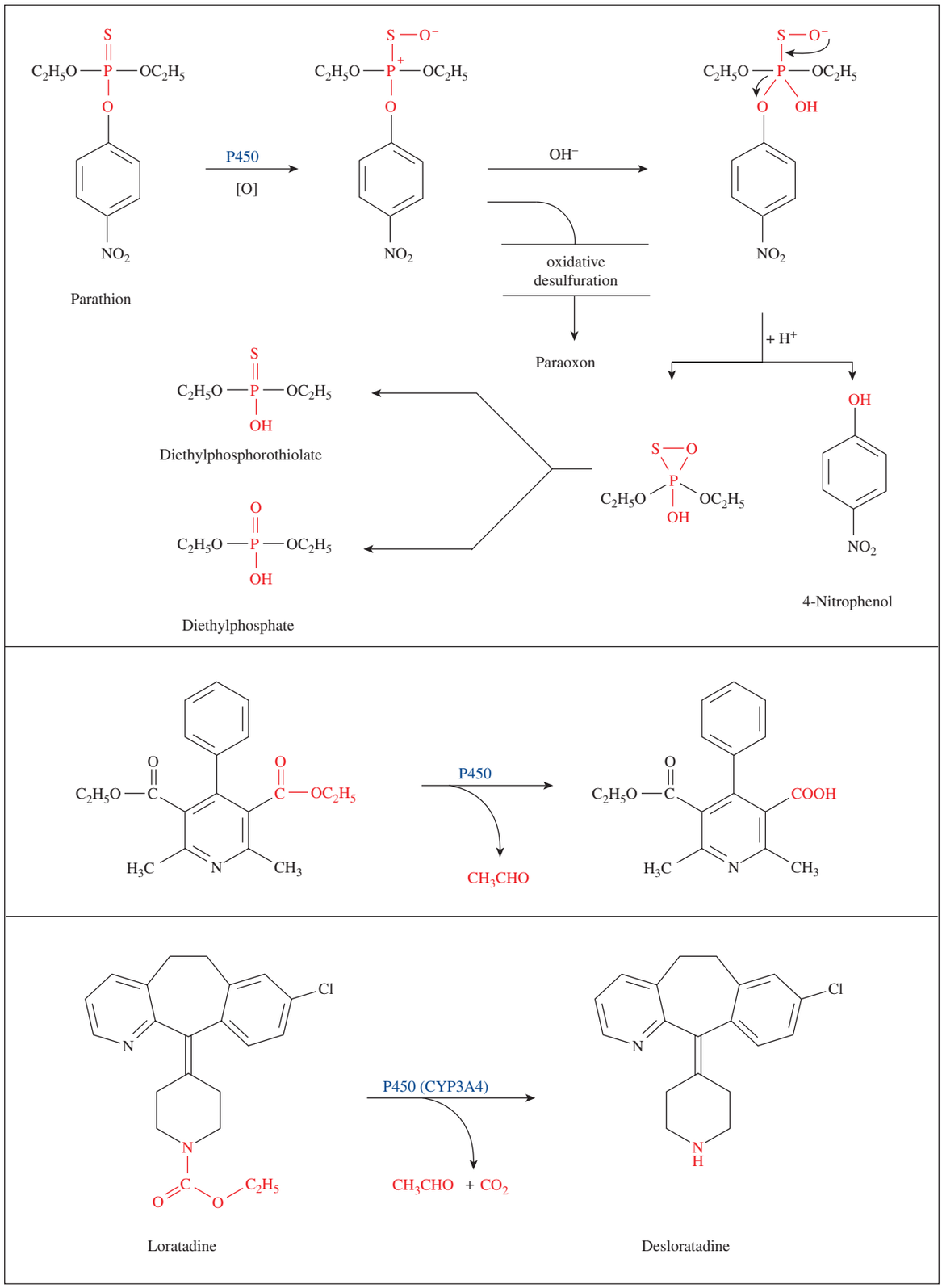


Figure 6-52. *Examples of reactions catalyzed by cytochrome P450 that resemble hydrolytic reactions: cleavage of a thiophosphate (parathion), a carboxylic acid ester (2,6-dimethyl-4-phenyl-3,5-pyridinecarboxylic acid diethyl ester), and a carbamate (loratadine).*

that underwent dehalogenation during its conjugation with GSH. Another example of oxidative defluorination is shown in Fig. 6-43 for the oxidative defluorination of 4-fluoro-*N*-methylaniline by FMO (Driscoll et al., 2010; Fan et al., 2016).

CYP can catalyze the reductive dehalogenation of halogenated alkanes (see Figs. 6-22 and 6-23) and the reduction of certain azo- and nitro-containing xenobiotics, although these latter reactions are largely catalyzed by gut microbiota (see Figs. 6-15 and 6-16). The ability of CYP to reduce xenobiotics can be understood from the catalytic cycle shown in Fig. 6-45. Binding of a substrate to CYP is followed by a one-electron reduction by POR. Under aerobic conditions, reduction of the heme iron to the ferrous state permits binding of oxygen. Anaerobic conditions, in contrast, interrupt the cycle at this point, which allows CYP to reduce those substrates capable of accepting an electron. Therefore, CYP can catalyze reduction reactions, such as azo-reduction, nitro-reduction, *N*-oxide reduction, sulfoxide reduction, and reductive dehalogenation, particularly under conditions of low oxygen tension. In effect, the substrate rather than molecular oxygen accepts electrons and is reduced. In fact, oxygen acts as an inhibitor of these reactions because it competes with the substrate for the reducing equivalents. The toxicity of many halogenated alkanes is dependent on their biotransformation by reductive dehalogenation. The first step in reductive dehalogenation is a one-electron reduction catalyzed by CYP, which produces a potentially toxic, carbon-centered radical and inorganic halide. The conversion of CCl_4 to a trichloromethyl radical and other toxic metabolites is shown in Fig. 6-22. The activation of the prodrug AQ4N by *N*-oxide reduction to form 4QA, a potent topoisomerase II inhibitor, by the hypoxia-inducible enzymes CYP2S1 and CYP2W1 represents a potentially new cancer chemotherapy, as discussed later in the section.

The oxidative desulfuration of parathion (see Fig. 6-51) involves the production of an intermediate that rearranges to paraoxon (as shown in Fig. 6-52). This same intermediate can decompose to 4-nitrophenol and diethylphosphorothioic acid, which are the same products formed by the hydrolysis of parathion (Fig. 6-52). In addition to facilitating the hydrolysis of phosphoric acid esters, CYP also catalyzes the cleavage of certain carboxylic acid esters and carbamates, as shown in Fig. 6-52. Carboxylic acid esters typically are cleaved by carboxylesterases and cholinesterases (see the section "Hydrolysis"), which results in the formation of an acid and an alcohol ($R_1COOCH_2R_2 + H_2O \rightarrow R_1COOH + R_2CH_2OH$). In contrast, CYP converts carboxylic acid esters to an acid plus aldehyde ($R_1COOCH_2R_2 + [O] \rightarrow R_1COOH + R_2CHO$), as shown in Fig. 6-52. The deacylation of loratadine, a carbamate, is the major route of biotransformation of this nonsedating antihistamine. The reaction is catalyzed predominantly by CYP (namely, CYP3A4 with a minor contribution from CYP2D6), with little contribution from carboxylesterases (Yumibe et al., 1996).

CYP can also catalyze the dehydrogenation of a number of compounds, including acetaminophen, nifedipine, and related dihydropyridine calcium channel blockers, sparteine, nicotine, and testosterone, as shown in Fig. 6-53. Dehydrogenation

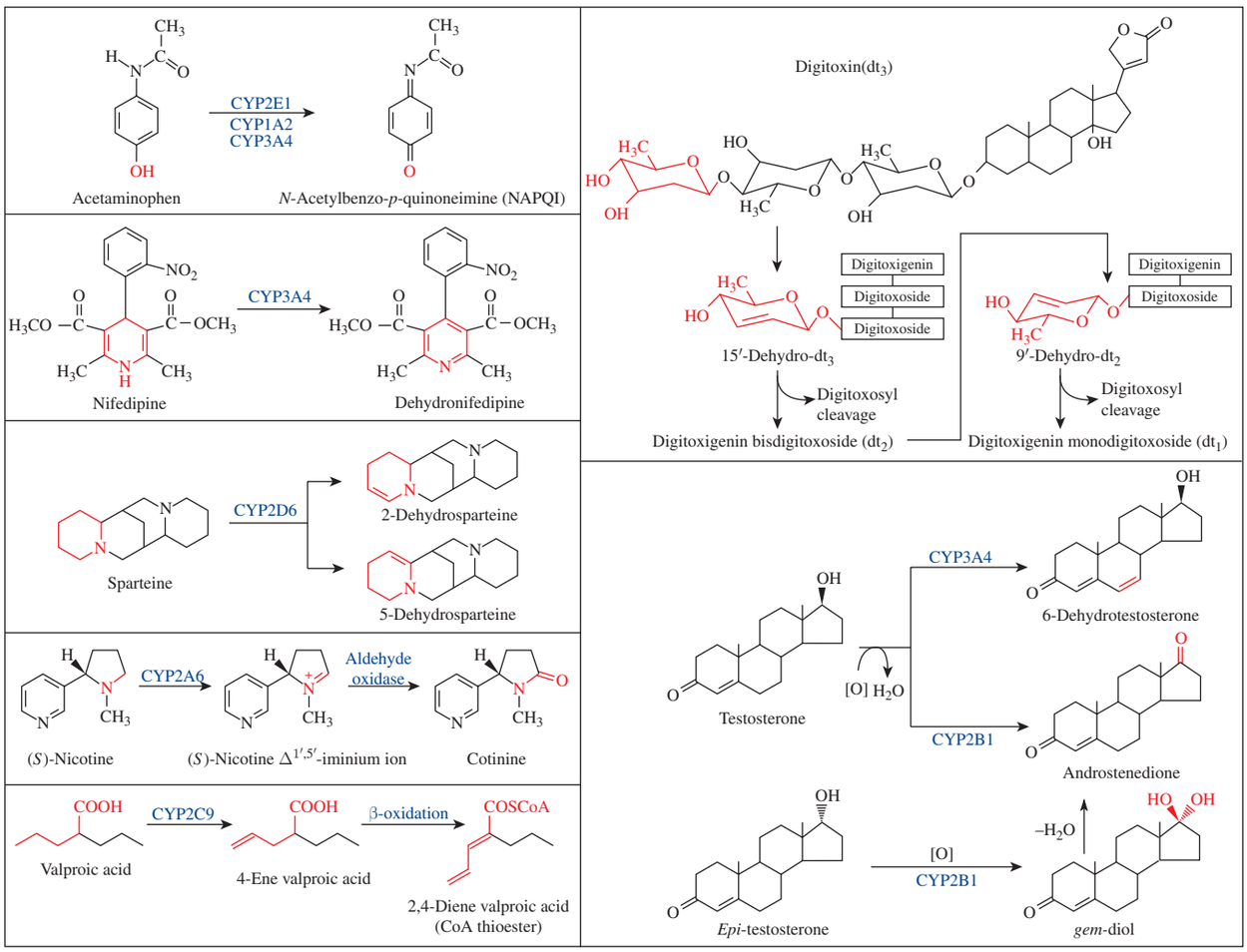


Figure 6-53. *Examples of reactions catalyzed by cytochrome P450: dehydrogenation.*

by CYP converts acetaminophen to its hepatotoxic metabolite *N*-acetylbenzo-*p*-quinoneimine (NAPQI), as shown in Fig. 6-39. The formation of a double bond during the conversion of digitoxin (dt_3) to 15′-dehydro-dt_3 leads to cleavage of the terminal sugar residue to produce digitoxigenin bisdigitoxoside (dt_2) (Fig. 6-53), which can similarly be converted to 9′-dehydro-dt_2, which undergoes digitoxosyl cleavage to digitoxigenin monodigitoxoside (dt_1). In contrast to digitoxin, the latter metabolite is an excellent substrate for glucuronidation. In rats, the CYP enzymes responsible for converting digitoxin to dt_1 (namely, the CYP3A enzymes) and the UGT responsible for glucuronidating dt_1 are inducible by dexamethasone, PCN, and spironolactone, all of which protect rats from the toxic effects of digitoxin. The dehydrogenation of nicotine produces nicotine $\Delta^{1',5'}$-iminium ion, which is oxidized by cytosolic AO to cotinine, a major metabolite of nicotine excreted in the urine of cigarette smokers (see Fig. 6-53). Although nicotine can be *N*-oxygenated by FMO, dehydrogenation by CYP2A6 is responsible for 80% of the clearance of nicotine from cigarette smoking (Hukkanen et al., 2005).

Testosterone is dehydrogenated by CYP to two metabolites: 6-dehydrotestosterone, which involves formation of a carbon–carbon double bond, and androstenedione, which involves formation of a carbon–oxygen double bond, as shown in Fig. 6-53. The conversion of testosterone to androstenedione is one of several cases where CYP converts a primary or secondary alcohol to an aldehyde or ketone, respectively. The reaction can proceed by formation of a *gem*-diol (two hydroxyl groups on the same carbon atom), with subsequent dehydration to a keto group, as shown in Fig. 6-29, for the conversion of ethanol to acetaldehyde. However, *gem*-diols are not obligatory intermediates in the oxidation of alcohols by CYP, and in fact the conversion of testosterone to androstenedione by CYP2B1 (the major phenobarbital-inducible CYP enzyme in rats) does not involve the intermediacy of a *gem*-diol but proceeds by direct dehydrogenation (Fig. 6-53). In contrast, a *gem*-diol is involved in the formation of androstenedione from *epi*-testosterone (which is identical to testosterone except the hydroxyl group at C_{17} is in the α-configuration, not the β-configuration) (Wood et al., 1988). The fact that formation of androstenedione from *epi*-testosterone involves formation of a *gem*-diol, whereas its formation from testosterone does not, makes it difficult to generalize the mechanism by which CYP converts alcohols to aldehydes and ketones.

Cytochrome P450 Nomenclature Liver microsomes from all mammalian species contain numerous CYP enzymes (Table 6-15), each with the potential to catalyze the various types of reactions shown in Figs. 6-46 to 6-53. In other words, all of the CYP enzymes expressed in liver microsomes have the potential to catalyze xenobiotic hydroxylation, epoxidation, dealkylation, oxygenation, dehydrogenation, and so forth. The broad and often overlapping substrate specificity of liver microsomal CYP enzymes precludes the possibility of naming these enzymes for the reactions they catalyze, which are now categorized into families and subfamilies and named individually on the basis of their amino acid sequence. As shown in Tables 6-14 and 6-15, the CYP enzymes involved in xenobiotic biotransformation belong mainly to the CYP1, CYP2, and CYP3 gene families, although on a case-by-case basis CYP enzymes in other gene families play a key role in xenobiotic biotransformation. The CYP enzymes involved in endobiotic metabolism generally have the same name in all mammalian species. Some of the xenobiotic-biotransforming CYP enzymes have the same name in all mammalian species, whereas others are named in a species-specific manner. For example, all mammalian species contain two CYP enzymes belonging to the CYP1A subfamily, and in all cases these are known as CYP1A1 and CYP1A2 because the function and regulation of these enzymes are highly conserved among mammalian species. The same is true of CYP1B1, CYP2E1, CYP2R1, CYP2S1, CYP2U1, and CYP2W1, all of which are highly conserved homologs that can be given the same name across mammalian species. In most other cases, functional or evolutionary relationships are not immediately apparent; hence, the CYP enzymes are named in a species-specific manner, and the names are assigned in chronological order regardless of the species of origin. For example, human liver microsomes express CYP2D6, but this is the only functional member of the CYP2D subfamily found in the human liver. CYP2D7 and CYP2D8 are human pseudogenes. The other members of this subfamily (i.e., CYP2D1 to CYP2D5 and CYP2D9 onward) are the names given to CYP2D enzymes in other species.

Genetic Variation and Drug Interactions Without exception, the levels and activity of each CYP enzyme have been shown to vary from one individual to the next, due to extrinsic (e.g., environmental) and/or intrinsic (e.g., genetic) factors. Decreased CYP enzymatic activity can result from (1) a genetic mutation that either blocks the synthesis of a CYP enzyme or leads to the synthesis of a catalytically compromised, inactive, or unstable enzyme, which gives rise to the PM and IM genotypes; (2) exposure to an environmental factor (such as an infectious disease or an inflammatory process) that suppresses CYP enzyme expression; or (3) exposure to a xenobiotic that inhibits or inactivates a preexisting CYP enzyme. By inhibiting CYP, one drug can impair the biotransformation of another, which may lead to an exaggerated pharmacological or toxicological response to the second drug. In this regard, inhibition of CYP by a drug (and suppression of CYP by infection, vaccination, or inflammation) essentially mimics the effects of a genetic deficiency in CYP enzyme expression (i.e., these environmental factors mimic the IM or PM genotype depending on the degree to which they decrease CYP activity). Genetic deficiencies in CYP expression, CYP inhibition, and, to a lesser extent, CYP suppression all contribute significantly to interindividual variability in drug metabolism and toxicity, and inhibition of CYP activity is a major cause of drug–drug interactions. Examples of the impact of these genetic and environmental factors on drug metabolism and toxicity are given later in this section in the overviews of individual CYP enzymes and in the section "Inhibition of Cytochrome P450."

Increased CYP enzymatic activity can result from (1) gene duplication leading to overexpression of a CYP enzyme, which gives rise to the UM genotype; (2) gene mutations in the coding or promoter region that increase expression, activity, or stability of CYP; (3) exposure to drugs and other xenobiotics that induce the synthesis or retard the degradation of CYP; or (4) exposure to drugs and other xenobiotics that stimulate the activity of a preexisting enzyme (a process known as homotropic or heterotropic activation depending on whether the drug stimulates its own metabolism or the metabolism of other drugs, respectively). Activation of CYP has been documented in vitro and in some in situ situations, such as the pronounced (up to 25-fold) activation of *R*-warfarin 10-hydroxylation by quinidine in rabbit liver microsomes and perfused rabbit liver (Chen et al., 2004a). However, in general, activation does not appear to be a major cause of drug–drug interactions. Although duplication of functional CYP2D6 genes has been documented (and shown to be relevant to drug metabolism and safety [see the section "CYP2D6"]), induction of CYP by xenobiotics is the most common mechanism by which CYP enzyme activity is increased to a pharmacologically relevant extent. By inducing CYP, one drug can stimulate the metabolism of a second drug and thereby decrease or ameliorate its therapeutic effect. Enzyme induction is a particular concern when it compromises the therapeutic effectiveness of drugs

that have a narrow therapeutic index and are being used to treat a life-threatening illness, such as anti-HIV drugs, antirejection drugs (such as cyclosporine and tacrolimus), and oral anticoagulants (such as warfarin), or when it is used with drugs that exhibit a quantal (all-or-nothing) dose–response relationship, such as oral contraceptive steroids (which either block or do not block ovulation and thereby provide or do not provide protection against pregnancy).

The environmental factors known to affect CYP levels include medications (e.g., prescription drugs and herbal remedies), foods (e.g., cruciferous vegetables and charcoal-broiled beef), social habits (e.g., alcohol consumption and cigarette smoking), and disease status (diabetes, infection, inflammation, vaccination, liver and kidney disease, and both hyperthyroidism and hypothyroidism). When environmental factors influence CYP enzyme levels, considerable variation may be observed during repeated measures of xenobiotic biotransformation (e.g., drug metabolism) in the same individual. Such variation is not observed when alterations in CYP activity are determined genetically.

Due to their broad substrate specificity, it is possible that two or more CYP enzymes can contribute to the metabolism of a single compound. Three human CYP enzymes, CYP1A2, CYP2E1, and CYP3A4, can convert the commonly used analgesic acetaminophen to its hepatotoxic metabolite NAPQI (Figs. 6-38 and 6-53). It is also possible for a single CYP enzyme to catalyze two or more metabolic pathways for the same drug. For example, CYP2D6 catalyzes both the *O*-demethylation and 5-hydroxylation (aromatic ring hydroxylation) of methoxyphenamine, and CYP3A4 catalyzes the 3-hydroxylation and *N*-oxygenation of quinidine, the 1′- and 4-hydroxylation of midazolam, the *tert*-butyl-hydroxylation and *N*-dealkylation of terfenadine, and several pathways of testosterone oxidation, including 1β-, 2β-, 6β-, and 15β-hydroxylation and dehydrogenation to 6-dehydrotestosterone (Figs. 6-46 and 6-53).

The pharmacological or toxic effects of certain drugs are exaggerated in a significant percentage of the population due to a heritable deficiency in a CYP enzyme (Meyer, 1994; Tucker, 1994; Zhou, 2009b). The observation that individuals who are genetically deficient in a particular CYP enzyme are PMs of one or more drugs illustrates a very important principle, namely, that the rate of elimination of drugs can be largely determined by a single CYP enzyme. This observation seems to contradict the fact that CYP enzymes have broad and overlapping substrate specificities. The resolution to this apparent paradox lies in the fact that although more than one human CYP enzyme can catalyze the biotransformation of a xenobiotic, they may do so with markedly different affinities. Consequently, xenobiotic biotransformation in vivo, where only low substrate concentrations are usually achieved, is often determined by the CYP enzyme with the highest affinity (lowest apparent K_m) for the xenobiotic. For example, the 5-hydroxylation of lansoprazole, which represents the key route of elimination of this proton pump inhibitor, is catalyzed by both CYP2C19 and CYP3A4. However, these reactions are catalyzed by CYP3A4 with such low affinity that the 5-hydroxylation (and hence the clearance) of lansoprazole in vivo is largely determined by CYP2C19 (Pearce et al., 1996b). When several CYP enzymes catalyze the same reaction, their relative contribution to xenobiotic biotransformation is determined by the kinetic parameter V_{max}/K_m, which is a measure of in vitro intrinsic metabolic clearance at low substrate concentrations (less than 10% of K_m).

A drug whose clearance is largely determined by a single CYP enzyme (or any single route of elimination) is said to have high *victim* or *object* potential, meaning its rate of clearance will be decreased by genetic polymorphisms and inhibitory drugs that result in a loss of that particular CYP activity and increased by drugs and other xenobiotics that induce that particular CYP enzyme. For example, a drug that is largely cleared by CYP2D6 will be slowly metabolized in CYP2D6 PMs and rapidly metabolized in CYP2D6 UMs, whereas a drug whose clearance is largely determined by CYP3A4 will be slowly metabolized in the presence of ketoconazole (and other CYP3A4 inhibitors) and rapidly metabolized in the presence of rifampin or St. John's wort (and other CYP3A4 inducers). The extent to which genetic polymorphisms and CYP inhibitors/inducers impact the disposition of a drug (or any other xenobiotic) is determined by fm, the fraction of clearance attributable to the affected enzyme. This principle applies to all xenobiotic-biotransforming enzymes and is explained in detail in Point 24 in the section "Principles of Xenobiotic Biotransformation." Point 24 also covers the principle of drug–drug interactions in terms of perpetrator–victim interactions (in which the disposition of one drug, the victim, is impacted by a single perpetrator such as an inhibitory drug or a genetic polymorphism) and perpetrator–perpetrator–victim interactions, otherwise known as "maximum exposure," which involves the dramatic (synergistic) impact of two perpetrators blocking two parallel pathways of clearance of the victim drug (Collins et al., 2006; Ogilvie and Parkinson, 2014).

Accordingly, for drugs under development, considerable attention is paid to identifying which CYP enzyme or enzymes are involved in eliminating the drug, a process known as *reaction phenotyping* or *enzyme mapping*. Four in vitro approaches have been developed for reaction phenotyping (Williams et al., 2003a; Ogilvie et al., 2008; Zientek and Youdim, 2015; Bohnert et al., 2016). Each has its advantages and disadvantages, and a combination of approaches is usually required to identify which human CYP enzyme is responsible for metabolizing a xenobiotic. The four approaches to reaction phenotyping are as follows:

1. Correlation analysis, which involves measuring the rate of xenobiotic metabolism by several samples of human liver microsomes and correlating reaction rates with the variation in the level or activity of the individual CYP enzymes in the same microsomal samples. This approach is successful because the levels of the CYP enzymes in human liver microsomes vary enormously from sample to sample as shown in Table 6-16.
2. Chemical inhibition, which involves an evaluation of the effects of known CYP enzyme inhibitors on the metabolism of a xenobiotic by human liver microsomes. Chemical inhibitors of CYP, which are discussed later, must be used cautiously because most of them can inhibit more than one CYP enzyme. Some chemical inhibitors are metabolism-dependent inhibitors that require biotransformation to a metabolite that inactivates or noncompetitively inhibits CYP.
3. Antibody inhibition, which involves an evaluation of the effects of inhibitory antibodies against selected CYP enzymes on the biotransformation of a xenobiotic by human liver microsomes. Due to the ability of antibodies to inhibit selectively and noncompetitively, this method alone can potentially establish which human CYP enzyme is responsible for biotransforming a xenobiotic.
4. Biotransformation by purified or recombinant (cDNA-expressed) human CYP enzymes, which can establish whether a particular CYP enzyme can or cannot biotransform a xenobiotic, but it does not address whether that CYP enzyme, contributes substantially to reactions catalyzed by human liver microsomes. The information obtained with purified or recombinant human CYP enzymes can be improved by taking into account large differences in the extent to which the individual CYP enzymes are expressed in human liver microsomes.

Table 6-16

Specific Content of Individual Cytochrome P450 (CYP) Enzymes in Human Liver Microsomes (HLM)

		INDIVIDUAL HLM (n = 17)[†]		
ENZYME	POOLED HLM[*]	MEAN	RANGE (MINIMUM–MAXIMUM)	FOLD VARIATION (MAXIMUM/MINIMUM)
CYP1A2	17.7	25.1	3.26–65.5	20.1
CYP2A6	49.2	56.2	5.45–168	30.8
CYP2B6	6.86	6.72	4.05–14.9	3.7
CYP2C8	29.3	30.0	5.66–83.5	14.8
CYP2C9	80.2	76.3	40.2–115	2.9
CYP2C19	3.64	5.31	2.08–22.2	10.7
CYP2D6	11.5	17.2	6.16–36.4	5.9
CYP2E1	51.3	66.1	36.3–147	4.0
CYP3A4	64.0	60.4	6.22–270	43.4
CYP3A5	3.54	3.86	2.48–17.1	6.9
CYP3A7	ND	2.40	BLQ–9.39	NA
CYP3A43	1.30	4.10	BLQ–6.42	NA
CYP4A11	ND	21.8	9.29–46.5	5.0
CYP4F2[‡]	ND	14.3	1.3–27.1	21
CYP4F3B[‡]	ND	11.3	6.4–20.9	3.3
P450 OR	ND	71.6	41.7–99.0	2.4

Abbreviations: ND, not determined; BLQ, below limit of quantitation; NA, not applicable; P450 OR, NADPH-cytochrome P450 reductase.

[*] *Data from Kawakami et al. (2011).*

[†] *Unless noted otherwise, data from Ohtsuki et al. (2012).*

[‡] *Data from Michaels and Wang (2014).*

The specific content of the major xenobiotic-biotransforming enzymes in human liver microsomes has been determined by mass spectrometry and the values are summarized in Table 6-16. The extrapolation of metabolic rates obtained with recombinant enzymes to those expected to occur in liver microsomes can be based on specific content, relative activity factors (RAF), or a combination of both called intersystem extrapolation factor (ISEF) (Proctor et al., 2004; Ogilvie et al., 2008).

Some CYP enzymes, such as CYP1A1 and CYP1B1, are expressed at such low levels in human liver microsomes that they do not contribute significantly to the hepatic biotransformation of xenobiotics that are excellent substrates for these enzymes. Other CYP enzymes are expressed in some but not all livers, as discussed later under each individual enzyme subheading. It should be emphasized that reaction phenotyping in vitro is not always carried out with pharmacologically or toxicologically relevant substrate concentrations. As a result, the CYP enzyme that appears responsible for biotransforming the drug in vitro may not be the CYP enzyme responsible for biotransforming the drug in vivo (Ogilvie et al., 2008). This may be particularly true of CYP3A4, which metabolizes several drugs with high capacity but low affinity.

Reaction phenotyping (often in conjunction with clinical observation) has been used to characterize the substrate specificity of many of the CYP enzymes expressed in human liver microsomes, as discussed later under each individual enzyme subheading. Examples of reactions catalyzed by individual human CYP enzymes are shown in Figs. 6-46 to 6-53, and lists of substrates, inhibitors, and inducers for each CYP enzyme are given in Table 6-17. The examples listed in Table 6-17 are largely based on examples cited by the FDA which has provided, where possible, the following lists to guide the conduct of in vitro and in vivo pharmacokinetic drug–drug interaction studies: (1) preferred and acceptable CYP substrates and chemical inhibitors for reaction phenotyping in vitro; (2) sensitive index substrates (formerly called probe substrates) to monitor CYP activity in vivo (a sensitive substrate is one whose clearance is largely [>80%] determined by a single CYP enzyme such that loss of that CYP enzyme causes a fivefold or higher increase in systemic exposure); (3) drugs that are strong, moderate, or weak inhibitors of CYP enzymes in vivo; (4) preferred and acceptable inhibitors and inducers for use as positive controls for CYP inhibition and induction studies in vitro; and (5) drugs that are effective inducers of CYP enzymes in vivo (FDA, 2017). Similar and more complete lists of CYP substrates, inhibitors, and inducers can be found at the University of Washington Drug Interaction Database (University of Washington DIDB, 2018).

The global features of the major xenobiotic-biotransforming human CYP enzymes are as follows:

1. Two CYP enzymes, namely, CYP2D6 and CYP3A4, metabolize the majority of orally effective drugs, but they often metabolize different drugs and they do not metabolize all drugs; consequently, there are many drugs whose clearance is largely determined by CYP enzymes other than CYP2D6 or CYP3A4. Interindividual variation in CYP2D6, which is largely confined to the liver, is largely determined by genetic factors, whereas interindividual variation in CYP3A4, which is

Table 6-17

Examples of Clinically Relevant Substrates, Inhibitors, and Inducers of the Major Human Liver Microsomal P450 Enzymes Involved in Xenobiotic Biotransformation

	CYP1A2	CYP2A6	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2E1
SUB-STRATES	Alosetron[*] Caffeine[*,‡,§] Duloxetine[*] 7-Ethoxyresorufin[‡] Melatonin[*] Phenacetin[†] Pirfenidone[*] Ramelteon[*] Selegiline[*] Tacrine[*,‡] Tasimelteon[*] Tizanidine[*,**] Theophylline[§,**]	Coumarin[†] Nicotine[†] Efavirenz Tegafur	Bupropion[*,†] Efavirenz[*,†,§] Propofol[‡] *(S)*-Mephenytoin[‡] Cyclophosphamide Ketamine Meperidine Nevirapine Ifosfamide Pethidine Methadone	Amodiaquine[‡] Cerivastatin Dasabuvir[*] GSK1278863[*] Montelukast Paclitaxel[†,**] Rosiglitazone[‡,§] Repaglinide[*,§]	Celecoxib[*] Diclofenac[†] Fluoxetine[‡] Flurbiprofen[‡] Meloxicam Peroxicam Phenytoin[**] Tolbutamide[†,§] *(S)*-Warfarin[†,§,**]	Clobazam[*] Diazepam Esomeprazole Fluoxetine[‡] Gliclazide *(R)*-Hexobarbital *(S)*-Mephenytoin[*,†,**] *(R)*-Mephobarbital Lansoprazole[*,§] Moclobemide Omeprazole[*,‡,§] Pantoprazole[§] Proguanil Tilidine[*]	Aniline[‡] Acetaminophen Chlorzoxazone[†] Lauric acid[‡] 4-Nitrophenol[‡]
INHIBI-TORS	Acyclovir[††] Allopurinol[††] Caffeine[††] Cimetidine[††] Ciprofloxacin[§§] Clinafloxacin[§§] Daidzein[††] Disulfiram[††] Echinacea[††] Enoxacin[§§] Famotidine[††] Fluvoxamine[§,§§] Furafylline[†] Methoxsalen[‡‡] Mexilitene[‡‡] α-Naphthoflavone[‡] Norfloxacin[††] Oltipraz[§§] Oral contraceptives[‡‡] Propafenone[††] Propranolol[††] Phenylpropanolamine[‡‡]	Methoxsalen[†] Pilocarpine[‡] Selegiline Tranylcypromine[†] Tryptamine[‡]	Boceprevir[††] Clopidogrel[‡,††] Clorgyline 3-Isopropenyl-3-methyl diamantane[‡] 2-Isopropenyl-2-methyladamantane[‡] Pargyline Phencyclidine[‡] Phenelzine Phenylethylpiperidine Prasugrel[††] Rolapitant[††] Selegiline Sertraline[‡] Tenofovir[††] Thio-TEPA[‡] Ticlopidine[‡,††] Voriconazole[††]	Clopidogrel glucuronide[§§] Deferasirox[‡‡] Fluvoxamine[††] Gemfibrozil glucuronide[‡,§,§§] Ketoconazole[††] Montelukast[†] Pioglitazone[‡] Quercetin[†] Rosiglitazone[‡] Rosuvastatin Teriflunomide[‡‡] Trimethoprim[‡,††]	Amiodarone[§,‡‡] Ataciguat[‡‡] Azapropazone[‡‡] Benzbromarone[‡‡] Bucolome[‡‡] Capecitabine[††] Cotrimoxazole[††] Etravirine[††] Fluconazole[‡,§,‡‡] Fluoxetine[‡] Fluvastatin[††] Fluvoxamine[‡,††] Metronidazole[††] Miconazole[‡‡] Oxandrolone[‡‡] Phenylbutazone[‡‡] Sulfaphenazole[†] Sulfinpyrazone[††] Tienilic acid Tigecycline[††] Voriconazole[††] Zafirlukast[††]	Allicin[††] Armodafinil[††] Carbamazepine[††] Cimetidine[††] Esomeprazole[‡‡] Efavirenz[‡‡] Etravirine[††] Felbamate[††] Fluconazole[§§] Fluoxetine[‡‡] Fluvoxamine[§,§§] Human growth hormone (rhGH)[††] Ketoconazole[††] Moclobemide[§,‡‡] Nootkatone[‡] Omeprazole[§,‡‡] Oral contraceptives[††] Ticlopidine[‡,§§] Voriconazole[e]	Clomethiazole[‡] Diallyldisulfide[‡] Diethyldithiocarbamate[‡] Disulfiram[§] Isoniazid[‡‡]

(*continued*)

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Table 6-17
(*Continued*)

	CYP1A2	CYP2A6	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2E1
	Rofecoxib[§§] Terbinafine[††] Thiabendazole[‡‡] Ticlopidine[††] Vemurafenib[‡‡] Verapamil[††] Zafirlukast[§§] Zileuton[‡‡]						
INDUCERS	Cigarette smoking[†††] Lansoprazole[‡] Omeprazole[†,†††] 3-Methylcholanthrene[†] Montelukast Moricizine[†††] β-Naphthoflavone[†] Phenobarbital[†††] Phenytoin[***] Rifampin[†††] Ritonivir[†††] TCDD Teriflunomide[†††]	Dexamethasone[†] Pyrazole[‡]	Carbamazepine[‡‡‡] Efavirenz[***] Nevirapine[†††] Phenobarbital[†] Phenytoin[‡] Rifampin[***] Ritonavir[†††] St. John's wort[***]	Carbamazepine[†††] Oral contraceptives[†††] Phenobarbital[‡] Rifampin[†,§,***]	Aprepitant[†††] Bosentan[†††] Carbamazepine[***] Enzalutamide[†††] Phenobarbital[†††] Rifampin[***] Ritonavir[†††] St. John's wort[†††]	Artemisinin[†††] Carbamazepine[***] Efavirenz[***] Enzalutamide[†††] Ginkgo[***] Phenobarbital Rifampin[†,§,***] St. John's wort[***]	Cigarette smoking[***] Ethanol[§] Isoniazid

	CYP2D6			CYP3A4			
SUBSTRATES	Atomoxetine[*,§] Amitriptyline Aripiprazole Brofaromine (±)-Bufuralol[†] (*S*)-Chlorpheniramine Chlorpromazine Clomipramine Codeine Debrisoquine[‡] Desipramine[*,§] Dextromethorphan[*,†,§] Dolasetron Doxepin Duloxetine Eliglustat[*] Encainide[*]	Methylphenidate Metoprolol[*] Mexiletine Morphine Nebivolol[*] Nicergoline[*] Nortriptyline Ondansetron Paroxetine Perhexilene Perphenazine[*] Pimozide[**] Propafenone (+)-Propranolol Risperidone[*] Sparteine Tamoxifen	Alfentanil[*,**] Alfuzosin Alisporivir[*] Almorexant Alprazolam Amlodipine Amprenavir Aplaviroc[*] Aprepitant[*] Artemether Astemiziole[**] Atazanavir Atorvastatin Avanafil[*] Azithromycin Barnidipine Bexarotene	Cyclosporine[**] Danoprevir[*] Darifenacin[*] Darunavir[*] Dasatinib[*] Depsipeptide Dexamethasone Dextromethorphan[‡] Diergotamine[**] α-Dihydroergocriptine Dihydroergotamine[**] Disopyramide Docetaxel Domperidone Dronedarone[*] Dutasteride Ebastine	Etoricoxib Felodipine[*,§] Fentanyl[**] Fluticasone[*] Gallopamil Gefitinib Gepirone Gestodene Granisetron Grazoprevir[*] Halofantrine Ibrutinib[*] Imatinib Indinavir[*] Isavuconazole[*] Isradipine Itraconazole	Methylprednisolone Mexazolam Midazolam[*,†,§] Midostaurin[*] Mifepristone Mosapride Naloxegol[*] Neratinib[*] Nicardipine Nifedipine[‡] Nimoldipine Nisoldipine[*] Nitrendipine Norethindrone Oxatomide Oxybutynin Paritaprevir[*]	Saquinavir[*] Sildenafil[*,§] Sibutramine Simeprevir[*] Simvastatin[*,§] Sirolimus[*,**] Sunitinib Tacrolimus[**] Tadalafil Telithromycin Terfenadine[‡,**] Testosterone[†] Tiagabine Ticagrelor[*] Tilidine[*] Tipranavir[*] Tirilazad

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	Fentanyl *(S)*-Fluoxetine[*] Haloperidol (reduced) Ibogaine[*] Imipramine Loperamide *(R)*-Metoprolol[*]	Thioridazine[**] Timolol Tolperisone[*] Tolterodine[*] Tramadol Traxoprodil[*] *(R)*-Venlafaxine[*] Vernakalant[*]	Bortezomib Bosutinib[*] Brecanavir Brotizolam Budesonide[*] Buspirone[*,§] Capravirine Carbamazepine Casopitant[*] Cibenzoline Cilastazol Cisapride[**] Clarithromycin Clindamycin Clopidogrel Cobimetinib[*] Conivaptan[*]	Eletriptan[*,§] Elvitegravir[*] Eplerenone[*] Ergotamine[**] Erlotinib Erythromycin[‡] Eplerenone Ethosuximide Etoperidone Everolimus[*] Ethinyl estradiol	Ivabradine[*] Ivacaftor[*] Karenitecin Ketamine Laquinimod Levomethadyl Lomitapide[*] Lonafarnib Loperamide Lopinavir[*] Lovastatin[*,§] Lumefantrine Lurasidone[*] Maraviroc[*] Medroxyprogesterone	Perospirone Pimozide[**] Pranidipine Praziquantel Quetiapine[*] Quinidine[**] Quinine Reboxetine Ridaforolimus[*] Rifabutin Ritonavir Rosuvastatin Ruboxistaurin Salmeterol	Tofisopam Tolvaptan[*] Triazolam[‡] Trimetrexate Ulipristal[*] Vardenafil[*] Vicriviroc[*] Vinblastine Vincristine Vinorelbine Voclosporin[*] Ziprasidone Zonisamide
INHIBITORS	Amiodarone[††] Buproprion[§§] Celecoxib[††] Chlorpheniramine Cimetidine[††] Cinacalet[‡‡] Clobazam[††] Clomipramine Dacomitinib[§§] Desvenlafaxine[††] Diltiazem[††] Diphenhydramine[††] Duloxetine[‡‡] Echinacea[††] Ecstasy[§§] Escitalopram[††] Febuxostat[††] Fluoxetine[§,§§] Gefitinib[††] Haloperidol	Hydralazine[††] Hydroxychloroquine[††] Imatinib[††] Methadone[††] Mibefradil Oral contraceptives[††] Paroxetine[§,§§] Pazopanib[††] Propafenone[††] Quinidine[†,§,§§] Ranitidine[††] Ritonavir[††] Sertraline[††] Telithromycin[††] Terbinafine[‡‡] Verapamil[††] Vemurafenib[††]	Alprazolam[††] Amiodarone[††] Amlodipine[††] Amprenavir[‡‡] Aprepitant[‡‡] Atazanavir[§,‡‡] Atorvastatin[††] Azamulin[‡] Bicalutamide[††] Bosentan Boceprevir[§§] Cilostazol[††]	Cimetidine[††] Ciprofloxacin[‡‡] Clarithromycin[§,§§] Cobicistat[§§] Conivaptan[§§] Crizotinib[‡‡] Cyclosporine[††] Darunavir[‡‡] Diltiazem[‡‡] Erythromycin[‡‡] Felbamate Fluconazole[‡‡] Fluoxetine[††]	Fluvoxamine[††] Fosamprenavir[‡‡] Gestodene Ginkgo[††] Goldenseal[††] Grapefruit Juice[‡‡,§§] Idelalisib[§§] Imatinib[‡‡] Indinavir[§,§§] Isoniazid[††] Itraconazole[†,§,§§] Ketoconazole[†,§,§§] Lapatinib[††]	Lopinavir[§§] Mibefradil[§§] Nefazodone[§,§§] Nelfinavir[§,§§] Nilotinib[††] Oral contraceptives[††] Pazopanib[††] Posaconazole[§§] Ranitidine[††] Ranolazine[††] Ritonavir[§,‡‡,§§] Roxithromycin	Saquinavir[§,§§] St. John's wort Telaprevir[§§] Telithromycin[§,§§] Ticagrelor[††] Tipranavir[††] Troleandomycin[‡] Verapamil[‡,‡‡] Voriconazole[§§] Zileuton[††]

(*continued*)

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Table 6-17
(Continued)

	CYP2D6	CYP3A4				
INDUCERS	Carbamazepine[***] Corticosteroids (pregnancy)[***] Phenobarbital[***] Rifampin[***]	Amprenavir[†††] Aprepitant[†††] Armodafinil[†††] Avasimibe[‡‡‡] Bosentan[***] Carbamazepine[§,‡‡‡] Clobazam Echinacea[†††] Clotrimazole Cyproterone acetate	Dabrafenib[†††] Dexamethasone[‡] Echinacea[†††] Efavirenz[***] Enzalutamide[‡‡‡] Etravirine[***] Etoposide Genistein[†††] Guggulsterone Hyperforin Lersivirine[†††] Lopinavir[†††] Lovastatin Modafinil[***]	Mifepristone Mitotane[‡‡‡] Nafcillin[***] Nelfinavir Nifedipine Omeprazole Paclitaxel[‡] PCBs Phenobarbital[‡] Phenytoin[‡,‡‡‡]	Pioglitazone[†††] Prednisone[†††] Rifabutin Rifampin[†,§,‡‡‡] Rifapentine[‡] Ritonavir Rufinamide[†††] Semagacestat[†††] St. John's wort[‡‡‡] Simvastatin	Spironolactone Sulfinpyrazole Talviraline[†††] Telotristat ethyl[†††] Thioridazine[†††] Topotecan Troglitazone[‡] Troleandomycin Vemurafenib[†††]

[*] *Classified by the FDA as a "Sensitive substrate" (i.e., drugs whose plasma AUC values have been shown to increase by more than or equal to fivefold when co-administered with a known CYP inhibitor).*
[†] *FDA-preferred in vitro substrate, inhibitor or inducer.*
[‡] *FDA-acceptable in vitro substrate, inhibitor or inducer.*
[§] *FDA-provided examples of in vivo substrates, inhibitors, or inducers for oral administration. Substrates in this category have plasma AUCs that are increased by at least twofold (fivefold for CYP3A4 substrates) when co-administered with inhibitors of the enzyme. Inhibitors in this category increase the AUC of substrates for that enzyme by at least twofold (fivefold for CYP3A4). Inducers in this category decrease the plasma AUC of substrates for that enzyme by at least 30%.*
[**] *Classified by the FDA as a "Substrate with narrow therapeutic range" (i.e., drugs whose exposure response indicates that increases in their exposure levels by concomitant use of CYP inhibitors may lead to serious safety concerns such as Torsades de Pointes).*
[††] *Classified by the FDA as a "Weak inhibitor" (i.e., caused a greater than or equal to 1.25-fold but less than twofold increase in plasma AUC or 20% to 50% decrease in the clearance of sensitive CYP substrates when the inhibitor was given at the highest approved dose and the shortest dosing interval in clinical evaluations).*
[‡‡] *Classified by the FDA as a "Moderate inhibitor" (i.e., caused a greater than or equal to twofold but less than fivefold increase in plasma AUC or 50% to 80% decrease in the clearance of sensitive CYP substrates when the inhibitor was given at the highest approved dose and the shortest dosing interval in clinical evaluations).*
[§§] *Classified by the FDA as a "Strong inhibitor" (i.e., caused a greater than or equal to fivefold increase in plasma AUC or greater than or equal to 80% decrease in the clearance of CYP substrates in clinical evaluations).*
[***] *Classified by the FDA as a "Moderate inducer" (i.e., caused a greater than or equal to 50% to 80% decrease in AUC of CYP substrates in clinical evaluations).*
[†††] *Classified by the FDA as a "Weak inducer" (i.e., caused a greater than or equal to 20% to 50% decrease in AUC of CYP substrates in clinical evaluations).*
[‡‡‡] *Classified by the FDA as a "Strong inducer" (i.e., caused a greater than or equal to 80% decrease in AUC of CYP substrates in clinical evaluations).*
Note: All FDA classifications are based on information available as of February 21, 2012 at the following URL: http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm.

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expressed in liver and small intestine, is largely determined by environmental factors (such as inhibitory and inducing drugs).

2. The induction of CYP3A4 is often associated with induction of CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP2C19. CYP1A2 and CYP2E1 are also inducible enzymes, but they are induced by different mechanisms and xenobiotics. CYP induction is invariably associated with the upregulation of other xenobiotic-biotransforming enzymes, as discussed later in the section "Induction of Cytochrome P450: Xenosensors." CYP2D6 is considered a noninducible enzyme despite in vitro and clinical data to the contrary (Spina et al., 1996; Madan et al., 2003; Farooq et al., 2016), as discussed later in the section "CYP2D6."
3. Genetic polymorphisms have been identified for all of the human CYP enzymes involved in drug metabolism. Zhou et al. (2009a) estimated that each human CYP gene contains an average of about 15 nonsynonymous SNPs, many of which are associated with altered drug metabolism or susceptibility to disease (a selection of which will be discussed later under each individual enzyme subheading). The incidence of CYP polymorphisms varies greatly among different ethnic groups. In the case of CYP2D6, genetic polymorphisms are common among Caucasian populations and impact the metabolism, safety, and efficacy of many drugs, as summarized in Point 23 in the section "Principles of Xenobiotic Biotransformation." Based on CYP2D6 genotype, individuals can be categorized as PMs, IMs, EMs, and UMs (and the EMs can be subdivided into low, medium, and high EMs), as shown in Table 6-7. There is a vast literature describing SNPs and other genetic polymorphisms affecting the human CYP enzymes involved in xenobiotic biotransformation, too large—and often too confusing—to be summarized here (reviewed by Zhou et al. [2009a]). Information on CYP genetic polymorphisms can be found at PharmVar, 2018. Genetic polymorphisms in the coding region can increase or decrease CYP activity, sometimes in a substrate-dependent manner, making generalizations difficult. They can also alter the stability of CYP enzymes without altering their enzymatic activity. Genetic polymorphisms in the promoter region can impair transcription (and thereby decrease CYP levels) or increase CYP levels by increasing its constitutive expression or inducibility by activated xenosensors. In many cases neither the impact of a particular SNP is known, nor it is known in many cases whether the SNP is in linkage disequilibrium with another genetic polymorphism. Studies on a genetic polymorphism in CYP2C8—the *3 allele—illustrate the complexity of this field of research. The *3 allele of CYP2C8 is in partial linkage disequilibrium with the *2 allele of CYP2C9 (i.e., individuals with one allelic variant will likely have the second too). Furthermore, based on in vitro studies CYP2C*3 was considered a *loss-of-function* allele, but in vivo studies with three different CYP2C8 substrates established that the CYP2C8*3 allele is a *gain-of-function* allele. These findings sound a word of caution about interpreting the impact of genetic polymorphisms on drug disposition, adverse effects, efficacy, and disease processes. That said, there are many cases of CYP genetic polymorphisms having a reproducible, clinically relevant impact on drug disposition, examples of which are given below under each individual enzyme subheading.

There are also many reports of CYP polymorphisms predisposing individuals to diseases or conferring protection against them. This literature is often confusing. For example, in the case of CYP2J2 (which converts arachidonic acid to EETs that are thought to be cardioprotective), a genetic polymorphism in the promoter region that causes a decrease in CYP2J2 expression was examined in four separate studies for its effects on coronary artery disease (Zordoky and El-Kadi, 2010). The CYP2J2 polymorphism was associated with an increased risk of coronary artery disease in one study (in Germans) and a decreased risk in another study (in African Americans) and had no discernible effect in two other studies (in Swedes and a Caucasian population). Four other studies on the impact of the same genetic polymorphism of CYP2J2 (i.e., lower expression) on hypertension gave similar results: the first showed increased risk (in Russians), second showed decreased risk (in Caucasians [but only in males]), and third and fourth showed no association. Given the incongruity of these studies, the impact of genetic polymorphisms on disease generally will not be discussed below with a few notable exceptions, such as the impact of CYP1A and CYP2A6 polymorphisms on cigarette-smoking-induced cancer of the lung and upper aerodigestive tract and selected other cases.

The salient features of each of the major xenobiotic-biotransforming CYP enzymes, with emphasis on the CYP enzymes in human liver microsomes, are summarized in the following subsections.

CYP1A1, CYP1A2, and CYP1B1 All mammalian species examined possess three members of the CYP1 family, namely, CYP1A1, CYP1A2, and CYP1B1. They have attracted considerable attention because they are highly inducible by AhR agonists such as dioxin and PAHs and because they play a major role in the activation/detoxication of carcinogenic/mutagenic compounds such as PAHs, aflatoxin B_1, various aromatic amines/amides, and PhIP and 2-amino-3-methylimidazol[4,5*f*]quinoline (IQ), two representatives of the many heterocyclic amines (products of amino acid pyrolysis) known as cooked food mutagens. A possible d'Artagnan to these three musketeers is CYP2S1, which resembles CYP1A1 and CYP1B1 in being an AhR-inducible, extrahepatic enzyme capable of metabolizing PAHs, but it will be discussed later in the section "CYP2R1, CYP2S1, CYP2U1, and CYP2W1." High levels of CYP1A2 are expressed in the liver (see Table 6-16) but not in extrahepatic tissues. In contrast, the liver contains low or undetectable levels of CYP1A1 and CYP1B1, but these enzymes are expressed in a great number of extrahepatic tissues (Shimada, 2006). Only CYP1A2 contributes significantly to the in vivo clearance of drugs (Zhou et al., 2010b).

The ability of CYP1 enzymes to activate a wide variety of compounds to mutagenic metabolites underscores the widespread practice of using liver microsomes from Aroclor 1254–induced rats in the Ames bacterial mutagenicity assay (because these microsomes have high levels of CYP1A1 and CYP1A2, among many other induced CYP enzymes). This and many other in vitro studies, including heterologous expression of CYP1 enzymes in mammalian cells, have established that CYP1A1 and CYP1B1 are adept at oxidizing PAHs such as B[*a*]P and DMBA to DNA-reactive diol epoxides, as illustrated in Fig. 6-14. (A second pathway of DMBA activation is shown later in the section "Sulfonation.") Similar in vitro studies have clearly established that CYP1A2 is highly effective in catalyzing the initial step (*N*-hydroxylation) in the activation of numerous carcinogenic aromatic amines (4-aminobiphenyl, 2-aminonaphthalene, 2-AF), aromatic amides (2-acetylaminofluorine), and heterocyclic amines (PhIP, IQ, etc.) to mutagenic metabolites (these activation pathways will be discussed in the sections "Glucuronidation and Formation of Acyl-CoA Thioesters" for 2-aminonaphthalene, "Sulfonation" for 2-AAF, and "Acetylation" for 2-aminofluorence and 2-AAF). In the case of PAHs, it was noted that these carcinogens are also AhR

agonists; hence, it was long assumed that PAHs can induce CYP1 enzymes and thereby induce their own activation to carcinogenic metabolites. Consequently, it came as a huge surprise when experiments with knockout mice indicated that these in vitro findings did not translate in a predictable manner to the in vivo situation (Nebert and Dalton, 2006; Shimada, 2006; Ma and Lu, 2007; Nebert et al., 2013). Compared with their wild-type littermates, CYP1A1, CYP1A2, and CYP1B1 knockout mice would be expected to be less susceptible and possibly resistant to the tumorigenic effects of PAHs such as B[*a*]P and DMBA, and to aromatic amines such as 4-aminobiphenyl and cooked food mutagens. However, contrary to expectation, deletion of CYP1A1 *potentiated* the toxicity of oral B[*a*]P, which caused increased spleen and thymus weight, leukocytopenia, extreme hypercellularity in the bone marrow, and death within 30 days in CYP1A1 knockout mice but not in wild-type mice. CYP1A1 knockout mice also had *higher* levels of B[*a*]P-DNA adducts in extrahepatic tissues. However, both wild-type mice and CYP1A1 knockout mice were susceptible to B[*a*]P toxicity when the PAH was administered by intraperitoneal injection instead of the oral route of administration. These findings strongly suggest that induction of CYP1A1 in the intestine and liver and metabolism of PAHs in these presystemic organs provide protection against orally administered PAHs (Nebert and Dalton, 2006; Shimada, 2006; Ma and Lu, 2007; Nebert et al., 2013).

Contrary to expectation, deletion of CYP1A2 similarly potentiates the toxicity and liver tumorigenicity of aromatic/heterocyclic amines. Deletion of CYP1A2 (CYP1A2 null mice) increased the toxicity of 4-aminobiphenyl (methemoglobinemia), adduct formation in the liver and bladder (two targets of arylamine carcinogenicity in rodents), and the incidence of hepatocellular tumors and preneoplastic foci. Deletion of CYP1A2 likewise increased adduct and tumor formation by the cooked food mutagens (heterocyclic amines) known as PhIP and IQ. These results strongly suggest that the role of CYP1A2 in aromatic/heterocyclic amine/amide metabolism is protective, and that enzymes other than CYP1A2 (such as peroxidases, extrahepatic CYP enzymes, and/or UGTs) play an important role in their activation (or, alternatively, it is the parent compound that causes tumor formation) (Nebert and Dalton, 2006; Shimada, 2006; Ma and Lu, 2007).

In contrast to the unexpected results observed with CYP1A1 and CYP1A2 knockout mice, gene deletion of CYP1B1 does in fact have the anticipated protective effect against PAH tumorigenicity. CYP1B1 mice are completely protected against DMBA tumor formation (Nebert and Dalton, 2006; Shimada, 2006; Ma and Lu, 2007). Similar protection against DMBA and/or B[*a*]P has been achieved with CYP1B1 inhibitors such as 1-ethinylpyrene (an irreversible inhibitor of both CYP1B1 and CYP1A1) and the specific (or more selective) CYP1B1 inhibitor 1,4-phenylenebis(methylene)-selonocyanate (*p*-XSC). These results suggest CYP1B1 plays a key role in activating PAHs to diol epoxides that cause skin and mammary tumors in experimental animals. Of particular interest is the finding that, although both CYP1A1 and CYP1B1 convert PAHs to DNA-reactive diol epoxides, the latter enzyme does so with higher affinity (Nebert and Dalton, 2006; Shimada, 2006; Ma and Lu, 2007).

The studies in knockout mice establish that induction of CYP1A1 protects against *oral* PAH toxicity (through first-pass [presystemic] elimination) but does not protect against parenterally administered PAH toxicity. In humans, the most prevalent exposure to parenterally administered PAHs is through cigarette smoking. Not surprisingly, therefore, a large number of studies have examined the impact of genetic polymorphisms in CYP1A1 and, to a lesser extent, in CYP1B1 on the incidence of lung and upper aerodigestive tract cancers in cigarette smokers and, in some cases, nonsmokers (Chen et al., 2010, 2011b; Zhan et al., 2011). Allelic variants that increase CYP1A1 and CYP1B1 have been investigated for their potential to predispose to environmental tobacco smoke (ETS)–induced lung, upper aerodigestive tract, and other cancers. In the case of CYP1A1, the focus has been on two mutually linked polymorphisms, namely, *2A and *2B; the former is a point mutation in the 3′-flanking region and is commonly called the *MspI* polymorphism. By itself *MspI* has no effect on CYP1A1 activity or expression. However, *MspI* (*2A) is linked to *2B, a point mutation in exon 7 that leads to an $Ile_{462}Val$ substitution, which is near the heme-binding site. The latter polymorphism is associated with increased activity or inducibility of CYP1A1 (Crofts et al., 1994). In the case of CYP1B1, the focus of attention has been on the *3 allele caused by a $Leu_{432}Val$ substitution that increases CYP1B1 activity (Li et al., 2000). Studies of the impact of these gain-of-function polymorphisms of CYP1A1 and CYP1B1 on the incidence of lung and other cancers are numerous and the outcomes varied. Within a study, an association between gain-of-function alleles and cancer incidence is often found in a subgroup based on ethnicity or gender, and in some studies the association (increased risk) is observed in both smokers and nonsmokers. In the case of CYP1A1, two meta-analysis studies, one based on 18,397 subjects (Zhan et al., 2011) and the other based on 30,368 subjects (Chen et al., 2011b), concluded that the overall risk of cigarette-smoking lung cancer posed by the gain-of-function polymorphism in CYP1A1 was low (odds ratio ~1.2) but that slightly higher risks could be discerned in subgroups based on ethnicity. In the case of CYP1B1, a meta-analysis based on 6501 subjects similarly concluded that the overall risk of cigarette-smoking lung cancer posed by the gain-of-function polymorphism in CYP1B1 was low (odds ratio 1.46) but variable among different ethnic groups (Chen et al., 2010). The authors concluded that the gain-of-function polymorphism in CYP1B1, which had a larger odds ratio than that of CYP1A1, was a low-penetrant risk for developing lung cancer.

The early studies of CYP1A1 induction were based on measurement of aryl hydrocarbon hydroxylase (AHH) activity, which took advantage of the conversion of B[*a*]P to the fluorescent metabolite 3-hydroxy-B[*a*]P (Conney, 1982). This assay was supplanted when it was discovered that CYP1A1 and CYP1A2 catalyze 7-ethoxyresorufin *O*-dealkylation (EROD) to form the highly fluorescent metabolite resorufin, which is one of the reactions shown in Fig. 6-50 (Burke and Mayer, 1974). These studies are of historical interest because they provided some of the earliest evidence for the existence of multiple forms of CYP, one of which (namely, CYP1A1) was highly inducible by 3-methylcholanthrene. Why was 3-methylcholanthrene studied for its effects on AHH activity? It was studied because of the observation reported in 1952 that treating rats with a low dose of one carcinogen, namely, 3-methylcholanthrene, could delay or entirely prevent liver tumor formation by a second carcinogen, namely, 3′-methyl-4-dimethylaminoazobenzene (Ma and Lu, 2007). Therefore, the more recent studies in knockout mice reinforced (arguably rediscovered) the protective effect of CYP1 induction (Nebert et al., 2013). The possibility that CYP1A induction is associated with adverse effects stemmed in part from the discovery that dioxin (or TCDD), an industrial and environmental chemical that is highly toxic to certain species, is an extraordinarily potent inducer of CYP1A enzymes in rodents, and that strain differences in dioxin toxicity in mice correlate with CYP inducibility (Poland and Glover, 1974). As outlined in the section "Induction of Cytochrome P450: Xenosensors," dioxin is the most

potent AhR agonist known, and it is this xenosensor, not the induction of CYP1A enzymes per se, that mediates the toxicity of dioxin, which is abrogated in AhR knockout mice. However, the impact of CYP1 gene deletion on the teratogenic effects of dioxin is interesting. Gene deletion of CYP1A1 or CYP1B1 has no effect on dioxin teratogenicity. However, gene deletion of CYP1A2 in dams predisposes to dioxin teratogenicity by exposing the embryos to higher levels of dioxin. The loss of CYP1A2 does not result in decreased metabolism of dioxin, which is essentially metabolically stable, but increased exposure to dioxin is likely due to loss of hepatic binding of dioxin to CYP1A2 (Dragin et al., 2006). In addition to metabolizing PAHs, CYP1B1 also catalyzes the 4-hydroxylation of 17β-estradiol, which produces a catechol that can potentially oxidize to a toxic *ortho*-quinone. Methylation of 4-hydroxyestradiol acts as a detoxication pathway in some tissues, as discussed in the section "Methylation."

CYP1 enzymes are regulated by AhR and its DNA-binding partner ARNT, which is discussed later in the section "Induction of Cytochrome P450: Xenosensors." Numerous endogenous compounds are AhR activators and substrates for CYP1 enzymes, indicating CYP1A enzymes play an important role in the disposition of endogenous compounds. Some of the most potent endogenous AhR activators identified to date (with binding affinities comparable to 3-MC and BNF) are various tryptophan derivatives such as kynurenine, kynurenic acid, FICZ (6-formylindolo[3,2-b]carbazole), ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester), and condensation products formed from indole-3-carbinol (I3C), such as DIM (3,3'-diindoylmethane, a dimer of I3C) and ICZ (indolo[3,2-*b*]carbazole, another of dimer of I3C) (Rannug et al., 1987; Bjeldanes et al., 1991; Heath-Pagliuso et al., 1998; Bergander et al., 2004; Henry et al., 2006; Chiaro et al., 2007; DiNatale et al., 2010; Opitz et al., 2011; Wincent et al., 2012). I3C is one of many dietary constituents that activate AhR and induce CYP1 enzymes. The role of intestinal CYP1A1 in preventing systemic exposure to phytochemicals was demonstrated in genetically modified mice with a selective loss of ARNT (the DNA-binding partner of AhR) from the intestine, which selectively blocks CYP1A1 expression in the intestine (Ito et al., 2007). In these mice, CYP1A1 was markedly induced in the liver and all extrahepatic tissues (including brain), a finding attributed to systemic exposure to dietary phytochemicals like I3C in the diet.

FICZ is a photoproduct of tryptophan; although it is a potent AhR activator it also is *rapidly* metabolized by CYP1 enzymes (Bergander et al., 2004; Wincent et al., 2012). In the eye and skin, FICZ is an auto-inducer; it activates AhR, induces CYP1A1 and CYP1B1, and thereby induces the metabolism of FIZC and other photoproducts of tryptophan. Like FICZ, another tryptophan product, namely melatonin, can potentially induce its own metabolism because melatonin is an AhR activator and a substrate for CYP1A1, CYP1A2, and CYP1B1 (Ma et al., 2005; Hu et al., 2007). CYP1B1 is expressed in the eye, and genetic polymorphisms that decrease CYP1B1 function are associated with primary congenital glaucoma, possibly due to impaired metabolism of FICZ and other photoproducts of tryptophan. CYP1B1 knockout mice also have structural eye defects, which suggest that CYP1B1 has an important physiological role in eye development.

In addition to being under the transcriptional control of AhR, the expression of human CYP1B1 is controlled posttranscriptionally by the microRNA known as miR-27b, a small noncoding RNA that blocks the synthesis of CYP1B1 by binding to near-perfect matching sequences in the 3′ untranslated region of CYP1B1. The level of miR-27b is decreased and the level of CYP1B1 is correspondingly increased (overexpressed) in breast cancerous cells (and other cancerous tissues) (Tsuchiya et al., 2006). The overexpression of CYP1B1 in malignant cells (coupled with its ability to activate xenobiotics to reactive metabolites) is being exploited to activate a prodrug to a cytotoxic metabolite as a possible cancer treatment. A related approach is the development of CYP1B1 DNA vaccine, which has been shown in clinical trials to kill CYP1B1-expressing cancer cells (Maecker et al., 2003).

Of the three CYP1 enzymes, only CYP1A2 contributes significantly to the in vivo clearance of drugs (Gunes and Dahl, 2008; Zhou et al., 2010b). Drugs and other xenobiotics that are substrates, inhibitors, or inducers of human CYP1A2 are shown in Table 6-17. CYP1A2 catalyzes the *O*-dealkylation of phenacetin and the 4-hydroxylation of acetanilide, both of which produce acetaminophen, which can be converted by CYP1A2 and other CYP enzymes to NAPQI, a toxic quinoneimine (Fig. 6-39). As shown in Fig. 6-50, CYP1A2 catalyzes the N_3-demethylation of caffeine to paraxanthine. By quantifying rates of formation of paraxanthine in blood, urine, or saliva or by quantifying the exhalation of isotopically labeled CO_2 from ^{13}C- or ^{14}C-labeled caffeine, the N_3-demethylation of caffeine can be used as an in vivo probe or index of CYP1A2 activity, which varies enormously from one individual to the next.

As in the case of CYP1A1 and CYP1B1, the function and regulation of CYP1A2 are reasonably conserved across mammalian species, but there are some notable differences. For example, furafylline is a metabolism-dependent inhibitor of human but not rodent CYP1A2 (Racha et al., 1998) and omeprazole and other proton pump inhibitors are inducers of human but not rodent CYP1A2 (Diaz et al., 1990).

Although the levels of CYP1A2 vary enormously from one individual to the next, genetic defects in CYP1A2 (i.e., CYP1A2 PMs) are extremely rare. Several clinical studies have reported gender differences in CYP1A2, with most—but not all—studies reporting slightly higher CYP1A2 in males compared with females (with wide variation in both genders). Rasmussen et al. (2002) reported that, based on a clinical study of caffeine metabolism in 378 individuals, males have higher CYP1A2 activity than females. In 2014 the FDA recommended that, due to their lower activity of CYP1A2, women should be administered half the dose administered to men for zolpidem (Intermezzo) and zolpidem-containing drugs such as Ambien, Edluar, and Zolpimist (FDA, 2018). Although oral contraceptive treatment reduces CYP1A2 activity, the gender difference in CYP1A2 was still significant even when women on oral contraceptive steroids were excluded from the analysis (Rasmussen et al., 2002). Rasmussen et al. (2002) also demonstrated a strong correlation in CYP1A2 activity between identical (monozygous) twins and concluded that, despite the large number of environmental factors that can potentially influence CYP1A2 expression (such as cigarette smoking and treatment with proton pump inhibitors, which induce CYP1A2, and oral contraceptive therapy, which suppresses CYP1A2), genetic factors account for 70% to 75% of the observed variation in CYP1A2 activity. Some but not all studies suggest an association between genetic polymorphisms that impair CYP1A2 function and elevated levels of clozapine. Decreased levels of clozapine are observed in cigarette smokers (Gunes and Dahl, 2008). In conjunction with caffeine consumption, a genetic polymorphism that decreases CYP1A2 expression and impairs caffeine metabolism is associated with a small increase in the risk of hypertension and nonfatal myocardial infarction (Zordoky and El-Kadi, 2010).

CYP2A6, CYP2A7, and CYP2A13 Enzymes belonging to the *CYP2A* gene family show marked species differences in catalytic function. For example, two of the three CYP2A enzymes expressed

in the rat liver, namely, CYP2A1 and CYP2A2, primarily catalyze the 7α- and 15α-hydroxylation of testosterone, respectively. In contrast, the CYP2A enzyme expressed in the human liver, namely, CYP2A6, catalyzes the 7-hydroxylation of coumarin, as shown in Fig. 6-47. Just as rat CYP2A1 and CYP2A2 have little or no capacity to 7-hydroxylate coumarin, human CYP2A6 has little or no capacity to hydroxylate testosterone. Mouse liver microsomes contain four CYP2A enzymes, one of which catalyzes the 7α-hydroxylation of testosterone (CYP2A12), one of which catalyzes the 15α-hydroxylation of testosterone (CYP2A4), and one of which catalyzes the 7-hydroxylation of coumarin (CYP2A5). Functionally, CYP2A5 can be converted to CYP2A4 by a single amino acid substitution ($Phe_{209}Leu$). In other words, this single amino substitution converts CYP2A5 from a coumarin 7-hydroxylating enzyme to a testosterone 15α-hydroxylating enzyme (Lindberg and Negishi, 1989). Differences in CYP2A function have important implications for the adverse effects of coumarin, which is hepatotoxic to rats but not humans. While coumarin is detoxified in humans by conversion to 7-hydroxycoumarin, which is subsequently conjugated with glucuronic acid and excreted, a major pathway of coumarin biotransformation in rats involves formation of the hepatotoxic metabolite, coumarin 3,4-epoxide, as shown in Fig. 6-48. In addition to catalyzing the 7-hydroxylation of coumarin, CYP2A6 converts 1,3-butadiene to butadiene monoxide and nicotine to nicotine $\Delta^{1',5'}$-iminium ion, which is further oxidized by AO to cotinine, as shown in Fig. 6-53.

Humans possess two functional CYP2A genes, namely, CYP2A6 and 2A13, and one nonfunctional CYP2A gene, namely, CYP2A7. CYP2A7 (originally named IIA4) is a full-length gene that encodes an enzyme incapable of incorporating heme, rendering it catalytically inactive (Yamano et al., 1990; Ding et al., 1995). CYP2A6 is expressed in the liver but few extrahepatic tissues (with the notable exception of mammary gland), whereas CYP2A13 is expressed mainly in the lung (bronchus and trachea) and bladder, with lower levels in reproductive organs such as the testis, ovary, uterus, and mammary gland. Hepatic CYP2A6 plays a major role in the metabolism of coumarin and nicotine, and plays a role in the metabolism of tegafur, fadrozole, methoxyflurane, and valproic acid. 8-Methoxypsoralen, a structural analog of coumarin, is a potent, metabolism-dependent inhibitor of CYP2A6 (and CYP2A13), whereas the aromatase inhibitor letrozole is a potent competitive inhibitor (Jeong et al., 2009). The MAO-B inhibitor selegiline is also a metabolism-dependent inhibitor of CYP2A6 (and CYP2B6). Selegiline is approved for the treatment of Parkinson disease, but it is also used for smoking cessation due, in part, to its ability to inhibit the CYP2A6-dependent metabolism of nicotine (Siu and Tyndale, 2008). Other inhibitors of CYP2A6 are listed in Table 6-17. CYP2A13 does not appear to contribute to drug clearance, but it may play an important role in the activation of xenobiotics to toxic and carcinogenic metabolites in lung and bladder. Both CYP2A6 and CYP2A13 activate nitrosamines to DNA-reactive (mutagenic) metabolites, especially those nitrosamines with bulky alkyl substituents such as NNK, *N*′-nitrosonorcotinine, and related nitrosamines found in tobacco smoke. (CYP2E1 also activates nitrosamines, but it preferentially activates nitrosamines with small alkyl substituents such as *N*-nitrosodimethylamine.)

The identification of three Japanese individuals who were PMs of SM-12502, an antiplatelet drug whose clearance is largely determined by CYP2A6, led to the identification of a genetic polymorphism that, in homozygous individuals, leads to the complete loss of CYP2A6 (CYP2A6*4/*4). The incidence of the CYP2A6 PM genotype is high in Asians (up to 15%) and relatively low in Caucasians (0% to 4.2%) and Africans and African Americans (0.9% to 1.9%) (Liu et al., 2011). Individuals who are genetically deficient in CYP2A6 have a considerably lower risk of cigarette-smoking-induced lung cancer, presumably because these individuals have a reduced capacity for activating tobacco-smoke nitrosamines to carcinogenic metabolites (Kamataki et al., 2005). In support of this interpretation, the CYP2A inhibitor 8-methoxypsoralen protects mice from NNK-induced lung adenomas. CYP2A6 PMs are not only PMs of nitrosamines but also PMs of nicotine. Therefore, decreased cigarette consumption may also contribute to the lowered risk of lung cancer in CYP2A6 PMs. CYP2A6 is detectable in human lung, but the levels are considerably less than those of CYP2A13, which can activate the same carcinogens as CYP2A6. Therefore, it is not immediately obvious why a genetic deficiency in CYP2A6 protects against lung cancer. A similar situation occurs with CYP2D6, inasmuch as individuals lacking this hepatic enzyme (i.e., CYP2D6 PMs) also have a slightly decreased risk of cigarette-smoking-induced lung cancer (Rostami-Hodjegan et al., 1998).

It seems somewhat paradoxical but just as CYP2A6 PMs have a decreased risk of lung cancer, so they have an increased risk of nasopharyngeal cancer, which is rare in Caucasians (Western countries) but endemic in Southern China and Southeast Asia. It has been postulated that, in the absence of CYP2A6, higher levels of nitrosamines and other carcinogens reach the nasal epithelium where they can be activated to carcinogenic metabolites by CYP2E1 and CYP2A13, both of which are expressed in nasal epithelium (Tiwawech et al., 2006).

Like CYP2A6, CYP2A13 can activate several carcinogens including many of the nitrosamines (e.g., NNK) and aromatic amines (e.g., 4-aminobiphenyl) that are present in cigarette smoke, and the mycotoxin aflatoxin B_1. CYP2A13 is suspected of playing a role in the activation of carcinogens in tobacco smoke because it is localized in the two sites where cigarette smoking causes cancer, namely, the lung, bronchus, and bladder (Nakajima et al., 2006).

In view of high prevalence of the CYP2A6 PM genotype in Chinese populations, it is noteworthy that the estimated 300 million cigarette smokers in China consume approximately one-third of the world's cigarettes. Although CYP2A6 PMs smoke fewer cigarettes than CYP2A6 EMs, the protective effect of this loss-of-function polymorphism does not prevent an estimated 1 million smokers dying annually from tobacco-related diseases each year in China, a number that is predicted to rise to 2.2 million by 2020 (Liu et al., 2011).

Of the 45 known CYP2A6 allelic variants, 6 are inactive (CYP2A6*3, CYP2A6*4, CYP2A6*12, CYP2A6*20, CYP2A6*27, and CYP2A6*34) and 1 variant (CYP2A6*9) is expressed at low levels due to a mutation in the TATA box. Many of the other allelic variants of CYP2A6 have up to 60% lower activity than CYP2A6*1, the wild-type enzyme (Hosono et al., 2017).

CYP2B6 CYP2B6 is expressed in the human liver and, to a lesser extent, in the kidney, intestine, and lung. It plays a significant role in the metabolism of the antidepressant bupropion, the antiretroviral drugs efavirenz and nevirapine, the anesthetics propofol and ketamine, the antineoplastic drugs cyclophosphamide, ifosfamide, and tamoxifen, the antimalarial artemisinin, the synthetic opioids methadone and pethidine (Demerol), the anti-Parkinsonian selegiline, and the endocrine disrupter methoxychlor (Wang and Tompkins, 2008; Hedrich et al., 2016). The activation of cyclophosphamide by CYP2B6 (and CYP3A4) is shown in Fig. 6-56. CYP2B6 also catalyzes the *N*-demethylation of *S*-mephenytoin but not the 4′-hydroxylation, which is catalyzed by CYP2C19. Some but not all of these substrates can be used in vitro or in vivo to assess CYP2B6 activity, as shown in Table 6-17. Several drugs (including many MAO

inhibitors) have been shown to inhibit CYP2B6 (Table 6-17), and many of them do so in a metabolism-dependent manner, such as clopidogrel, clorgyline, pargyline, phenelzine, selegiline, ticlopidine, and thio-TEPA, many of which inactivate other CYP enzymes (Sridar et al., 2012; Nirogi et al., 2015). 2-Phenyl-2-(1-piperidinyl) propane (PPP) is a potentially selective inactivator of CYP2B6 for in vitro studies (Walsky et al., 2006; Walsky and Obach, 2007). Several organothiophosphate pesticides are also metabolism-dependent inhibitors of CYP2B6, including chlorpyrifos, chlorpyrifos-methyl, diazinon, parathion-methyl, and aziophos-methyl (D'Agostino et al., 2015).

CYP2B6 is an inducible enzyme (Fahmi et al., 2016); it is generally coinduced with CYP3A4 and several CYP2C enzymes by xenobiotics that activate CAR and/or PXR, such as rifampin, efavirenz, ritonavir, clotrimazole, nevirapine, the enzyme-inducing antiepileptic drugs (EIAEDs) phenobarbital, phenytoin, carbamazepine, and felbamate, and the herbal preparation of St. John's wort, which is discussed later (see the section "Induction of Cytochrome P450: Xenosensors").

There is considerable interindividual variation in CYP2B6 activity, partly due to environmental factors (such as treatment with enzyme inducers) and partly due to genetic factors. Several SNPs that, to varying degrees, impair CYP2B6 expression and/or activity have been identified. The CYP2B6*6 allele ($Gln_{172}His$, $Lys_{262}Arg$) is common in Asians (15% to 40%), Caucasians (20% to 30%), and African Americans (over 50%), which is associated with a marked decrease in CYP2B6 activity. One functional consequence of the CYP2B6*6 polymorphism is impaired clearance of efavirenz, an antiretroviral drug, leading to increased plasma levels and adverse effects (Wang and Tompkins, 2008; Mukonzo et al., 2014; Mukonzo et al., 2016). CYP2B6*6 is also associated with impaired cyclophosphamide metabolism and *S*-mephenytoin *N*-demethylation. Another loss-of-function allele, namely, CYP2B6*5 ($Arg_{487}Cys$), is common in Caucasians (14% to 25%) and curiously results in a decrease in CYP2B6 expression and activity but only in females (Wang and Tompkins, 2008).

CYP2C Enzymes Humans express four CYP2C enzymes, namely, CYP2C8, CYP2C9, 2C18, and 2C19, the genes for which are clustered in about 390-kb segment of chromosome 10q23.3. With the exception of CYP2C18, these CYP2C enzymes play a significant role in drug metabolism, and their inhibition or induction is the basis of a number of clinically important drug interactions. In the case of CYP2C9 and 2C19, genetic polymorphisms give rise to a relatively common IM or even PM phenotype, which also impacts the metabolism and safety or efficacy of certain drugs. As a general rule, CYP2C8 metabolizes large, acidic drugs, including several glucuronide conjugates, whereas CYP2C9 metabolizes (or is inhibited by) smaller acid- or sulfonamide-containing drugs, and CYP2C19 metabolizes neutral or weakly basic drugs often with a high degree of stereoselectivity (i.e., it preferentially metabolizes one enantiomer over the other). All four enzymes appear to be inducible by CAR/PXR agonists such as phenobarbital, rifampin, and St. John's wort (hyperforin); hence, they tend to be coinduced together with CYP2B6 and CYP3A4. Substrates, inhibitors, and inducers of CYP2C8, CYP2C9, and CYP2C19 are listed in Table 6-17.

CYP2C8, CYP2C9, and CYP2C19 are abundant enzymes in the human liver (see Table 6-16) but low levels of CYP2C8 and CYP2C9 are also expressed in a wide range of tissue and in vascular endothelium where, together with CYP2J2, they epoxidate arachidonic acid to vasoactive EETs (see the sections "CYP2J2" and "Epoxide Hydrolases") (Chen and Goldstein, 2009; Zordoky and El-Kadi, 2010). CYP2C8 and CYP2C9 can also hydroxylate *all-trans*-retinoic acid and 9-*cis*- and 13-*cis*-retinoic acid (Marill et al., 2000, 2002).

CYP2C8 and CYP2C9 show a marked preference for metabolizing large and small acidic drugs, respectively, and these substrates are ionized (negatively charged) at physiological pH and highly bound to albumin in plasma. In many cases substrates for CYP2C8, which metabolizes large acidic drugs, are also substrates for one or more of the organic anion-transporting polypeptides (OATPs) in the intestine and liver. Accordingly, drug interactions with CYP2C8 may also involve drug interactions at the level of OATP, especially if transporter-mediated uptake into the liver becomes a rate-limiting step in clearance.

As shown in Table 6-15, rats and mice express a large number of CYP2C enzymes (13 and 15, respectively). The expression of CYP2C11 in adult male but not adult female rats (together with male-predominant expression of CYP3A2, which also has a broad substrate specificity) is a key factor underlying many gender differences in xenobiotic and steroid metabolism in mature rats (Waxman and Holloway, 2009).

CYP2C8 CYP2C8 has been implicated in the metabolism of over 100 drugs including the thiazolidinediones rosiglitazone, pioglitazone, and troglitazone (withdrawn), amiodarone, amodiaquine, carbamazepine, cerivastatin (withdrawn), chloroquine, cilostazol, dasabuvir, enzalutamide, *R*-ibuprofen, imatinib, loperamide, lovastatin, montelukast, morphine, paclitaxel (Taxol®), repaglinide, rosuvastatin, simvastatin, tazarotene (a retinoid derivative), torsemide, verapamil, and zopiclone (Lai et al., 2009; Backman et al., 2016). However, with the exception of the antidiabetic drug, repaglinide, all of the above drugs are metabolized to a significant extent by enzymes other than CYP2C8, and in the case of repaglinide its disposition is influenced by the transporter OATP1B1. Repaglinide is classified by the FDA as a sensitive CYP2C8 substrate with the qualification that it is only suitable as an in vivo probe or index substrate for drug–drug interaction studies if the investigational drug being evaluated as a CYP2C8 inhibitor does not inhibit OATP1B1. In the presence of the CYP2C8 inhibitor gemfibrozil, exposure to repaglinide (based on plasma AUC) increases about eightfold, which is greater than the increase observed with other CYP2C8 substrates with the exception of dasabuvir (ABT-333) and daprodustat (GSK1278863A), whose respective plasma AUC values increase 11.3- and 18.6-fold following inhibition of CYP2C8 by gemfibrozil (Backman et al., 2016).

Substrates for CYP2C8 tend to be large and acidic as evidenced by the observation that CYP2C8 metabolizes several glucuronides (Nishihara et al., 2012). For example, while 17β-estradiol and diclofenac are metabolized by CYP2C9, the glucuronide metabolites of these compounds (namely, estradiol-17β-glucuronide and diclofenac acyl glucuronide) are metabolized by CYP2C8 (at the same site as that metabolized by CYP2C9, which is distal to the glucuronide moiety).

Cerivastatin (Baycol), a cholesterol-lowering drug, was withdrawn from the U.S., European, and Japanese markets in 2001 after postmarketing surveillance revealed a high incidence of rhabdomyolysis, a muscle disorder potentially caused by all statins, and 99 fatalities, most of which were judged to be probably or possibly the result of treatment with cerivastatin. Of the 99 fatalities, 36 cases involved coadministration of cerivastatin with gemfibrozil and 18 cases involved coadministration with clopidogrel (EMA, 2002). At the time of its worldwide market withdrawal, the mechanism by which gemfibrozil and clopidogrel increase systemic exposure to cerivastatin and increase its risk of causing rhabdomyolysis was not known, although inhibition of hepatic uptake (OATP) transporters and/or inhibition of CYP2C8 and possibly CYP3A4, the

major and minor enzymes involved in cerivastatin metabolism, were suspected. The mechanism of interaction is unusual and involves metabolism-dependent (irreversible) inhibition of CYP2C8 by the acyl glucuronides of gemfibrozil and clopidogrel, which represent rare cases of CYP inhibition by a Phase 2 conjugate of drugs that otherwise have little inhibitory potential (Ogilvie et al., 2006; Baer et al., 2009; Tornio et al., 2014; Itkonen et al., 2016; Kim et al., 2016). During the benzylic hydroxylation of gemfibrozil glucuronide, CYP2C8 forms a benzylic radical (as shown previously in the scheme for the mechanism of carbon hydroxylation [this section]) that evades oxygen rebound and alkylates the heme moiety (at the α-meso position), which inactivates the enzyme (Baer et al., 2009). Clopidogrel (the parent drug) is a metabolism-dependent (irreversible) inhibitor of CYP2B6 due to formation of a reactive *S*-oxide or thiophene epoxide that alkylates the heme moiety and, to a lesser extent, the apoprotein (at Cys_{475}) (Zhang et al., 2011a). Heme alkylation by a reactive *S*-oxide or thiophene epoxide is also thought to be the mechanism by which clopidogrel glucuronide inactivates CYP2C8 (Tornio et al., 2014). In contrast to gemfibrozil (a carboxylic acid–containing drug), clopidogrel is an ester that is hydrolyzed by CES1 to a carboxylic acid before converted to an acyl glucuronide. The metabolism of clopidogrel is discussed further in the section "CYP2C19."

The ability of CYP2C8 to metabolize the glucuronide conjugates of certain drugs held the answer to a long-standing mystery: What enzyme converts desloratadine, a nonsedating long-lasting antihistamine, to 3-hydroxydesloratadine, a human-predominant metabolite that is not formed in certain individuals. In the general population such poor metabolizers occur with a frequency of 6% but such PMs are most frequent in African American (17%) compared with Caucasian (2%), Native American (8%), Hispanic (2%), and Jordanian (3%) populations. Kazmi and colleagues established that the 3-hydroxylation of desloratadine is catalyzed by CYP2C8 but prior *N*-glucuronidation of desloratadine by UGT2B10 is an obligatory first step, as shown in Fig. 6-54 (Kazmi et al., 2015a, 2015b). The metabolite formed by CYP2C8, namely 3-hydroxydesloratadine *N*-glucuronide, is not a circulating metabolite because the *N*-glucuronide moiety is unstable and undergoes rapid hydrolysis (deconjugation) to release 3-hydroxydesloratadine. Genetic polymorphisms leading to loss of CYP2C8 or UGT2B10 activity could give rise to the PM phenotype because both enzymes are indispensable to 3-hydroxydesloratadine formation. A splice site mutation that prevents synthesis of functional UGT2B10 occurs with high frequency in individuals of African origin (45%), with moderate frequency in Asians (8%), and rarely in Caucasians (<1%), which resembles the frequency of the desloratadine PM phenotype (Fowler et al., 2015). Likewise, CYP2C8*2 (c.805A > T, p.Cys266Phe) is a low-activity allelic variant of CYP2C8 that is common in certain African populations but rare in Caucasian and Asian populations (Backman et al., 2016).

A large number of genetic polymorphisms have been identified in the CYP2C8 gene, one of which is particularly noteworthy, namely, the *3 allele, which is common in Caucasians (about 12%) but rare in Asians, Africans, and African Americans (Daily and Aquilante, 2009). CYP2C8*3 is a double mutation ($Arg_{139}Lys$ and $Lys_{399}Arg$). It is of particular interest for two reasons. First, the *3 allele of CYP2C8 is in partial linkage disequilibrium with the *2 allele of CYP2C9, meaning individuals often carry both allelic variants, which complicates the clinical assessment of either one of these genetic polymorphisms on drug metabolism or disease processes. Second, CYP2C8*3 is of particular interest because of the disconnect between in vitro and in vivo findings. In vitro studies identified the CYP2C8*3 as a *loss-of-function* allelic variant. However, clinical studies established that *3 is a *gain-of-function* allelozyme that is associated with increased clearance of repaglinide, rosiglitazone, and pioglitazone, but not paclitaxel (Daily and Aquilante, 2009). Both the linkage between the *3 allele for CYP2C8 and the *2 allele for CYP2C9 and the discrepancy between in vitro and in vivo findings on CYP2C8*3 sound a word of caution about interpreting the impact of genetic polymorphisms on drug disposition, adverse effects, efficacy, and disease processes.

CYP2C8 is one of several CYP enzymes including CYP2B6, CYP2C9, CYP2C19, and CYP3A4 that are all inducible by activators of the xenosensors PXR and CAR. As discussed in "Induction of Cytochrome P450: Xenosensors," there is a growing body of in vitro evidence that CYP2C8 along with CYP3A4 and CYP1A1 but not the other aforementioned CYP enzymes are inducible by activators of human PPARα, the xenosensor linked with induction

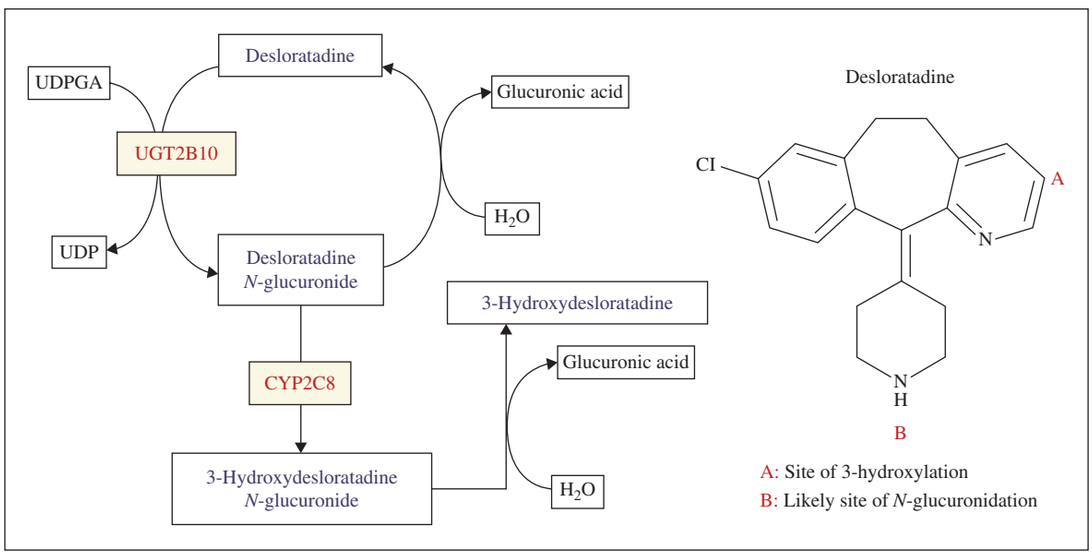


Figure 6-54. *Role of CYP2C8 and UGT2B10 in the conversion of desloratadine to the human-predominant metabolite 3-hydroxydesloratadine.* CYP2C8 catalyzes the 3-hydroxylation of desloratadine only after desloratadine is *N*-glucuronidated by UGT2B10. Following hydroxylation, the *N*-glucuronide moiety of 3-hydroxydesloratadine-*N*-glucuronide hydrolyzes rapidly to release 3-hydroxydesloratadine, the major circulating metabolite of desloratadine in humans.

of CYP4A enzymes in rodents (Seree et al., 2004; Prueksaritanont et al., 2005; Klein et al., 2012; Thomas et al., 2013, 2015; Makia and Goldstein, 2016). Whether fibrates and other drugs that activate PPARα cause clinically relevant induction of CYP2C8 (or CYP1A1 and CYP3A4) is not known.

CYP2C9 CYP2C9 plays a role in the metabolism of over 100 drugs including NSAIDs (e.g., diclofenac, celecoxib, ibuprofen, flurbiprofen, meloxicam, naproxen, piroxicam, mefenamic acid, and suprofen), oral hypoglycemics (e.g., glyburide, glipizide, glimepiride, tolbutamide), oral anticoagulants (e.g., *S*-warfarin, *S*-acenocoumarol, and phenprocoumon), diuretics and uricosurics (e.g., sulfinpyrazone sulfide, torsemide, and tienilic acid [ticrynafen]), angiotensin II blockers (e.g., candesartan, irbesartan, and losartan), anticonvulsants (e.g., phenytoin), and many others (Zhou et al., 2009b). It also metabolizes Δ^9-tetrahydrocannabinol. Substrates and inhibitors of CYP2C9 tend to be carboxylic acid-, tetrazole-, or sulfonamide-containing compounds, but there are exceptions to this general rule. In fact, the most potent CYP2C9 inhibitors known, namely, benzbromarone and 2-methyl-3-(3′,5′-diiodo-4′-hydroxybenzoyl)benzofuran, are neither acid- nor sulfonamide-containing compounds but they resemble these agents in that they are phenolic anions (negatively charged) at physiological pH (Rettie and Jones, 2005).

CYP2C9 is a polymorphically expressed enzyme. Of the more than 30 variants identified, 2 particular loss-of-function alleles, the *2 and *3 variants, are recognized for their clinically significant impact on drug disposition and safety (Zhou, 2009b; Van Booven et al., 2010; Niinuma et al., 2014). CYP2C9*2 ($Arg_{144}Cys$) is prevalent in Caucasians (10% to 15%) but not in Asians or African Americans, as is the case with the *3 allele of CYP2C8. These two alleles (on different enzymes) are in partial linkage disequilibrium as noted above in the preceding section. CYP2C9*3 ($Ile_{359}Leu$) is widespread across all ethnic groups, being highest in Caucasians (5% to 16%) followed by African Americans (about 10%) and Asians (1% to 5%) (Van Booven et al., 2010). CYP2C9*2 and CYP2C9*3 have moderately and markedly decreased catalytic activity, respectively. Individuals who are homozygous for the *3 allele are considered CYP2C9 PMs. They are PMs of *S*-warfarin, tolbutamide, and phenytoin. These three drugs have a low therapeutic index, and their dose must be reduced in CYP2C9 PMs to prevent adverse drug events. Dosage adjustment is also required when these drugs are combined with a CYP2C9 inhibitor or inducer, examples of which are given in Table 6-17. In the case of the anticoagulant warfarin, CYP2C9 genotype is one of three important genetic factors that affect dosing requirements, the second being the therapeutic target of warfarin vitamin K epoxide reductase complex 1 (VKORC1) (McDonald et al., 2009; Lurie et al., 2010). A third genetic factor is a genetic polymorphism in CYP4F2 ($Val_{433}Met$) that impairs the metabolism of vitamin K_1. Individuals with this CYP4F2 polymorphism require a higher warfarin dose to compete with the increased levels of the vitamin K_1 in the liver (McDonald et al., 2009; Lurie et al., 2010).

Tienilic acid, suprofen, and silybin are all metabolism-dependent inhibitors of CYP2C9. Tienilic acid is of interest because it was withdrawn from the market due to its hepatotoxicity (see Table 6-6). CYP2C9 converts tienilic acid to an electrophilic thiophene sulfoxide that can react either with water to give 5-hydroxytienilic acid or with a nucleophilic amino acid in CYP2C9 to form a covalent adduct, which inactivates the enzyme (Lecoeur et al., 1994). Antibodies directed against the adduct between CYP2C9 and tienilic acid are thought to be responsible for the immunoallergic hepatitis that develops in about 1 out of every 10,000 patients treated with this uricosuric diuretic drug. As shown in Table 6-6, these autoantibodies against CYP2C9 are known as anti-LKM_2 because they are directed against *l*iver and *k*idney *m*icrosomes. The subscript number 2 distinguishes these antibodies against CYP2C9 from those against CYP2D6, which are known as anti-LKM_1. It should be noted that tienilic acid can also be activated to a reactive acyl glucuronide, as outlined in the section "Glucuronidation and Formation of Acyl-CoA Thioesters."

CYP2C18 In contrast to the other three CYP2C enzymes expressed in the human liver, CYP2C18 plays little or no role in drug metabolism. CYP2C18 is the most abundant CYP2C enzyme in the skin and lung, and it is also expressed in the brain, uterus, mammary gland, kidney, and duodenum. CYP2C18 is more than an order of magnitude more active than either CYP2C9 or CYP2C19 at catalyzing the *para*-hydroxylation of phenytoin to HPPH, and CYP2C18 can further hydroxylate HPPH to a catechol (Kinobe et al., 2005). Autoxidation of this catechol to an *ortho*-quinone is thought to be responsible, at least in part, for rare incidences of hypersensitivity reactions to phenytoin. Therefore, although hepatic CYP2C9 and CYP2C19 are largely responsible for determining the systemic clearance of phenytoin, it is possible that CYP2C18 is responsible for activating phenytoin by the catechol/quinone pathway in the skin, the tissue where the first signs of hypersensitivity to phenytoin are observed.

CYP2C19 CYP2C19 is a polymorphically expressed enzyme that metabolizes several drugs with a high degree of stereospecificity such that, in the case of drugs administered as a racemic mixture, it metabolizes one enantiomer faster than the other, as in the case of mephenytoin (Mesantoin®), which is of historical interest with respect to CYP2C19. A genetic polymorphism for the metabolism of *S*-mephenytoin was first described in 1984 (Wilkinson et al., 1989). The deficiency affects the 4′-hydroxylation (aromatic ring hydroxylation) of this anticonvulsant drug (see Fig. 6-47). The other major pathway of *S*-mephenytoin metabolism, namely, *N*-demethylation to *S*-nirvanol, is not affected. (This pathway was subsequently shown to be catalyzed by CYP2B6.) Consequently, PMs excrete little or no 4′-hydroxymephenytoin in their urine, but they do excrete increased amounts of the *N*-demethylated metabolite, *S*-nirvanol (*S*-phenylethylhydantoin). The enzyme responsible for this genetic polymorphism is CYP2C19, and it is highly stereoselective for the *S*-enantiomer of mephenytoin (de Morais et al., 1994). In contrast to the *S*-enantiomer, the *R*-enantiomer is not converted to 4′-hydroxymephenytoin, but it is *N*-demethylated to *R*-nirvanol (*R*-phenylethylhydantoin). Individuals who are genetically deficient in CYP2C19 are at increased risk of adverse events from Mesantoin® (a racemic mixture of the *S*- and *R*-enantiomers of mephenytoin). An exaggerated central response has been observed in CYP2C19 PMs administered Mesantoin® at doses that were without effect in CYP2C19 EMs.

CYP2C19 plays an important role in the metabolism of the proton pump inhibitors omeprazole, lansoprazole, pantoprazole, and rabeprazole, representative structures of which are shown in Fig. 6-49. The sulfoxide group in proton pump inhibitors is a chiral center, and CYP2C19 preferentially metabolizes one enantiomer over the other: it preferentially hydroxylates the *R*-enantiomer in the case of omeprazole, and it preferentially hydroxylates the *S*-enantiomer in the case of lansoprazole. These reactions are equivalent inasmuch as these two proton pump inhibitors are hydroxylated on opposite ends of the molecule (on the pyridine ring in the case of omeprazole and on the benzimidazole ring in the case of lansoprazole), meaning the sulfoxide in each case binds to the active site of CYP2C19 in the same orientation (Kim et al., 2003). CYP2C19 PMs are poor metabolizers of omeprazole and lansoprazole. However, CYP2C19 PMs are not at increased risk for ADEs but actually derive greater therapeutic benefit (better control

of GERD) from standard doses of proton pump inhibitors (Furuta et al., 2005). Esomeprazole, the *S*-enantiomer of omeprazole, was approved for the treatment of GERD and related gastric acid disorders. Compared with the *R*-enantiomer, esomeprazole is not rapidly metabolized by CYP2C19, and its improved pharmacokinetic behavior provides CYP2C19 EMs with the same therapeutic benefit as CYP2C19 PMs administered omeprazole. This is due in part to the ability of esomeprazole to function as a metabolism-dependent inhibitor of CYP2C19 and inhibit its own metabolism in CYP2C19 EMs (Ogilvie et al., 2011). Other substrates, inhibitors, and inducers of CYP2C19 are listed in Table 6-17.

Two genetic polymorphisms, the *2 and *3 alleles (and their variants *2A, *2B, and *2C, and *3A and *3B), are responsible for the majority of CYP2C9 PMs (Zhou et al., 2009a). Both the *2 and *3 are null alleles; hence, individuals homozygous for either allele (*2/*2 and *3/*3) and heterozygotes (*2/*3) are devoid of functional CYP2C19. The incidence of the CYP2C19 PM genotype varies considerably from one ethnic group to the next: it ranges from 13% to 23% in Japanese, Chinese, Korean, Vietnamese, and Indian populations, but the incidence is considerably lower in Africans and African Americans (3% to 5%), and in Caucasians (1% to 7% depending on geographical location) (Zhou et al., 2009a). A high incidence of CYP2C19 PMs is found in Australian aboriginals (about 26%) and in South Pacific Polynesian populations (about 14%). In the Polynesian island of Vanuatu, 79% of the population has been identified as CYP2C19 PMs. When administered standard drug doses, CYP2C19 PMs have elevated plasma levels of proton pump inhibitors (omeprazole, lansoprazole, pantoprazole, and, to a lesser extent, rabeprazole), diazepam, escitalopram, sertraline, and voriconazole, not to mention the *S*-enantiomer of mephenytoin in individuals administered Mesantoin, which was the original observation that led to the discovery of the CYP2C19 PM genotype. No CYP2C19 UM individuals with functional CYP2C19 gene duplications have been identified; however, CYP2C19*17 ($Ile_{331}Val$) has been identified as a gain-of-function allele with allele frequencies of about 20% in Caucasians and Africans but only about 2% in Asian populations (Li-Wan-Po et al., 2010). Based on the magnitude of the increase in clearance of drugs such as escitalopram, CYP2C19 activity in individuals who are homozygous for this gain-of-function allele (*17/*17) can be considered high-activity EMs rather than UMs (Li-Wan-Po et al., 2010). CYP2C19 is regulated by CAR/PXR; hence, its activity in IMs and EMs can be increased by enzyme-inducing drugs (see Table 6-17).

CYP2C19 has attracted considerable attention for its role in converting the antiplatelet drug clopidogrel to its pharmacologically active metabolite. This activation process is shown in Fig. 6-55. It is unusual for several reasons. First, the therapeutically active component is a reactive metabolite that binds covalently to the ADP-binding receptor ($P2Y_{12}$) on platelets. Second, the oxidative pathway leading to formation of this active metabolite is in competition with two hydrolytic pathways, one that hydrolyzes clopidogrel, which is catalyzed by CES1 (a carboxylesterase), and another that hydrolyzes the first oxidative metabolite (a thiolactone), which is catalyzed by PON1 (a paraoxonase). Third, the active metabolite formed by CYP and the inactive metabolite formed from the intermediary thiolactone by PON1 are isomers, as shown in Fig. 6-55 (Dansette et al., 2012). Fourth, clopidogrel provides an example of Type F toxicity (Aronson and Ferner, 2003) whereby PMs (individuals who fail to produce the active metabolite) derive diminished therapeutic benefit and, in the case of clopidogrel, are at increased risk of thrombosis and coronary artery disease, whereas high-activity EMs that produce too much of the active metabolite (and hence too much platelet inhibition) are at increased risk from bleeding and hemorrhage. Much of the pharmacogenomic data strongly implicate CYP2C19 as the most important enzyme for clopidogrel activation, based on poor response to clopidogrel in carriers of the loss-of-function CYP2C19 alleles (i.e., the *2 and *3 alleles), and also increased bleeding in carriers of the gain-of-function CYP2C19*17 allele (Sibbing et al., 2010; Scott et al., 2011). A dominant role for PON1 in clopidogrel activation has been proposed (Bouman et al., 2011). This rather compelling (but controversial) proposal was explained when Dansette et al. (2012) showed that the ring-opened thiol produced by PON1 (with the double bond in the α/β position relative to the thiol) is an inactive isomer of the true active metabolite produced by CYP2C19 (with the double bond in the β/γ position relative to the thiol), as shown in Fig. 6-55.

Because of its tendency to cause gastric bleeding as an adverse event, clopidogrel was often prescribed together with a proton pump inhibitor (to raise gastric pH and lessen the severity of gastric bleeding). It was established that omeprazole and its *S*-enantiomer esomeprazole, but not pantoprazole or lansoprazole, compromise the antiplatelet effect of clopidogrel. It was subsequently established that omeprazole (and its *S*-enantiomer esomeprazole), but not pantoprazole or lansoprazole, is a metabolism-dependent inhibitor of CYP2C19 (Ogilvie et al., 2011). This drug–drug interaction and the pharmacogenomic data strongly implicate CYP2C19 as the major enzyme responsible for converting clopidogrel to its pharmacologically active metabolite.

CYP2D6 In the late 1950s, clinical trials in the United States established that sparteine was as potent as oxytocin for inducing labor at term. However, the duration and intensity of action of sparteine were dramatically increased in about 7% of all patients tested. The exaggerated response to sparteine included prolonged (tetanic) uterine contraction and abnormally rapid labor. In some cases, sparteine caused the death of the unborn baby. The drug was not recommended for clinical use because these adverse effects were unpredictable and occurred at doses that were well tolerated by other patients. The antihypertensive drug debrisoquine was subsequently found to cause a marked and prolonged hypotension in 5% to 10% of patients, and a genetic polymorphism for the metabolism of debrisoquine and sparteine was discovered during 1977 to 1979. PMs lack CYP2D6, which catalyzes the 4-hydroxylation of debrisoquine and the dehydrogenation (Δ^2- and Δ^5-oxidation) of sparteine (see Fig. 6-53).

Genetic polymorphisms of CYP2D6 have been so extensively studied that it is possible not only to categorize individuals into four phenotypes, namely, PMs, IMs, EMs, and Ums, but also to subdivide the EMs into three categories, namely, High-Activity EM, Medium-Activity EM, and Low-Activity EM, as summarized in Table 6-7. More than 100 allelic variants of CYP2D6 have been identified (Zhou et al., 2009a; PharmVar, 2018). Many of the allelic variants that result in a partial or complete loss of CYP2D6 activity are listed in the legend to Table 6-7. The incidence of the CYP2D6 PM genotype varies considerably from one ethnic group to the next: it is 5% to 7% in Caucasians, 2% to 4% in Africans and African Americans, and 1% to 2% in Asian (Thai, Chinese, and Japanese) populations. Similarly, the incidence of the CYP2D6 gene duplication, which gives rise to the CYP2D6 UM genotype, varies among ethnic groups: it is up to 29% in Ethiopians, 20% in Saudi Arabians, 5% to 10% in Southern European Caucasians, but only 1% to 2% in Northern European Caucasians, 5% to 10% in Turkish subjects, and 2% or less in African, African American, and Asian subjects.

CYP2D6 plays a major role in drug metabolism, second only to CYP3A4 in terms of the sheer number of drugs it metabolizes (Zhou, 2009a, 2009b; Zhou et al., 2009a). CYP2D6 metabolizes 13% of drugs ($n = 860$) and 6% of natural products/endobiotics

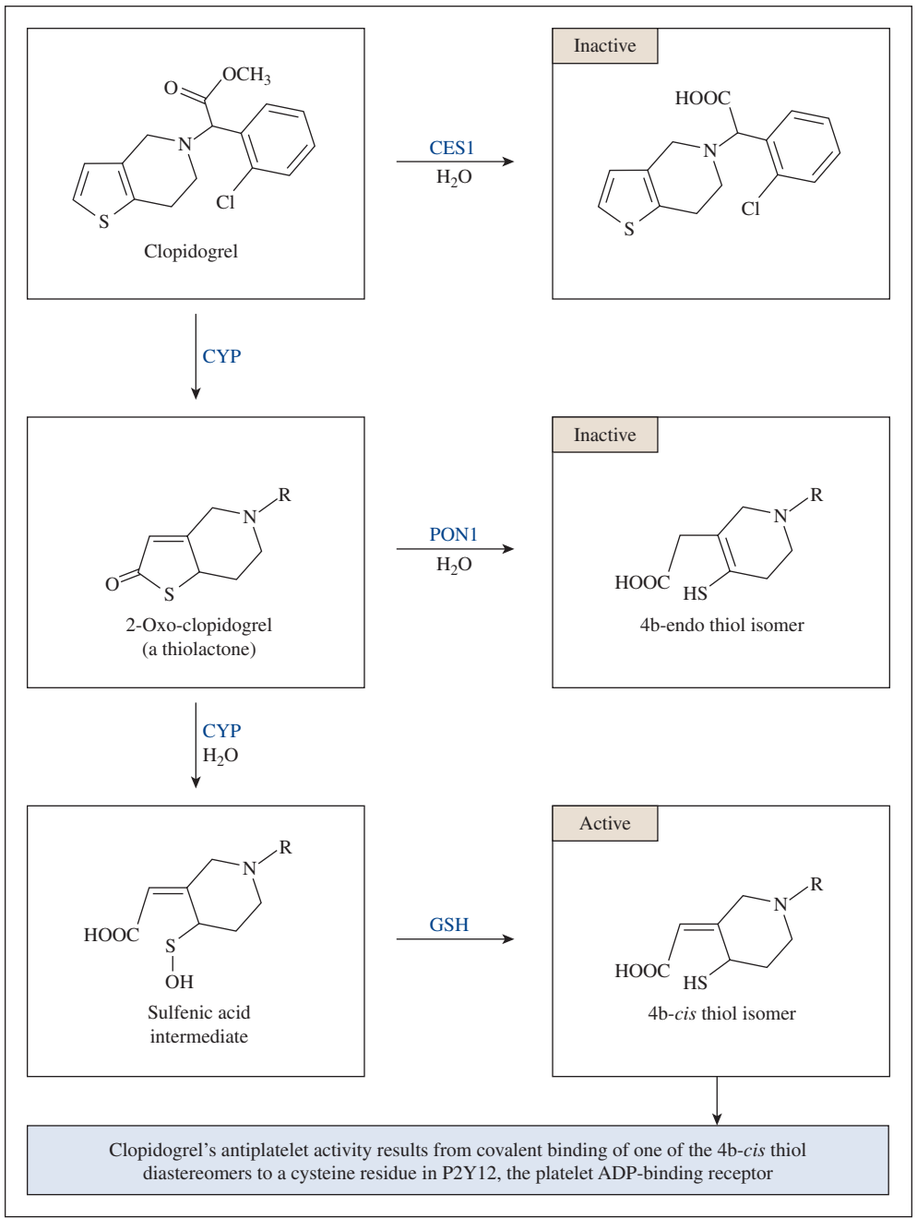


Figure 6-55. *Biotransformation of clopidogrel to inactive metabolites by carboxylesterase (CES1) and paraoxonase (PON1) and its active, antiplatelet metabolite by cytochrome P450 (especially CYP2C19).*

($n = 331$ compounds) (Rendic and Guengerich, 2015). CYP2D6 prefers to metabolize lipophilic amines, which is the fundamental structure of most cardiovascular and CNS drugs. Accordingly, CYP2D6 metabolizes various tricyclic antidepressants, nontricyclic antidepressants, SSRIs, neuroleptics, β-blockers, antiarrhymthics, antiemetics, opioids, and antihistamines, examples of which are given in Table 6-17. CYP2D6 plays an important role in tamoxifen metabolism (discussed later in this section). CYP2D6 also metabolizes several drugs of abuse including methamphetamine ("meth" or "ice"), methylenedioxymethamphetamine (NMDA or "ecstasy"), *N*-ethyl-3,4-methylenedioxyamphetamine (MDE or "eve"), and 3,4-methylenedioxyamphetamine (MDA or "the love drug"). Drugs of abuse containing a methylenedioxy (benzodioxole) moiety are also metabolism-dependent inhibitors of CYPD6 (Van et al., 2006). CYP2D6 also metabolizes the neurotoxin MPTP (see Fig. 6-33 and the section "Amine Oxidases"). Individuals lacking CYP2D6 have an exaggerated response to some but not all the drugs mentioned above or shown in Table 6-17. Large increases (more than fivefold) in systemic exposure are observed in CYP2D6 PMs with atomoxetine, desipramine, dextromethorphan, metoprolol, perphenazine, and risperidone, some of which are useful in vivo probe/index substrates of CYP2D6 activity. Drugs whose clearance is impaired in CYP2D6 PMs are cleared faster in UMs.

Whether the impact of CYP2D6 polymorphisms on the pharmacokinetics of a drug translate into changes in pharmacodynamic response depends on the nature of the polymorphism (ranging from PM to UMs), the magnitude of the PK response (which is determined by fm_{CYP2D6}, the fractional contribution of CYP2D6 to drug

clearance [see Point 24 in the section "Principles of Xenobiotic Biotransformation"]), and the pharmacological and toxicological properties of the metabolites. For example, the metabolites produced by CYP2D6 from the antiarrhythmic drug encainide are more potent than those of the parent drug; hence, QT prolongation is more apparent in CYP2D6 EMs than PMs. On the hand, propafenone is a more potent β-blocker than its metabolites; hence, PMs experience prolonged hypotension from a standard dose of propafenone. CYP2D6 status affects the pharmacokinetics of most β-blockers, but many of them have active metabolites such that changes in pharmacokinetics have little or no effect on their overall pharmacological activity (Tucker, 1994; Turner et al., 2001; Ingelman-Sundberg, 2005; Zhou, 2009b). CYP2D6 PMs are at increased risk from the hepatotoxic and other adverse effects (sedation, extrapyramidal, and psychomotor symptoms) of perhexiline, for which reason perhexiline joined sparteine and debrisoquine in being denied regulatory approval by the U.S. FDA.

The analgesic codeine (which is *O*-demethylated to morphine) and the anticancer drug tamoxifen (which is hydroxylated and *N*-demethylated to endoxifen) are converted to pharmacologically active metabolites by CYP2D6. Consequently, CYP2D6 PMs derive less therapeutic benefit than CYP2D6 EMs. In contrast, in the case of codeine, CYP2D6 UMs are at increased risk from morphine toxicity. The death of an elderly man administered a recommended dose of codeine and the death of baby who was breastfed by a woman on codeine have been attributed to the CYP2D6 UM genotype that causes rapid conversion of codeine to morphine (which causes respiratory suppression) (Gasche et al., 2004; Koren et al., 2006). CYP2D6 converts the breast cancer drug tamoxifen to endoxifen (which is 30- to 100-fold more potent than tamoxifen in suppressing estrogen-dependent cell proliferation); hence, CYP2D6 PMs are at increased risk for breast cancer recurrence following tamoxifen adjuvant therapy (Goetz et al., 2005; Schroth et al., 2009). The effects of codeine in CYP2D6 UMs (who form too much active metabolite) and tamoxifen in PMs (who form too little active metabolite) represent examples of Type A and Type F toxicity, respectively (Aronson and Ferner, 2003).

The propensity for CYP2D6 to metabolize so many cardiovascular and CNS drugs (and other lipophilic amines) can be attributed in large part to the binding of the amine function to anionic residues (Glu216 and Asp301) in the enzyme's substrate-binding site. Substrate oxidation usually occurs 5 to 7.5 Å from the basic nitrogen atom. Other substrate binding orientations are possible, which is why CYP2D6 can *N*-dealkylate drugs, even drugs that contain a single amine function (i.e., drugs that appear to bind upside down in the active site) (Bonn et al., 2008; Unwalla et al., 2010).

Quinidine is a potent inhibitor of CYP2D6 because it interacts favorably with the anionic sites on CYP2D6 but it cannot be oxidized at a site 5 to 7.5 Å from its basic nitrogen atoms. Quinidine, paroxetine, and fluoxetine (and especially norfluoxetine, its *N*-demethylated metabolite) are strong CYP2D6 inhibitors in vivo, and a PM phenotype can be induced pharmacologically with these particular inhibitors. Quinine, the levorotatory diastereomer of quinidine, is not a potent inhibitor of CYP2D6, and neither drug is a potent inhibitor of the CYP2D enzymes expressed in rats. Compared with other SSRIs, fluoxetine causes surprisingly prolonged inhibition of CYP2D6; this is attributable in part to the long half-life of norfluoxetine (a primary amine), which also inhibits CYP2D6 (Liston et al., 2002). Paroxetine has a relatively short plasma half-life (16 to 24 hours) but causes prolonged inhibition of CYP2D6 because it contains a methylenedioxy (benzodioxole) moiety that causes irreversible inhibition (MDI) of CYP2D6 (Venkatakrishnan and Obach, 2005). Because of the low dose of paroxetine (20 mg), maximum inactivation of CYP2D6 activity is not achieved without 7 to 10 days of paroxetine treatment. Bupropion, a commonly used in vitro probe or index of CYP2B6 activity, causes clinically relevant inhibition of CYP2D6 even though it is a relatively weak inhibitor of the enzyme in vitro (in human liver microsomes) (Reese et al., 2008). Bupropion represents one of a handful of examples of system-dependent inhibition (Parkinson et al., 2010) (see the section "Inhibition of Cytochrome P450"). The ability of bupropion to inhibit CYP2D6 in vivo is attributable in large part to its reduction to inhibitory metabolites (erythrohydrobupropion and threohydrobupropion). Accordingly, the in vivo inhibition of CYP2D6 by bupropion is better predicted in human hepatocytes than in human liver microsomes (Parkinson et al., 2010).

The prominent determinants of CYP2D6 activity in vivo are genetic polymorphisms and treatment with inhibitory drugs. CYP2D6 is considered a noninducible enzyme despite in vitro and clinical data to the contrary (Spina et al., 1996; Madan et al., 2003; Farooq et al., 2016). For example, phenobarbital decreases systemic exposure to desipramine, a CYP2D6 probe/index drug, by 40% in CYP2D6 EMs but not in CYP2D6 PMs, suggesting induction of CYP2D6 is the mechanism by which phenobarbital increases desipramine clearance (Spina et al., 1996). Rifampin increases the oral clearance of the CYP2D6 substrates sparteine, dextromethorphan, and debrisoquine approximately twofold, whereas carbamazepine causes a similar increase in the oral clearance of risperidone (Farooq et al., 2016). CYP2D6 levels increase during pregnancy (Sit et al., 2010) and this effect has been attributed to induction of CYP2D6 by corticosteroids, which rise during gestation (Farooq et al., 2016). In contrast, renal impairment is associated with a decrease in CYP2D6 levels (Rostami-Hodjegan et al., 1999). Although these data establish that CYP2D6 can be induced (and repressed) to a clinically significant extent, it should be emphasized that genetic polymorphisms and duplication of the CYP2D6 gene, giving rise to PMs and UMs, respectively, are the major contributor to interindividual differences in CYP2D6 activity.

Compared with hepatic levels, low levels of CYP2D6 are present in several tissues including the brain, kidney, lung, intestine, breast, and placenta (Zhou, 2009a, 2009b). The metabolism of CNS drugs by CYP2D6 in the brain has attracted considerable interest, as has its ability to convert *m*- and *p*-tyramine to dopamine and to catalyze the 21-hydroxylation of certain steroid hormones such as progesterone.

Individuals lacking CYP2D6 have an unusually low incidence of some chemically induced neoplastic diseases (Idle, 1991). For example, CYP2D6 PMs appear to be relatively resistant to lung cancer, whereas CYP2D6 UMs appear to be at increased risk, although the effects are modest (Rostami-Hodjegan et al., 1998; Agundez et al., 2001). It has been hypothesized that CYP2D6 may play a role in the metabolic activation of chemical carcinogens, such as those present in the environment, in the diet, and/or in cigarette smoke. According to this hypothesis, CYP2D6 PMs have a low incidence of cancer because they fail to activate chemical carcinogens, whereas CYP2D6 UMs have an increased risk because they rapidly and/or extensively activate procarcinogens. However, CYP2D6 plays little or no role in the activation of known chemical carcinogens to DNA-reactive or mutagenic metabolites, with the notable exception of the tobacco-smoke-specific nitrosamine, NNK, which is also activated by numerous other CYP enzymes (see the section "CYP2A6, CYP2A7, and CYP2A13").

CYP2E1 As shown in Fig. 6-29, CYP2E1 was first identified as the microsomal ethanol oxidizing system (MEOS) (Lieber, 1999). It is now recognized as a key enzyme involved in the activation of acetaminophen (Tylenol) to its reactive metabolite NAPQI (see

Fig. 6-39), a leading cause of drug-induced liver injury (Kaplowitz, 2005). CYP2E1 also biotransforms a large number of volatile halogenated alkanes (chloroform, carbon tetrachloride, vinyl chloride, and many more), halogenated anesthetics (halothane, enflurane, and others), small aromatic and nitrogen aromatic compounds (benzene, styrene, toluene, pyridine, pyrazole, and others), alkanes (ethane–hexane), alcohols (ethanol–pentanol), the colon carcinogen azoxymethane, and numerous mutagenic/carcinogenic nitrosamines (Pohl and Scinicariello, 2011). In many cases CYP2E1 converts the aforementioned xenobiotics to reactive (toxic/mutagenic/carcinogenic) metabolites, as shown in Table 6-18 and as illustrated for carbon tetrachloride in Fig. 6-22, halothane in Fig. 6-23, benzene in Fig. 6-36, and *N*-nitrosamine, urethane (ethyl carbamate), trichloroethylene, and tetrachloroethylene in Fig. 6-56 (Guengerich, 1991). CYP2E1 activates acetylhydrazine, a metabolite formed following the *N*-acetylation of isoniazid, to a hepatotoxic metabolite. The severity of isoniazid hepatotoxicity is influenced by genetic polymorphisms that decrease *N*-acetyltransferases (NAT) and increase CYP2E1 activity, as discussed in the section "Acetylation."

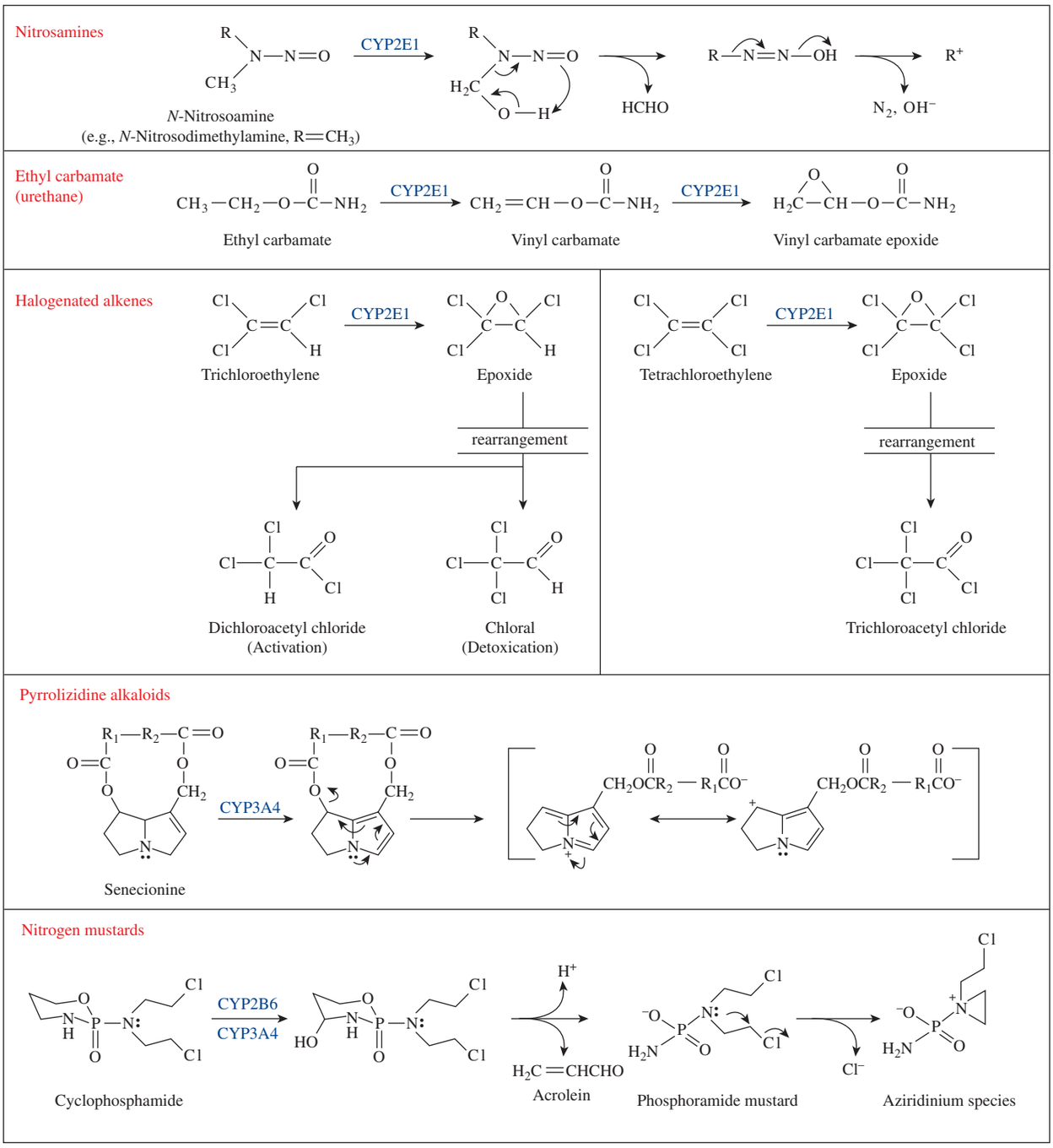


Figure 6-56. *Additional mechanisms of cytochrome P450–dependent activation of xenobiotics to reactive (electrophilic) metabolites.*

Table 6-18

Examples of Xenobiotics Activated by Human P450

CYP1A2	CYP2E1
Acetaminophen	Acetaminophen
2-Acetylaminofluorene	Acrylonitrile
4-Aminobiphenyl	Benzene
2-Aminofluorene	Carbon tetrachloride
2-Naphthylamine	Chloroform
NNK	Dichloromethane
Amino acid pyrolysis products	1,2-Dichloropropane
(DiMeQx, MelQ, MelQx, Glu P-2, IQ, PhlP, Trp P-1, Trp P-2)	Ethylene dibromide Ethylene dichloride
Tacrine	Ethyl carbamate
CYP2A6 and 2A13	Halothane
NNK and bulky nitrosamines	*N*-Nitrosodimethylamine
N-Nitrosodiethylamine	Styrene
Aflatoxin B_1	Trichloroethylene
CYP2B6	Vinyl chloride
6-Aminochrysene	CYP3A4
Cyclophosphamide	Acetaminophen
Ifosfamide	Aflatoxin B_1 and G_1
CYP2C8, 9, 18, 19	6-Aminochrysene
Tienilic acid	Benzo[*a*]pyrene 7,8-dihydrodiol
Phenytoin	Cyclophosphamide
Valproic acid	Ifosfamide
CYP2D6	1-Nitropyrene
NNK	Sterigmatocystin
CYP2F1	Senecionine
3-Methylindole	*Tris*(2,3-dibromopropyl)phosphate
Acetaminophen	CYP4B1 (in animals but not humans)*
Valproic acid	Ipomeanol
CYP1A1 and 1B1	3-Methylindole
Benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons	2-Aminofluorene

Abbreviation: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific nitrosamine.
** Due to lack of heme incorporation human CYP4B1 is probably inactive.*
SOURCE: Data adapted from Guengerich and Shimada (1991).

Studies in CYP2E1 knockout mice have established an important role for CYP2E1 in the in vivo activation of some of the aforementioned xenobiotics. Compared with wild-type mice, CYP2E1 knockout mice are protected (partially or completely) from the hepatotumorigenic effects of urethane (ethyl carbamate), the preneoplastic changes induced in the colon by azoxymethane, the myelotoxicity of benzene, and the hepatotoxicity of acetaminophen (Gonzalez, 2003, 2007; Ghanayem, 2007). CYP2E1 is one of three enzymes that catalyze the conversion of acetaminophen to NAPQI, the others being CYP1A2 and CYP3A4. Double knockout mice, in which both CYP2E1 and CYP1A2 are deleted, are even more resistant to acetaminophen hepatotoxicity than CYP2E1 knockout mice (Gonzalez, 2007). These findings in mice are thought to translate to the situation in humans. It is interesting to note, however, that human CYP2E1 knockin mice (transgenic mice harboring human CYP2E1 in place of the mouse enzyme, also known as CYP2E1-humanized mice) are more resistant to acetaminophen hepatotoxicity than wild-type mice, suggesting mouse CYP2E1 may be more active than human CYP2E1 in converting acetaminophen to NAPQI (Cheung and Gonzalez, 2008). The role of CYP2E1 in acetaminophen hepatotoxicity is discussed later in this section.

The only orally administered drug (as opposed to volatile anesthetics) that is primarily metabolized by CYP2E1 is chlorzoxazone, an FDA-approved muscle relaxant (Paraflex®). The

urinary excretion of 6-hydroxychlorzoxazone and the plasma ratio of 6-hydroxychlorzoxazone to chlorzoxazone have been used as in vivo probes of CYP2E1. In human liver microsomes, CYP2E1 activity can be conveniently measured by the 6-hydroxylation of chlorzoxazone and the hydroxylation of 4-nitrophenol (Table 6-17). The 6-hydroxylation of chlorzoxazone can also be catalyzed by CYP1A1, but this enzyme is rarely expressed in the human liver and only low levels are present in extrahepatic tissues. CYP2E1 is one of the CYP enzymes that require cytochrome b_5, which lowers the K_m for certain substrates (Levin et al., 1986).

The function and regulation of CYP2E1 are relatively well conserved across mammalian species. CYP2E1 is expressed constitutively in the liver and several extrahepatic tissues, such as the kidney, pancreas, brain, lung, intestine, nasal epithelium, bone marrow, and lymphocytes. The levels of CYP2E1 in human liver microsomes do not show the high degree of interindividual variation characteristic of other enzymes, as shown in Table 6-16. Numerous genetic polymorphisms in the CYP2E1 gene have been identified but none impact function and/or expression in a manner that gives rise to a PM or UM phenotype (Pohl and Scinicariello, 2011). In humans and most other mammalian species, CYP2E1 is moderately inducible (less than fivefold) by ethanol, isoniazid, pyrazole, acetone, and other ketone bodies (which form during uncontrolled diabetes). In contrast to the induction of most other CYP enzymes, the induction of CYP2E1 often involves increased translation of preexisting mRNA and/or protein stabilization rather than transcriptional activation of the CYP2E1 gene; hence, induction of CYP2E1 often occurs in the absence of an increase in mRNA levels (Gonzalez, 2007). An exception to this general rule is the induction of CYP2E1 by diabetes, which involves transcriptional activation and a marked increase in CYP2E1 mRNA. CYP2E1 can be inhibited by 4-methylpyrazole (fomepizole), which also inhibits ADH (class I ADH), and by disulfiram (Antabuse), which also inhibits ALDH (ADH2). Disulfiram is one of many sulfur-containing agents that competitively or irreversibly inhibit CYP2E1, such as chlormethiazole, diallylsulfide, diethyldithiocarbamate, and phenethylisothiocyanate (Pohl and Scinicariello, 2011; Stresser et al., 2016).

Alcohol can potentiate the hepatotoxicity of acetaminophen. Several mechanisms have been proposed, including increased activation of acetaminophen due to the induction of CYP2E1, increased oxidative stress (reactive oxygen species production), and decreased inactivation of NAPQI and reactive oxygen species due to a lowering of GSH levels. Likewise fasting potentiates acetaminophen hepatotoxicity, presumably by inducing CYP2E1 (which is inducible by ketone bodies) and lowering UDPGA levels, which impairs the direct conjugation of acetaminophen and directs more drug toward the formation of NAPQI (Whitcomb and Block, 1994).

CYP2E1 is receiving considerable attention for several reasons that impact our understanding of its role in acetaminophen hepatotoxicity and alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD) (Abdelmegeed et al., 2015). First, during its catalytic cycle CYP2E1 is prone to uncoupling (see Fig. 6-45), such that, during the metabolism of ethanol (alcohol) and acetaminophen, roughly 50% of its catalytic cycles result in the production of superoxide anion and related reactive oxygen species instead of metabolite formation (Gonzalez, 2005; Lu and Cederbaum, 2008). Second, in the rat liver, approximately 40% of CYP2E1 is phosphorylated and localized to the inner membrane of mitochondria (Knockaert et al., 2011a, 2011b). Like all microsomal enzymes, CYP2E1 has a canonical N-terminal signal recognition peptide (SRP) that targets its translation to the rough endoplasmic reticulum (in ribosomes bound to the endoplasmic reticulum), but the process is relatively inefficient such that about half of CYP2E1 is transcribed membrane-free ribosomes. CYP2E1 contains a mitochondrial targeting signal (two positively charged amino acids, Lys_{24} and Lys_{25}) and a serine residue (Ser_{129}) that, when phosphorylated by cAMP-dependent protein kinase A (PKA), complexes with heat-shock proteins (Hsp70 and Hsp90) and is translocated to the inner membrane of mitochondria (Knockaert et al., 2011b). Cell culture experiments in which CYP2E1 is expressed only in mitochondria or in both mitochondria and microsomes established that mitochondrial CYP2E1 is catalytically active (it is presumably supported by ferredoxin/ferredoxin reductase, the electron transporters that support endobiotic biotransformation by mitochondrial CYP enzymes) and is sufficient to cause oxidative stress, GSH depletion, and cell toxicity in response to acetaminophen or alcohol (Knockaert et al., 2011a). A variety of evidence points to mitochondrial toxicity by NAPQI and reactive oxygen species as the key event in acetaminophen hepatotoxicity (Jaeschke et al., 2012). For example, studies with radioactive acetaminophen (4′-hydroxyacetanilide) and its nonhepatotoxic *meta* isomer 3′-hydroxyacetanilide show comparable levels of overall covalent binding to mouse liver proteins but covalent binding of acetaminophen is predominantly in mitochondria, whereas binding of the nonhepatotoxic regioisomer is in the cytoplasm (Jaeschke et al., 2012). The mitochondrial localization of CYP2E1 and its ability to form NAPQI and reactive oxygen species in the mitochondrion appear to be important determinants of acetaminophen hepatotoxicity. It is of interest that several CYP enzymes in addition to CYP2E1 have been located inside mitochondria (Knockaert et al., 2011a, 2011b).

There is growing evidence that the potentially deleterious reactive oxygen species produced by microsomal CYP2E1 and other CYP enzymes (notably CYP1A2; the other high-spin enzyme that, like CYP2E1, can produce reactive oxygen species even in the absence of substrate) is actively inhibited during the process known as ER stress, which is triggered by the accumulation of unfolded proteins (the ER plays a key role in protein folding and posttranslational modification of proteins prior to their exit to the Golgi and other destinations) , which occurs in response to ischemia/reperfusion injury and exposure to certain xenobiotics such as tunicamycin (an *N*-glycosylation inhibitor) and thapsigargin (an ER calcium ATPase inhibitor) (Rasheva and Domingos, 2009; Tabas and Ron, 2011). Extensive ER stress (i.e., a prolonged unfolded protein response [UPR], which is mediated by PERK, ATF6, and IRE1) results in the activation of caspases and Bcl-2, which leads to apoptotic cell death. However, mild ER stress activates Nrf2 and the antiapoptotic factor BI-1. Both activation of Nrf2 (through its phosphorylation by PERK, one of the three main ER stress responders) and BI-1 protect the endoplasmic reticulum from further damage by inhibiting the production of reactive oxygen species by CYP2E1 (and presumably other CYP enzymes such as CYP1A2) by two distinct mechanisms (Lee et al., 2007; Kim et al., 2009). In the case of Nrf2, this transcription factor causes a marked (about 10-fold) induction of heme oxygenase (among other antioxidant defense enzymes), which inhibits CYP activity by competing for electrons from POR (which serves both enzymes) (Reed et al., 2011). Heme oxygenase can also inactivate certain CYP enzymes by converting their heme moiety to biliverdin (Kutty et al., 1988). BI-1 also inhibits CYP but does so by complexing with POR (the two proteins coimmunoprecipitate), which inhibits reactive oxygen species production by CYP2E1 (Kim et al., 2009). From these studies, it is emerging that CYP2E1 and possibly CYP1A2, the two high-spin CYP enzymes, produce reactive oxygen species and contribute to oxidative stress, processes that are augmented by xenobiotics such as acetaminophen and alcohol and processes that are particularly damaging during ER stress.

CYP2F1 CYP2F1 is a human enzyme; its homologs in mouse and rat are CYP2F2 and CYP2F4, respectively. These enzymes are expressed in the respiratory tract (lung and nasal mucosa), although CYP2F1 is expressed in human lung at much lower levels than CYP2F2 is expressed in mice. In mice CYP2F2 plays an important role in activating various pulmonary/nasal toxicants including 3-methylindole, naphthalene, 1-nitronaphthalene, styrene, benzene, B[*a*]P, dichloroethylene, and trichloroethylene. For example, CYP2F2 knockout mice are resistant to the toxic effects of naphthalene on the lung and nasal olfactory mucosa (Li et al., 2011a) and they are similarly resistant to the toxic effects of styrene on lung Clara cells and terminal bronchial cells (Cruzan et al., 2012, 2013). Studies on the metabolic activation of 3-methylindole (a constituent in tobacco smoke) are interesting for illustrating how two respiratory enzymes CYP2F2 and CYP2A5 (the homologs of human CYP2F1 and CYP2A13) can make the same initial reactive metabolites with different subsequent fates (Zhou et al., 2012). Both CYP2F2 and CYP2A convert 3-methylindole to a carbon radical and epoxide, but these are further converted by CYP2A5 to stable metabolites: the carbon radical is converted to indole-3-carbinol by oxygen rebound and the epoxide is converted to an amide (3-methyloxindole) by rearrangement. In contrast, CYP2F2 converts these intermediates to reactive metabolites: the carbon radical is converted by dehydrogenation to a Michael acceptor, 3-methyleneindolenine, whereas the epoxide is either released or converted to iminium ions, all of which react with GSH. In animals, 3-methylindole can also be activated by another pulmonary enzyme, namely, CYP4B1, an enzyme that is functionally inactive in humans due to lack of heme incorporation (see the section "CYP4 Enzymes").

CYP2G1 CYP2G1 is not expressed in humans but it is expressed in the olfactory mucosa of rodents and other animals. Numerous xenobiotics cause nasal cytotoxicity and cancer in rodents (Jeffrey et al., 2006). Along with other CYP enzymes that are highly expressed in olfactory mucosa, such as CYP2A5 and CYP2F2 in mice and CYP2A3 and CYP2F4 in rats, CYP2G1 is a potential contributor to the formation of reactive metabolites in this target of xenobiotic toxicity in rodents.

CYP2J2 CYP2J2 is of interest for a number of reasons. First, it is expressed in vascular epithelium and high levels are found in the heart and many other tissues. CYP2J2 is the most abundant CYP expressed in heart and left ventricular levels are about 1.5-fold higher in women than in men (Michaud et al., 2010). Based on mRNA levels (which may not reflect protein levels), the expression of CYP2J2 is highest in small intestine followed by heart, skeletal muscle, kidney, salivary gland, lung, and liver. Second, CYP2J2 metabolizes many of the same drugs that are substrates for CYP3A4 (although not always to the same metabolites) and it is inhibited by many of the same drugs that inhibit CYP3A4 (Lee et al., 2010a, 2012). Various derivatives of terfenadine and other compounds have been identified as selective competitive or metabolism-dependent inhibitors of CYP2J2 (Lafite et al., 2006, 2007; Karkhanis et al., 2016; Phuc et al., 2017). However, given the relatively low levels of intestinal and hepatic CYP2J2 compared with CYP3A4, Lee et al. (2010a) concluded that the contribution of CYP2J2 to presystemic clearance of drugs is probably low compared with that of CYP3A4. Furthermore, in contrast to CYP3A4, CYP2J2 is not inducible by CAR/PXR agonists (Xu et al., 2013).

The third notable aspect is that CYP2J2 is the major enzyme in vascular endothelial cells responsible for the epoxidation of arachidonic acid to four EETs (5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid) with a minor contribution from CYP2C8 and CYP2C9 (Liu et al., 2006a; Spiecker and Liao, 2006; Deng et al., 2012; Xu et al., 2013; Karkhanis et al., 2017). EETs are endothelin-derived hyperpolarizing factors (EDHFs) (i.e., vasodilators) that possess anti-inflammatory properties and protect tissues from ischemic injury. Hydrolysis of EETs by sEH terminates their vasodilatory and anti-inflammatory effects (see the section "Epoxide Hydrolases") (Fretland and Omiecinski, 2000; Morisseau and Hammock, 2005; Imig and Hammock, 2009; Fleming, 2014; Kodani and Hammock, 2015; He et al., 2016). The acute vascular inflammatory response to endotoxin in transgenic mice can be attenuated either by overexpressing CYP2J2 (or CYP2C8) or by deleting sEH (sEH knockout mice) (Deng et al., 2012).

The fourth and final notable aspect is that CYP2J2 is overexpressed in many human cancers and cancer-derived cell lines (Chen et al., 2011a; Xu et al., 2011). In human-derived tumor cell lines, overexpression of CYP2J2 (or direct application of EETs) stimulates cell proliferation and inhibits apoptosis, whereas the opposite effects are achieved by inhibiting CYP2J2 with terfenadine-like inhibitors or silencing CYP2J2 expression with antisense oligonucleotides (Chen et al., 2009, 2011a; Xu et al., 2011). Inhibitors of CYP2J2 are under consideration as anticancer drugs, but by blocking EET formation in vascular endothelial cells they may potentially predispose to hypertension and coronary artery disease (Chen et al., 2011a; Xu et al., 2011, 2013; Karkhanis et al., 2017), whereas inhibitors of sEH are under consideration as cardioprotective drugs, but by inhibiting the hydrolysis of EETs in cancer cells they may potentially predispose to tumor development and metastatic tumorigenesis (Imig and Hammock, 2009; Wang et al., 2010c; Xu et al., 2013; Karkhanis et al., 2017).

In addition to its role in arachidonic acid metabolism, CYP2J2 can also catalyze the 25-hydroxylation of vitamin D_1, D_2, and D_3, the first of two steps in the formation of the active metabolite 1α,25-dihydroxyvitamin D (Aiba et al., 2006).

CYP2R1, CYP2S1, CYP2U1, and CYP2W1 CYP2R1, CYP2S1, CYP2U1, and CYP2W1 are four of the eight members of the CYP1 and CYP2 families that are so well conserved from an evolutionary perspective that they have been given the same names across all mammalian species (the other members are CYP1A1, CYP1A2, CYP1B1, and CYP2E1). These enzymes have been conserved during evolution presumably because they perform an important but in some cases a poorly defined physiological function. The hepatic enzyme CYP2R1 was identified as a vitamin D 25-hydroxylating enzyme in 2004, but other CYP enzymes in human liver microsomes (CYP27A1, CYP2J2, and CYP3A4) have been implicated in catalyzing this first step in the activation of vitamin D to 1α,25-dihydroxyvitamin D (Cheng et al., 2004; Schuster, 2011). However, based on an analysis of genetic determinants of vitamin D deficiency, two groups established that genetic polymorphism of CYP2R1 is associated with circulating 25-hydroxyvitamin D, which provides strong evidence that CYP2R1is the main enzyme responsible for the critical first step in vitamin D activation (Ahn et al., 2010; Wang et al., 2010b).

CYP2U1 is highly expressed in the brain and thymus and to a lesser extent in the kidney, lung, and heart (Devos et al., 2010). CYP2U1 metabolizes arachidonic acid, docosahexaenoic acid (DHA), and other long-chain fatty acids. CYP2U1 catalyzes the ω-1 and ω-hydroxylation of arachidonic acid 19- and 20-HETE. The latter metabolite, 20-HETE, is a powerful vasoconstrictor in the kidney and brain, which suggests CYP2U1 may play a role in the regulation of blood flow in these tissues. However, several CYP4A and CYP4F enzymes can also catalyze the ω-1 and ω-hydroxylation of arachidonic acid to 19- and 20-HETE. Genetic polymorphisms in CYP2U1 have been identified but they do not appear to alter its function or expression (Devos et al., 2010).

CYP2S1 and CYP2W1 are intriguing enzymes because they are expressed in certain tumors and because they have unusual catalytic properties. CYP2S1 is expressed in several extrahepatic tissues such as the lung, trachea, stomach, small intestine, colon, spleen, bladder, and skin (Saarikoski et al., 2005). It is also expressed in psoriatic skin, ovarian cancer, and colorectal tumors where its presence is associated with poor prognosis (Kumarakulasingham et al., 2005). CYP2W1 is expressed in a large number of tumors including colorectal and adrenal tumors, but it does not appear to be expressed to a significant extent in normal (i.e., noncancerous) hepatic or extrahepatic tissues (Karlgren et al., 2006). Both enzymes are induced by hypoxia, which may account for their upregulation in tumors. CYP2S1 is also inducible by AhR agonists and joins CYP1A1, CYP1A2, and CYP1B1 as a dioxin-inducible enzyme (Saarikoski et al., 2005). There is some evidence to suggest that, at least in certain cell lines, CYP2W1 is also inducible by AhR ligands (Tan et al., 2011).

There is considerable controversy surrounding the catalytic function of CYP2S1, but this controversy has been resolved (at least in part) by understanding the importance of oxygen tension in the function of CYP2S1. Under aerobic conditions, CYP2S1 can catalyze the oxidative metabolism of several carcinogens (PAHs and their diol epoxides, aflatoxin B_1, naphthalene, and styrene) but only if these reactions are supported by lipid peroxides or hydrogen peroxide (by the peroxide shunt) and not if the enzyme is supported by NADPH and O_2 together with POR (Wu et al., 2006; Bui and Hankinson, 2009; Bui et al., 2009). However, under anaerobic conditions, CYP2S1 can efficiently use POR and NADPH to catalyze reductive reactions, as can CYP2W1 (discussed later in this section) (Nishida et al., 2010). These reactions are inhibited by oxygen. The ability of CYP2S1-expressing cells to catalyze the oxidative metabolism of carcinogens is suspect of being due to the presence of intracellular lipid peroxides and hydrogen peroxide that support the peroxide shunt (Bui and Hankinson, 2009; Bui et al., 2009).

CYP2W1 catalyzes the oxidative metabolism of a wide variety of PAHs and their diol epoxides, aflatoxin B_1, and indoline to cytotoxic/mutagenic metabolites (Wu et al., 2006; Gomez et al., 2010). CYP2W1 also hydroxylates and epoxidates a variety of lysophospholipids and catalyzes the 4-hydroxylation of *all-trans* retinoic acid and *all-trans* retinol (Xiao and Guengerich, 2012; Zhao et al., 2016). It is unusual, however, for being one of the few CYP enzymes to undergo glycosylation, which places CYP2W1 on the luminal side of the endoplasmic reticulum, which is the wrong side for an interaction with POR, which faces the cytoplasmic side (Gomez et al., 2010). Nevertheless, CYP2W1 expressed in cells is functional, either because it uses lipid peroxides/hydrogen peroxide (as does CYP2S1) or because it can use electron donors other than POR. As a result of its luminal orientation in the endoplasmic reticulum (where it becomes glycosylated), approximately 8% of CYP2W1 is expressed on the plasma membrane (Gomez et al., 2010).

Because of their presence and upregulation in hypoxic tumors, CYP2S1 and especially CYP2W1 have been targeted for their potential to activate anticancer drugs (Travica et al., 2013; Choong et al., 2015; Chung et al., 2016). AQ4N is a prodrug (a di-*N*-oxide) that undergoes *N*-oxide reduction to 4QA, a potent topoisomerase II inhibitor. The reaction is catalyzed by both CYP2S1 and CYP2W1 under anaerobic conditions. The reductive activation by CYP2S1 and CYP2W1 requires POR but the reaction is inhibited by oxygen.

Phortress (a prodrug) is representative of a new class of anticancer drugs that are derivatives of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole, better known as 5F-203. Phortress is in clinical trials as an anticancer drug. It is an AhR agonist that induces CYP1A1 and induces its own activation to one or more cytotoxic metabolites (Brantley et al., 2004). Like CYP1A1, CYP2W1 also activates 5F-203 (the active metabolite of Phortress) and the related fluorinated benzothiazole GW-610 to cytotoxic metabolites, whereas CYP2S1 converts 5F-203 and GW-610 to inactive metabolites (Tan et al., 2011). 5F-203 and GW-610 are AhR agonists that induce CYP1A1, CYP2S1, and, in certain cell lines, CYP2W1 (Tan et al., 2011). CYP2W1 is inducible by imatinib and can activate duocarmycin to cytotoxic metabolites suggesting that a combination of these two anticancer drugs might be a promising approach to treat colorectal cancer (Choong et al., 2015).

CYP3A Enzymes In terms of the number of drugs it metabolizes, CYP3A4 is the most important CYP enzyme (Rendic and Guengerich, 2015). Numerous drugs, herbals, and food constituents inhibit or induce CYP3A4, making the enzyme the center of many drug interactions. The high levels in the liver and small intestine (where it works in conjunction with efflux transporters including P-gp, BCRP, and MRP2) allow CYP3A4 to play a key role in the presystemic elimination of drugs and other xenobiotics. CYP3A5 is a polymorphically expressed enzyme expressed in the liver, small intestine, and kidney. It is unusual because, in several ethnic groups, the CYP3A5*3 allele that gives rise to a PM genotype is more prevalent than the wild-type genotype (the *1 allele), such that the majority of Caucasians, Asians, and Mexicans are CYP3A5 PMs. CYP3A5 plays a significant role in the disposition of tacrolimus but few other drugs. CYP3A7 is expressed in the fetal liver. It is also expressed in the adult liver and the levels increase with age (Ohtsuki et al., 2012). CYP3A43 is the major CYP3A enzyme in the brain where it plays an important role in the disposition of a select few drugs.

The function and regulation of the CYP3A enzymes are fairly well conserved among mammalian species, with some notable exceptions. For example, rifampin is an inducer of the CYP3A enzymes in humans and rabbits but not rats or mice, whereas the opposite is true of PCN. In adult rats, the levels of CYP3A2 in males are much greater (more than 10-fold) than in females (Waxman and Holloway, 2009). In humans, men do not have higher levels of CYP3A4 than women; in fact, there is evidence to suggest that the reverse is true—that women have slightly higher levels of CYP3A4 than men, although it should be noted that CYP3A4 levels vary widely in both men and women (Parkinson et al., 2004; Soldin et al., 2011).

CYP3A4 CYP3A4 is abundantly expressed in the liver and small intestine, where it biotransforms an extraordinary array of xenobiotics and endobiotics (such as steroid hormones, sterols, and vitamin D), including drugs from numerous therapeutic classes as shown in Table 6-17. CYP3A4 metabolizes 27% of drugs ($n = 860$) and 15% of natural products/endobiotics ($n = 331$ compounds) (Rendic and Guengerich, 2015). A large number of clinically significant drug–drug, drug–herbal, and drug–food interactions involve the inhibition or induction of hepatic and/or intestinal CYP3A4. Three victim drugs whose clearance is mainly determined by CYP3A4, namely, terfenadine (Seldane), cisapride (Propulsid), and astemizole (Hismanal), have been withdrawn from the market because of their potential to cause ventricular arrhythmias (and on rare occasions heart attacks) when their metabolism by CYP3A4 was inhibited by drugs such as ketoconazole and erythromycin (Huang et al., 2008; Zhang et al., 2009). One perpetrator drug, namely, mibefradil (Posicor), has been withdrawn from the market because of its ability to cause marked and prolonged inhibition of CYP3A4 by virtue of its ability to cause irreversible inhibition (MDI) of CYP3A4 (which persists after the inhibitory drug is withdrawn and until new enzyme is synthesized) (Foti et al., 2011).

Intestinal CYP3A4 contributes significantly to the presystemic elimination of drugs because drugs in the small intestine are recycled by efflux transporters such as P-gp, BCRP, and MRP2 on the luminal surface of enterocytes, which delays absorption and increases the opportunity for xenobiotic biotransformation by CYP3A4 (Benet, 2009; Galetin et al., 2010). Cobicistat is a structural analog of ritonavir. Despite being devoid of antiviral activity, cobicistat shares ritonavir's ability to inhibit CYP3A4 and P-gp and accordingly is approved as a pharmacokinetic booster for combination with various antiviral drugs to increase and prolong their systemic exposure (Deeks, 2014; Marzolini et al., 2016). Like ritonavir, cobicistat causes particularly large increases in systemic exposure to drugs whose intestinal absorption is normally restricted by P-gp efflux and metabolism by intestinal CYP3A4 and whose systemic clearance is determined largely by hepatic metabolism by CYP3A4. For example, ritonavir can increase systemic exposure to atazanavir and saquinavir more than 25-fold (University of Washington DIDB, 2018). CYP3cide is a metabolism-dependent inhibitor that inactivates CYP3A4 but not CYP3A5; it is used in vitro to distinguish the relative contribution of CYP3A4 and CYP3A5 to the metabolism of drugs by human liver microsomes, which is discussed in the next section "CYP3A5" (Tseng et al., 2014).

Furanocoumarins in grapefruit juice inhibit intestinal but not hepatic CYP3A4 and yet grapefruit juice increases systemic exposure to felodipine and other drugs that undergo first-pass metabolism by CYP3A4 (Paine et al., 2006). The impact of the CYP3A4 inhibitor ketoconazole on the disposition of the CYP3A4 substrate midazolam depends on whether midazolam is given orally or intravenously. When midazolam is given intravenously, such that its clearance is dependent only on hepatic metabolism, ketoconazole causes a three- to fivefold increase in the plasma AUC of midazolam. However, when midazolam is given orally, ketoconazole causes a 10- to 15-fold increase in plasma AUC, the difference reflecting the significant role of intestinal metabolism to the presystemic clearance of midazolam. In rats, administration of the general CYP inhibitor 1-aminobenzotriazole (ABT) by the intravenous route (which inhibits only hepatic CYP enzymes) and the oral route (which inhibits both intestinal and hepatic CYP enzymes) can be used to assess the relative role of the intestine and liver to the first-pass metabolism of a drug or other xenobiotic (Strelevitz et al., 2006; Boily et al., 2015).

The induction of CYP3A4 is mediated by CAR and PXR, agonists for which include several antibiotics, such as rifampin and rifabutin, several EIAEDs, such as phenobarbital, phenytoin, carbamazepine, and felbamate, and the herbal St. John's wort (active inducer hyperforin), as shown in Table 6-17. The induction of intestinal and hepatic CYP3A4 (and conjugating enzymes, as outlined in the sections "Glucuronidation and Formation of Acyl-CoA Thioesters" and "Sulfonation") by these drugs can result in a loss of therapeutic efficacy of oral contraceptive steroids, anti-HIV, and anti-organ rejection drugs. It is an important mechanism of drug–drug and drug–herbal interactions (Huang et al., 2008). Prolonged induction of CYP3A4 also adversely impacts the metabolism of vitamin D, as discussed in the section "Induction of Cytochrome P450: Xenosensors." As mentioned earlier in the section "CYP2C8," there is in vitro evidence that CYP3A4 along with CYP2C8 and CYP1A1 are inducible by activators of human PPARα, the xenosensor linked with induction of CYP4A enzymes in rodents (Seree et al., 2004; Prueksaritanont et al., 2005; Klein et al., 2012; Thomas et al., 2013, 2015; Makia and Goldstein, 2016). Whether fibrates and other drugs that activate PPARα cause clinically relevant induction of CYP3A4 (or CYP1A1 and CYP2C8) is not known.

CYP3A4 metabolizes more drugs than any other xenobiotic-biotransforming enzyme, which is apparent from the large list of CYP3A4 substrates listed in Table 6-17. Like CYP2C8, CYP3A4 can metabolize large substrates, such as paclitaxel (Taxol®), which is a substrate for both enzymes. The active site of CYP3A4 is sufficiently large to bind two drugs simultaneously (Ekroos and Sjögren, 2006), and individual drugs tend to bind to discrete regions, which has several consequences. First, the binding of two substrates to CYP3A4 allows for homotropic activation (where a substrate stimulates its own metabolism, as in the case of testosterone and diazepam) and heterotropic activation (where one compound, such as α-naphthoflavone, stimulates the metabolism of another). These effects give rise to non-Michaelis–Menten or atypical enzyme kinetics, which is characteristic of the metabolism of several drugs by CYP3A4 (Atkins, 2005). The binding of one ligand (substrate, inhibitor, or activator) to CYP3A4 can cause conformational changes that increase the size of the active site by 80% or more, which allows additional ligands to bind, possibly in a stacked or side-by-side configuration (Ekroos and Sjögren, 2006). Second, substrates can bind to relatively discrete sites within the active site. Testosterone and midazolam appear to bind to distinct sites (called the steroid and benzodiazepine binding site, respectively); consequently, these two prototypical substrates only weakly inhibit each other's metabolism by CYP3A4 (Schrag and Wienkers, 2001; Galetin et al., 2003). As a result of these different substrate-binding sites or orientations, it is possible for a drug to inhibit CYP3A4 in a substrate-dependent manner (Schrag and Wienkers, 2001; Galetin et al., 2003). However, inhibitors that coordinate directly with the heme moiety of CYP3A4 will inhibit the metabolism of all CYP3A4 substrates, as will most metabolism-dependent inhibitors of CYP3A4 (see the section "Inhibition of Cytochrome P450"). Although the active site of CYP3A4 is sufficiently large to bind substrates in apparently discrete sites and to bind two molecules simultaneously, it nevertheless often catalyzes reactions with a high degree of regioselectivity and stereoselectivity. In the case of testosterone, for example, CYP3A4 catalyzes hydroxylation at several sites on the β-face of the molecule (e.g., 1β-, 2β-, 6β-, and 15β-hydroxylation) but none on the α-face.

More than 40 SNPs have been identified in the CYP3A4 gene, some of which contribute to the large interindividual variation in CYP3A4 activity (Staatz et al., 2010). An individual with low CYP3A4 activity was found to be heterozygous for a null allele (CYP3A4*20) and was characterized as a genetically determined IM (Westlind-Johnsson et al., 2006). The *20 variant was the first null allele identified for CYP3A4. A genetic polymorphism in intron 6 (CYP3A4*22; rs35599367, C > T) affects the expression and, hence, the activity of CYP3A4 such that individuals who are homozygous for the *1 allele (CC carriers) have 1.7 times more CYP3A4 activity than heterozygotes (*1/*22 or CT) and 2.5 times more CYP3A4 activity than individuals homozygous for the *22 allele (TT carriers) (Wang et al., 2011a). Compared with *1/*1 (wild-type) carriers, individuals carrying one or two of the loss-of-function *22 alleles require lower doses (40% to 80% less) of atorvastatin, simvastatin, and lovastatin for optimal lipid control (Wang et al., 2011a). The same genetic polymorphism impacts the disposition of cyclosporine and tacrolimus (Elens et al., 2011a, 2011b). In individuals who lack CYP3A5 (the majority of Caucasians, for example), carriers of the *22/*22 (TT) genotype (low CYP3A4) and *1/*22 (TC) genotype (intermediate CYP3A4) have dose-adjusted concentrations of cyclosporine that are, respectively, 1.5- and 2.2-fold greater than in carriers of the *1/*1 (CC) genotype (high CYP3A4); in the case of tacrolimus they are 1.6- and 4.1-fold greater (Elens et al., 2011b).

The most widely studied CYP3A4 allelic variant (392A>G) is designated CYP3A4*1B. This allelic variant encodes a functional CYP3A4 enzyme that is expressed to the same extent (or perhaps to a slightly greater extent) than the wild-type enzyme (CYP3A4*1). The prevalence of the *1B allele varies from one ethnic group to the next as follows: 2% to 9.6% in Caucasians, 35% to 67% in Africans, 9.3% to 11% in Hispanics, and 0% in Asians. This polymorphism is linked to the *1 genetic polymorphism in CYP3A5 such that CYP3A5 EMs tend to express the CYP3A4*1B variant, as discussed in the next section.

CYP3A4*1G (rs2242480, 20239G>A) is the most prevalent allelic variant of CYP3A4 in Asian populations (up to about 35%), but the functional consequences of this genetic polymorphism appear to be substrate dependent. Clinical pharmacokinetic studies in genotyped Chinese subjects have shown that, compared with wild-type (*1/*1) individuals, individuals who are heterozygous or homozygous for the *1G allele (i.e., *1/*1G or *1G/*1G) have increased activity toward atorvastatin, cyclosporine, diltiazem, tacrolimus, and methadone but decreased activity toward fentanyl with little or no change in activity toward midazolam, cortisol, or ticagrelor (Qiu et al., 2008; Zhang et al., 2010; Miura et al., 2011; Uesugi et al., 2013; He et al., 2014; Chan et al., 2016; Zhou et al., 2016; Liu et al., 2017b).

CYP3A5 CYP3A5 is a polymorphically expressed enzyme whose role in drug metabolism is a matter of considerable debate. The relative role of CYP3A4 versus CYP3A5 in the disposition of drugs is not easy to ascertain from in vitro or in vivo studies. In general, both enzymes are inducible by the same drugs, both enzymes metabolize the same drugs, and both are inhibited by the same drugs, although the two enzymes may differ in the affinity with which they bind to substrates and inhibitors. For example, when evaluated as inhibitors of recombinant human CYP3A enzymes, 14 out of 14 compounds inhibited CYP3A4 more potently than they inhibited CYP3A5 by a factor of 3.9- to 142-fold (Ekins et al., 2003). CYP3cide is a selective irreversible inhibitor of CYP3A4 (discussed later in this section) (Tseng et al., 2014). A large number of drugs are metabolized in vitro by both CYP3A4 and CYP3A5. Some drugs appear to be metabolized by CYP3A4 but not CYP3A5; however, all of the drugs known to be metabolized by CYP3A5 are also metabolized by CYP3A4. Accordingly, there is no in vitro or in vivo probe drug or index substrate to selectively measure CYP3A5 activity.

Assessing the relative contribution of CYP3A4 and CYP3A5 to the metabolism of drugs is complicated by several factors. For example, studies with human liver microsomes are complicated by conflicting reports on the relative levels of CYP3A5 and CYP3A4; some reports indicate that the levels of CYP3A5 in human liver microsomes are uniformly low (Patki et al., 2003; Westlind-Johnsson et al., 2003), whereas others indicate that, in some liver samples, the levels of CYP3A5 can approach those of CYP3A4 (Lin et al., 2002). Analysis of the specific content of CYP enzymes by mass spectrometry indicates that, on average, CYP3A4 levels are considerably higher (>10 times) than the levels of CYP3A5, as shown in Table 6-16, but in individual livers the levels of CYP3A5 can approach those of CYP3A4 (Ohtsuki et al., 2012). There are also conflicting reports on the relative rate of drug and steroid metabolism by recombinant CYP3A5 and CYP3A4. Early studies with recombinant enzymes generally showed that CYP3A4 is considerably more active than CYP3A5 in the metabolism of a wide range of substrates. However, these studies were criticized later by Huang et al. (2004) on the basis that few of them were conducted with recombinant enzymes in the presence of *both* POR and cytochrome b_5. Under such conditions, the catalytic activity of CYP3A5 can rival or even surpass that of CYP3A4. Of the eight substrates examined by Huang et al. (2004), midazolam represented the extreme case: in the presence of both POR and cytochrome b_5, recombinant CYP3A5 was three times more active than CYP3A4 at catalyzing the 1′-hydroxylation of midazolam, based on estimates of in vitro intrinsic metabolic clearance (i.e., V_{max}/K_m). However, when this same group evaluated the metabolism of the same eight substrates in two pools ($n = 10$) of human liver microsomes each with the same levels of CYP3A4 but with markedly different levels of CYP3A5 (to mimic the CYP3A5 genetic polymorphism), it was found that CYP3A5 contributed nothing to the metabolism of testosterone, carbamazepine, lidocaine, terfenadine, dextromethorphan, or itraconazole, but contributed 27%, 26%, and 35% to the metabolism of midazolam, erythromycin, and flunitrazepam, respectively.

The selective CYP3A4 inhibitor CYP3cide was used with human liver microsomes from individuals expressing CYP3A4 but not CYP3A5 (i.e., livers from CYP3A5*3/*3 PMs) and individuals expressing both CYP3A4 and CYP3A5 (i.e., livers from CYP3A5*1/*1 EMs) to assess the relative contribution of these two CYP3A enzymes to the metabolic clearance of 32 drugs (Tseng et al., 2014). CYP3A5 contributed less than 25% to the metabolic clearance of 19 of 32 drugs (with less than 10% contribution to 11 of them). However, CYP3A5 contributed 25% to 50% to the metabolic clearance of 11 drugs, including midazolam (49%), and contributed considerably more than 50% to the metabolic clearance of two drugs, namely vincristine (75%) and atazanavir (82%) (Tseng et al., 2014). Based on these findings CYP3A5 EMs would be expected, for example, to have twice the oral clearance (CL/F) of midazolam compared with CYP3A5 PMs; however, the results of 11 clinical studies show a range of CL/F values ranging from –18% to +33% (Tseng et al., 2014).

Huang et al. (2004) demonstrated that, under certain in vitro conditions, the catalytic activity of CYP3A5 can surpass that of CYP3A4 in a substrate-dependent manner, and they further predicted that CYP3A5 contributes to the in vivo metabolism of midazolam, a drug whose disposition is known to be highly dependent on CYP3A-catalyzed metabolism in the liver and small intestine. However, two clinical studies have shown that CYP3A5 genotype has no influence on the disposition of midazolam in vivo (Goh et al., 2002; Shih and Huang, 2002). Williams et al. (2003b) have reviewed possible reasons for the discrepancy between the in vitro prediction of the contribution of CYP3A5 to drug metabolism and the in vivo observation.

The relative role of CYP3A4 and CYP3A5 in the metabolism of the calcineurin inhibitors cyclosporine and tacrolimus has been studied in vitro (Staatz et al., 2010). These two anti-organ rejection drugs have a narrow therapeutic index and factors that affect their disposition are of great clinical interest. Studies with recombinant CYP3A4 and CYP3A5 and studies with human liver microsomes that contain both CYP3A4 and CYP3A5 or that contain only CYP3A4 suggest that the metabolism of cyclosporine is predominantly catalyzed by CYP3A4, whereas the metabolism of tacrolimus is predominantly catalyzed by CYP3A5 when this enzyme is present in human liver microsomes. Estimates of in vitro intrinsic metabolic clearance suggest that CYP3A4 contributes 2.3-fold more to the metabolism of cyclosporine than CYP3A5, whereas CYP3A5 contributes 2.0-fold more to the metabolism of tacrolimus than CYP3A4. Although these in vitro studies suggest a role for CYP3A5 in the metabolism of tacrolimus and, to a lesser extent, cyclosporine, it should be remembered that identical studies by Huang et al. (2004) predicted a role for CYP3A5 in the metabolism of midazolam, a prediction that subsequent clinical studies proved to be false.

In the case of CYP3A5, 11 SNPs have been identified, one of which (the *3 allele) causes a complete loss of CYP3A5 expression and, hence, a complete loss of CYP3A5 activity (Staatz et al., 2010). Individuals who are homozygous for the *3 allele can be classified as CYP3A5 PMs; they are genetically deficient in CYP3A5. Individuals with at least one wild-type allele (*1/*1 or *1/*3) are considered CYP3A5 EMs.

The *3 allele that gives rise to the CYP3A5 PM genotype is unusual in that it is more prevalent than the wild-type allele (*1), at least in most populations. The prevalence of the wild-type allele (CYP3A5*1) varies from one ethnic group to the next as follows: 5% to 15% in Caucasians, 55% to 73% in Africans, about 25% in Mexicans, and 15% to 35% in Asians. In Caucasians, Mexicans, and Asians, most individuals are CYP3A5 PMs, which creates an atypical situation with respect to drug metabolism, one that contrasts sharply with genetic polymorphisms in other CYP enzymes where, to take CYP2D6 as an example, most Caucasians are EMs and only a minority (about 7%) are PMs. In the case of a drug that is largely cleared by CYP2D6, for example, the dose that confers therapeutic benefit to the largest number of people is a relatively *high* dose because most individuals are EMs. In the small number of individuals who are PMs (such as the 7% of Caucasians who lack CYP2D6), this dose may be too high; it may cause extended pharmacological effects (as seen with debrisoquine and sparteine) or adverse side effects (as seen with perhexiline). However, in the case of a drug that is primarily cleared by CYP3A5, the dose that confers therapeutic benefit to the largest number of people (in most ethnic populations) is a relatively *low* dose because most individuals are PMs. In the small number of individuals who are EMs (such as the 5% to 15% of Caucasians who are CYP3A5 EMs), this dose may be too low; it may fail to confer a therapeutic benefit, which is a major concern for an anti-organ rejection drug such as tacrolimus.

Several studies have shown linkage disequilibrium between the CYP3A4*1B allelic variant and the CYP3A5*1 wild-type allele, as evidenced by the observation that 67% of Caucasians and 100% of Africans expressing the CYP3A4*1B variant allele also express the CYP3A5*1 wild-type allele (Staatz et al., 2010). There is also evidence of linkage disequilibrium between the P-gp 3435C > T variant allele and the CYP3A5*3 variant allele. Such linkages underscore the need for caution in attributing an effect to one allelic variant when in fact the effect may be caused by another allele in close linkage.

Numerous studies have been conducted to assess the impact of genetic polymorphisms of CYP3A4 and CYP3A5 (as well as P-gp) on the pharmacokinetics of cyclosporine and tacrolimus. These studies were extensively reviewed by Staatz et al. (2010), and their findings can be summarized as follows:

1. The CYP3A4*1B allele has no effect on the disposition of cyclosporine.
2. The CYP3A4*1B allele increases the metabolism of tacrolimus, but this association likely reflects its linkage with CYP3A5*1 (the EM genotype).
3. The CYP3A5*3 allele has no consistent effect on the disposition of cyclosporine; some studies suggest the lack of functional CYP3A5 actually increases the rate of metabolism of cyclosporine, but others show no effect or a slight decrease.
4. The CYP3A5*3 allele impairs the metabolism of tacrolimus. The dose-adjusted trough level of tacrolimus in CYP3A5 PMs (*3/*3) is roughly twice that in CYP3A5 EMs (*1/*1 and *1/*3). Accordingly, to achieve the same blood levels, the dose of tacrolimus administered to CYP3A5 EMs is twice that administered to CYP3A5 PMs.

Overall, the results establish that the disposition of tacrolimus, but not cyclosporine, is affected by genetic polymorphisms of CYP3A5. Individuals with one or two copies of the wild-type *1 allele (EMs) metabolize tacrolimus twice as quickly as individuals with two copies of the *3 allele (PMs). In contrast, genetic polymorphisms of CYP3A4 (such as the loss-of-function *22 allele) impact the disposition of both cyclosporine and tacrolimus, as outlined in the section "CYP3A4."

CYP3A7 CYP3A7 is expressed in the fetal liver where it may play a role in the activation of drugs and other xenobiotics to teratogenic metabolites (Li et al., 1997; Pang et al., 2012). This is a concern because the laboratory animals used in drug safety testing (rats, mice, dogs, and monkeys) do not express a fetal CYP3A enzyme, raising the possibility that teratogenic metabolites formed by CYP3A7 may go undetected during reproductive toxicity testing in animals. In the fetus CYP3A7 is also expressed in extrahepatic tissue such as intestine, endometrial, placenta, adrenal gland, prostate, and lung in the fetus.

CYP3A7 is also expressed in most adult human livers and the levels increase with age (Ohtsuki et al., 2012). CYP3A7 shows high activity toward endobiotics such as dehydroepiandrosterone (DHEA), estrone, and retinoic acid but low activity toward the majority of drugs metabolized by CYP3A4 (Williams et al., 2002; Pang et al., 2012). The 16α-hydroxylation of DHEA is catalyzed by CYP3A7 but not by CYP3A4. A null allelic variant (CYP3A7*3, caused by a thymidine insertion [4011insT] that prematurely terminates translation) has been identified in a Korean subject (Lee et al., 2010b).

CYP3A43 CYPA43 is expressed in the human liver but higher levels are found in the brain, testis, and ovary (Westlind et al., 2001). It is upregulated in prostate, breast, and ovarian carcinomas. There are reports of an association between the loss-of-function CYP3A43*3 polymorphism ($Pro_{340}Ala$) and the risk of prostate cancer and the grade and size of benign prostatic hyperplasia but there is no association with breast cancer risk (Justenhoven et al., 2010). A genetic polymorphism (an intronic SNP) in CYP3A43 (rs472660) accounts for the long recognized ethnic difference in the metabolism of olanzapine, an antipsychotic drug associated with a high rate of discontinuation due to lack of efficacy or the prevalence of adverse effects. Although the functional consequences of the SNP remain to be determined, 67% of African Americans carry the A variant and have higher rates of olanzapine clearance than Caucasians, only 14% of whom carry the A variant. Among the AA carriers 89% are African Americans (compared with 11% of Caucasians), whereas 95% of GG carriers are Caucasians compared with only 5% of African Americans (Justenhoven et al., 2010).

The expression CYP3A43 in brain impacts the metabolism of the antianxiety drug alprazolam (Agarwal et al., 2008). In the liver, CYP3A4 mainly converts alprazolam to the pharmacologically inactive metabolite 4-hydroxy-alprazolam, whereas in the brain CYP3A43 converts alprazolam to both 4-hydroxy-alprazolam and the pharmacologically active metabolite α-hydroxy-alprazolam.

CYP4 Enzymes Humans express 11 members of the CYP4 family, namely, CYP4A11, CYP4A22, CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12, CYP4F22, CYP4V2, CYP4X1, and CYP4Z1. CYP4B1 is often considered a 12th member, but the human CYP4B1 gene, in contrast to that in other mammalian species, encodes a protein that does not incorporate heme; hence, human CYP4B1 is catalytically inactive (Baer and Rettie, 2006; Edson and Rettie, 2013; Johnson et al., 2015). For the most part, the CYP4 enzymes can be divided into two groups: a large group that is known to metabolize fatty acids and/or eicosanoids and

a small group (CYP4A22 and CYP4X1) with no known function (so-called orphan enzymes), as shown in Table 6-14. The first group (CYP4A11, CYP4F2, CYP4F3, CYP4F8, CYP4F12, CYP4F22, CYP4V2, and CYP4Z1) is part of a larger group of fatty acid–metabolizing and eicosanoid-metabolizing enzymes, which includes CYP2C8, CYP2C9, and CYP2J2. However, these latter enzymes are fatty acid/eicosanoid epoxygenases; they oxidize arachidonic acid to four regioisomers of EETs, which are vasodilators. In contrast, the CYP4 enzymes hydroxylate the terminal methyl group (ω-hydroxylation) of fatty acids and eicosanoids. In the case of arachidonic acid, ω-hydroxylation by CYP4 enzymes produces 20-HETE, which is a vasoconstrictor. Hydroxylation of the terminal methyl group (ω-hydroxylation) by CYP4 enzymes is thermodynamically unfavorable compared with hydroxylation of a methylene group (e.g., ω-1 hydroxylation), which some CYP4 enzymes also catalyze (Baer and Rettie, 2006; Kalsotra and Strobel, 2006; Johnston et al., 2011; Edson and Rettie, 2013). Following ω-hydroxylation, the terminal hydroxymethyl group can be further oxidized to convert the original fatty acid/eicosanoid to a dicarboxylic acid. These dicarboxylic acids are then catabolized by fatty acid β-oxidation (chain shortening) and eliminated. Hepatic CYP4A enzymes are upregulated in humans and mice with nonalcoholic fatty liver disease (NAFLD) and CYP4A14 knockout mice are protected against NAFLD induced by a high-fat diet or a diet deficient in methionine and choline (Zhang et al., 2017).

Unlike other CYP families, the majority of CYP4 enzymes have a covalently attached heme moiety in which the heme forms an ester bond with a conserved glutamic acid residue (Ortiz de Montellano, 2008). However, CYP4F8 and CYP4F12 are exceptions (and CYP4X1 probably also lacks a covalent heme because it lacks the critical glutamate residue). It is noteworthy that CYP4F8 and CYP4F12 preferentially hydroxylate fatty acids in the ω-1 or ω-2 position, which are more energetically favorable than ω-hydroxylation but which do not lead to the formation of dicarboxylic acids (Stark et al., 2005). It has been postulated that the purpose of the covalently bound heme in CYP4 ω-hydroxylases is to help restrict the orientation of the fatty acid in the active site to direct hydroxylation toward the ω-position (Ortiz de Montellano, 2008; Johnston et al., 2011).

Several CYP4 enzymes have been shown to metabolize drugs and other xenobiotics, but in nearly all cases the drugs that are metabolized by the CYP4 enzymes are also metabolized more extensively by CYP1, CYP2, or CYP3 family members. A notable exception is the antiparasitic prodrug pafuramidine (DB-289), which is *O*-demethylated in human liver microsomes by CYP4F2 and CYP4F3 with negligible contribution from CYP enzymes in the 1, 2, or 3 family (Wang et al., 2006). *O*-Demethylation converts pafuramidine (DB-289) to an amidoxime, which undergoes dehydroxylation by mARC/cytochrome b_5/NADH-cytochrome b_5 reductase as shown for other amidoximes in Fig. 6-26. Other examples of CYP4-mediated drug metabolism include the CYP4F12-dependent hydroxylation of ebastine and terfenadine (Cauffiez et al., 2004), and the CYP4F2-dependent ω-hydroxylation of fingolimod (FTY720, Gilenya), an FDA-approved immunosuppressive drug for the treatment of multiple sclerosis (Zollinger et al., 2011).

Another notable exception is CYP4B1, which is expressed in a wide range of extrahepatic tissues and which activates several protoxicants including the pneumotoxins ipomeanol and 3-methylindole, the bladder carcinogen 2-AF, as well as aromatic amines in animals (Baer and Rettie, 2006; Parkinson et al., 2012). However, CYP4B1 appears to be inactive in humans due to a mutation that interferes with heme incorporation; the mutation is located in the meander region and encodes for a serine residue at amino acid 427. This amino acid is an evolutionarily conserved proline in other species where CYP4B1 is known to be active including cow, mouse, rat, rabbit, and dog. Introducing a $Pro_{427}Ser$ mutation into rabbit CYP4B1 interferes with heme incorporation, and reverting Ser_{427} to the conserved proline in the human CYP4B1 gene yields a functional enzyme that incorporates heme (Zheng et al., 1998). The $Pro_{427}Ser$ mutation likely explains why attempts to express human CYP4B1 in a recombinant system have yielded inactive enzyme lacking heme.

CYP4F2 has been implicated in the clearance of both vitamins K and E (Parker et al., 2004; Sontag and Parker, 2007; McDonald et al., 2009). Both of these compounds have phytyl-derived, long alkyl chains attached to a polar moiety (which makes them roughly structurally analogous to fatty acids). Urinary metabolites of vitamins K and E that lack the phytyl chain likely arise from CYP4F2-catalyzed ω-hydroxylation followed by further oxidation to the carboxylic acid with subsequent chain shortening by β-oxidation. A genetic polymorphism in CYP4F2 ($Val_{433}Met$) that impairs the metabolism of vitamin K_1 is clinically relevant because individuals with this loss-of-function allelic variant require a higher warfarin dose to compete with the increased levels of the vitamin K_1 in the liver (McDonald et al., 2009; Lurie et al., 2010).

Genetic defects in CYP4V2 and CYP4F22 have been linked to diseases with symptoms possibly caused by disruption of fatty acid homeostasis and/or disruption of eicosanoid signaling. Mutations in CYP4V2 are linked to Bietti crystalline corneoretinal dystrophy (Li et al., 2004; Lockhart et al., 2014; Ng et al., 2016), a late-onset eye disease characterized by night blindness and eventually blindness caused by crystalline deposits in the retinal epithelium and cornea. Mutations in CYP4F22 are associated with autosomal recessive congenital ichthyosis (Lefevre et al., 2006), which is characterized by large dark scales on the skin without exfoliative dermatitis. CYP4F22 is likely involved in the metabolism of hepoxilins or trioxilins, signaling molecules that are part of the arachidonic acid cascade (Kelly et al., 2011).

Endogenous Biomarkers of Cytochrome P450 The ability of many CYP enzymes (and other drug-metabolizing enzymes) to biotransform endogenous substrates (in addition to xenobiotics) opens the possibility that their activity in vivo can be monitored with biomarkers (i.e., metabolites of endogenous compounds formed by a specific CYP or other drug-metabolizing enzyme). In principle, biomarkers could be used, for example, to assess the impact of a new investigational drug, herbal medication, or any other xenobiotic to which humans are exposed on one or more enzymes involved in xenobiotic biotransformation. In the case of investigational drugs, this assessment could be conducted early in the clinical phase of drug development to assess the potential of a new drug candidate to cause clinically relevant inhibition or induction of CYP enzymes. A large number (>20) of metabolites formed from androgens, estrogens, corticosteroids, cholesterol, and vitamin D_3 are potential biomarkers of CYP3A activity (Tracy et al., 2016). Changes in the ratio of plasma 6β-hydroxycholesterol/cholesterol and urinary 6β-hydroxycortisol/cortisol were used (in addition to changes in the plasma pharmacokinetics of midazolam) to assess the CYP3A-inducing potential of PF-06282999, an investigational drug that inhibits myeloperoxidase (Dong et al., 2017). Metabolomic studies identified an endogenous urinary biomarker for CYP2D6 (Tay-Sontheimer et al., 2014). This biomarker, designated M1 (m/z 444.3102), the structure of which is unknown, is undetectable in urine from CYP2D6 PMs. In CYP2D6 EMs, the levels of M1 excreted in urine decrease by almost an order of magnitude following treatment with the CYP2D6 inhibitor fluoxetine. These examples illustrate the potential for biomarkers to provide

valuable information on interindividual differences in the activity of xenobiotic-biotransforming enzymes and the potential for drugs and other xenobiotics to cause clinically relevant inhibition or induction of these enzymes.

Activation of Xenobiotics by Cytochrome P450 Biotransformation by CYP does not always lead to detoxication, and several examples have been given previously where the toxicity or tumorigenicity of a chemical depends on its activation by CYP. The role of individual human CYP enzymes in the activation of selected procarcinogens and protoxicants is summarized in Table 6-18 (Guengerich, 1991). A variety of CYP-dependent reactions are involved in the activation of the chemicals listed in Table 6-18. The conversion of PAHs to tumor-forming metabolites involves the formation of bay-region diol epoxides, as shown in Fig. 6-14, for the conversion of B[*a*]P to B[*a*]P 7,8-dihydrodiol-9,10-epoxide. Epoxidation generates hepatotoxic metabolites of chlorobenzene and coumarin (Fig. 6-48), and generates a hepatotumorigenic metabolite of aflatoxin B1 (Fig. 6-38).

The initial step in the conversion of aromatic amines and amides to tumor-forming metabolites involves *N*-hydroxylation, as shown for 2-amino-6-nitrobenzylalcohol (Fig. 6-16), 2-AF, and 2-AAF (Fig. 6-67). The aromatic amide phenacetin, a discontinued drug, undergoes *N*-hydroxylation by CYP (and peroxidases) to form a hydroxamic acid. Under acidic conditions (such as in urine), the hydroxamic acid can lose water to form reactive nitrenium ion, which is thought to contribute to the renal toxicity of phenacetin (Testa and Krämer, 2008, 2010). In the case of acetaminophen, activation to hepatotoxic metabolite involves dehydrogenation to NAPQI, as shown in Fig. 6-39. A similar reaction converts BHT to a toxic quinone methide, as shown in Fig. 6-40. The myelotoxicity of benzene depends on its conversion to phenol and hydroquinone (Fig. 6-36). The toxicity of several OP insecticides involves oxidative group transfer to the corresponding organophosphate, as shown for the conversion of parathion to paraoxon in Figs. 6-51 and 6-52. The hepatotoxicity of carbon tetrachloride involves reductive dechlorination to a trichloromethyl free radical, which binds to protein and initiates lipid peroxidation, as shown in Fig. 6-22. The hepatotoxicity and nephrotoxicity of chloroform involve oxidative dechlorination to phosgene (Fig. 6-22). Both oxidative and reductive dehalogenation play a role in the activation of halothane, although hepatotoxicity in rats is more dependent on reductive dehalogenation, whereas the immune hepatitis in humans is largely a consequence of oxidative dehalogenation, which leads to the formation of neoantigens (Pohl et al., 1989). Formation of neoantigens (by covalent binding to CYP2C9) is also the mechanism by which the uricosuric diuretic drug, tienilic acid, causes immune hepatitis (Lecoeur et al., 1994).

Some of the chemicals listed in Table 6-18 are activated to toxic or tumorigenic metabolites by mechanisms not mentioned previously. For example, *N*-nitrosodimethylamine, which is representative of a large class of tumorigenic nitrosamines, is activated to an alkylating electrophile by *N*-demethylation, as shown in Fig. 6-56. The activation of ethyl carbamate (urethane) involves two sequential reactions catalyzed by CYP (CYP2E1): dehydrogenation to vinyl carbamate followed by epoxidation, as shown in Fig. 6-56. CYP2E1 is one of several CYP enzymes that can catalyze the epoxidation of tetrachloroethylene. The rearrangement of this epoxide to a carbonyl is accompanied by migration of chlorine, which produces the highly reactive metabolite, trichloroacetyl chloride, as shown in Fig. 6-56. The toxic pyrrolizidine alkaloids, such as senecionine, are cyclic arylamines that are dehydrogenated by CYP (CYP3A4) to the corresponding pyrroles. Pyrroles themselves are nucleophiles, but electrophiles are generated through the loss of substituents on the pyrrolizidine nucleus, as shown in Fig. 6-56. Cyclophosphamide and ifosfamide are examples of chemicals designed to be activated to toxic electrophiles for the treatment of malignant tumors and other proliferative diseases. These drugs are nitrogen mustards, which have a tendency to undergo intramolecular nucleophilic displacement to form an electrophilic aziridinium species. In the case of cyclophosphamide and ifosfamide, the nitrogen mustard is stabilized by the presence of a phosphoryl oxygen, which delocalizes the lone pair of nitrogen electrons required for intramolecular nucleophilic displacement. For this reason, formation of an electrophilic aziridinium species requires hydroxylation by CYP, as shown in Fig. 6-56, for cyclophosphamide. Hydroxylation of the carbon atom next to the ring nitrogen leads spontaneously to ring opening and elimination of acrolein. In the resultant phosphoramide mustard, delocalization of the lone pair of nitrogen electrons to the phosphoryl oxygen is now disfavored by the presence of the lone pair of electrons on the oxygen anion; hence, the phosphoramide undergoes an intramolecular nucleophilic elimination to generate an electrophilic aziridinium species. This reaction is catalyzed by CYP2B6 and CYP3A4. Activation of cyclophosphamide by CYP enzymes in the skin would generate a cytotoxic metabolite at the base of hair follicles, which may be the reason why hair loss is one of the side effects of cyclophosphamide treatment.

Many of the chemicals listed in Table 6-18 are also detoxified by CYP by biotransformation to less toxic metabolites. In some cases, the same CYP enzyme catalyzes both activation and detoxication reactions. For example, CYP3A4 activates aflatoxin B_1 to the hepatotoxic and tumorigenic 8,9-epoxide, but it also detoxifies aflatoxin B_1 by 3-hydroxylation to aflatoxin Q_1. Similarly, CYP3A4 activates senecionine by converting this pyrrolizidine alkaloid to the corresponding pyrrole, but it also detoxifies senecionine through formation of an *N*-oxide (a reaction mainly catalyzed by FMO3). Epoxidation of trichloroethylene by CYP2E1 is both an activation and a detoxication pathway, as shown in Fig. 6-56. Rearrangement of trichloroethylene epoxide can be accompanied by migration of chlorine, which produces chloral (trichloroacetaldehyde), or hydrogen, which produces dichloroacetylchloride. Chloral is much less toxic than dichloroacetylchloride; hence, migration of the chlorine during epoxide rearrangement is a detoxication reaction, whereas migration of the hydrogen is an activation reaction. These few examples serve to underscore the complexity of factors that determine the balance between xenobiotic activation and detoxication.

Structural alerts (functional groups) for the bioactivation of xenobiotics to reactive metabolites by CYP include the following: acetylenes, activated aromatics, 3-alkly indoles, anilines, cyclopropylamines, formamides, furans, hydrazines, polyaromatics, pyrroles, nitro aromatics, sulfonylureas, thiazolidinones, thioamides, thiophenes, and thioureas (Walsh and Miwa, 2011). As outlined more fully in Point 12 (see the section "Principles of Xenobiotic Biotransformation"), the prominence of cytochrome P450 in converting xenobiotics to reactive and potentially harmful metabolites is illustrated by the widespread use of liver microsomes in the in vitro safety evaluation of drug candidates. Liver microsomes are used in (1) the Ames bacterial mutagenicity assay, (2) an assessment of the formation of reactive metabolites that bind covalently to protein or GSH (or a related nucleophile such as *N*-acetylcysteine [NAC]), and (3) an assessment of a drug candidate's potential to cause irreversible inhibition of the major drug-metabolizing CYP enzymes. The latter application is the topic of the next section.

Inhibition of Cytochrome P450 Inhibition of CYP is a major cause of drug–drug interactions (and occasionally the withdrawal of regulatory approval), as illustrated by the examples cited earlier (terfenadine, cisapride, astemizole, cerivastatin, and mibefradil) in

the sections "CYP3A4" and "CYP2C8" (Ogilvie et al., 2008). The magnitude of the drug–drug interaction depends on the degree of CYP inhibition by the perpetrator drug and the fractional metabolism (fm) of the victim drug by the affected enzyme. This principle is explained in Point 23 in the section "Principles of Xenobiotic Biotransformation." In summary, complete inhibition of a CYP enzyme that contributed 50% to the clearance of Drug A (fm = 0.5) and 90% to the clearance of Drug B (fm = 0.9) would cause a twofold increase in systemic exposure (plasma AUC) to Drug A, and a 10-fold increase in exposure to Drug B. The concept of "maximum exposure," where two perpetrators inhibit two parallel pathways of clearance and cause dramatic increases in systemic exposure to the victim drug, has been explained in Point 24 in the section "Principles of Xenobiotic Biotransformation." Maximum exposure often occurs when a genetic polymorphism affects one pathway of clearance and a perpetrator drug inhibits a second, parallel pathway of clearance, as illustrated in Point 24 for the weak effect (about twofold) of the CYP3A4 inhibitor ritonavir on voriconazole exposure in CYP2C19 EMs (individuals in whom CYP3A4 plays a minor role in voriconazole metabolism) and the dramatic (26-fold) increase in voriconazole AUC in CYP2C19 PMs (individuals who lack CYP2C19 and in whom CYP3A4 plays the major role in voriconazole clearance) (Collins et al., 2006; Ogilvie and Parkinson, 2014). A single drug can inhibit multiple CYP enzymes and thereby inhibit two or more parallel pathways of drug clearance. An exceptional case is the interaction between fluvoxamine and ramelteon. The three major enzymes involved in the clearance of ramelteon are CYP1A2, CYP2C19, and CYP3A4, all of which are inhibited by fluvoxamine. The product label for ramelteon (Rozerem, Takeda Pharmaceuticals, Osaka, Japan) describes a drug–drug interaction study in which fluvoxamine caused a 190-fold increase in the plasma AUC of ramelteon, which is the largest drug interaction reported. Interestingly, the magnitude of the interaction surpasses that predicted from in vitro studies (Obach and Ryder, 2010).

Inhibitory drug interactions generally fall into two categories, namely reversible or irreversible. Reversible (direct) inhibitors can be competitive, noncompetitive, or uncompetitive (or mixed) inhibitors. Irreversible inhibitors are metabolism-dependent inhibitors (MDIs), which can be completely irreversible or quasi-irreversible (reversible under certain conditions). Direct inhibition can be subdivided into two types. The first involves competition between two drugs that are metabolized by the same CYP enzyme. The second type of direct inhibition is when the inhibitor is not a substrate for the affected CYP enzyme. The inhibition of dextromethorphan biotransformation by quinidine is a good example of this type of drug interaction. Dextromethorphan is *O*-demethylated by CYP2D6, and the clearance of dextromethorphan is impaired in individuals lacking this polymorphically expressed enzyme. The clearance of dextromethorphan is similarly impaired when this antitussive drug is taken with quinidine, a potent inhibitor of CYP2D6. However, quinidine is not biotransformed by CYP2D6, even though it binds to this enzyme with high affinity (K_i ~100 nM). Quinidine is actually biotransformed by CYP3A4, and is a weak competitive inhibitor of this enzyme (K_i >100 μM), although its effects are highly dependent on the CYP3A4 substrate employed.

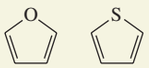
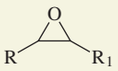
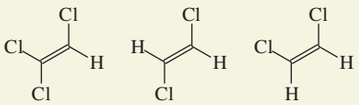
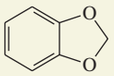
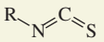
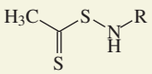
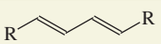
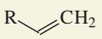
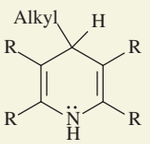
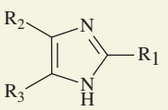
As the name implies, MDI occurs when CYP converts a xenobiotic to a metabolite that is a more potent inhibitor than the parent compound. In some cases, the metabolite, once formed, functions as a potent, reversible inhibitor, as occurs when fluoxetine is *N*-demethylated to norfluoxetine. The contribution of norfluoxetine to the inhibitory effects of fluoxetine illustrates a common finding that drug metabolites often function as reversible inhibitors of CYP and thereby contribute significantly to drug–drug interactions (Isoherranen et al., 2009; Yeung et al., 2011; Callegari et al., 2013; Yu and Tweedie, 2013; Yu et al., 2015). In other cases, however, the metabolite functions as an irreversible inhibitor (which occurs when the metabolite alkylates the heme or apoprotein moiety of CYP) or a quasi-irreversible inhibitor (which occurs when the metabolite coordinates tightly but not covalently with the ferrous heme to form what is known as a metabolite-inhibitory complex [MIC]). Metabolism-dependent inhibitors that cause irreversible inactivation of CYP include 8-methoxypsoralen (CYP2A6), clopidogrel (CYP2B6), tienilic acid (CYP2C9), and ticlopidine (CYP2C19), all of which contain a furan or thiophene that is activated by CYP to a reactive metabolite that inactivates the enzyme (suicide inactivation). Metabolism-dependent inhibitors that form metabolite inhibitory complexes and cause quasi-irreversible inhibition include the CYP3A4 inhibitor troleandomycin, which contains a tertiary amine ($R–N(CH_3)_2$) that is converted by several successive oxidative reactions to a putative nitroso metabolite (R–N=O) that coordinately binds to the ferrous heme iron. This interaction resembles the binding of CO to the ferrous heme iron, and indeed compounds that form inhibitory metabolite complexes with CYP can be detected spectrophotometrically based on an absorbance peak at around 455 nm (which is similar to the peak at about 450 nm when CO binds to CYP). The chemical structures commonly associated with MDI of CYP are shown in Table 6-19.

In some cases, MDI involves metabolism by enzymes other than the affected CYP enzyme, which can give rise to system-dependent inhibition in in vitro studies (whereby the results obtained in human liver microsomes or recombinant enzymes do not predict as well as studies performed with human hepatocytes) (Parkinson et al., 2010). For example, the inhibition of CYP2C8 by gemfibrozil and clopidogrel requires the formation of an acyl glucuronide by UGT, after which gemfibrozil glucuronide and clopidogrel glucuronide function as MDIs of CYP2C8 (Ogilvie et al., 2006; Tornio et al., 2014). The reduction of bupropion to erythrohydrobupropion and threohydrobupropion is largely responsible for its ability to cause clinically relevant inhibition of CYP2D6 (Reese et al., 2008). Likewise, the oxidation of decernotinib (VX-509) by aldehyde oxidase produces a metabolite (M3) that, in contrast to the parent drug, is an MDI of CYP3A4 (Zetterberg et al., 2016). Experiments with human liver microsomes and recombinant CYP enzymes underpredict the inhibition of CYP2C8 by gemfibrozil and clopidogrel, the inhibition of CYP2D6 by bupropion, and the inhibition of CYP3A4 by decernotinib, whereas hepatocytes do not because they support the necessary glucuronidation, reduction, and oxidation pathways, respectively. Conversely, experiments with human liver microsomes and recombinant CYP enzymes overpredict the inhibition of CYP3A4 by ezetimibe. In the clinic (and in hepatocytes) rapid glucuronidation of ezetimibe protects CYP3A4 from MDI by ezetimibe (Parkinson et al., 2010). Other issues that can arise during the in vitro evaluation of drug candidates as MDIs of CYP enzymes are described elsewhere (Parkinson et al., 2011). An extensive database on drug–drug interactions is available at the University of Washington Metabolism and Transport Drug Interaction Database (University of Washington DIDB, 2018).

There are cases where inhibition of CYP has proved advantageous, as in the case of certain combinations of anti-HIV drugs. For example, by inhibiting CYP3A4, the HIV protease inhibitor ritonavir and the structurally related drug cobicistat (which lacks antiviral activity) improve the pharmacokinetic profile of saquinavir, lopinavir, and several other antiviral drugs that are otherwise cleared so rapidly by CYP3A4 that blood levels easily fall below therapeutically effective concentrations (Deeks, 2014; Marzolini et al., 2016).

Table 6-19

Structures Associated with Metabolism-Dependent Inhibition of Cytochrome P450 (CYP) Enzymes

CHEMICAL GROUPS (EXAMPLES)	STRUCTURES
Terminal (ω) and ω-1 acetylenes (gestodene)	
Furans and thiophenes (8-methoxypsoralen and tienilic acid)	
Epoxides (*R*-bergamottin-6′,7′-epoxide)	
Dichloro- and trichloro-ethylenes (1,2,-dichloroethylene and trichloroethylene)	
Secondary amines (fluoxetine)	
Benzodioxoles (paroxetine)	
Isothiocyanates (phenethyl isothiocyanate)	
Thioamides (methimazole)	
Dithiocarbamates (disulfiram)	
Conjugated structures (rhapontigenin)	
Terminal alkenes (tiamulin)	
4-Alkyl-1,4-dihydropyridines (3,5-diethoxycarbonyl-1, 4-dihydro-2,6-dimethyl-4-ethylpyridine [DDEP]),	
2-Alkyl-1*H*-imidazoles (furafylline)	

SOURCES: Data from Fontana et al. (2005) and Testa and Kramer (2008, 2010).

Induction of Cytochrome P450: Xenosensors The induction (upregulation) of xenobiotic-biotransforming enzymes and transporters is a receptor-mediated, adaptive process that augments xenobiotic elimination during periods of high xenobiotic exposure. It is not a toxicological or pathological response, but enzyme induction is often associated with liver enlargement (due to both hepatocellular hypertrophy and hyperplasia), and it may be associated with toxicological and pharmacological consequences, especially for the safety evaluation of drug candidates in laboratory animals and for clinical practice in humans. In animals and humans, enzyme induction may be associated with pharmacokinetic tolerance, whereby the xenobiotic induces its own elimination. Carbamazepine is one of many EIAEDs that induced its own metabolism. Because of autoinduction it is often necessary to increase the dose of carbamazepine at a rate of 200 mg every 1 to 2 weeks to achieve the desired antiseizure threshold. During safety testing in animals, autoinduction may require increasing dosages of the drug candidate to achieve the same degree of systemic exposure over time. In rodents, lifetime exposure to enzyme inducers may be associated with liver or thyroid tumor formation, as discussed later in this section. In humans, enzyme induction may also be associated with pharmacokinetic tolerance (autoinduction). Long-term treatment of humans with enzyme inducers can cause osteomalacia due to increased inactivation of 1,25-$(OH)_2$-D_3, the active metabolite

of vitamin D (discussed later in this section), but it is not associated with liver or thyroid tumor formation. However, in humans, enzyme induction by one drug (the perpetrator) can augment the clearance of a concomitantly administered drug (the victim), which is a cause of drug–drug interactions.

As an underlying cause of serious adverse events, enzyme induction is generally less important than enzyme inhibition because the latter can cause a rapid and profound increase in the blood concentration of a victim drug, which can cause an exaggerated pharmacological or toxicological effect. In contrast, enzyme induction lowers blood concentration, which usually does not cause an exaggerated pharmacological or toxicological response to the drug. However, enzyme induction may be associated with a loss of therapeutic effectiveness, which is a particular concern when it compromises the therapeutic effectiveness of drugs that have a narrow therapeutic index and are being used to treat a life-threatening illness, such as anti-HIV drugs, anti-organ rejection drugs (such as cyclosporine and tacrolimus), and oral anticoagulants (such as warfarin), or when it is used with drugs that exhibit a quantal (all-or-nothing) dose–response relationship, such as oral contraceptive steroids (which either block or do not block ovulation and thereby provide or do not provide protection against pregnancy).

CYP induction does not necessarily enhance the biotransformation of the inducer, in which case the induction is said to be gratuitous. Consequently, lack of autoinduction cannot be taken as evidence that a xenobiotic does not cause enzyme induction. For example, in humans, omeprazole induces CYP1A2, even though the disposition of this acid-suppressing drug is largely determined by CYP2C19 and, to a lesser extent, CYP3A4. Some of the most effective inducers of CYP are polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated derivatives of dibenzo-*p*-dioxin (PCDDs), dibenzofurans (PCDFs), azobenzenes and azoxybenzenes, biphenyl (PCBs), and naphthalene. In general, highly chlorinated compounds are resistant to biotransformation and cause a prolonged induction of CYP and other enzymes. Some inducers are potent direct-acting or metabolism-dependent inhibitors such that they mask the activity of one or more of the enzymes they induce. Such dual-acting xenobiotics include macrolide antibiotics (e.g., erythromycin and troleandomycin), methylenedioxy-containing compounds (e.g., tadalafil [Cialis®], safrole, isosafrole), and imidazole antimycotics (e.g., clotrimazole, ketoconazole, and miconazole). In the case of dual-acting drugs (such as the induction/inhibition of CYP3A4 by ritonavir and several other HIV protease inhibitors, and the induction/inhibition of CYP2E1 by isoniazid), the initial effect—and often the effect observed during the period of drug treatment—is CYP inhibition, with induction becoming evident after prolonged drug treatment or following drug cessation. However, when ritonavir induces CYP3A4, for example, it also induces several other CYP enzymes, as well as conjugating enzymes and transporters that are regulated by CAR/PXR. Like ritonavir, cobicistat inhibits CYP3A4 and P-gp and is approved for use as a pharmacokinetic booster for combination with various antiviral drugs to increase and prolong their systemic exposure. In contrast to ritonavir, cobicistat is not an enzyme inducer (Deeks, 2014; Marzolini et al., 2016). Some dual-acting xenobiotics, such as allylisopropylacetamide, cause certain types of porphyria (a disorder of heme synthesis) because they induce CYP enzymes and destroy the CYP heme moiety, which greatly increases the demand for heme synthesis (discussed later in this section).

In general, CYP induction is mediated by four ligand-activated receptors, namely, AhR, CAR, PXR, and PPARα, as summarized in Table 6-20 (Tirona and Kim, 2005). These so-called xenosensors resemble other nuclear receptors, such as steroid and thyroid hormone receptors, which has consequences for receptor interactions (cross-talk among xenosensors and cross-talk between xenosensors and other nuclear receptors), the role that some xenosensors play in responding to endobiotics and regulating their metabolism (e.g., bilirubin, bile acids, fatty acids, and 1,25-$(OH)_2$-D_3), and the role that some nuclear receptors (such as farnesoid X receptor [FXR], VDR, small heterodimer partner-1 [SHP], NF-κB) play in inducing or suppressing the expression of xenobiotic-biotransforming enzymes and transporters, as discussed later in this section. Details of the nomenclature system of this superfamily of DNA-binding transcriptional factors have been reviewed (Germain et al., 2006). Xenosensors have a ligand-binding domain (LBD) and a highly conserved cysteine-rich DNA-binding domain (DBD). In general, CYP induction involves the following steps (with steps 2 and 3 reversed in the case of AhR): (1) binding of ligand (xenobiotic) to the receptor, which triggers conformational changes that promote its dissociation from accessory proteins (such as corepressors, chaperones, and cytoplasm retention proteins) and promote its association with coactivators; (2) dimerization of the ligand-bound receptor with a partner protein to form a DNA-binding heterodimer (which is analogous to the two halves of a clothespin coming together to form a functional unit); (3) translocation of the functional receptor heterodimer from the cytoplasm to the nucleus; (4) binding of the functional receptor heterodimer to discrete regions of DNA (response elements) that are typically located in the 5′-promoter region of the gene (which is analogous to a clothespin being fastened to a clothes line); (5) recruitment of other transcription factors and coactivators (such as histone and RNA methyltransferases, histone and chromatin deacetylases, and histone remodeling helicases) and RNA polymerase to form a transcription complex; and (6) gene transcription, which leads to increased levels of CYP mRNA and protein (as well as other xenobiotic-biotransforming enzymes and transporters). As is the case with all nuclear receptors, the details of the process of activating a xenosensor to its transcriptionally active form are complex and multifaceted. Over 200 coactivators/coregulators of nuclear receptor function have been identified (Lonard and O'Malley, 2006), some of which are known to play a critical but undefined role in xenosensor activation.

As shown in Table 6-20, the major ligand-activated receptors mediating CYP induction are (1) AhR, which partners with aryl hydrocarbon receptor nuclear translocator (ARNT) to induce CYP1A1, CYP1A2, CYP1B1, and CYP2S1; (2) CAR (aka the constitutively active receptor), which partners with RXRα (the retinoid X receptor, which binds 9-*cis*-retinoic acid) to induce several members of the CYP2A, CYP2B, CYP2C, and CYP3A subfamilies; (3) PXR (aka SXR, the steroid X receptor) that, like CAR, partners with RXRα and induces several members of the CYP2A, CYP2B, CYP2C, and CYP3A subfamilies; and (4) PPARα, which partners with RXRα and induces CYP4A enzymes in rodents and CYP1A1, CYP2C8, and CYP3A4 in humans. The response elements (aka consensus sequences) to which xenosensors bind are generally a pair of hexanucleotide sequences in a direct repeat (DR), inverted repeated (IR), or everted repeat (ER) orientations, separated by a zero-to-eight nucleotide spacer. As shown in Tables 6-20 and 6-21, activation of a xenosensor leads to the induction of multiple enzymes, which is called a pleiotypic response. However, in terms of fold induction, CYP enzymes tend to be the most inducible and, consequently, the most studied enzymes (although activation of PPARα has a more pronounced effect on the expression of peroxisomal enzymes involved in fatty acid oxidation). Like induction of CYP enzymes, induction of conjugating and other non-CYP enzymes is an important mechanism of drug–drug and drug–endobiotic interactions. For example, induction of various conjugating

Table 6-20

Receptors Mediating the Induction (or Suppression) of Cytochrome P450 Enzymes and Other Xenobiotic-Biotransforming Enzymes and Transporters

NUCLEAR RECEPTOR	RESPONSE ELEMENT(S)	RECEPTOR ACTIVATORS	REGULATED GENES*
AhR	XRE	PAHs, TCDD (other PHAHs), β-naphthoflavone, indigoids, tryptophan metabolites, omeprazole, lansoprazole, laquinimod, roquinimex	CYP1A1, 1A2, 1B1, 2S1, UGT1A1, UGT1A6, AKR1A1, AKR1C1–4
CAR	DR-3 DR-4 ER-6	Phenobarbital, phenytoin, carbamazepine, CITCO (human), TCPOBOP (mouse), clotrimazole, Yin Zhi Wuang (many PXR agonists are also CAR agonists, and vice versa)	CYP2A6, 2B6, 2C8, 2C9, 2C19, 3A4, UGT1A1, SULT1A1, AKR1D1, ALAS, MRP2, MRP3, MRP4
PXR	DR-3 DR-4 ER-6 ER-8	Amprenavir, avasimibe, bosentan, bile acids, carbamazepine, clindamycin, clotrimazole, cortisol, cyproterone acetate, dicloxacillin, efavirenz, etoposide, dexamethasone, griseofulvin, guggulsterone, hyperforin (SJW), indinavir, lovastatin, mifepristone, nafcillin, nelfinavir, nifedipine, omeprazole, paclitaxel, PCBs, phenobarbital, phthalate monoesters, 5β-pregnane-3,20-dione, rifabutin, rifampin, ritonavir, saquinavir, simvastatin, spironolactone, sulfinpyrazole, TAO, tetracycline, topotecan, transnanoclor, troglitazone, verapamil, vitamin E, vitamin K_2	CYP2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A7, 4F12, 7A1↓, CES2, SULT2A1, UGT1A1, 1A3, 1A4, 1A6, GSTA1, AKR1D1, PAPSS2, ALAS, MDR1, MRP2, AhR
PPARα	DR-1	Fibrates, WY-14643, perfluorodecanoic acid	CYP4A, UGT1A9, UGT2B4 in rodents
			CYP1A1, 2C8, and 3A4 in humans
Nrf2	ARE	β-Naphthoflavone, oltipraz, phenolic antioxidants (e.g., BHA and BHT), phenylisothiocyanate, acetaminophen, and various glutathione depletors (diethyl maleate, phorone)	NQO1, mEH, AKR7A, UGTs, GSTA1, γ-GCL, MRP1
GR	GRE	Glucocorticoids (e.g., dexamethasone)	CYP2C9, 2B6, 2D6, 3A4, 3A5, CAR, PXR
FXR	IR-1	Bile acids, GW4064, AGN29, AGN31	BSEP, I-BABP, MDR3, UGT2B4, SULT2A1, OATP8, OSTα/β, PPARα, SHP
LXRα	DR-4	GW3965, T0901317, paxiline, F_3methylAA,† acetylpodocarpic dimer (APD)	LRH1, SHP, CYP7A, LXRα, CYP3A4↓↓, 2B6↓
VDR	DR-3 ER-6 IR-0	1α,25-Dihydroxyvitamin D_3, lithocholate	CYP2B6, 2C9, 3A4, SULT2A1
HNF-1α	Not named‡		OATP-C, OATP8, CYP7A1, UGT1A6, 1A8, 1A9, 1A10, HNF4α, PXR, kidney specific expression of OAT1, OAT3, URAT1
HNF-4α	DR		CYP2A6, 2B6, 2C9, 2D6, 3A4, DD4, MDR1, PXR, CAR, FXR, PPARα, HNF1α
LRH-1	DR-4		CYP7A, ASBT
SHP	None		Targets of PPARα↓, AhR↓, PXR↓, CAR↓, LRH-1↓, HNF4α↓, LXRα↓, GR↓

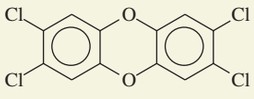
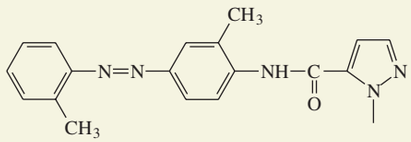
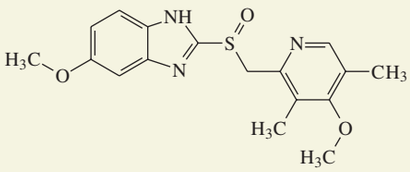
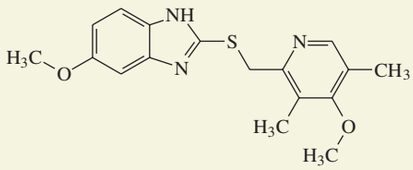
** A downward arrow indicates downregulation (suppression). All others are upregulated (induced).*
† [3-Chloro-4-(3-(7-propyl-3-trifluoromethyl-6-(4,5)-isoxazolyl)propylthio)-phenylacetic acid].
‡ The HNF-1α consensus sequence is GTTTAATNATTAAC.
SOURCES: Data from Jin et al. (2012), Penning and Drury (2007), and Tirona and Kim (2005).

enzymes (both SULT and UGT enzymes) plays a key role in the metabolism of oral contraceptive steroids in humans, which is associated with a loss of therapeutic effect. In rodents induction of conjugating enzymes is responsible for increased metabolism of thyroid hormone, which is associated with thyroid follicular cell hyperplasia and tumor formation.

As shown in Table 6-20, AhR, CAR, PXR, and PPARα are not the only receptors that regulate CYP expression in response to xenobiotics (or certain endobiotics). Nuclear factor erythroid 2–related factor 2 (Nrf2) plays a key role in regulating the induction of several enzymes in response to electrophilic metabolites, oxidative stress, or GSH depletion, as discussed previously (see Point

Table 6-21

Examples of Agonists and Antagonists for the Receptors That Mediate Enzyme Induction (AhR, CAR, and PXR) and PXR/CAR Target Genes in Humans and Mice

	AGONISTS			ANTAGONISTS		
AhR	 **TCDD**			 **CH-223191**		
	 **Omeprazole**			 **Omeprazole sulfide**		
CAR	Agonists	Human	Mouse	Inverse agonists	Human	Mouse
	Chlorpromazine	No	Yes	Chlorpromazine	Yes	No
	Meclizine	No	Yes	Meclizine	Yes	No
	Clotrimazole	No	Yes			
	CITCO	Yes	No			
	TCPOBOP	No	Yes			
	Indirect Activators					
	Phenobarbital	Yes	Yes			
	Phenytoin	Yes	Yes			
PXR	Agonists	Human	Mouse	Antagonists	Human	Mouse
	Artemisinin	Yes	No	Ketoconazole	Yes	Unknown
	Clotrimazole	Yes	No	Trabectedin (ET-743)	Yes	Unknown
	Hyperforin	Yes	Yes			
	Nicardipine	Yes	No			
	Nifedipine	Yes	No			
	Phenobarbital	Yes	Yes			
	PCN	Weak	Yes			
	Rifampin	Yes	No			
	TCPOBOP	Yes	Unknown			

XENOSENSOR	HUMAN PXR/CAR TARGET GENES*		MOUSE PXR/CAR TARGET GENES*	
PXR and CAR	CYP2A6		CYP2B10	SULT1E1
	CYP2B6		CYP3A11	SULT2A1
	CYP2C9		ALDH1	SULT2A2
	CYP2C19		AKR1B7	MDR1a
	CYP3A4		UGT1A1	MRP2
	UGT1A1		UGT1A9	MRP3
	MDR1		GSTA1/2	
	MRP2			
PXR only	CYP3A7	UGT1A3	CES6	
	CYP4F12	UGT1A6	UGT2B5	
	CYP7A1↓	GSTA1	GST(α,π,μ)	
	AKR1C1/2		MDR1b	
	CES2		OATP1A4	
CAR only	MRP3		CYP1A1	EST1
	MRP4		CYP1A2	UGT1A6
			CYP2A4	UGT2B1
			CYP2C29	MRP1
			CYP2C37	MRP4

Abbreviations: CITCO: 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; PCN, pregnenolone-16α-carbonitrile; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCPOBOP: 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene.

** Data from Tolson and Wang (2010).*

9 in the section "Principles of Xenobiotic Biotransformation" and sections "Epoxide Hydrolases" and "Quinone Reduction: NQO1 and NQO2"). The enzymes regulated by Nrf2 include several conjugating but few CYP enzymes (notable exceptions are CYP2C55 and CYP2U1 in mice), whereas those regulated by AhR, CAR, PXR, and PPARα include both conjugating and CYP enzymes. In mice, activation of AhR leads to activation of Nrf2; accordingly, the enzymes induced by the AhR agonist dioxin are the result of increased transcription of two classes of genes, namely those controlled by AhR and those controlled by Nrf2 (Yeager et al., 2009). A role for HNF-1α in the kidney-specific expression of drug transporters, OAT1, OAT3, and URAT1, has been documented (Jin et al., 2012). Some of the other receptors listed in Table 6-20 are discussed later in this section. CYP induction can also involve mechanisms other than receptor-mediated transcriptional activation. Increased translation of mRNA is the mechanism by which several xenobiotics increase the levels of CYP2E1, and increased translational efficiency and protein stabilization play important roles in the induction of several CYP enzymes including CYP1A2, CYP2E1, and CYP3A enzymes (Gonzalez, 2007).

It might be assumed that xenobiotics can be divided into four categories of inducers represented by the xenosensors AhR, CAR, PXR, and PPARα. This is largely true of rats where 3-methylcholanthrene, phenobarbital, PCN, and clofibric acid are prototypical inducers of CYP1A, CYP2B, CYP3A, and CYP4A by virtue of their ability to activate AhR, CAR, PXR, and PPARα, respectively. In fact, rats have five classes of inducible CYP enzymes with the fifth class represented by CYP2E1, which is inducible by the prototypical inducer isoniazid. However, the situation in other species (including rats) on a xenobiotic-by-xenobiotic basis is not so straightforward because (1) CAR and PXR have similar but not identical LBDs such that ligands that activate one receptor activate the other and vice versa (although they can do so with markedly different affinities) and (2) CAR and PXR have similar but not identical DBDs such that, once activated, CAR and PXR bind to some of the same response elements and induce the same enzymes. It was once thought that CYP2B induction was mediated only by CAR, and that CYP3A induction was mediated only by PXR. However, the PXR agonist dexamethasone can maximally induce both CYP2B10 and CYP3A11 in wild-type and CAR knockout mice, indicating that PXR can maximally induce both CYP2B and CYP3A enzymes in mice. Similarly, the CAR activator phenobarbital can maximally induce CYP2B10 and induce CYP3A11 (albeit submaximally) in wild-type and PXR knockout mice, indicating that CAR can also induce both CYP2B and CYP3A enzymes (Kodama and Negishi, 2006). The same is true of humans: PXR can maximally induce both CYP2B6 and CYP3A4, whereas CAR can maximally induce CYP2B6 and induce CYP3A4 submaximally due to its relatively weaker binding and functional activation of the CYP3A4 ER6 (Faucette et al., 2006). Furthermore, there are species and strain differences in the LBDs, DBDs, and DNA-response elements that give rise to species and strain differences in enzyme induction, and even interindividual differences in humans. Some species differences in enzyme induction reflect differences in the catalytic activity of CYP enzymes. For example, although 7-methoxyresorufin and 7-ethoxyresorufin are useful substrates to study CYP1A enzyme induction in all mammalian species, 7-pentoxyresorufin is useful for studying CYP2B only in mouse and rat (not hamster, dog, monkey, or humans). However, many species, strain, and individual differences in enzyme induction reflect differences in xenosensor function, as discussed below (this section). The following descriptions of the individual xenosensors focus largely on the mouse and human receptors; studies of the latter have been driven by the desire to investigate drug–drug interactions, whereas studies of the former have been facilitated by the ability to create transgenic mice in which a particular xenosensor has been deleted (so-called knockout or null mice) or replaced with the human receptor (so-called humanized mice).

Aryl Hydrocarbon Receptor AhR and ARNT are cytosolic and nuclear transcription factors, respectively, that are expressed in most tissues and many cell lines. The binding of an agonist such as dioxin (the structure of which is shown in Table 6-21) to AhR initiates a number of changes (such as dissociation from heat-shock protein [hsp90] and other chaperones, dissociation from proteins that retain the unbound receptor in the cytoplasm, and phosphorylation by tyrosine kinase) that culminate in (1) the translocation of AhR to the nucleus, (2) the dimerization of AhR with ARNT (which is somewhat misnamed as the *aryl hydrocarbon receptor nuclear translocator* because it is a nuclear, not a cytoplasmic protein that complexes with AhR after it translocates to the nucleus), (3) the binding of AhR–ARNT to DNA-response elements known as XRE, and (4) the increased transcription of CYP1A1 and numerous other genes listed in Table 6-20. While members of the nuclear receptor superfamily (such as CAR, PXR, and PPARα) are "zinc finger" proteins, AhR and ARNT are "bHLH-PAS" proteins, so-named because they belong to the per-arnt-sim (PAS) class of receptors and they have a basic helix–loop–helix (bHLH) domain near their N-terminus, which is involved in protein–protein interactions.

Dioxin is a high-affinity ligand for AhR (K_D values tend to be in the nanomolar-to-submicromolar range), and AhR mediates the toxicity of dioxin and related PHAHs (such as various chlorinated or brominated dibenzodioxins, dibenzofurans, and biphenyls), which is characterized by a wasting syndrome (progressive weight loss or cachexia resulting in death), immunosuppression (thymic atrophy), tumor promotion, fetal abnormalities (cleft palate and other teratogenic disorders), and, in some species, liver enlargement and a skin condition called chloracne. Dioxin binds to AhR and induces CYP1A1 in all mammalian species, but it causes lethal cachexia in some species (such as guinea pig, where the LD_{50} is about 1 μg/kg) but not others (such as hamsters, where the LD_{50} exceeds 1000 μg/kg). This greater than 1000-fold species difference in dioxin toxicity with only marginal differences in CYP1A1 induction raised the possibility that AhR does not mediate the toxicity of dioxin, but several lines of evidence established that AhR does in fact mediate many, perhaps all, of the toxic effects of dioxin and related compounds. First, naturally occurring strain differences in the responsiveness of mice (such as so-called responsive C57 and nonresponsive DBA mice) and rats (such as responsive Long–Evans and nonresponsive Han/Wistar rats) to dioxin toxicity are associated with polymorphisms in AhR (Pohjanvirta et al., 1999). Second, AhR knockout mice, mice harboring a modified form of AhR that cannot be translocated to the nucleus, and mice harboring a hypomorphic (reduced expression) form of ARNT are all resistant to dioxin-induced toxicity, whereas transgenic mice that express a constitutively active form of AhR (CA-AhR mice) spontaneously display numerous signs of dioxin toxicity (even though the CYP1A1 levels in the liver of CA-AhR mice are elevated to a lesser extent than those in dioxin-treated wild-type mice) (Bunger et al., 2003, 2008; Brunnberg et al., 2006, 2011). Third, the AhR antagonist CH-223191 (see Table 6-21) blocks the enzyme-inducing effects and various toxic effects of dioxin (Kim et al., 2006). Although AhR mediates both CYP1A1 induction and dioxin toxicity, it is noteworthy that the dose of dioxin required to cause 50% lethality to various strains of rats and mice (LD_{50}) is at least two orders of magnitude greater than the dose required to achieve 50% of maximum CYP1A1 induction (ED_{50}) (Watson et al., 2014). For

example, the ED_{50} for induction of CYP1A1 mRNA by dioxin is 0.04 and 0.01 μg dioxin/kg body weight in Long–Evans and Han/Wistar rats, whereas LD_{50} after 28 days is 17.7 and greater than 9600 μg dioxin/kg body weight, respectively. These findings not only indicate that acute toxicity requires much higher doses of dioxin than those required to induce CYP1A1, but also indicate that dioxin is equally effective at inducing CYP1A1 in both strains of rats despite a marked difference (more than 100-fold) in dioxin toxicity. It remains to be determined why prolonged and sustained activation of AhR by dioxin and dioxin-like compounds (DLCs) causes an irreversible cachexia that leads to death after a latency period that varies from one species to the next (and even one strain to the next) but that cannot be shortened by administering "superlethal" doses of dioxin. Another enduring mystery is why numerous other AhR agonists, including many endogenous compounds, activate AhR but do not cause the toxic effects characteristic of dioxin and dioxin-like compounds (DLCs).

AhR agonists include PHAHs (such as dioxin), PAHs (such as 3-methylcholanthrene and B[*a*]P), flavonoids (such as β-naphthoflavone), and various acid-catalyzed condensation products or UV-induced derivatives of naturally occurring indoles (such as indole-3-carbinol and tryptophan). Some of the most potent endogenous AhR activators identified to date (with binding affinities comparable to 3-MC and BNF) are various tryptophan derivatives such as kynurenine, kynurenic acid, FICZ (6-formylindolo[3,2-b] carbazole), ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester) and condensation products formed from indole-3-carbinol (I3C), such as DIM (3,3′-diindoylmethane, a dimer of I3C) and ICZ (indolo[3,2-*b*]carbazole, another of dimer of I3C) (Rannug et al., 1987; Bjeldanes et al., 1991; Heath-Pagliuso et al., 1998; Bergander et al., 2004; Henry et al., 2006; Chiaro et al., 2007; DiNatale et al., 2010; Opitz et al., 2011; Wincent et al., 2012). I3C is one of many dietary constituents that activate AhR and induce CYP1 enzymes. The role of intestinal CYP1A1 in preventing systemic exposure to phytochemicals was demonstrated in genetically modified mice with a selective loss of ARNT (the DNA-binding partner of AhR) from the intestine, which selectively blocks CYP1A1 expression in the intestine (Ito et al., 2007). In these mice, CYP1A1 was markedly induced in the liver and all extrahepatic tissues (including brain), a finding attributed to systemic exposure to dietary phytochemicals such as I3C in the diet.

FICZ is a photoproduct of tryptophan; although it is a potent AhR activator it also is *rapidly* metabolized by CYP1 enzymes (Bergander et al., 2004; Wincent et al., 2012). In the eye and skin, FICZ is an auto-inducer; it activates AhR, induces CYP1A1 and CYP1B1, and thereby induces the metabolism of FIZC and other photoproducts of tryptophan. Like FICZ, another tryptophan product, namely melatonin, can potentially induce its own metabolism because melatonin is an AhR activator and a substrate for CYP1A1, CYP1A2, and CYP1B1 (Ma et al., 2005; Hu et al., 2007).

In general, AhR agonists induce CYP1A1 and other enzymes across all mammalian and many nonmammalian species. A notable exception is a class of benzimidazole-containing compounds that includes the proton pump inhibitors omeprazole and lansoprazole. These drugs are not typical AhR agonists because they induce CYP1A enzymes in humans but not in rats or mice, which is one of the few species differences in AhR activation by xenobiotics (Diaz et al., 1990). In addition to agonists and antagonists there is a third class of AhR ligands known as selective AhR modulators (SAhRMs) as exemplified by SGA360. In contrast to AhR agonists that induce both XRE-driven transcriptional activity and repression of acute-phase gene expression, SAhRMs are only capable of mediating repression of acute-phase genes (Murray et al., 2010). The SAhRM-mediated suppression of cytokine-mediated acute-phase genes is a non-XRE-driven, cytoplasmic event. This process illustrates an emerging role of AhR in the regulation of inflammatory process and liver homeostasis. AhR influences T-cell differentiation and influences the balance of T_H1 and T_H2 cells, as was established through the use of an AhR agonist M50367 that preferentially promotes the production of T_H1 over T_H2 cells. Exposure of mice to M50367 leads to a decrease in IgE synthesis and peritoneal eosinophilia in allergic experimental models (Negishi et al., 2005).

Several compounds can disrupt AhR signaling at a variety of levels. CH-223191 (Table 6-21), GNF351, and α-naphthoflavone are receptor antagonists that bind to the LBD of AhR without activating the receptor (Kim et al., 2006; Smith et al., 2011). CH-223191 blocks CYP1 enzyme induction by dioxin and dioxin-related compounds (DLCs) and protects mice against the acute toxicity of dioxin (Kim et al., 2006), but it has no effect (or a marginal effect) on AhR activation by 3-methylcholanthrene, β-naphthoflavone, and other non-DLCs (Zhou et al., 2010b). Although CH-223191 does not antagonize CYP1A1 induction by non-DLCs (i.e., the XRE properties of AhR) it does antagonize their ability to stimulate the differentiation of regulatory T cells (i.e., the SAhRM properties of AhR) (Mezrich et al., 2010; Rouse et al., 2013). In contrast, GNF351 antagonizes both the XRE- and SAhRM-mediated effects of AhR (Smith et al., 2011). Omeprazole sulfide (which forms by reduction of the sulfoxide group in omeprazole [see Table 6-21]) antagonizes CYP1A1 induction by stabilizing AhR in an inactive conformation (Gerbal-Chaloin et al., 2006). In cell-based systems, the ability of omeprazole to induce CYP1A1 is compromised by formation of omeprazole sulfide unless the cells express CYP3A4, which can sulfoxidate omeprazole sulfide back to omeprazole. After AhR is activated, its translocation to the nucleus is blocked by 3′-methoxy-4-nitroflavone, its binding to XRE is blocked by salicylamide, and its ability to recruit transcription factors and initiate gene transcription is blocked by resveratrol (an antioxidant in red wine) (Gerbal-Chaloin et al., 2006). The presence of exogenous (and perhaps endogenous) AhR antagonists is one of the many factors that are known or suspected of causing clinically observed interindividual differences in CYP1A induction. These factors have been reviewed by Ma and Lu (2003), who have documented that differences in the magnitude of CYP1A induction in humans can be attributed to (1) genetic polymorphisms in AhR, ARNT, or XRE; (2) altered levels or function of accessory proteins (some of which are associated with inflammation and other disease states that activate NF-κB, as discussed later in this section); (3) gender differences in basal CYP1A2 activity (which tends to be lower in females compared with males); and (4) variation in the intracellular concentration of the inducing drug, as exemplified by variation in the metabolism of omeprazole. The clearance of omeprazole is largely determined by its rate of metabolism by the polymorphically expressed enzyme CYP2C19. Based on measurements of caffeine metabolism, a daily dose of 40 mg omeprazole causes CYP1A2 induction in CYP2C19 PMs but not in CYP2C19 EMs, which require higher doses (120 mg) to achieve CYP1A2 induction. In a clinical setting, the formation of omeprazole sulfide (an AhR antagonist) does not prevent CYP1A induction by omeprazole (an agonist) partly because reduction to the sulfide occurs slowly and partly because the sulfide can be converted back to the sulfoxide (parent drug) by CYP3A4. However, it is possible that the formation of rabeprazole sulfide, which occurs rapidly and extensively (by nonenzymatic means), explains why rabeprazole is one of the proton pump inhibitors that does not induce CYP1A.

Although strain differences in CYP1A inducibility have been linked to genetic polymorphisms in the mouse and rat AhR gene,

species differences have been attributed either to the unusual interaction of AhR with benzimidazole-containing compounds such as omeprazole and lansoprazole or to species differences in the location of XRE. For example, rat UGT1A6 and UGT1A7 are highly inducible (more than 20-fold) enzymes because these conjugating genes are under the control of XRE in rats but in few other species.

Increased expression of AhR is correlated with tumor invasiveness of cigarette-smoke-associated urothelial carcinoma cells in vitro, presumably due to enhanced expression of MMP-1 and MMP-9, matrix metalloproteinases involved in tumor invasion (Ishida et al., 2010). The involvement of AhR signaling in tumor invasiveness is further supported by a positive correlation between nuclear localization of the receptor and histopathological characteristics of the neoplasm and long-term survival of urothelial carcinoma patients.

AhR and ARNT are evolutionarily conserved across invertebrate and vertebrate species, suggesting they have important physiological functions (Guyot et al., 2013). Deletion of the gene encoding AhR affects the development of mice and rats, but it affects them in different ways (Harrill et al., 2013). AhR knockout mice have alterations in serum analytes indicative of compromised hepatic function, patent ductus venosus of the liver, and persistent hyaloid arteries in the eye. In contrast, AhR knockout rats display pathological alterations to the urinary tract, such as bilateral renal dilation (hydronephrosis), secondary medullary tubular and uroepithelial degenerative changes, and bilateral ureter dilation (hydroureter). These results suggest AhR plays significant roles in tissue development and homeostasis but does so in a species-dependent manner. This may account for some of the species differences in dioxin toxicity. ARNT is also known as hypoxia-inducible factor-1β (HIF-1β). In addition to partnering with AhR, ARNT complexes with HIF-1α to form the nuclear receptor known as hypoxia-inducible factor-1 (HNF-1), which regulates gene expression in response to hypoxia. In contrast to the situation with AhR, deletion of the gene encoding ARNT is 100% lethal, suggesting ARNT has a critical physiological function. It should be noted that deletion of the AhR gene is also lethal to about half of newborn mice but the other half survive despite the closure of the fetal liver ductus venosus, which leads to defective development of adult liver vasculature, which likely contributes to their low liver and body weight compared with wild-type mice.

With growing appreciation for AhR's important but incompletely understood physiological functions, particularly its immunomodulatory effects, and with the identification of numerous endogenous AhR agonists, such as the numerous tryptophan derivatives that potently activate AhR, attention has turned to the possibility that AhR is a potential pharmacological target for the treatment of certain diseases including cancer, multiple sclerosis, inflammatory skin disorders, Crohn disease, and colitis (Quintana and Sherr, 2013; Wheeler et al., 2017). When administered orally or parenterally, the tryptophan product ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester), which is an endogenous AhR agonist, protects mice from experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis, by inducing immunological tolerance by direct effects on dendritic cells in the CNS and peripheral T cells (Quintana et al., 2010). Circulating levels of endogenous AhR agonists are decreased in patients with relapsing-remitting multiple sclerosis (Rothhammer et al., 2017). Laquinimod, a drug under development for the treatment of multiple sclerosis, arrests EAE (i.e., multiple sclerosis) in mice by activating AhR, as indicated by a loss of therapeutic activity in AhR knockout mice (Berg et al., 2016; Kaye et al., 2016). Laquinimod (Nervanta) is structurally similar to roquinimex (Linomide), which also activates AhR and was once under development for the treatment of multiple sclerosis. Clinical studies with roquinimex were halted because of concerns over serious cardiovascular events including myocardial infarction, pleuritis, and pericarditis (Tan et al., 2000) and, likewise, clinical studies with high doses of laquinimod were halted for the same reason (Teva, 2016). Whether cardiotoxicity limits the usefulness of all drugs that activate AhR remains to be seen. Laquinimod, roquinimex, tasquinimod (an investigational drug for the treatment of prostate cancer), and IMA-08401 are all nonplanar compounds that can be converted by *N*-dealkylation or amide hydrolysis to coplanar metabolites that potentially play a key role in their ability to activate AhR (Mahiout et al., 2017).

Constitutive Androstane Receptor and Pregnane X Receptor

CAR and PXR are members of the nuclear receptor family 1 (NR1I2 and NR1I3, respectively). In contrast to AhR, which is expressed in most tissues and numerous cell lines, CAR and PXR are expressed in relatively few organs—liver, small intestine, and colon—and in few cell lines. Both CAR and PXR are cytoplasmic nuclear receptors; both are activated by some of the same compounds, and both dimerize with RXRα to form DNA-binding proteins that recognize some of the same response elements, as shown in Tables 6-20 and 6-21. Consequently, there is considerable cross-talk between these two xenosensors, and it can sometimes be difficult to ascertain whether induction is mediated by CAR, PXR, or both receptors. Both humans and mice have drug-metabolizing enzymes and transporters that are regulated by CAR only, PXR only, and both xenosensors, but the enzymes/transporters are inducible in a species-dependent manner, as shown for humans and mice in Table 6-21 (Tolson and Wang, 2010).

As shown in Table 6-20, three factors combine to make CAR and PXR the most important human xenosensors in terms of drug–drug interactions. First, CAR and PXR regulate several CYP enzymes (e.g., CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4), conjugating enzymes (several UGTs and SULTs), and transporters (such as P-gp, MRP2, and MRP4). Second, a large number of xenobiotics (drugs and the herbal preparations, St. John's wort, and Yin Zhi Huang) and certain endobiotics (such as bilirubin, bile acids, and 1,25-$(OH)_2$-D_3) activate CAR and/or PXR. Third, some of the genes regulated by CAR and PXR encode proteins with broad substrate specificities, such as CYP3A4 and P-gp, such that the induction mediated by CAR and PXR impacts the disposition of a large number of xenobiotics and certain endobiotics (see Table 6-17). In terms of drug–drug interactions, human xenosensors can be rank-ordered (from most important to least) as follows: CAR/PXR > AhR > "CYP2E1" > PPARα. PPARα is ranked last because its activation in humans, in contrast to its activation in rodents, does not lead to induction of CYP4 enzymes (a family of enzymes that plays a limited role in drug metabolism compared with CYP1, CYP2, and CYP3 enzymes), and although activation of human PPARα in vitro leads to induction of CYP1A1, CYP2C8, and CYP3A4, this effect has not be demonstrated in a clinical setting (see the section "Peroxisome Proliferator–Activated Receptor Alpha"). CYP2E1 is an inducible enzyme in humans by mechanisms that do not appear to involve any of the xenosensors listed in Table 6-20, such as induction by activators of the WNT/β-catenin pathway (Gerbal-Chaloin et al., 2014), but relatively few drugs are inducers of—or substrates for—CYP2E1. AhR is less important than CAR/PXR because there are relatively few drugs that activate AhR and because the major hepatic enzyme regulated by AhR, namely, CYP1A2, plays a more limited role in drug metabolism than the hepatic and intestinal CYP enzymes regulated by CAR/PXR, namely, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and other enzymes and transporters (see Tables 6-20 and 6-21).

The activation of PXR follows the canonical steps outlined earlier in this section, beginning with (1) ligand binding to LBD, (2) dissociation of PXR from cytoplasmic retention protein and corepressors (such as nuclear receptor corepressor [NCOR] and silencing mediator for retinoid and thyroid hormone receptors [SMRT]), (3) association with coactivators (such as SRC1, GRIP-1, PGC-1, C/EBPα, and RAC3), (4) dimerization with RXRα, (5) translocation to the nucleus, and (6) binding to a set of core gene promoter elements within xenobiotic-responsive enhancer modules that typically contain DR-3 or ER-6 motifs, which culminates in transcriptional activation of multiple genes, as shown in Tables 6-20 and 6-21 (Chang and Waxman, 2006; Stanley et al., 2006; Gao and Xie, 2010; Tolson and Wang, 2010; Omiecinski et al., 2011a). Sequence variation in the LBD of PXR accounts for certain species differences in CYP3A induction. For example, PCN is a prototypical inducer of CYP3A in rats and mice, but not humans, whereas the opposite is true of rifampin. Transgenic mice with human PXR in place of mouse PXR (PXR-humanized mice) lose the ability to respond to PCN and gain the ability to respond to rifampin. Rifampin can also bind and activate rat PXR once the opening to the LBD is slightly widened (by changing a phenylalanine to a leucine residue). As shown in Table 6-21, rifampin, artemisinin, clotrimazole, nicardipine, and nifedipine are agonists for human PXR but not mouse PXR, which exemplifies why enzyme induction studies in rodents often fail to predict enzyme induction in humans. Some agonists bind to human PXR with relatively high affinity (with K_D values in the [sub] micromolar range), including rifampin and hyperforin, the inducing agent in St. John's wort. In general, activation of CAR/PXR shows little or no stereospecificity, although the *C*-cyclopropylalkylamide known as S20 is a rare exception: (−)-C20 preferentially activates mouse PXR, whereas (+)-C20 preferentially activates human PXR. This difference further underscores the lack of predictability of rodent data to the human situation.

Tang and colleagues have reviewed the factors that give rise to differences in the magnitude of enzyme induction by PXR/CAR agonists in humans (Tang et al., 2005); such differences can be attributed to (1) genetic polymorphisms or splicing variation in PXR or CAR and/or polymorphism in their DNA-response elements; (2) altered levels or function of accessory proteins (some of which are associated with inflammation and other disease states that activate NF-κB, as discussed later in this section); (3) gender differences in basal CYP3A4 activity (which tends to be lower in males compared with that in females); and (4) variation in the intracellular concentration of the inducing drug, as exemplified by variation in the metabolism of propafenone and celecoxib. The COX-2 inhibitor celecoxib is a PXR agonist whose in vivo clearance is largely determined by CYP2C9. At recommended clinical doses, celecoxib induces CYP3A4 in CYP2C9 PMs but not in CYP2C9 EMs due to the higher levels of celecoxib in the PMs (Rodrigues et al., 2006).

The activation of CAR is considerably more complex than that of PXR (Chang and Waxman, 2006; Kodama and Negishi, 2006; Stanley et al., 2006; Mutoh et al., 2009; Tolson and Wang, 2010; Omiecinski et al., 2011b; Osabe and Negishi, 2011; Kobayashi et al., 2015; Negishi, 2017). CAR is constitutively active, meaning that, in the absence of an exogenous ligand, it can complex with RXRα, translocate to the nucleus, bind to DNA-response elements (known as PBREM [for phenobarbital response elements] and containing DR4 and DR5 elements), and activate gene transcription, which is what generally happens when CAR is expressed in cell lines in vitro. In the in vivo situation (and in primary cultures of hepatocytes), CAR is prevented from being transcriptionally active by its retention in the cytoplasm by cytoplasmic CAR retention protein (CCRP), also known as DNAJC7, and chaperones such as hsp90, and possibly by its binding to an endogenous reverse agonist such as androstanol (5α-androstan-3α-ol) and androstenol (5α-androst-16-en-3α-ol), the androstanes for which CAR is named (reverse agonists are discussed later in this section). The retention of CAR in the cytoplasm is achieved by its phosphorylation (at threonine 38 in human CAR and threonine 48 in mouse CAR) by protein kinase C (PKC) (Mutoh et al., 2009). Once phosphorylated, CAR complexes with P-ERK1/2, the phosphorylated (active) form of extracellular-signal-regulated kinase (also known as mitogen-activated protein kinase p42/p44), which binds to the so-called xenochemical response signal (XRS), a peptide sequence (^{313}LGLLAEL^{319}) near the C-terminus of the CAR receptor (Osabe and Negishi, 2011). Similar XRSs are present in RXR, VDR, peroxisome proliferator–activated receptors (PPARs), and estrogen receptor. Binding of P-ERK1/2 to XRS prevents the hydrolysis of the phosphate group at threonine 38 (human CAR) or threonine 48 (mouse CAR). The activation of CAR by xenobiotics involves the dephosphorylation of the threonine residue by protein phosphatase 2A, as evidenced by the observation that the protein phosphatase 2A inhibitor okadaic acid blocks CAR translocation in mouse hepatocytes (Mutoh et al., 2009). Treatment with CAR activators also initiates ubiquitination of CCRP and thereby promotes its degradation, which releases CAR from its cytosolic complex, followed by translocation of CAR to the nucleus (Kobayashi et al., 2015).

A factor that long complicated our understanding of the mechanism of CAR activation by xenobiotics was the realization that CAR activators can be divided into *direct activators*, which bind directly to the LBD of CAR, and *indirect activators*, which do not bind to CAR. CITCO and TCPOBOP are direct activators of human CAR and mouse CAR, respectively, and they are selective CAR activators, meaning they do not activate PXR. In humans and mice, phenobarbital (along with other EIAEDs) is the canonical indirect activator of CAR. The direct binding of CITCO and TCPOBOP to CAR triggers its dephosphorylation by protein phosphatase 2A, leading to the translocation of CAR to the nucleus. Phenobarbital treatment also leads to dephosphorylation (and hence activation) of CAR but it does so by inhibiting a membrane-bound receptor, namely epidermal growth factor (EGF) (Meyer and Jirtle, 2013; Mutoh et al., 2013). Inhibition of EGF leads to the loss of phosphorylated RACK1 (receptor for activated C kinase 1). Once dephosphorylated, RACK1 can complex with and activate protein phosphatase 2A, which then dephosphorylates (and hence activates) CAR. Because CAR is constitutively active but retained in the cytoplasm, ligands can function as agonists, reverse agonists, or antagonists. Agonists activate CAR by displacing endogenous reverse agonists (e.g., androstanes) (Chang and Waxman, 2006; Kodama and Negishi, 2006; Stanley et al., 2006; Mutoh et al., 2009; Tolson and Wang, 2010; Omiecinski et al., 2011b; Osabe and Negishi, 2011) or by promoting the dissociation of corepressors (such as P-ERK1/2 and NCOR) and the recruitment of coactivators (such as SRC-1, SP1, ASC-2, and PBP). Reverse agonists have the opposite effect: they promote the dissociation of coactivators and the recruitment of corepressors, and they may also decrease the transcriptional activity of CAR. Antagonists bind to the same LBD as agonists and reverse agonists; they neither activate nor deactivate CAR, but they block the binding of other ligands. Examples of agonists, reverse agonists, or antagonists of CAR are shown in Table 6-21.

Xenobiotics can function as CAR agonists or antagonists in a species-specific manner. For example, chlorpromazine and meclizine are agonists for mouse CAR, but they are inverse agonists for human CAR. TCPOBOP and CITCO are potent direct activators of CAR, but they function as CAR agonists in a species-specific manner: TCPOBOP is a potent agonist for mouse CAR, whereas

CITCO is a potent agonist for human CAR (Table 6-21). These selective CAR agonists must be used in a species-specific manner, as underscored by the fact that the mouse CAR agonist TCPOBOP is *not* an agonist for human CAR but it *is* an agonist for human PXR. Phenobarbital is an indirect activator of mouse and human CAR, but it is also an activator of human PXR (but not mouse or rat PXR). In contrast, another enzyme-inducing antiepileptic drug phenytoin does not activate human PXR and, like CITCO, is a selective activator of human CAR. There are also species differences in the genes regulated specifically by CAR, specifically by PXR, and by both xenosensors, as shown for mouse and human in Table 6-21. These species differences in CAR, such as those described earlier in this section for PXR, illustrate why, during the safety evaluation of drug candidates, enzyme induction studies in rodents are often of limited value in predicting enzyme induction in humans.

Alternative splicing of CAR mRNA gives rise to CAR2 and CAR3, which are not constitutively active but are ligand-activated forms of the receptor. CAR2 and CAR3 contain four and five amino acid inserts, respectively, within the LBD of the receptor. The inserts interfere with the ligand-binding pocket affinity of CAR2 but not CAR3. As a result, the ubiquitous plasticizer and environmental pollutant di(2ethylhexyl)phthalate (DEHP) is a highly potent and selective CAR2 activator. Alternative splicing of CAR mRNA leads to the expression of tissue-specific variants, which can increase CAR's functional diversity and broaden the human receptor's repertoire of responses to xenobiotics (DeKeyser et al., 2009, 2011). The splice variants detected for human CAR are not present in rats or mice, which further underscores why rodents may not be adequate to assess CAR-mediated events in humans.

Certain cofactors/enzymes are required for activation of CAR to its transcriptionally active form. For example, the coactivator PBP/MED1 (PPAR-binding protein/TRAP220/DRIP205/mediator subunit 1) is required for CAR activation (as well as PPARα activation) (Pyper et al., 2010), as is AMP-activated protein kinase (AMPK) (Rencurel et al., 2006). The ability of phenobarbital to induce CYP2B10 and CYP3A11 is abolished in transgenic mice lacking CAR, PBP/MED1, or the α-subunit of AMPK. Activation of CAR by direct ligands is dependent on p38 mitogen–activated protein kinase (MAPK) and the low levels of MAPK in various cell lines compared with primary hepatocytes contributes to the relatively low degree of CYP2B induction in, for example, HepG2 cells (Saito et al., 2013). Phenobarbital activates the upstream kinase LKB1, which in turn phosphorylates and activates AMPK, which phosphorylates transcriptional coactivators such as p300 (a nuclear phosphoprotein with histone acetylase activity), TORC2 (transducer of regulated cAMP response element–binding protein [CREB]), and peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α) (Blattler et al., 2007; Shindo et al., 2007).

The role of AMPK in CAR activation and CYP2B induction is not clear. Metformin, a drug used to lower fasting blood glucose levels in patients with noninsulin-dependent (type 2) diabetes, is an activator of AMPK. Metformin has been reported to be both an inducer of CYP2B6 (Rencurel et al., 2006) and a suppressor of CYP2B6 induction in human hepatocytes (Yang et al., 2014). In the latter study, metformin suppressed the induction of CYP2B6 in human hepatocytes by both direct-acting and indirect-acting CAR activators (CITCO and phenobarbital, respectively) which was associated with AMPK-dependent phosphorylation of CAR at Thr-38, leading to its inactivation. Another activator of AMPK, namely, 5-amino-4-carboxamide-1-β-ribofuranoside (AICAR), does not induce CYP2B in rat hepatocytes (Kanno et al., 2010) and metformin does not induce CYP2B in vivo in mice or rats (Shindo et al., 2007). Furthermore, metformin does not cause clinically significant CYP2B6 or CYP3A4 induction in humans (Blattler et al., 2007). Despite the initial finding that metformin was an activator of CAR, the weight of evidence suggests metformin actually suppresses CYP2B induction by activating AMPK and keeping CAR in the phosphorylated (inactive) state.

AMPK is sensitive to energy charge; consequently, it is activated by an increase in the ratio of AMP to ATP, which occurs, for example, during fasting. AMPK, PGC-1α, and CAR are all activated by fasting, which is associated with the induction of CYP2B10 and other CAR-regulated enzymes and transporters in mice (Ding et al., 2006). Like metformin, phenobarbital also lowers glucose levels in patients with type 2 diabetes and improves glucose tolerance and insulin sensitivity in mice (Rencurel et al., 2006; Dong et al., 2009). This glucose-lowering effect involves, at least in part, the binding of activated CAR to forkhead box protein O1 (FOXO1), a transcription factor that binds to insulin-response sequences (IRS) in the promoter region of gluconeogenic genes such as phosphoenolpyruvate carboxykinase-1 (PEPCK1) and glucose-6-phosphatase (G6Pase). Inactivation of FOXO1 by CAR leads to the suppression of PEPCK1 and G6Pase, which in turn leads to decreased gluconeogenesis (i.e., decreased glucose synthesis) (Kodama and Negishi, 2006; Rencurel et al., 2006). Consistent with this mechanism of action, phenobarbital decreases glucose levels in wild-type mice but not in CAR knockout mice. PXR agonists can also suppress gluconeogenesis either by the binding of activated PXR to FOXO1, which impairs the ability of FOXO1 to activate IRS-regulated gluconeogenic enzymes such as PEPCK1 and G6Pase, or by the binding of activated PXR to phosphorylated CREB that prevents CREB-dependent transcription of G6Pase (Kodama et al., 2004; Gao and Xie, 2010). In addition to suppressing gluconeogenic enzymes (PEPCK1 and G6Pase), CAR activators also induce hexokinase and glucose metabolism by the pentose phosphate pathway, which supports drug metabolism by generating the NADPH required by CYP, FMO, and certain other xenobiotic-biotransforming enzymes (Kodama et al., 2004; Rencurel et al., 2006; Dong et al., 2009).

The interaction between CAR and transcription factor FOXO1 has opposing effects on each other's transcriptional activity: CAR is a *corepressor* of FOXO1, whereas FOXO1 is a *coactivator* of CAR. Activation of FOXO1 by diabetes increases CYP2B and CYP3A expression in rats and mice, and this effect is reversed by insulin treatment (which stimulates the phosphorylation of FOXO1 and thereby decreases its activity). By inactivating FOXO1 (which removes a coactivator for CAR), insulin blunts the induction of CYP2B enzymes by phenobarbital and represses the gluconeogenic pathway (Kodama and Negishi, 2006; Moreau et al., 2008). The interactions between CAR and FOXO1 have also been described for PXR and FOXO1 (Kodama et al., 2004; Gao and Xie, 2010).

In addition to playing a role in glucose homeostasis (by suppressing gluconeogenic enzymes), CAR also plays a role in lipid homeostasis (largely by suppressing lipogenic enzymes). Multiple mechanisms have been identified that contribute to the suppression of lipogenesis by CAR activators (Gao and Xie, 2010). CAR activation and its associated activation of AMPK lead to the induction of Insig-1, a microsomal protein that, together with Insig-2, plays a key role in the control of hepatic triglyceride and cholesterol synthesis (Roth et al., 2008). Induction of Insig-1 results in repression of sterol regulatory element–binding protein-1 (SREBP-1), which in turn results in the downregulation of lipogenic enzymes and, hence, a decrease in hepatic triglyceride and, to a lesser extent, cholesterol levels. In addition to Insig-1 induction, the mechanism of SREBP-1 repression may also occur by an indirect mechanism that involves induction of CAR-regulated SULT2B enzyme(s) that sulfonate and

inactivate the oxysterols that function as agonists for the liver X receptor-α (LXRα), which is an inducer of SREBP-1. This indirect pathway may play a significant role in suppressing SREBP-1 expression based on the observation that the ability of CAR activators to suppress triglyceride/cholesterol synthesis is substantially impaired in SULT1B1b knockout mice (Dong et al., 2009). In addition to suppressing lipogenesis, CAR activators also induce fatty acid β-oxidation. The mechanism is distinct from the induction of fatty acid β-oxidation by PPARα agonists, which are the main regulators of lipid catabolism (discussed in the next section). CAR activators promote fatty acid β-oxidation by repressing the expression of acetyl-coenzyme A (acetyl-CoA) carboxylase (both ACC1 and ACC2; gene symbols ACACA and ACACB), which results in a decrease in the levels of malonyl-CoA, the initial intermediate in lipogenesis and an allosteric inhibitor of the carnitine-dependent transport of fatty acids into mitochondria for β-oxidation (Dong et al., 2009). From the foregoing it can be appreciated that there is growing interest in CAR as a potential therapeutic target in type 2 diabetes and fatty liver disease.

Just as activation of CAR suppresses lipogenesis so excess lipid in the liver suppresses CAR activity. Obesity, steatosis (fatty liver), and high-fat diets all suppress CAR activation and CYP2B induction (Blouin et al., 1993; Gao and Xie, 2010). The effects are mediated, at least in part, by suppression of CAR (and PXR) by the key lipogenic factors SREBP-1 and LXRα presumably by competition for nuclear receptor coactivators. Although CAR and PXR have the same effect on gluconeogenesis (both of them suppress glucose synthesis), these xenosensors have opposing effects on lipid homeostasis: activation of CAR leads to repression of lipogenesis, whereas activation of PXR leads to the promotion of lipogenesis (Gao and Xie, 2010). Transgenic mice expressing a constitutively activated PXR develop hepatomegaly with marked steatosis, as do PXR-humanized mice treated with rifampin, albeit to a lesser extent (Zhou et al., 2006b). PXR activation causes fatty liver by inducing CD36 (a transporter of free fatty acids), PPARγ (a lipogenic peroxisome proliferation activation receptor discussed in the next section), and several accessory lipogenic enzymes including stearoyl-CoA desaturase-1 (SDC-1) and long-chain fatty acid elongase and by suppressing enzymes involved in β-oxidation (Zhou et al., 2006b). Both PXR and CAR agonists induce thyroid hormone-responsive spot 14 (gene symbol THRSP), the expression of which correlates with lipogenesis, which is consistent with the aforementioned role of PXR in lipogenesis and which suggests that the role of CAR in lipid homeostasis may be complex because paradoxically CAR appears to induce both lipid synthesis and catabolism. In the clinical setting, a number of PXR agonists (rifampin, nifedipine, and carbamazepine) and a number of CAR activators (phenobarbital, phenytoin, and valproic acid) have been shown to alter lipid homeostasis and/or cause steatosis (fatty liver) (Zhou et al., 2006b).

CAR and PXR are activated by certain endobiotics, and the enzymes regulated by these xenosensors play important roles in terminating the hormonal activity of 1,25-$(OH)_2$-D_3 and attenuating the toxicity of high levels of bilirubin and bile acids. The active form of vitamin D, namely, 1,25-$(OH)_2$-D_3, binds to VDR to induce the synthesis of calbindin-D9K, a calcium-binding protein that facilitates the uptake of calcium from the intestinal lumen to blood. VDR also induces CYP enzymes that further hydroxylate and thereby inactivate 1,25-$(OH)_2$-D_3, which establishes a negative feedback loop. Two VDR-inducible CYP enzymes have been shown to inactivate 1,25-$(OH)_2$-D_3 by hydroxylating the active hormone in the 24-position and, to a lesser extent, the 23-position. The first is CYP24A1, which is expressed mainly in the kidney, and which catalyzes the 24*R*-hydroxylation of 1,25-$(OH)_2$-D_3. The second is CYP3A4, which is expressed mainly in the intestine and liver, and which catalyzes the 24*S*-hydroxylation of 1,25-$(OH)_2$-D_3. The stereochemical differences in the 24-hydroxylation reaction helped to establish that CYP3A4 is the principal inactivator of 1,25-$(OH)_2$-D_3 in the intestine, with little or no contribution from CYP24A1. These findings are important for two reasons. First, the ability of VDR to mimic PXR/CAR and induce CYP3A4 is an example of the cross-talk that can occur between a xenosensor and other nuclear receptors. Second, the key role played by CYP3A4 in the inactivation of 1,25-$(OH)_2$-D_3 in the intestine provides an explanation for the clinical observation that long-term treatment of patients with rifampin, phenobarbital, phenytoin, or carbamazepine can lead to osteomalacia, a bone disorder that is symptomatic of vitamin D deficiency. These PXR/CAR agonists are thought to cause osteomalacia by inducing CYP3A4 in the intestine, which accelerates the inactivation of 1,25-$(OH)_2$-D_3 and thereby impairs calcium absorption (Xu et al., 2006; Zhou et al., 2006a).

CAR plays an important role in detoxifying high levels of bilirubin, as might occur in patients with hemolytic anemia. Like phenobarbital, bilirubin is an indirect activator of CAR. When activated, CAR induces UGT1A1 and several SULTs, the major hepatic enzymes responsible for conjugating bilirubin, as well as transporters of bilirubin/bilirubin conjugates (MRP2 and MRP4). When hemolytic anemia is induced by treating mice with phenylhydrazine, bilirubin levels rise to a greater extent in CAR knockout mice compared with wild-type mice, which demonstrates the important role CAR plays in responding to high levels of bilirubin. Phenobarbital was once used to treat neonatal jaundice, and the Chinese herbal Yin Zhi Huang is still used for this purpose. Phenobarbital and scoparone (6,7-dimethylesculetin), the active ingredient in Yin Zhi Huang, are CAR activators that, like bilirubin, induce the major bilirubin-conjugating enzyme UGT1A1 and related transporters (Chang and Waxman, 2006; Kodama and Negishi, 2006). In contrast, CAR appears to play a role in acetaminophen hepatotoxicity presumably by allowing acetaminophen to induce its own activation to a toxic quinoneimine. CAR knockout mice are relatively resistant to acetaminophen hepatotoxicity compared with both wild-type and CAR-humanized mice, which suggests early treatment with a CAR antagonist might be a useful adjunct treatment for acetaminophen overdose.

CAR, PXR, and the VDR appear to act as backup receptors to prevent bile acid accumulation and toxicity when the principal bile acid sensor, FXR, is overloaded. In the mouse ileum and colon, bile acid activation of FXR and, to a lesser extent, VDR and PXR, promotes bile acid detoxication by three mechanisms: (1) metabolism by AKR1B7 and CYP3A11; (2) intracellular binding to ileal bile acid–binding protein (IBABP); and (3) secretion by the organic solute transporter OSTα/β (Ballatori et al., 2009b; Degirolamo et al., 2011; Schmitt et al., 2011). FXR induces the expression of AKR1B7 in murine small intestine, colon, and liver by binding directly to a response element in the AKR1B7 promoter and this AKR metabolizes 3-keto bile acids to the corresponding 3β-hydroxy bile acids, which are less toxic than their 3α-hydroxy isomers. High concentrations of bile acids activate FXR, which suppresses the expression of CYP7A1, the cholesterol 7α-hydroxylase that catalyzes the rate-limiting step in bile acid synthesis, and which induces the expression of bile acid conjugating enzymes, namely UGT2B4 and SULT2A1, and bile acid transporters, such as the bile salt export pump (BSEP). When hepatocytes contain low concentrations of bile acids, FXR is inactive and the expression of CYP7A1 is stimulated by liver receptor homolog-1 (LRH-1), a constitutively active nuclear receptor. When hepatocytes contain high concentrations of bile acids, FXR is activated, and it inactivates LRH-1 by

inducing the synthesis of small heterodimer partner-1 (SHP), so named because it lacks a DNA-binding domain (hence its small size) and because it forms heterodimers with LRH-1 and numerous other nuclear receptors including AhR, CAR, PXR, LXRα, HNF4α, and the glucocorticoid receptor (GR). Because it lacks a DBD, SHP inactivates all of these receptors; in the case of LRH-1, this results in a loss of CYP7A1 expression and a decrease in bile acid synthesis, which represents a negative feedback loop. FXR knockout mice are more sensitive than wild-type mice to bile acid toxicity, and treatment with the FXR ligand GW4064 further protects wild-type mice against experimentally induced extrahepatic and intrahepatic cholestasis. Cholic acid is both an FXR and PXR agonist; it suppresses CYP7A1 expression by activating first FXR and then PXR. In contrast to the situation in wild-type or single receptor knockout mice, 1% dietary cholic acid is lethal to FXR/PXR double knockout mice. PXR and CAR play a similar role in attenuating the toxicity of lithocholic acid, which is both a PXR agonist and a substrate for many of the enzymes regulated by PXR, including CYP3A, SULT, and transporters involved in the hepatic uptake and efflux of lithocholic acid. In PXR/CAR double knockout mice, lithocholic acid causes lethal hepatotoxicity at doses that are well tolerated by wild-type mice. The mouse PXR agonist PCN can markedly suppress CYP7A1 expression and bile acid synthesis in mice, and the human PXR agonist rifampin can markedly suppress CYP7A1 expression in human hepatocytes. The ability of PXR/CAR to function as a backup system for FXR may be compromised by the expression of SHP, which represses the activity of these xenosensors. This complex interplay is one of the likely reasons why there are some conflicting results from studies of the role of PXR/CAR in bile acid detoxication. The overall role of FXR is to provide protection against the toxic and tumorigenic effects of bile acids in the liver and colon; consequently, agonists of this receptor are being considered as potential colon cancer chemotherapeutic drugs (Degirolamo et al., 2011). Obeticholic acid (Ocaliva), a bile acid analog (6α-ethyl-chenodeoxycholic acid) and potent activator of FXR, received FDA approval in 2016 for the treatment of primary biliary cholangitis. Obeticholic acid lowers bile acids in the liver by inducing BSEP (to transport bile acids into bile) and OSTα/β (to transport bile acids into blood), and by suppressing CYP7A1, the enzyme that catalyzes the rate-limiting step in bile acid synthesis (Zhang et al., 2017). Activation of FXR by obeticholic acid in human hepatocytes is associated with suppression of various CYP enzymes and drug transporters but treatment with the clinical dose of obeticholic acid is associated only with slight suppression of CYP1A2 (based on a about 50% increase in systemic exposure to caffeine), which could be relevant to CYP1A2 substrates with a narrow therapeutic index, such as theophylline (Edwards et al., 2017). Conversely, enzyme inducers that activate PXR/CAR can decrease the activity of FXR and thereby suppress the expression of BSEP and OSTα/β, leading to concern that prolonged treatment with PXR/CAR agonists could be a cause of drug-induced cholestasis, which can also result from direct inhibition of BSEP (Garzel et al., 2014).

As the preceding examples in this section illustrate, there is a complex interplay between many of the nuclear receptors listed in Table 6-20. Some nuclear receptors, such as the hepatic nuclear factors HNF-1α and HNF-4α, and the coactivator PBP/MED1 (PPAR-binding protein/TRAP220/DRIP205/mediator subunit 1), play a critical permissive role in gene regulation by CAR, PXR, and other receptors, whereas others, such as SHP, play a widespread role in suppressing nuclear receptor activity. HNF-1α is instrumental for the expression of high levels of CYP2E1 mRNA in the liver, although it is not the only factor because disruption of the WNT/β-catenin pathway in mice greatly reduces CYP2E1 mRNA levels in the liver in the absence of changes in HNF-1α expression (Gonzalez, 2007). Conversely, activation of the WNT/β-catenin pathway causes a pronounced induction of CYP2E1 (Gerbal-Chaloin et al., 2014). Interestingly, the cross-talk between transcription factors, particularly between HNF-4α, CAR, and PXR, is markedly increased in tissues from patients with severe viral hepatitis with pronounced inflammation and fibrosis (Congiu et al., 2009).

The glucocorticoid receptor (GR) plays an important role in inducing the synthesis of CAR and PXR (and individual xenobiotic-biotransforming enzymes such as CYP2C9). The role of GR in regulating the synthesis of xenosensors and the interplay among nuclear receptors are instrumental to the mechanism by which infections and inflammatory diseases suppress the expression and blunt the induction of xenobiotic-biotransforming enzymes and, conversely, the mechanism by which PXR agonists cause immunosuppression.

The expression and induction of xenobiotic-biotransforming enzymes by AhR, CAR, PXR, and PPARα are all suppressed by infection (viral, bacterial, and parasitic), vaccination, inflammation, and treatment with endotoxin (lipopolysaccharide or LPS) or proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-6. The suppression involves (1) an increase in proinflammatory cytokines, (2) a decrease in the levels of PXR and CAR due to a decrease in GR activity, and (3) a decrease in the activity of GR, PXR, CAR, AhR, PPARα, and several other nuclear receptors including the androgen, estrogen, and mineralocorticoid receptors (De Bosscher et al., 2006; Pascual and Glass, 2006; Zhou et al., 2006b). The decrease in receptor activity is caused by NF-κB, which is activated during infection and inflammatory disease, and which induces the synthesis of COX-2, TNF-α, ICAM-1, and several interleukins. NF-κB binds to the DBD of RXRα and thereby decreases the transcriptional activity of all nuclear receptors that form heterodimers with RXRα (such as PXR, CAR, PPARα, and GR). It also decreases the transcriptional activity of AhR even though AhR forms heterodimers with ARNT, not RXRα. By lowering the levels and/or inhibiting the activity of the major xenosensors and numerous other nuclear factors, NF-κB mediates the widespread suppression of xenobiotic-biotransforming enzymes associated with infection and inflammatory disease. However, the converse is also true inasmuch as certain nuclear receptors, when activated, can complex NF-κB and thereby repress the expression of NF-κB-regulated genes (COX-2, TNF-α, ICAM-1, and interleukins). This is the basis, at least in part, for the immunosuppressive effect of corticosteroids, which activate GR, and the immunosuppressive effect of PXR ligands such as rifampin and mifepristone (RU-486). PXR plays a role in suppressing NF-κB activity in the small intestine. PXR knockout mice show signs of inflammation of the small intestine, and certain genetic polymorphisms and splicing variants of PXR are associated with inflammatory bowel disease. Similarly, during periods of inflammation, the levels of mRNA encoding PXR and CYP3A4 are decreased in the intestine, and are decreased in children with active Crohn disease (a type of inflammatory bowel disease) (Shakhnovich et al., 2016). Common environmental AhR agonists can suppress the response to bacterial LPS through downregulation of IL-6. This suppression occurs at the level of IL-6 gene transcription and may be regulated by NF-κB (Jensen et al., 2003). AhR can also attenuate cytokine-mediated induction of acute-phase response genes in the liver in a XRE-independent manner. This function of the receptor suggests its role in maintaining liver homeostasis during inflammation. The role of PXR in regulating NF-κB activity, responding to high levels of bile acids, and terminating the actions of 1,25-$(OH)_2$-D_3, the role of CAR in responding to high level of bilirubin, and the role of CAR and PXR in glucose and lipid (energy) metabolism all illustrate that

these so-called xenosensors do not simply respond to xenobiotics but play key roles in endobiotic homeostasis.

PXR is expressed in a variety of tumors, which has been linked to drug resistance, cell proliferation, apoptosis, and cancer prognosis. The possibility of using a PXR antagonist to downregulate drug-metabolizing enzymes and transporters under the control of PXR in order to sensitize tumor cells to anticancer drugs has been proposed (Mani et al., 2013; Qiao et al., 2013; Pondugula et al., 2016).

Peroxisome Proliferator–Activated Receptor Alpha An increase in the size and number of rat hepatic peroxisomes was first observed in response to the hypolipidemic drug clofibrate. Peroxisome proliferation is accompanied by hepatomegaly, and induction of fatty acid oxidation in peroxisomes, mitochondria, and microsomes. With long-term administration of hypolipidemic drugs, peroxisome proliferation is accompanied by hepatocarcinogenicity. Induction of microsomal fatty acid oxidation is due to the induction of CYP4A enzymes that play a limited role in xenobiotic biotransformation but that convert fatty acids to dicarboxylic acids by ω-hydroxylation (see the section "CYP4 Enzymes"). Numerous xenobiotic "peroxisome proliferators" have been identified including hypolipidemic fibrate drugs (e.g., clofibrate, fenofibrate, ciprofibrate, bezafibrate, nafenopin, and methylcofenapate), various NSAIDs, aspirin, leukotriene receptor antagonists (MK-0571 and RG 7512), organic solvents (trichloroacetic acid), phthalate ester plasticizers, herbicides (haloxyfab, lactyofen, and 2,4-dichlorophenoxyacetic acid [2,4-D]), perfluorodecanoic acid, nicotinic acid, cinnamyl anthranilate, and the potent and widely used experimental peroxisome proliferator–activated receptor α (PPARα) agonist Wy-14643 (Lake, 2009). Peroxisome proliferation by some of these xenobiotics occurs stereoselectively. The receptor that mediates peroxisome proliferation by these xenobiotics is PPARα. PPARα (NR1C1) is one of a family of three nuclear receptors; the other members are PPARβ/δ (NR1C2) and PPARγ (NR1C3). All three are type II nuclear receptors that reside in the nucleus (in contrast to AhR, CAR, and PXR, which reside in the cytoplasm) (Pyper et al., 2010).

The distinction between xenobiotic- and endobiotic-sensing functions is particularly blurred in the case of PPARα, which is activated by the aforementioned xenobiotics and by several endogenous fatty acids (e.g., notably the acyl-CoA and enoyl-CoA derivatives of long-chain fatty acid), dicarboxylic acids (such as those formed by CYP4-dependent ω-hydroxylation of long-chain fatty acids and arachidonic acid [HETEs]), LTB_4, products formed by fatty acid synthase (such as 1-palmityl-2-oleolyl-*sn*-glycerol-3-phosphocholine [16:0/18:1-GPC]), and DHEA sulfate. From this list of endogenous ligands it is apparent that fatty acids (generally in the form of acyl-CoA thioesters) can activate PPARα and induce their own metabolism. All three receptors play an important role in lipid (energy) metabolism. In general, PPARα and PPARβ/δ are regulators of fatty acid catabolism (catabolic energy metabolism) that are highly expressed in the liver, heart, kidney, brown adipose tissue, muscle, and the small and large intestines (Issemann and Green, 1990; Braissant et al., 1996; Bookout et al., 2006). PPARγ, which is expressed in at least three forms (e.g., PPARγ1, PPARγ2, and PPARγ3), regulates lipid synthesis and is abundant in white and brown adipose tissues (Tontonoz et al., 1994; Fajas et al., 1998). The pattern of tissue expression of PPARα is similar in rodents and humans (Bookout et al., 2006). PPARα exerts a dominant role in regulating fatty acid catabolism and ketone body synthesis in the liver. PPARβ/δ also participates in the regulation of fatty acid oxidation, but its effects are more prominent in muscle and heart; however, PPARβ/δ also regulates certain lipid-metabolizing enzymes in the liver (Sanderson et al., 2009). PPARα functions as the major xenobiotic and lipid sensor to regulate fatty acid catabolism, hepatic steatosis, lipoprotein synthesis, inflammation, and hepatocarcinogenesis; hence, this xenosensor is the major focus of discussion here.

Activation of PPARα follows the canonical steps outlined earlier in this section. Binding of ligands to the LBD of PPARα results in (1) dissociation of corepressors (such as NCOR and SMRT), (2) dimerization with RXRα, (3) association with coactivators (p300, SRC/p160 family members, PBP/MED1, PGC-1α, and many others), and (4) binding of activated PPARα to response elements (PPREs) that usually reside in the promoter (5′-region) of target genes. The PPRE is a direct repeat (DR1) composed of two copies of the consensus hexamer sequence AGG(A)TCA separated by a single base pair. PPARα activity is increased by phosphorylation of serine residues 12 and 21. Insulin stimulates the phosphorylation and, hence, the transcriptional activity of PPARα. The expression of PPARα is increased by fasting/starvation to provide energy through the oxidation of fatty acids released from adipose tissue. During starvation, PPARα is essential for the upregulation of the genes necessary for fatty acid oxidation, although PPARβ/δ can also upregulate some of the same enzymes independently of PPARα (Sanderson et al., 2009). The β-oxidation of fatty acids produces acetyl-CoA that either enters the mitochondrial tricarboxylic acid cycle or undergoes condensation reactions to form ketone bodies that are released into blood for energy (ATP) production in extrahepatic tissues. PPARα is also regulated by stress, growth hormone, leptin, and glucocorticoids. Direct binding of glucose to PPARα may be an important mechanism of stimulating fatty acid oxidation during uncontrolled diabetes (Pyper et al., 2010).

Once activated, PPARα induces the expression of key enzymes involved in the oxidation of fatty acids in peroxisomes, mitochondria, and endoplasmic reticulum (the three major organelles involved in lipid catabolism). Oxidation of fatty acids in peroxisomes and mitochondria involves β-oxidation, whereas their oxidation in microsomes involves ω- and ω-1 hydroxylation by CYP (especially CYP4 enzymes). Peroxisomal β-oxidation targets very long straight-chain fatty acids, 2-methyl branched fatty acids, prostanoids, and dicarboxylic acids. The latter are formed by the ω-hydroxylation of fatty acids by CYP4 enzymes. Compared with their precursors (monocarboxylic acids), dicarboxylic acids are highly toxic; hence, the coordinate induction of microsomal ω-hydroxylation and peroxisomal β-oxidation is an important function of PPARα. There are two peroxisomal β-oxidation systems: the first system targets long-chain fatty acids and is inducible by PPARα, with the initial step being catalyzed by the highly inducible enzyme peroxisomal acyl-coenzyme A oxidase 1 (ACOX1). The second system is a noninducible system that targets 2-methyl-branched fatty acids (with the initial step catalyzed by ACOX2). Peroxisomal β-oxidation of long-chain fatty acids (C_{20+}) produces chain-shortened fatty acids (approximately C_{10}) that are transported to mitochondria for further β-oxidation. The three enzymes involved in peroxisomal β-oxidation of long-chain fatty acids, namely, ACOX1, the bifunctional enzyme L-PBE/MFP1 (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase), and PTL (3-ketoacyl-CoA thiolase), are all inducible by PPARα. PPARα also induces mitochondrial carnitine palmitoyl transferase-1, which facilitates the entry of fatty acyl carnitine into mitochondria, and long-chain acyl-CoA synthetases, which initiate β-oxidation of fatty acids.

Fatty liver is the most common liver disease. It encompasses steatosis and steatohepatitis, diseases that can progress to cirrhosis and hepatocellular carcinoma. Fatty liver disease is divided into *alcoholic fatty liver disease* (due to excessive alcohol consumption) and

nonalcoholic fatty liver disease (due to obesity, which may occur with or without insulin resistance). During fasting PPARα knockout mice develop steatosis, hyperlipidemia, hypoketonemia, hypoglycemia, and hypothermia because they are unable to induce the peroxisomal, mitochondrial, and microsomal enzymes required to metabolize lipids released from adipose tissue. PPARα knockout mice also develop severe steatohepatitis if fed a choline- and methionine-deficient diet. Ethanol-fed PPARα knockout mice develop marked hepatomegaly, steatohepatitis, hepatocellular proliferation, and cell death. In wild-type mice and humans, ethanol impairs PPARα function, which is thought to contribute to the mechanism by which alcohol causes alcoholic fatty liver disease (Pyper et al., 2010).

In addition to playing a key role in protecting the liver against fatty liver disease, PPARα plays an important role in reducing inflammation. The proinflammatory eicosanoid LTB_4 is an activator of PPARα and induces its own metabolism by β- and ω-oxidation. PPARα ligands significantly reduce the levels of other proinflammatory cytokines/enzymes including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), COX-2, and inducible nitric oxide synthase (NOS-2) by inhibiting NF-κB and by decreasing phosphorylation of the c-Jun subunit of AP-1 (Pyper et al., 2010).

Paradoxically, in addition to inducing peroxisomal, mitochondrial, and microsomal enzymes involved in fatty acid catabolism, PPARα can upregulate the expression of hepatic lipogenic genes by activating SREBP-1 and liver X receptor α (LXRα) (Browning and Horton, 2004; Hebbachi et al., 2008). The situation is analogous to CAR that also upregulates lipid synthesis and catabolism (see the preceding section). The upregulation of lipogenic genes by PPARα is possibly a fail-safe mechanism to save fatty acids by incorporating them into triglycerides during starvation.

Species differences in PPARα and peroxisome proliferation are a complex issue. Activation of human PPARα in humans and other primates (and guinea pigs) is generally considered to cause little or no induction of microsomal CYP4A enzymes (Lake, 2009). There is in vitro evidence that CYP1A1, CYP2C8, and CYP3A4 are inducible by activators of human PPARα, the xenosensor (Seree et al., 2004; Prueksaritanont et al., 2005; Klein et al., 2012; Thomas et al., 2013, 2015; Makia and Goldstein, 2016). Whether fibrates and other drugs that activate PPARα cause clinically relevant induction of CYP3A4 (or CYP1A1 and CYP2C8) is not known. In rodents, CYP induction by fibrates is not exclusively mediated by PPARα. For example, the ability of clofibric acid to induce CYP2B enzymes in rats is due to activation of CAR, not PPARα (Ibrahim et al., 2014).

Activation of human PPARα in humans and other primates (and guinea pigs) generally causes minimal peroxisome proliferation, although peroxisome proliferation does occur in rhesus and cynomolgus monkeys treated with supertherapeutic doses of ciprofibrate (Lake, 2009). The lack of hepatomegaly and peroxisome proliferation in humans administered PPARα agonists is attributed to dose and the levels of hepatic PPARα, which are about an order of magnitude lower in humans compared with those in rodents. PPARα-humanized mice (i.e., mice harboring human PPARα instead of the mouse receptor) are resistant to peroxisome proliferation but the overexpression of human PPARα in mice restores peroxisome proliferation, the pleiotropic induction response, and cell proliferation (Pyper et al., 2010). These findings are pertinent to the risk of hepatocellular carcinogenesis posed by fibrate drugs and other PPARα agonists, which is discussed in the next section.

Enzyme Induction and Chemical Carcinogenesis In humans, induction of CYP2E1 and the associated increased activation of acetaminophen to *N*-acetylbenzo-*p*-quinoneimine (NAPQI) is one of the mechanisms by which alcohol consumption and fasting potentiate acetaminophen hepatotoxicity (other contributing mechanisms include the impaired conjugation of acetaminophen due to lowered levels of UDPGA and PAPS, and the impaired detoxication of NAPQI due to lowered levels of GSH). By analogy, induction of CYP enzymes would be expected to increase the activation of procarcinogens to DNA-reactive metabolites, leading to increased tumor formation. However, contrary to expectation, treatment of rodents with a CYP inducer prior to treatment with a known proximate carcinogen (tumor initiators such as aflatoxin, various nitrosamines, or PAHs) is generally associated with a decrease, not an increase, in tumor incidence (Parkinson and Hurwitz, 1991; Nebert and Dalton, 2006; Shimada, 2006; Ma and Lu, 2007; Nebert et al., 2013). The route of exposure to the carcinogen can affect the impact of enzyme induction; it protects against orally administered drugs but may increase the tumorigenicity of carcinogens applied directly to their site of action (Nebert et al., 2004). Although treatment of rats and mice with a CYP inducer *before* treatment with an initiator (a genotoxic carcinogen) generally decreases tumor incidence, treatment with a CYP inducer *after* the initiator (such as diethylnitrosamine) generally increases tumor incidence because CYP inducers generally function as tumor promoters in rodents.

Phenobarbital is representative of a large number of compounds that appear to promote liver tumor formation in mice and, to a lesser extent, in rats by a nongenotoxic mechanism, including phenytoin, carbamazepine, chlordecone, butylated hydroxytoluene, DDT, dieldrin, hexachlorocyclohexane, certain polychlorinated and polybrominated biphenyls, loratadine, doxylamine, lansoprazole, musk xylene, and fenbuconazole (Williams and Iatropoulos, 2002; Juberg et al., 2006; Elcombe et al., 2014; Lake et al., 2015). Although the exact mechanism of tumor formation remains unknown, it is critically dependent on CAR activation, as evidenced by the finding that phenobarbital and related compounds do not function as tumor promoters in CAR knockout mice (Gonzalez and Yu, 2006). Replicative DNA synthesis and possibly inhibition of apoptosis are mechanistically important. Phenobarbital does not function as a tumor promoter in Syrian hamsters (even following treatment with a genotoxic agent such as DMN, DEN, or methylazoxymethanol acetate). Phenobarbital is a CYP2B inducer in hamsters but it does not induce replicative DNA synthesis and does not inhibit apoptosis in hamster hepatocytes (either spontaneous or transforming growth factor-β1 [TGF-β1]–induced apoptosis) (Lake, 2009). In mice and rats phenobarbital and related compounds only cause liver tumors at doses that cause liver enlargement due to an increase in cell number (hepatocellular hyperplasia) and cell size (hepatocellular hypertrophy). The liver changes induced by phenobarbital, including an increase in DNA synthesis within 24 hours, a wave of mitotic activity after 1 to 3 days, enzyme induction (e.g., CYP2B enzymes), and a progressive increase in liver weight, all exhibit nonlinear dose–response relationships, suggesting that there is a threshold dose below which no activation of CAR and no liver tumor promotion occur.

Although induction of CYP2B enzymes (and other xenobiotic-biotransforming enzymes and transporters) is a common feature of phenobarbital and related tumor-promoting chemicals, the two events do not appear to be mechanistically linked (Williams and Iatropoulos, 2002). Activation of CAR is critical to liver tumor development in mice, although upregulation of xenobiotic-biotransforming enzymes is less important than other xenosensor-dependent events, such as, in the cases of the indirect (phenobarbital) or direct mouse CAR-activator (TCPOBOP), altered DNA methylation of numerous genes, upregulation of GADD45B (an antiapoptotic factor) and Mdm2 (a negative

regulator of the tumor suppressor p53), and the downregulation of gap junctional proteins (connexin 32), which diminishes cell–cell communication (Omiecinski et al., 2011b). Connexin 32 null mice are resistant to the tumor-promoting effects of phenobarbital, although it remains to be determined whether connexin 32 is a CAR-regulated signal molecule (Kobayashi et al., 2015). Although phenobarbital and other CAR activators induce CYP2B6 in human hepatocytes (both in vitro and in vivo), they neither cause replicative DNA synthesis nor inhibit spontaneous or TGF-β1-induced apoptosis (Lake, 2009).

Prolonged treatment of rodents with clofibrate, fenofibrate, ciprofibrate, bezafibrate, nafenopin, methylcofenapate, Wy-14643, DEHP, DEHA, cinnamyl anthranilate, or trichloroethylene is associated with hepatic peroxisome proliferation and liver tumor (adenoma and/or carcinoma) formation (Lake, 2009). PPARα plays a critical role in tumor formation by peroxisome proliferators as evidenced by the finding that Wy-14643 does not function as a tumor promoter in PPARα knockout mice (Gonzalez and Yu, 2006). Furthermore, there is an excellent agreement between the potency with which agonists activate PPARα and the potency (in terms of latency period and tumor incidence) with which they promote liver tumor formation in rodents (which in turn is correlated with their ability to induce the proliferation of peroxisomes, induce peroxisomal enzymes involved in the β-oxidation of fatty acids, and induce microsomal CYP4A enzymes). Like CAR agonists, PPARα agonists are considered nongenotoxic tumorigens because they are not mutagenic and they are not known to be converted to mutagenic metabolites. Another similarity with CAR agonists is that, in rodents, PPARα agonists cause replicative DNA synthesis in association with hepatomegaly (due to early hyperplastic and hypertrophic growth of hepatocytes) and they inhibit apoptosis. Just as it is sensitive to the enzyme-inducing/endoplasmic reticulum proliferative effects but not the tumor-promoting effects of CAR agonists, so the Syrian hamster is sensitive to the enzyme-inducing/peroxisome proliferating effects of PPARα agonists, but is resistant to their tumor-promoting effects (Lake, 2009). PPARα agonists cause replicative DNA synthesis and inhibit apoptosis in rat and mouse hepatocytes, but not in hepatocytes from hamsters, guinea pigs, monkeys, and humans (Lake, 2009). Treatment of mice with Wy-14643 represses the expression of let-7C microRNA, which results in increased expression (derepression) of c-myc oncogene expression (Shah et al., 2007). Interestingly, suppression of let-7C miRNA does not occur in PPARα-humanized mice treated with Wy-14643 (Yang et al., 2008).

Although PPARα agonists are not genotoxic, the dramatic (more than 20-fold) induction of peroxisomal ACOX1 by Wy-14643 and other peroxisome proliferators (dramatic when compared with the modest [two- to fivefold] induction of peroxisomal catalase) leads to increased production of hydrogen peroxide, which can cause oxidative stress and DNA damage that possibly play a role in some of the downstream events leading to tumor promotion (Pyper et al., 2010). Oxidative DNA damage is evident from the fact that treatment of rodents with PPARα agonists leads to the induction of long-patch base excision DNA repair, the principal pathway for removing oxidized DNA bases (Rusyn et al., 2004).

Activation of PPARα is required but apparently not sufficient to lead to liver tumor formation. Other key events include increased oxidant production (such as increased peroxisomal H_2O_2 production), increased cell proliferation (which fixes mutations), and suppression of apoptosis (which prevents the removal of genetically damaged cells and permits clonal expansion) (Klaunig et al., 2003; Lai, 2004; Bosgra et al., 2005). Kupffer cells appear to play an important role in liver tumor formation by releasing mitogenic cytokines and possibly by contributing to oxidative stress by releasing superoxide anion, although it is not clear how PPARα agonists trigger these events. Inhibition of gap junctional intercellular communication plays an important role in liver tumor formation by PPARα agonists, just as it does in the promotion of liver tumors by phenobarbital-type inducers.

Epidemiological studies designed to assess the human cancer risk posed by PPARα agonists have produced inconclusive results. Clofibrate has been reported to cause a statistically significant increase in cancer mortality rate during a 5- to 8-year treatment period. However, there was no significant increase in cancer rate in a follow-up study, which included a posttreatment period (Lai, 2004). Three compelling lines of evidence suggest that PPARα agonists will not cause liver tumors in humans. First, PPARα agonists do not cause peroxisome proliferation and microsomal CYP4A induction in human hepatocytes in vitro or in primates in vivo at therapeutic doses. Second, PPARα agonists do not cause replicative DNA synthesis or inhibit apoptosis in human hepatocytes (or in hepatocytes from hamsters and guinea pigs, two species that are resistant to the hepatocarcinogenic effects of PPARα agonists). Third, treatment of PPARα-humanized mice (transgenic mice with human PPARα in place of mouse PPARα) with Wy-14643 or fenofibrate neither results in the hepatocellular proliferation characteristic of wild type nor results in the repression of let-7C miRNA and the upregulation of the c-myc oncogene (Gonzalez and Yu, 2006; Yang et al., 2008; Lake, 2009; Pyper et al., 2010).

The mechanism of thyroid tumor formation is better understood. In rodents, CAR activators (as well as PXR and PPARα agonists) induce UGT and SULT enzymes and transporters that accelerate the conjugation and elimination of thyroid hormones, which triggers a compensatory increase in thyroid stimulating hormone (TSH). T4 is a substrate for UGT1A enzymes, whereas T3 is a substrate for UGT2B2 and an as yet unidentified UGT enzyme (van Raaij et al., 1993; Visser et al., 1993; Richardson and Klaassen, 2010). Induction of T3 glucuronidation, rather than induction of T4 glucuronidation, is strongly associated with a compensatory increase in TSH and thyroid follicular hyperplasia. The PXR agonist PCN induces T3 glucuronidation in both Sprague–Dawley rats and UGT2B2-deficient Fischer 344 rats, which establishes that at least two PCN-inducible UGTs are involved in T3 glucuronidation (Richardson and Klaassen, 2010).

Sustained stimulation of the thyroid gland by TSH leads to the development of thyroid follicular tumors, the development of which can be blocked by administering T4, which blocks the release of TSH and thereby abrogates the hormonal stimulation of the thyroid gland. It is not clear why sustained stimulation of the thyroid gland by TSH results in tumor formation in rodents, but such tumors develop in other rodent organs following prolonged hormonal stimulation: LH causes Leydig cell tumors, gastrin causes stomach tumors, and corticosteroids cause pancreatic tumors. In addition, just as proliferation of the hepatic endoplasmic reticulum and peroxisomes by phenobarbital and Wy-14643 is associated with liver tumor formation, so the proliferation of hepatic lysosomes by Ponceau S and the proliferation of mitochondria by methapyrilene are also associated with liver tumor formation (Grasso et al., 1991).

Epidemiological studies of epileptic patients treated for more than 35 years with phenobarbital or phenytoin have established that chronic liver microsomal enzyme induction in humans does not increase the incidence of thyroid tumor formation (Singh et al., 2005). Prolonged elevation of TSH in humans does not lead to tumor formation but causes goiter, a reversible enlargement of the thyroid gland associated with iodide deficiency and treatment with

drugs that block thyroid hormone synthesis. In humans and monkeys, circulating T4 is largely bound to thyroxine-binding globulin (TBG). This high-affinity binding protein is not present in rodents, for which reason T4 is rapidly conjugated and excreted in bile in rodents. Accordingly, the plasma half-life of T4 in rats (12 to 24 hours) is considerably shorter than in humans (5 to 9 days). Similar differences are observed for T3. To compensate for the increased turnover of thyroid hormones, the rat pituitary secretes more TSH. While the baseline plasma TSH concentration in humans is about 2.2 μU/mL, TSH concentrations in rats range from 55.5 to 65 μU/mL in males and 36.5 to 41 μU/mL in females (Hill et al., 1989). It is estimated that rats require a 10-fold higher rate of T4 production (on a per kilogram body weight basis) than do humans to maintain physiological T4 and T3 concentrations. These differences in plasma half-life, protein binding, thyroid hormone metabolism, and TSH secretion between rats and humans are thought to be reasons for the greater sensitivity of rats to developing hyperplastic and/or neoplastic nodules in response to chronic TSH stimulation (Capen, 1997).

Enzyme Induction and Porphyria Due to the increased demand for heme, persistent induction of CYP can lead to porphyria, a disorder characterized by excessive accumulation of intermediates in the heme biosynthetic pathway. In 1956, widespread consumption of wheat contaminated with the fungicide hexachlorobenzene caused an epidemic of porphyria cutanea tarda in Turkey. Another outbreak occurred in 1964 among workers at a factory in the United States manufacturing 2,4,5-trichlorophenoxyacetic acid (the active ingredient in several herbicides and in the defoliant, Agent Orange). The outbreak of porphyria cutanea tarda was caused not by the herbicide itself but by a contaminant, namely, dioxin. Drugs that cause P450 induction have not been shown to cause porphyria cutanea tarda under normal circumstances, but phenobarbital, phenytoin, and alcohol are recognized as *precipitating factors* because they cause episodes of porphyria in individuals with an inherited deficiency in the heme-biosynthetic enzyme, uroporphyrinogen decarboxylase. Studies conducted in mice suggest that the use of flurane-based anesthetics, such as enflurane and isoflurane, which affect heme metabolism, should be avoided in porphyria patients (Sampayo et al., 2009). Due to the increased demand for heme, induction of CYP enzymes in vivo or in vitro (in hepatocytes) is associated with induction of δ-aminolevulinic acid synthase (ALA synthase), the enzyme that catalyzes the first and rate-limiting step in heme synthesis.

Enzyme Suppression The suppression (downregulation) of drug-metabolizing enzymes and transporters is an adaptive process that reduces xenobiotic elimination during periods of inflammation, infection, and postvaccination or in response to certain therapeutic proteins. The phenomenon may be associated with pharmacological and toxicological consequences, especially in the case of drugs with a narrow therapeutic index, and it can be a manifestation of a drug–disease interaction. Suppression of xenobiotic-biotransforming enzymes, a consequence of influenza infection, resulted in severe toxicity of theophylline, a CYP1A2 substrate with a narrow therapeutic index. A number of children infected with the influenza virus and receiving theophylline, a prophylactic asthma treatment, experienced convulsions, and cardiac conduction anomalies associated with elevated plasma levels of the drug (which increased up to fivefold in association with a prolongation of plasma half-life from 4 to up to 20 hours) (Kraemer et al., 1982). Suppression of xenobiotic-biotransforming enzymes, as a result of vaccination or treatment with certain therapeutic protein, may cause drug–drug interactions involving a small drug molecule as victim and the biological drug as perpetrator. Blood trough levels of tacrolimus, a narrow therapeutic index immunosuppressant and a substrate of CYP3A4/5, increased by 63% in patients treated with basiliximab, a chimeric monoclonal antibody to the interleukin-2 (IL-2) receptor (Sifontis et al., 2002). Similar effects of basiliximab on the calcineurin inhibitor cyclosporine A were observed in children following kidney transplantation. Within the first 10 days following transplantation, substantially less cyclosporine was required in the basiliximab treatment group than in controls, but it resulted in higher trough concentrations and was associated with liver and kidney toxicity (Strehlau et al., 2000). The proposed mechanism of these interactions is an IL-2-mediated suppression of CYP3A4. This mechanism is in agreement with CYP suppression initiated by proinflammatory cytokines, such as IL-1β, IL-6, interferon-γ (INF-γ), or TNF-α, released in response to infection or inflammation. These proinflammatory cytokines, in turn, modulate expression and activity of transcription factors and specific nuclear receptors determining the levels of CYP mRNA (Morgan, 2009). In the liver proinflammatory cytokines can induce NOS-2 and the increased production of NO can directly inhibit CYP enzymes. Accordingly infection and inflammation can both suppress and inhibit CYP enzymes (Morgan et al., 2008; Zhou and Mascelli, 2011). Therapeutic proteins that are cytokines or cytokine modulators deserve special scrutiny during the drug development process because they can suppress CYP enzymes without stimulating the release of endogenous cytokines. All the major drug-metabolizing CYP enzymes, including the noninducible CYP2D6, are subject of suppression by cytokines (IL-1, IL-2, IL-6, IL-10, IFN-α, IFNα-2b, IFN-β, and TNF-α) or cytokine modulators (tocilizumab, basiliximab, and muromonab-CD3) (Huang et al., 2010). By reversing the disease process—such as lessening the inflammation associated with rheumatoid arthritis—some biologics, for example, tocilizumab (a humanized monoclonal antibody against the interleukin-6 receptor), can reverse the suppression of drug-metabolizing enzymes and restore their activity to normal levels (Morgan, 2009; Schmitt et al., 2011).

CONJUGATION

Conjugation reactions include glucuronidation, sulfonation (often called sulfation), acetylation, methylation, conjugation with glutathione (GSH; mercapturic acid synthesis), and conjugation with coenzyme A (CoA) followed by conjugation with amino acids such as glycine, taurine, and glutamine. The cofactors (or cosubstrates) for these reactions, which are shown in Fig. 6-57, react with functional groups that are either present on the xenobiotic or introduced/exposed during oxidation, reduction, or hydrolysis. With the exception of methylation and acetylation, conjugation reactions result in a large increase in xenobiotic hydrophilicity and total polar surface area (TPSA), so they greatly promote the excretion of foreign chemicals (see Table 6-3 and discussion in the section "Introduction'). Because of their high water solubility, most xenobiotic conjugates cannot readily diffuse across the plasma membrane; hence, their exit from hepatocytes, for example, generally involves transport across the canalicular membrane into bile (for elimination in feces) or the sinusoidal membrane into blood (for elimination in urine) (see the section "Introduction" and Point 6 in the section "Principles of Xenobiotic Biotransformation"). Glucuronidation, sulfonation, acetylation, and methylation involve reactions with activated or "high-energy" cofactors, whereas conjugation with amino acids or GSH involves reactions with activated xenobiotics. Most conjugation enzymes are located in the cytosol; a notable exception is the UDP-glucuronosyltransferases (UGTs), which are microsomal enzymes (Table 6-4).

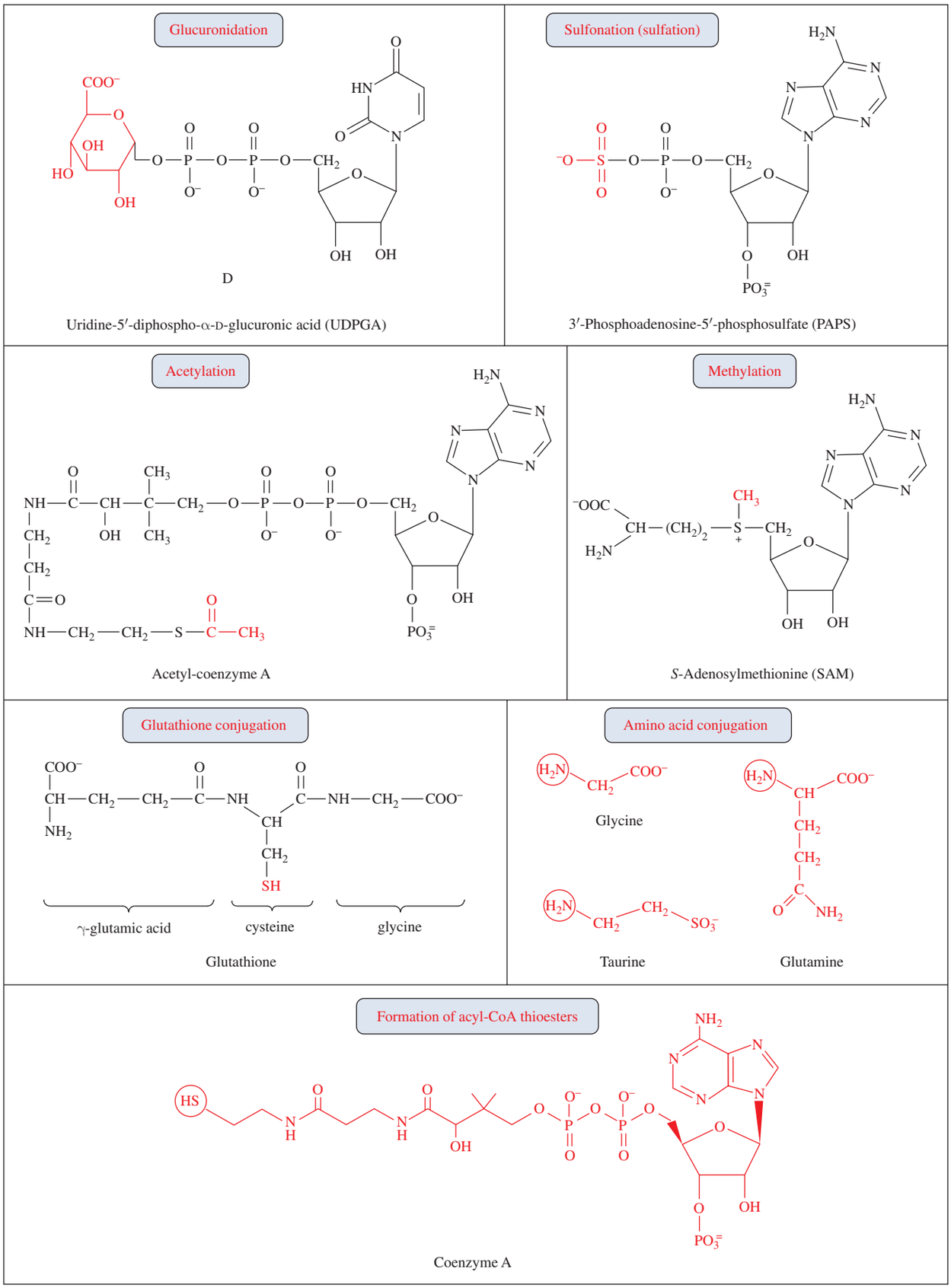


Figure 6-57. *Structures of cofactors for conjugation reactions. The functional group that reacts with or is transferred to the xenobiotic is shown in red.*

Glucuronidation and Formation of Acyl-CoA Thioesters

Glucuronidation is a major pathway of xenobiotic biotransformation in mammalian species except for members of the cat family (lions, lynxes, civets, and domestic cats) (Tukey and Strassburg, 2000; Court, 2013). It requires primarily the cofactor UDP-glucuronic acid (UDPGA), but can also use UDP-glucose, UDP-xylose, and UDP-galactose. The reaction is catalyzed by UGTs, which are located predominantly in the endoplasmic reticulum of the liver and other tissues, such as the kidney, gastrointestinal tract, lungs, prostate, mammary glands, skin, brain, spleen, and nasal mucosa (Fig. 6-58). Examples of xenobiotics that are glucuronidated are shown in Fig. 6-59. The site of glucuronidation is generally an electron-rich (nucleophilic) O, N, or S heteroatom. Glucuronidation reactions mediated by UGTs are S_N2 substitution reactions where the nucleophilic heteroatom of the substrate attacks the C_1 atom of UDPGA, which results in a xenobiotic glucuronide in the β-configuration (Yin et al., 1994). Therefore, substrates for glucuronidation are typically small lipophilic compounds that contain functional groups such as aliphatic alcohols and phenols (which form *O*-glucuronide acetals), carboxylic acids (which form acyl glucuronides), primary and secondary aromatic and aliphatic amines (which form *N*-glucuronides), and free sulfhydryl groups (which form *S*-glucuronides). In humans and monkeys (but in few other species), numerous tertiary amines (including tripelennamine, cyclobenzaprine, and imipramine) are substrates for *N*-glucuronidation, which leads to the formation of positively charged quaternary ammonium glucuronides, some of which may be carcinogenic (see below in this section) (Hawes, 1998). Certain xenobiotics, such as phenylbutazone, sulfinpyrazone, suxibuzone, ethchlorvynol, Δ^6-tetrahydrocannabinol, and feprazone, contain carbon atoms that are sufficiently nucleophilic to form *C*-glucuronides. Coumarin and certain other carbonyl-containing compounds are glucuronidated to form arylenol-glucuronides.

In addition to the typical conjugation reactions above, UGTs can form unusual conjugates: (1) bisglucuronides (e.g., bilirubin and morphine), where two different functional groups on the same molecule are glucuronidated; (2) diglucuronides (e.g., 5α-dihydroxytestosterone; DHT), where two glucuronides are attached in tandem to a single site; (3) *N*-carbamoyl glucuronides (e.g., sertraline and varenicline), where carbonate incorporated in the glucuronide; and (4) glycosidation with UDP-sugars other than UDPGA (e.g., glucosidation of barbiturates). Two examples are shown in Fig. 6-60. These reactions will be covered in greater detail later in this section. In addition to numerous xenobiotics, substrates for glucuronidation include several endogenous compounds, such as bilirubin, steroid hormones, and thyroid hormones. Table 6-22 provides examples of marker substrates for the major human UGTs. Several review articles provide extensive descriptions of UGT substrates, K_m and V_{max} values, and specificity of substrates and inhibitors for individual UGT enzymes (Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000; Kiang et al., 2005; Miners et al., 2010; Rowland et al., 2013; Oda et al., 2015).

The cofactor for glucuronidation, UDPGA, is synthesized from glucose-1-phosphate, and the linkage between glucuronic acid and UDP has an α-configuration, as shown in Fig. 6-58. This configuration protects the cofactor from hydrolysis by β-glucuronidase.

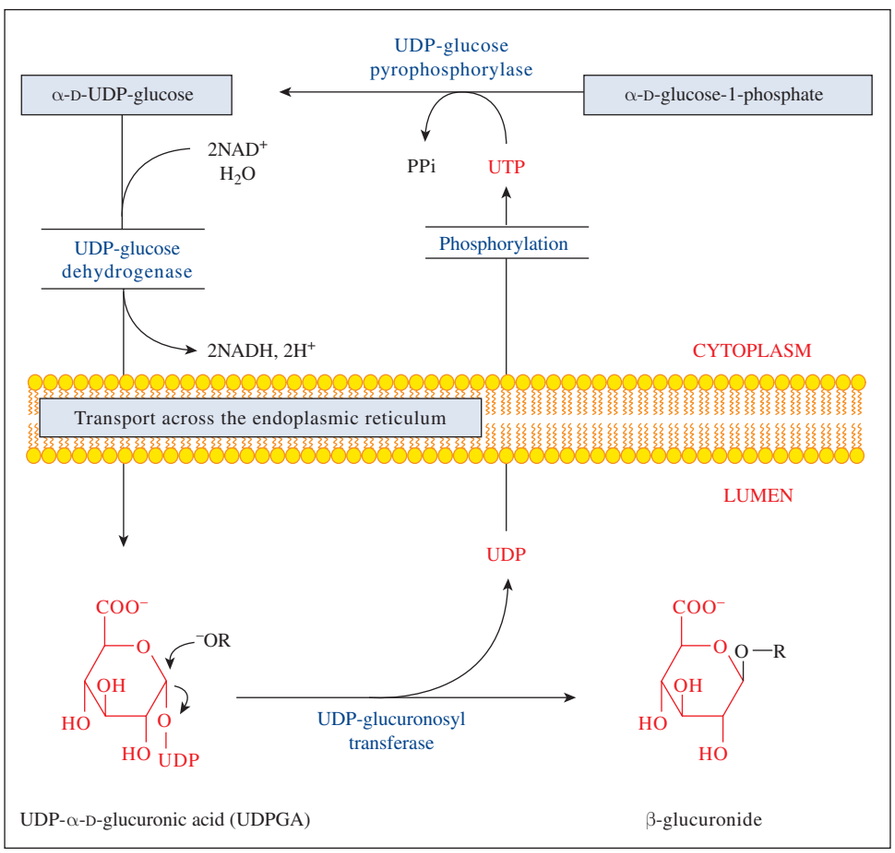


Figure 6-58. *Synthesis of UDP-glucuronic acid and inversion of configuration (α → β) during glucuronidation of a phenolic xenobiotic (designated RO—).*

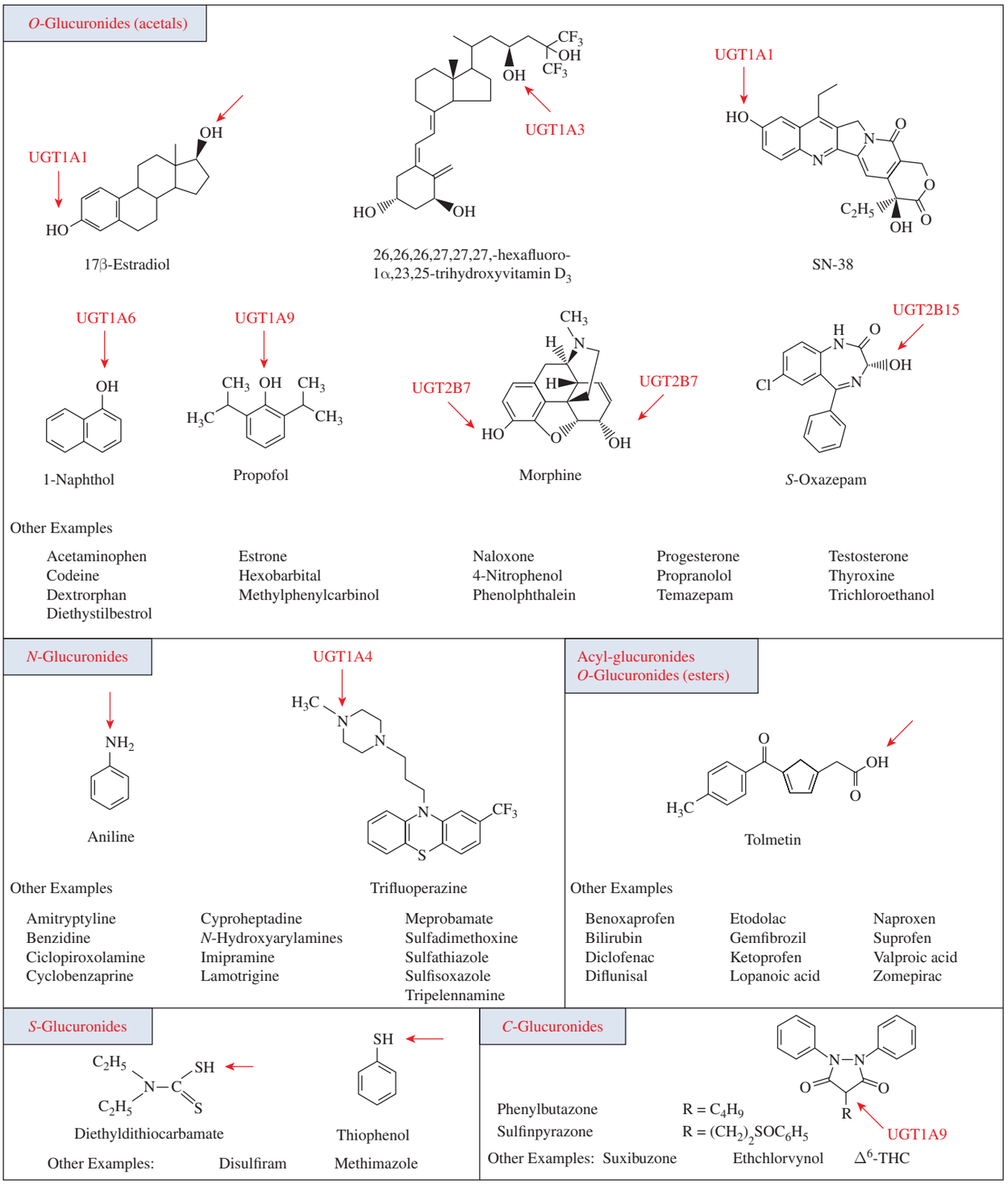


Figure 6-59. *Examples of xenobiotics and endogenous substrates that are glucuronidated.* The arrow indicates the site of glucuronidation, with the UGT enzyme if selective.

However, glucuronides of xenobiotics have a β-configuration. This inversion of configuration occurs because glucuronides are formed by nucleophilic attack by an electron-rich atom (usually O, N, or S) on UDPGA, and this attack occurs on the opposite side of the linkage between glucuronic acid and UDP, as shown in Fig. 6-58. In contrast to the UDPGA cofactor, xenobiotics conjugated with glucuronic acid are substrates for β-glucuronidase. Although present in the lysosomes of some mammalian tissues, considerable

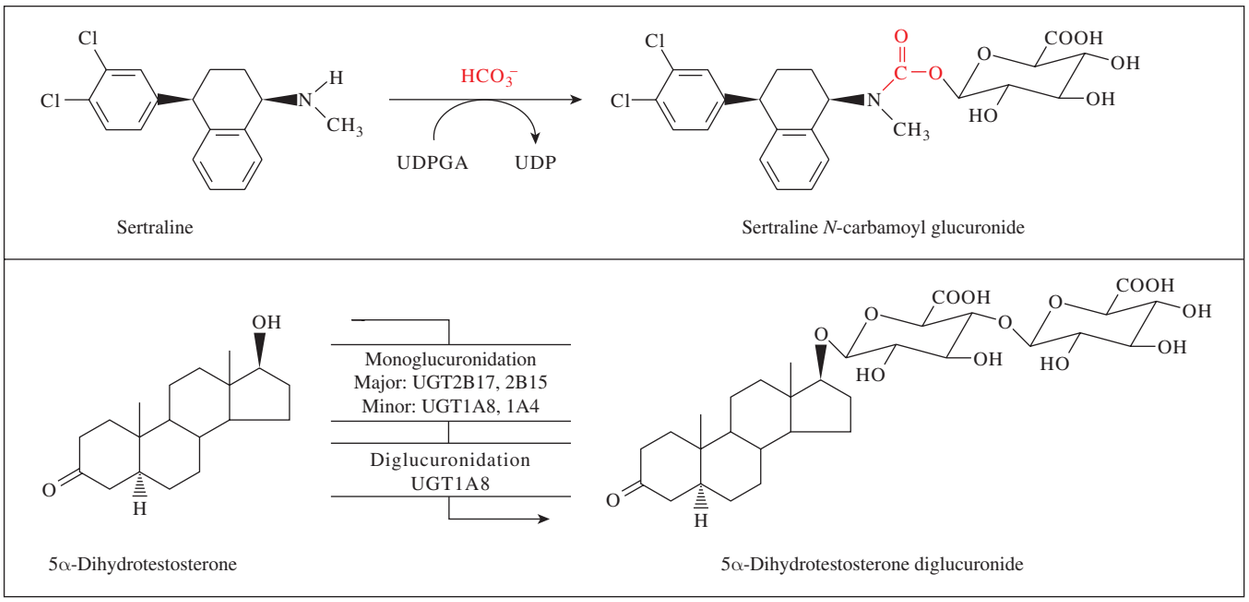


Figure 6-60. *Examples of unusual glucuronide conjugates.*

Table 6-22

Major Human UDP-Glucuronosyltransferase Enzymes

UGT	TISSUE	SPECIFIC CONTENT IN HUMAN MICROSOMES*	SELECTIVE SUBSTRATES (BOLD), OTHER SUBSTRATES, AND SELECTIVE INHIBITORS (ITALICS)
1A1	Liver, small intestine, colon	Liver: 45 pmol/mg Intestine: 23 pmol/mg	**Bilirubin, 17β-estradiol (3-glucuronidation), etoposide, ezetimibe**, acetaminophen, belinostat, *R*-carvedilol, efavirenz, ethinyl estradiol, ezetimibe, levothyroxine, raloxifene, SN-38 (active metabolite of irinotecan), telmisartan, tranilast. *Atazanavir, erlotinib*
1A3	Liver, small intestine, colon	Liver: 32 pmol/mg Intestine: 1.9 pmol/mg	**Hexafluoro-1α,23(s),25-trihydroxyvitamin D_3, vitamin D_3, R-lorazepam**, beviramat, bile acids (chenodeoxycholic acid, lithocholic acid, hyodeoxycholic acid), 17β-estradiol, ezetimibe, ibuprofen, fenoprofen, ketoprofen, ketotifen, naproxen, norbuprenorphine, telmisartan, valproic acid, zolosartan
1A4	Liver, small intestine, colon	Liver: 55 pmol/mg Intestine: 1.6 pmol/mg	**Trifluoperazine, 1′-hydroxymidazolam**, afloqualone, amitriptyline, benzidine, clozapine, *S*-cotinine, cyclobenzaprine, diphenhydramine, imipramine, ketoconazole, ketotifen, lamotrigine, levomedetomidine, midazolam, *S*-nicotine, olanzapine, tamoxifen. *Hecogenin*
1A5	Liver	Liver: 0.1 pmol/mg Intestine: Not determined	Unknown
1A6	Liver, small intestine, colon, stomach	Liver: 43 pmol/mg Intestine: 2.3 pmol/mg Kidney: 14 pmol/mg	**1-Naphthol, serotonin, deferiprone,** acetaminophen, deferiprone, diclofenac, 4-methylumbelliferone, ibuprofen, methotrexate, 4-nitrophenol, SN-38 (active metabolite of irinotecan)
1A7	Esophagus, stomach, lung	Liver: <0.2 pmol/mg Intestine: 8.4 pmol/mg	4-Hydroxybiphenyl, 4-hydroxyestrone, hydroxybenzo[*a*]pyrene, mycophenolic acid, octylgallate, SN-38 (active metabolite of irinotecan)
1A8	Colon, small intestine, kidney	Liver: <0.2 pmol/mg Intestine: 6.1 pmol/mg	Anthraquinone, ciprofibric acid, clofibric acid, diflunisal, catechol estrogens, entacapone, furosemide, 8-hydroxyquinoline, mycophenolic acid, niflumic acid, raloxifene, troglitazone, valproic acid
1A9	Liver, colon, kidney	Liver: 32 pmol/mg Intestine: 6.6 pmol/mg Kidney: 171 pmol/mg	**Propofol, mycophenolic acid, phenylbutazone, sulfinpyrazone**, acetaminophen, bumetanide, diclofenac, diflunisal, edaravone, entacapone, fenofibrate, fenoprofen, furosemide, ibuprofen, indomethacin, ketoprofen, mycophenolic acid, naproxen, *R*-oxazepam, propofol, salicylic acid, scopoletin, sorafenib. Thyroid hormones. *Niflumic acid*

Table 6-22
(Continued)

UGT	TISSUE	SPECIFIC CONTENT IN HUMAN MICROSOMES*	SELECTIVE SUBSTRATES (BOLD), OTHER SUBSTRATES, AND SELECTIVE INHIBITORS (ITALICS)
1A10	Stomach, small intestine, colon	Liver: <0.1 pmol/mg Intestine: 4.7 pmol/mg	**Dopamine**, furosemide, mycophenolic acid, 1-naphthol, raloxifene, troglitazone
2A1	Olfactory	Not determined	Ibuprofen, valproic acid
2A2	Unknown	Not determined	Unknown
2A3	Liver, small intestine, colon, adipose tissue	Not determined	Bile acids
2B4	Liver, small intestine	Not determined	Androsterone, bile acids such as hyodeoxycholic acid, codeine, carvedilol, estriol
2B7	Kidney, small intestine, colon	Liver: 98 pmol/mg Intestine: 16 pmol/mg Kidney: 95 pmol/mg	**Zidovudine (AZT), morphine (6-glucuronidation†), carbamazepine**, benoxaprofen, bile acids (hyocholic acid, hyodeoxycholic acid), buprenorphine, *S*-carvedilol, clofibric acid, codeine, diclofenac, denopamine, epirubicin, efavirenz, flurbiprofen, gemfibrozil, hydroxyprogesterone (6α- and 21-), ibuprofen, ketoprofen, naproxen, naloxone, *R*-oxazepam, RO5263397, tiaprofenic, valproic acid, zomepirac. *Fluconazole*
2B10	Liver, ileum, prostate	Liver: 20 pmol/mg Intestine: <0.1 pmol/mg	**S-Nicotine, cotinine, levomedetomidine**, amitriptyline, clomipramine, desloratadine, diphenhydramine, imipramine, trimipramine. *S-Nicotine, desloratadine*
2B11	Mammary, prostate, others	Not determined	2-Aminophenol, 3α-androgens and pregnanes, 4-hydroxybiphenyl, 4-nitrophenol, 1-naphthol, estriol and hydroxylated estrogens
2B15	Liver, small intestine, prostate	Liver: 52 pmol/mg Intestine: <0.1 pmol/mg	**S-Oxazepam, S-lorazepam**, androgens, diclofenac, entacapone, estriol, flavonoids, 4-hydroxytamoxifen, sipoglitazar, SN-38 (active metabolite of irinotecan), tolcapone, vorinostat
2B17	Liver, prostate	Liver: 18 pmol/mg Intestine: 112 pmol/mg	Androgens, eugenol, galangin, ibuprofen, MK-7246, scopoletin, vorinostat.
2B28	Liver, mammary	Not determined	17β-Estradiol, testosterone

**Specific content of UGT enzymes (pmol/mg protein) was determined in up to 126 samples of microsomes prepared from human liver, intestine, or microsomes by LC-MS/MS (Oda et al., 2015).*
†UGT2B7 also catalyzes the 3-glucuronidation of morphine, but this reaction is also catalyzed by other UGTs.
Note: Bold text represents selective substrates (or reactions) for hepatic UGTs. These compounds may be glucuronidated in extrahepatic tissues by other UGT enzymes. Italic text represents selective inhibitors.
SOURCES: Data from Fisher et al. (2000), Williams et al. (2004), Kiang et al. (2005), Miners et al. (2006), Court et al. (2008), Miners et al. (2010), Kaivosaari et al. (2011), Ohtsuki et al. (2012), and Oda et al. (2015).

β-glucuronidase activity is present in the intestinal microbiota. The intestinal enzyme can release the aglycone, which can be reabsorbed and thereby enter a cycle called *enterohepatic circulation*, which delays the elimination of xenobiotics. In general, *N*-glucuronides are hydrolyzed relatively slowly by β-glucuronidase (with some *N*-glucuronides being resistant to hydrolysis by β-glucuronidase) but some *N*-glucuronides undergo hydrolysis in acidic conditions (hence, some *N*-glucuronides are hydrolyzed in urine, which can give the impression that the parent compound was eliminated in urine unchanged). In contrast, acyl glucuronides are stable under acidic conditions; unless the samples are acidified (to pH ~5), acyl glucuronides in plasma and other biological matrices can isomerize or hydrolyze to a significant extent. The instability of *N*-glucuronides under acidic conditions has implications for the tumorigenicity of *N*-glucuronides of compounds such as benzidine (discussed later in this section).

Glucuronide conjugates of xenobiotics and endogenous compounds are polar (typically anionic) with a pK_a(A) of 3 to 3.5, water-soluble metabolites that are eliminated from the body in urine or bile. Whether glucuronides are excreted from the body in bile (following their canalicular transport by MRP2) or urine (following their sinusoidal transport into blood by MRP3) depends, in part, on the size of the aglycone (i.e., the parent compound or its unconjugated metabolite). In rat, glucuronides are preferentially excreted in urine if the molecular weight of the aglycone is less than 250, whereas glucuronides of larger molecules (aglycones with molecular weight greater than 350) are preferentially excreted in bile. Molecular weight cutoffs for the preferred route of excretion vary among mammalian species. Yang et al. (2009) conducted a statistical analysis of the molecular weight cutoff for biliary excretion of anionic, cationic, zwitterionic, and neutral compounds and concluded that, for anionic compounds, the molecular weight cutoff was approximately 400 and 475 Da in rats and humans, respectively. The carboxylic acid moiety of glucuronic acid (pK_a(A) 3–3.5), which is ionized at physiological pH, promotes excretion because (1) it increases the aqueous solubility and TPSA of the xenobiotic and (2) it is recognized by the biliary and renal organic anion transport systems, which enable glucuronides to be actively

secreted into urine and bile. Furthermore, as anions, glucuronides generally bind extensively to albumin, which, in addition to their large TPSA, confines glucuronides to plasma (although some glucuronides are actively taken up by the liver, as described below).

As discussed above, glucuronide metabolites are often substrates of transmembrane transporters that mediate the vectoral transport of conjugates into systemic circulation or into bile for fecal excretion or enterohepatic circulation. These transport proteins efflux the glucuronide either across the canalicular membrane for biliary excretion or across the sinusoidal membrane into the blood for excretion in urine. Transport of glucuronides across the sinusoidal membrane into the blood is mediated largely by MRP3 and, to a lesser extent, by MRP1 and MRP4. The transport of glucuronides across the canalicular membrane into bile is largely mediated by MRP2 and, to a lesser extent, by BCRP. Glucuronides can also be taken up by hepatocytes through the action of OATP1B1 and OATP1B3 on the sinusoidal membrane (Giacomini and Sugiyama, 2006; Giacomini et al., 2010).

Bilirubin monoglucuronide and diglucuronide, certain steroid conjugates (such as estradiol-17β-glucuronide), and certain bile acids are also substrates for MRP2 and MRP3, although bile acids are mainly effluxed into bile by the bile salt export pump (BSEP). The important role of MRP2 in transporting conjugated bilirubin into bile is evident from the observation that rats deficient in MRP2 (such as TR$^-$/GY and EHBR rats) have high plasma levels of monoconjugated and diconjugated bilirubin. In humans, a deficiency of MRP2 also leads to conjugated hyperbilirubinemia, and this inherited disease is called Dubin–Johnson syndrome. When MRP2 function is impaired (due to a genetic deficiency or due to drug-induced cholestasis or bile duct ligation), MRP3 levels increase. In this way, when the biliary efflux of xenobiotics and endobiotics by MRP2 is impaired, their efflux into blood is increased (as if the back door is opened wider when the front door is closed). The upregulation of MRP3 in response to a loss of MRP2 augments the basolateral efflux of drug, steroid and bilirubin conjugates, and certain bile acids during cholestasis. MRP2 can be highly effective at transporting glucuronides and anionic compounds into bile as evidenced by bile-to-blood concentration ratios greater than 1 (and commonly between 10 and 1000) (Brauer, 1959). However, the elimination of drug conjugates in bile may not result in the elimination of the drug in feces because the conjugates may be hydrolyzed in the gut by bacterial β-glucuronidase and the parent drug may be reabsorbed or eliminated in feces. For orally administered drugs, the former process (biliary excretion of conjugates, hydrolysis in the gut, and reabsorption of the parent drug) gives rise to the phenomenon of enterohepatic circulation, whereas the latter process (biliary excretion of conjugates, hydrolysis in the gut, and elimination of the parent drug in feces) can give the impression of incomplete intestinal absorption.

The mammalian UGT gene superfamily contains four families, UGT1, UGT2, UGT3, and UGT8. The major UGTs involved in xenobiotic conjugation are 9 members of the UGT1 family and 10 members of the UGT2 family (3 and 7 in the UGT2A and UGT2B subfamilies, respectively). Families 1 and 2 preferentially utilize UDPGA as the cofactor, but may use other UDP-sugars such as UDP-glucose and UDP-xylose (Mackenzie et al., 2005). UGT3A1 and UGT3A2 were identified in the human genome, and are about 30% similar to UGT1 and UGT2 sequences but prefer *N*-acetylglucosamine as a cofactor (Mackenzie et al., 2005; Meech and Mackenzie, 2010). UGT8A1 utilizes UDP-galactose and is involved in the synthesis of membrane components, which is beyond the scope of this chapter. The total number of functional human UGT enzymes is 22.

The major xenobiotic-metabolizing human UGT enzymes are products of either a single UGT1A gene locus (see Fig. 6-61) or multiple individual UGT2 genes (with the exception of UGT2A1 and 2A2, see below). The human UGT1A locus encodes 13 potential UGT enzymes (namely, UGT1A1, 1A2, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 1A11, 1A12, and 1A13), 9 of which are expressed in vivo; the 4 transcripts that are not expressed are UGT1A2, 1A11, 1A12, and 1A13, which are pseudogenes because they lack an open reading frame (Mackenzie et al., 2005). Each of the UGT1A enzymes is encoded by five exons, four of which (exons 2 to 5) are the same and encode the cofactor-binding site. Because of the cassette nature of the UGT1A locus, the individual UGT1A enzymes differ in only the first exon, which encodes the substrate-binding site. Because they share four out of five exons, all the UGT1A enzymes share extensive amino acid sequence identity.

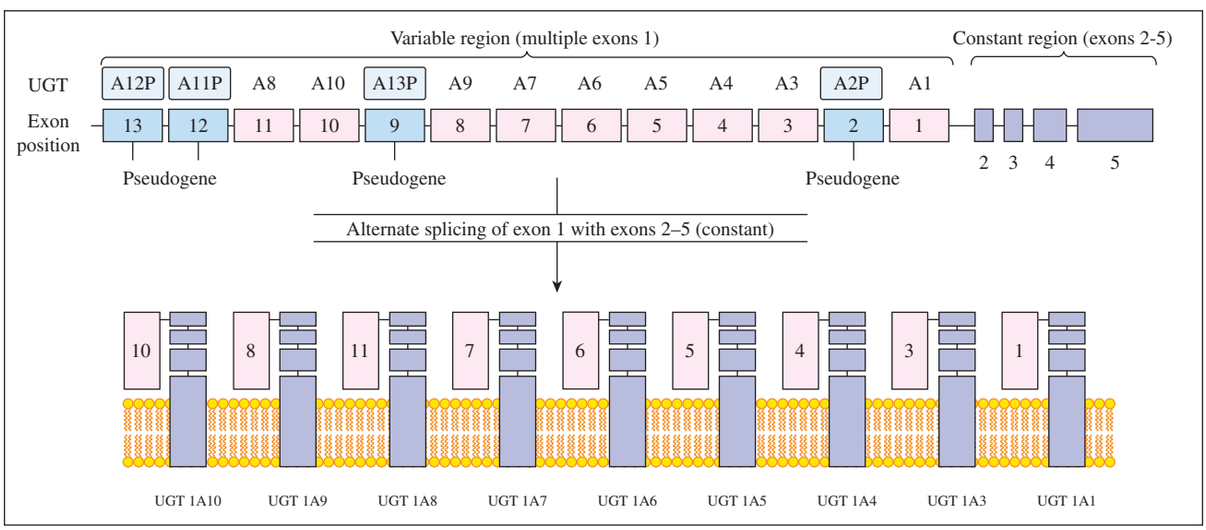


Figure 6-61. *Structure of the human UGT1 locus that encodes multiple forms of UDP-glucuronosyltransferase.* Note that these microsomal enzymes face the lumen of the endoplasmic reticulum.

The UGT2 genes expressed in humans are UGT2A1, 2A2, 2A3, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28. Human UGT2B24 to UGT2B27 and UGT2B29 are pseudogenes. The UGT2 genes are made up of six exons each, which are not shared between subfamily members, with the exception of UGT2A1 and UGT2A2, and these two genes are more than 70% similar (Mackenzie et al., 2005). UGT2A1 and UGT2A2 share five exons with a variable first exon, similar to the UGT1A enzymes. The human UGT3 family contains two members UGT3A1 and UGT3A2. UGT3A1 mRNA is expressed in the liver and, to a lesser extent, in the kidney, stomach, duodenum, colon, and testes. UGT3A2 mRNA is expressed at high levels in kidney and testes. UGT3A1 utilizes *N*-acetylglucosamine as a sugar donor and is implicated in the formation of *N*-acetylglucosamide conjugates of various steroidal substrates, such as steroid hormones and bile acids (Meech and Mackenzie, 2010).

The C-terminus of all UGTs contains a membrane-spanning domain that anchors the enzyme in the endoplasmic reticulum. The enzyme faces the lumen of the endoplasmic reticulum, which places UGTs on the opposite side of the endoplasmic reticulum from CYP. As outlined in the section "Introduction" and in Table 6-3, biotransformation of xenobiotics by CYP generally results in a modest increase in TPSA and hydrophilicity; hence, metabolites formed by CYP enzymes can generally diffuse through the lipid bilayer of the endoplasmic reticulum to access UGTs on the luminal side of the membrane. The luminal orientation of UGT poses a problem because UDPGA is a water-soluble cofactor synthesized in the cytoplasm. One or more transporters are suspected of shuttling UDPGA from the cytoplasm to the lumen of the endoplasmic reticulum, and they may also shuttle UDP (the by-product of glucuronidation) back into the cytoplasm for synthesis of UDPGA, as shown in Fig. 6-58 (Rowland et al., 2013).

Historically, studies on the ontogeny and inducibility of various UGT enzyme activities in rats contributed considerably to our understanding of the multiplicity of UGT enzymes. These studies predated widespread gene sequencing and genomic analysis and they were reviewed in previous versions of this chapter. Today we know that the genes encoding the xenobiotic-metabolizing UGT enzymes in rats are organized like those in humans: multiple UGT1A enzymes are encoded in cassette fashion by a single gene locus and multiple UGT2B enzymes are encoded by numerous individual genes. Three historical findings in rats are worth noting. First, in contrast to the situation in humans, some of the rat UGT enzymes are highly inducible. For example, AhR ligands such as dioxin and 3-methylcholanthrene cause more than a 10-fold induction of UGT1A6 and UGT1A7 in rats but not in humans because the human UGT genes lack the XRE activated by AhR. Second, activators of rat PXR, such as dexamethasone and PCN, cause more than a 10-fold induction of a UGT enzyme that conjugates digitoxigenin monodigitioxoside (dt_1), a metabolite of digitoxin (dt_3) formed by CYP3A (see Fig. 6-53). The specific rat UGT1A enzyme responsible for glucuronidating dt_1 has not been identified. Third, all UGT1A enzymes are deficient in Gunn rats. In this hyperbilirubinemic rat, a mutation at codon 415 introduces a premature stop signal, so that all forms of UGT encoded by the UGT1 locus are truncated and functionally inactive. The UGTs known to be encoded by the rat UGT1 locus include the 3-methylcholanthrene-inducible enzyme that conjugates planar molecules such as 1-naphthol (UGT1A6 and UGT1A7), the phenobarbital- and clofibric acid–inducible enzyme that conjugates bilirubin (UGT1A1), and the PCN-inducible enzyme that conjugates dt_1. While Gunn rats are genetically defective in all UGT1A enzymes, Wistar-LA rats are selectively defective in UGT2B2, which allowed this enzyme to be identified as the principal enzyme responsible for glucuronidating androsterone and triiodothyronine (T_3) in rats (Burchell, 1999).

The tissue distribution and substrate/inhibitor specificity of the human UGT1 and UGT2 enzymes are summarized in Table 6-22 (Rowland et al., 2013; Oda et al., 2015; Yang et al., 2017). With the exception of UGT1A5, 1A7, 1A8, 1A10, 2A1 and possibly 2A2, all of the UGT1 and UGT2 members are expressed in human liver microsomes, with mean specific contents ranging from 18 pmol/mg protein (UGT2B17) to 98 pmol/mg protein (UGT2B7), as shown in Table 6-22. Of the hepatically expressed UGT enzymes, UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10, 2B15 and 2B17 are considered to be the UGT enzymes most important for hepatic drug metabolism (Miners et al., 2006, 2010; Kaivosaari et al., 2011). UGT1A7, 1A8, 1A10, and 2A1 also play a role in drug metabolism, and these four UGT enzymes are expressed in the stomach and/or small intestine but not the liver, as shown in Table 6-22, which has implications for the common practice of using human liver microsomes to investigate the role of glucuronidation in drug metabolism. Numerous UGT1 and UGT2 enzymes are expressed throughout the gastrointestinal tract (stomach and/or small intestine), where they contribute significantly to the first-pass (presystemic) elimination of numerous xenobiotics. The major UGT enzymes expressed in human kidney are UGT1A6, 1A9, and 2B7; they are expressed in human kidney microsomes at levels that are comparable to or greater than those in human liver microsomes, as shown in Table 6-22. Other UGTs expressed in human kidney include UGT1A5, 1A7, 2B4, and 2B17 (with low or undetectable levels of mRNA encoding UGT1A1, 1A3, 1A4, 1A8, 1A10, 2B10, 2B11, 2B15, and 2B28). UGT2A1 is primarily expressed in nasal epithelia, whereas UGT2A3 is a polymorphic enzyme expressed in the liver, gastrointestinal tract, and kidneys (Jedlitschky et al., 1999; Court et al., 2008). Several UGT2B enzymes, notably UGT2B15 and UGT2B17, are expressed in steroid-sensitive tissues, such as prostate and mammary gland, where they glucuronidate various steroid hormones.

Various epigenetic/transcriptional factors have been identified that explain, at least in part, the tissue distribution of UGT enzymes including the hepatic nuclear factors HNF-1α, HNF-4α, and HNF-3α (FOXA1), caudal homeodomain-containing 2 (Cdx2), and the degree of methylation of CpG-rich regions in the promoter regions of individual UGTs (Rowland et al., 2013; Oda et al., 2015; Yang et al., 2017). For example, HNF-1α, which is predominantly expressed in the liver and kidney, promotes the expression of several UGTs in these two organs including UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B17. An HNF-4α-binding site in the UGT1A9 promoter region that is important for its expression in the liver and kidney is absent from UGT1A7, 1A8, and 1A10, which are not expressed in the liver. Cdx2, which is expressed in small intestine and colon but not liver or stomach, promotes the expression of UGT1A8, 1A10, and 2B7 in the intestine (but not UGT1A7, which is not regulated by Cdx2 and which is expressed mainly in stomach and esophagus). HNF-3α (FOXA1) promotes the expression of UGT2B15 and UGT2B17 in prostate. A CpG-rich sequence in the promoter region of UGT1A1 is hypermethylated in the kidney but hypomethylated in the liver, which coincides with the high expression of UGT1A1 in the liver but not kidney. Conversely, CpG-rich regions in UGT1A8 and UGT1A10 are hypermethylated in the liver but hypomethylated in the small intestine, which coincides with high expression of UGT1A8 and UGT1A10 in the intestine but not liver (Rowland et al., 2013; Oda et al., 2015; Yang et al., 2017). The regulation of UGT expression by xenosensors and bile acids is discussed later in this section.

Probe drugs (index substrates) and selective inhibitors have been identified for most but not all of the human UGTs, as

summarized in Table 6-22. Some drugs are glucuronidated by all of the UGTs involved in drug conjugation, albeit at different rates. For example, all UGTs form acyl glucuronides of several NSAIDs including diclofenac, ibuprofen, and indomethacin, and all UGTs with the exception of UGT2B15 form acyl glucuronides of flurbiprofen and ketoprofen (Iwamura et al., 2017). Some UGTs glucuronidate drugs stereoselectively. For example, *R*- and *S*-carvedilol are preferentially glucuronidated by UGT1A1 and UGT2B7, respectively; *R*- and *S*-lorazepam are preferentially glucuronidated by UGT1A3 and UGT2B15, respectively, whereas *R*- and *S*-oxazepam are preferentially glucuronidated by both UGT1A9 and 2B7 and 2B15, respectively. Some drugs are converted to more than one glucuronide. For example, the glucuronidation of morphine by UGT2B7 involves conjugation of the phenolic 3-hydroxyl and the alcoholic 6-hydroxyl group in a 7:1 ratio. The 6-*O*-glucuronide is 600 times more potent an analgesic than the parent drug, whereas the 3-*O*-glucuronide is devoid of analgesic activity. UGT2B7 is present in the brain, where it might facilitate the analgesic effect of morphine through formation of the 6-*O*-glucuronide, which presumably does not readily cross the blood–brain barrier and may be retained in the brain longer than morphine (Tukey and Strassburg, 2000). Only UGT2B7 catalyzes the 6-glucuronidation of morphine, whereas several UGTs including UGT1A1, 1A3, 1A6, 1A8, 1A9, 1A10, as well as 2B7 can catalyze the 3-glucuronidation (Stone et al., 2003). Some UGTs conjugate the same drugs but with markedly different affinities. For example, in general, UGT1A4 catalyzes quaternary *N*-glucuronide formation with low affinity, whereas UGT2B10 does so with high affinity. Consequently, CYP2B10 is primarily responsible for the *N*-glucuronidation of various tricyclic antidepressants (e.g., amitriptyline, clomipramine, imipramine, and trimipramine) at low, pharmacologically relevant substrate concentrations (Zhou et al., 2010a; Kaivosaari et al., 2011; Oda et al., 2015).

Despite their broad and overlapping substrate specificities, some generalities can be made regarding the types of substrates conjugated by certain UGT enzymes. For instance, UGT1A4 and UGT2B10 are largely responsible for glucuronidating tertiary amines (Kaivosaari et al., 2011). For UGT1A1 and UGT1A4, the site of glucuronidation is always adjacent to a hydrophobic region of the substrate, with another hydrophobic region 6 to 8 Å from the site of glucuronidation (Smith et al., 2004). UGT1A9 is similar, except that the distal hydrophobic region also contains a hydrogen-bond acceptor (Smith et al., 2004). UGT1A6 prefers to glucuronidate less bulky phenolic substrates. Specifically, rat UGT1A6 enzyme has restricted selectivity toward planar phenols with a molecular thickness less than 4 Å and a total length of more than 6.3 Å (Smith et al., 2004). In a series of 24 *para*-substituted phenols, a methyl or ether in any position increased the rate of glucuronidation in the human liver (in vitro) compared with phenol itself (Temellini et al., 1991). A hydrogen bond acceptor 10 Å from the nucleophile is common to UGT2B4 substrates (Sorich et al., 2004). The *C*-glucuronidation of the enolic form of phenylbutazone is catalyzed specifically by UGT1A9 (Nishimura and Naito, 2006). Additionally, Sorich et al. (2008) reviewed a number of pharmacophores and their importance as determinants in enzyme-specific glucuronidation with in silico modeling.

Kaivosaari et al. (2011) reviewed *N*-glucuronidation of drugs and xenobiotics. Historically, UGT1A4 was considered the major UGT enzyme responsible for the formation of *N*-glucuronides of primary, secondary, and tertiary amines, but it is now known that several UGT enzymes including UGT1A3, 1A9, 2B4, 2B7, and 2B10 can also form *N*-glucuronides (Zhou et al., 2010a; Kaivosaari et al., 2011). UGT1A4 and UGT2B10 are considered low- and high-affinity enzymes specific for *N*-glucuronidation in the liver, especially for tertiary amines, which are converted to positively charged quaternary ammonium glucuronides. In vitro, many tertiary amine substrates, such as imipramine and amitriptyline, exhibit biphasic kinetics with UGTB10 as the high-affinity, low-capacity component and UGT1A4 as the low-affinity, high-capacity component (Zhou et al., 2010a). Many in vitro studies that previously identified other UGT enzymes as key enzymes involved in *N*-glucuronidation may have overlooked the role of UGT2B10 because they employed substrate concentrations several times higher than those observed in vivo. *N*-Glucuronidation can be divided into four categories based on the structure of the aglycone: (1) primary amines and hydroxylamines, (2) amides and sulfonamides, (3) tertiary amines, and (4) aromatic *N*-heterocycles. In the case of primary amines, hydroxylamines, amides, and sulfonamides, UGT1A4 is the primary *N*-glucuronidating enzyme, with other enzymes such as UGT1A1, 1A3, 1A9, 1A10, and 2B7 playing a secondary role. In the case of tertiary amines and *N*-heterocycles, UGT2B10 and UGT1A4 are the primary high- and low-affinity enzymes, respectively, whereas several other enzymes have the potential to form *N*-glucuronides with these substrates, namely, UGT1A1, 1A3, and 2B7 (Kaivosaari et al., 2011).

Kaivosaari et al. (2011) also summarized species differences in *N*-glucuronide formation based on the structure of the aglycone. Most species are capable of *N*-glucuronidating primary amines and hydroxylamines, although rats preferentially acetylate these compounds. Primary amine *N*-glucuronides are generally labile under acidic conditions and can undergo hydrolysis in urine (discussed in more detail later in this section). There are marked species differences in the *N*-glucuronidation of tertiary amines. Formation of quaternary *N*-glucuronides is observed in humans and higher primates but is generally not observed in rats, mice, guinea pigs, and dogs, presumably due to the negligible contribution of UGT1A4 (a pseudogene in rodents). *N*-Glucuronidation of *N*-heterocycles is variable between species. For instance, the pyridine derivative nicotine is *N*-glucuronidated in humans, rhesus monkeys, cynomolgus monkeys, and guinea pigs (in descending order based on rate), but this reaction is undetectable in dogs, rats, mice, and rabbit. In most cases, heterocyclic amines form *N*-glucuronides in humans and certain higher primates but are highly variable among all other species, particularly rats and dogs, two species that are commonly used to conduct safety toxicology studies for new drug candidates.

N-Carbamoyl glucuronidation has been reported for relatively few primary amines, or the demethylated metabolites of secondary and tertiary amines, and includes drugs such as sertraline (Fig. 6-60), carvedilol, varenicline, mofegiline, garenoxacin, tocainide, and sibutramine (Gipple et al., 1982; Tremaine et al., 1989; Beconi et al., 2003; Hayakawa et al., 2003; Link et al., 2006; Obach et al., 2006). Marked species difference have been found in the formation of *N*-carbamoyl glucuronides, and humans have only been found to produce these conjugates from even fewer drugs, including sertraline, varenicline, and mofegiline. To form this type of conjugate in vitro, the incubation must be performed under a CO_2 atmosphere, in a carbonate buffer. Although not directly demonstrated, it has been hypothesized that a transient carbamic acid intermediate is formed by the interaction of the amine with the dissolved CO_2, followed by glucuronidation (Obach et al., 2005). Because the intermediate is not stable, the hypothesis that UGT also catalyzes the formation of the carbamic acid cannot be disproved. However, in the case of sertraline and varenicline, it is predominantly UGT2B7 that forms the *N*-carbamoyl glucuronide, which also conjugates various carboxylic acids (Obach, et al., 2005, 2006). Given that the in vitro formation of *N*-carbamoyl glucuronides occurs only under

special incubation conditions that are not typically employed, it is possible that many other primary and secondary amines or their oxidative metabolites can be converted to such conjugates but have not been detected because of the unusual incubation conditions required to support the formation of *N*-carbamoyl glucuronides.

As mentioned above and as shown in Fig. 6-60, UGTs can form unusual conjugates including bisglucuronides, diglucuronides, *N*-carbamoyl glucuronides, *N*-glucosides, and other glycoside conjugates. Bisglucuronides (i.e., a glucuronide in which two separate functional groups on the aglycone are glucuronidated) are more common than diglucuronides, and include the bisglucuronides of bilirubin, morphine, octylgallate, diosmetin, phenolphthalein, and hydroxylated PAHs (such as hydroxylated chrysene and B[*a*]P) (Murai et al., 2006). A diglucuronide is a glucuronide in which a single functional group on the aglycone is conjugated twice resulting in two glucuronosyl groups in tandem (Murai et al., 2006). Diglucuronides of the xenobiotics nalmefene and 4-hydroxybiphenyl and of the endogenous steroids androsterone, DHT, 17β-estradiol, estriol, estrone, and testosterone have previously been detected in dogs. Rat liver microsomes do not form diglucuronides of these steroids, whereas monkey liver microsomes form detectable levels of the DHT, testosterone, and 17 β-estradiol diglucuronides, with human liver microsomes forming only the diglucuronide of DHT (see Fig. 6-60) (Murai et al., 2005). In all cases, it is the 2-hydroxyl group of the first glucuronide moiety that is subject to additional glucuronidation. In the case of DHT, only human UGT1A8 (an intestinal UGT) has been found to produce the diglucuronide from DHT itself, although UGT1A4, 2B15, and 2B17 can produce the monoglucuronide, and UGT1A1 and 1A9 can produce the diglucuronide when the monoglucuronide is the substrate (Murai et al., 2006). Human intestinal microsomes form the diglucuronide more efficiently than human liver microsomes, reflecting the fact that UGT1A8, an extrahepatic UGT, is the predominant enzyme involved in the diglucuronidation of DHT.

Although human UGTs typically are highly selective in the use of UDPGA as a sugar donor, they can accommodate the use of other sugar donors such as UDP-glucose, UDP-galactose, and UDP-xylose in an aglycone-dependent manner, as mentioned previously (Tang and Ma, 2005). For instance, recombinant human UGT1A1 can utilize UDPGA, UDP-xylose, or UDP-glucose to form glycosides, but only with bilirubin (Tang and Ma, 2005). Recombinant human UGT2B7 can glycosidate Compound A [(+)-(5*S*,6*R*,7*R*)-2-isopropylamino-7-[4-methoxy-2-((2*R*)-3-methoxy-2-methylpropyl)-5-(3,4-methylenedioxyphenyl)cyclopenteno[1,2-*b*] pyridine 6-carboxylic acid], an endothelin ET_A receptor antagonist, with UDPGA, UDP-glucose, and UDP-galactose, but can glycosidate diclofenac with only UDPGA (Tang and Ma, 2005). In the case of AS-3201, an aldose reductase inhibitor, it was found that UGT2B4, 2B7, and especially 2B15 all *preferentially* utilize UDP-glucose over UDPGA (Toide et al., 2004). Several other compounds have been found to be glucosidated in mammals, including 5-aminosalicylic acid, bromfenac, pranoprofen, pantothenic acid, hyodeoxycholic acid, mycophenolic acid, sulfadimidine, sulfamerazine, sulfamethoxazole, and various barbiturates (e.g., phenobarbital and amobarbital) (Tang and Carro-Ciampi, 1980; Nakano et al., 1986; Arima, 1990; Kirkman et al., 1998; Toide et al., 2004; Picard et al., 2005). In the case of the carboxyl-containing amine, bromfenac, the aglycone was observed in rat bile after base hydrolysis, and it was concluded that it was formed by hydrolysis of an acyl glucuronide (Kirkman et al., 1998). In later studies to characterize the stability of the putative acyl glucuronide, an *N*-glucoside was detected (Kirkman et al., 1998). 5-Aminosalicylic acid is structurally similar to bromfenac in that both NSAIDs contain a primary amine near the carboxyl group, and both are converted to *N*-glucosides (Kirkman et al., 1998). In the human metabolism of barbiturates, *N*-glucosides are the major metabolites found in urine, and glucuronides have not been detected. Accordingly, it was theorized that enzymes other than UGTs may be involved in the conjugation of barbiturates in humans (Toide et al., 2004). This theory is interesting because liver homogenates from cats, which lack UGT activity, can *N*-glucosidate amobarbital (however, it should be noted that glucose conjugates can sometimes form nonenzymatically) (Carro-Ciampi et al., 1985). However, Toide et al. (2004) demonstrated that the *N*-glucosidation of amobarbital in human liver microsomes correlates highly with the *N*-glucosidation of AS-3201, but not its *N*-glucuronidation, indicating that UGT2B15 is probably the predominant human UGT responsible for the glucosidation of amobarbital.

In general, in vivo hepatic clearance is often estimated from kinetic parameters (K_m and V_{max}) determined in vitro with human hepatocytes, liver microsomes, or S9 fraction. The process is commonly used for in vitro–in vivo extrapolation (IVIVE). However, for drugs that are primarily eliminated by glucuronidation, these in vitro studies conducted with liver microsomes and, to a lesser extent, hepatocytes consistently underpredict UGT-mediated clearance in vivo (Miners et al., 2010). There are several intrinsic factors that influence the clearance of glucuronidated compounds in vitro, such as (1) the permeability of the microsomal membranes to allow access of both the aglycone substrate and cofactor UDPGA, (2) the influence of long-chain unsaturated fatty acids released in microsomal incubations, and (3) the homodimerization and heterodimerization of UGT proteins in the endoplasmic reticulum membrane. Additionally, the kinetic properties of UGTs vary in vitro with changes in the incubation conditions (extrinsic factors), such as the concentration of cofactor, membrane composition, partitioning into microsomes, type of buffer, ionic strength, pH, organic solvents (used for the addition of substrates), β-glucuronidase activity, and the stability of the glucuronide in the incubation buffer (Miners et al., 2010). Many of these extrinsic factors are minimized with appropriate experimental conditions. For instance, cofactor availability is overcome with saturating concentrations of UDPGA, typically 2 to 5 mM. However, Fujiwara et al. (2008) reported that UDP, the by-product of glucuronidation reactions, is a competitive inhibitor that blocks binding of UDGPA. Therefore, for in vitro experiments, the concentrations of cofactor must be maintained at very high levels (up to 20 mM UDPGA) to overcome competitive inhibition of the UDPGA binding site by free UDP produced during the incubation, especially for high-turnover UGT substrates such as 1-naphthol. Other common in vitro conditions for microsomal assays include the addition of the pore-forming peptide alamethicin or zwitterionic detergent CHAPS (described below), the addition of saccharic acid-1,4-lactone to inhibit β-glucuronidase, and the acidification (pH 5 to 6.8) of the organic solvent used to stop reactions when acyl glucuronide formation is expected (Miners et al., 2010).

In vitro intrinsic metabolic clearance (CL_{int}) values (measured as V_{max}/K_m) for zidovudine (AZT) glucuronidation in human liver microsomes were shown to vary sixfold depending on incubation conditions, but even under conditions that produced the greatest CL_{int}, the in vivo clearance rate was underpredicted by three- to fourfold (Miners et al., 2006). This in vitro underprediction of the in vivo rate of clearance of drugs that are glucuronidated is typical when human liver microsomes are used to assess CL_{int}, and is likely due to a number of factors including the presence or absence of albumin, the effects of long-chain unsaturated fatty acids, correction for membrane partitioning, atypical in vitro kinetics, active uptake into hepatocytes, and significant extrahepatic expression of various UGTs (Miners et al., 2010). The prediction of the in vivo clearance

of drugs that are glucuronidated by hepatocytes is generally more accurate than predictions made with microsomes, but underprediction is still the common outcome. Zidovudine is an exception to this rule because its in vivo clearance is accurately predicted from in vitro studies with hepatocytes (Miners et al., 2006). The use of either microsomes or recombinant UGT2B7 also underpredicts the in vivo magnitude of the *inhibitory* interaction between fluconazole and zidovudine by 5- to 10-fold (Miners et al., 2006). However, when 2% bovine serum albumin (BSA) is added to either test system, there is a decrease in the K_i value of 85% which results in a much improved prediction of the in vivo interaction (Miners et al., 2006). The effect of BSA is due to the sequestration of inhibitory fatty acids released from membranes during the incubation. The BSA or "albumin" effect is also observed with fatty-acid-free human serum albumin (HSA-FAF) added to incubations. The K_m values of reactions catalyzed by UGT1A9 and UGT2B7 are markedly influenced by the presence of free fatty acids released from microsomal membranes (notably arachidonic, linoleic, and oleic acids) and albumin serves as a fatty acid scavenger. Similarly, the addition of intestinal fatty acid–binding protein (IFABP) to microsomal incubations decreased the K_m values for zidovudine glucuronidation, a UGT2B7 substrate (Rowland et al., 2009). Unsaturated long-chain fatty acids are potent competitive inhibitors of some, but not all, UGT enzymes and can lead to overestimation of K_m values and, therefore, underprediction of CL_{int}. Furthermore, the effect of fatty acids varies depending on the membrane composition of the enzyme source (recombinant vs. microsomes vs. hepatocytes) inasmuch as kinetic constants for a specific reaction may vary based on the test system. Unlike UGT1A9 and UGT2B7, fatty acids have little to no effect on reactions catalyzed by UGT1A1, 1A4, and 1A6 (Miners et al., 2010).

The glucuronidation of xenobiotics by liver microsomes in vitro displays a property known as latency inasmuch as it can be stimulated by detergents (e.g., CHAPS and Brij-58) and the pore-forming peptide alamethicin, which disrupt the lipid bilayer of the endoplasmic reticulum and allow UGTs free access to UDPGA. High concentrations of detergent, especially nonionic detergents such as Brij, can inhibit certain UGTs, presumably by disrupting their interaction with phospholipids, which are important for catalytic activity (Fisher et al., 2000). Treatment of microsomes with detergents also virtually eliminates CYP activity; hence, detergents cannot be used to study the possible coupling of oxidation reactions catalyzed by CYP with conjugation reactions catalyzed by UGT. This is not a limitation of alamethicin which does not inhibit CYP. Furthermore, in contrast to certain detergents, alamethicin increases V_{max} without affecting K_m. From a review of the literature, Miners et al. (2006) reported that alamethicin and nonionic detergents such as Brij-58 generally result in the highest UGT activity and that alamethicin is the preferred activator because the effects of detergents are not reproducible between substrates. However, in incubations designed strictly to measure UGT activity (and not both UGT and CYP activity), the zwitterionic detergent, CHAPS, can activate certain UGT activities to a comparable or even greater extent than alamethicin.

The kinetic properties of UGTs are possibly influenced by the formation of homodimers and heterodimers among UGT enzymes and by the formation of heterodimers with other microsomal enzymes such as various CYP enzymes, POR, or epoxide hydrolase (Ishii et al., 2010). For instance, it has been demonstrated that the ratio of morphine-3-glucuronide to morphine-6-glucuronide formed by UGT2B7 is altered by the presence of CYP3A4. The K_m of UGT2B7-catalyzed formation of morphine-3-glucuronide was increased 9.8-fold (0.38 to 3.7 μM) when coexpressed with CYP3A4 (Takeda et al., 2005; Ishii et al., 2010). Fremont et al. (2005) reported that UGT1A1, 1A6, and 2B7 coimmunoprecipitated with CYP3A4 in solubilized human liver microsomes. If there are functional consequences to the associations between different UGT enzymes and/or the association between UGT and CYP enzymes, then such associations would have implications for studies with recombinant UGT enzymes, which are invariably expressed individually.

Advances in mass spectrometry have enabled the quantification of UGT proteins in samples of human liver, intestinal and kidney microsomes, recombinant UGT enzymes, and cultured hepatocytes (see Table 6-22), which permits extrapolation of rates of glucuronidation from these in vitro systems to the in vivo situation as is done with CYP enzymes (through the application of relative activity factors [RAF] or intersystem extrapolation factors [ISEF]) (Harbourt et al., 2012; Ohtsuki et al., 2012; Fallon et al., 2013; Achour et al., 2014; Sato et al., 2014). The role of heteromeric or homomeric expression of UGTs in vivo is largely undefined. Several publications have investigated the coexpression of UGT enzymes in vitro with cell lines overexpressing various UGTs with mixed results: enhancement of some activities and impairment of others (Ishii et al., 2010). Finally, posttranslational modifications to UGTs that occur in vivo in humans (e.g., phosphorylation and *N*-glycosylation) may not occur in the cell expression system chosen to produce the recombinant UGTs (i.e., bacterial systems), which can impact activity in a substrate-dependent manner (Miners et al., 2006). All of these findings suggest that the use of recombinant human UGT enzymes may not provide accurate indications of the extent to which a given UGT can glucuronidate a given substrate.

Glucuronidation can be impaired by cofactor availability, polymorphisms, drug–drug interactions, or the effects of endogenous or dietary compounds, any of which can result in toxicities due to decreased elimination of certain compounds (e.g., bilirubin). Cofactor availability can limit the rate of glucuronidation of drugs that are administered in high doses and are conjugated extensively, such as aspirin and acetaminophen. In experimental animals, the glucuronidation of xenobiotics can be impaired in vivo by factors that reduce or deplete UDPGA levels, such as diethyl ether, borneol, and galactosamine. The lowering of UDPGA levels by fasting, such as might occur during a severe toothache or oral cancer, is thought to predispose individuals to the hepatotoxic effects of acetaminophen, although even then hepatotoxicity only occurs with higher-than-recommended doses of this analgesic (Whitcomb and Block, 1994; Rumack, 2004).

Drug–drug interactions that are at least partly due to inhibition of UGTs have been reported; however, the drug–drug interactions caused by UGT inhibition rarely cause increases in the plasma AUC of the victim drug more than twofold (Williams et al., 2004). Valproic acid, which inhibits the clearance of lamotrigine, lorazepam, and ziduvodine), and probenecid, which inhibits the clearance of acetaminophen, clofibrate, lorazepam, and ziduvodine, are the two drugs most commonly associated with clinically relevant inhibition of UGT activity (Rowland et al., 2013). Particular attention is paid to investigational drugs that inhibit UGT1A1, the major bilirubin-conjugating enzyme. Administration of the protease inhibitors atazanavir and indinavir to HIV patients can lead to reversible hyperbilirubinemia due to inhibition of UGT1A1 (Zhang et al., 2005). The potential for atazanavir to cause hyperbilirubinemia is increased in individuals with genetic polymorphisms that decrease the activity of UGT1A1, namely UGT1A1*28 and UGT1A1*37 (discussed later in this section), and already have elevated levels of

bilirubin (Gammal et al., 2016). Clinically relevant drug–drug interactions due to induction of UGT enzymes have also been observed. Rifampin coadministration increases mycophenolic acid clearance by 30%, and increases the AUC of its acyl glucuronide (formed by UGT2B7) and its 7-*O*-glucuronide (formed by various UGT1 enzymes) by more than 100% and 20%, respectively (Naesens et al., 2006). Similarly, rifampin coadministration in humans causes a decrease in the systemic AUC of some UGT substrates, including raltagravir (UGT1A1; decreases AUC by 40%) and zidovudine (UGT2B7; decreases AUC 30% to 50%) (Gallicano et al., 1999; Wenning et al., 2009). Coadministration of other drugs, such as the enzyme-inducing antiepileptics (e.g., phenobarbital), and certain physiological conditions (e.g., increased levels of estrogens during pregnancy) can cause an increase of hepatic UGT enzymes and, therefore, the clearance of some UGT substrates. The CAR agonists phenobarbital and the Chinese herbal Yin Zhi Huang (active ingredient scoparone [6,7-dimethylesculetin]) have been used for the treatment of neonatal jaundice due to their ability to induce the UGT1A1-dependent glucuronidation of bilirubin (a treatment no longer practiced with phenobarbital). In general, the magnitude of drug–drug interactions due to UGT enzyme induction is less than those reported for CYP-mediated induction. Although inhibition and induction of UGT enzymes are associated with modest changes (usually less than twofold) in the clearance of drugs that undergo glucuronidation, certain genetic polymorphisms have a profound impact (more than 100-fold) on systemic exposure to certain investigational drugs that are substrates for UGT2B10 or UGT2B17, as discussed later in this section.

Human UGT1A6 glucuronidates acetaminophen. This reaction is enhanced by cigarette smoking and dietary cabbage and Brussels sprouts, which suggests that human UGT1A6 is inducible by PAHs and derivatives of indole 3-carbinol, but not to the extent observed in rats (Bock et al., 1994). Ligands for AhR, such as those present in cigarette smoke, induce CYP1A2, which would be expected to enhance the hepatotoxicity of acetaminophen. Increased acetaminophen glucuronidation may explain why cigarette smoking does not enhance the hepatotoxicity of acetaminophen. Conversely, decreased glucuronidation may explain why some individuals with Gilbert syndrome are predisposed to the hepatotoxic effects of acetaminophen (de Morais et al., 1992). Low rates of glucuronidation predispose newborns to jaundice and to the toxic effects of chloramphenicol; the latter was once used prophylactically to prevent opportunistic infections in newborns until it was found to cause severe cyanosis and even death (gray baby syndrome).

Some glucuronide conjugates have been found to act as substrates for further biotransformation by oxidation or even by further conjugation. For instance, in male WAG/Rij rats, estradiol 17β-glucuronide can be sulfonated by one or more sulfotransferases (SULTs) to estradiol 3-sulfate-17 β-glucuronide (Sun et al., 2006). In addition, the acyl glucuronide of 4-hydroxydiclofenac can be formed either by a combination of 4′-hydroxylation followed by acyl glucuronidation or by a combination of acyl glucuronidation followed by 4′-hydroxylation. In the former case, the 4′-hydroxylation of diclofenac is catalyzed by CYP2C9, whereas in the latter case the 4′-hydroxylation of diclofenac acyl glucuronide is catalyzed by CYP2C8 (Kumar et al., 2002). Glucuronidation converts several other CYP2C9 substrates into CYP2C8 substrates or inhibitors. For instance, CYP2C8 catalyzes the oxidation of several glucuronides, the aglycones of which are CYP2C9 substrates, including estradiol 17β-glucuronide and the acyl glucuronides of naproxen, the PPARα/agonist MRL-C, gemfibrozil (Delaforge et al., 2005; Kochansky et al., 2005; Ogilvie et al., 2006). Desloratadine undergoes 3-hydroxylation by CYP2C8 (to form 3-hydroxydesloaratdine, a human-predominant metabolite) but only after desloratadine is *N*-glucuronidated by UGT2B10, as shown in Fig. 6-54 (see the section "CYP2C8"). Despite its instability, desloratadine *N*-glucuronide is an obligatory intermediate in the conversion of desloratadine to 3-hydroxydesloratadine by CYP2C8 (Kazmi et al., 2015a, 2015b).In the case of gemfibrozil and clopidogrel (following ester hydrolysis to its acidic metabolite), glucuronidation produces metabolites, namely acyl glucuronides, that cause irreversible (metabolism-dependent) inhibition of CYP2C8, which is the mechanism by which gemfibrozil and clopidogrel increase systemic exposure to cerivastatin leading to increased myotoxicity of this statin (see the section "CYP2C8") (Ogilvie et al., 2006; Tornio et al., 2014). Similar to its inhibition of CYP2C8, gemfibrozil and its glucuronide are inhibitors (and substrates) of the organic anion transporter OATP1B1 on the sinusoidal membrane of hepatocytes. Inhibition of OATP1B1 by gemfibrozil and gemfibrozil glucuronide accounts, at least in part, for the pharmacokinetic drug–drug interaction between gemfibrozil and several statin drugs whose systemic clearance is predominately through OATP-mediated hepatic uptake, namely, pravastatin and rosuvastatin (Tornio et al., 2008). Although inactivation of CYP2C8 by gemfibrozil glucuronide and clopidogrel glucuronide are the only known examples of CYP inactivation by the glucuronide metabolites of clinically used drugs, Kazmi et al. (2010) identified a carbamoyl-glucuronide of an investigational drug that caused irreversible (metabolism-dependent) inhibition of CYP2C8 in vitro, the clinical significance of which is unknown.

Glucuronidation is important for the conversion of atorvastatin acid to its pharmacologically inactive lactone form at physiological pH; however, the formation of atorvastatin lactone is also associated with dose-limiting toxicities (Riedmaier et al., 2011). Atorvastatin (Lipitor) is administered as a free acid but is found in systemic circulation at levels similar to its pharmacologically inactive lactone based on plasma AUC. The major cause of adverse events associated with atorvastatin therapy is myopathy (skeletal muscle toxicity) which, in severe cases, can result in rhabdomyolysis and fatal kidney failure. In vitro studies with the acid and lactone forms of atorvastatin in primary skeletal muscle cells established that the lactone form is 14 times more potent than the acid form at inducing cell death. Both atorvastatin acid and lactone can undergo oxidative metabolism by CYP3A4 and CYP3A5 to form *ortho*- and *para*-hydroxylated metabolites. Atorvastatin acid also undergoes glucuronidation to form atorvastatin acyl glucuronide. The conversion of atorvastatin acid to its pharmacologically inactive lactone occurs nonenzymatically at low pH (e.g., intestine) or enzymatically at physiological pH (7.4); the latter is dependent on formation of the acyl glucuronide (Riedmaier et al., 2011). The glucuronide moiety is a good leaving group and is lost during the lactonization reaction. Based on in vitro and in vivo studies, Riedmaier et al. (2010) identified UGT1A3 as the enzyme largely responsible for the lactonization of atorvastatin and further demonstrated that the paraoxonases PON1 and PON3 are responsible for the hydrolysis of atorvastatin lactone back to the acid form. Taken together, UGT1A3, PON1, and PON3 are important determinants of the atorvastatin acid–lactone ratio in vivo and, hence, the potential for atorvastatin myopathy and rhabdomyolysis.

Kumar et al. (2002) demonstrated that failure to take into account the formation of the acyl glucuronide of 4′-hydroxydiclofenac leads to an underestimation of hepatic clearance of diclofenac. It may also be that oxidation of glucuronide metabolites can lead to toxicity. A case report described the formation of an IgM antibody that bound erythrocytes, but only in the presence of

the 4-hydroxydiclofenac acyl glucuronide in a patient who developed hemolysis (hemolytic anemia) during diclofenac treatment (Shipkova et al., 2003). A determination of the absolute amount of diclofenac acyl glucuronide formed in vivo relative to the amount of 4-hydroxydiclofenac formed in vivo would be confounded by the rapid hydrolysis of the glucuronide to the aglycone, and it would therefore be likely that detection of 4-hydroxydiclofenac acyl glucuronide would be attributed to oxidative metabolism occurring prior to conjugation. Two reports suggest that direct glucuronidation with subsequent oxidation (by a combination of UGT2B7 and CYP2C8 in humans) may be the major determinants of diclofenac clearance in humans (possibly as high as 75%) and monkeys (more than 90%) (Kumar et al., 2002; Prueksaritanont et al., 2006), as opposed to earlier in vivo data that suggested oxidative metabolism by CYP2C9 alone is the major determinant of clearance (Stierlin and Faigle, 1979; Stierlin et al., 1979). In support of this interpretation, there are no clinical reports that implicate pharmacokinetic interactions between diclofenac and potent CYP2C9 inhibitors or inducers (Prueksaritanont et al., 2006). Taken together, these observations suggest that the CYP-mediated oxidation of glucuronide metabolites has implications not only for the prediction of in vivo drug–drug interactions from in vitro data (i.e., gemfibrozil and clopidogrel) but also for the prediction of in vivo clearance (i.e., diclofenac), and possibly also toxicity, as in the case of immune-mediated toxicity of diclofenac.

In humans, Crigler–Najjar syndrome (types I and II) and Gilbert disease are congenital defects in bilirubin conjugation. The diseases have historically been differentiated largely on the basis of the severity of symptoms and total plasma concentrations of bilirubin (e.g., Crigler–Najjar type I: 310 to 855 μM, Crigler–Najjar type II: 100 to 430 μM, and Gilbert disease: 20 to 100 μM). The major bilirubin-conjugating enzyme in humans is UGT1A1. Genetic polymorphisms in exons 2 to 5 (which affect all enzymes encoded by the UGT1A locus), in exon 1 (which affect only UGT1A1), in the promoter regions, and in introns 1 and 3 have been identified in patients with Crigler–Najjar syndrome or Gilbert disease. More than 60 genetic polymorphisms are associated with these diseases. A current list of all UGT polymorphisms and phenotypes (when known) can be found at https://www.pharmacogenomics.pha.ulaval.ca/ugt-alleles-nomenclature/. Some polymorphisms cause the introduction of a premature stop codon in one of the UGT1A common exons 2 to 5 (which causes a loss of all UGT1A enzymes, analogous to the Gunn rat) and are associated with type I Crigler–Najjar syndrome, a severe form of the disease characterized by a complete loss of bilirubin-conjugating activity and marked hyperbilirubinemia. Type I Crigler–Najjar syndrome is also associated with various frameshifts and deletions in exons 1 to 5, and in at least three cases with changes in introns 1 and 3 that affect splice donor or acceptor sites. Other polymorphisms are associated with the less severe type II Crigler–Najjar syndrome (i.e., UGT1A1*7–9, 12, 26, 30, 33-38, 42, 48, 51, 52, 59, and 64). Individuals with Gilbert disease have an occasionally transient, and generally mild, hyperbilirubinemia that is often caused by the addition of one "TA" segment in the TATA promoter region (i.e., UGT1A1*28: $A(TA)_6$**TAA** → **A(TA)**$_7$TAA). There is some overlap between Crigler–Najjar type II and Gilbert disease, not only in terms of the plasma concentrations of bilirubin but also in the type of polymorphism that underlies the disease. In addition to the *28 allele, the *6, *27, *29, *60, and *62 alleles are associated with Gilbert disease, and some of these polymorphisms affect coding regions of the UGT1A1 gene. In addition, the UGT1A1*37 allele produces a $A(TA)_8$TAA promoter defect that results in Crigler–Najjar type II disease. A Korean individual heterozygous for three UGT1A1 alleles associated with Gilbert disease (i.e., likely *6, *28, and *60) was found to have total bilirubin concentrations as high as 193 μM (Seo et al., 2007), which is a concentration typically associated with Crigler–Najjar type II disease. Crigler–Najjar type II and Gilbert diseases (in contrast to Crigler–Najjar type I disease) typically respond to some extent to phenobarbital treatment, which stimulates bilirubin conjugation by inducing UGT1A1. Type I Crigler–Najjar syndrome is also associated with impaired glucuronidation of propofol, ethinylestradiol, and various phenolic substrates for UGT1A enzymes.

Genetic variants that impair UGT1A1 activity, notably the *6, *28, and *37 allelic variants, are clinically important due to their impact on the toxicity of the topoisomerase I inhibitor irinotecan, which is used primarily to treat colorectal cancer. The disposition of irinotecan is complex. Irinotecan (a prodrug) is administered intravenously with hydrolysis to the active metabolite, SN-38, occurring mainly by tissue carboxylesterases, namely, CES2, and hydrolytic enzymes in plasma. The active metabolite SN-38 is subsequently glucuronidated in the liver primarily by UGT1A1 after which the glucuronide conjugate is excreted into bile. Other UGT enzymes that contribute to glucuronidation, at least in part, include hepatic UGT1A6 and UGT1A9 and intestinal UGT1A7 and UGT1A10 (Nagar and Blanchard, 2006; Wallace et al., 2010). The UGT1A1*28 variant has now been referenced in the Camptosar® prescribing information, which notes that patients with reduced UGT1A1 activity have a higher exposure to SN-38 (which is 50 to 100 times more toxic than the glucuronide), and that the dose of irinotecan should be adjusted downward accordingly (Nagar and Blanchard, 2006). The toxicity of SN-38 primarily manifests as severe diarrhea and myelosuppression (in the form of leucopenia, severe thrombocytopenia, severe anemia, or grade 3 to 4 neutropenia) (Nagar and Blanchard, 2006). Several studies have demonstrated grade 3 to 4 neutropenia and/or grade 3 to 4 diarrhea upon irinotecan administration in patients with at least one UGT1A1*28 or UGT1A*27 allele, and one study implicated *high*-activity variants of UGT1A7 and UGT1A9 with diarrhea (Nagar and Blanchard, 2006).

Inhibition of bacterial β-glucuronidase is a potential pharmacological approach to overcome the severe diarrhea associated with irinotecan (CPT-11) chemotherapy (Wallace et al., 2010). Hydrolysis of SN-38-*O*-glucuronide (SN-38G) by bacterial β-glucuronidase in the gastrointestinal tract releases the pharmacologically active SN-38 into the intestinal lumen where it can be reabsorbed and cause dose-limiting diarrhea. The β-glucuronidase enzyme responsible for hydrolysis of SN-38G in the gut is found in the symbiotic microbiota. Wallace et al. (2010) identified four compounds that potently (K_i values in the nanomolar range) and specifically inhibit *Escherichia coli* β-glucuronidase, but not human β-glucuronidase activity, by targeting a 17-residue loop in the bacterial enzyme that is not present in mammalian forms (Wallace et al., 2010). Inhibition of *E. coli*–specific β-glucuronidase protected mice from the GI toxicity of irinotecan (Wallace et al., 2010). Taken together, these results suggest that β-glucuronidase may be a potential pharmacological target to limit pharmacokinetic or toxicological concerns associated with hydrolysis of glucuronides in the gastrointestinal tract, which may lead to reabsorption of, and prolonged exposure to, the aglycone.

Genetic polymorphisms have been identified in UGT2B4, 2B7, 2B10, 2B15, 2B17, and 2B28. Until recently, these were thought to have a modest impact on systemic exposure to drugs (Rowland et al., 2013; Mazerska et al., 2016). However, that view changed with reports of dramatic changes (more than 25-fold

increase) in systemic exposure to two investigational drugs that were associated with genetic polymorphisms in either UGT2B10 or UGT2B17. A genetic polymorphism in UGT2B10 (caused by a splice site mutation that prevents synthesis of functional enzyme) was identified as the cause of a 136-fold increase in systemic exposure to RO5263397, a drug candidate under investigation for the treatment of schizophrenia, in an individual enrolled in a clinical trial (Fowler et al., 2015). Further clinical studies identified two additional PMs, all of African descent and all with the same splice variant of UGT2B10. Interestingly, in vitro reaction phenotyping studies with a panel of recombinant human UGT enzymes pointed to UGT1A4 as the enzyme primarily responsible for the *N*-glucuronidation of RO5263397, but this study did not include UGT2B10, which was subsequently identified as a high-affinity enzyme capable of catalyzing the *N*-glucuronidation of RO5263397 (Fowler et al., 2015). The UGT2B10 splice variant (rs2942857 variant C allele) was found in Africans with a frequency of 45%, compared with 7% in Native Americans, 8% in Asians, and 0% in Caucasians. As discussed in the section "CYP2C8," this variant is a likely cause of (or at least a contributor to) the PM phenotype for desloratadine 3-hydoxylation, a reaction that requires *N*-glucuronidation of desloratadine by UGT2B10 (see Fig. 6-54) and is impaired mainly in individuals of African descent, although genetic polymorphisms that impair CYP2C8 could give rise to the same PM phenotype.

The second example of a genetic polymorphism of UGT greatly impacting systemic exposure to an investigational drug involves MK-7246, a carboxylic acid–containing compound under development for the treatment of respiratory diseases based on its ability to antagonize the chemoattractant receptor on T helper type 2 cells (a CRTH2 antagonist). Clearance of MK-7246 is mainly due to acyl glucuronidation by UGT2B17. In individuals with no UGT2B17 activity (UGT2B17*2/*2 carriers), the plasma AUC and C_{max} of MK-7246 was 25- and 82-fold greater than in individuals harboring the wild-type enzyme (UGT2B17*/*1) (Wang et al., 2012). UGT2B17 (as well as UGT2B15) also plays an important role in the glucuronidation of dihydrotestosterone, testosterone, and androsterone, which terminates androgen signaling in prostate and other androgen target cells. Genetic polymorphisms that decrease UGT2B17 activity or expression, like the *2 allele, have attracted attention for their impact on circulating androgen levels, such as sports doping and some instances of an increased risk of prostate cancer, among others (Guillemette et al., 2010; Gauthier-Landry et al., 2015).

Oxazepam is glucuronidated by UGT2B15, which preferentially glucuronidates *S*-oxazepam over its *R*-enantiomer. Ten percent of the population appear to be poor glucuronidators of *S*-oxazepam, and one study has implicated the low-activity UGT2B15*2 allele as a possible determinant of such variation (Nagar and Remmel, 2006). Such polymorphisms also appear to be the underlying cause of alterations in hyodeoxycholate glucuronidation in gastric mucosa (Tukey and Strassburg, 2000).

In some cases, glucuronidation represents an important event in the toxicity of xenobiotics. For example, the aromatic amines that cause bladder cancer (such as benzidine, 2-aminonaphthalene, and 4-aminobiphenyl) undergo *N*-hydroxylation in the liver (*aryl*-NH_2 → *aryl*-NHOH) followed by *N*-glucuronidation (not *O*-glucuronidation) of the resultant hydroxylamine. Direct *N*-glucuronidation (prior to *N*-hydroxylation) also occurs, and is a competing pathway of hepatic metabolism. In the case of 4-aminobiphenyl, the competing pathways of ring hydroxylation and *O*-esterification (i.e., *O*-glucuronidation, *O*-sulfonation, or *O*-acetylation) are detoxication pathways, whereas *N*-esterification catalyzed by UGTs, SULTs, or NATs represent activating pathways (see the section "Acetylation") (Cohen et al., 2006). Benzidine and 2-aminonaphthalene are particularly tumorigenic. The risk of bladder cancer may increase by up to 100-fold in workers exposed to these substances in the course of their occupation in various manufacturing processes (Al-Zoughool et al., 2006). The *N*-glucuronides of such carcinogens, which accumulate in the urine of the bladder, are unstable in acidic pH and thus are hydrolyzed to the corresponding unstable, tumorigenic hydroxylamines, as shown in Fig. 6-62. Under acidic conditions, hydroxylamines can produce highly electrophilic aromatic nitrenium ions (*aryl*-NHOH + H^+ → *aryl*-N^+ + H_2O) that can bind to DNA and other macromolecules, or they can be converted to reactive acetoxy metabolites directly in the bladder epithelium by NAT-mediated *O*-acetylation (*aryl*-NH–O–CO–CH_3), which also leads to the formation of aromatic nitrenium ions (Al-Zoughool et al., 2006). Because *N*-glucuronidation of aromatic amines can also occur directly without prior oxidation, this reaction competes with oxidation, and therefore a decrease in UGT activity in the liver would favor *N*-hydroxylation, with subsequent *O*-acetylation by NAT and spontaneous formation of aromatic nitrenium ions in the liver, rather than the bladder. In contrast, decreased acetylation with normal UGT activity would lead to a greater accumulation of *N*-glucuronides (of the parent aromatic amine and its hydroxylamine) in the bladder with increased bladder tumor formation. Concordant with this scenario, benzidine induces predominantly liver tumors in rats (fast acetylators) but bladder cancer in dogs (poor acetylators) (Al-Zoughool et al., 2006). In humans, there are sex differences in aromatic amine carcinogenicity. Irrespective of ethnicity or race, men are 2.5 to 5 times more likely to develop bladder cancer than women in general. In particular male smokers, hairdressers, and dye and textile workers who are exposed to aromatic amines have several times increased risk relative to their female counterparts. Male mice, which *N*-glucuronidate 4-aminobiphenyl faster than females, were found during the treatment with this carcinogen to have a 2.2-fold higher rate of DNA-adduct formation, and increased rates of bladder tumor formation relative to females, and female mice were found to have tumors only in the liver (Al-Zoughool et al., 2006). In mice, coadministration of 4-aminobiphenyl with hecogenin, which in humans has been found to inhibit hepatic UGT1A4 (Uchaipichat et al., 2006), was found to increase DNA-adduct formation in a statistically significant manner in the livers of male mice, and to slightly decrease adduct formation in the bladders of both male and female mice (Al-Zoughool et al., 2006). The available literature regarding sex differences in human UGT activity is conflicting. Glucuronidation of temazepam, oxazepam, propranolol, and salicylic acid was 20% to 60% higher in men than in women (Al-Zoughool et al., 2006), whereas 4-methyumbelliferone glucuronidation in cryopreserved human hepatocytes was found to be an average of 40% higher in female samples ($n = 33$) than in male samples ($n = 31$). The carcinogenicity of aromatic amines is multifactorial, and involves not only hepatic *N*-glucuronidation but also hepatic oxidation, hepatic and bladder acetylation, and possibly peroxidation of *N*-hydroxy-*N*-acetyl aromatic amines in the bladder (Al-Zoughool et al., 2006). Therefore, sex differences in UGT activity alone may not fully explain the sex differences observed in the carcinogenicity of aromatic amines. A similar mechanism may be involved in colon tumor formation by aromatic amines, although in this case hydrolysis of the *N*-glucuronide is probably catalyzed by β-glucuronidase in intestinal microbiota. Aromatic amides (such as phenacetin) can also be *N*-hydroxylated by CYP (and peroxidases) to form hydroxamic acids: *aryl*-N(OH)–$COCH_3$. Under acidic conditions (such as in urine), these can lose water to form reactive nitrenium ions that are thought to contribute to the

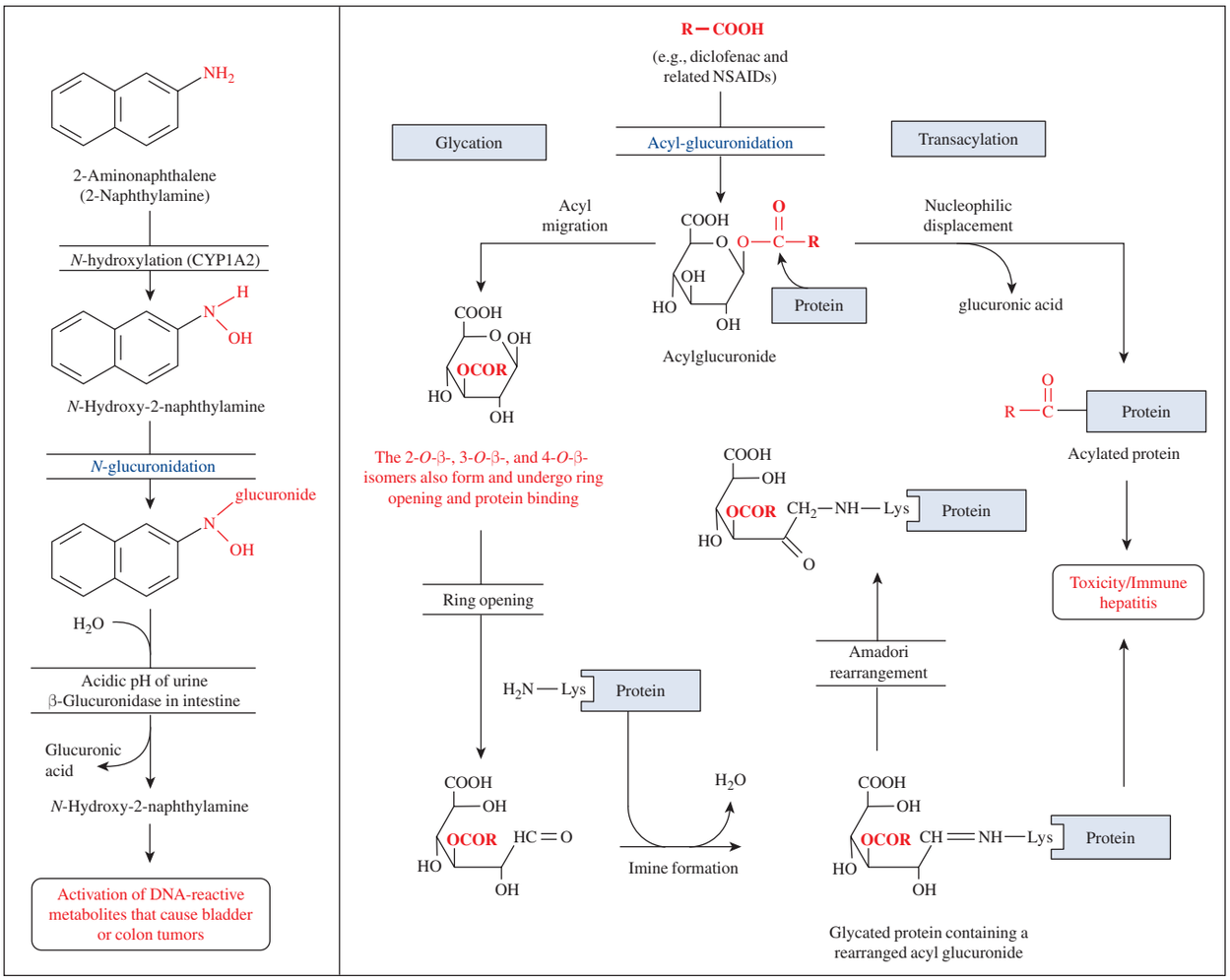


Figure 6-62. *Role of glucuronidation in the activation of xenobiotics to toxic metabolites.*

renal toxicity of phenacetin. The N–OH group can also be conjugated, which introduces a good leaving group. This represents a second pathway to nitrenium ion formation (Testa and Krämer, 2008, 2010).

Steroid hormones glucuronidated on the D-ring (but not the A-ring) cause cholestasis. For instance, 17β-estradiol-glucuronide causes cholestasis in rats by inhibition of MRP2 and BSEP, which are transporters located on the apical membrane of hepatocytes and are important for biliary efflux of endobiotics and xenobiotics, and formation of bile (Huang et al., 2000; Stieger et al., 2000). Induction of UGT activity has been implicated as a nongenotoxic mechanism of thyroid tumor formation in rodents (McClain, 1989; Curran and DeGroot, 1991). Inducers of UGTs cause a decrease in plasma thyroid hormone levels, which triggers a compensatory increase in thyroid stimulating hormone (TSH). During sustained exposure to the enzyme-inducing agent, prolonged stimulation of the thyroid gland by TSH (more than 6 months) results in the development of thyroid follicular cell neoplasia. Glucuronidation followed by biliary excretion is a major pathway of thyroxine (T4) biotransformation in rodents, whereas deiodination is the major pathway (up to 85%) of T4 metabolism in humans. In contrast to the situation in rodents, prolonged stimulation of the thyroid gland by TSH in humans will result in malignant tumors only in exceptional circumstances and possibly only in conjunction with some thyroid abnormality. Therefore, chemicals that cause thyroid tumors in rats or mice by inducing UGT activity are unlikely to cause such tumors in humans. In support of this conclusion, extensive epidemiological data in epileptic patients suggest that phenobarbital and other anticonvulsants do not function as thyroid (or liver) tumor promoters in humans (Parkinson et al., 2006; Elcombe et al., 2014; Lake et al., 2015).

Earlier in this chapter the metabolic activation of drugs to reactive metabolites was discussed on the basis of structural class (acidic vs. neutral/basic) and is illustrated in Fig. 6-63. In particular, acidic drugs (e.g., NSAIDs) can undergo conjugation by UGTs to form acyl glucuronides and/or conjugation with amino acids through the formation of acyl-CoA thioesters catalyzed by mitochondrial acyl-CoA ligases (see the section "Amino Acid Conjugation"). Both conjugation pathways potentially lead to formation of chemically reactive metabolites that have the intrinsic ability to covalently modify proteins by glycation (acyl glucuronides) and/or transacylation (acyl glucuronides and acyl-CoA

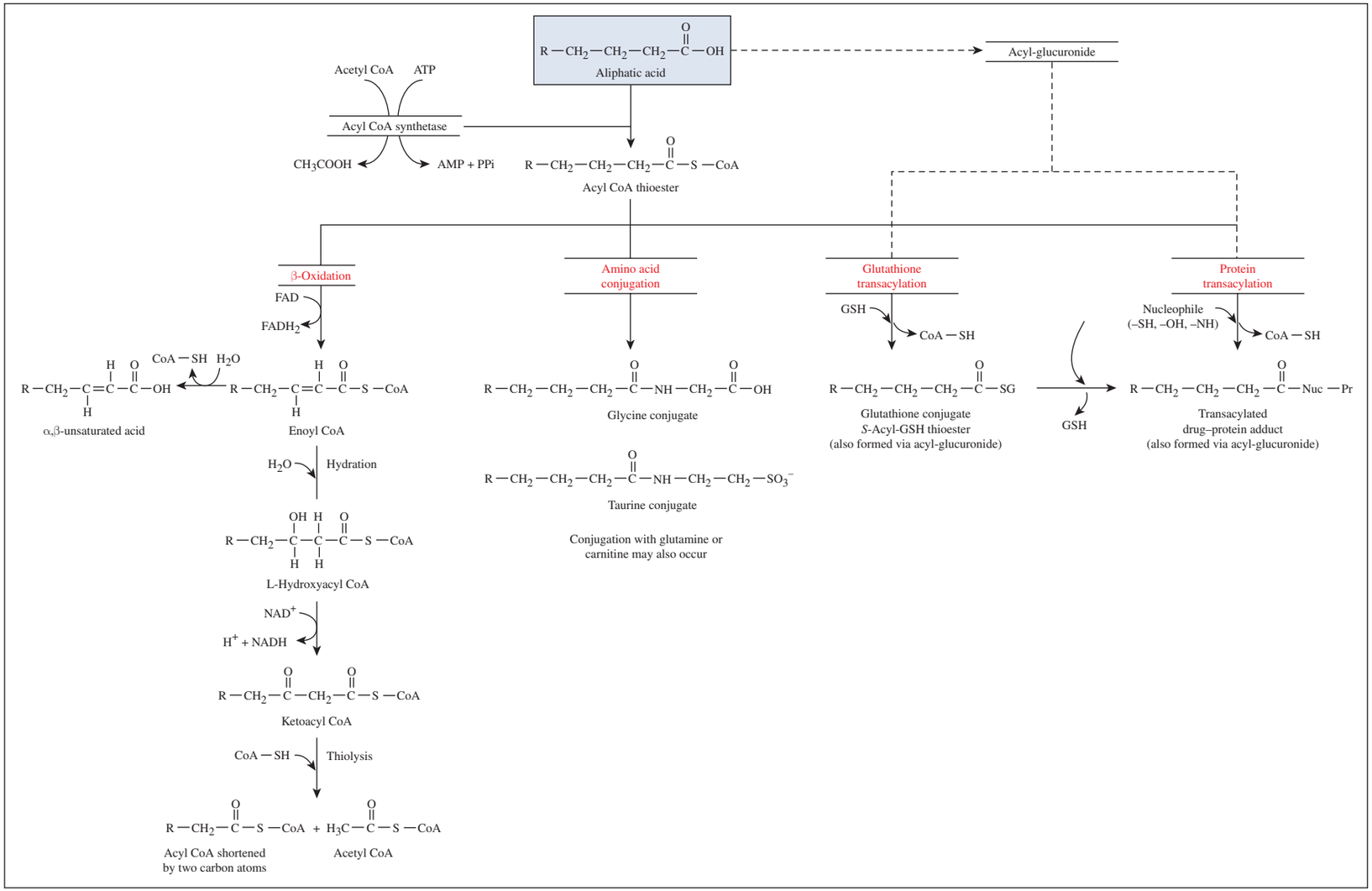


Figure 6-63. *Conversion of carboxylic acid–containing xenobiotics to acyl-CoA thioesters and their subsequent biotransformation by chain shortening by β-oxidation, conjugation with amino acid, and transacylation with glutathione and protein.*

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thioesters) (Iwamura et al., 2017; Van Vleet et al., 2017). Reactive acyl glucuronides glycate amine group of lysine residues through Schiff base formation (in which case the glucuronide moiety covalently modifies the protein) and they can also transacylate proteins by nucleophilic displacement reactions (in which case the glucuronic acid moiety serves as the leaving group and is not covalently bound to the protein), as shown in Fig. 6-63 (Regan et al., 2010). Similarly, acyl-CoA thioesters can transacylate proteins at nucleophilic sites (such as lysine, serine, cysteine, and tyrosine residues), in which case the thio-CoA moiety is the leaving group (Grillo, 2011). An important difference between acyl glucuronides and acyl-CoA thioesters is that only the former are transported into bile where they can damage cholangiocytes and other bile duct cells (discussed later in this section). Several drugs, including the NSAIDs benoxaprofen, bromfenac, diclofenac, diflunisal, etodolac, fenoprofen, flurbiprofen, indoprofen, ketoprofen, ketorolac, loxoprofen, sulindac, suprofen (very similar to the diuretic, tienilic acid), tiaprofenic acid, tolmetin, and zomepirac, contain a carboxylic acid moiety that can be glucuronidated to form a reactive acyl glucuronide that can form covalent adducts with proteins. Acyl glucuronides vary widely in their reactivity, from the highly reactive zomepirac and tolmetin acyl glucuronides to the less reactive acyl glucuronides of ibuprofen and salicylic acid (Shipkova et al., 2003). A relationship between the reactivity of acyl glucuronides and the substitution near the carboxylic acid has been found. In general, α-unsubstituted acetic acid derivatives such as zomepirac, tolmetin, and diclofenac exhibit the highest degree of covalent binding, whereas mono-α-substituted acetic acids such as fenoprofen show intermediate levels, and fully substituted α-acetic acids such as furosemide, ketoprofen, ibuprofen, and suprofen exhibit lower levels of covalent binding (Bolze et al., 2002). The chemical reactivity of acyl glucuronides (as measured by glycation of albumin or a trapping agent such as the dipeptide phenylalanine lysine) is highly correlated with its rate of isomerization whereby the xenobiotic sequentially migrates from the initial 1-*O*-β position to the 2-, 3-, and 4-positions (which can be in either the α- or β-configuration) (Wang et al., 2004). It is only after the xenobiotic migrates from the 1-position that the glucuronide moiety can transiently open to its hemiacetal form and form a Schiff base with lysine residues. When incubated in protein-free phosphate buffer at pH 7.4, those acyl glucuronides with a half-life of less than 30 minutes (with the loss of the 1-*O*-β isomer occurring by a combination of isomerization and hydrolysis) include the acyl glucuronides of the particularly hepatotoxic NSAIDs zomepirac and tolmetin. Isomerization is slowed by the addition of one or two substituents on the α-carbon (next to the carboxylic acid moiety), which correlates with their reduced toxicity. However, a direct correlation between the ability of acyl glucuronides to give rise to covalent adducts with proteins such as albumin and their ability to cause drug-related toxicity has not been firmly established, and other mechanisms may come into play. For instance, diclofenac is still a widely used drug in spite of the fact that its acyl glucuronide is very reactive, whereas zomepirac, which is less reactive than these other drugs, was withdrawn from the market in 1983 (Chen et al., 2006; Regan et al., 2010).

Neoantigens formed by binding of acyl glucuronides to protein might be the cause of rare cases of NSAID-induced immune hepatitis. Covalent adducts with proteins in the liver, kidneys, colon, small intestine, skeletal muscle, and bladder were detected in rats administered diflunisal, and in the liver, lungs, and spleen of rats administered diclofenac (Shipkova et al., 2003). Covalent binding of acyl glucuronides to proteins can be selective, with diclofenac acyl glucuronide forming adducts with dipeptidyl peptidase in the rat liver, and with aminopeptidase N and sucrase-isomaltase in rat intestine (Shipkova et al., 2003). Human and rat liver UGTs are themselves targets of adducts formed by ketoprofen acyl glucuronide, which may cause nonspecific irreversible inhibition (Shipkova et al., 2003). Both acyl glucuronides and acyl-CoA thioesters can transacylate proteins either directly or after their conversion to GSH adducts. Based on in vitro trapping studies with *N*-acetylcysteine (NAC) as the nucleophile, the reactivity of acyl-CoA thioesters is dependent on the substitution of the α-carbon and β-carbon of the xenobiotic (Grillo, 2011). The addition of methyl groups to the α-carbon and/or oxygen to the β-carbon decreases the reactivity of acyl-CoA thioesters just as it decreases the reactivity of acyl glucuronides. In terms of binding to NAC (an indicator of transacylation reactivity), acyl-CoA thioesters and their corresponding acyl-GSH thioesters can be orders of magnitude more reactive than acyl glucuronides, for which reason the formation of acyl-CoA thioesters may contribute to the hepatotoxicity of NSAIDs and other carboxylic acid–containing drugs (Grillo, 2011).

Knights et al. (2007) reviewed the ability of acyl-CoA conjugates of acidic drugs to cause mitochondrial toxicity by interruption of the mitochondrial β-oxidation of fatty acids, which is dependent on their conversion to fatty acyl-CoA thioesters. Xenobiotic-CoA conjugates that are resistant to hydrolysis and are not conjugated with amino acids (e.g., valproic acid) can sequester the pool of mitochondrial CoA and disrupt fatty acid β-oxidation, which are key events in the hepatotoxicity of valproic acid. Valproic acid is metabolized in mitochondria (major pathway) and microsomes (where it is dehydrogenated to 4-ene-valporic acid by CYP2C9 [Fig. 6-53]). In mitochondria, valproic acid is converted to an acyl-CoA thioester that in turn is converted to metabolites that resemble intermediates in the β-oxidation of fatty acids, as shown in Fig. 6-63. Valproic acid is present in mitochondria primarily in the form of its acyl-CoA thioester, which is too polar to diffuse out of the mitochondrion. Taken together, these studies suggest that the conversion of carboxylic acid–containing drugs to acyl-CoA thioesters can play an important role in cell toxicity by causing mitochondrial damage (by depleting CoA and disrupting fatty acid β-oxidation) or by binding covalently to proteins (either directly or following conversion to acyl-GSH thioesters) (Knights et al., 2007; Grillo, 2011).

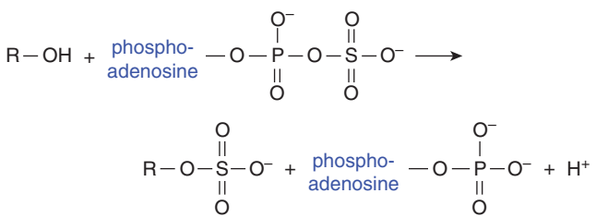
The intrinsic reactivity of acyl glucuronides and acyl-CoA thioesters is not the only factor that contributes to the toxicity of carboxylic acid–containing drugs and metabolites. Extrinsic factors also influence the potential for toxicity, such as dose/exposure, disposition of the parent drug and its metabolites, hydrolysis of acyl glucuronides in the gut (or even in plasma), and reexposure to the aglycone via enterohepatic circulation, transport mechanisms for the efflux of acyl glucuronides from liver into bile or blood, and, perhaps most importantly, targets of these reactive species. Dose or daily body burden is a concern for many acidic drugs (e.g., NSAIDs) because hundreds of milligrams—or even gram quantities—are consumed daily. The reactivity of these metabolites can be assessed with relatively simple in vitro techniques as described above. Intrinsic reactivity can be predicted, to some extent, with structural alerts; the substitution pattern of the α-carbon is particularly informative. Transport proteins also play a crucial role in the disposition of acidic drugs and their acyl glucuronides. MRP2 and MRP3 play critical roles in the efflux of acyl glucuronides into bile and blood, respectively. Shifts in the vectoral transport of these acyl glucuronides may dictate whether toxicity is limited to the cells in which they are formed or whether these reactive metabolites enter bile where they can cause damage to biliary endothelial cells or enter the systemic circulation where they can cause damage to other

tissues or blood. Just as efflux transporters are crucial for transporting these anionic compounds into blood, uptake transporters (OATPs) are involved in tissue-mediated uptake of acyl glucuronides as well as active renal elimination. Expression patterns of certain transporters may predispose or preclude certain cell types to toxicities caused by acyl glucuronides. It should be noted that unlike acyl glucuronides, acyl-CoA thioester metabolites are not transporter substrates and do not leave the cell in which they are formed. This suggests that toxicities caused by acylation of proteins by acyl-CoA thioester metabolites are restricted to the cell where the metabolite is formed (e.g., formed in the hepatocyte and causes hepatotoxicity). In the case of acyl glucuronides and acyl-CoA thioesters, the identity of the protein adducted may determine whether or not these acidic drugs are toxic.

Sulfonation

Many of the xenobiotics and endogenous substrates that undergo *O*-glucuronidation also undergo *O*-sulfonation, as illustrated in Fig. 6-39, for acetaminophen (Mulder, 1981; Paulson et al., 1986). Sulfonation generally produces a highly water-soluble sulfuric acid ester (see Table 6-3). The reaction is catalyzed by sulfotransferases (SULTs), a large multigene family of enzymes that are widely distributed throughout the body (Chen et al., 2015a). In mammals, there are two major classes of SULTs: (1) membrane-bound SULTs in the Golgi apparatus and (2) soluble SULTs in the cytoplasm (Gamage et al., 2006). The membrane-bound SULTs are responsible for the sulfonation of various glycosaminoglycans, proteins, and peptides. At least five different Golgi-resident *N*-acetylglucosamine 6-*O*-sulfotransferases have been identified in humans (Grunwell and Bertozzi, 2002). Brachymorphic mice, which have a global defect in SULT activity due to an impairment in the synthesis of PAPS, the cofactor for sulfonation (see below in this section), are undersized because of their defective sulfonation of glycosaminoglycans and proteoglycans, such as heparin and chondroitin, which are important components of cartilage. These particular sulfonation reactions are catalyzed by membrane-bound SULTs, which play no known role in xenobiotic sulfonation (Wang and James, 2006). This section focuses on the cytosolic SULTs, which are known for the sulfonation of various drugs, mutagens, flavonoids, and other xenobiotics, as well as endogenous substrates such as bile acids, thyroid hormones, catecholamine neurotransmitters, and steroids.

The cofactor for the sulfonation reaction is 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the structure of which is shown in Fig. 6-57. The sulfonation of aliphatic alcohols and phenols, R-OH, proceeds as follows:



Sulfonation involves the transfer of sulfonate, not sulfate (i.e., SO_3^-, not SO_4^-) from PAPS to the xenobiotic. The reaction catalyzed by a SULT enzyme involves simultaneous binding of PAPS and substrate (S), and can be depicted as follows:

$$\text{Enzyme} + \text{PAPS} + \text{S} \rightarrow [\text{Enzyme-PAPS-S}] \rightarrow [\text{Enzyme-PAP}] + \text{S-sulfonate}$$

Sulfonation of the substrate involves the conversion of PAPS to PAP. Dissociation of PAP from the SULT enzyme is relatively slow and generally determines the overall rate of sulfonation (i.e., dissociation of PAP is the rate-limiting step in the catalytic cycle). In addition to PAPS, several sulfonated compounds, such as 4-nitrophenyl sulfate, can support the sulfonation of substrates by converting PAP to PAPS in the active site of SULT, which effectively bypasses the rate-limiting step in sulfonation. For this reason, sulfonation reactions supported by both PAPS and 4-nitrophenyl sulfate are an order of magnitude faster than those supported by PAPS alone (Duffel and Jakoby, 1981).

PAPS is synthesized from inorganic sulfate (SO_4^{2-}) and ATP in a two-step reaction. The first reaction is catalyzed by ATP sulfurylase, which converts ATP and SO_4^{2-} to adenosine-5′-phosphosulfate (APS) and pyrophosphate. The second reaction is catalyzed by APS kinase, which transfers a phosphate group from ATP to the 3′-position of APS. These two enzymes are domains on a single enzyme called PAPS synthetase or PAPSS. Mammals express two forms of PAPS synthetase, PAPSS1 and PAPSS2 (Coughtrie, 2016). Mutations in PAPSS2 are linked to rare musculoskeletal and endocrine disorders in humans (Coughtrie, 2016). The major source of sulfate required for the synthesis of PAPS is derived from cysteine through a complex oxidation sequence. Because the concentration of free cysteine is limited, the cellular concentrations of PAPS (4 to 80 μM) are considerably lower than those of UDPGA (200 to 350 μM) and GSH (5 to 10 mM). This topic has been thoroughly reviewed, and is outside the scope of this chapter (Klaassen and Boles, 1997). The relatively low concentration of PAPS limits the capacity for xenobiotic sulfonation. In general, sulfonation is a high-affinity but low-capacity pathway of xenobiotic conjugation, whereas glucuronidation is a low-affinity but high-capacity pathway. Acetaminophen is one of the several xenobiotics that are substrates for both SULTs and UGTs (see Fig. 6-39). The relative amount of sulfonate and glucuronide conjugates of acetaminophen is dependent on dose. At low doses, acetaminophen sulfonate is the main conjugate formed due to the high affinity of SULTs. As the dose of acetaminophen increases, the proportion of acetaminophen conjugated with sulfonate or glucuronic acid decreases and increases, respectively. In some cases, even the absolute amount of xenobiotic conjugated with sulfonate can decrease at high doses due to substrate inhibition of SULT, which can occur by two mechanisms. First, at high concentrations, substrates can bind to the Enzyme-PAP complex (rather than the Enzyme-PAPS complex) and form a dead-end complex. Second, two substrates can bind simultaneously to SULT either with both substrates in the large substrate-binding (active) site or with one substrate in the active site and another in an allosteric binding site (Chen et al., 2015a). Binding of two different substrates to a single SULT can alter the regiospecificity of sulfonation. For example, in the absence of other chemicals, SULT2A1 catalyzes the sulfonation of 17α-ethinylestradiol in the 3-position, but in the presence of high concentrations of celecoxib this switches to sulfonation in the 17-position (Cui et al., 2004).

Table 6-23 lists some examples of xenobiotics and endogenous compounds that are sulfonated without prior biotransformation by oxidative enzymes. An even greater number of xenobiotics are sulfonated after a hydroxyl group is exposed or introduced during hydrolytic or oxidative biotransformation. Phenols and aliphatic alcohols (which are often the products of oxidative or hydrolytic biotransformation) are converted to *O*-sulfonates, and these represent the largest groups of substrates for SULTs. However, in some cases, SULTs can form *N*-sulfonates, otherwise known as sulfamates. For example, aniline and 2-aminonaphthalene, two

Table 6-23

Properties of the Human Cytosolic Sulfotransferases (SULTs)

HUMAN SULT	POLYMORPHIC?*	TISSUE DISTRIBUTION	MAJOR SUBSTRATES†,‡
SULT1A1	Yes*** *1–*4	Liver, platelets, placenta, adrenal gland, endometrium, colon, small intestine, leukocytes, brain	**4-Nitrophenol**, acetaminophen, apomorphine, 4-cresol, dopamine, epinephrine, 17β-estradiol (and other phenolic steroids), 4-ethylphenol, genistein, *N*-hydroxy-PhIP, minoxidil, morphine, naloxone, 2-naphthol (and other phenols), 2-nitropropane, numerous aromatic amines, hydroxylamines, hydroxamic acids, and benzylic alcohols, T2, T3, troglitazone
SULT1A2	Yes *1–*6	Liver, kidney, brain, gastrointestinal tract, leukocytes, bladder tumors	4-Nitrophenol, *N*-hydroxy-2-acetylaminofluorene, minoxidil, morphine, naloxone, 2-naphthol, various aromatic hydroxylamines and hydroxamic acids
SULT1A3	Yes *1–*4	Small intestine and colon, liver, platelets, placenta, brain, leukocytes, fetal liver	**Dopamine**, albuterol, dobutamine, 1-hydroxymethylpyrene, minoxidil, morphine, 4-nitrophenol, norepinephrine, salbutamol, vanillin
SULT1A4		Liver, pancreas, colon, brain	Likely similar to SULT1A3
SULT1B1		Gastrointestinal tract, liver, leukocytes, skeletal muscle	Benzylic alcohols, dopamine, 4-nitrophenol, T2, T3, r-T3, T4
SULT1C2	Yes *1–*5	Kidney, gastrointestinal tract, thyroid gland, heart, fetal lung and kidney	Aromatic hydroxylamines, *N*-hydroxy-2-acetylaminofluorene, 4-nitrophenol, thyroid hormones
SULT1C3		Appendix, lymph nodes, thymus, adrenal gland, fetal liver	Unknown
SULT1C4		Kidney, ovary, spinal cord, fetal kidney and lung	Bisphenol-A, diethylstilbestrol, estrone, 1-hydroxymethylpyrene, *N*-hydroxy-2-acetylaminofluorene, 4-nitrophenol, nonylphenol, 4-octylphenol
SULT1D1		Not characterized	
SULT1E1		Liver, small intestine, endometrium, heart, adrenal gland, mammary epithelial cells, and fetal liver, lung, and kidney	**17β-Estradiol**, dehydroepiandrosterone, diethylstilbestrol, equilenin, estrone, ethinyl estradiol, 2- and 4-hydroxyestrone, 2- and 4-hydroxyestradiol, 4-hydroxylonazolac, 1-naphthol, naringenin, pregnenolone, raloxifene, tamoxifen, thyroid hormones
SULT2A1	Yes *1–*3	Liver, adrenal gland, ovaries, prostate, small intestine, kidney, brain	**Dehydroepiandrosterone (DHEA)**, afitoxifene, bile acids, budesonide, buprenorphine, estradiol (and other hydroxysteroids), hycanthone, 1-hydroxymethylpyrene, 6-hydroxymethylbenzo[*a*]pyrene, 4-hydroxytamoxifen, pregnenolone, raloxifene, testosterone (and other androgens)
SULT2B1		Tongue, placenta, liver, prostate, trachea, skin, skeletal muscle, spleen, thymus, kidney, ovary, adrenal gland, gastrointestinal tract	**Cholesterol**, dehydroepiandrosterone, pregnenolone and other oxysterols and hydroxysteroids
SULT4A1		Brain	Estrone, 2-naphthol, 2-naphthylamine, 4-nitrophenol, T3, T4
SULT6B1		Testis, kidney	Unknown

**Data from Lindsay et al. (2008).*

†Substrates in bold are reported to be selective probe substrates for the SULT listed. Data from Coughtrie and Fisher (2003), Blanchard et al. (2004), Bradley and Benner (2005), Gamage et al. (2006), Wang and James (2006), Lindsay et al. (2008), Riches et al. (2009), and Salman et al. (2009).

‡T4 is thyroxine. T2 and T3 are di- and tri-iodothyronine. r-T3 is reverse tri-iodothyronine.

aromatic amines, can undergo sulfonation to the corresponding sulfamates, which involves formation of a nitrogen-sulfur bond ($R–NH_2 + PAPS \rightarrow R–NH–SO_3H + PAP$). The primary amines in cisapride and DPC423 (an inhibitor of coagulation factor Xa) can also be directly *N*-sulfonated to sulfamates. *N*-Oxides and hydroxylamines can also be *O*-sulfonated by SULTs. The *N*-oxide group in minoxidil can be sulfonated to form a pharmacologically active metabolite, and in this case the sulfonate group is attached to the oxygen atom, not the nitrogen atom ($R_1R_2R_3 - N^+ - O^- + PAPS \rightarrow R_1R_2R_3 - N^+ - O - SO_3^- + PAP$).

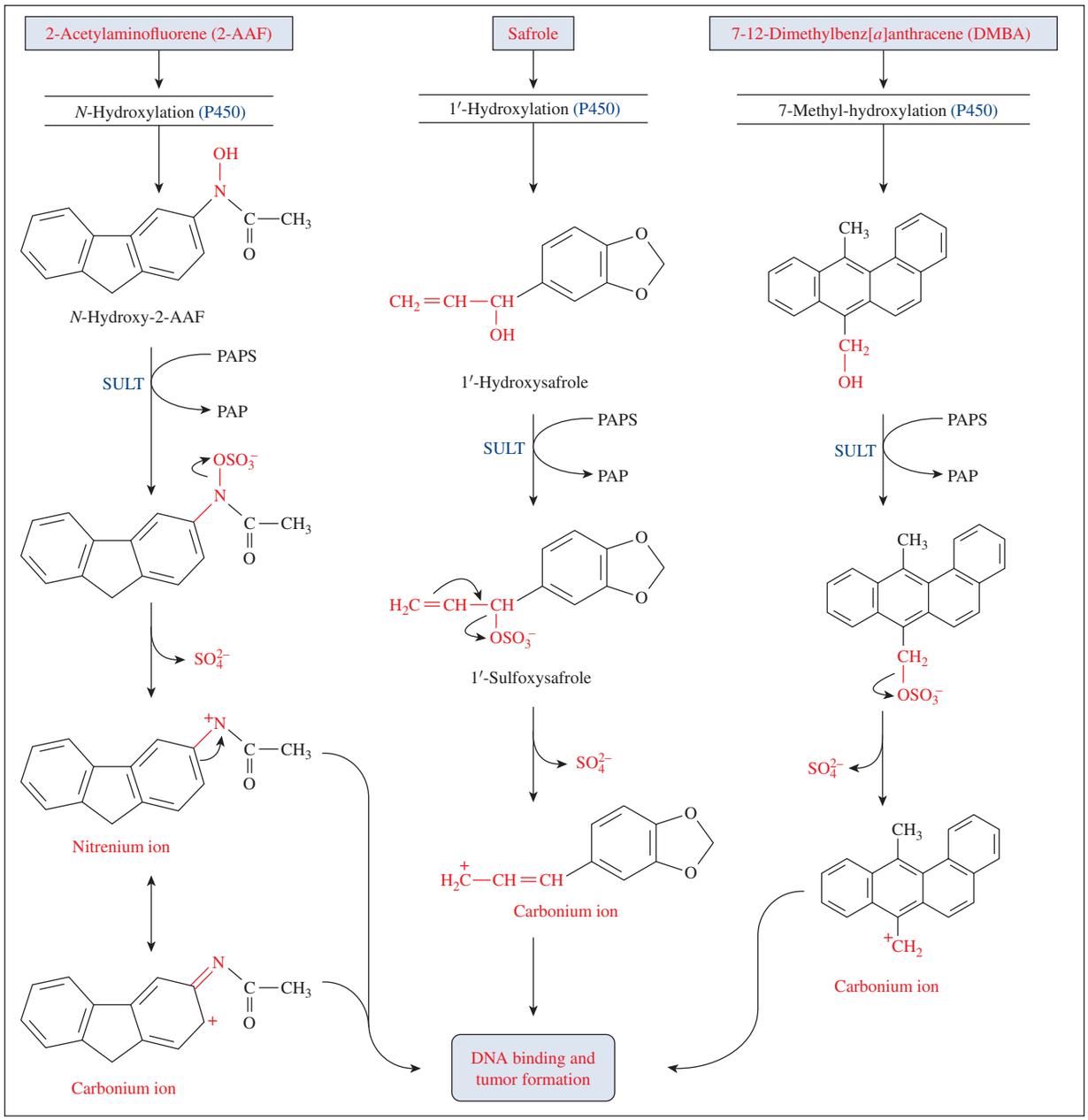


Figure 6-64. *Role of sulfonation in the generation of tumorigenic metabolites (nitrenium or carbonium ions) of 2-acetylaminofluorene, safrole, and 7,12-dimethylbenz[a]anthracene (DMBA).*

Similarly, the *N*-hydroxy group in *N*-hydroxy-2-aminonaphthalene and hydroxy-2-acetylaminofluorene (NOH-AAF) can be sulfonated, as shown for NOH-AAF in Fig. 6-64. In all cases, the conjugation reaction involves nucleophilic attack of oxygen or nitrogen on the electrophilic sulfur atom in PAPS with cleavage of the phosphosulfate bond.

Sulfonation and glucuronidation are often viewed as equal solutions to the problem of biotransforming xenobiotics and their phase 1 metabolites to water-soluble conjugates that can be readily eliminated from the body. However, in terms of forming reactive and potentially toxic/carcinogenic metabolites, sulfonation and glucuronidation differ in several aspects. As discussed in the previous section "Glucuronidation," formation of reactive metabolites that undergo glucuronidation is a concern with carboxylic acid–containing xenobiotics, which can be converted to potentially reactive acyl glucuronides, but it is not a concern with aliphatic alcohols and phenols that undergo *O*-glucuronidation. Acidic drugs are not conjugated by SULTs; hence, the conversion of carboxylic acid–containing xenobiotics to potentially reactive acyl sulfonates is not a concern. However, in contrast to *O*-glucuronidation, *O*-sulfonation can produce reactive and potentially toxic/carcinogenic metabolites. This is because addition of a sulfonate moiety to the oxygen atom of an alcohol, phenol, or *N*-hydroxylamine produces a sulfate group that, being a good leaving group, can dissociate to form a

carbon- or nitrogen-centered anion (i.e., a carbonium or nitrenium ion), as shown below:

$$R - OH(\text{alcohol or phenol}) \rightarrow R - O - SO_3^- \rightarrow R^+(\text{carbonium ion}) + {}^-O - SO_3^-(\text{sulfate})$$

$$R - NH - OH(\text{hydroxylamine}) \rightarrow R - NH - O - SO_3^- \rightarrow R - NH^+(\text{nitrenium ion}) + {}^-O - SO_3^-(\text{sulfate})$$

Examples of xenobiotics that are converted to reactive metabolites by pathways that involve *O*-sulfonation are shown in Fig. 6-64. *O*-Sulfonation plays an important role in the activation of aromatic amines, such as the hepatocarcinogen 2-acetylaminofluorene (2-AAF), methyl-substituted PAHs, such as the lung and skin carcinogen dimethylbenzanthracene (DMBA), and safrole, a hepatotumorigen. Activation of 2-AAF involves CYP-dependent *N*-hydroxylated to a hydroxylamine (NOH-AAF) followed by *O*-sulfonation to a conjugate that can lose the sulfate moiety to form a reactive nitrenium ion (which can rearrange to form a carbonium ion), as shown in the first panel of Fig. 6-64. The same reactions (i.e., *N*-hydroxylation followed by *O*-sulfonation) are the final steps in the metabolic activation of the hepatotumorigen 2,6-dinitrotoluene, as shown in Fig. 6-16. To exert its tumorigenic effect in rodents, safrole must be hydroxylated by CYP to 1′-hydroxysafrole, which is then sulfonated to the electrophilic and tumor-initiating metabolite 1′-sulfooxysafrole, as shown in the middle panel of Fig. 6-64. 1′-Hydroxysafrole is a more reactive and a more potent hepatotumorigen than safrole due to its ability to form a reactive carbonium ion. Two lines of evidence support a major role for sulfonation in the hepatotumorigenic effect of 1′-hydroxysafrole. First, the hepatotumorigenic effect of 1′-hydroxysafrole can be inhibited by treating mice with the SULT inhibitor pentachlorophenol (discussed later in this section). Second, the hepatotumorigenic effect of 1′-hydroxysafrole is markedly reduced in brachymorphic mice, which have a diminished capacity to sulfonate xenobiotics because of a genetic defect in PAPS synthesis (Boberg et al., 1983). A similar pathway, namely, benzylic hydroxylation followed by *O*-sulfonation, converts DMBA to a sulfate conjugate that can lose sulfate to form a reactive carbonium ion, as shown in the third panel in Fig. 6-64. The sulfonate conjugates of benzylic and allylic alcohols, aromatic hydroxylamines, and hydroxamic acids (including those in cooked meat) are short-lived electrophiles capable of reacting with nucleophilic substances including proteins and DNA (Wang and James, 2006). In addition to 1′-hydroxysafrole, the benzylic alcohol of DMBA, and NOH-AAF, sulfonation has been implicated in converting 1-hydroxymethylpyrene (1-HMP), and the cooked food mutagen *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo(4,5-*b*) pyridine (*N*-OH-PhIP) to electrophilic nitrenium or carbocation intermediates linked to mutagenesis or tumorigenesis (Wang and James, 2006). In rats, gender differences in the tumorigenesis of various aromatic amines, arylhydroxamic acids, and the benzylic alcohols of polycyclic aromatic hydrocarbons have been linked to gender differences in the expression of multiple SULT enzymes (discussed later in this section) (Coughtrie, 2016).

Benzylic hydroxylation and subsequent *O*-sulfonation are key steps in the metabolic activation of safrole and DMBA, as shown in Fig. 6-64. These same pathways are also responsible for activating nevirapine to a reactive metabolite in skin, which is the underlying cause of skin rashes (red blotches, itchy lumps, and/or speckles) in about one in five people starting nevirapine treatment, which qualifies as a serious (life-threatening) idiosyncratic reaction in approximately 0.3% of patients on this antiretroviral drug. Covalent binding to protein and rash can be induced by nevirapine in the human and rat skin, but not in the mouse skin, which does not sulfonate the benzyl alcohol metabolite of nevirapine. In the human and rat skin, covalent binding and rash caused by nevirapine or its benzyl alcohol metabolite can be blocked by local application of 1-phenyl-1-hexanol, a SULT inhibitor (Uetrecht and Naisbitt, 2013). These studies underscore the importance of SULTs in extrahepatic tissues in the formation of reactive metabolites.

Although carboxylic acids can be conjugated with glucuronic acid, they cannot be conjugated with sulfonate. However, a number of carboxylic acids, such as benzoic acid, naphthoic acid, naphthylacetic acid, salicylic acid, and naproxen, are inhibitors of SULTs (Rao and Duffel, 1991). Pentachlorophenol and 2,6-dichloro-4-nitrophenol are potent SULT inhibitors because they bind to the enzyme but cannot initiate a nucleophilic attack on PAPS due to the presence of electron-withdrawing substituents in the *ortho*- and *para*-positions on the aromatic ring. In addition to these direct inhibitors, salicylamide and 1-phenyl-1-hexanol, both of which are SULT substrates that undergo rapid sulfonation, are used in vitro and in animals to cause generalized inhibition of SULT activity by depleting PAPS, which was exploited, for example, in studies of the role of SULT in an idiosyncratic reaction to nevirapine (namely skin rash), as discussed earlier in this section. Inhibition of SULTs can occur with exposure to drugs such as mefenamic acid, salicylic acid, clomiphene, danazol, environmental chemicals, such as hydroxylated PCBs, hydroxylated PAHs, pentachlorophenol, triclosan, and bisphenol A, and dietary constituents, such as catechins, colorants, phytoestrogens, and flavonoids. Adverse effects on human health can potentially result from SULT inhibition, such as the thyroid hormone disruption, that occurs with exposure to hydroxylated PCBs (Wang and James, 2006). In contrast, given that some sulfonate conjugates are chemically reactive (discussed earlier in this section), inhibition of their formation may be protective. There are a few reports of drug–drug interactions due to SULT inhibition. It has been reported that the sulfonation rates of both acetaminophen and salicylamide are decreased when these drugs are administered together, and dapsone and lamivudine have been found to decrease acetaminophen sulfonation (Wang and James, 2006). Coadministration of acetaminophen with ethinyl estradiol increases its AUC by up to 54% and decreases the AUC of ethinyl estradiol sulfate by about 40%, indicating that acetaminophen may directly inhibit one or more SULTs (Rogers et al., 1987). Drug–drug interactions involving induction of SULT are detailed later in this section.

Sulfonate conjugates of xenobiotics are excreted mainly in urine. Those excreted in bile may be hydrolyzed by aryl sulfatases present in gut microbiota, which contributes to the enterohepatic circulation of certain xenobiotics. Sulfatases are also present in the endoplasmic reticulum and lysosomes, where they primarily hydrolyze sulfonates of endogenous compounds, presumably in a manner analogous to that described for microsomal β-glucuronidase (Dwivedi et al., 1987) (see comments on egasyn in the section "Carboxylesterases"). Like glucuronide conjugates, some sulfonate conjugates are substrates for further biotransformation. For instance, the 7- and 4-sulfates of daidzein and genistein can be sulfonated by SULT1E1 to disulfates (Nakano et al., 2004). Other examples include the oxidation of sulfonate conjugates of testosterone and estrogens. Dehydroepiandrosterone-3-sulfate is 16α-hydroxylated by CYP3A7, the major CYP enzyme expressed in the human fetal liver (Ingelman-Sundberg et al., 1975; Kitada et al., 1987; Ohmori et al., 1998). CYP2C12, which is expressed in female but not male rats, catalyzes the oxidation of a steroid *di*-sulfate (namely, 5α-androstane-3α,17β-diol-3,17-disulfate) (Ryan et al., 1984). Sulfonation facilitates the deiodination of T4 and

triiodothyronine (T3) and can determine the rate of elimination of thyroid hormones in some species. Efavirenz sulfate is conjugated with GSH in rodents but not primates and accounts for the rodent-specific nephrotoxicity of this antiretroviral drug, as described in the section "Glutathione Conjugation."

Multiple SULTs have been identified in all mammalian species examined and they are arranged into gene families that share at least 45% amino acid sequence identity, which are subdivided into subfamilies that are at least 60% identical (Blanchard et al., 2004; Gamage et al., 2006). The SULTs expressed in mammals belong to six families (SULT1 to SULT6). Humans express SULT only in families 1, 2, 4, and 6. SULT3 is expressed only in mice and rabbits, whereas SULT5 is expressed only in mice (Chen et al., 2015a). SULT1 and SULT2 are the only gene families subdivided into multiple subfamilies: five in the case of SULT1 (SULT1A to SULT1E), and two in the case of SULT2 (SULT2A and SULT2B). As shown in Table 6-23, humans express 14 SULTs, namely, SULT1A1, 1A2, 1A3, 1A4, 1B1, 1C2, 1C3, 1C4, 1D1, 1E1, 2A1, 2B1, 4A1, and 6B1. Human SULT4A1 is expressed in the brain and SULT6B1 is expressed in the testis and kidney. Their role in xenobiotic sulfonation is largely unknown, as is their presumptive physiological function. Sulfonation of xenobiotics is catalyzed by various members of the SULT1 and SULT2 families. SULT1 enzymes catalyze the sulfonation of phenols, isoflavones, the procarcinogen NOH-AAF, endogenous compounds such as 17β-estradiol (including its glucuronide conjugate; see the section "Glucuronidation and Formation of Acyl-CoA Thioesters"), and other steroids, thyroid hormones, endogenous catecholamines, and eicosanoids. The SULT2 enzymes catalyze the sulfonation of the 3β-hydroxy groups of steroids (in contrast to UGTs, which glucuronidate the 3α-hydroxy groups of steroids), bile acids, benzylic alcohols of PAHs, and other primary and secondary alcohols.

SULTs are expressed in a wide range of tissues. Based on quantitative immunoblotting techniques, Riches et al. (2009) quantified five of the major SULT enzymes involved in xenobiotic sulfonation (SULT1A1, 1B1, 1E1, 2A1, and 1A3) in cytosol from the human liver, small intestine, kidney, and lung. In human liver cytosol, the rank order of protein expression was as follows: SULT1A1 (53%) > 2A1 (27%) > 1B1 (14%) > 1E1 (6%) > 1A3 (0%). The kidney was similar to the liver inasmuch as SULT1A1 was the major enzyme expressed (40%), followed by SULT1B1 (31%) > 1A3 (28%) > 2A1 (1%) > 1E1 (0%). In the small intestine, SULT1B1 and SULT1A3 were most highly expressed enzymes (36% and 31%, respectively), followed by SULT1A1 (19%) > 1E1 (8%) > 2A1 (6%). Lastly, SULT1E1 protein was most highly expressed in lung (40%), followed by SULT1A1 (20%) > 1A3 (19%) > 1B1 (12%) > 2A1 (9%). In humans, SULTs are predominantly expressed in extrahepatic tissues. Based on microarray analysis of 79 human tissues, mRNA encoding most SULT enzymes was detected in the liver but in no case did the liver have the highest levels of an individual SULT mRNA (Chen et al., 2015a). Further details on the tissue expression of SULTs are summarized in Table 6-23.

Our current understanding of the multiplicity of SULTs is based on genome sequencing, as is the case with all other classes of xenobiotic biotransforming enzymes. Two prior classifications that provided insight into the multiplicity of SULTs are briefly described here for historical reasons. In human liver cytosol, SULT activity toward phenolic compounds, the so-called phenol sulfotransferase or PST, was found to exist in two forms that could be distinguished by their thermal stability; hence, they were known as TS-PST (*thermally stable*) and TL-PST (*thermally labile*) (Weinshilboum, 1992a; Weinshilboum et al., 1997). It is now known that TS-PST largely reflects the activity of SULT1A1 and SULT1A2, whereas TL-PST largely reflects the activity of SULT1A3 and SULT1A4 (Gamage et al., 2006).

Historically, the SULT enzymes were previously categorized into five classes based on their catalytic activity. These five functional classes were as follows: *arylsulfotransferase*, which sulfonates numerous phenolic xenobiotics; *alcohol SULT*, which sulfonates primary and secondary alcohols including nonaromatic hydroxysteroids (for which reason these enzymes are also known as hydroxysteroid SULTs); *estrogen SULT*, which sulfonates estrone and other aromatic hydroxysteroids; *tyrosine ester SULT*, which sulfonates tyrosine methyl ester and 2-cyanoethyl-*N*-hydroxythioacetamide; and *bile salt SULT*, which sulfonates conjugated and unconjugated bile acids. The *arylsulfotransferase* and *estrogen SULT* are composed largely of SULT1 enzymes, which catalyze the sulfonation of phenolic xenobiotics, catechols, and aromatic (phenolic) steroids. The *alcohol SULT* and *bile salt SULT* are composed largely of SULT2 enzymes, which catalyze the sulfonation of a variety of primary and secondary alcohols, bile acids, and hydroxysteroids (such as DHEA).

Genetic polymorphisms have been described for human SULTs. They can affect SULT function and/or expression in a tissue-specific manner. For example, an allelic variant of SULT1A1 known as SULT1A1*2 ($Arg_{213} \rightarrow His_{213}$) is associated with decreased expression in platelets but not the liver, and decreased thermal stability (Glatt and Meinl, 2004a). This particular genetic polymorphism is common in both Caucasians and Nigerians (with an allele frequency of 31% and 37%, respectively), and is correlated with interindividual variation in the sulfonation of acetaminophen. SULT1A1 catalyzes the sulfonation of the 4-hydroxymetabolite of tamoxifen, a metabolite identified as a potent inducer of apoptosis in certain breast cancer cell lines. Contrary to expectation, the lower activity SULT1A1*2 allele did not improve the efficacy of tamoxifen in breast cancer patients by decreasing clearance of 4-hydroxytamoxifen. It was the wild-type (*1) allele that, paradoxically, was correlated with improved outcomes with tamoxifen therapy (Nowell and Falany, 2006). Human SULT1B1, like the corresponding enzyme in other species, catalyzes the sulfonation of thyroid hormones, 2-naphthol, and dopamine. SULT1B1 levels in human liver cytosol vary widely, possibly due to polymorphisms (e.g., $Glu_{186} \rightarrow Gly_{186}$ and $Glu_{204} \rightarrow Asp_{204}$) (Glatt and Meinl, 2004a). Polymorphisms with consequences for the bioactivation of xenobiotics have been reported. For instance, the human SULT1A*Arg (*1) allelozyme expressed in *S. typhimurium* is 12- to 350-fold more active in the sulfonation of 2-acetylamino-4-hydroxyaminotoluene, 2-nitropropoane, 2,4-dinitrobenzylalcohol, (–)-1-(α-hydroxyethyl) pyrene, and 1-hydroxymethlpyrene to mutagens than are cells expressing SULT1A1*His (Gamage et al., 2006). Enantioselective sulfonation of promutagens has been reported, as in the case of 1-(α-hydroxyethyl) pyrene, for which SULT2A1 exhibits a 15-fold preference for the (+)-enantiomer, and SULT1E1 exhibits a 160-fold preference for the (–)-enantiomer (Gamage et al., 2006).

Human SULT1E1 has been identified as a high-affinity estrogen SULT. SULT1A1 also catalyzes the sulfonation of estrogens, such as 17β-estradiol, but it does so with a much lower affinity than does SULT1E1. The sulfonation of ethinyl estradiol in human hepatocytes is inducible by rifampin (Li et al., 1999). In addition to the human liver, SULT1E1 is expressed in the placenta, breast, brain, testes, adrenal glands, and uterine tissue. SULT1E1 has been studied in SULT1E1-deficient mice, and it has been shown that these mice had spontaneous fetal loss caused by placental thrombosis, which was reversible by administration of antiestrogens (Gamage et al., 2006).

SULT2A1 is the human alcohol SULT long known as DHEA-ST (for its ability to sulfonate dehydroepiandosterone [DHEA]). In addition to DHEA, substrates for SULT2A1 include steroid hormones, bile acids, and cholesterol. Furthermore, SULT2A1 converts several procarcinogens to electrophilic metabolites, including hydroxymethyl PAHs, NOH-AAF, and 1-hydoxysafrole, as shown in Fig. 6-64. The thermal stability of SULT2A1 is intermediate between that of the four phenol SULTs (SULT1A1/2 and SULT1A3/4), and the enzyme is resistant to the inhibitory effects of 2,6-dichloro-4-nitrophenol. In humans, SULT2A1 selectively catalyzes the *N*-sulfonation of quinolone drugs, such as ciprofloxacin, moxifloxacin, garenoxacin, and other amine drugs such as desipramine and metoclopramide (Senggunprai et al., 2009). SULT2A1 is not expressed in blood platelets, but the activity of this enzyme has been measured in human liver cytosol. It is also expressed in adrenal cortex, brain, and intestine (Blanchard et al., 2004). SULT2A1 is bimodally distributed, possibly due to a genetic polymorphism that apparently lies outside of the coding region, and perhaps outside of the SULT2A1 gene itself, with a high-activity group composed of about 25% of the population (Glatt and Meinl, 2004a). Several SULT2A1 SNPs have been identified, but the underlying basis for the high-activity group remains to be determined as these polymorphisms appear to be too rare to explain the bimodal distribution.

Human SULT2B1 is also a DHEA sulfotransferase. It is expressed in the placenta, prostate, and trachea. The SULT2B1 gene can be transcribed from one of the two exons, both of which contain coding sequences; hence, two forms of SULT2B1 (known as 2B1_v1 and 2B1_v2) with different N-terminal amino acid sequences can be transcribed by alternate splicing of precursor mRNA. This situation is analogous to the alternative splicing of multiple exons 1 in the UGT1 gene family (see Fig. 6-61). SULT2B1_v1 catalyzes the sulfonation of pregnenolone, and SULT2B1_v2 can catalyze the sulfonation of both pregnenolone and cholesterol (Blanchard et al., 2004).

Extrapolation of animal data with regard to biotransformation by SULTs is confounded by the number of SULTs, the expression pattern of SULTs, and pronounced gender differences in rodents (Gamage et al., 2006; Coughtrie, 2016). For instance, expression of SULTs in humans is largely extrahepatic, whereas SULT expression in rodents is predominantly hepatic. Based on microarray analysis of 79 human tissues, mRNA encoding most SULT enzymes was detected in the liver but in no case did the liver have the highest levels of an individual SULT mRNA (Chen et al., 2015a). Additionally, humans express four members of the SULT1A subfamily (1A1 to 1A4), whereas rodents express only one. In contrast, rats express four members of the SULT2A subfamily, whereas humans have only a single SULT2A gene (SULT2A1). Equivalents of mouse SULT3A1 and SULT5A1 are not expressed in humans (Gamage et al., 2006). There are also significant differences between other mammalian species. For instance, SULT activity is generally low in pigs but high in cats. The high SULT activity in cats offsets their low capacity to conjugate xenobiotics with glucuronic acid.

In rats, SULT activity varies considerably with sex and age. In mature rats, expression of various SULTs in the liver is greater in males than females: approximately twofold in the case of SULT1A1 and 10-fold in the case of SULT1C1. SULT1E1 is undetectable in the female rat liver and qualifies as a male-specific SULT in adult rats (Coughtrie, 2016). In contrast, various SULT2 enzymes are expressed to a greater extent in mature female rats and mice compared with their male counterparts (Coughtrie, 2016). Sex differences in the developmental expression of individual SULTs are the result of a complex interplay between gonadal, thyroidal, and pituitary hormones (particularly growth hormone), which similarly determine sex differences in CYP enzyme expression. However, compared with CYP enzymes, the SULTs are refractory or only marginally responsive to the enzyme-inducing effects of AhR and CAR activators such as 3-methylcholanthrene and phenobarbital, respectively, although one or more individual SULT2 enzymes are inducible by PXR agonists such as PCN. Likewise, SULT1A1, 2A1, or 2E1 expressed in Caco-2 cells are refractory to various PAHs, and 3-methylcholanthrene has no effect on SULT1A1 and SULT1A3 mRNA levels in primary human hepatocytes (Gamage et al., 2006). From rodent studies, it is generally held that AhR agonists have suppressive effects on SULT regulation. 2-AAF, dioxin, 3-methylcholanthrene, and β-naphthoflavone markedly suppress SULT1A1 and SULT12A activities and mRNA levels in rat livers (Gamage et al., 2006). In contrast, there is evidence that the rat liver hydroxysteroid SULTs (SULT2s) may be inducible by tamoxifen and estrogens (Gamage et al., 2006). For mouse SULT2A2, a functional nuclear response element responsive for CAR has been reported (Gamage et al., 2006). Human SULT2A1 has been reported to be regulated by FXR, PXR, VDR, and PPARα, whereas SULT1A1, 2A1, and 2A9 genes in mice are regulated by CAR (Tirona and Kim, 2005; Gamage et al., 2006). There are conflicting data regarding the influence of CAR ligands on SULT1A1 and SULT1A3. One study reports an 11-fold increase in SULT1A1 mRNA in primary human hepatocytes by the CAR ligand, CITCO, whereas another study could not reproduce these results for either SULT1A1 or SULT1A3 (Gamage et al., 2006). The glucocorticoid dexamethasone has been reported to induce both murine and human SULT2A1 through PXR and GR activation (Gamage et al., 2006). Human SULT2A1 mRNA and protein have also been reported to be induced by rifampin, vitamin D_3, phenobarbital, TCPOBOP, and the PPARα-agonist ciprofibrate (Runge-Morris and Kocarek, 2005). Recent reviews reinforce the complexity of the role of xenosensors (including FXR and LXR) in the induction and suppression of SULT enzymes, which often occurs in a species-, gender- and tissue-specific manner (Kodama and Negishi, 2013; Runge-Morris et al., 2013).

Induction of SULTs by rifampin may be clinically relevant. Rifampin (600 mg q.d.) has been reported to cause up to a 190% increase in the clearance of ethinyl estradiol (35 μg q.d.) (Barditch-Crovo et al., 1999). The interaction between ethinyl estradiol–containing oral contraceptives and antibiotics such as rifampin is often attributed to the induction of CYP3A4, which is the major CYP involved in the *oxidative* metabolism of ethinyl estradiol (e.g., Ortho-Evra® prescribing information). Several lines of evidence suggest that induction of CYP3A4 is not the predominant mechanism by which rifampin increases the clearance of ethinyl estradiol. First, Li et al. (1999) reported that treatment of primary cultures of human hepatocytes with rifampin (33.3 nM) caused up to a 3.3-fold increase in ethinyl estradiol 3-*O*-sulfate formation. Second, SULTs 1A1, 1A2, 1A3, 1E1, and 2A1 catalyze the 3-*O*-sulfonation of ethinyl estradiol with K_m values ranging from 6.7 to 4500 nM, nearer the pharmacologically relevant concentrations (Schrag et al., 2004). Finally, it is known that ethinyl estradiol is predominantly excreted in bile and urine as the 3-sulfate and, to a lesser extent, the 3-glucuronide (Li et al., 1999), which suggests that 3-sulfonation is the major pathway of ethinyl estradiol metabolism. Taken together, these data suggest that induction of SULTs can be clinically relevant at least for low-dose drugs that can be sulfonated with high affinity.

Some drugs must be converted to a sulfonate conjugate to exert their desired effect, including triamterene, cicletanine, and minoxidil (Wang and James, 2006). Sulfonation (as well as glucuronidation) converts morphine to more potent analgesics than the parent, with morphine-6-sulfate being 30 times more potent, and with morphine-6-glucuronide being 45 to 800 times more potent than morphine itself in rats (Wang and James, 2006). Similarly, several

sulfonated steroids such as pregnenolone sulfate and DHEA sulfate interact directly with neurotransmitter receptors. It has been found that pregnenolone sulfate and DHEA sulfate enhance memory in mice. Prevention of hydrolysis of these sulfates by the steroid sulfatase inhibitor (*para*-*O*-sulfamoyl)-*N*-tetradecanoyl tyramine increases the memory enhancement caused by DHEA sulfate in rats, which suggests there is an important role of these sulfates in the central nervous system (Wang and James, 2006). Other examples of pharmacologically active sulfonated drugs and endogenous compounds have been reported (Chen et al., 2015a).

Methylation

Methylation is a common but generally minor pathway of xenobiotic biotransformation. It differs from most other conjugation reactions by causing only modest changes in log *P* and TPSA, as shown in Table 6-3, which can decrease, rather than increase, the aqueous solubility of xenobiotics. One exception to this rule is the *N*-methylation of pyridine-containing xenobiotics, such as nicotine, which produces quaternary ammonium ions that are water-soluble and readily excreted. Another exception is the *S*-methylation of thioethers to form positively charged sulfonium ions, a reaction catalyzed by thioether methyltransferase (TEMT), which has only been identified in mice (Weinshilboum et al., 1999). Methylation masks functional groups (–OH, –SH, and –NH_2) that might otherwise be metabolized by other conjugating enzymes, which raises the question: Why did methylation evolve as a pathway of xenobiotic biotransformation? In the preceding section "Sulfonation," conjugation of certain alcohols and phenols with sulfonic acid was identified as a mechanism of activation of certain xenobiotics (e.g., those shown in Fig. 6-64). In contrast, methylation is not a mechanism of xenobiotic activation with the possible exception of arsenic (discussed later in the section). Furthermore, in the case of catechols, which are redox active and can nonenzymatically generate reactive oxygen species, methylation represents an important pathway of detoxication.

The cofactor for methylation is *S*-adenosylmethionine (SAM), the structure of which is shown in Fig. 6-57. The methyl group bound to the sulfonium ion in SAM has the characteristics of a carbonium ion and is transferred to xenobiotics and endogenous substrates by nucleophilic attack from an electron-rich heteroatom (*O*, *N*, or *S*). Consequently, the functional groups involved in methylation reactions are phenols, catechols, aliphatic and aromatic amines, *N*-heterocyclics, and sulfhydryl-containing compounds. The conversion of benzo[*a*]pyrene (B[*a*]P) to 6-methylbenzo[*a*]pyrene is a rare example of *C*-methylation. Another reaction that appears to involve *C*-methylation, the conversion of cocaine to ethylcocaine, is actually a transesterification reaction catalyzed by CES1, as shown in Fig. 6-9. Metals can also be methylated. Both inorganic mercury and arsenic can be dimethylated, and inorganic selenium can be trimethylated. The selenium atom in ebselen is methylated following the ring opening of this anti-inflammatory drug. Some examples of xenobiotics and endogenous substrates that undergo *O*-, *N*-, or *S*-methylation are shown in Fig. 6-65. During

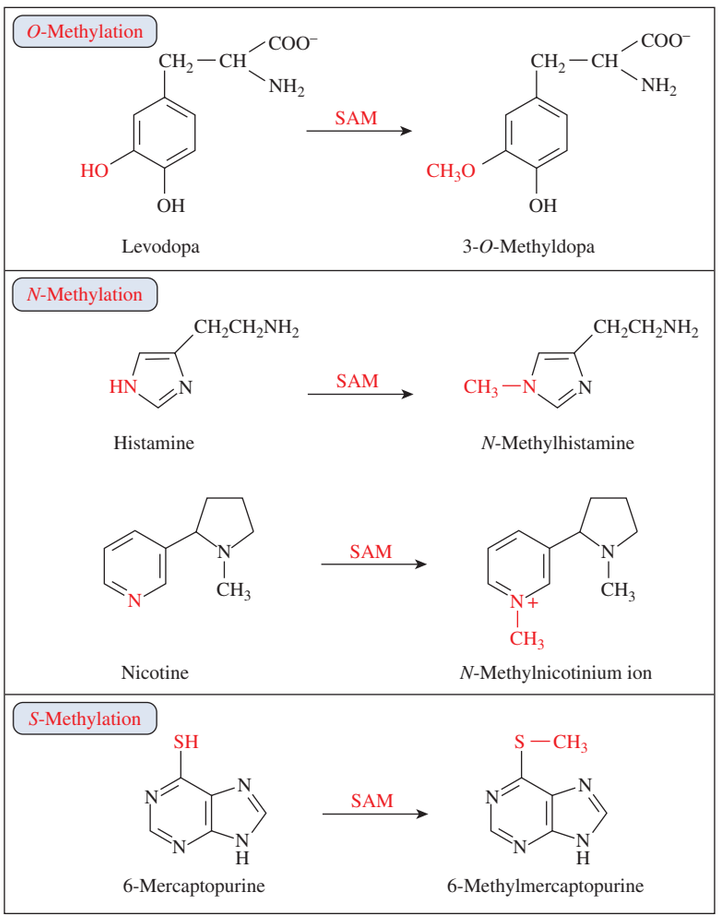


Figure 6-65. *Examples of compounds that undergo O-, N-, or S-methylation.*

these methylation reactions, SAM is converted to *S*-adenosyl-L-homocysteine (SAH). This section will cover the following methyltransferases: arsenic (III) methyltransferase (AS3MT), catechol-*O*-methyltransferase (COMT), glycine *N*-methyltransferase (GNMT), histamine *N*-methyltransferase (HNMT), indolethylamine *N*-methyltransferase (INMT), nicotinamide *N*-methyltransferase (NNMT), phenylethanolamine *N*-methyltransferase (PNMT), phenol *O*-methyltransferase (POMT) (thiol methyltransferase [TMT]), TEMT, and thiopurine methyltransferase (TPMT). Other methyltransferases that have been implicated as drug-metabolizing enzymes include guanidinoacetate *N*-methyltransferase (GAMT) and phosphatidylethanolamine *N*-methyltransferase (PEMT) (Nishimura and Naito, 2006).

The *O*-methylation of phenols and catechols is catalyzed by two different enzymes known as COMT and the enzyme historically termed POMT (Weinshilboum, 1989, 1992b). POMT is a microsomal enzyme that methylates phenols but not catechols, and COMT is both a cytosolic and microsomal enzyme with the converse substrate specificity, that is, an absolute requirement for catechol substrates (Weinshilboum, 2006). COMT plays a greater role in the biotransformation of catechols than POMT plays in the biotransformation of phenols. It should be noted that there is strong evidence to suggest that the membrane-bound POMT is the same enzyme as TMT (Weinshilboum, 2006). COMT was originally described as a cytosolic, Mg^{2+}-requiring, monomeric enzyme (M_r 25,000). However, in rats and humans, the enzyme is encoded by the COMT gene (on chromosome 22 in humans) with two different promoters and transcription initiation sites. Transcription at one site produces a cytosolic form of COMT, whereas transcription from the other site produces a membrane-bound form by adding 50 hydrophobic amino acids to the N-terminus of the microsomal COMT, which targets this form to the endoplasmic reticulum (Weinshilboum et al., 1999; Weinshilboum, 2006). The microsomal COMT is expressed at high levels in the brain and lymphocytes (Weinshilboum, 2006). The cytosolic form of COMT is present in virtually all tissues, including erythrocytes, but the highest concentrations are found in the liver and kidney. The membrane-bound form is more highly expressed in brain.

Substrates for COMT include several catecholamine neurotransmitters, such as epinephrine, norepinephrine, and dopamine; and catechol drugs, such as the anti-Parkinson disease agent levodopa (L-3,4-dihydroxyphenylalanine) and the antihypertensive drug methyldopa (α-methyl-3,4-dihydroxyphenylalanine). Catechol estrogens, which are formed by 2- or 4-hydroxylation of the steroid A-ring, are substrates for COMT, as are drugs that are converted to catechols by two consecutive hydroxylation reactions (as in the case of phenobarbital, duloxetine, and diclofenac), by ring opening of a phenyl methylenedioxy (benzodioxole) group (as in the case of stiripentol and 3,4-methylenedioxymethamphetamine), or by hydrolysis of vicinal esters (as in the case of ibopamine). Formation of catechol estrogens, particularly 4-hydroxyestradiol, has been suggested to play an important role in estrogen-induced tumor formation in hamster kidney, rat pituitary, and mouse uterus (Zhu and Liehr, 1993) (see the section "CYP1B1"). These tissues contain high levels of epinephrine or dopamine, which inhibit the *O*-methylation of 4-hydroxyestradiol by COMT. Nontarget tissues do not contain high levels of catecholamines, which suggests that 4-hydroxyestradiol induces tumor formation in those tissues that fail to methylate and detoxify this catechol estrogen. These observations in animals are especially intriguing in view of subsequent epidemiological evidence demonstrating that low COMT activity appears to increase the risk of breast cancer, with odds ratios ranging from 1.7 to 3.0 (Weinshilboum, 2006).

In the 1970s, when COMT levels in erythrocytes (predominantly the cytosolic form) were measured in human subjects, it was apparent that there was a subpopulation that displayed low levels of this enzyme. Segregation analysis indicated that erythrocyte COMT activity was an autosomal codominant trait, and that erythrocyte levels correlated with relative COMT levels in liver and lymphocyte cytosol (Weinshilboum, 2006). It was subsequently found that COMT is encoded by a single gene with alleles for a low-activity form ($COMT^L$) and high-activity form ($COMT^H$) (Weinshilboum, 1989, 1992b, 2006; Weinshilboum et al., 1999). This polymorphism results from a single G → A transition in exon 4 that results in the substitution Val_{108}Met in cytosolic COMT and Val_{158}Met in microsomal COMT (Weinshilboum et al., 1999; Weinshilboum, 2006). The presence of methionine at position 108 in the cytosolic enzyme not only decreases the catalytic activity of COMT but also decreases the thermal stability of the enzyme, which has long been used to differentiate $COMT^L$ (thermolabile) from $COMT^H$ (thermostable). In Caucasians, these allelic variants are expressed with roughly equal frequency, so that 25% to 30% of the population is homozygous for Met/Met, the low activity COMT, 20% to 25% is homozygous for Val/Val, the high activity COMT, and 50% is heterozygous (Met/Val) and has intermediate COMT activity. The Val/Val form of COMT is three- to fourfold more active than the Met/Met form of COMT. COMT activity is generally higher in Asians and African Americans due to a higher frequency of $COMT^H$ (the Val allele) (about 75% for Asians and African Americans compared with about 50% for Caucasians) (McLeod et al., 1994). Subsequent resequencing of the COMT gene has revealed numerous SNPs, with at least eight that occur with a frequency of more than 10% in Caucasians, and 11 such SNPs in African Americans. Several of these SNPs are found in the intronic regions. A list of COMT alleles can be found at http://alfred.med.yale.edu/alfred/index.asp.

The genetically determined levels of COMT in erythrocytes correlate with individual differences in the proportion of levodopa converted to 3-*O*-methyldopa and the proportion of methyldopa converted to its 3-*O*-methyl metabolite. *O*-Methylation is normally a minor pathway of levodopa biotransformation, but 3-*O*-methyldopa is the major metabolite when levodopa is administered with a dopa decarboxylase inhibitor, such as carbidopa or benserazide, which is a common clinical practice. High COMT activity, resulting in extensive *O*-methylation of levodopa to 3-*O*-methyldopa, has been associated with poor therapeutic management of Parkinson disease and an increased incidence of drug-induced toxicity (dyskinesia). A large number of epidemiological studies have been performed to examine the effects of the COMT $Val_{108/158}$Met polymorphism, and there is no evidence that the genetic polymorphism in COMT represents a risk modifier for the development of Parkinson disease (Weinshilboum et al., 1999). Talcopone, entacapone, and opicapone are drugs that inhibit COMT and are used (together with inhibitors of dopa decarboxylase) to slow the metabolism and lower the dose of levodopa in Parkinson patients (Muller, 2015). All three COMT inhibitors are nitrocatechols (catechols with an adjacent nitro group).

Egan et al. (2001) reported that COMT genotype is related in an allele-dosage manner to cognitive performance, with Met/Met individuals ($COMT^L$ phenotype) demonstrating increased executive cognition, as measured by the Wisconsin Card Sorting Test. The impact of the low-activity COMT allele was attributed to decreased dopamine catabolism in the prefrontal cortex, which results in enhanced neuronal function. Conversely, those individuals who are homozygous for Val_{108} ($COMT^H$ phenotype) appear to have decreased executive cognition, and may be at a slightly increased risk of developing schizophrenia (Egan et al., 2001). Modafinil, a

drug approved for the treatment of narcolepsy and other sleep disorders, allows better executive function performance and better vigilance throughout sleep deprivation in individuals with high COMT activity (the Val/Val allelic variant). Due to their higher brain levels of dopamine, individuals with low COMT activity (the Met/Met allelic variant) respond to lower doses of modafinil than individuals with intermediate (Val/Met) or high (Val/Val) COMT activity (Dauvilliers et al., 2015).

Several *N*-methyltransferases have been described in humans and other mammals, including PNMT, which catalyzes the *N*-methylation of the neurotransmitter norepinephrine to form epinephrine; HNMT, which specifically methylates the imidazole ring of histamine and closely related compounds (Fig. 6-65); and NNMT, which methylates compounds containing a pyridine ring, such as nicotinamide, or an indole ring, such as tryptophan and serotonin (Weinshilboum, 1989, 1992b; Weinshilboum et al., 1999). PNMT is a cytosolic enzyme expressed at high levels in adrenal medullary chromafin cells, and in neurons of the medulla oblongata, hypothalamus, as well as in sensory nuclei of the vagus nerve and the retina, and is not thought to play a significant role in the biotransformation of xenobiotics (Ji et al., 2005; Testa and Krämer, 2008, 2010).

HNMT is a cytosolic enzyme (M_r 33,000) that is highly expressed in kidney, liver, colon, prostate, ovary, and spinal cord cells (Horton et al., 2005). Its activity (which can be measured in erythrocytes) varies sixfold among individuals due to a genetic polymorphism (C → T) that results in a point mutation, namely, $Thr_{115}Ile$. The latter allele (Ile_{115}) is quite common in Caucasians and Han Chinese (10% frequency) and encodes a variant of HNMT with decreased catalytic activity and thermal stability. HNMT may influence efficacy of some drugs by a mechanism that is not yet fully understood. For instance, individuals who are heterozygous for the Ile_{115} allele have been found to exhibit significantly decreased methylprednisolone-induced cortisol suppression relative to (Thr_{115})-homozygous individuals (Hon et al., 2006). Several other polymorphisms in the noncoding region of the HNMT gene have also been identified. HNMT can be inhibited by several antihistamines, quinacrine, amodiaquine, metoprine, and tacrine (Horton et al., 2005).

NNMT is a monomeric, cytosolic enzyme (M_r ~30,000) that appears to be a member of a family of methyltransferases that includes PNMT and TEMT (the thioether *S*-methyltransferase present in mouse lung). It catalyzes the *N*-methylation of nicotinamide and structurally related pyridine compounds (including pyridine itself) to form positively charged pyridinium ions. Nicotinic acid (niacin), a commonly used lipid-lowering agent, is converted to nicotinamide in vivo, which is then methylated by NNMT (or it is incorporated into nicotinamide adenine dinucleotide [NAD]). In contrast to many other methyltransferases, NNMT is not expressed in erythrocytes.

NNMT activity in the human liver, like HNMT activity in erythrocytes, varies considerably from one individual to the next, and has a trimodal distribution dependent on variations in mRNA and protein levels, and up to 25% of the general population has high NNMT levels (Souto et al., 2005; Williams et al., 2005). It is not known to what extent genetic polymorphisms account for this variation. However, 10 SNPs in the untranslated regions of the gene have been detected in a Spanish population (Souto et al., 2005). A genome-wide scan for genes associated with plasma homocysteine levels determined that there was a statistically significant association with the NNMT gene, and, moreover, that 1 SNP (dbSNP ID#: rs694539) has a greater statistically significant association with homocysteine levels ($P = .017$) (Souto et al., 2005). Homocysteine plasma levels are an independent intermediate risk marker for osteoporotic fractures, congestive heart failure, venous thrombosis, myocardial infarction, stroke, and Alzheimer disease (Souto et al., 2005). In humans, the only source of homocysteine is from the demethylation of methionine in a multistep pathway that involves SAM-dependent methyltransferases to form *S*-adenosylhomocysteine, the immediate precursor to homocysteine. Taken together, these data suggest that high methyltransferase activity could contribute to hyperhomocysteinemia. Of the many SAM-dependent methyltransferase genes examined for an association with homocysteine levels, only NNMT (which is highly expressed in the liver) was found to show a significant association (Souto et al., 2005). NNMT is reported to be expressed in the brain and has been implicated as a component of the etiology of idiopathic Parkinson disease because it can convert 4-phenylpyridine to MPP^+, which is known to cause Parkinson disease symptoms due to its toxic effect on neuronal mitochondria (see the section "Amine Oxidases" and Fig. 6-33) (Williams et al., 2005).

There are numerous other human *N*-methyltransferases (as well as *O*-, *S*-, and *C*-methyltransferases) that appear to play relatively specific roles in the methylation of endogenous compounds, and most have not been well characterized with regard to their capability to methylate xenobiotics (there are at least 39 SAM-dependent methyltransferases in humans) (Souto et al., 2005). For instance, INMT catalyzes the *N*-methylation of tryptamine and structurally related compounds (Thompson et al., 1999). Other such enzymes that were initially thought to play a role only in the *N*-methylation of endogenous compounds were later found to play a role, albeit a minor one, in the *N*-methylation of one or more xenobiotics. Amine *N*-methyltransferase (AMNT, also called arylamine *N*-methyltransferase or nicotine *N*-methyltransferase), which is highly expressed in the human thyroid and is also found in the adrenal gland and lung, exhibits some activity toward tryptamine and has been also found to preferentially methylate the pyridine nitrogen of *R*-nicotine, which gives rise to nicotine isomethonium ions (Hukkanen et al., 2005). GNMT is thought to play an important role in the regulation of methyl group metabolism in the liver and pancreas through regulation of the ratio between *S*-adenosyl-L-methionine and SAH. Rat data show that the tetrameric form of the GNMT has catalytic activity, and the dimeric form binds PAHs. There is also evidence that the dimeric form of human GNMT sequesters B[*a*]P, and thereby decreases its cytotoxic effects (Chen et al., 2004b; Lee et al., 2006).

The system that is used to classify human *N*-methyltransferases may not be appropriate for other species. In guinea pigs, for example, both nicotine and histamine are methylated by a common *N*-methyltransferase. Guinea pigs have an unusually high capacity to methylate histamine and xenobiotics. The major route of nicotine biotransformation in the guinea pig is methylation, although *R*-nicotine is preferentially methylated over its *S*-enantiomer (Cundy et al., 1985). Guinea pigs also methylate the imidazole ring of cimetidine.

S-Methylation is an important pathway in the biotransformation of sulfhydryl-containing xenobiotics, such as the antihypertensive drug captopril, the antirheumatic agent D-penicillamine, the antineoplastic and immunosuppressive drugs 6-mercaptopurine, 6-thioguanine, and azathioprine, metabolites of the alcohol deterrent disulfiram, and the deacetylated metabolite of the antidiuretic, spironolactone. In humans, *S*-methylation is catalyzed by at least two enzymes TPMT and TMT (which may be the same enzyme as POMT).

TPMT is a cytoplasmic enzyme that preferentially methylates aromatic and heterocyclic compounds such as the thiopurine

drugs 6-mercaptopurine, 6-thioguanine, and azathioprine. TMT is a microsomal enzyme that preferentially methylates aliphatic sulfhydryl compounds such as captopril, D-penicillamine, and disulfiram derivatives. It has also been found to methylate the heterocyclic thiol-containing leaving groups of some cephalosporins (Wood et al., 2002), the thiazolidinedione drug, MK-0767, a dual α/γ PPAR agonist (Karanam et al., 2004), dithiothreitol (Weinshilboum, 2006), and some thiofuran flavoring agents (Lake et al., 2003). Although a gene that encodes TMT has not yet been definitively identified, there is strong evidence to suggest that the membrane-bound POMT is the same enzyme as TMT, which means that TMT could also catalyze the *O*-methylation of phenols (Weinshilboum, 2006).

Both TMT and TPMT are present in erythrocytes at levels that reflect the expression of TPMT and TMT in liver and other tissues. Although TPMT and TMT are independently regulated, their expression in erythrocytes is largely determined by genetic factors. TPMT is encoded by a single gene with alleles for a low-activity form ($TPMT^L$) and for a high-activity form ($TPMT^H$). The allele frequencies of $TPMT^L$ and $TPMT^H$ are 0.06 and 0.94, respectively, which produces a trimodal distribution of TPMT activity with low, intermediate, and high activity expressed in 0.3%, 11.1%, and 88.6% of Caucasians, respectively. At least 28 separate genetic polymorphisms have been identified and most are associated with low TPMT activity, with the *2, *3A, *3B, and *3C alleles accounting for 80% to 95% of the $TPMT^L$ phenotype (Weinshilboum, 2006; Ujiie et al., 2008; Wang et al., 2010a; Asadov et al., 2017; Liu et al., 2017a; Tamm et al., 2017). In Caucasians, the allele that is most commonly associated with the $TPMT^L$ phenotype is TPMT*3A (5%), which contains two nonsynonymous SNPs: $Ala_{154}Thr$ and $Tyr_{240}Cys$ (Wood et al., 2006). These amino acid changes (and those in the TPMT*3B and TPMT*3C variants) lead to aggregation and rapid degradation of expressed TPMT by a ubiquitin/proteasome-dependent mechanism (Wang et al., 2005). Another variant TPMT*4 results in alternative TPMT mRNA splicing and reduced enzyme expression, leading to low TPMT activity in individuals carrying this allele (Wang et al., 2010a).

Thiopurine drugs such as 6-mercaptopurine and its prodrug azathioprine are widely used in the treatment of cancer, such as acute lymphoblastic leukemia, inflammatory disorders, such as inflammatory bowel disease, and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythromatosus, autoimmune hepatitis, and generalized eczematous disorders. Patients with low TPMT activity are at increased risk of thiopruine toxicity, which includes myelosuppression (bone marrow toxicity), gastrointestinal disturbances (nausea and vomiting), hepatotoxicity, and pancreatitis, which can lead to discontinuation of treatment with 6-merceptopurine or azathioprine in up to one-third of patients (Weinshilboum, 1989, 1992b; Liu et al., 2015b; Abaji and Krajinovic, 2017; Asadov et al., 2017). The thiopurine drugs metabolized by TPMT have a relatively narrow therapeutic index. They are metabolized by xanthine oxidoreductase (in addition to TPMT), but because xanthine oxidoreductase is not present in hematopoietic tissues, TPMT activity in these tissues is more important in the avoidance of life-threatening myelosuppression at standard doses (Weinshilboum, 2006). Phenotyping for the TPMT genetic polymorphism represents one of the first examples in which testing for a genetic variant has entered standard clinical practice (Weinshilboum et al., 1999; Liu et al., 2015b; Abaji and Krajinovic, 2017; Asadov et al., 2017). The clinical relevance of TPMT polymorphisms is reflected by the inclusion of TPMT as a "valid biomarker" for pharmacogenomics, along with CYP2D6 polymorphisms, in the FDA's 2005 "Guidance for Pharmacogenomic Data Submission." TPMT can be inhibited by benzoic acid derivatives, which also complicates therapy with drugs that are metabolized by TPMT. Patients with inflammatory bowel disorders such as Crohn disease are often treated with thiopurine drugs, which are metabolized by TPMT, and with sulfasalazine or olsalazine, which are potent TPMT inhibitors. The combination of these drugs can lead to thiopurine-induced myelosuppression.

A genetic polymorphism for TMT also has been described, but its pharmacological and toxicological significance remains to be determined. The molecular basis for the polymorphism has not been determined, but studies have shown that 98% of the fivefold individual variation in erythrocyte TMT activity is due to inheritance, with an allele for high TMT activity having a frequency of 12%. TMT is relatively specific for aliphatic sulfhydryl compounds such as 2-mercatoethanol, captopril, D-penicillamine, and *N*-acetylcysteine. It also rapidly methylates a dihydro metabolite of ziprasidone that is formed by aldehyde oxidase, as shown in Fig. 6-7 (Obach et al., 2005). TMT is present at high levels in the colonic mucosa and is also expressed in liver microsomes and erythrocyte membranes. It is not inhibited by benzoic acid derivatives, but it is inhibited by the CYP inhibitor proadifen (aka SKF-525A) (Weinshilboum et al., 1999).

As shown in Fig. 6-55, clopidogrel is a prodrug that is converted to its active metabolite, a ring-opened thiol, by sequential CYP- and GSH-dependent steps. The active metabolite, H4, is one of four diastereomers formed during this process. Due to stereopreferential metabolism by CYP enzymes, clopidogrel is converted to H3, an inactive metabolite, to a greater extent (about threefold) than H4 but the circulating levels of H3 and H4 are roughly the same. This is because microsomal TMT preferentially methylates H3 over H4 by almost 100-fold (Liu et al., 2015a).

Some of the hydrogen sulfide produced by anaerobic bacteria in the intestinal tract is converted by *S*-methyltransferases to methane thiol and then to dimethylsulfide. Another source of substrates for *S*-methyltransferases are the thioethers of GSH conjugates. GSH conjugates are hydrolyzed to cysteine conjugates, which can either be acetylated to form mercapturic acids or cleaved by cysteine-conjugate β-lyase (CCBL1). This β-lyase pathway converts the cysteine conjugate to pyruvate, ammonia, and a sulfhydryl-containing xenobiotic, which is a potential substrate for *S*-methylation.

Methylation can also lead to increased toxicity. AS3MT is a methyltransferase (previously called Cyt19) that methylates inorganic arsenic to form methylarsonic and dimethylarsonic acids, which are more cytotoxic and genotoxic than arsenate and arsenite (Wood et al., 2006). As many as 27 polymorphisms have been identified in this gene, with two rare alleles that cause markedly decreased activity and immunoreactive protein levels, and one frequent allele (i.e., about 10% in both African Americans and Caucasians) that causes increased activity and immunoreactive protein levels (Wood et al., 2006). Up to 1% of African Americans and Caucasians would be expected to be homozygous for the allele that encodes the high-activity AS3MT, and this may potentially lead to increased arsenic toxicity in such individuals. Contrary to expectation, AS3MT knockout mice are more susceptible, not less susceptible, than wild-type mice to the toxicity of arsenite (such as urinary bladder hyperplasia) (Antonelli et al., 2014). It is possible that methylation of inorganic arsenic by AS3MT represents a detoxication process in mice and an activation process in humans.

Acetylation

N-Acetylation is a major route of biotransformation for xenobiotics containing an aromatic amine (phenyl-NH_2) or a hydrazine group (R–NH–NH_2), which are converted to aromatic amides (phenyl-NH–$COCH_3$) and hydrazides (R–NH–NH–$COCH_3$), respectively

(Evans, 1992). *N*-Acetylation of aromatic amines and hydrazines occurs on nitrogen atoms with a low $pK_a(B)$, usually less than 5. Xenobiotics containing primary aliphatic amines, most of which have $pK_a(B)$ values greater than 8, are rarely substrates for *N*-acetylation. Notable exceptions are the antibacterial agent trovafloxacin, the primary metabolite of the β-adrenoreceptor blocker propranolol, and cysteine conjugates, which are formed from GSH conjugates and converted to mercapturic acids by *N*-acetylation in the kidney (see the section "Glutathione Conjugation") (Testa and Krämer, 2008, 2010). *N*-acetylation of aliphatic amines lowers their $pK_a(B)$ value from above 8 to below 6, which lowers their ion-partitioning into lysosomes and reduces their volume of distribution, as described in the section "Introduction." In a few cases, the *N*-acetylation of aliphatic acidic amines (such as the amine in a sulfonamide, which is present as an anion, not a cation) has also been documented, an example of which is *N*-acetylation of the anticonvulsant drug zonisamide (Testa and Krämer, 2008, 2010). Like methylation, *N*-acetylation has a negligible effect on log *P* and TPSA, as shown in Table 6-3; hence, *N*-acetylation has a negligible effect on aqueous solubility and does little to promote the renal and biliary excretion of xenobiotics.

The *N*-acetylation of xenobiotics is catalyzed by *N*-acetyltransferases (NATs) and requires the cofactor acetyl coenzyme A (acetyl-CoA), the structure of which is shown in Fig. 6-57. The reaction occurs in two sequential steps according to a *ping-pong Bi–Bi* mechanism (Hein, 1988). In the first step, the acetyl group from acetyl-CoA is transferred to a cysteine residue in the NAT active site with release of CoA ($E–SH + CoA–SCOCH_3 \rightarrow E–S–COCH_3 + CoA–SH$). In the second step, the acetyl group is transferred from the acylated enzyme to the amino group of the substrate with regeneration of the enzyme. For "strongly" basic amines ($pK_a(B) > 8$), the rate of *N*-acetylation is determined by the first step (acetylation of the enzyme), whereas the rate of *N*-acetylation of "weakly" basic amines ($pK_a(B) < 6$) is determined by the second step (transfer of the acetyl group from the acylated enzyme to the acceptor amine). In certain cases (discussed later in this section), NATs can catalyze the *O*-acetylation of hydroxylamines.

NATs are cytosolic enzymes found in the liver and many other tissues of most mammalian species, with the notable exception of the dog, fox, and musk shrew (*Suncus marinus*) which are unable to acetylate xenobiotics (a notable exception being the ability of dogs to *N*-acetylate cyanamide, which contains an acidic amine group) (Nakura et al., 1995; Trepanier et al., 1997; Sim et al., 2008; Testa and Krämer, 2008, 2010). In contrast to other xenobiotic-biotransforming enzymes, the number of NATs known to play a role in xenobiotic metabolism is limited (Vatsis et al., 1995; Boukouvala and Fakis, 2005). Rabbits and hamsters express only two NAT enzymes, known as NAT1 and NAT2, whereas mice and rats express three enzymes, namely, NAT1, NAT2, and NAT3. The two xenobiotic-acetylating human enzymes are NAT1 and NAT2, which are encoded by two highly polymorphic genes located on chromosome 8. Other human NAT genes are NAT6, NAT8 to NAT10, NAT14, and NAT16, located on other chromosomes. The activities and expression pattern of these enzymes have not yet been definitively characterized, although some of these genes have been associated with atopic dermatitis or psoriasis (NAT9) and nasopharyngeal cancer (NAT6) (Helms et al., 2003; Bowcock and Cookson, 2004; Duh et al., 2004; Yamada and Ymamoto, 2005; Morar et al., 2006). Individual NATs and their allelic variants were named in the order of their description in the literature, which makes for a somewhat confusing nomenclature system (Vatsis et al., 1995). For example, in humans, the "wild-type" NAT1 and NAT2 alleles are designated NAT1*4 and NAT2*4 (Hein, 2006; McDonagh et al., 2014). Information on allelic variants of NATs is available at http://nat.mbg.duth.gr/.

In each species examined, NAT1 and NAT2 are closely related proteins (79% to 95% identical in amino acid sequence) with an active-site cysteine residue (Cys_{68}) in the N-terminal region (Grant et al., 1992; Vatsis et al., 1995). In addition to the active-site cysteine residue, the presence of a histidine residue (His_{107}) and an aspartate residue (Asp_{122}) are required for activity, forming a catalytic triad necessary for the transfer of an acetyl group from acetyl-CoA to the substrate (Sandy et al., 2005; Sim et al., 2008). Human NAT1 and NAT2 genes are composed of intronless open reading frames of 870 bp on the same chromosome with a NAT pseudogene (NATP1) between them, and encode proteins of 290 amino acids that share 87% homology in the coding region (Boukouvala and Fakis, 2005; Hein, 2006).

Despite their proximity on the same chromosome, NAT1 and NAT2 are independently regulated proteins. For instance, human NAT1 protein and/or mRNA have been detected in every tissue examined (e.g., liver, gastrointestinal tract, leukocytes, erythrocytes, bladder, uroepithelial cells, mammary, lung, placenta, kidney, pineal gland, skeletal muscle, heart, brain, and pancreas) and are present from the blastocyst stage, whereas NAT2 is mainly expressed in the liver, small intestine, and codon. However, most (but not all) of the tissues that express NAT1 also express low levels of NAT2 mRNA (Debiec-Rychter et al., 1999).

NAT1 and NAT2 have different but overlapping substrate specificities, although no substrate is exclusively *N*-acetylated by one enzyme or the other. Substrates preferentially *N*-acetylated by human NAT1 include 4-aminobenzoic acid (PABA), 4-aminosalicylic acid, sulfamethoxazole, and sulfanilamide, whereas substrates preferentially *N*-acetylated by human NAT2 include isoniazid, hydralazine, procainamide, dapsone, aminoglutethimide, and sulfamethazine. Some investigators have used PABA as a selective probe/index substrate for wild-type NAT1, and either sulfamethazine or sulfadiazine as selective probe/index substrate for wild-type NAT2 (Winter and Unadkat, 2005). Some xenobiotics, such as the carcinogenic aromatic amine, 2-aminofluorene (2-AF), are *N*-acetylated equally well by NAT1 and NAT2. The calcium-sensitizing agent levosimendan, developed for the treatment of congestive heart failure, has a pharmacologically active *N*-acetylated metabolite produced by NAT2 (Testa and Krämer, 2008, 2010). Other drugs that are substrates for either NAT1 or NAT2 include acebutolol, amantadine, amonafide, amrinone, benzocaine, declopramide, metamizole, and phenelzine (Gonzalez and Tukey, 2006; Sirot et al., 2006; Sim et al., 2008).

Several drugs are *N*-acetylated following their biotransformation by hydrolysis, reduction, or oxidation. For example, caffeine is N_3-demethylated by CYP1A2 to paraxanthine (Fig. 6-50), which is then *N*-demethylated to 1-methylxanthine and *N*-acetylated to 5-acetylamino-6-formylamino-3-methyluracil (AFMU) by NAT2. The ratio of AFMU to 1-methylxanthine can be used to determine acetylator phenotype (Testa and Krämer, 2008, 2010). Other drugs converted to metabolites that are *N*-acetylated by NAT2 include sulfasalazine, nitrazepam, and clonazepam. Examples of drugs that are *N*-acetylated by NAT1 and NAT2 are shown in Fig. 6-66. It should be noted, however, that there are species differences in the substrate specificity of NATs. For example, PABA is preferentially *N*-acetylated by NAT1 in humans and rabbits but by NAT2 in mice and hamsters.

Genetic polymorphisms for *N*-acetylation have been documented in humans, hamsters, rabbits, and mice (Evans, 1992; Vatsis et al., 1995; Hirvonen, 1999; Hein et al., 2000; Sim et al., 2008). The impact of genetic polymorphisms of human NATs on drug

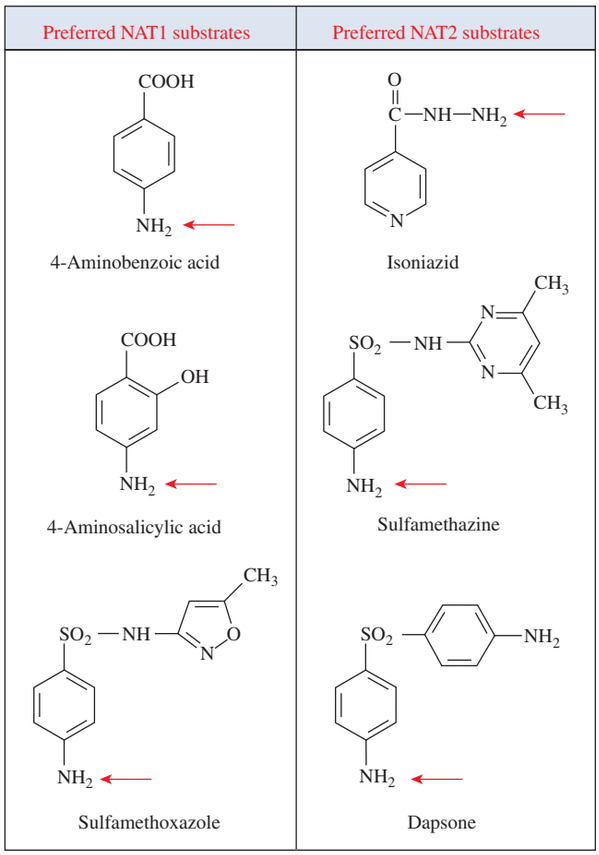


Figure 6-66. *Examples of substrates for the human N-acetyltransferases, NAT1, and the highly polymorphic NAT2.*

toxicity and environmental carcinogenesis, such as the incidence of cancer associated with cigarette smoking and the consumption of cooked meat, has attracted considerable attention. However, the literature is replete with contradictory findings (McDonagh et al., 2014). A further complication is that some genetic polymorphisms affect the *N*-acetylation of compounds in a substrate-dependent manner (McDonagh et al., 2014). NAT1 and NAT2 used to be referred to as *monomorphic* and *polymorphic* NATs because only the latter enzyme was thought to be genetically polymorphic. However, at least 28 allelic variants have now been documented for the human NAT1 gene, compared with 88 for NAT2 (McDonagh et al., 2014). In general, NAT1 allelic variants are less prevalent than NAT2 allelic variants; hence, there is less genetically determined variation in the metabolism of "NAT1 substrates."

A series of clinical observations in the 1950s established the existence of *slow* and *fast acetylators* of the antitubercular drug isoniazid, which is now known to be primarily acetylated by NAT2. In general, about 50% of patients treated with isoniazid have adverse events such as peripheral neuropathy and hepatotoxicity. These adverse effects are more pronounced in NAT2 slow acetylators, although some early reports suggested fast acetylators were at increased risk of isoniazid-induced liver injury (discussed later in this section). NAT2 slow acetylators also exhibit a higher incidence of adverse events with clonazepam, hydralazine, procainamide, and sulfonamides, such as sulfamethoxazole and sulfasalazine (Sirot et al., 2006; McDonagh et al., 2014). The incidence of the NAT2 slow acetylator phenotype is high in Middle Eastern populations (e.g., about 92% in Egyptians), intermediate in Caucasian and African populations (e.g., about 50% to 59% in Caucasian Americans, Australians, and Europeans; about 41% in African Americans; about 50% to 60% in Africans), and low in Asian populations (e.g., ~=about 20% in Chinese; about 8% to 10% in Japanese) (Sirot et al., 2006). Many studies have demonstrated the typical bimodal distribution of fast and slow acetylators with drugs such as isoniazid, but other drugs, such as caffeine and sulfamethazine, exhibit slow, intermediate, and fast acetylator phenotypes (Hein, 2006). Parkin et al. (1997) also demonstrated that isoniazid elimination is trimodal, and that phenotype and genotype were concordant.

The NAT2 slow acetylator phenotype is caused by various single nucleotide polymorphisms in the NAT2 gene that decrease NAT2 activity, decrease enzyme stability, or target the enzyme for proteasomal degradation. At least 88 allelic variants of human NAT2 have been documented. The wild-type enzyme is NAT2*4. Allelic variants that do NOT appear to affect NAT2 activity are *11A, *12A–C, *13, and *18. Common allelic variants associated with the NAT2 poor acetylator phenotype include *5 (rs1801280), *6 (rs1799930), *7 (rs1799931), and *14 (rs1801279) (McDonagh et al., 2014). NAT2*5 is a point mutation $T_{341}C$ (which causes the amino acid substitution $Ile_{114}Thr$) that decreases V_{max} for *N*-acetylation without altering the K_m for substrate binding or the stability of NAT2. The NAT2*5 allele is the most common cause of the slow acetylator phenotype in Caucasians (e.g., frequency of about 50% in the United Kingdom) but is rarely observed in Koreans (i.e., less than 5%) (Hein, 2006). There are 10 known NAT2*5 alleles, designated NAT2*5A through NAT*5J. The alleles containing only the $Ile_{114}Thr$ substitution are NAT2*5A (which also contains a silent $C_{481}T$ nucleotide change) and NAT2*5D. The others contain this and at least one other nucleotide and/or amino acid substitution, and NAT2*5B confers the "slowest acetylator" phenotype (Patin et al., 2006). The NAT2*7 allele is more prevalent in Asians than Caucasians or Africans (Hein, 2006), and involves either a point mutation $G_{857}A$ (i.e., NAT2*7A) or both this change and $C_{282}T$ (i.e., NAT2*7B). Both of these changes cause the amino acid substitution $Gly_{286}Glu$, which decreases the stability, rather than the activity, of NAT2. NAT2*6 (more common in southern India) and NAT2*14 (present mainly in Africa and Spain) also result in one or more amino acid changes that lead to decreased NAT2 activity, whereas the amino acid change in NAT2*12 (present mainly in Africa and Spain) does not decrease activity (Hein, 2006). Within the slow NAT2 acetylator phenotype there is considerable variation in rates of xenobiotic *N*-acetylation. This is partly because different mutations in the NAT2 gene have different effects on NAT2 activity and/or enzyme stability, heterozygotes retain moderate NAT2 activity, and the *N*-acetylation of "NAT2-substrates" by NAT1 becomes significant in slow NAT2 acetylators.

The slowest acetylator haplotype, namely NAT2*5B, which has the strongest association with bladder cancer, appears to have been positively selected during the last 6500 years in western and central Eurasians, which suggests that slow acetylation conferred an evolutionary advantage within this population (Patin et al., 2006). Moreover, because most of the NAT2 polymorphisms that lead to slow acetylation are present at high frequencies in various populations, it would seem that varied rates of acetylation may be an important human adaptation to environmental variables. Because acetylation can be very important in the determination of the interaction between human populations and many xenobiotics in their particular environment (e.g., through different diets and lifestyles), it is not surprising that they are recent targets for natural selection. Today, slow acetylation can be a disadvantage inasmuch as it predisposes to the toxicity of several drugs.

At least 28 allelic variants have now been documented for the human NAT1 gene, although these variants are less prevalent than

the NAT2 allelic variants; hence, there is less genetically determined variation in the metabolism of "NAT1 substrates." Nevertheless, there is evidence that phenotypic differences in the *N*-acetylation of 4-aminosalicylic acid are distributed bimodally, consistent with the existence of low- and high-activity forms of NAT1. Furthermore, an extremely slow acetylator of 4-aminosalicylic acid has been identified with mutations in both NAT1 alleles: one that decreases NAT1 activity and stability and one that encodes a truncated and catalytically inactive form of the enzyme. Two NAT1 variants, NAT1*10 and NAT1*11, are gain-of-function polymorphisms that lead to increased efficiency of protein translation (Wang et al., 2011b). Individuals homozygous for NAT1*10 or heterozygous for NAT1*11 are classified as fast acetylators, whereas those rare individuals who are homozygous for NAT1*11 have been classified as ultrafast acetylators (Wang et al., 2011b). Both NAT*10 and NAT*11 alleles are risk modifiers of sulfamethoxazole-induced hypersensitivity in HIV/AIDS patients, such that individuals who are NAT1 *fast* acetylators but are also NAT2 *slow* acetylators are protected against this type of hypersensitivity (Wang et al., 2011b). It has also been demonstrated that NAT1*16 but not NAT1*10 or NAT1*11 causes a 50% decrease in NAT1 activity and NAT1 protein levels in COS-1 cell cytosol (Soucek et al., 2004). Cascorbi et al. (2001) found that individuals who express NAT1*10 and NAT2*4 (i.e., intermediate NAT1 activity and normal NAT2 activity) were at a significantly lower risk of bladder cancer, especially when exposed to environmental carcinogens (Cascorbi et al., 2001). It is therefore likely that the interplay between NAT1 and NAT2 phenotypes is relevant to susceptibility to certain carcinogens.

Genetic polymorphisms in NAT2 have a number of pharmacological and toxicological consequences for drugs that are *N*-acetylated by this enzyme. The pharmacological effects of the antihypertensive drug hydralazine are more pronounced in slow NAT2 acetylators. Slow NAT2 acetylators are predisposed to several drug toxicities, including nerve damage (peripheral neuropathy) from isoniazid and dapsone, systemic lupus erythematosus from hydralazine and procainamide, and the toxic effects of coadministration of the anticonvulsant phenytoin with isoniazid. Slow NAT2 acetylators who are also deficient in glucose-6-phosphate dehydrogenase are particularly prone to hemolysis from certain sulfonamides. HIV-infected individuals of the slow acetylator phenotype suffer more often from adverse drug events (ADEs) and, among patients with Stevens–Johnson syndrome, the overwhelming majority are slow acetylators. Fast NAT2 acetylators are predisposed to the myelotoxic effects of amonafide because *N*-acetylation retards the clearance of this antineoplastic drug, which is thought to be due to inhibition of CYP1A2 by *N*-acetylamonofide (McDonagh et al., 2014).

Early epidemiological studies suggested that rapid NAT2 acetylators were at increased risk for the development of isoniazid-induced liver toxicity; however, the current consensus is that slow NAT2 acetylation is a risk modifier for isoniazid-induced hepatotoxicity while rapid acetylators are protected from toxicity (Metushi et al., 2016; Wang et al., 2016a). Following its acetylation by NAT2, isoniazid can be hydrolyzed to isonicotinic acid and acetylhydrazine ($CH_3CO–NHNH_2$). The latter metabolite can be *N*-hydroxylated by FMO or CYP to a reactive intermediate, as shown in Fig. 6-43. The generation of a reactive metabolite from acetylhydrazine would seem to provide a mechanistic basis for enhanced isoniazid hepatotoxicity in fast acetylators. However, acetylhydrazine can be further acetylated to diacetylhydrazine ($CH_3CO–NHNH–COCH_3$), and this detoxication reaction is also catalyzed by NAT2. Therefore, acetylhydrazine is both produced and detoxified by NAT2; hence, slow acetylation, not fast acetylation, becomes the risk modifier for isoniazid-induced hepatotoxicity, just as it is for isoniazid-induced peripheral neuropathy. Isoniazid can also be directly hydrolyzed to yield isonicotinic acid and hydrazine ($H_2N–NH_2$). Hydrazine (also a toxic substance used as rocket propellant) has been proposed to be the hepatotoxic metabolite of isoniazid as slow acetylators have significantly higher plasma concentrations of hydrazine relative to rapid acetylators (Testa and Krämer, 2008, 2010; Metushi et al., 2011). Rifampin and alcohol have been reported to enhance the hepatotoxicity of isoniazid. These drug interactions support a growing body of evidence that isoniazid itself is converted to one or more reactive metabolites that result in covalent binding to protein (hapten formation), which in severe cases of isoniazid-induced liver injury trigger an immune response. In support of this interpretation, isoniazid hepatotoxicity is more severe in NAT2 slow acetylator who also carry the high-activity CYP2E1 c1/c1 genotype (Metushi et al., 2011). Furthermore, circulating antibodies against isoniazid-adducted proteins (antidrug antibodies) and CYP enzymes (antibodies against CYP2E1, 3A4, and 2C9) have been found in patients with severe isoniazid-induced liver injury (Metushi et al., 2014). Isoniazid is an antitubercular drug, and its inactivation by NAT2 is interesting from the perspective that several organisms, including *Mycobacterium tuberculosis*, express a NAT2-like enzyme, which has implications for isoniazid resistance in tuberculosis.

Aromatic amines can be both activated and deactivated by NATs (Kato and Yamazoe, 1994; Hirvonen, 1999; Hein et al., 2000). The NATs detoxify aromatic amines by converting them to the corresponding amides because aromatic amides are less likely than aromatic amines to be activated to DNA-reactive metabolites by CYP, peroxidases such as PHS, and UGTs (see the section "Glucuronidation and Formation of Acyl-CoA Thioesters"). However, NATs can activate aromatic amines if they are first *N*-hydroxylated by CYP because NATs can also function as *O*-acetyltransferases and convert hydroxylamines to acetoxy esters (*aryl*-NHOH → *aryl*-NH–O–$COCH_3$). As shown in Fig. 6-16, the acetoxy esters of hydroxylamines, like the corresponding sulfonate esters (Fig. 6-64), can break down to form highly reactive nitrenium and carbonium ions that bind to DNA. NATs catalyze the *O*-acetylation of *N*-hydroxyaromatic amines by two distinct mechanisms. The first reaction, which is exemplified by the conversion of *N*-hydroxyaminofluorene to *N*-acetoxyaminofluorene, requires acetyl-CoA and proceeds by the same mechanism previously described for *N*-acetylation. The second reaction is exemplified by the conversion of 2-acetylaminofluorene (2-AAF) to *N*-acetoxyaminofluorene, which does not require acetyl-CoA but involves an intramolecular transfer of the *N*-acetyl group from nitrogen to oxygen. These reactions are shown in Fig. 6-67.

Genetic polymorphisms in NAT2 have been reported to influence susceptibility to aromatic amine–induced bladder and colon cancer (see the section "Glucuronidation and Formation of Acyl-CoA Thioesters") (Evans, 1992; Kadlubar, 1994; Hirvonen, 1999; Hein et al., 2000; Sim et al., 2008; Ma et al., 2016). Bladder cancer is thought to be caused by bicyclic aromatic amines (benzidine, 2-aminonaphthalene, and 4-aminobiphenyl), whereas colon cancer is thought to be caused by heterocyclic aromatic amines, such as the products of amino acid pyrolysis (e.g., 2-amino-6-methylimidazo[4,5-*b*]pyridine or PhIP, and others listed in Table 6-18). Epidemiological studies suggest that slow NAT2 acetylators are more likely than fast NAT2 acetylators to develop bladder cancer from cigarette smoking and from occupational exposure to bicyclic aromatic amines. The possibility that slow NAT2 acetylators are at increased risk for aromatic amine–induced cancer is supported by the finding that dogs, which are poor acetylators, are highly prone to aromatic amine–induced bladder cancer. By comparison, fast NAT2

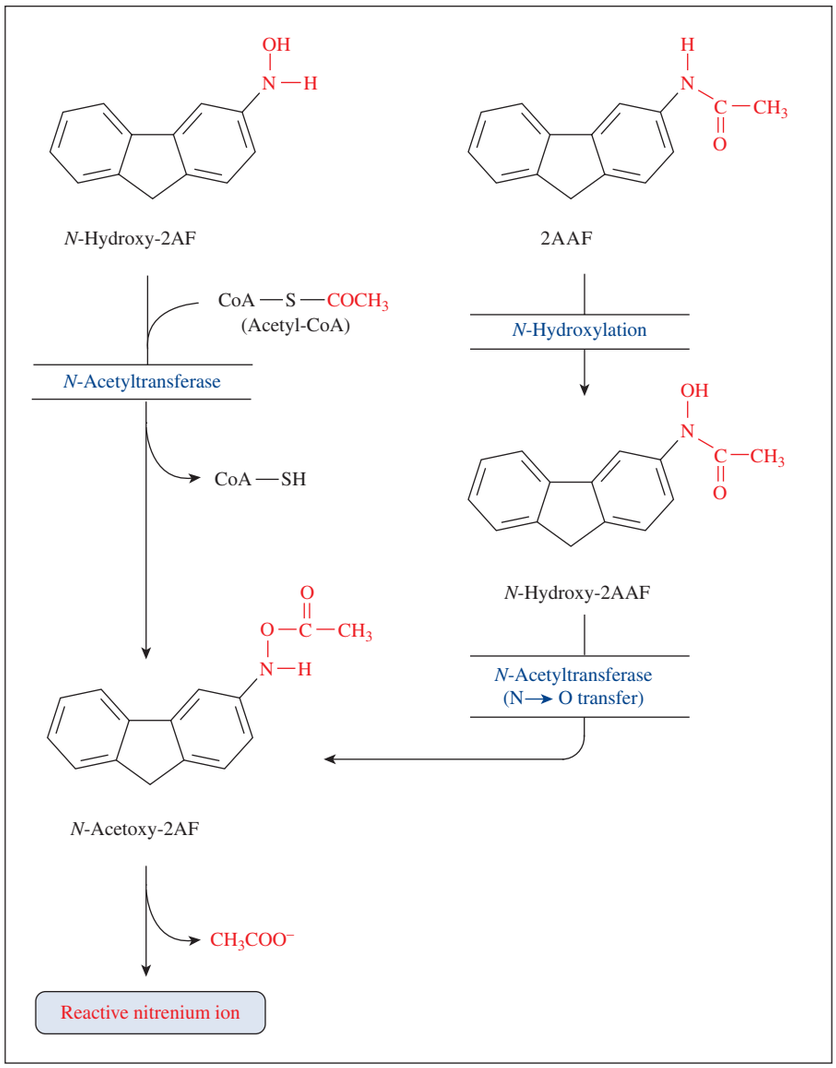


Figure 6-67. *Role of N-acetyltransferase in the O-acetylation of N-hydroxy-2-aminofluorene (N-hydroxy-2-AF) and the intramolecular rearrangement of N-hydroxy-2-acetylaminofluorene (N-hydroxy-2-AAF).*

acetylators appear to be at increased risk for colon cancer from heterocyclic aromatic amines.

The influence of acetylator phenotype on susceptibility to aromatic amine–induced cancer can be rationalized on the ability of NATs to activate and detoxify aromatic amines and by differences in the substrate specificity and tissues distribution of NAT1 and NAT2 (recall that NAT1 is expressed in virtually all tissues, whereas NAT2 is expressed mainly in the liver and gastrointestinal tract). Both NAT1 and NAT2 catalyze the *O*-acetylation (activation) of *N*-hydroxy bicyclic aromatic amines, whereas the *O*-acetylation of *N*-hydroxy heterocyclic aromatic amines is preferentially catalyzed by NAT2. Bicyclic aromatic amines can also be *N*-acetylated (detoxified) by NAT1 and NAT2, but heterocyclic aromatic amines are poor substrates for both enzymes. Therefore, the fast acetylator phenotype protects against aromatic amine–induced bladder cancer because NAT2 (as well as NAT1) catalyzes the *N*-acetylation (detoxication) of bicyclic aromatic amines in the liver. In slow acetylators, a greater proportion of the bicyclic aromatic amines are activated through *N*-hydroxylation by CYP1A2. These *N*-hydroxylated aromatic amines can be activated by *O*-acetylation, which can be catalyzed in the bladder itself by NAT1. A high level of NAT1 in the bladder is a risk modifier for aromatic amine–induced bladder cancer. In addition, the fast acetylator phenotype potentiates the colon cancer–inducing effects of heterocyclic aromatic amines. These aromatic amines are poor substrates for NAT1 and NAT2, so that high levels of NAT2 in the liver do little to prevent their *N*-hydroxylation by CYP1A2. The *N*-hydroxylated metabolites of heterocyclic aromatic amines can be activated by *O*-acetylation, which can be catalyzed in the colon itself by NAT2. The presence of NAT2 (and NAT1) in the colons of fast acetylators probably explains why this phenotype is a risk modifier for the development of colon cancer.

Whether fast acetylators are protected from or predisposed to the cancer-causing effects of aromatic amines depends on the nature of the aromatic amine (bicyclic vs. heterocyclic) and on other important risk modifiers. Both the activation of aromatic amines by *N*-glucuronidation and the activation of *N*-hydroxy aromatic amines (hydroxylamines) by sulfonation are suspected of playing an important role in the incidence of bladder and colon cancer. These and other risk modifiers may explain why some epidemiological studies,

contrary to expectation, have shown that slow NAT2 acetylators are at increased risk for aromatic amine–induced bladder cancer, as was demonstrated for benzidine manufacturers in China (Hayes et al., 1993).

The *N*-acetylation of aromatic amines (a detoxication reaction) and the *O*-acetylation of *N*-hydroxy aromatic amines (an activation reaction) can be reversed by a microsomal enzyme called arylacetamide deacetylase (gene symbol AADAC) (Probst et al., 1994). This enzyme is similar to but distinct from the microsomal carboxylesterases that hydrolyze esters and amides, as described in the section "Carboxylesterases." AADAC can potentially alter the overall balance between detoxication and activation of aromatic amines.

Overall, it would appear that low NAT2 activity increases the risk of bladder, breast, liver, and lung cancers, and decreases the risk of colon cancer, whereas low NAT1 activity increases the risk of bladder and colon cancers and decreases the risk of lung cancer (Hirvonen, 1999). The individual risks associated with particular NAT1 and/or NAT2 acetylator genotypes are small, but they increase when considered in conjunction with other susceptibility genes and/or exposure to carcinogenic aromatic and heterocyclic amines. Because of the relatively high frequency of allelic variants of NAT1 and NAT2, the attributable risk of cancer in the population may be high (Hein et al., 2000).

Amino Acid Conjugation

There are two principal pathways by which xenobiotics are conjugated with amino acids, as illustrated in Fig. 6-68. The first involves conjugation of xenobiotics containing a carboxylic acid group with the *amino group* of amino acids such as glycine, glutamine, and taurine (see Fig. 6-57). This pathway involves activation of the xenobiotic by conjugation with coenzyme A (A), which produces a xenobiotic-CoA thioester that reacts with the *amino group* of an amino acid to form an amide linkage. The second pathway involves conjugation of xenobiotics containing an aromatic hydroxylamine (*N*-hydroxy aromatic amine) with the *carboxylic acid group* of such amino acids as serine and proline. This pathway involves activation of an amino acid by aminoacyl-tRNA synthetase, which reacts with an aromatic hydroxylamine to form a reactive *N*-ester (Kato and Yamazoe, 1994).

The conjugation of benzoic acid with glycine to form hippuric acid (see Fig. 6-4) was discovered in 1842, making it the first biotransformation reaction discovered (Knights et al., 2007; Badenhorst et al., 2014). The first step in this conjugation reaction involves activation of benzoic acid to an acyl-CoA thioester. This reaction requires ATP and is catalyzed by acyl-CoA synthetase (ATP-dependent acid:CoA ligase [AMP forming]). The second step is catalyzed by acyl-CoA:amino acid *N*-acyltransferase, which transfers the acyl moiety of the xenobiotic to the amino group of the acceptor amino acid. The reaction proceeds by a *ping-pong Bi–Bi* mechanism, and involves transfer of the xenobiotic to a cysteine residue in the enzyme with release of CoA, followed by transfer of the xenobiotic to the acceptor amino acid with regeneration of the enzyme. The second step in amino acid conjugation is analogous to amide formation during the acetylation of aromatic amines by NAT. Substrates for amino acid conjugation are restricted to certain aliphatic, aromatic, heteroaromatic, cinnamic, and arylacetic acids.

The ability of xenobiotics to undergo amino acid conjugation depends on steric hindrance around the carboxylic acid group, and by substituents on the aromatic ring or aliphatic side chain. In rats, ferrets, and monkeys, the major pathway of phenylacetic acid biotransformation is amino acid conjugation. However, due to steric hindrance, diphenylacetic acid cannot be conjugated with an amino acid, so the major pathway of diphenylacetic acid biotransformation in these three species is acyl glucuronidation. Bile acids are endogenous substrates for glycine and taurine conjugation. However, the activation of bile acids to an acyl-CoA thioester is catalyzed by a microsomal enzyme, cholyl-CoA synthetase, and conjugation with glycine or taurine is catalyzed by the cytosolic enzyme, bile acid-CoA:amino acid *N*-acyltransferase (BACAT; gene symbol BAAT) (Falany et al., 1994; Testa and Krämer, 2008, 2010). BACAT is expressed at high levels in the liver, kidney, gallbladder, and intestine, with lower levels in the adrenal gland, muscle, lung, and brain, which suggests that it may have substrates other than bile acids (O'Byrne et al., 2003). In vitro studies show that BACAT can form glycine conjugates of long-chain fatty acids, and also possesses thioesterase activity toward the same substrates (O'Byrne et al., 2003). In contrast to bile acids, the conjugation of xenobiotics with CoA occurs mainly in mitochondria, which appear to contain multiple acyl-CoA ligases. There are three acid-thiol ligases that are involved in the formation of a xenobiotic acyl-CoA intermediate, namely, the short-, medium-, and long-chain acyl-CoA ligase (Testa and Krämer, 2008, 2010). These enzymes are also involved in endogenous fatty acid activation. Of particular importance to amino acid conjugation is the medium-chain [butyrate]-CoA ligase present in the mitochondrial matrix, which converts medium-chain fatty acids (C4-C12) to CoA thioesters to initiate β-oxidation (Knights et al., 2007). In humans, two distinct forms of medium-chain fatty acid CoA ligases have been characterized, HXM-A (48 kDa) and HXM-B (49 kDa), each of which has overlapping substrate specificities. Common substrates of HXM-A and HXM-B include benzoate, propionate, hexanoate, octanoate, valproate, and salicylate (Knights et al., 2007). The formation of xenobiotic acyl-CoA conjugates is interesting because of their potential to cause mitochondrial toxicity by inhibiting fatty-acid β-oxidation due to a combination of CoA sequestration and direct inhibition of acyl-CoA ligases (Knights et al., 2007; Testa and Krämer, 2008, 2010). Some xenobiotic acyl-CoA conjugates can also act as reactive electrophiles and form protein adducts with immunogenic potential (see the section "Glucuronidation and Formation of Acyl-CoA Thioesters"). The second step in the conjugation of xenobiotics with amino acids is catalyzed by cytosolic and/or mitochondrial forms of *N*-acyltransferase, expressed primarily in the liver and kidney (Knights et al., 2007). Amino acid conjugation is apparently catalyzed by separate *N*-acyltransferases specific for each amino acid (Gopaul et al., 2003). Glycine-*N*-acyltransferase and glutamine-*N*-phenylacetyltransferase have been isolated from human hepatic mitochondria (Gopaul et al., 2003).

There are several examples of xenobiotics that undergo amino acid conjugation as a major metabolic pathway, such as the hypolipidemic drug nicotinic acid (niacin) that is conjugated with glycine to form nicotinuric acid (Iwaki et al., 1996; Testa and Krämer, 2008, 2010). In humans, over 90% of a dose of acetylsalicylic acid (aspirin) is hydrolyzed to salicylic acid, of which 75% is excreted in urine as the glycine conjugate, salicyluric acid (Knights et al., 2007; Testa and Krämer, 2008, 2010). While benzoic acids and small analogs generally are conjugated with glycine, larger substrates such as 4-phenylbutanoic acid are generally conjugated with glutamine. 4-Phenylbutanoic acid is a drug used in the treatment of hyperammonemia caused by genetic errors in urea synthesis. Conjugation of 4-phenylbutanoic acid with glutamine contributes to nitrogen elimination under conditions of impaired urea synthesis (Kasumov et al., 2004; Testa and Krämer, 2008, 2010). Taurine (along with glycine) plays an important role in the conjugation of bile acids. The herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) forms both taurine and glycine conjugates in dogs, but only trace glycine conjugates in rats (Lappin et al., 2002; Testa and Krämer, 2008,

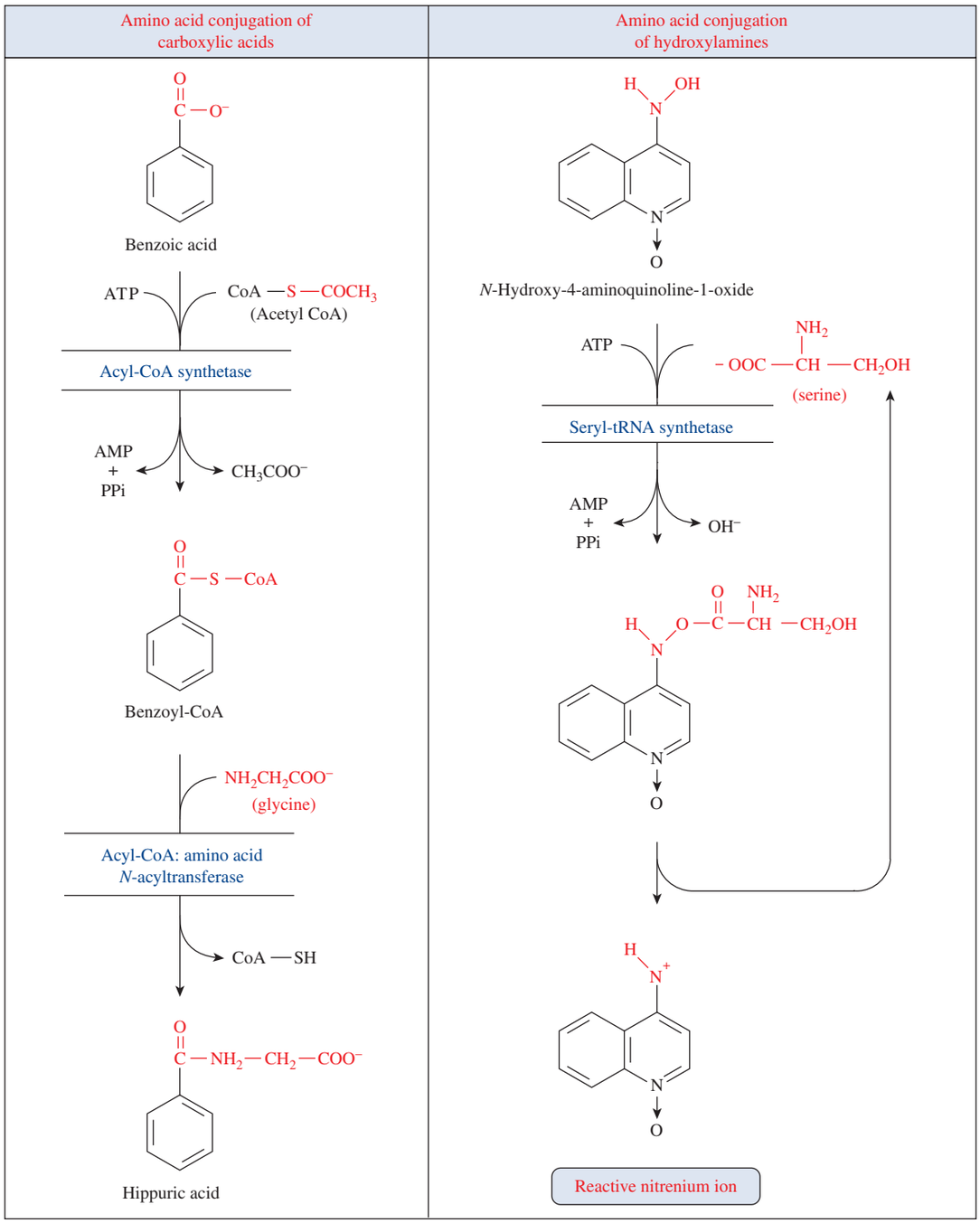


Figure 6-68. *Conjugation of xenobiotics with amino acids.*

2010). The PPARα agonist MRL-II exhibits dose-dependent formation of acyl glucuronides and taurine conjugates, major biliary metabolites in dogs, with lower doses favoring the formation of the taurine conjugate (Kim et al., 2004; Testa and Krämer, 2008, 2010). While few reports of xenobiotic taurine conjugates in humans exist, taurine conjugation of the NSAID ibuprofen is a minor metabolite eliminated in urine almost solely as the *S*-enantiomer (Shirley et al., 1994; Testa and Krämer, 2008, 2010).

An important difference between the amino acid conjugates of xenobiotics and bile acids is their route of elimination: bile acids are secreted into bile, whereas amino acid conjugates of xenobiotics are eliminated primarily in urine. The addition of an endogenous amino acid to xenobiotics may facilitate this elimination by increasing their ability to interact with the tubular organic anion transport system in the kidney. The precursor to amino acid conjugation, namely, the xenobiotic acyl-CoA thioester, is not transported out of the cell and is not detected in the bile, plasma, or urine, but it can be detected in tissue samples and in vitro test systems. The formation of xenobiotic acyl-CoA thioesters in adipose tissue (and their further metabolism to more complex lipid conjugates)

can be a relatively long-lived repository for certain acidic drugs. Consequently, in mass balance studies (i.e., in studies of the disposition of a radiolabeled drug) the formation of acyl-CoA thioesters and their lipid derivatives in adipose tissue can contribute to incomplete recovery of radioactivity in the case of acidic drugs and drugs that are extensively metabolized to one or more carboxylic acid–containing metabolites.

In addition to glycine, glutamine, and taurine, acceptor amino acids for xenobiotic conjugation include ornithine, arginine, histidine, serine, aspartic acid, and several dipeptides, such as glycylglycine, glycyltaurine, and glycylvaline. The acceptor amino acid used for conjugation is both species- and xenobiotic-dependent. For benzoic, heterocyclic, and cinnamic acids, the acceptor amino acid is glycine, except in birds and reptiles, which use ornithine. Arylacetic acids are also conjugated with glycine except in primates, which use glutamine. In mammals, taurine is generally an alternative acceptor to glycine. Taurine conjugation is well developed in nonmammalian species and carnivores. While most species conjugate bile acids with both glycine and taurine, cats and dogs conjugate bile acids only with taurine.

Amino acid conjugation of carboxylic acid–containing xenobiotics is an alternative to glucuronidation. Conjugation of carboxylic acid–containing xenobiotics with amino acids has long been considered a predominantly detoxication reaction. However, as outlined in the section "Glucuronidation and Formation of Acyl-CoA Thioesters," there is growing evidence that the toxicity of certain NSAIDs is due in part to the formation of reactive acyl-CoA thioesters (the precursors to amino acid conjugates), which represents an alternative activation pathway to the formation of reactive acyl glucuronides, as illustrated in Fig. 6-63. Amino acid conjugation of ibuprofen, ketoprofen, and related *profens* (2-substituted propionic acid NSAIDs) is significant for two reasons: it limits the formation of potentially toxic acyl glucuronides and it leads to chiral inversion (the interconversion of *R*- and *S*-enantiomers) (Shirley et al., 1994). The latter reaction requires conversion of the profen to its acyl-CoA thioester, which undergoes chiral inversion by 2-arylpropionyl-CoA epimerase (this involves the intermediacy of a symmetric, conjugated enolate anion). Chiral inversion may explain why the *R*- and *S*-enantiomers of several profen NSAIDs have comparable anti-inflammatory effects in vivo, even though the *S*-enantiomers are considerably more potent than their antipodes as in vitro inhibitors of cyclooxygenase (the target of NSAID therapy). There is also evidence that the acyl-CoA thioesters of the *R*-enantiomers are pharmacologically active and contribute to the therapeutic effect by inhibiting COX-2 (Neupert et al., 1997; Levoin et al., 2004; Testa and Krämer, 2008, 2010). In general, amino acid conjugation is a high-affinity–low-capacity reaction, whereas glucuronidation is a high-capacity–low-affinity reaction. At low substrate concentrations, amino acid conjugation can be the predominant reaction.

Amino acid conjugation of *N*-hydroxy aromatic amines (hydroxylamines) is an activation reaction because it produces *N*-esters that can degrade to form electrophilic nitrenium and carbonium ions (Anders, 1985; Kato and Yamazoe, 1994). Conjugation of hydroxylamines with amino acids is catalyzed by cytosolic aminoacyl-tRNA synthetases and requires ATP (Fig. 6-68). Hydroxylamines activated by aminoacyl-tRNA synthetases include *N*-hydroxy-4-aminoquinoline 1-oxide, which is conjugated with serine, and *N*-hydroxy-Trp-P-2, which is conjugated with proline. (*N*-Hydroxy-Trp-P-2 is the *N*-hydroxylated metabolite of Trp-P-2, a pyrolysis product of tryptophan [see Table 6-18].) It is now apparent that the hydroxylamines formed by the CYP-dependent *N*-hydroxylation of aromatic amines can potentially be activated by numerous reactions, including *N*-glucuronidation by UGTs (Fig. 6-62), *O*-acetylation by NAT (Fig. 6-16), *O*-sulfonation by SULT (Fig. 6-64), and conjugation with amino acids by seryl- or prolyl-tRNA synthetase (Fig. 6-68).

Various amino acid conjugates of valproic acid, including glutamine, glutamate, and glycine conjugates, have been detected in urine, plasma, and cerebrospinal fluid (CSF) of patients administered this antiepileptic drug (Gopaul et al., 2003). CSF concentrations of the glutamine and glutamate conjugates of valproic acid were five and nine times higher than in plasma, which suggests that these conjugation reactions in the brain decrease levels of the excitatory neurotransmitter glutamic acid and decrease the incidence of seizure (Gopaul et al., 2003). Taken together, these data suggest that amino acid conjugation in the brain may play a role in the mechanism of action of valproic acid.

Glutathione Conjugation

The preceding section described the conjugation of xenobiotics with certain amino acids, including some simple dipeptides, such as glycyltaurine. This section describes the conjugation of xenobiotics with the tripeptide glutathione (GSH) in a reaction also known as glutathionylation, which is fundamentally different from other conjugation reactions. While glucuronidation, sulfonation, and all of the other previously described conjugation reactions involve conjugation of nucleophilic xenobiotics or their nucleophilic metabolites, GSH conjugation occurs with electrophilic xenobiotics or their electrophilic metabolites. GSH is composed of glycine, cysteine, and glutamic acid; the latter being linked to cysteine via the γ-carboxyl group, not the usual α-carboxyl group, as shown in Fig. 6-57. Glutathione transferase (GST) activity was first discovered as the enzyme-catalyzed conjugation of GSH with halogenated compounds such as chloronitrobenzenes and bromosulfophthalein (Mannervik et al., 2005; Higgins and Hayes, 2011). The activity was initially designated GSH *S*-aryltransferase, and GST activities toward other substrates such as alkyl halides and epoxides were called *S*-alkyl and *S*-epoxide transferases. The original names were later replaced with letter designations once the overlapping substrate specificity was described, and it was recognized that GSTs do not simply conjugate xenobiotics with GSH but also catalyze reactions such as hydroperoxide reduction (an example of which is shown in Fig. 6-22 as part of the metabolism of carbon tetrachloride), dehydrohalogenation (as shown in Fig. 6-24 [general mechanisms] and Fig. 6-25 [dechlorination of DDT]), thiolysis (the general mechanism for disulfide cleavage is shown in Fig. 6-18), disulfide interchange, and isomerization (Mannervik et al., 2005; Higgins and Hayes, 2011). Although the abbreviation "GST" has survived to describe the enzyme family, the term "glutathione *S*-transferase" is technically incorrect because the glutathionyl group (GS-) is transferred rather than a single sulfur atom (Mannervik et al., 2005); hence, the correct term is glutathione transferase. GSTs function endogenously as part of a defense mechanism against reactive oxygen species; GSTs reduce the formation of hydroperoxides of fatty acids, phospholipids, and cholesterol and protect against the redox cycling of many quinone-containing compounds by conjugating them with GSH (Hayes et al., 2005; Testa and Krämer, 2010). GSTs also play a role in other endogenous functions such as the degradation of aromatic amino acids, steroid hormone synthesis, eicosanoid synthesis (leukotrienes are GSH conjugates), and modulation of signaling pathways (Hayes et al., 2005; Higgins and Hayes, 2011; Oakley, 2011)

Substrates for glutathionylation include an enormous array of electrophilic xenobiotics, or xenobiotics that can be biotransformed to electrophiles. GSH conjugates are thioethers that form

by nucleophilic attack of GSH thiolate anion (GS^-) with an electrophilic carbon or heteroatom (*O*, *N*, and *S*) in the xenobiotic or its metabolite. Michael acceptors are also conjugated with GSH (discussed later in this section). The sulfhydryl group in GSH has a pK_a(A) value of ~9.0; hence, at physiological pH, only about 1% of GSH is present in the thiolate anion (GS^-) form. A primary function of GSTs is to lower the pK_a(A) of GSH by more than two orders of magnitude (to pK_a(A) 6 to 7), such that most (more than 50%) of the GSH bound to GST is in the reactive anionic form, which is stabilized by hydrogen bonding with a tyrosine, serine, or cysteine residue in the active site (Testa and Krämer, 2008, 2010). GSH, cysteine, *N*-acetylcysteine (NAC), and various analogs (esters and amides) are used in vitro to trap electrophilic metabolites formed by CYP in liver microsomes. In this system, which lacks the cytosolic GSTs, only about 1% of GSH is present in the thiolate anion (GS^-), compared with 6% of cysteine and up to 16% for certain cysteine esters, which is why chemicals other than GSH are widely used to trap electrophilic metabolites formed by CYP in vitro (Reed, 1985). Cysteine has sixfold greater ionization than GSH at physiological pH; however, the concentration of GSH in liver is about 50 times greater than that of cysteine, and its ionization is greatly increased by binding to GSTs.

The synthesis of GSH involves formation of the peptide bond between cysteine and glutamic acid, followed by peptide bond formation with glycine. The first and overall rate-limiting reaction is catalyzed by γ-glutamylcysteine synthetase (γ-GCL), the second by GSH synthetase. At each step, ATP is hydrolyzed to ADP and inorganic phosphate (Testa and Krämer, 2008, 2010). Due to the sheer multiplicity of GSTs, they cannot be collectively inhibited or induced; therefore, the importance of glutathionylation in xenobiotic toxicity in vivo is often assessed by altering levels of GSH. This can be achieved by (1) inhibiting GSH synthesis, (2) depleting GSH, or (3) increasing GSH levels through Nrf2 activation. Inhibition of GSH synthesis can be achieved with buthionine-*S*-sulfoximine (BSO), which inhibits γ-GCL, the enzyme that catalyzes the first reaction in GSH synthesis (Ballatori et al., 2009a). Michael acceptors such as diethyl maleate (DEA) and phorone are commonly used in vivo to deplete GSH levels in experimental animals. Other chemicals, such as 2-cyclohexen-1-one and 2-cyclohepten-1-one, selectively decrease cerebral GSH levels in comparison with BSO treatment (Masukawa et al., 1989; Yoneyama et al., 2008). Lastly several chemicals can activate Nrf2, which induces γ-GCL, the rate-limiting step in GSH synthesis. Nrf2 activators include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), ethoxyquin, and low doses of acetaminophen (Higgins and Hayes, 2011).

The conjugation of xenobiotics with GSH is catalyzed by GSTs in cytosol, microsomes, and mitochondria (Hayes et al., 2005; Higgins and Hayes, 2011; Oakley, 2011). Most GSTs were originally found in the soluble cell fraction, and they are referred to as cytosolic or soluble GSTs, even though they may also be found in the nucleus or peroxisomes (Mannervik et al., 2005; Higgins and Hayes, 2011; Oakley, 2011). The only mitochondrial GST found to date has traditionally been referred to as Kappa GST (detailed later in this section), and although it dimerizes (as do the cytosolic GSTs), Kappa GST has a structure that is distinct from the cytosolic GSTs and is also present in peroxisomes, which suggests that it is involved in fatty acid β-oxidation (Hayes et al., 2005; Mannervik et al., 2005; Morel and Aninat, 2011). The microsomal GSTs or MAPEGs, which stands for membrane-associated proteins in eicosanoid and glutathione metabolism, are an independent group of GSTs expressed in microsomes and mitochondria. It should also be noted that some GSTs have nonenzymatic functions such as binding of zeaxanthin in the retina and c-Jun N-terminal kinase 1 by GST P1-1, and were initially designated by other names (Mannervik et al., 2005). There are also other proteins that are homologous to some GSTs but are not yet known to serve as detoxication enzymes, such as the chloride intracellular channels (CLIC) (Mannervik et al., 2005; Littler et al., 2010). Proteins that were characterized and named were subsequently identified as GSTs, as was the case with GSH-dependent hematopoietic prostaglandin D synthase (HPGDS), now determined to be GST S1-1 due to similarities with the Sigma class GSTs expressed in nonmammalian organisms. The GSTs are present in most tissues, with high concentrations in the liver, intestine, kidney, testis, adrenal, and lung.

Substrates for GST share three common features: they are hydrophobic, they contain an electrophilic atom, and they react nonenzymatically with GSH at some measurable rate. The mechanism by which GST increases the rate of GSH conjugation involves deprotonation of GSH to GS^- by an active-site tyrosine or serine, which functions as a general base catalyst (Atkins et al., 1993; Dirr et al., 1994). In the case of the Omega class GSTs, the active site contains a cysteine residue (Mukherjee et al., 2006; Board, 2011). The concentration of GSH in the liver is extremely high (5 to 10 mM) relative to plasma (0.5 to 10 μM); hence, the nonenzymatic conjugation of certain xenobiotics with GSH can be significant (Testa and Krämer, 2008, 2010). However, some xenobiotics are conjugated with GSH stereoselectively, indicating that the reaction is largely catalyzed by GST. Like GSH, the GSTs are themselves abundant cellular components, accounting for up to 10% of the total cellular protein. These enzymes bind, store, and/or transport a number of compounds that are not substrates for GSH conjugation. The cytoplasmic protein formerly known as ligandin, which binds heme, bilirubin, steroids, azo-dyes, polycyclic aromatic hydrocarbons (PAHs), and thyroid hormones, is an Alpha class GST (Oakley, 2011).

As shown in Fig. 6-69, substrates for GSH conjugation can be divided into two groups: those that are sufficiently electrophilic to be conjugated directly and those that must first be biotransformed to an electrophilic metabolite prior to conjugation. The second group of substrates for GSH conjugation includes reactive intermediates (often formed by CYP) such as oxiranes (arene oxides and alkene epoxides), nitrenium ions, carbonium ions, and free radicals. The conjugation reactions themselves can be divided into two types: *displacement reactions*, in which GSH displaces an electron-withdrawing group, and *addition reactions*, in which GSH is added to an activated double bond or strained ring system. Table 6-24 provides examples of typical substrates for each of the characterized GSTs.

The displacement of an electron-withdrawing group by GSH typically occurs when the substrate contains halide, sulfate, sulfonate, phosphate, or a nitro group (i.e., good *leaving groups*) attached to an allylic or benzylic carbon atom. Displacement of an electron-withdrawing group from aromatic xenobiotics is decreased by the presence of other substituents that donate electrons to the aromatic ring ($–NH_2$, –OH, –OR, and –R). Conversely, such displacement reactions are increased by the presence of other electron-withdrawing groups (–F, –Cl, –Br, –I, $–NO_2$, –CN, –CHO, and –COOR). This explains why 1,2-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene, each of which contains three electron-withdrawing groups, are commonly used as substrates for measuring GST activity in vitro, and one or more members of all three GST families can catalyze this conjugation reaction (Hayes et al., 2005; Higgins and Hayes, 2011). GST can catalyze the *O*-demethylation of dimethylvinphos and other methylated organophosphorus compounds. The reaction is analogous to the interaction between methyliodide and GSH, which produces methylglutathione and iodide ion ($GS^- + CH_3I \rightarrow GS–CH_3 + I^-$). In this case, iodide is the leaving group. In

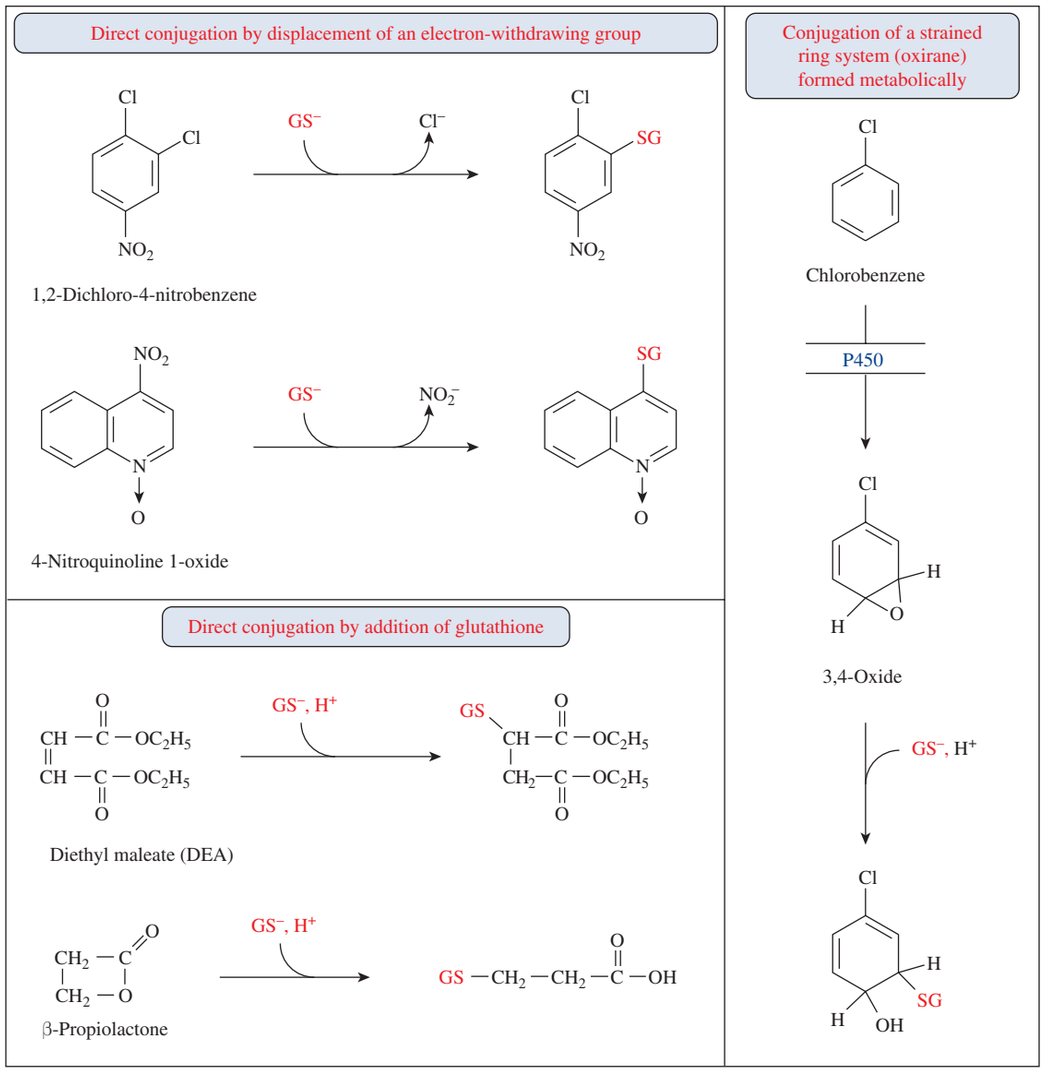


Figure 6-69. *Examples of glutathione conjugation of xenobiotics with an electrophilic carbon. GS− represents the anionic form of glutathione.*

the case of dimethylvinphos, the entire organophosphate molecule (minus the methyl group) functions as the leaving group.

The addition of GSH to a carbon–carbon double bond is also facilitated by the presence of a nearby electron-withdrawing group; hence, substrates for this reaction typically contain a double bond attached to –CN, –CHO, –COOR, or –COR (i.e., they are Michael acceptors). The double bond in diethyl maleate (DEA) is attached to two electron-withdrawing groups and readily undergoes a Michael addition reaction with GSH, as shown in Fig. 6-69. The loop diuretic, ethacrynic acid, contains an α/β-unsaturated ketone that readily reacts with GSH and other sulfhydryls by Michael addition. The conversion of acetaminophen to a GSH conjugate involves addition of GSH to an activated double bond, which is formed during the CYP-dependent dehydrogenation of acetaminophen to NAPQI, as shown in Fig. 6-39.

An interesting feature of GSH conjugation is the potential for nonenzymatic GSH conjugation in xenobiotic metabolism. The mechanism for this nonenzymatic activity is based on electrophilicity or nucleophilicity of potential reactants (Testa and Krämer, 2008, 2010). Electrophiles and nucleophiles are scaled according to their hardness or softness, and this determines whether a nonenzymatic or GST-catalyzed enzymatic reaction will be favored. Hard electrophilic sites are highly localized positive charges that maintain a high charge density during the approach of a nucleophilic reactant (i.e., they are not readily polarized). In contrast, soft electrophilic sites have a low charge density (i.e., a delocalized charge) and are easily polarized by an approaching nucleophilic reactant (Testa and Krämer, 2008, 2010). Because GSH acts as a soft nucleophile, it will react spontaneously with soft electrophiles, such as in the case of 1-4(nitrophenoxy)propane-2,3-oxide, which is a poor GST substrate, but reacts spontaneously with GSH (Testa and Krämer, 2008, 2010). Hard electrophiles are unlikely to react spontaneously with GSH and require the catalytic facilitation of GSTs (Testa and Krämer, 2008, 2010).

Arene oxides and alkene epoxides, which are often formed by CYP-dependent oxidation of aromatic hydrocarbons and alkenes, are examples of strained ring systems that open during the addition of GSH (Fig. 6-69). In many cases, conjugation of arene oxides with GSH proceeds stereoselectively, as shown in Fig. 6-70 for the 1,2-oxides of naphthalene. The GSH conjugates of arene oxides

Table 6-24

Human Glutathione Transferase Enzymes

GST FAMILY	CLASS	GENE	EXAMPLE ENZYMES	EXAMPLE SUBSTRATES
Cytosolic	Alpha	GSTA1	GST A1-1	Δ^5-ADD, BCDE, BPDE, busulfan, chlorambucil, DBADE, DBPDE, BPhDE, *N*-acetoxy-PhIP
		GSTA2	GST A2-2	CuOOH, DBPDE, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole
		GSTA3	GST A3-3	Δ^5-ADD, Δ^5-pregnene-3,20-dione, DBPDE
		GSTA4	GST A4-4	COMC-6, EA, 4-hydroxynonenal, 4-hydroxydecenal
		GSTA5	GST A5-5	Unknown
	Mu	GSTM1	GST M1-1	*trans*-4-Phenyl-3-buten-2-one, BPDE, CDE, DBADE, *trans*-stilbene oxide, styrene-7,8-oxide
		GSTM2	GST M2-2	COMC-6, 1,2-dichloro-4-nitrobenzene, aminochrome, dopa O-quinone, $PGH_2 \rightarrow PGE_2$
		GSTM3	GST M3-3	BCNU, $PGH_2 \rightarrow PGE_2$
		GSTM4	GST M4-4	CDNB
		GSTM5	GST M5-5	CDNB
	Pi	GSTP1	GST P1-1	Acrolein, base propenals, BPDE, CDE, chlorambucil, COMC-6, EA, thio-TEPA
	Sigma	HPGDS	GST S1-1*	$PGH_2 \rightarrow PGD_2$
	Theta	GSTT1	GST T1-1	BCNU, butadiene epoxide, CH_2Cl_2, EPNP, ethylene oxide
		GSTT2	GST T2-2	CuOOH, menaphthyl sulfonate
	Zeta	GSTZ1	GST Z1-1	Dichloroacetate, fluoroacetate, 2-chloropropionate, maleylacetoacetate
	Omega	GSTO1	GST O1-1	Monomethylarsonic acid, dehydroascorbic acid
		GSTO2	GST O2-2	Monomethylarsonic acid, dehydroascorbic acid
Mitochondrial	Kappa	GSTK1	GSTK1-1	CDNB, CuOOH, (S)-15-hydroperoxy-5,8,11,13-eicosatetraenoic acid
Microsomal (MAPEGs)	Clan 1	MGST2	MGST2	CDNB, $LTA_4 \rightarrow LTC_4$, (*S*)-5-hydroperoxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid
		MGST3	MGST3	CDNB, $LTA_4 \rightarrow LTC_4$,(S)-5-hydroperoxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid
		LTC4S	LTC_4S	$LTA_4 \rightarrow LTC_4$
	Clan 2	PTGEs	PGES1	$PGH_2 \rightarrow PGE_2$
		MGST1	MGST1	CDNB, CuOOH, hexachlorobuta-1,3-diene
	Clan 3	ALOX5AP	FLAP	Arachidonic acid binding (nonenzymatic)

Abbreviations: Δ^5-ADD, Δ^5-androstene-3,17-dione; BCDE, benzo[g]chrysene diol epoxide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BPDE, benzo[a]pyrene diol epoxide; BPhDE, benzo[c]phenanthrene diol epoxide; CDE, chrysene-1,2-diol 3,4-epoxide; CDNB, 1-chloro-2,4-dinitrobenzene; COMC-6, crotonyloxymethyl-2-cyclohexenone; CuOOH, cumene hydroperoxide; DBADE, dibenz[a,h]anthracene diol epoxide; DBPDE, dibenzo[a,l]pyrene diol epoxide; EA, ethacrynic acid; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; N-acetoxy-PhIP, N -acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

**GST S1-1 is also the glutathione-dependent prostaglandin D_2 synthase.*

SOURCES: Data from Hayes et al. (2005), Mannervik et al. (2005), and Higgins and Hayes (2011).

may undergo rearrangement reactions, which restore aromaticity and possibly lead to migration of the conjugate to the adjacent carbon atom (through formation of an episulfonium ion), as shown in Fig. 6-70. Conjugation of quinones and quinoneimines with GSH also restores aromaticity, as shown in Fig. 6-39 for NAPQI, the reactive metabolite of acetaminophen. Compared with glucuronidation and sulfonation, conjugation with GSH is a minor pathway of acetaminophen biotransformation, even though the liver contains high levels of both GSH and GSTs. The relatively low rate of GSH conjugation reflects the slow rate of formation of NAPQI, which is catalyzed by CYP enzymes (Fig. 6-39).

GSH can also conjugate xenobiotics with an electrophilic heteroatom (*O*, *N*, and *S*) as shown in Fig. 6-71. In each of the examples shown in Fig. 6-71, the initial conjugate formed between GSH and the heteroatom is cleaved by a second molecule of GSH to form oxidized GSH, which is also known as glutathione disulfide (GSSG). The initial reactions shown in Fig. 6-71 are catalyzed by GST, whereas the second reaction (which leads to GSSG formation) generally occurs nonenzymatically. Analogous reactions leading to the reduction and cleavage of disulfides have been described previously (see Fig. 6-18). Some of the reactions shown in Fig. 6-71, such as the reduction of hydroperoxides to alcohols, can also be catalyzed by glutathione peroxidase (GPX), which is a selenium-dependent enzyme. For their role in the reduction of hydroperoxides, the GSTs are sometimes called nonselenium-requiring GPXs. For instance, one or more members of the mammalian cytosolic, microsomal, and mitochondrial GSTs exhibit GPX activity toward cumene hydroperoxide (Hayes et al., 2005). Cleavage of the nitrate esters of nitroglycerin releases nitrite, which can be converted to the potent vasodilator, nitric oxide (NO). The ability of sulfhydryl-generating chemicals to partially prevent or reverse tolerance to nitroglycerin suggests that GSH-dependent denitration may play

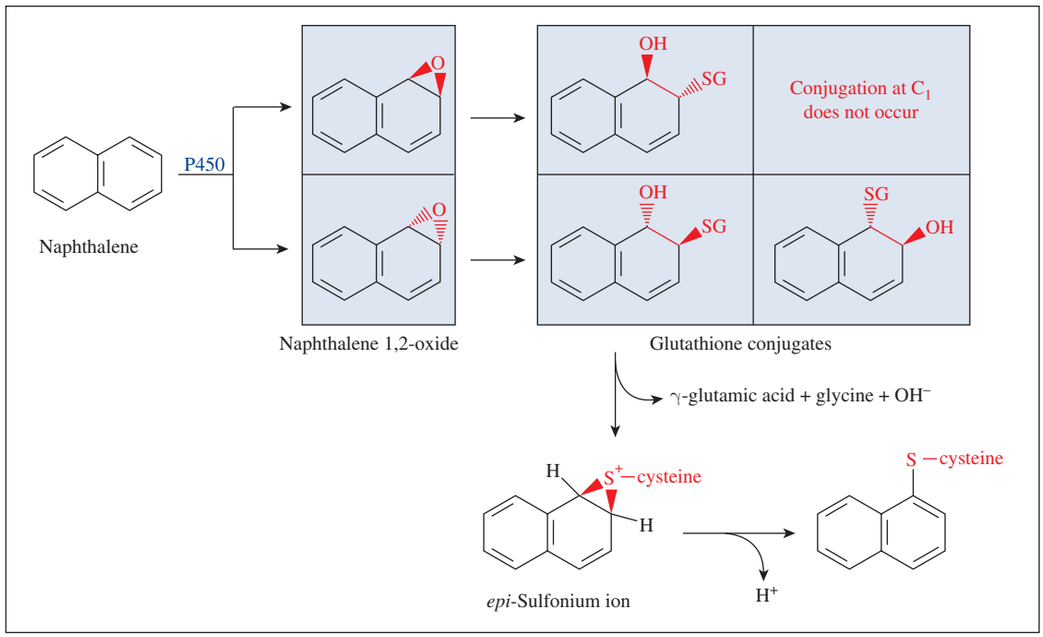


Figure 6-70. *Stereoselective conjugation of naphthalene 1,2-oxide and rearrangement of 2-naphthyl to 1-naphthyl conjugates.*

a role in nitroglycerin-induced vasodilation, although ALDH2 is recognized as the principal enzyme responsible for activating nitroglycerin as discussed in the section "Aldehyde Dehydrogenase" (Marchitti et al., 2008).

Cytosolic or mitochondrial GSTs catalyze two important isomerization reactions, namely, the conversion of the endoperoxide, PGH_2, to the prostaglandins PGD_2 and PGE_2 and the conversion of Δ^5 steroids to Δ^4 steroids, such as the formation of androstenedione from androst-5-ene-3,17-dione. Another physiological function of GST is the synthesis of leukotriene C_4, which is catalyzed by the microsomal GSTs, namely, MGST2, MGST3, and LTC_4S (see Table 6-24).

GSH conjugates formed in the liver can be effluxed into bile by MRP2 on the canalicular membrane, or they can be transported into blood by various transporters on the sinusoidal membrane such as MRP1, MRP3, MRP4, MRP5, and MRP6 (Giacomini and Sugiyama, 2006; Ballatori et al., 2009a; Klaassen and Aleksunes, 2010). MRP7 and MRP8 have also been implicated in the transport of GSH conjugates (Ballatori et al., 2009a). GSH itself is often cotransported with other xenobiotics or xenobiotic metabolites, namely, by MRP2 into bile, and by MRP1, MRP4, and MRP5 into blood (Ballatori et al., 2009a). This cotransport is exemplified by drugs such as vincristine, etoposide, and vinblastine, each of which stimulates MRP-mediated GSH transport (Ballatori et al., 2009a). BCRP is also involved in GSH transport (Brechbuhl et al., 2010). Furthermore, GST and MRP overexpression can act synergistically to confer multiple-drug resistance in many cancers, and can affect the efficacy of chemotherapeutic drugs such as chlorambucil, etoposide, ethacrynic acid, vincristine, and doxorubicin (Sau et al., 2010).

GSH conjugates can be converted to mercapturic acids (*N*-acetylcysteine conjugates) in the kidney and excreted in urine. As shown in Fig. 6-72, the conversion of GSH conjugates to mercapturic acids involves the sequential cleavage of glutamic acid and glycine from the GSH moiety, followed by *N*-acetylation of the resulting cysteine conjugate. The first two steps in mercapturic acid synthesis are catalyzed outside the cell by two membrane-bound enzymes on the luminal surface of the proximal tubule of the kidney (and the bile ducts of the liver), namely γ-glutamyltransferase (GGT; aka γ-glutamyltranspeptidase), which cleaves the glutamic acid residue, and alanyl aminopeptidase (ANPEP), which cleaves the glycine residue. After hydrolysis, the xenobiotic, now conjugated only to cysteine, is transported into the proximal tubule, where it is *N*-acetylated to a mercapturic acid by cytosolic NAT or deconjugated by mitochondrial β-lyase, as shown in Fig. 6-72. Mercapturic acids are transported back across the luminal surface of the proximal tubules for elimination in urine. Unconjugated GSH is also hydrolyzed by GGT and ANPEP to prevent loss of circulating GSH in urine. The process is remarkably efficient, as evidenced by the observation that treatment of rats with L-γ-glutamyl-(*O*-carboxy)-phenyl-hydrazine, an irreversible inhibitor of GGT, resulted in a 3000-fold increase in the urinary excretion of GSH (Griffith and Meister, 1979). Sequential hydrolysis by GGT and ANPEP is involved in leukotriene metabolism. Leukotriene C_4, a GSH conjugate, is hydrolyzed by GGT to leukotriene D_4, a cysteinylglycine conjugate, which in turn is hydrolyzed by ANPEP to leukotriene E_4, a cysteine conjugate.

Hydrolysis of GSH conjugates at the luminal membrane of proximal tubules followed by active uptake of the resulting cysteine conjugates into the proximal cells of the kidney plays a key role in the nephrotoxicity of certain xenobiotics. Nephrotoxicity is the dose-limiting toxicity of the anticancer drug cisplatin, which is conjugated with GSH. After hydrolysis by GGT and ANPEP in the lumen of the proximal tubule, the cysteine conjugate of cisplatin is actively transported into the proximal tubular cells, where it is converted by β-lyase to a highly reactive thiol that is toxic to kidney cells (Zhang and Hanigan, 2003). Cisplatin nephrotoxicity is not observed in GGT knockout mice or in mice treated with the GGT inhibitor acivicin (Hanigan et al., 1994, 2001). The antiretroviral drug efavirenz is nephrotoxic to rats and mice but not monkeys or humans. A sulfate conjugate of efavirenz is conjugated with GSH by a rodent-specific GST. Hydrolysis of the GSH conjugate by GGT and ANPEP followed by active transport of the cysteine

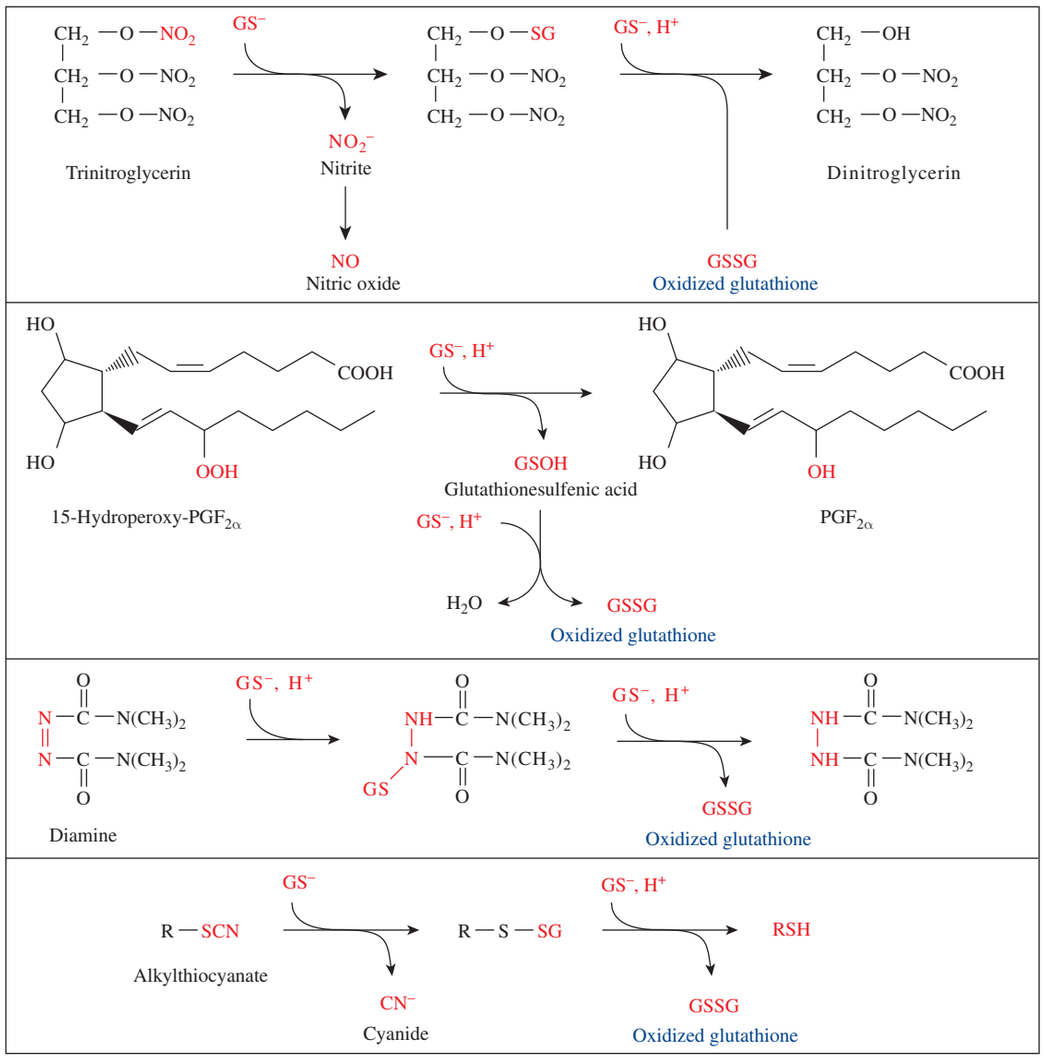


Figure 6-71. *Examples of glutathione conjugation of electrophilic heteroatoms.*

conjugate into the proximal cells of the kidney efficiently delivers high concentrations of efavirenz sulfate to the kidney, which results in nephrotoxicity in rodents but not primates (Mutlib et al., 1999, 2000). A similar mechanism (i.e., hydrolysis of GSH conjugates by membrane-bound GGT and ANPEP followed by active transport of the cysteine conjugate) accounts for the nephrotoxicity of a number of halogenated compounds, such as trichloroethene and hexachloro-1,3-butadiene, and other xenobiotics, as discussed later in this section (Dekant, 2001).

Cytosolic GSTs are dimers, typically composed of identical subunits (M_r 23–29 kDa), although some forms are heterodimers. Each subunit contains 199 to 244 amino acids and one catalytic site. The general structural features of cytosolic GSTs include an N-terminal thioredoxin-like domain (with βαβαβαβα topology) and a C-terminal domain consisting of α-helices (Oakley, 2011). Mitochondrial GSTs are also dimeric with 226 amino acids per subunit. The microsomal GSTs vary in their ability to form complex aggregates across the three groups of human MAPEGs. Numerous GST subunits have been cloned and sequenced, which forms the basis of a nomenclature system, as shown in Table 6-24 (Hayes et al., 2005; Mannervik et al., 1992; Hayes and Pulford, 1995; Whalen and Boyer, 1998; Higgins and Hayes, 2011). Each cytosolic and mitochondrial enzyme is assigned a two-digit number to designate its subunit composition. For example, the homodimers of subunits 1 and 2 are designated 1-1 and 2-2, respectively, whereas the heterodimer is designated 1-2. The soluble GSTs were initially arranged into four classes designated A, M, P, and T (which refer to Alpha, Mu, Pi, and Theta). Four additional classes were subsequently identified, namely, K (Kappa; the mitochondrial form), S (Sigma), Z (Zeta), and O (Omega) (Hayes and Pulford, 1995; Whalen and Boyer, 1998; Strange et al., 2000; Hayes et al., 2005; Higgins and Hayes, 2011; Oakley, 2011). None of these eight gene classes corresponds to the microsomal GSTs (MAPEGs), which appear to have evolved independently. By definition, the subunits in the different classes share less than 50% amino acid sequence identity. Generally, the subunits within a class are about 70% identical, but can share up to 90% sequence identity, and can form heterodimers, whereas the subunits in different classes are generally only about 30% identical. On the basis of structural similarity as well as the residues in the GSH-binding site that are involved in thiolate anion formation, the cytosolic GSTs can be divided into two groups: the more recently evolved Alpha, Mu, Pi, and Sigma classes that use

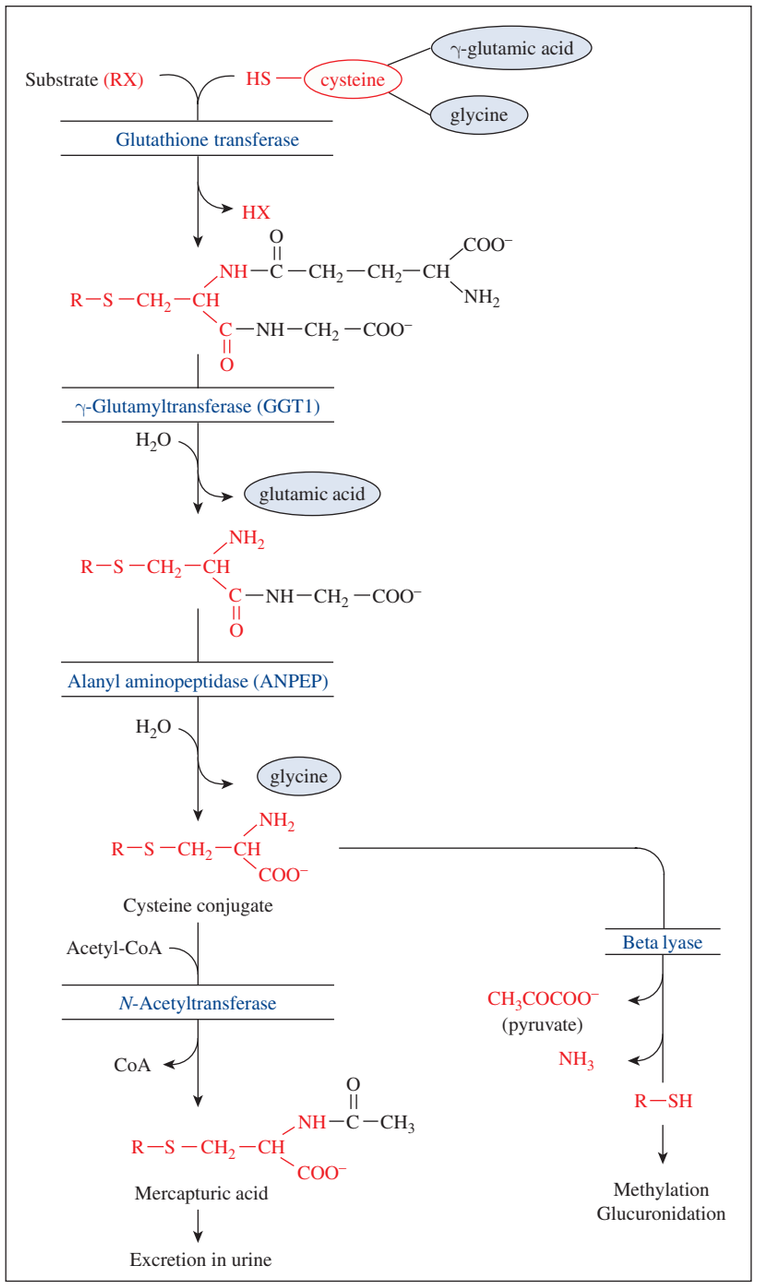


Figure 6-72. *Glutathione conjugation and mercapturic acid biosynthesis.*

a tyrosine residue near the N-terminus to activate GSH, and the older Theta, Omega, and Zeta classes that use either an N-terminal proximate serine or cysteine residue to activate GSH (Higgins and Hayes, 2011). Members of the Alpha and Mu classes can also form heterodimers with each other (Hayes et al., 2005). Affinity labeling studies with rat Alpha GSTs demonstrated the presence of a high-affinity, nonsubstrate binding site within the cleft between the two subunits of the dimer. Presumably, the cleft in heterodimers would be different from that in homodimers, which suggests the possibility that there is an evolutionary reason that the formation of heterodimers between Alpha and Mu subunits has been conserved (Hayes et al., 2005).

Several GSTs (as well as GGT) are overexpressed in various cancers, such as Alpha, Mu, Theta, Pi, and microsomal GSTs, which can confer resistance against many chemotherapeutic drugs (Hollman et al., 2016). For example, GST Alpha overexpression has been correlated with resistance to alkylating drugs (Sau et al., 2010). GST Mu overexpression is associated with chlorambucil resistance and a poor prognosis in childhood acute lymphoblastic leukemia (Sau et al., 2010). The microsomal GSTs have been shown to confer resistance to chlorambucil, melphalan, cisplatin, and doxorubicin treatment (Sau et al., 2010). GSTP1-1 overexpression, the most highly expressed GST in various cancers, is often associated with a poor prognosis and chemotherapeutic resistance

in ovarian, non-small-cell lung, breast, and colorectal cancers (Sau et al., 2010; Tew and Townsend, 2011). Two strategies have been adopted in the development of cancer therapies that specifically target cells that overexpress GSTs, namely (1) inhibition of GSTs and (2) activation of prodrugs by GSTs (or GGT) to form reactive metabolites (Ramsay and Dilda, 2014). Ezatiostat (Telintra or TLK199), which is currently in clinical trials for the treatment of myelodysplastic syndrome, is a GSH-peptidomimetic prodrug that is activated by cellular esterases to the selective GSTP1-1 inhibitor TLK117 (Sau et al., 2010; Tew and Townsend, 2011; Ramsay and Dilda, 2014). Although not commonly used due to its diuretic effects, ethacrynic acid inhibition of GSTs sensitizes cancer cells to chemotherapeutics (Sau et al., 2010). A synthetic GSH analog specifically inhibits GSTA1-1, whereas the 7-nitro-2,1,3-benzoxadiazole derivative NBDHEX is an inhibitor of GSTP1-1 that is not effluxed by transporters (Sau et al., 2010). Several other drugs have been shown to inhibit GSTs, such as drugs that act as NO donors (PABA/NO and oxathiazolylum-5-olate derivatives) and bombesin-sulfonamide derivatives (Sau et al., 2010). Canfosfamide (Telcyta or TLK286) is a GST-activated nitrogen mustard. This modified GSH analog is metabolized by GSTP1-1, which results in the release of two electrophilic fragments, GSH vinyl sulfone and tetrakis(chloroethyl)phosphorodiamidate, that induce apoptosis (Townsend and Tew, 2003; Rosen et al., 2004; Gonzalez and Tukey, 2006; Tew and Townsend, 2011). Clinical development of canfosfamide, for treatment of ovarian cancer, was halted by the FDA in 2007. Other drugs have also been developed to take advantage of activation by GSTs, such as brostallicin and ethacraplatin, which are derivatives of 2-crotonyloxymethyl-2-cycloalkenone (Sau et al., 2010). Like GST, GGT is also overexpressed in various cancer cells. Arsenic-based prodrugs (GSAO and darinaparsin) have been developed, which are activated by GGT (Ramsay and Dilda, 2014).

As is the case with many of the other xenobiotic-metabolizing enzymes, all of the GST enzymes are polymorphic and this likely contributes to interindividual differences in drug response or toxicity (Hollman et al., 2016). For example, the null phenotypes for GST Mu and GST Theta, GSTM1*0 and GSTT1*0, respectively, contribute to the drug-associated toxicities of several xenobiotics, such as carbamazepine, valproic acid, and troglitazone (Saruwatari et al., 2010; Ikeda, 2011). Therefore, individuals who are polymorphic for these enzymes (and also potentially for other GST allelic variants) are at an increased risk of toxicity by xenobiotics that are selectively biotransformed by specific GSTs. A large number of associations between genetic variants of GSTs (A1, M1, M3, M4, M5, O1, O2, P1, T1, and Z1) and multiple types of cancer, inflammatory diseases, immunological disorders, neurological disorders, age-related or metabolic disorders, and reproductive disorders have been described (Hollman et al., 2016).

The alpha GSTs are the major GSTs in the liver and kidney. They have basic isoelectric points. Rats express four GSTA genes, GSTA1–A4, whereas humans express five GSTA genes, GSTA1–A5. GST1*A and GST1*B are common variants of human GSTA1 (Hayes et al., 2005). GSTA1*A expression is higher than GSTA1*B, and individuals with the latter loss-of-function variant have improved rates of breast cancer survival, presumably due to increased efficacy of chemotherapeutic drugs (Ekhart et al., 2009). In contrast, GSTA2 variants *A to *D do not show much difference in enzymatic activity, whereas GSTA2*E is a loss-of-function variant (McIlwain et al., 2006). Rats express at least seven functional Mu subunits, designated GSTM1 through GSTM7. Humans express five subunits belonging to the Mu class of GSTs, designated GSTM1 through GSTM5. Human GSTM1, 3, and 4 are known to be polymorphic. In the case of GSTM1, the *A and *B allelic variants differ by a single amino acid; *A is a "basic variant" with lysine at residue 173, whereas *B is an "acidic variant" with asparagine at the corresponding site. There is also a GSTM1 deletion (*0) and a duplication (*1 × 2) (Hayes et al., 2005). The incidence of the GSTM1 deletion is high in Pacific Islander and Malaysian populations (i.e., 62% to 100%), intermediate in several other populations (i.e., 35% to 62% in those of European descent; 32% to 53% in those of Asian descent; 40% to 53% in those of Hispanic descent), and low in those of African descent (i.e., 23% to 41%) (Geisler and Olshan, 2001; Piacentini et al., 2011). GSTM1*0 has only a modest effect on the incidence of the lung, head, and neck cancers consistent with its high prevalence in various populations. The GSTM1*0 polymorphism has been linked to an increased susceptibility to certain inflammatory diseases (e.g., asthma) (Hayes et al., 2005). At least two allelic variants of GSTM3 and GSTM4 have also been identified. Members of the Mu class of GSTs have neutral isoelectric points. Human GSTM2 and GSTM3 are expressed in muscle and brain, respectively. The GSTM1*0 polymorphism is a risk factor for carbamazepine and valproic acid hepatotoxicity, whereas the double null phenotype of GSTM1*0 and GSTT1*0 is a risk factor for troglitazone hepatotoxicity (Ikeda, 2011; Saruwatari et al., 2010).

Humans and rats express one subunit belonging to the Pi class of GSTs, GSTP1, whereas mice have been found to express two Pi subunits, GSTP1 and GSTP2 (provisional). The human Pi enzyme is polymorphic, with at least four allelic variants reported (Hayes et al., 2005). The subunit encoded by GSTP1*B is seven times more active than that encoded by GSTP1*A in conjugating diol epoxides of polycyclic aromatic hydrocarbons (PAHs) (Strange et al., 2000). GSTP polymorphisms are known to influence susceptibility to carcinogens (including some pesticides), affect the response to chemotherapy used in the treatment of metastatic colon cancer and multiple myeloma, and modify the risk of acute myeloid leukemia which results from the successful treatment of Hodgkin disease, non-Hodgkin lymphoma, breast cancer, and ovarian cancer (Hayes et al., 2005; Liu et al., 2006b). GSTP polymorphisms that result in decreased activity have been implicated in an increased susceptibility to certain inflammatory diseases (e.g., asthma) (Hayes et al., 2005). In some cancers, such as prostate, breast, liver and blood cancers, GSTP1 is silenced by hypermethylation of a CpG-rich promoter region, which has been linked to the promotion of neoplastic transformation, such that low GSTP1 expression is a biomarker for multistep cancer development (Schnekenburger et al., 2014). Members of the Pi class of GSTs have acidic isoelectric points. They are expressed in the placenta, lung, gut, and other extrahepatic tissues. In rats, GSTP1 is one of several proteins (so-called preneoplastic antigens) that are overexpressed in chemical-induced tumors.

The Sigma class of GSTs is widely distributed in nature, and is present in many species including humans, mice, rats, chickens, insects, flatworms, and mollusks (Flanagan and Smythe, 2011). Sigma GSTs, also known as hematopoietic prostaglandin D synthases (HPGDS), are involved in the synthesis of prostaglandin in mammals, and catalyze the conversion of the cyclooxygenase product PGH_2 to PGD_2. Sigma subunits are also capable of conjugating GSH to a variety of aryl halide substrates and organic isothiocyanates and also have GPX activity toward cumene hydroperoxide (Flanagan and Smythe, 2011). The human Sigma class GST (HPGDS) is different from other cytosolic GSTs in that it is activated by divalent metal ions, such as Mg^{2+} and Ca^{2+}, binding at the center of the dimer interface formed by a group of six conserved Asp residues (Inoue et al., 2003; Flanagan and Smythe, 2011). Rats and humans appear to express a single Sigma GST (GSTS1, also

known as the GSH-dependent HPGDS). The tissue distribution of Sigma GST occurs in a species-specific manner. In mammals, high levels of Sigma GST are present in the spleen, bone marrow, placenta, lung, adipose, oviduct, and both dermal and epidermal layers of skin (Flanagan and Smythe, 2011). In rats, Sigma GST is highly expressed in bone marrow and spleen, whereas in humans it is highly expressed in the adipose tissue, placenta, lung, and fetal liver (Flanagan and Smythe, 2011). Immunohistochemical studies of Sigma GST (i.e., HPGDS) in rat, mouse, and human have shown that expression of the enzyme is limited to select cell types, namely, antigen-presenting cells, resident tissue and infiltrating mast cells, and T-helper (Th)-2 cells (Flanagan and Smythe, 2011). Sigma GST can play multiple roles in the immune response because the PGD_2 produced by Sigma GST (i.e., by HPGDS) can exert both proinflammatory and anti-inflammatory effects (Flanagan and Smythe, 2011). To block PGD_2 production associated with allergic inflammation, drugs have been or are currently being developed as Sigma GST (HPGDS) inhibitors, such as tranilast (*N*-(3,4-dimethoxycinnamoyl) anthranilic acid), which is an inhibitor of both HPGDS and LPGDS (lipocalin prostaglandin D synthase) (Flanagan and Smythe, 2011).

Humans express two subunits belonging to the Theta class of GSTs (GSTT1 and GSTT2), whereas rats and mice each express four subunits (GSTT1–4). GST Theta catalyzes the conjugation of mono and dihaloalkanes with GSH, in contrast to α-haloalkanoates and α, α-dihaloalkanoates which tend to be substrates of GST Zeta (Josephy, 2010). Due to the small active site of GST Theta, small xenobiotics are typical substrates of this enzyme, such as dichloromethane (Josephy, 2010). Additionally, analogous to the reaction shown in Fig. 6-24, GSTT1 is involved in the bioactivation of ethylene dibromide (an insecticide and gasoline additive) to an electrophilic sulfonium ion that can form adducts with macromolecules (Josephy, 2010). A GSTT1 deletion has been identified (GSTT1*0), which has a modest effect on the incidence of lung, head, and neck cancers, and has also been implicated in an increased susceptibility to certain inflammatory diseases (e.g., asthma) (Hayes et al., 2005) and coronary heart disease, especially in cigarette smokers (Song et al., 2017). The incidence of the GSTT1*0 is present in several populations, at a low to intermediate frequency (10% to 43%) in those of European and Mediterranean descent and a slightly higher frequency (20% to 45%) in those of African descent (Piacentini et al., 2011). The GSTT1 null allele, in conjunction with GST Mu deficiency, predisposes to troglitazone hepatotoxicity (Ikeda, 2011). Human GSTT2-2 conjugates ethacrynic acid with GSH and catalyzes the GSH-dependent reduction of cumene hydroperoxide, as well as the GSH-dependent sulfatase activity of 1-menaphthyl sulfate (Higgins and Hayes, 2011).

Zeta class GSTs are found in many species, such as plants, fungi, and mammals. Within mammals, there is also a wide tissue distribution of the protein with high levels predominantly in the liver and kidney (Board and Anders, 2011). A single Zeta subunit is expressed in rats, mice, and humans (GSTZ1), which dimerizes to form the functional enzyme GSTZ1-1. GSTZ1-1 is the same enzyme as maleylacetoacetate isomerase (MAAI), the enzyme that catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate, the penultimate step in tyrosine and phenylalanine catabolism (Board and Anders, 2011). GSTZ1-1 also catalyzes the GSH-dependent biotransformation of dichloroacetate (DCA) to glyoxylate ($CHCl_2$–COOH → CHO–COOH) (Board and Anders, 2011). Although GSH is required for the biotransformation of DCA to glyoxylate, it is not consumed as the GSTZ1-1-catalyzed nucleophilic attack of GSH on DCA yields *S*-(α-chlorocarboxymethyl) glutathione (GS–CHClCOOH), which in turn undergoes dehalogenation and facile hydrolysis to produce glyoxylate and GSH (Board and Anders, 2011). In all species examined, DCA inhibits its own metabolism by irreversibly inhibiting GSTZ1-1 through covalent modification of Cys_{16} by *S*-(α-chlorocarboxymethyl) glutathione (Board and Anders, 2011; James et al., 2017). DCA has several potential therapeutic applications (such as the treatment of inherited mitochondrial disorders that result in lactic acidosis, pulmonary hypertension and, by restoring aerobic glycolysis, several solid tumors) based on its ability to inhibit pyruvate dehydrogenase kinase. However, treatment with DCA is complicated by its auto-inhibition, which results in increased exposure to DCA with repeat dosing. While monohaloalkanes and dihaloalkanes tend to be substrates for GST Theta, a number of α-haloalkanoates and α,α-dihaloalkanoates have been identified as substrates for GSTZ1-1 (Board and Anders, 2011). At least four polymorphic variants of GSTZ1-1 have been identified in humans, with the haplotypes designated as GSTZ1A (Lys_{32}, Arg_{42}, Thr_{82}), GSTZ1B (Lys_{32}, Gly_{42}, Thr_{82}), GSTZ1C (Glu_{32}, Gly_{42}, Thr_{82}), and GSTZ1D (Glu_{32}, Gly_{42}, Met_{82}) (Board and Anders, 2011). GSTZ1C shows the highest allele frequency and has been designated the wild-type enzyme. GSTZ1A shows the highest rate of conversion of DCA to glyoxylate and is resistant to inactivation by DCA (Board and Anders, 2011). Genetically determined differences in DCA turnover may be significant if DCA is utilized in cancer therapy (Michelakis et al., 2010; Board and Anders, 2011). GSTZ1 knockout mice have increased levels of GSTA1, A2, M1, M5, P1, P2, and NQO1 in the liver, and undergo rapid weight loss, leucopenia, and death when provided 2% phenylalanine in drinking water (recall that GSTZ1-1 is the same enzyme as MAAI, the enzyme that catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate, the penultimate step in tyrosine and phenylalanine catabolism) (Board and Anders, 2011).

The Omega class of GST was initially identified through bioinformatics analysis of the expressed sequence tag (EST) database. Omega GSTs are present in many species including plants, yeast, nematodes, insects, and mammals (Board, 2011). Two Omega subunits have been identified in mice, rats, and humans (GSTO1 and GSTO2). GSTO1 and GSTO2 are also highly expressed in the mouse liver, heart, and kidney with wide distribution in other tissues (Board, 2011). The GSTOs differ from the other cytosolic GSTs in that they do not display the typical activity toward substrates such as 1-chloro-2,4-dinitrobenzene, dichloromethane, cumene hydroperoxide, or ethacrynic acid, but have some characteristics of glutaredoxins (GLRXs) (Board, 2011; Mukherjee et al., 2006). Interestingly, a mutation in GSTO1 (Cys_{32}Ala) significantly improves its GSH-conjugating activity with 1-chloro-2,4-dinitrobenzene (Board, 2011). Human GSTO1 shows thioltransferase activity and can catalyze the reduction of *S*-phenacylglutathione (a metabolite of tear gas), dehydroascorbate, and methylated arsenic species (Board, 2011). In fact, GSTO2 was originally identified in rats as dehydroascorbate reductase. The major component in tear gas 2-chloroacetophenone is converted to a GSH conjugate (*phenyl*-CO–CH_2Cl + GSH → *phenyl*-CO–CH_2–SG + HCl) that is further metabolized by GSTO1 to acetophenone (*phenyl*-CO–CH_2–SG + GSH → *phenyl*-CO–CH_3 + GSSG) (Board, 2011). Inasmuch as the reaction with *S*-phenacylglutathione is highly specific to GSTO1, a novel substrate was developed, namely, *S*-(4-nitrophenacyl)glutathione, that can be used in spectrophotometric assays to determine GSTO1 activity (Board, 2011). GSTO2 also exhibits thioltransferase activity and can catalyze the reduction of monomethylarsenate and dehydroascorbate, but not *S*-phenacylglutathiones (Board, 2011). Sulfonylurea cytokine-release inhibitory drugs (CRIDs) can bind to GSTO1 and inhibit IL-1β activation, suggesting GSTO1 involvement in a proinflammatory response (Board, 2011). GSTO1 can catalyze the glutathionylation and deglutathionylation of proteins.

This reversible, posttranslational modification of proteins may explain the requirement for functional GSTO1 in LPS-stimulated inflammatory signaling through the TLR4 receptor. The observation that ML175, a selective GSTO1 inhibitor, can block LPS-stimulated inflammatory signaling suggests that GSTO1 inhibitors represent a potential new class of anti-inflammatory drugs for the treatment of toxic shock and other inflammatory disorders (Board and Menon, 2016). Other chemicals have been shown to inhibit GSTO1 activity, such as tocopherol succinate, phenylsulfonate, omeprazole, and rifampicin (Bachovchin et al., 2009; Board, 2011). As Omega GSTs can reduce monomethylarsenic acid, they have been suggested to play a key role in arsenic biotransformation through the arsenic methylation pathway (Board, 2011). However, several studies have not supported a major role for GSTOs in overall arsenic biotransformation (Board, 2011). While most GSTs have a tyrosine or serine at the active site, GSTOs have a cysteine and an additional 19 amino acids at the N-terminus (Mukherjee et al., 2006; Board, 2011). Many polymorphisms have been detected in both GSTO1 and GSTO2, with five and four polymorphisms that result in amino acid changes, respectively (Mukherjee et al., 2006; Board, 2011). GSTO genes have also been associated with several neurological diseases, suggesting that variation of these GSTs may modify disease susceptibility or age of onset through a common pathological mechanism (Board, 2011).

The Kappa class of GSTs is an ancient protein family and represents a separate evolutionary pathway that has significant differences in function, localization, and structure than other soluble GSTs (Morel and Aninat, 2011). Harris et al. (1991) initially discovered and isolated GST Kappa from rat liver mitochondrial matrix. The Kappa subunit is a soluble, dimeric protein with a 36% sequence identity to GST Theta, and localized to both the mitochondria and peroxisomes (Morel and Aninat, 2011). A single Kappa subunit is expressed in mouse, rat, and humans, with the gene designated as GSTK1 in each species. The three-dimensional structure of the Kappa subunit is more similar to bacterial GSH-dependent and disulfide-bond-forming oxidoreductase (DsbA) than to the cytosolic GSTs. The Kappa subunit contains a thioredoxin-like domain but with a DsbA-like α-helical domain inserted between the α2 helix and the β3 strand (Oakley, 2011). GSTK1 is ubiquitously expressed in humans with the kidney, liver, adrenal gland, and adipose tissue expressing the most abundant levels (Morel and Aninat, 2011). Differential expression of GSTK1 has been seen between fetal and adult liver and brain tissues, indicating possible ontogenic regulation of GST Kappa (Morel and Aninat, 2011). In rodents it is present at high levels in the liver and stomach; moderate levels in the kidney, heart, and lung; and subsequent decreasing levels in the duodenum, jejunum, and ileum (Thomson et al., 2004; Knight et al., 2007; Morel and Aninat, 2011). GSTK1 expression in the heart shows marked gender differences, with females expressing twice as much Kappa subunit than males (Knight et al., 2007; Morel and Aninat, 2011).

Two human single nucleotide polymorphisms (SNPs) of GSTK1 have been identified in the 5′-flanking region of the GSTK1 gene located −1308 and −1032 bp from the transcription start site (Shield et al., 2010; Morel and Aninat, 2011). The −1308 SNP corresponds to a G to T transition, whereas the −1032 SNP corresponds to an alteration in a CpG methylation site (Shield et al., 2010; Morel and Aninat, 2011). The T allele variant (−1308 SNP) occurs with a 20% frequency in Asian populations (Morel and Aninat, 2011). The −1032 SNP has been demonstrated to cause a 38% decrease in GSTK1 promoter activity in HEK293 and HepG2 cells (Shield et al., 2010; Morel and Aninat, 2011). GSTK1 expression has been correlated with obesity; hence, GST Kappa may represent a new target for xenobiotics in the treatment of insulin resistance and related metabolic disorders (Morel and Aninat, 2011).

The microsomal GSTs are distinct from the soluble enzymes and have been referred to as the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Higgins and Hayes, 2011). Six human microsomal GSTs divided into three clans have been identified (see Table 6-24), which differ in their ability to form aggregates. For instance, MGST1 exists as a trimer, as does the other clan 2 MAPEG, namely, PGES1, whereas the clan 1 MAPEGs, LTC_4S, and MGST2, as well as the clan 3 MAPEG 5-lipoxygenase-activating protein (FLAP), can function as monomers, dimers, trimers, or more complex aggregates (Hayes et al., 2005; Martinez Molina et al., 2008; Higgins and Hayes, 2011). MGST1 conjugates xenobiotics with GSH and probably functions solely as a detoxication enzyme. It is a highly abundant microsomal protein (1% of total ER protein) in the human liver and the outer mitochondrial membrane (5% of outer mitochondrial membrane protein) in the rat liver (Morgenstern et al., 2011). MGST1 plays a role in the biotransformation of certain lipophilic reactive electrophiles and the reduction of membrane-embedded hydroperoxides with substrates that include halogenated hydrocarbons and phospholipid hydroperoxides (Morgenstern et al., 2011). The denitration of glyceryl trinitrate is another role for MGST1 (Morgenstern et al., 2011). While no specific substrates for MGST1 have been found, this MAPEG can be selectively activated with *N*-ethylmaleimide to determine its contribution toward overall GST activity (Morgenstern et al., 2011). Furthermore, MGST1 is the only GST that can utilize *N*-acetyl-L-cysteine as a cofactor instead of GSH (Morgenstern et al., 2011). MGST1 is involved in the formation of the mitochondrial permeability transition (MPT), suggesting a role for MGST1 in mitochondria-mediated cell death (Aniya and Imaizumi, 2011). It is also upregulated in many tumor types, overexpressed in many stem cells and stem-cell-like tumor cells, where it may be playing a protective role for these cells (Morgenstern et al., 2011). Other MAPEGs, such as MGST2 and MGST3, contribute to detoxication and synthesis of leukotriene C_4 (Hayes et al., 2005). FLAP does not have catalytic activity, but binds arachidonic acid and is essential for leukotriene synthesis. LTC_4S and PGES1 do not appear to be involved in xenobiotic metabolism (Hayes et al., 2005). Many SNPs have been identified in MGST1 and FLAP as well as diallelic variants in MGST3 in certain populations, but the biological significance has not yet been determined (Hayes et al., 2005).

The conjugation of certain xenobiotics with GSH is catalyzed by most classes of GST. For example, members of the Alpha, Kappa, Mu, and Pi classes of human cytosolic GSTs, as well as several of the MAPEG GSTs, all catalyze the conjugation of 1-chloro-2,4-dinitrobenzene. Other reactions are fairly specific for one class of enzymes (Hayes and Pulford, 1995). For example, the Alpha GSTs preferentially isomerize Δ^5 steroids to Δ^4 steroids and reduce linoleate and cumene hydroperoxide to their corresponding alcohols. The Mu GSTs preferentially conjugate certain arene oxides and alkene epoxides, such as styrene-7,8-epoxide. The Pi GSTs preferentially conjugate ethacrynic acid. Additional examples are shown in Table 6-24. However, individual members within a class of GSTs can differ markedly in their substrate specificity. In mice, for example, the Alpha GSTs composed of GSTA3 subunits can rapidly conjugate aflatoxin B_1 8,9-epoxide, whereas those composed of GSTA1 subunits are virtually incapable of catalyzing this reaction (Eaton and Gallagher, 1994).

In rodents, individual members of the Alpha and Mu classes of GSTs are inducible (generally two- to threefold) by 3-methylcholanthrene, phenobarbital, corticosteroids, oltipraz, and

various antioxidants (such as ethoxyquin and BHA). Several GST substrates (i.e., Michael acceptors) are GST inducers, as are certain nonsubstrates, such as hydrogen peroxide and other reactive oxygen species, all of which activate Nrf2 (Rushmore et al., 1991; Daniel, 1993; Nguyen et al., 1994; Hayes and Pulford, 1995; Hayes et al., 2005; Higgins and Hayes, 2011). Induction is usually associated with increased levels of mRNA due to transcriptional activation of the gene encoding a subunit of GST. Not all subunits are induced to the same extent (Higgins and Hayes, 2011).

The enhancer regions of the genes encoding some of the rodent GSTs (such as rat GSTA2-2) have been shown to contain a xenobiotic-responsive element (XRE), a putative phenobarbital-responsive element, a glucocorticoid-responsive element (GRE), and an antioxidant-responsive element (ARE, which is also known as the electrophile-responsive element [see Point 9 in the section "Principles of Xenobiotic Biotransformation"]). Accordingly, in rodents, certain GST subunits are regulated by both AhR and Nrf2 activators (i.e., by both *monofunctional* and *bifunctional* agents), as described previously for DT-diaphorase (see the sections "Quinone Reduction: NQO1 and NQO2" and "Induction of Cytochrome P450: Xenosensors"). Induction of GSTs by the Nrf2 activator sulforaphane is thought to be responsible, at least in part, for the anticancer effects of broccoli (Zhang et al., 1992; Higgins and Hayes, 2011). GSTA4 is induced in mice administered α-angeliclactone, butylated hydroxyanisole (BHA), ethoxyquin, indole-3-carbinol, limettin, and oltipraz (Hayes et al., 2005; Higgins and Hayes, 2011). In addition to inducing one or more GSTs, activation of Nrf2 by oxidative stress or exposure to electrophiles induces γ-GCL, which increases GSH levels in response to an initial decrease in GSH levels (see Point 9 in the section "Principles of Xenobiotic Biotransformation").

Factors that regulate the expression of GSTs in rodents may have similar effects in humans, but some differences have been noted. For example, the 5′ promoter region of Alpha GST in humans (GSTA1) lacks the ARE and XRE consensus sequences through which the corresponding rat enzyme is induced. However, such sequences appear to be present in the promoter region of human GSTM4 and GSTP1 genes (Hayes and Pulford, 1995; Whalen and Boyer, 1998; Higgins and Hayes, 2011). (A functional ARE is also present in the promoter region of human DT-diaphorase [NQO1].) Therefore, certain subunits of GSTs are inducible by a variety of mechanisms in rats, and other subunits appear to be inducible by similar mechanisms in humans. Species differences in GST regulation may also stem from differences in xenobiotic biotransformation, especially differences in the formation of electrophiles, Michael acceptors, and/or the production of oxidative stress. For example, coumarin is thought be an inducer of GSTP1 in rats because it is converted in the rat liver to reactive metabolites, namely, coumarin 3,4-epoxide and *ortho*-hydroxyphenylacetaldehyde (see Fig. 6-48). In contrast, the major route of coumarin biotransformation in humans is by 7-hydroxylation, which would not be expected to be associated with GST induction.

Conjugation with GSH represents an important detoxication reaction because electrophiles are potentially toxic species that can bind to critical nucleophiles, such as proteins and nucleic acids, and cause cellular damage and genetic mutations. All the enzymes involved in xenobiotic biotransformation have the potential to generate reactive intermediates, most of which are detoxified to some extent by conjugation with GSH. GSH is also a cofactor for GPXs, which play an important role in protecting cells against lipid and hemoglobin peroxidation. Resistance to toxic compounds is often associated with an overexpression of GST. Examples include the resistance of insects to DDT (see Fig. 6-25), of corn to atrazine, and of cancer cells to chemotherapeutic drugs.

GST is the major determinant of certain species differences in chemical-induced toxicity. For example, low doses of aflatoxin B_1 cause liver toxicity and tumor formation in rats but not mice, even though rats and mice convert aflatoxin B_1 to the highly reactive 8,9-epoxide at similar rates (this reaction is shown in Fig. 6-38). This species difference arises because mice express high levels of an Alpha class GST (GSTA3) enabling them to conjugate aflatoxin B_1 8,9-epoxide with GSH up to 50 times faster than rats (or humans, which are also considered a susceptible species) (Eaton and Gallagher, 1994). Mice become sensitive to the adverse effects of aflatoxin B_1 following treatment with chemicals that decrease GSH levels, such as diethyl maleate (DEA) (which depletes GSH) or buthionine-*S*-sulfoximine (BSO) (which inhibits GSH synthesis). Conversely, treatment of rats with inducers of certain GSTs, such as ethoxyquin, BHA, oltipraz, and phenobarbital, protects them from the hepatotoxic/tumorigenic action of aflatoxin B_1 (Hayes et al., 1994)

The conjugation of aflatoxin B_1 8,9-epoxide with GSH provides an interesting example of the stereospecificity with which certain GSH conjugation reactions can occur. CYP converts aflatoxin B_1 to a mixture of *exo*- and *endo*-8,9-epoxides (only a generic 8,9-epoxide is shown in Fig. 6-38, meaning the figure does not indicate whether the oxygen atom is above or below the plane of the ring system). Both enantiomeric epoxides are formed by liver microsomes from mice, rats, and humans, but only the *exo*-epoxide binds extensively to DNA (where it binds to the N^7 position of guanine). One or more mouse Alpha GSTs rapidly conjugate the *exo*-epoxide, which accounts for the resistance of this species to aflatoxin-induced hepatotoxicity and tumorigenicity (as described above). Rat and human GSTAs do not rapidly conjugate either the *exo*- or the *endo*-epoxide (with the exception of the inducible rat GST, which is not constitutively expressed in rats to any great extent). However, human GSTM1-1 can conjugate aflatoxin B_1 8,9-epoxide, but it preferentially conjugates the relatively innocuous *endo*-isomer (Wang et al., 2000).

Species differences in the detoxication of aflatoxin B_1 8,9-epoxide suggest that individual differences in GST may determine susceptibility to the toxic effects of certain chemicals. In support of this interpretation, a genetic polymorphism for GSTM1 has been identified, and individuals who are homozygous for the null allele (i.e., those with low GST activity due to complete deletion of the GSTM1 gene) appear to be at a moderately increased risk for cigarette-smoking-induced lung cancer, head and neck cancer, and possibly bladder cancer (Hayes and Pulford, 1995; Whalen and Boyer, 1998; Strange et al., 2000; Hayes et al., 2005). Depending on the ethnic group, 22% to 100% of the population is homozygous for the GSTM1 null genotype, which results in a complete lack of GSTM1 activity in all tissues. However, there is evidence that GSTM1 confers significant protection from breast cancer in individuals homozygous for a functional GSTM1 allele (Hayes et al., 2005). GSTT1 activity is absent from 11% to 58% of the population (depending on ethnicity) due to deletion of the GSTT1 gene, which appears to increase susceptibility to development of astrocytoma, meningioma, and myelodysplasia. When examined for their individual effect, these null genotypes generally have a small effect on disease susceptibility, with an odds ratio of 2 or less. However, the odds ratio can increase dramatically when these null GST genotypes are examined in conjunction with other genotypes or with environmental factors (such as exposure to carcinogens). For example, when the GSTM1 null genotype is combined with cigarette smoking and a particular CYP1A1 allele, the odds ratio can increase to 8.9 (in one study) or 21.9 (in another study). Polymorphisms that result in amino acid substitutions have been reported for most

human GST genes, some of which alter GST function. Some of these polymorphisms may also be risk modifiers for certain diseases in an analogous manner to the GSTM1 and GSTT1 null genotypes.

In some cases, conjugation with GSH enhances the toxicity of a xenobiotic (Monks et al., 1990; Dekant and Vamvakas, 1993; Testa and Krämer, 2008, 2010). Five mechanisms of GSH-dependent activation of xenobiotics have been identified, with the first four shown in Fig. 6-73. These mechanisms are (1) formation of GSH conjugates of haloalkanes, organic thiocyanates, and nitrosoguanides that release a toxic metabolite; (2) formation of GSH conjugates of vicinal dihaloalkanes that are inherently toxic because they can form electrophilic sulfur mustards; (3) formation of GSH conjugates of halogenated alkenes that are degraded to toxic metabolites by β-lyase in the kidney; (4) formation of GSH conjugates of quinones, quinoneimines, and isothiocyanates that are degraded to toxic metabolites by GGT1 and ANPEP in the kidney; and (5) cyclic GSH conjugation that leads to GSH depletion.

The first mechanism is illustrated by dichloromethane, which is conjugated with GSH to form the highly unstable *S*-chloromethylglutathione, which then breaks down to formaldehyde. Both formaldehyde and the GSH conjugate are reactive metabolites, and either or both may be responsible for dichloromethane-induced tumorigenesis in sensitive species. The rate of conjugation of dichloromethane with GSH is considerably faster in mice, which are susceptible to dichloromethane-induced tumorigenesis, than in rats or hamsters, which are resistant species. Other examples include the 1,1,3,3,3-pentafluoro-2-(fluromethoxy)prop-1-ene breakdown product of the general anesthetic sevoflurane. This breakdown product and its derivatives are conjugated to GSH, biotransformed to cysteine conjugates and mercapturic acids, which are then converted through β-lyase cleavage and thiol rearrangement to the highly reactive thioacyl fluoride (Testa and Krämer, 2008, 2010). This highly reactive metabolite can form adducts with proteins and may explain its associated nephrotoxicity (Testa and Krämer, 2008, 2010).

The second mechanism accounts for the toxicity of dichloroethane and dibromoethane. These vicinal dihaloalkanes are converted to GSH conjugates that can rearrange to form mutagenic and nephrotoxic episulfonium ions (sulfur half-mustards) (Fig. 6-73). Dichloroethane and dibromoethane can also be oxidized by CYP

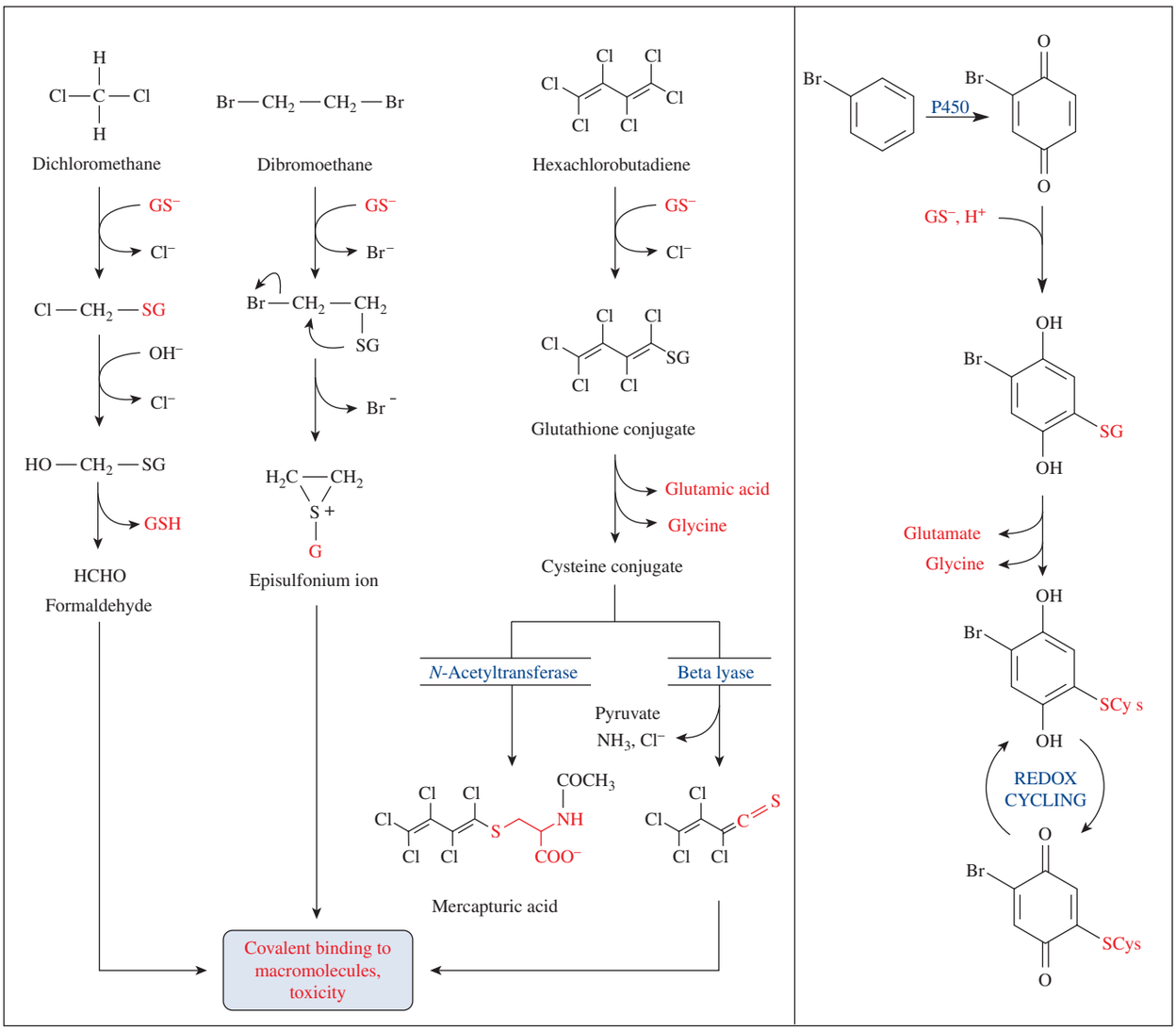


Figure 6-73. *Role of glutathione conjugation in the activation of xenobiotics to toxic metabolites.*

to chloroacetaldehyde and bromoacetaldehyde (by reactions analogous to those shown in Fig. 6-56). Either pathway can potentially account for the toxic and tumorigenic effects of these dihaloalkanes. However, the toxicity and DNA binding of dihaloalkanes are increased by factors that decrease their oxidation by CYP and increase their conjugation with GSH.

The third mechanism accounts for the nephrotoxicity of several halogenated alkenes. Several halogenated alkenes, such as hexachlorobutadiene, cause damage to the kidney tubules in rats, which leads to carcinoma of the proximal tubules. These nephrotoxic halogenated alkenes are conjugated with GSH and transported to the kidney for processing to mercapturic acids. The cysteine conjugates, which form by removal of glutamic acid and glycine, are substrates for *N*-acetyltransferase (NAT), which completes the synthesis of mercapturic acids, and cysteine-conjugate β-lyase (CCBL1), which removes pyruvate and ammonia from the cysteine conjugate to produce thionylacyl halides, thiiranes, thiolactones, and thioketenes. The early damage to renal mitochondria caused by halogenated alkenes is probably because CCBL1 is a mitochondrial enzyme.

The fourth mechanism accounts for the nephrotoxicity of bromobenzene, which causes damage to the proximal tubules in rats. Bromobenzene is oxidized by CYP in the liver to bromohydroquinone, which is conjugated with GSH and transported to the kidney (Fig. 6-73). The GSH conjugate is converted to the cysteine derivative by GGT1 and membrane ANPEP. Substitution of bromohydroquinones with cysteine lowers their redox potential and thereby facilitates their oxidation to toxic quinones. The cysteine conjugates of bromohydroquinone are thought to undergo redox cycling and cause kidney damage through the generation of reactive oxygen species. 4-Aminophenol is thought to cause kidney damage by a similar mechanism, except a benzoquinoneimine is involved in conjugation with GSH and subsequent damage to proximal tubules of the kidney. Treatment of rats with the GSH depletor, buthionine-*S*-sulfoximine (BSO), protects them against the nephrotoxic effects of 4-aminophenol, which implicates GSH conjugation in the activation of this compound.

The fifth mechanism occurs with moderately toxic allyl-, benzyl-, and phenethylisothiocyantes as well as sulforaphane formed from plant glucosinolates. These compounds are reversibly conjugated with GSH to form thiocarbamates which spontaneously degrade to their isothiocyanates upon export from the cell, which releases GSH. The isothiocyanate is then taken up by the cells again and the cycle can repeat until intracellular GSH is depleted. Once GSH levels are low, the compounds will tend to thiocarbamylate proteins, which can lead to cell death (Hayes et al., 2005).

Thiosulfate Sulfurtransferase (Rhodanese)

Cyanide forms naturally in leucocytes and neural cells, and also as a result of vitamin B_{12} metabolism, and is also encountered as a xenobiotic in plants containing cyanogenic glycosides, such as cassava, tobacco smoke, and pyrolysis of nitrile polymers or plastic (Billaut-Laden et al., 2006a; Libiad et al., 2015). Thiosulfate sulfurtransferase (TST) is a mitochondrial enzyme encoded by the TST gene that converts cyanide to the far less toxic metabolite, thiocyanate. The reaction involves transfer of sulfur from thiosulfate (or another sulfur donor) as follows:

$$\underset{\text{Cyanide}}{CN^-} + \underset{\text{Thiosulfate}}{S_2O_3^{2-}} \longrightarrow \underset{\text{Thiocyanate}}{SCN^-} + \underset{\text{Sulfite}}{SO_3^{2-}}$$

The sulfite produced by this reaction can be converted to sulfate by the molybdoenzyme, sulfite oxidase (see the section "Molybdenum Hydroxylases (Molybdoenzymes)"). Taking advantage of the TST reaction, thiosulfate has been used as an antidote against cyanide poisoning (Libiad et al., 2015). Cyanide can also be "detoxified" by binding to methemoglobin (the oxidized or ferric form of hemoglobin). 4-Dimethylaminophenol is used to induce methemoglobinemia as an antidote to cyanide poisoning because methemoglobin competes with cytochrome oxidase for the cyanide ion. However, 4-dimethylaminophenol is nephrotoxic to rats, presumably by a mechanism similar to that described above for the structural analog 4-aminophenol (see the preceding section and the section "Glutathione Conjugation"). TST also catalyzes the transfer of sulfane sulfur from glutathione persulfide (GSSH) to sulfite to form thiosulfate as part of the mitochondrial sulfide oxidation pathway (Libiad et al., 2015). The disulfide glutathione persulfide is formed by the transfer of sulfur from hydrogen sulfide to glutathione by sulfide quinone oxidoreductase (SQR).

In humans, TST is expressed at high levels in the liver, kidney, and particularly the colon where it plays a major role in the detoxication of hydrogen sulfide (which can reach concentrations of more than 3 mM) produced by anaerobic bacteria (Billaut-Laden et al., 2006a; Libiad et al., 2015). TST is also widely distributed in the central nervous system and may also play a role in the import of 5S rRNA into mitochondria (Smirnov et al., 2010). Several factors have been shown to induce TST, including resveratrol, α-lipoic acid, fungal (*Phellinus linteus*) polysaccharide extracts, and long-term low-dose irradiation, implicating TST in protection against persistent oxidative stress (Nakajima, 2015). Six allelic variants of TST have been identified, namely, TST*1B to TST*1F and TST*2, with the latter showing significantly reduced intrinsic metabolic clearance for thiocyanate formation (Billaut-Laden et al., 2006a). TST polymorphisms that decrease cyanide and hydrogen sulfide detoxication may ultimately become important in understanding certain diseases, such as ulcerative colitis and ALS. For instance, hydrogen sulfide release has been found to be three to four times higher in patients with ulcerative colitis when compared with healthy subjects, and is positively correlated with disease severity (Billaut-Laden et al., 2006a). Furthermore, it has been postulated that individuals with ALS display a disorder in cyanide metabolism because of the higher blood and urine levels of cyanide (Billaut-Laden et al., 2006a). Mimori et al. (1984) significantly decreased TST activity in cervical and thoracic spinal cords of patients with ALS compared with healthy subjects. Finally, TST has also been proposed as a target for the treatment of obesity and type 2 diabetes based on the observed inverse relationship between TST expression in adipose tissue and diabetes in rodents (Morton et al., 2016).

3-Mercaptopyruvate Sulfurtransferase

An additional pathway of cyanide detoxication involves the enzyme 3-mercaptopyruvate sulfurtransferase (encoded by the gene MPST) that converts cyanide to thiocyanate by the transfer of sulfur from 3-mercaptopyruvate as follows:

$$CN^- + \text{3-Mercaptopyruvate} \rightarrow \text{Thiocyanate} + \text{Pyruvate}.$$

The 3-mercaptopyruvate in this reaction is produced during cysteine metabolism by cysteine aminotransferase (Billaut-Laden et al., 2006b; Nagahara et al., 2015). MPST is both a cytosolic and mitochondrial enzyme and is expressed at high levels in the kidney, liver, heart, and erythrocytes (Billaut-Laden et al., 2006b).

Three allelic variants of MPST have been identified, namely, MPST*1B, MPST*1C, and MPST*2, with the latter showing little-to-no activity because it is a nonsense allele (Billaut-Laden et al., 2006b). While TST and MPST are related enzymes due to their

shared amino acid sequence identity and function in cyanide detoxification, MPST enzymatic activity has been shown to be induced specifically by diallyl disulfide, suggesting a separate mechanism of regulation for each enzyme (Nakajima, 2015).

Unusual Conjugation Reactions

In rare cases, xenobiotics can be biotransformed to unusual conjugates which can contribute to xenobiotic clearance. As shown in Fig. 6-74, the immunosuppressive drug 6-mercaptopurine (as well as its precursor azathioprine) is converted to 6-thioinosine monophosphate (TIMP) by hypoxanthine guanine phophoribosyltransferase (HGPRT) with phophoribosyl pyrophosphate (PRPP) as the phophoribosyl donor (Wielinga et al., 2002; Gearry et al., 2010; Hofmann et al., 2012). Similarly, to 6-mercaptopurine, 6-thiopurine can also be converted to a thionucleotide monophosphate (TNMP) (Wielinga et al., 2002). While the 6-thioguanine nucleotides are responsible for immunosuppressant activity, 6-methylmercaptopurine nucleotides have been associated with hepatotoxicity at high doses (Gearry et al., 2010).

Another example of an unusual conjugate is that of the immunosuppressant macrolide everolimus, which forms a phosphocholine conjugate as a major metabolite (ATG181) in both animals and humans (Zollinger et al., 2008). While phosphocholine esters of endogenous lipids, such as sphingomyelins or phosphatidylcholines, are common, phosphocholine conjugates of xenobiotics are rare. The addition of activated phosphocholine is catalyzed by the enzymes cholinephosphotransferase found in the Golgi apparatus as well as choline/ethanolaminephosphotransferase found in the endoplasmic reticulum and nuclear envelope; however, it is not known whether these enzymes also catalyze the phosphocholine conjugation of everolimus (Zollinger et al., 2008). Hepatitis C inhibitors containing a bicycle[1.1.1]pentane moiety are also conjugated with phosphocholine (Zhuo et al., 2016), whereas pimasertib, an inhibitor of MEK1/2 (the mitogen-activated protein kinase signaling pathway), is conjugated with phosphoethanolamine (Scheible et al., 2017).

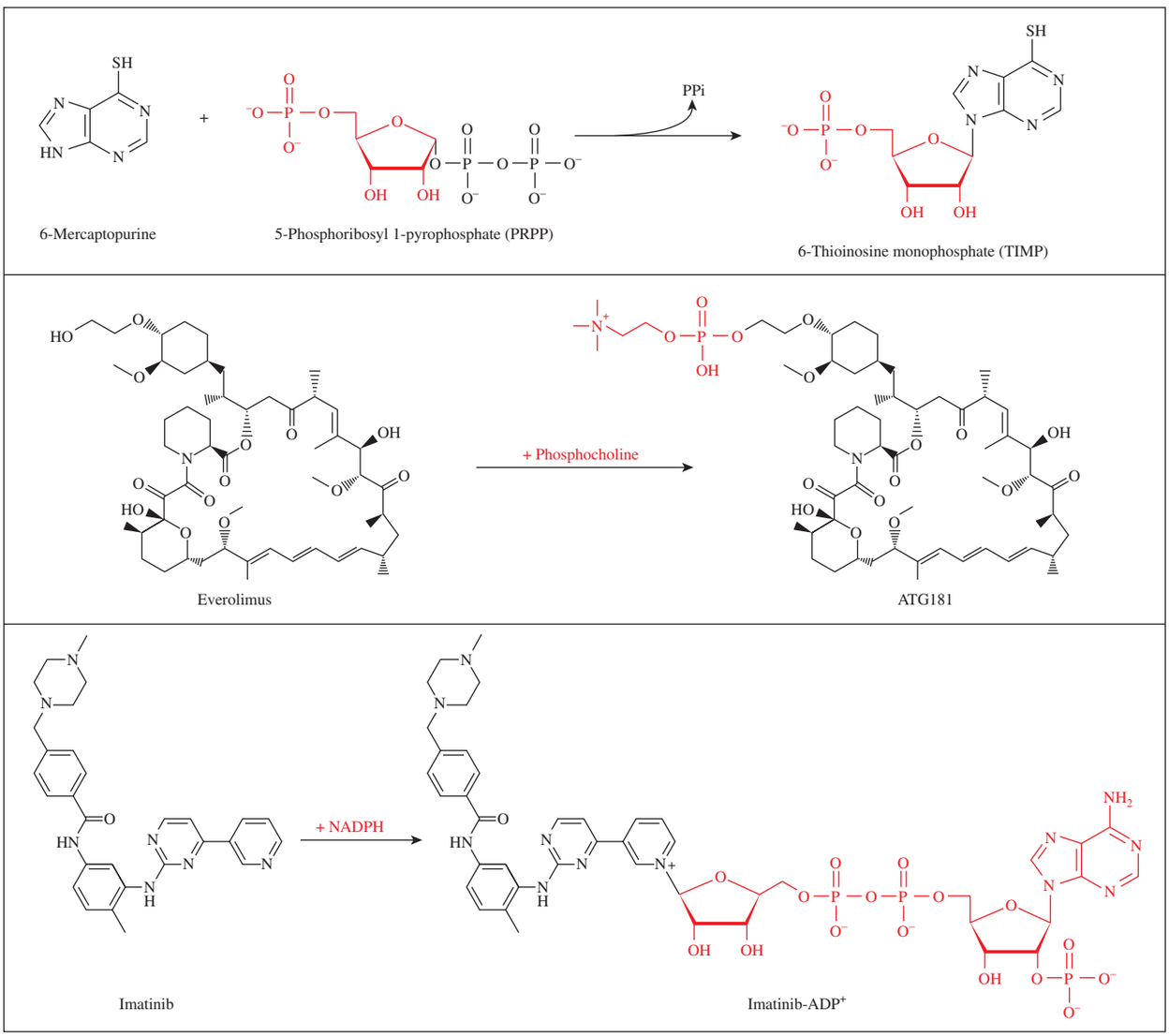


Figure 6-74. *Examples of unusual conjugation reactions.*

The hydroxyl group or a nitrogen atom in azoheterocyclic xenobiotics can be conjugated with ADP-ribose, the ADP moiety of which can be hydrolyzed by phosphatases to convert the ADP-ribose conjugate to a ribose conjugate (Fan et al., 2016). The ADP-ribosyltransferases (ART) responsible for the ADP-ribosylation of xenobiotics is known variously as NAD^+ hydrolase, NAD-glycohydrolase, nucleosidase, or NADase (EC 3.2.2.5), which use various nicotiminade cofactors including NAD, NADP, and/or NMN. Examples of substrates that undergo ADP-ribosylation are rifampin, imatinib, otenabant, continine, nicotinic acid, 4-ipomeanol, and nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Different forms of ART are thought to be responsible for ADP-ribosylation of xenobiotics in liver cytosol and endoplasmic reticulum (Fan et al., 2016). ADP-ribosylation is highly species specific and is mainly observed in rodents (i.e., imatinib, ipomeanol, and NNK/NNAL) but not in dogs, monkeys, or humans (Fan et al., 2016).

While single amino-acid conjugation is a well characterized metabolic pathway for xenobiotic metabolism (see the section "Amino Acid Conjugation"), xenobiotic conjugation to a peptide moiety other than glutathione is rare. The metabolism of lucanthone is an example of this unusual peptide conjugation. In human liver microsomes and in incubations with fungi, lucanthone undergoes desethylation or 4-methyl hydroxylation to yield desethyl lucanthone and hycanthone, respectively, and these products can be further metabolized (Mitchell et al., 2014). Lucanthone can also undergo sulfoxidation to yield lucanthone sulfoxide, the major urinary metabolite excreted by monkey. Lucanthone sulfoxide is conjugated with a peptide moiety, named miracil X, which was shown to be a unique human urinary metabolite (Mitchell et al., 2014). There are several other examples of peptide conjugation to xenobiotics, namely 3-phenoxybenzoic acid conjugation to dipeptide glycylvaline, quinaldic acid conjugates with glycylglycine and glycyltaurine, polyglutamyl conjugates of methotrexate and aminopterin, CCNU ((2-chloroethyl)-3-cyclohexyl-1-nitrosourea) peptide conjugate, DDA (4,4′-dichlorodiphenylacetic acid) peptide conjugate, and polypeptide conjugates of phenothiazine (Mitchell et al., 2014). However, the enzymatic pathways involved and the significance of these conjugates are not well established.

Succinylation has been shown to be a posttranslational modification of proteins (typically at lysine residues); however, succinylation of xenobiotics is rare (Zhang et al., 2011b). The only reported case of this conjugation reaction is that of mephedrone, which is succinylated in humans. In studies with human volunteers dosed with mephedrone, a novel mephedrone succinate conjugate was detected in urine, and proposed to form from the conjugation of succinic acid to nor-mephedrone (Pozo et al., 2015). Succinylation of xenobiotics is typically used as a prodrug strategy to produce drugs with favorable properties; however, succinylation as a mechanism of xenobiotic clearance is highly unusual (Pozo et al., 2015).

Another unusual conjugate is that of investigational anticancer agent β-lapachone, which forms a glucosylsulfate conjugate in mammals (Savage et al., 2008). Glucosidation and sulfonation of xenobiotics can readily occur (see the sections "Glucuronidation and Formation of Acyl-CoA Thioesters" and "Sulfonation") by UGT and SULT enzymes. However, typically these Phase 2 reactions are independent of each other and are not sequential to each other at the same metabolic site. In the case of β-lapachone, reduction by NOQ to a hydroquinone is followed by glucosidation, which in turn is followed by sulfonation of the glucose moiety (suspected to occur at glucose C6) forming the unusual glucosylsulfate conjugate (Savage et al., 2008). Glucosylsulfate conjugates have previously been observed in cotton plants treated with the insecticide profenofos and 2,4-dicholorphenoxyacetic acid; in sugar beets treated with phenmedipham, and in crustaceans exposed to 1-hydroxypyrene (Savage et al., 2008).

Lastly, the mechanism of xenobiotic conjugation to urea has been an area of some debate in the literature. These unusual conjugates have largely been considered as artifacts formed in urine through a nonenzymatic chemical reaction; however, there are reports of xenobiotic-urea metabolites found in bile and feces. An example of this is that of rosaramicin, where its urea metabolite 20-bisureidorosaramicin was found in dog bile and feces, as well as human feces, suggesting an enzymatic process in the formation of this metabolite (Mitchell, 2014). Xenobiotics that form urea conjugates include aminopyrine, formaldehyde, oxazepam, caroxazone, laropiprant, torcetrapib, isoproturon, and andrographolide (Mitchell, 2014). The significance of these conjugates and their mechanism of formation (chemical vs. metabolic) remain unclear.

Phosphorylation

Some drugs are intentionally designed to be phosphorylated by intracellular enzymes. For example, the anti-HIV drug zidovudine (AZT) is converted to a triphosphate nucleoside by enzymes in the salvage pathway (nucleoside kinase, nucleoside monophosphate kinase [NMK], and nucleoside diphosphate kinase [NDK]). A rare example of a non-nucleoside mimetic that undergoes phosphorylation is AZ2381, a tankyrase inhibitor, which is phosphorylated at its primary alcohol moiety by red blood cells from mice and, to a lesser extent, rat, dog, and human (Gu et al., 2017). However, phosphorylation of xenobiotics is the exception rather than the rule. All conjugation reactions ultimately require ATP, either to activate the xenobiotic for conjugation with GSH or amino acids or to synthesize high-energy cofactors such as UDPGA and PAPS. The process is inefficient in that several ATP molecules (or their equivalent) are used to synthesize each cofactor molecule. The question arises: why is ATP not used directly by conjugating enzymes? In other words, why are xenobiotics rarely phosphorylated directly (with the exception of nucleoside mimetics like zidovudine and the tankyrase inhinitor AZ2381), which would require less ATP and would achieve the goal of converting xenobiotics to water-soluble conjugates? It is difficult to be certain why this does not occur, but four reasons suggest themselves. First, if xenobiotics could be phosphorylated, high intracellular levels of a xenobiotic might consume so much ATP as to jeopardize cell viability, whereas UDPGA and PAPS can be depleted without killing cells. Second, phosphorylation of endogenous substrates, such as glucose, is a mechanism for trapping endogenous substrates inside a cell. This works because the plasma membrane of all cells is a barrier to the passage of polar compounds by virtue of its hydrophobic properties (lipid bilayer) and its general lack of transporters that efflux phosphorylated compounds out of the cell. The same is true for the phosphorylated metabolites of zidovudine and related antiviral drugs; they are retained inside the cell that forms them. A lipid bilayer is also a physical barrier to other water-soluble conjugates, such as glucuronides and sulfonates, but these are transported out of the cell by various transporters. Third, phosphorylation of both small molecules (such as inositol) and proteins (such as membrane-bound receptors and various transcription factors) plays an important role in intracellular and intranuclear signaling. It is possible that some xenobiotics, if they were phosphorylated, might interfere with these regulatory systems and thereby disrupt cellular homeostasis. Fourth, even if xenobiotics were phosphorylated in the liver and excreted in bile, they would be rapidly hydrolyzed by high levels of alkaline phosphatase lining the surface of enterocytes in the small intestine, which would promote reabsorption of the xenobiotic (by the very process exploited in the design of phosphorylated prodrugs such as fosamprenavir). Whatever the reason, there appears to be strong evolutionary pressure against the conjugation of xenobiotics with phosphoric acid.

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REFERENCES

Abaji R, Krajinovic M. Thiopurine *S*-methyltransferase polymorphisms in acute lymphoblastic leukemia, inflammatory bowel disease and autoimmune disorders: influence on treatment response. *Pharmgenomics Pers Med*. 2017;10:143–156.

Abdelmegeed MA, Ha SK, Choi Y, Akbar M, Song BJ. Role of CYP2E1 in mitochondrial dysfunction and hepatic tissue injury in alcoholic and non-alcoholic diseases. *Curr Mol Pharmacol*. 2015;10:207–225.

Achour B, Russell MR, Barber J, Rostami-Hodjegan A. Simultaneous quantification of the abundance of several cytochrome P450 and uridine 5′-diphospho-glucuronosyltransferase enzymes in human liver microsomes using multiplexed targeted proteomics. *Drug Metab Dispos*. 2014;42:500–510.

Adamson RH, Bridges JW, Evans ME, Williams RT. Species differences in the aromatization of quinic acid in vivo and the role of gut bacteria. *Biochem J*. 1970;116:437–443.

Agarwal DP. Pharmacogenetics of alcohol dehydrogenase. In: Kalow W, ed. *Pharmacogenetics of Drug Metabolism*. New York: Pergamon; 1992.

Agarwal V, Kommaddi RP, Valli K, et al. Drug metabolism in human brain: high levels of cytochrome P4503A43 in brain and metabolism of anti-anxiety drug alprazolam to its active metabolite. *PLoS One*. 2008;3:e2337.

Agostinelli E, Arancia G, Vedova LD, et al. The biological functions of polyamine oxidation products by amine oxidases: perspectives of clinical applications. *Amino Acids*. 2004;27:347–358.

Agundez JA, Gallardo L, Ledesma MC, et al. Functionally active duplications of the CYP2D6 gene are more prevalent among larynx and lung cancer patients. *Oncology*. 2001;61:59–63.

Ahmed MM, Wang T, Luo Y, et al. Aldo-keto reductase-7A protects liver cells and tissues from acetaminophen-induced oxidative stress and hepatotoxicity. *Hepatology*. 2011;54:1322–1332.

Ahn J, Yu K, Stolzenberg-Solomon R, et al. Genome-wide association study of circulating vitamin D levels. *Hum Mol Genet*. 2010;19:2739–2745.

Aiba I, Yamasaki T, Shinki T, et al. Characterization of rat and human CYP2J enzymes as vitamin D 25-hydroxylases. *Steroids*. 2006;71:849–856.

Al-Zoughool M, Succop P, Desai P, et al. Effect of *N*-glucuronidation on urinary bladder genotoxicity of 4-aminobiphenyl in male and female mice. *Environ Toxicol Pharmacol*. 2006;22:153–159.

Albericio F, Kruger HG. Therapeutic peptides. *Future Med Chem*. 2012;4:1527–1531.

Aldridge WN. Serum esterases. I. Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. *Biochem J*. 1953;53:110–117.

Alfaro JF, Jones JP. Studies on the mechanism of aldehyde oxidase and xanthine oxidase. *J Org Chem*. 2008;73:9469–9472.

Alfirevic A, Pirmohamed M. Drug-induced hypersensitivity reactions and pharmacogenomics: past, present and future. *Pharmacogenomics*. 2010;11:497–499.

Amano T, Ochi N, Sato H, Sakaki S. Oxidation reaction by xanthine oxidase: theoretical study of reaction mechanism. *J Am Chem Soc*. 2007;129:8131–8138.

Anders MW. *Bioactivation of Foreign Compounds*. New York: Academic Press; 1985.

Anders MW, Ratnayake JH, Hanna PE, Fuchs JA. Thioredoxin-dependent sulfoxide reduction by rat renal cytosol. *Drug Metab Dispos*. 1981;9:307–310.

Aniya Y, Imaizumi N. Mitochondrial glutathione transferases involving a new function for membrane permeability transition pore regulation. *Drug Metab Rev*. 2011;43:292–299.

Annalora AJ, Marcus CB, Iversen PL. Alternative splicing in the cytochrome P450 superfamily expands protein diversity to augment gene function and redirect human drug metabolism. *Drug Metab Dispos*. 2017;45:375–389.

Antonelli R, Shao K, Thomas DJ, et al. AS3MT, GSTO, and PNP polymorphisms: impact on arsenic methylation and implications for disease susceptibility. *Environ Res*. 2014;132:156–167.

Arima N. Acyl glucuronidation and glucosidation of pranoprofen, a 2-arylpropionic acid derivative, in mouse liver and kidney homogenates. *J Pharmacobiodyn*. 1990;13:724–732.

Armstrong RN. Kinetic and chemical mechanism of epoxide hydrolase. *Drug Metab Rev*. 1999;31:71–86.

Arner ES, Nordberg J, Holmgren A. Efficient reduction of lipoamide and lipoic acid by mammalian thioredoxin reductase. *Biochem Biophys Res Commun*. 1996;225:268–274.

Aronson JK, Ferner RE. Joining the DoTS: new approach to classifying adverse drug reactions. *BMJ*. 2003;327:1222–1225.

Asadov C, Aliyeva G, Mustafayeva K. Thiopurine *S*-methyltransferase as a pharmacogenetic biomarker: significance of testing and review of major methods. *Cardiovasc Hematol Agents Med Chem*. 2017;15(1):23–30.

Atkins WM. Non-Michaelis-Menten kinetics in cytochrome P450-catalyzed reactions. *Annu Rev Pharmacol Toxicol*. 2005;45:291–310.

Atkins WM, Wang RW, Bird AW, Newton DJ, Lu AY. The catalytic mechanism of glutathione *S*-transferase (GST). Spectroscopic determination of the pKa of Tyr-9 in rat alpha 1-1 GST. *J Biol Chem*. 1993;268:19188–19191.

Augustinsson KB. The nature of an "anionic" site in butyrylcholinesterase compared with that of a similar site in acetylcholinesterase. *Biochim Biophys Acta*. 1966;128:351–362.

Ayesh R, Smith RL. Genetic polymorphism of trimethylamine *N*-oxidation. In: Kalow W, ed. *Pharmacogenetics of Drug Metabolism*. New York: Pergamon; 1992.

Bachovchin DA, Brown SJ, Rosen H, Cravatt BF. Identification of selective inhibitors of uncharacterized enzymes by high-throughput screening with fluorescent activity-based probes. *Nat Biotechnol*. 2009;27:387–394.

Backman JT, Filppula AM, Niemi M, Neuvonen PJ. Role of cytochrome P450 2C8 in drug metabolism and interactions. *Pharmacol Rev*. 2016;68:168–241.

Badenhorst CP, Erasmus E, van der Sluis R, et al. A new perspective on the importance of glycine conjugation in the metabolism of aromatic acids. *Drug Metab Rev*. 2014;46:343–361.

Baer BR, DeLisle RK, Allen A. Benzylic oxidation of gemfibrozil-1-*O*-beta-glucuronide by P450 2C8 leads to heme alkylation and irreversible inhibition. *Chem Res Toxicol*. 2009;22:1298–1309.

Baer B, Rettie A. CYP4B1: an enigmatic P450 at the interface between xenobiotic and endobiotic metabolism. *Drug Metab Rev*. 2006;38:451–476.

Bafort F, Parisi O, Perraudin JP, Jijakli MH. Mode of action of lactoperoxidase as related to its antimicrobial activity: a review. *Enzyme Res*. 2014;2014:517164.

Baillie TA, Rettie AE. Role of biotransformation in drug-induced toxicity: influence of intra- and inter-species differences in drug metabolism. *Drug Metab Pharmacokinet*. 2011;26:15–29.

Bains OS, Grigliatti TA, Reid RE, Riggs KW. Naturally occurring variants of human aldo-keto reductases with reduced in vitro metabolism of daunorubicin and doxorubicin. *J Pharmacol Exp Ther*. 2010;335:533–545.

Ballard JE, Prueksaritanont T, Tang C. Hepatic metabolism of MK-0457, a potent aurora kinase inhibitor: interspecies comparison and role of human cytochrome P450 and flavin-containing monooxygenase. *Drug Metab Dispos*. 2007;35:1447–1451.

Ballatori N, Krance SM, Marchan R, Hammond CL. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. *Mol Aspects Med*. 2009a;30:13–28.

Ballatori N, Li N, Fang F, et al. OST alpha-OST beta: a key membrane transporter of bile acids and conjugated steroids. *Front Biosci (Landmark Ed)*. 2009b;14:2829–2844.

Barditch-Crovo P, Trapnell CB, Ette E, et al. The effects of rifampin and rifabutin on the pharmacokinetics and pharmacodynamics of a combination oral contraceptive. *Clin Pharmacol Ther*. 1999;65:428–438.

Barr JT, Choughule KV, Nepal S, et al. Why do most human liver cytosol preparations lack xanthine oxidase activity? *Drug Metab Dispos*. 2014;42:695–699.

Bauman JN, Kelly JM, Tripathy S, et al. Can in vitro metabolism-dependent covalent binding data distinguish hepatotoxic from nonhepatotoxic drugs? An analysis using human hepatocytes and liver S-9 fraction. *Chem Res Toxicol*. 2009;22:332–340.

Bayrak A, Bayrak T, Tokgozoglu SL, et al. Serum PON-1 activity but not Q192R polymorphism is related to the extent of atherosclerosis. *J Atheroscler Thromb*. 2012;19:376–384.

Beasley DE, Koltz AM, Lambert JE, et al. The evolution of stomach acidity and its relevance to the human microbiome. *PLoS One*. 2015; 10:e0134116.

Beconi MG, Mao A, Liu DQ, et al. Metabolism and pharmacokinetics of a dipeptidyl peptidase IV inhibitor in rats, dogs, and monkeys with selective carbamoyl glucuronidation of the primary amine in dogs. *Drug Metab Dispos*. 2003;31:1269–1277.

Beedham C, Miceli JJ, Obach RS. Ziprasidone metabolism, aldehyde oxidase, and clinical implications. *J Clin Psychopharmacol*. 2003;23:229–232.

Beetham JK, Grant D, Arand M, et al. Gene evolution of epoxide hydrolases and recommended nomenclature. *DNA Cell Biol*. 1995;14:61–71.

Benedetti MS. Biotransformation of xenobiotics by amine oxidases. *Fund Clin Pharmacol*. 2001;15:75–84.

Benedetti MS, Dostert P. Contribution of amine oxidases to the metabolism of xenobiotics. *Drug Metab Rev*. 1994;26:507–535.

Benet LZ. The drug transporter-metabolism alliance: uncovering and defining the interplay. *Mol Pharm*. 2009;6:1631–1643.

Berg J, Mahmoudjanlou Y, Duscha A, et al. The immunomodulatory effect of laquinimod in CNS autoimmunity is mediated by the aryl hydrocarbon receptor. *J Neuroimmunol*. 2016;298:9–15.

Bergander L, Wincent E, Rannug A, et al. Metabolic fate of the Ah receptor ligand 6-formylindolo[3,2-b]carbazole. *Chem Biol Interact*. 2004;149:151–164.

Billaut-Laden I, Allorge D, Crunelle-Thibaut A, et al. Evidence for a functional genetic polymorphism of the human thiosulfate sulfurtransferase (Rhodanese), a cyanide and H2S detoxification enzyme. *Toxicology*. 2006a;225:1–11.

Billaut-Laden I, Rat E, Allorge D, et al. Evidence for a functional genetic polymorphism of the human mercaptopyruvate sulfurtransferase (MPST), a cyanide detoxification enzyme. *Toxicol Lett*. 2006b;165:101–111.

Bjeldanes LF, Kim JY, Grose KR, et al. Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol in vitro and in vivo: comparisons with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Proc Natl Acad Sci U S A*. 1991;88:9543–9547.

Bjornsson TD, Callaghan JT, Einolf HJ, et al. The conduct of in vitro and in vivo drug–drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab Dispos*. 2003; 31:815–832.

Blanchard RL, Freimuth RR, Buck J, et al. A proposed nomenclature system for the cytosolic sulfotransferase (SULT) superfamily. *Pharmacogenetics*. 2004;14:199–211.

Blattler SM, Rencurel F, Kaufmann MR, Meyer UA. In the regulation of cytochrome P450 genes, phenobarbital targets LKB1 for necessary activation of AMP-activated protein kinase. *Proc Natl Acad Sci U S A*. 2007;104:1045–1050.

Blouin RA, Bandyopadhyay AM, Chaudhary I, et al. Cytochrome P450 2B enzyme (CYP2B) induction defect following phenobarbital treatment in the fa/fa Zucker rat: molecular characterization. *Arch Biochem Biophys*. 1993; 303:313–320.

Board PG. The omega-class glutathione transferases: structure, function, and genetics. *Drug Metab Rev*. 2011; 43:226–235.

Board PG, Anders MW. Glutathione transferase zeta: discovery, polymorphic variants, catalysis, inactivation, and properties of Gstz1–/– mice. *Drug Metab Rev*. 2011;43:215–225.

Board PG, Menon D. Structure, function and disease relevance of Omega-class glutathione transferases. *Arch Toxicol*. 2016;90:1049–1067.

Boberg EW, Miller EC, Miller JA, Poland A, Liem A. Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1′-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver. *Cancer Res.* 1983;43:5163–5173.

Bock KW, Schrenk D, Forster A, et al. The influence of environmental and genetic factors on CYP2D6, CYP1A2 and UDP-glucuronosyltransferases in man using sparteine, caffeine, and paracetamol as probes. *Pharmacogenetics*. 1994;4:209–218.

Bohnert T, Patel A, Templeton I, et al. Evaluation of a new molecular entity as a victim of metabolic drug-drug interactions—an industry perspective. *Drug Metab Dispos*. 2016;44:1399–1423.

Boily MO, Chauret N, Laterreur J, et al. In vitro and in vivo mechanistic studies toward understanding the role of 1-aminobenzotriazole in rat drug–drug interactions. *Drug Metab Dispos*. 2015;43:1960–1965.

Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ. Role of quinones in toxicology. *Chem Res Toxicol*. 2000;13:135–160.

Bolze S, Bromet N, Gay-Feutry C, et al. Development of an in vitro screening model for the biosynthesis of acyl glucuronide metabolites and the assessment of their reactivity toward human serum albumin. *Drug Metab Dispos*. 2002;30:404–413.

Bonn B, Masimirembwa CM, Aristei Y, Zamora I. The molecular basis of CYP2D6-mediated *N*-dealkylation: balance between metabolic clearance routes and enzyme inhibition. *Drug Metab Dispos*. 2008;36:2199–2210.

Bookout AL, Jeong Y, Downes M, et al. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell*. 2006;126:789–799.

Borst P, Zelcer N, van de Wetering K. MRP2 and 3 in health and disease. *Cancer Lett*. 2006; 234:51–61.

Bosgra S, Mennes W, Seinen W. Proceedings in uncovering the mechanism behind peroxisome proliferator-induced hepatocarcinogenesis. *Toxicology*. 2005;206:309–323.

Boukouvala S, Fakis G. Arylamine *N*-acetyltransferases: what we learn from genes and genomes. *Drug Metab Rev*. 2005;37:511–564.

Boulton DW, Fawcett JP. Pharmacokinetics and pharmacodynamics of single oral doses of albuterol and its enantiomers in humans. *Clin Pharmacol Ther*. 1997;62:138–144.

Bouman HJ, Schomig E, van Werkum JW, et al. Paraoxonase-1 is a major determinant of clopidogrel efficacy. *Nat Med*. 2011;17:110–116.

Bowcock AM, Cookson WO. The genetics of psoriasis, psoriatic arthritis and atopic dermatitis. *Hum Mol Genet*. 2004;13(suppl 1):R43–R55.

Bradley ME, Benner SA. Phylogenomic approaches to common problems encountered in the analysis of low copy repeats: the sulfotransferase 1A gene family example. *BMC Evol Biol*. 2005;5:22.

Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology*. 1996;137:354–366.

Brantley E, Trapani V, Alley MC, et al. Fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles induce CYP1A1 expression, become metabolized, and bind to macromolecules in sensitive human cancer cells. *Drug Metab Dispos*. 2004;32:1392–1401.

Brauer RW. Mechanisms of bile secretion. *J Am Med Assoc*. 1959;169:1462–1466.

Brechbuhl HM, Gould N, Kachadourian R, et al. Glutathione transport is a unique function of the ATP-binding cassette protein ABCG2. *J Biol Chem*. 2010; 285:16582–16587.

Brennan P, Lewis S, Hashibe M, et al. Pooled analysis of alcohol dehydrogenase genotypes and head and neck cancer: a HuGE review. *Am J Epidemiol*. 2004;159:1–16.

Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*. 2004;114:147–152.

Brunnberg S, Andersson P, Lindstam M, et al. The constitutively active Ah receptor (CA-Ahr) mouse as a potential model for dioxin exposure—effects in vital organs. *Toxicology*. 2006;224:191–201.

Brunnberg S, Andersson P, Poellinger L, Hanberg A. The constitutively active Ah receptor (CA-AhR) mouse as a model for dioxin exposure—effects in reproductive organs. *Chemosphere*. 2011;85:1701–1706.

Bui PH, Hankinson O. Functional characterization of human cytochrome P450 2S1 using a synthetic gene-expressed protein in *Escherichia coli*. *Mol Pharmacol*. 2009;76:1031–1043.

Bui PH, Hsu EL, Hankinson O. Fatty acid hydroperoxides support cytochrome P450 2S1-mediated bioactivation of benzo[*a*]pyrene-7, 8-dihydrodiol. *Mol Pharmacol*. 2009;76:1044–1052.

Bunger MK, Glover E, Moran SM, et al. Abnormal liver development and resistance to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity in mice carrying a mutation in the DNA-binding domain of the aryl hydrocarbon receptor. *Toxicol Sci*. 2008;106:83–92.

Bunger MK, Moran SM, Glover E, et al. Resistance to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity and abnormal liver development in mice carrying a mutation in the nuclear localization sequence of the aryl hydrocarbon receptor. *J Biol Chem*. 2003;278:17767–17774.

Burchell B. Transformation reactions: glucuronidation. In: Woolf TF, ed. *Handbook of Drug Metabolism*. New York: Marcel Dekker; 1999.

Burczynski ME, Penning TM. Genotoxic polycyclic aromatic hydrocarbon ortho-quinones generated by aldo-keto reductases induce CYP1A1 via nuclear translocation of the aryl hydrocarbon receptor. *Cancer Res*. 2000; 60:908–915.

Burke MD, Mayer RT. Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab Dispos*. 1974;2:583–588.

Bushueva OY, Bulgakova IV, Ivanov VP, Polonikov AV. Association of flavin monooxygenase gene E158K polymorphism with chronic heart disease risk. *Bull Exp Biol Med*. 2015;159:776–778.

Callegari E, Kalgutkar AS, Leung L, et al. Drug metabolites as cytochrome p450 inhibitors: a retrospective analysis and proposed algorithm for evaluation of the pharmacokinetic interaction potential of metabolites in drug discovery and development. *Drug Metab Dispos*. 2013;41:2047–2055.

Capen CC. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. *Toxicol Pathol*. 1997;25:39–48.

Carbone V, Hara A, El-Kabbani O. Structural and functional features of dimeric dihydrodiol dehydrogenase. *Cell Mol Life Sci*. 2008;65:1464–1474.

Carro-Ciampi G, Jurima M, Kadar D, Tang BK, Kalow W. *N*-Glucosidation of amobarbital in the cat. *Can J Physiol Pharmacol*. 1985;63:1263–1266.

Cascorbi I, Roots I, Brockmoller J. Association of NAT1 and NAT2 polymorphisms to urinary bladder cancer: significantly reduced risk in subjects with NAT1*10. *Cancer Res*. 2001;61:5051–5056.

Cashman JR. Role of flavin-containing monooxygenase in drug development. *Expert Opin Drug Metab Toxicol*. 2008;4:1507–1521.

Cashman JR. Structural and catalytic properties of the mammalian flavin-containing monooxygenase. *Chem Res Toxicol*. 1995;8:166–181.

Cashman JR. In vitro metabolism: FMO and related oxygenations. In: Woolf TE, ed. *Handbook of Drug Metabolism*. New York: Marcel Dekker; 1999.

Cashman JR, Akerman BR, Forrest SM, Treacy EP. Population-specific polymorphisms of the human FMO3 gene: significance for detoxication. *Drug Metab Dispos*. 2000;28:169–173.

Cashman JR, Zhang J. Human flavin-containing monooxygenases. *Annu Rev Pharmacol Toxicol*. 2006;46:65–100.

Casida JE, Quistad GB. Serine hydrolase targets of organophosphorus toxicants. *Chem Biol Interact*. 2005;157–158:277–283.

Catterson ML, Preskorn SH. Pharmacokinetics of selective serotonin reuptake inhibitors: clinical relevance. *Pharmacol Toxicol*. 1996; 78: 203–208.

Caudle KE, Thorn CF, Klein TE, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for dihydropyrimidine dehydrogenase genotype and fluoropyrimidine dosing. *Clin Pharmacol Ther*. 2013;94:640–645.

Cauffiez C, Klinzig F, Rat E, et al. Human CYP4F12 genetic polymorphism: identification and functional characterization of seven variant allozymes. *Biochem Pharmacol*. 2004;68:2417–2425.

Cerf C, Mesguish M, Gabriel I, Amselem S, Duvaldestin P. Screening patients with prolonged neuromuscular blockade after succinylcholine and mivacurium. *Anesth Analg*. 2002;94:461–466, table of contents.

Chalasani N, Bjornsson E. Risk factors for idiosyncratic drug-induced liver injury. *Gastroenterology*. 2010;138:2246–2259.

Chalmers RA, Bain MD, Michelakakis H, Zschocke J, Iles RA. Diagnosis and management of trimethylaminuria (FMO3 deficiency) in children. *J Inherit Metab Dis*. 2006;29:162–172.

Chan SW, Xiao Y, Hu M, et al. Associations of the CYP3A5*3 and CYP3A4*1G polymorphisms with the pharmacokinetics of oral midazolam and the urinary 6beta-hydroxycortisol/cortisol ratio as markers of CYP3A activity in healthy male Chinese. *J Clin Pharm Ther*. 2016;41:552–558.

Chandrasena RE, Vatsis KP, Coon MJ, Hollenberg PF, Newcomb M. Hydroxylation by the hydroperoxy-iron species in cytochrome P450 enzymes. *J Am Chem Soc*. 2004;126:115–126.

Chang TK, Waxman DJ. Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metab Rev*. 2006;38:51–73.

Chen J, Mannargudi BM, Xu L, Uetrecht J. Demonstration of the metabolic pathway responsible for nevirapine-induced skin rash. *Chem Res Toxicol*. 2008;21:1862–1870.

Chen B, Qiu LX, Li Y, et al. The CYP1B1 Leu432Val polymorphism contributes to lung cancer risk: evidence from 6501 subjects. *Lung Cancer*. 2010;70:247–252.

Chen BH, Wang CC, Hou YH, Mao YC, Yang YS. Mechanism of sulfotransferase pharmacogenetics in altered xenobiotic metabolism. *Expert Opin Drug Metab Toxicol*. 2015a;11:1053–1071.

Chen C, Hanson E, Watson JW, Lee JS. P-glycoprotein limits the brain penetration of nonsedating but not sedating H1-antagonists. *Drug Metab Dispos*. 2003;31:312–318.

Chen C, Li G, Liao W, et al. Selective inhibitors of CYP2J2 related to terfenadine exhibit strong activity against human cancers in vitro and in vivo. *J Pharmacol Exp Ther*. 2009;329:908–918.

Chen C, Wei X, Rao X, et al. Cytochrome P450 2J2 is highly expressed in hematologic malignant diseases and promotes tumor cell growth. *J Pharmacol Exp Ther*. 2011a;336:344–355.

Chen F, Ma B, Yang ZC, Lin G, Yang D. Extraordinary metabolic stability of peptides containing alpha-aminoxy acids. *Amino Acids*. 2012a;43:499–503.

Chen H, Ge L, Sui Q, Lin M. Systematic review and meta-analysis of the relationship between EPHX1 polymorphisms and the risk of head and neck cancer. *PLoS One*. 2015b;10:e0123347.

Chen J, Lin Y, Zhang R, Huang ZJ, Pan XG. Contribution of NAD(P)H quinone oxidoreductase 1 (NQO1) Pro187Ser polymorphism and risks of colorectal adenoma and cancer for Caucasians: a meta-analysis. *Arch Med Res*. 2012b;43(1):58–66.

Chen Q, Doss GA, Tung EC, et al. Evidence for the bioactivation of zomepirac and tolmetin by an oxidative pathway: identification of glutathione adducts in vitro in human liver microsomes and in vivo in rats. *Drug Metab Dispos*. 2006;34:145–151.

Chen Q, Tan E, Strauss JR, et al. Effect of quinidine on the 10-hydroxylation of R-warfarin: species differences and clearance projection. *J Pharmacol Exp Ther*. 2004a;311:307–314.

Chen S-Y, Lin JR, Darbha R, et al. Glycine *N*-methyltransferase tumor susceptibility gene in the benzo(a)pyrene-detoxification pathway. *Cancer Res*. 2004b;64:3617–3623.

Chen S, Wu K, Knox R. Structure-function studies of DT-diaphorase (NQO1) and NRH:quinone oxidoreductase (NQO2). *Free Radic Biol Med*. 2000;29:276–284.

Chen X, Xue L, Hou S, et al. Long-acting cocaine hydrolase for addiction therapy. *Proc Natl Acad Sci U S A*. 2016;113:422–427.

Chen Y, Goldstein JA. The transcriptional regulation of the human CYP2C genes. *Curr Drug Metab*. 2009;10:567–578.

Chen Z, Li Z, Niu X, et al. The effect of CYP1A1 polymorphisms on the risk of lung cancer: a global meta-analysis based on 71 case-control studies. *Mutagenesis*. 2011b;26:437–446.

Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc Natl Acad Sci U S A*. 2004;101:7711–7715.

Cheung C, Gonzalez FJ. Humanized mouse lines and their application for prediction of human drug metabolism and toxicological risk assessment. *J Pharmacol Exp Ther*. 2008;327:288–299.

Chiaro CR, Patel RD, Marcus CB, Perdew GH. Evidence for an aryl hydrocarbon receptor-mediated cytochrome p450 autoregulatory pathway. *Mol Pharmacol.* 2007;72:1369–1379.

Chladek J, Martinkova J, Sispera L. An in vitro study on methotrexate hydroxylation in rat and human liver. *Physiol Res*. 1997;46:371–379.

Cho CE, Caudill MA. Trimethylamine-*N*-oxide: friend, foe, or simply caught in the cross-fire? *Trends Endocrinol Metab*. 2017;28:121–130.

Choong E, Guo J, Persson A, et al. Developmental regulation and induction of cytochrome P450 2W1, an enzyme expressed in colon tumors. *PLoS One*. 2015;10:e0122820.

Chung FF, Mai CW, Ng PY, Leong CO. Cytochrome P450 2W1 (CYP2W1) in colorectal cancers. *Curr Cancer Drug Target*. 2016;16:71–78.

Chung KT. The etiology of bladder cancer and its prevention. *J Cancer Sci Ther*. 2013;5:346–361.

Ciraulo DA, Shader RI, Greenblatt DJ. Clinical pharmacology and therapeutics of antidepressants. In: Ciraulo DA, Shader RI, eds. *Pharmacotherapy of Depression*. Totowa, NJ: Springer Science+Business Media; 2011:33–124:chap 2.

Claus SP, Guillou H, Ellero-Simatos S. The gut microbiota: a major player in the toxicity of environmental pollutants? *NPJ Biofilms Microbiomes*. 2016;2:16003.

Clayden J, Moran WJ, Edwards PJ, LaPlante SR. The challenge of atropisomerism in drug discovery. *Angew Chem Int Ed Engl*. 2009;48:6398–6401.

Cohen S, Boobis A, Meek M, Preston R, McGregor D. 4-Aminobiphenyl and DNA reactivity: case study within the context of the 2006 IPCS Human Relevance Framework for Analysis of a Cancer Mode of Action for Humans. *Crit Rev Toxicol*. 2006;36:803–819.

Cole TB, Jansen K, Park S, et al. The toxicity of mixtures of specific organophosphate compounds is modulated by paraoxonase 1 status. *Adv Exp Med Bio*. 2010;660:47–60.

Collins C, Levy R, Ragueneau-Majlessi I, Hachad H. Prediction of maximum exposure in poor metabolizers following inhibition of nonpolymorphic pathways. *Curr Drug Metab*. 2006;7:295–299.

Congiu M, Mashford ML, Slavin JL, Desmond PV. Coordinate regulation of metabolic enzymes and transporters by nuclear transcription factors in human liver disease. *J Gastroenterol Hepatol*. 2009;24:1038–1044.

Conney AH. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons. *Cancer Res*. 1982;42:4875–4917.

Coughtrie MW. Function and organization of the human cytosolic sulfotransferase (SULT) family. *Chem Biol Interact*. 2016;259:2–7.

Coughtrie MW, Fisher MB. The role of sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs) in human drug clearance and bioactivation. In: Obach RS, Lee JS, Fisher MB, eds. *Drug Metabolizing Enzymes*. New York: Marcel Dekker; 2003.

Court MH. Feline drug metabolism and disposition: pharmacokinetic evidence for species differences and molecular mechanisms. *Vet Clin North Am Small Anim Pract*. 2013;43:1039–1054.

Court MH, Hazarika S, Krishnaswamy S, Finel M, Williams JA. Novel polymorphic human UDP-glucuronosyltransferase 2A3: cloning, functional characterization of enzyme variants, comparative tissue expression, and gene induction. *Mol Pharmacol*. 2008;74:744–754.

Crofts F, Taioli E, Trachman J, et al. Functional significance of different human CYP1A1 genotypes. *Carcinogenesis*. 1994;15:2961–2963.

Cronin A, Decker M, Arand M. Mammalian soluble epoxide hydrolase is identical to liver hepoxilin hydrolase. *J Lipid Res*. 2011;52:712–719.

Cruzan G, Bus J, Hotchkiss J, et al. CYP2F2-generated metabolites, not styrene oxide, are a key event mediating the mode of action of styrene-induced mouse lung tumors. *Regul Toxicol Pharmacol*. 2012;62:214–220.

Cruzan G, Bus J, Hotchkiss J, et al. Studies of styrene, styrene oxide and 4-hydroxystyrene toxicity in CYP2F2 knockout and CYP2F1 humanized mice support lack of human relevance for mouse lung tumors. *Regul Toxicol Pharmacol*. 2013;66:24–29.

Cui D, Booth-Genthe CL, Carlini E, Carr B, Schrag ML. Heterotropic modulation of sulfotransferase 2A1 activity by celecoxib: product ratio switching of ethynylestradiol sulfation. *Drug Metab Dispos*. 2004;32:1260–1264.

Cundy KC, Sato M, Crooks PA. Stereospecific in vivo *N*-methylation of nicotine in the guinea pig. *Drug Metab Dispos*. 1985;13:175–185.

Curran PG, DeGroot LJ. The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. *Endocr Rev*. 1991;12:135–150.

D'Agostino J, Zhang H, Kenaan C, Hollenberg PF. Mechanism-based inactivation of human cytochrome P450 2B6 by chlorpyrifos. *Chem Res Toxicol*. 2015;28:1484–1495.

Dahabreh IJ, Kitsios GD, Kent DM, Trikalinos TA. Paraoxonase 1 polymorphisms and ischemic stroke risk: a systematic review and meta-analysis. *Genet Med*. 2010;12:606–615.

Daily EB, Aquilante CL. Cytochrome P450 2C8 pharmacogenetics: a review of clinical studies. *Pharmacogenomics*. 2009;10:1489–1510.

Daly AK. Pharmacogenetics. In: Woolf TF, ed. *Handbook of Drug Metabolism*. New York: Marcel Dekker; 1999.

Daniel V. Glutathione *S*-trasferases: gene struture and regulation of expression. *Crit Rev Biochem Mol Biol*. 1993;28:173–207.

Dansette PM, Rosi J, Bertho G, Mansuy D. Cytochromes P450 catalyze both steps of the major pathway of clopidogrel bioactivation, whereas paraoxonase catalyzes the formation of a minor thiol metabolite isomer. *Chem Res Toxicol*. 2012;25:348–356.

Dauvilliers Y, Tafti M, Landolt HP.Catechol-*O*-methyltransferase, dopamine, and sleep-wake regulation. *Sleep Med Rev*. 2015;22:47–53.

Davies NM, Skjodt NM. Choosing the right nonsteroidal anti-inflammatory drug for the right patient: a pharmacokinetic approach. *Clin Pharmacokinet*. 2000;38:377–392.

Dawson JH. Probing structure-function relations in heme-containing oxygenases and peroxidases. *Science*. 1988;240:433–439.

De Bosscher K, Vanden Berghe W, Haegeman G. Cross-talk between nuclear receptors and nuclear factor kappaB. *Oncogene*. 2006;25:6868–6886.

De Bruyn T, Stieger B, Augustijns PF, Annaert PP. Clearance prediction of HIV protease inhibitors in man: role of hepatic uptake. *J Pharm Sci*. 2016; 105:854–863.

de Morais SM, Wilkinson GR, Blaisdell J, et al. The major genetic defect responsible for the polymorphism of *S*-mephenytoin metabolism in humans. *J Biol Chem*. 1994;269:15419–15422.

de Morais SM, Uetrecht JP, Wells PG. Decreased glucuronidation and increased bioactivation of acetaminophen in Gilbert's syndrome. *Gastroenterology*. 1992;102:577–586.

Debiec-Rychter M, Land SJ, King CM. Histological localization of acetyltransferases in human tissue. *Cancer Lett*. 1999;143:99–102.

Decker CJ, Cashman JR, Sugiyama K, Maltby D, Correia MA. Formation of glutathionyl-spironolactone disulfide by rat liver cytochromes P450 or hog liver flavin-containing monooxygenases: a functional probe of two-electron oxidations of the thiosteroid? *Chem Res Toxicol*. 1991;4:669–677.

Deeks ED. Cobicistat: a review of its use as a pharmacokinetic enhancer of atazanavir and darunavir in patients with HIV-1 infection. *Drugs*. 2014;74:195–206.

Degirolamo C, Modica S, Palasciano G, Moschetta A. Bile acids and colon cancer: solving the puzzle with nuclear receptors. *Trends Mol Med*. 2011;17:564–572.

Dekant W. Chemical-induced nephrotoxicity mediated by glutathione *S*-conjugate formation. *Toxicol Lett*. 2001;124:21–36.

Dekant W, Vamvakas S. Glutathione-dependent bioactivation of xenobiotics. *Xenobiotica*. 1993;23:873–887.

DeKeyser JG, Laurenzana EM, Peterson EC, Chen T, Omiecinski CJ. Selective phthalate activation of naturally occurring human constitutive androstane receptor splice variants and the pregnane X receptor. *Toxicol Sci*. 2011;120:381–391.

DeKeyser JG, Stagliano MC, Auerbach SS, et al. Di(2-ethylhexyl) phthalate is a highly potent agonist for the human constitutive androstane receptor splice variant CAR2. *Mol Pharmacol*. 2009;75:1005–1013.

Delaforge M, Pruvost A, Perrin L, Andre F. Cytochrome P450-mediated oxidation of glucuronide derivatives: example of estradiol-17{beta}-glucuronide oxidation to 2-hydroxy-estradiol-17{beta}-glucuronide by CYP 2C8. *Drug Metab Dispos*. 2005;33:466–473.

Deng LH, Zeng YL, Feng P, et al. Clinical characteristics of critical patients with pandemic influenza A (H1N1) virus infection in Chengdu, China. *J Zhejiang Univ Sci B*. 2012;13:49–55.

Devarajan A, Bourquard N, Hama S, et al. Paraoxonase 2 deficiency alters mitochondrial function and exacerbates the development of atherosclerosis. *Antioxid Redox Signal*. 2011;14:341–351.

Devos A, Lino Cardenas CL, Glowacki F, et al. Genetic polymorphism of CYP2U1, a cytochrome P450 involved in fatty acids hydroxylation. *Prostaglandins Leukot Essent Fatty Acids*. 2010;83:105–110.

Diaz D, Fabre I, Daujat M, et al.Omeprazole is an aryl hydrocarbon-like inducer of human hepatic cytochrome P450. *Gastroenterology*. 1990;99:737–747.

Dick RA, Kanne DB, Casida JE. Nitroso-imidacloprid irreversibly inhibits rabbit aldehyde oxidase. *Chem Res Toxicol*. 2007;20:1942–1946.

DiNatale BC, Murray IA, Schroeder JC, et al. Kynurenic acid is a potent endogenous aryl hydrocarbon receptor ligand that synergistically induces

interleukin-6 in the presence of inflammatory signaling. *Toxicol Sci*. 2010;115:89–97.
Ding S, Lake BG, Friedberg T, Wolf CR. Expression and alternative splicing of the cytochrome P-450 CYP2A7. *Biochem J*. 1995;306(pt 1):161–166.
Ding X, Lichti K, Kim I, Gonzalez FJ, Staudinger JL. Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor alpha, and the coactivator peroxisome proliferator-activated receptor gamma coactivator-1alpha. *J Biol Chem*. 2006;281:26540–26551.
Dirr H, Reinemer P, Huber R. X-Ray crystal structures of cytosolic glutathione *S*-transferases. *Eur J Biochem*. 1994;220:645–661.
Dolphin CT, Beckett DJ, Janmohamed A, et al. The flavin-containing monooxygenase 2 gene (FMO2) of humans, but not of other primates, encodes a truncated, nonfunctional protein. *J Biol Chem*. 1998;273:30599–30607.
Dolton MJ, Roufogalis BD, McLachlan AJ. Fruit juices as perpetrators of drug interactions: the role of organic anion-transporting polypeptides. *Clin Pharmacol Ther*. 2012;92:622–630.
Dong B, Saha PK, Huang W, et al. Activation of nuclear receptor CAR ameliorates diabetes and fatty liver disease. *Proc Natl Acad Sci U S A*. 2009;106:18831–18836.
Dong JQ, Gosset JR, Fahmi OA, et al. Examination of the human cytochrome P4503A4 induction potential of PF-06282999, an irreversible myeloperoxidase inactivator: integration of preclinical, in silico, and biomarker methodologies in the prediction of the clinical outcome. *Drug Metab Dispos*. 2017;45:501–511.
Draganov DI, La Du BN. Pharmacogenetics of paraoxonases: a brief review. *Naunyn Schmiedebergs Arch Pharmacol*. 2004;369:78–88.
Dragin N, Dalton TP, Miller ML, Shertzer HG, Nebert DW. For dioxin-induced birth defects, mouse or human CYP1A2 in maternal liver protects whereas mouse CYP1A1 and CYP1B1 are inconsequential. *J Biol Chem*. 2006;281:18591–18600.
Driscoll JP, Aliagas I, Harris JJ, et al. Formation of a quinoneimine intermediate of 4-fluoro-*N*-methylaniline by FMO1: carbon oxidation plus defluorination. *Chem Res Toxicol*. 2010;23:861–863.
Dubé C, Rostom A, Lewin G, et al. The use of aspirin for primary prevention of colorectal cancer: a systematic review prepared for the U.S. Preventive Services Task Force. *Ann Intern Med*. 2007;146:365–375.
Duell EJ, Sala N, Travier N, et al. Genetic variation in alcohol dehydrogenase (ADH1A, ADH1B, ADH1C, ADH7) and aldehyde dehydrogenase (ALDH2), alcohol consumption, and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. *Carcinogenesis*. 2012;33(2):361–367.
Duffel MW, Jakoby WB. On the mechanism of aryl sulfotransferase. *J Biol Chem*. 1981;256:11123–11127.
Duh FM, Fivash M, Moody M, et al. Characterization of a new SNP c767A/T (Arg222Trp) in the candidate TSG FUS2 on human chromosome 3p21.3: prevalence in Asian populations and analysis of association with nasopharyngeal cancer. *Mol Cell Probes*. 2004;18:39–44.
Duysen EG, Koentgen F, Williams GR, et al. Production of ES1 plasma carboxylesterase knockout mice for toxicity studies. *Chem Res Toxicol*. 2011;24:1891–1898.
Duysen EG, Li B, Darvesh S, Lockridge O. Sensitivity of butyrylcholinesterase knockout mice to (– –)-huperzine A and donepezil suggests humans with butyrylcholinesterase deficiency may not tolerate these Alzheimer's disease drugs and indicates butyrylcholinesterase function in neurotransmission. *Toxicology*. 2007;233:60–69.
Dwivedi C, Downie AA, Webb TE. Net glucuronidation in different rat strains: importance of microsomal beta-glucuronidase. *FASEB J*. 1987;1:303–307.
Eaton DL, Gallagher EP. Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol*. 1994;34:135–172.
Edenberg HJ. The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Res Health*. 2007;30:5–13.
Edmondson DE, Mattevi A, Binda C, Li M, Hubalek F. Structure and mechanism of monoamine oxidase. *Curr Med Chem*. 2004;11:1983–1993.
Edson KZ, Rettie AE. CYP4 enzymes as potential drug targets: focus on enzyme multiplicity, inducers and inhibitors, and therapeutic modulation of 20-hydroxyeicosatetraenoic acid (20-HETE) synthase and fatty acid omega-hydroxylase activities. *Curr Top Med Chem*. 2013;13:1429–1440.
Edwards J, Eliot L, Parkinson A, Karan S, MacConell L. Assessment of pharmacokinetic interactions between obeticholic acid and caffeine, midazolam, warfarin, dextromethorphan, omeprazole, rosuvastatin, and digoxin in healthy subjects. *Adv Ther*. 2017;34(9):2120–2138.
Egan MF, Goldberg TE, Kolachana BS, et al. Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc Natl Acad Sci U S A*. 2001;98:6917–6922.
Eichenbaum G, Skibbe J, Parkinson A, et al. Use of enzyme inhibitors to evaluate the conversion pathways of ester and amide prodrugs: a case study example with the prodrug ceftobiprole medocaril. *J Pharm Sci*. 2012;101:1242–1252.
Ekhart C, Rodenhuis S, Smits PH, Beijnen JH, Huitema AD. An overview of the relations between polymorphisms in drug metabolising enzymes and drug transporters and survival after cancer drug treatment. *Cancer Treat Rev*. 2009;35:18–31.
Ekins S, Stresser DM, Williams JA. In vitro and pharmacophore insights into CYP3A enzymes. *Trends Pharmacol Sci*. 2003;24:161–166.
Ekroos M, Sjögren T. Structural basis for ligand promiscuity in cytochrome P450 3A4. *Proc Natl Acad Sci U S A*. 2006;103:13682–13687.
Elcombe CR, Peffer RC, Wolf DC, et al. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: a case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. *Crit Rev Toxicol*. 2014;44:64–82.
Elens L, Bouamar R, Hesselink DA, et al. A new functional CYP3A4 intron 6 polymorphism significantly affects tacrolimus pharmacokinetics in kidney transplant recipients. *Clin Chem*. 2011a;57:1574–1583.
Elens L, van Schaik RH, Panin N, et al. Effect of a new functional CYP3A4 polymorphism on calcineurin inhibitors' dose requirements and trough blood levels in stable renal transplant patients. *Pharmacogenomics*. 2011b;12:1383–1396.
EMA. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Cerivastatin_36/WC500011752.pdf. 2002.
EMA. *Guideline on the Investigation of Drug Interactions (Final)*. London: European Medicines Agency; 2012.
Enright EF, Gahan CG, Joyce SA, Griffin BT. The impact of the gut microbiota on drug metabolism and clinical outcome. *Yale J Biol Med*. 2016;89:375–382.
Eriksson T, Björkman S, Roth B, Fyge A, Höglun P. Enantiomers of thalidomide: blood distribution and the influence of serum albumin on chiral inversion and hydrolysis. *Chirality*. 1998;10:223–228.
Ertl P, Rohde B, Selzer P. Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties. *J Med Chem*. 2000;43:3714–3717.
Evans DAP. *N*-acetyltransferase. In: Kalow W, ed. *Pharmacogenetics of Drug Metabolism*. New York: Pergamon; 1992.
Evans DC, O'Connor D, Lake BG, Evers R, Allen C, Hargreaves R. Eletriptan metabolism by human hepatic CYP450 enzymes and transport by human P-glycoprotein. *Drug Metab Dispos*. 2003; 31:861–869.
Evans MJ, Cravatt BF. Mechanism-based profiling of enzyme families. *Chem Rev*. 2006;106:3279–3301.
Fahmi OA, Shebley M, Palamanda J, et al. Evaluation of CYP2B6 induction and prediction of clinical drug–drug interactions: considerations from the IQ Consortium Induction Working Group—an industry perspective. *Drug Metab Dispos*. 2016;44:1720–1730.
Fajas L, Fruchart JC, Auwerx J. PPARgamma3 mRNA: a distinct PPARgamma mRNA subtype transcribed from an independent promoter. *FEBS Lett*. 1998;438:55–60.
Falany CN, Johnson MR, Barnes S, Diasio RB. Glycine and taurine conjugation of bile acids by a single enzyme. Molecular cloning and expression of human liver bile acid CoA:amino acid *N*-acyltransferase. *J Biol Chem*. 1994;269:19375–19379.
Fallon JK, Neubert H, Goosen TC, Smith PC. Targeted precise quantification of 12 human recombinant uridine-diphosphate glucuronosyl transferase 1A and 2B isoforms using nano-ultra-high-performance liquid chromatography/tandem mass spectrometry with selected reaction monitoring. *Drug Metab Dispos*. 2013;41: 2076–2080.

Fan PW, Zhang D, Halladay JS, Driscoll JP, Khojasteh SC. Going beyond common drug metabolizing enzymes: case studies of biotransformation involving aldehyde oxidase, gamma-glutamyl transpeptidase, cathepsin B, flavin-containing monooxygenase, and ADP-ribosyltransferase. *Drug Metab Dispos*. 2016;44: 1253–1261.

Farooq M, Kelly EJ, Unadkat JD. CYP2D6 is inducible by endogenous and exogenous corticosteroids. *Drug Metab Dispos*. 2016;44: 750–757.

Faucette SR, Sueyoshi T, Smith CM, et al. Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *J Pharmacol Exp Ther*. 2006;317:1200–1209.

FDA. *Guidance for Industry. In vitro Metabolism- and Transporter-Mediated Drug-Drug Interaction Studies*. Silver Spring, MD: FDA; 2017.

FDA. Available at: https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm581965.pdf.

Feng C, Tollin G, Enemark JH. Sulfite oxidizing enzymes. *Biochim Biophys Acta*. 2007;1774:527–539.

Fennema D, Phillips IR, Shephard EA. Trimethylamine and trimethylamine *N*-oxide, a flavin-containing monooxygenase 3 (FMO3)-mediated host-microbiome metabolic axis implicated in health and disease. *Drug Metab Dispos*. 2016;44:1839–1850.

Fisher MB, Campanale K, Ackermann BL, Vandenbrande M, Wrighton SA. In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. *Drug Metab Dispos*. 2000;28: 560–566.

Flanagan JU, Smythe ML. Sigma-class glutathione transferases. *Drug Metab Rev*. 2011;43:194–214.

Fleming I. The pharmacology of the cytochrome P450 epoxygenase/soluble epoxide hydrolase axis in the vasculature and cardiovascular disease. *Pharmacol Rev*. 2014;66:1106–1140.

Fontana E, Dansette PM, Poli SM. Cytochrome p450 enzymes mechanism based inhibitors: common sub-structures and reactivity. *Curr Drug Metab*. 2005;6:413–454.

Foti RS, Dalvie DK. Cytochrome P450 and non-cytochrome P450 oxidative metabolism: contributions to the pharmacokinetics, safety, and efficacy of xenobiotics. *Drug Metab Dispos*. 2016;44:1229–1245.

Foti R, Rock DA, Pearson JT, Wahlstrom JL, Wienkers LC. Mechanism-based inactivation of cytochrome P450 3A4 by mibefradil through heme destruction. *Drug Metab Dispos*. 2011;39:1188–1195.

Fowler S, Kletzl H, Finel M, et al. A UGT2B10 splicing polymorphism common in African populations may greatly increase drug exposure. *J Pharmacol Exp Ther*. 2015;352:358–367.

Francois AA, Nishida CR, de Montellano PR, Phillips IR, Shephard EA. Human flavin-containing monooxygenase 2.1 catalyzes oxygenation of the antitubercular drugs thiacetazone and ethionamide. *Drug Metab Dispos*. 2009;37:178–186.

Franks ME, Macpherson GR, Figg WD. Thalidomide. *Lancet*. 2004;363: 1802–1811.

Fremont JJ, Wang RW, King CD. Coimmunoprecipitation of UDP-glucuronosyltransferase isoforms and cytochrome P450 3A4. *Mol Pharmacol*. 2005;67:260–262.

Fretland AJ, Omiecinski CJ. Epoxide hydrolases: biochemistry and molecular biology. *Chem Biol Interact*. 2000;129:41–59.

Frezza M, di Padova C, Pazzato G, et al. High blood alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. *N Engl J Med*. 1990;322:95–99.

Fu C, Di L, Han X, et al. Aldehyde oxidase 1 (AOX1) in human liver cytosols: quantitative characterization of AOX1 expression level and activity relationship. *Drug Metab Dispos*. 2013;41:1797–1804.

Fujikawa Y, Satoh T, Suganuma A, et al. Extremely sensitive biomarker of acute organophosphorus insecticide exposure. *Hum Exp Toxicol*. 2005;24:333–336.

Fujiwara R, Nakajima M, Yamanaka H, Katoh M, Yokoi T. Product inhibition of UDP-glucuronosyltransferase (UGT) enzymes by UDP obfuscates the inhibitory effects of UGT substrates. *Drug Metab Dispos*. 2008;36:361–367.

Fukami T, Iida A, Konishi K, Nakajima M. Human arylacetamide deacetylase hydrolyzes ketoconazole to trigger hepatocellular toxicity. *Biochem Pharmacol*. 2016;116:153–161.

Fung M, Thornton A, Mybeck A, et al. Evaluation of the characteristics of safety withdrawal of prescription drugs from worldwide pharmaceutical markets 1960 to 1999. *Drug Inf J*. 2001;35:293–317.

Furlong CE, Suzuki SM, Stevens RC,et al. Human PON1, a biomarker of risk of disease and exposure. *Chem Biol Interact*. 2010;187:355–361.

Furukawa M, Nishimura M, Ogino D, et al. Cytochrome p450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver. *Cancer Sci*. 2004;95:520–529.

Furuta T, Shirai N, Sugimoto M, et al. Influence of CYP2C19 pharmacogenetic polymorphism on proton pump inhibitor-based therapies. *Drug Metab Pharmacokinet*. 2005;20:153–167.

Gaedigk A, Simon SD, Pearce RE, et al. The CYP2D6 activity score: translating genotype information into a qualitative measure of phenotype. *Clin Pharmacol Ther*. 2008;83:234–242.

Galetin A, Clarke SE, Houston JB. Multisite kinetic analysis of interactions between prototypical CYP3A4 subgroup substrates: midazolam, testosterone, and nifedipine. *Drug Metab Dispos*. 2003;31:1108–1116.

Galetin A, Gertz M, Houston JB. Contribution of intestinal cytochrome p450-mediated metabolism to drug-drug inhibition and induction interactions. *Drug Metab Pharmacokinet*. 2010;25:28–47.

Gallicano KD, Sahai J, Shukla VK, et al. Induction of zidovudine glucuronidation and amination pathways by rifampicin in HIV-infected patients. *Br J Clin Pharmacol*. 1999;48:168–179.

Gamage N, Barnett A, Hempel N, et al. Human sulfotransferases and their role in chemical metabolism. *Toxicol Sci*. 2006;90:5–22.

Gammal RS, Court MH, Haidar CE, et al. Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline for UGT1A1 and atazanavir prescribing. *Clin Pharmacol Ther*. 2016;99:363–369.

Gao J, Xie W. Pregnane X receptor and constitutive androstane receptor at the crossroads of drug metabolism and energy metabolism. *Drug Metab Dispos*. 2010;38:2091–2095.

Garattini E, Fratelli M, Terao M. Mammalian aldehyde oxidases: genetics, evolution and biochemistry. *Cell Mol Life Sci*. 2008;65:1019–1048.

Garattini E, Terao M. Increasing recognition of the importance of aldehyde oxidase in drug development and discovery. *Drug Metab Rev*. 2011;43:374–386.

Garzel B, Yang H, Zhang L, et al. The role of bile salt export pump gene repression in drug-induced cholestatic liver toxicity. *Drug Metab Dispos*. 2014;42:318–322.

Gasche Y, Daali Y, Fathi M, et al. Codeine intoxication associated with ultrarapid CYP2D6 metabolism. *N Engl J Med*. 2004;351:2827–2831.

Gauthier-Landry L, Belanger A, Barbier O. Multiple roles for UDP-glucuronosyltransferase (UGT)2B15 and UGT2B17 enzymes in androgen metabolism and prostate cancer evolution. *J Steroid Biochem Mol Biol*. 2015;145:187–192.

Gearry RB, Day AS, Barclay ML, Leong RW, Sparrow MP. Azathioprine and allopurinol: a two-edged interaction. *J Gastroenterol Hepatol*. 2010;25:653–655.

Geisler SA, Olshan AF. GSTM1, GSTT1, and the risk of squamous cell carcinoma of the head and neck: a mini-HuGE review. *Am J Epidemiol*. 2001;154:95–105.

Gentilucci L, De Marco R, Cerisoli L. Chemical modifications designed to improve peptide stability: incorporation of non-natural amino acids, pseudo-peptide bonds, and cyclization. *Curr Pharm Des*. 2010;16:3185–3203.

Gerbal-Chaloin S, Dume AS, Briolotti P, et al. The WNT/beta-catenin pathway is a transcriptional regulator of CYP2E1, CYP1A2, and aryl hydrocarbon receptor gene expression in primary human hepatocytes. *Mol Pharmacol*. 2014;86:624–634.

Gerbal-Chaloin S, Pichard-Garcia L, Fabre JM, et al. Role of CYP3A4 in the regulation of the aryl hydrocarbon receptor by omeprazole sulphide. *Cell Signal*. 2006;18:740–750.

Gerlach M, Riederer P, Przuntek H, Youdim MB. MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. *Eur J Pharmacol*. 1991;208:273–286.

Germain P, Staels B, Dacquet C, Spedding M, Laudet V. Overview of nomenclature of nuclear receptors. *Pharmacol Rev*. 2006;58:685–704.

Geshi E, Kimura T, Yoshimura M, et al. A single nucleotide polymorphism in the carboxylesterase gene is associated with the responsiveness

to imidapril medication and the promoter activity. *Hypertens Res*. 2005;28:719–725.
Ghanayem BI. Inhibition of urethane-induced carcinogenicity in cyp2e1–/– in comparison to cyp2e1+/+ mice. *Toxicol Sci*. 2007;95:331–339.
Giacomini KM, Huang SM, Tweedie DJ, et al. Membrane transporters in drug development. *Nat Rev Drug Discov*. 2010;9:215–236.
Giacomini KM, Sugiyama Y. Membrane transporters and drug response. In: Goodman LS, Gilman A, Brunton LL, et al., eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. New York: McGraw-Hill; 2006.
Gingell R, Bridges JW, Williams RT. The role of the gut flora in the metabolism of prontosil and neoprontosil in the rat. *Xenobiotica*. 1971;1:143–156.
Gipple KJ, Chan KT, Elvin AT, Lalka D, Axelson JE. Species differences in the urinary excretion of the novel primary amine conjugate: tocainide carbamoyl *O*-beta-D-glucuronide. *J Pharm Sci*. 1982;71:1011–1014.
Glatt H, Meinl W. Pharmacogenetics of soluble sulfotransferases (SULTs). *Naunyn Schmiedebergs Arch Pharmacol*. 2004a;369:55–68.
Glatt H, Meinl W. Use of genetically manipulated *Salmonella typhimurium* strains to evaluate the role of sulfotransferases and acetyltransferases in nitrofen mutagenicity. *Carcinogenesis*. 2004b;25:779–786.
Goedde HW, Agarwal DP. Pharmacogenetics of aldehyde dehydrogenase. In: Kalow W, ed.. *Pharmacogenetics of Drug Metabolism*. New York: Pergamon; 1992.
Goetz MP, Rae JM, Suman VJ, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol*. 2005;23:9312–9318.
Goh BC, Lee SC, Wang LZ, et al. Explaining interindividual variability of docetaxel pharmacokinetics and pharmacodynamics in Asians through phenotyping and genotyping strategies. *J Clin Oncol*. 2002;20:3683–3690.
Goldring CE, Kitteringham NR, Elsby R, et al. Activation of hepatic Nrf2 in vivo by acetaminophen in CD-1 mice. *Hepatology*. 2004;39:1267–1276.
Gomez A, Nekvindova J, Travica S, et al. Colorectal cancer-specific cytochrome P450 2W1: intracellular localization, glycosylation, and catalytic activity. *Mol Pharmacol*. 2010;78:1004–1011.
Gonzalez FJ. Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. *Mutat Res*. 2005;569:101–110.
Gonzalez FJ. The 2006 Bernard B. Brodie Award lecture. Cyp2e1. *Drug Metab Dispos*. 2007;35:1–8.
Gonzalez FJ, Tukey RH. Drug metabolism. In: Goodman LS, Gilman A, Brunton LL, et al. eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. New York: McGraw-Hill; 2006.
Gonzalez FJ. Role of gene knockout and transgenic mice in the study of xenobiotic metabolism. *Drug Metab Rev*. 2003;35:319–335.
Gonzalez FJ, Yu A-M. Cytochrome P450 and xenobiotic receptor humanized mice. *Annu Rev Pharmacol Toxicol*. 2006;46:41–64.
Gonzalez-Covarrubias V, Ghosh D, Lakhman SS, Pendyala L, Blanco JG. A functional genetic polymorphism on human carbonyl reductase 1 (CBR1 V88I) impacts on catalytic activity and NADPH binding affinity. *Drug Metab Dispos*. 2007;35:973–980.
Gopaul VS, Tang W, Farrell K, Abbott FS. Amino acid conjugates: metabolites of 2-propylpentanoic acid (valproic acid) in epileptic patients. *Drug Metab Dispos*. 2003;31:114–121.
Gorell JM, Rybicki BA, Johnson CC, Peterson EL. Smoking and Parkinson's disease: a dose–response relationship. *Neurology*. 1999;52:115–119.
Grant DM, Blum M, Meyer UA. Polymorphisms of *N*-acetyltransferase genes. *Xenobiotica*. 1992;22:1073–1081.
Grasso P, Sharratt M, Cohen AJ. Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans. *Annu Rev Pharmacol Toxicol*. 1991;31:253–287.
Griffith OW, Meister A. Translocation of intracellular glutathione to membrane-bound gamma-glutamyl transpeptidase as a discrete step in the gamma-glutamyl cycle: glutathionuria after inhibition of transpeptidase. *Proc Natl Acad Sci U S A*. 1979;76:268–272.
Grillo MP. Drug-*S*-acyl-glutathione thioesters: synthesis, bioanalytical properties, chemical reactivity, biological formation and degradation. *Curr Drug Metab*. 2011;12:229–244.
Grompe M, Strom S. Mice with human livers. *Gastroenterology*. 2013;145:1209–1214.
Grose KR, Bjeldanes LF. Oligomerization of indole-3-carbinol in aqueous acid. *Chem Res Toxicol*. 1992;5:188–193.
Grunwell JR, Bertozzi CR. Carbohydrate sulfotransferases of the GalNAc/Gal/GlcNAc6ST family. *Biochemistry*. 2002;41:13117–13126.
Gu Z, Feng X, Dong X, Chan P. Smoking, genes encoding dopamine pathway and risk for Parkinson's disease. *Neurosci Lett*. 2010;482:31–34.
Gu C, Wen S, Doig P, Gangl E, Zheng X, Wang Y, Johannes JW. Mouse red blood cell-mediated rare xenobiotic phosphorylation of a drug molecule not intended to be a kinase substrate. *Drug Metab Dispos*. 2017;45:1345–1353.
Guengerich FP. Reactions and significance of cytochrome P-450 enzymes. *J Biol Chem*. 1991;266:10019–10022.
Guengerich FP. Uncommon P450-catalyzed reactions. *Curr Drug Metab*. 2001a;2:93–115.
Guengerich FP. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol*. 2001b;14:611–650.
Guengerich FP. Mechanisms of cytochrome P450 substrate oxidation: mini review. *J Biochem Mol Toxicol*. 2007;21:163–168.
Guengerich FP, Shimada T. Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chem Res Toxicol*. 1991;4:391–407.
Guengerich FP, Tang Z, Salamanca-Pinzon SG, Cheng Q. Characterizing proteins of unknown function: orphan cytochrome p450 enzymes as a paradigm. *Mol Interv*. 2010;10:153–163.
Guidotti G, Brambilla L, Rossi D. Cell-penetrating peptides: from basic research to clinics. *Trends Pharmacol Sci*. 2017;38:406–424.
Guillemette C, Levesque E, Harvey M, Bellemare J, Menard V. UGT genomic diversity: beyond gene duplication. *Drug Metab Rev*. 2010;42:24–44.
Gunes A, Dahl ML. Variation in CYP1A2 activity and its clinical implications: influence of environmental factors and genetic polymorphisms. *Pharmacogenomics*. 2008;9:625–637.
Gupta N, Gill K, Singh S. Paraoxonases: structure, gene polymorphism & role in coronary artery disease. *Indian J Med Res*. 2009;130:361–368.
Gupta RA, DuBois RN. Aspirin, NSAIDs, and colon cancer prevention: mechanisms? *Gastroenterology*. 1998;114:1095–1098.
Gutierrez PL. The role of NAD(P)H oxidoreductase (DT-Diaphorase) in the bioactivation of quinone-containing antitumor agents: a review. *Free Radic Biol Med*. 2000;29:263–275.
Guyot E, Chevallier A, Barouki R, Coumoul X. The AhR twist: ligand-dependent AhR signaling and pharmaco-toxicological implications. *Drug Discov Today*. 2013;18:479–486.
Hagihara K, Kazui M, Kurihara A, Kubota K, Ikeda T. Glutaredoxin and thioredoxin can be involved in producing the pharmacologically active metabolite of a thienopyridine antiplatelet agent, prasugrel. *Drug Metab Dispos*. 2011;39:208–214.
Hakkinen MR, Hyvonen MT, Auriola S, et al. Metabolism of *N*-alkylated spermine analogues by polyamine and spermine oxidases. *Amino Acids*. 2010;38:369–381.
Hamman M, Haehner-Daniels B, Wrighton S, Rettie A, Hall S. Stereoselective sulfoxidation of sulindac sulfide by flavin-containing monooxygenases. *Biochem Pharmacol*. 2000;60:7–17.
Han Y, Oota H, Osier MV, et al. Considerable haplotype diversity within the 23kb encompassing the ADH7 gene. *Alcohol Clin Exp Res*. 2005;29:2091–2100.
Hanigan MH, Gallagher BC, Taylor PT Jr, Large MK. Inhibition of gamma-glutamyl transpeptidase activity by acivicin in vivo protects the kidney from cisplatin-induced toxicity. *Cancer Res*. 1994;54:5925–5929.
Hanigan MH, Lykissa ED, Townsend DM, et al. Gamma-glutamyl transpeptidase-deficient mice are resistant to the nephrotoxic effects of cisplatin. *Am J Pathol*. 2001;159:1889–1894.
Haramaki N, Han D, Handelman GJ, Tritschler HJ, Packer L. Cytosolic and mitochondrial systems for NADH- and NADPH-dependent reduction of alpha-lipoic acid. *Free Radic Biol Med*. 1997;22:535–542.
Harbourt DE, Fallon JK, Ito S, et al. Quantification of human uridine-diphosphate glucuronosyl transferase 1A isoforms in liver, intestine, and kidney using nanobore liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2012;84:98–105.
Harrill JA, Hukkanen RR, Lawson M, et al. Knockout of the aryl hydrocarbon receptor results in distinct hepatic and renal phenotypes in rats and mice. *Toxicol Appl Pharmacol*. 2013;272:503–518.
Harris JM, Meyer DJ, Coles B, Ketterer B. A novel glutathione transferase (13-13) isolated from the matrix of rat liver mitochondria

having structural similarity to class theta enzymes. *Biochem J*. 1991;278 (pt 1):137–141.
Hartmann T, Terao M, Garattini E, et al. The impact of single nucleotide polymorphisms on human aldehyde oxidase. *Drug Metab Dispos*. 2012;40(5):856–864.
Havemeyer A, Grunewald S, Wahl B, et al. Reduction of *N*-hydroxysulfonamides, including *N*-hydroxy-valdecoxib, by the molybdenum-containing enzyme mARC. *Drug Metab Dispos*. 2010;38:1917–1921.
Havemeyer A, Lang J, Clement B. The fourth mammalian molybdenum enzyme mARC: current state of research. *Drug Metab Rev*. 2011;43:524–539.
Hawes EM. *N*+-glucuronidation, a common pathway in human metabolism of drugs with a tertiary amine group. *Drug Metab Dispos*. 1998;26:830–837.
Hayakawa H, Fukushima Y, Kato H, et al. Metabolism and disposition of novel des-fluoro quinolone garenoxacin in experimental animals and an interspecies scaling of pharmacokinetic parameters. *Drug Metab Dispos*. 2003;31:1409–1418.
Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. *Annu Rev Pharmacol Toxicol*. 2005;45:51–88.
Hayes JD, Nguyen T, Judah DJ, Petersson DG, Neal GE. Cloning of cDNAs from fetal rat liver encoding glutathione *S*-transferase Yc polypeptides. The Yc2 subunit is expressed in adult rat liver resistant to the hepatocarcinogen aflatoxin B1. *J Biol Chem*. 1994;269:20707–20717.
Hayes JD, Pulford DJ. The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol*. 1995;30:445–600.
Hayes RB, Bi W, Rothman N, et al. *N*-acetylation phenotype and genotype and risk of bladder cancer in benzidine-exposed workers. *Carcinogenesis*. 1993;14:675–678.
He BX, Shi L, Qiu J, Zeng XH, Zhao SJ. The effect of CYP3A4*1G allele on the pharmacokinetics of atorvastatin in Chinese Han patients with coronary heart disease. *J Clin Pharmacol*. 2014;54:462–467.
He J, Wang C, Zhu Y, Ai D. Soluble epoxide hydrolase: a potential target for metabolic diseases. *J Diabetes*. 2016;8:305–313.
Heath-Pagliuso S, Rogers WJ, Tullis K, et al. Activation of the Ah receptor by tryptophan and tryptophan metabolites. *Biochemistry*. 1998;37:11508–11515.
Hebbachi AM, Knight BL, Wiggins D, Patel DD, Gibbons GF. Peroxisome proliferator-activated receptor alpha deficiency abolishes the response of lipogenic gene expression to re-feeding: restoration of the normal response by activation of liver X receptor alpha. *J Biol Chem*. 2008;283:4866–4876.
Hedrich WD, Hassan HE, Wang H. Insights into CYP2B6-mediated drug–drug interactions. *Acta Pharm Sin B*. 2016;6:413–425.
Hein DW. Acetylator genotype and arylamine-induced carcinogenesis. *Biochim Biophys Acta*. 1988;948:37–66.
Hein DW. *N*-acetyltransferase 2 genetic polymorphism: effects of carcinogen and haplotype on urinary bladder cancer risk. *Oncogene*. 2006;25:1649–1658.
Hein DW, Doll MA, Fretland AJ, et al. Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. *Cancer Epidemiol Biomarkers Prev*. 2000;9:29–42.
Helms C, Cao L, Krueger JG, et al. A putative RUNX1 binding site variant between SLC9A3R1 and NAT9 is associated with susceptibility to psoriasis. *Nat Genet*. 2003;35:349–356.
Henry EC, Bemis JC, Henry O, Kende AS, Gasiewicz TA. A potential endogenous ligand for the aryl hydrocarbon receptor has potent agonist activity in vitro and in vivo. *Arch Biochem Biophys*. 2006;450:67–77.
Higgins LG, Hayes JD. Mechanisms of induction of cytosolic and microsomal glutathione transferase (GST) genes by xenobiotics and pro-inflammatory agents. *Drug Metab Rev*. 2011;43:92–137.
Hill RN, Erdreich LS, Paynter OE, et al. Thyroid follicular cell carcinogenesis. *Fundam Appl Toxicol*. 1989;12:629–697.
Hines RN, Hopp KA, Franco J, Saeian K, Begun FP. Alternative processing of the human FMO6 gene renders transcripts incapable of encoding a functional flavin-containing monooxygenase. *Mol Pharmacol*. 2002;62:320–325.
Hinrichs AL, Murphy SE, Wang JC, et al. Common polymorphisms in FMO1 are associated with nicotine dependence. *Pharmacogenet Genomics*. 2011;21:397–402.
Hirvonen A. Polymorphic NAT's and cancer predisposition. In: Ryder W, ed. *Metabolic Polymorphisms and Susceptibility to Cancer (IARC)*. Lyon: IARC Scientific Publications; 1999.
Hofmann U, Heinkele G, Angelberger S, et al. Simultaneous quantification of eleven thiopurine nucleotides by liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2012;84(3):1294–1301.
Holcapek M, Kolarova L, Nobilis M. High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. *Anal Bioanal Chem*. 2008;391:59–78.
Hollman AL, Tchounwou PB, Huang HC. The association between gene–environment interactions and diseases involving the human GST superfamily with SNP variants. *Int J Environ Res Public Health*. 2016;13:379.
Holmes RS, Wright MW, Laulederkind SJ, et al. Recommended nomenclature for five mammalian carboxylesterase gene families: human, mouse, and rat genes and proteins. *Mamm Genome*. 2010;21:427–441.
Hon YY, Jusko WJ, Spratlin VE, Jann MW. Altered methylprednisolone pharmacodynamics in healthy subjects with histamine *N*-methyltransferase C314T genetic polymorphism. *J Clin Pharmacol*. 2006;46:408–417.
Horton JR, Sawada K, Nishibori M, Cheng X. Structural basis for inhibition of histamine *N*-methyltransferase by diverse drugs. *J Mol Biol*. 2005;353:334–344.
Hosono H, Kumondai M, Maekawa M, et al. Functional characterization of 34 CYP2A6 allelic variants by assessment of nicotine C-oxidation and coumarin 7-hydroxylation activities. *Drug Metab Dispos*. 2017;45:279–285.
Hrycay EG, Bandiera SM. The monooxygenase, peroxidase, and peroxygenase properties of cytochrome P450. *Arch Biochem Biophys*. 2012;522:71–89.
Hu R, Xu C, Shen G, et al. Identification of Nrf2-regulated genes induced by chemopreventive isothiocyanate PEITC by oligonucleotide microarray. *Life Sciences*. 2006;79:1944–1955.
Hu W, Sorrentino C, Denison MS, Kolaja K, Fielden MR. Induction of cyp1a1 is a nonspecific biomarker of aryl hydrocarbon receptor activation: results of large scale screening of pharmaceuticals and toxicants in vivo and in vitro. *Mol Pharmacol*. 2007;71:1475–1486.
Huang L, Smit JW, Meijer DK, Vore M. Mrp2 is essential for estradiol-17beta(beta-D-glucuronide)-induced cholestasis in rats. *Hepatology*. 2000;32:66–72.
Huang SM, Strong JM, Zhang L, et al. New era in drug interaction evaluation: US Food and Drug Administration update on CYP enzymes, transporters, and the guidance process. *J Clin Pharmacol*. 2008;48: 662–670.
Huang SM, Zhao H, Lee JI, et al. Therapeutic protein–drug interactions and implications for drug development. *Clin Pharmacol Ther*. 2010;87(4):497–503.
Huang W, Lin YS, McConn DJ II, et al. Evidence of significant contribution from CYP3A5 to hepatic drug metabolism. *Drug Metab Dispos*. 2004;32:1434–1445.
Hukkanen J, Jacob P III, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev*. 2005;57:79–115.
Hyland R, Roe EG, Jones BC, Smith DA. Identification of the cytochrome P450 enzymes involved in the *N*-demethylation of sildenafil. *Br J Clin Pharmacol*. 2001;51:239–248.
Ibrahim ZS, Ahmed MM, El-Shazly SA, Ishizuka M, Fujita S. Clofibric acid induces hepatic CYP 2B1/2 via constitutive androstane receptor not via peroxisome proliferator activated receptor alpha in rat. *Biosci Biotechnol Biochem*. 2014;78:1550–1559.
Idle JR. Is environmental carcinogenesis modulated by host polymorphism? *Mutat Res*. 1991;247:259–266.
Ikeda T. Drug-induced idiosyncratic hepatotoxicity: prevention strategy developed after the troglitazone case. *Drug Metab Pharmacokinet*. 2011;26:60–70.
Imig JD, Hammock BD. Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nat Rev Drug Discov*. 2009;8:794–805.
Ingelman-Sundberg M, Rane A, Gustafsson JA. Properties of hydroxylase systems in the human fetal liver active on free and sulfoconjugated steroids. *Biochemistry*. 1975;14:429–437.

Ingelman-Sundberg M. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J*. 2005;5:6–13.

Inoue T, Irikura D, Okazaki N, et al. Mechanism of metal activation of human hematopoietic prostaglandin D synthase. *Nat Struct Biol*. 2003;10:291–296.

Ishida M, Mikami S, Kikuchi E, et al. Activation of the aryl hydrocarbon receptor pathway enhances cancer cell invasion by upregulating the MMP expression and is associated with poor prognosis in upper urinary tract urothelial cancer. *Carcinogenesis*. 2010;31:287–295.

Ishii Y, Takeda S, Yamada H. Modulation of UDP-glucuronosyltransferase activity by protein-protein association. *Drug Metab Rev*. 2010;42:145–158.

Ishizuka T, Fujimori I, Nishida A, et al. Paraoxonase 1 as a major bioactivating hydrolase for olmesartan medoxomil in human blood circulation: molecular identification and contribution to plasma metabolism. *Drug Metab Dispos*. 2012;40:374–380.

Isin EM, Guengerich FP. Complex reactions catalyzed by cytochrome P450 enzymes. *Biochim Biophys Acta*. 2007;1770:314–329.

Iskander K, Jaiswal AK. Quinone oxidoreductases in protection against myelogenous hyperplasia and benzene toxicity. *Chemico-Biological Interactions*. 2005;153–154:147–157.

Islam MR, Waheed A, Shah GN, Tomatsu S, Sly WS. Human egasyn binds beta-glucuronidase but neither the esterase active site of egasyn nor the C terminus of beta-glucuronidase is involved in their interaction. *Arch Biochem Biophys*. 1999;372:53–61.

Isoherranen N, Hachad H, Yeung CK, Levy RH. Qualitative analysis of the role of metabolites in inhibitory drug–drug interactions: literature evaluation based on the metabolism and transport drug interaction database. *Chem Res Toxicol*. 2009;22:294–298.

Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*. 1990;347:645–650.

Itkonen MK, Tornio A, Neuvonen M, et al. Clopidogrel markedly increases plasma Concentrations of CYP2C8 Substrate Pioglitazone. *Drug Metab Dispos*. 2016;44:1364–1371.

Ito S, Chen C, Satoh J, Yim S, Gonzalez FJ. Dietary phytochemicals regulate whole-body CYP1A1 expression through an arylhydrocarbon receptor nuclear translocator-dependent system in gut. *J Clin Invest*. 2007;117:1940–1950.

Iwaki M, Ogiso T, Hayashi H, Tanino T, Benet LZ. Acute dose-dependent disposition studies of nicotinic acid in rats. *Drug Metab Dispos*. 1996;24:773–779.

Iwamura A, Nakajima M, Oda S, Yokoi T. Toxicological potential of acyl glucuronides and its assessment. *Drug Metab Pharmacokinet*. 2017;32:2–11.

Iwata H, Fujita K, Kushida H, et al. High catalytic activity of human cytochrome P450 co-expressed with human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Biochem Pharmacol*. 1998;55:1315–1325.

Jack DB. *Handbook of Clinical Pharmacokinetic Data*. Houndmils, UK: Macmillan; 1992:chap 2 (pharmacokinetic tables).

Jaeschke H, Williams CD, Ramachandran A, Bajt ML. Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. *Liver Int*. 2012;32:8–20.

James MO, Jahn SC, Zhong G, et al. Therapeutic applications of dichloroacetate and the role of glutathione transferase zeta-1. *Pharmacol Ther*. 2017;170:166–180.

Jedlitschky G, Cassidy AJ, Sales M, Pratt N, Burchell B. Cloning and characterization of a novel human olfactory UDP-glucuronosyltransferase. *Biochem J*. 1999;340(pt 3):837–843.

Jeffrey AM, Iatropoulos MJ, Williams GM. Nasal cytotoxic and carcinogenic activities of systemically distributed organic chemicals. *Toxicol Pathol*. 2006;34:827–852.

Jensen BA, Leeman RJ, Schlezinger JJ, Sherr DH. Aryl hydrocarbon receptor (AhR) agonists suppress interleukin-6 expression by bone marrow stromal cells: an immunotoxicology study. *Environ Health*. 2003;2:16.

Jeong S, Woo MM, Flockhart DA, Desta Z. Inhibition of drug metabolizing cytochrome P450s by the aromatase inhibitor drug letrozole and its major oxidative metabolite 4,4′-methanol-bisbenzonitrile in vitro. *Cancer Chemother Pharmacol*. 2009;64:867–875.

Jez JM, Penning TM. The aldo-keto reductase (AKR) superfamily: an update. *Chem Biol Interact*. 2001;130–132:499–525.

Ji Y, Salavaggione OE, Wang L, et al. Human phenylethanolamine *N*-methyltransferase pharmacogenomics: gene re-sequencing and functional genomics. *J Neurochem*. 2005;95:1766–1776.

Jin L, Kikuchi R, Saji T, Kusuhara H, Sugiyama Y. Regulation of tissue-specific expression of renal organic anion transporters by hepatocyte nuclear factor 1 alpha/beta and DNA methylation. *J Pharmacol Exp Ther*. 2012;340:648–655.

Jin S, Bryson TA, Dawson JH. Hydroperoxoferric heme intermediate as a second electrophilic oxidant in cytochrome P450-catalyzed reactions. *J Biol Inorg Chem*. 2004;9:644–653.

Johnson AL, Edson KZ, Totah RA, Rettie AE. Cytochrome P450 omega-hydroxylases in inflammation and cancer. *Adv Pharmacol*. 2015;74:223–262.

Johnston JB, Ouellet H, Podust LM, Ortiz de Montellano PR. Structural control of cytochrome P450-catalyzed ω-hydroxylation. *Arch Biochem Biophys*. 2011;507:86–94.

Josephy PD. Genetic variations in human glutathione transferase enzymes: significance for pharmacology and toxicology. *Hum Genomics Proteomics*. 2010;2010:876940.

Jourova L, Anzenbacher P, Anzenbacherova E. Human gut microbiota plays a role in the metabolism of drugs. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2016;160:317–326.

Juberg DR, Mudra DR, Hazelton GA, Parkinson A. The effect of fenbuconazole on cell proliferation and enzyme induction in the liver of female CD1 mice. *Toxicol Appl Pharmacol*. 2006;214:178–187.

Just W, Zeller J, Riegert C, Speit G. Genetic polymorphisms in the formaldehyde dehydrogenase gene and their biological significance. *Toxicol Lett*. 2011;207:121–127.

Justenhoven C, Winter S, Hamann U, et al. The frameshift polymorphism CYP3A43_74_delA is associated with poor differentiation of breast tumors. *Cancer*. 2010;116:5358–5364.

Kadlubar FF. Biochemical individuality and its implications for drug and carcinogen metabolism: recent insights from acetyltransferase and cytochrome P4501A2 phenotyping and genotyping in humans. *Drug Metab Rev*. 1994;26:37–46.

Kaitaniemi S, Elovaara H, Gron K, et al. The unique substrate specificity of human AOC2, a semicarbazide-sensitive amine oxidase. *Cell Mol Life Sci*. 2009;66:2743–2757.

Kaivosaari S, Finel M, Koskinen M. *N*-glucuronidation of drugs and other xenobiotics by human and animal UDP-glucuronosyltransferases. *Xenobiotica*. 2011;41:652–669.

Kalsotra A, Strobel HW. Cytochrome P450 4F subfamily: at the crossroads of eicosanoid and drug metabolism. *Pharmacol Ther*. 2006;112:589–611.

Kamataki T, Fujieda M, Kiyotani K, Iwano S, Kunitoh H. Genetic polymorphism of CYP2A6 as one of the potential determinants of tobacco-related cancer risk. *Biochem Biophys Res Commun*. 2005;338:306–310.

Kanamitsu SI, Ito K, Okuda H, et al. Prediction of in vivo drug–drug interactions based on mechanism-based inhibition from in vitro data: inhibition of 5-fluorouracil metabolism by (E)-5-(2-bromovinyl) uracil. *Drug Metab Dispos*. 2000;28:467–474.

Kang SJ, Scott WK, Li YJ, et al. Family-based case-control study of MAOA and MAOB polymorphisms in Parkinson disease. *Mov Disord*. 2006;21:2175–2180.

Kanno Y, Inoue Y, Inouye Y. 5-Aminoimidazole-4-carboxamide-1-beta-ribofuranoside (AICAR) prevents nuclear translocation of constitutive androstane receptor by AMP-activated protein kinase (AMPK) independent manner. *J Toxicol Sci*. 2010;35:571–576.

Kaplowitz N. Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov*. 2005;4:489–499.

Karanam BV, Hop CE, Liu DQ, et al. In vitro metabolism of MK-0767 [(+/–)-5-[(2,4-dioxothiazolidin-5-yl)methyl]-2-methoxy-*N*-[[(4-trifluoromet hyl) phenyl]methyl]benzamide], a peroxisome proliferator-activated receptor alpha/gamma agonist. I. Role of cytochrome P450, methyltransferases, flavin monooxygenases, and esterases. *Drug Metab Dispos*. 2004;32:1015–1022.

Karkhanis A, Hong Y, Chan ECY. Inhibition and inactivation of human CYP2J2: implications in cardiac pathophysiology and opportunities in cancer therapy. *Biochem Pharmacol*. 2017;135:12–21.

Karkhanis A, Lam HY, Venkatesan G, et al. Multiple modes of inhibition of human cytochrome P450 2J2 by dronedarone, amiodarone and their active metabolites. *Biochem Pharmacol*. 2016;107:67–80.

Karlgren M, Gomez A, Stark K, et al. Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1. *Biochem Biophys Res Commun*. 2006;341:451–458.

Kasumov T, Brunengraber LL, Comte B, et al. New secondary metabolites of phenylbutyrate in humans and rats. *Drug Metab Dispos*. 2004;32:10–19.

Kato R, Yamazoe Y. Metabolic activation of *N*-hydroxylated metabolites of carcinogenic and mutagenic arylamines and arylamides by esterification. *Drug Metab Rev*. 1994;26:413–429.

Kavanagh KL, Jornvall H, Persson B, Oppermann U. Medium- and short-chain dehydrogenase/reductase gene and protein families : the SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. *Cell Mol Life Sci*. 2008;65:3895–3906.

Kawakami H, Ohtsuki S, Kamiie J, et al. Simultaneous absolute quantification of 11 cytochrome P450 isoforms in human liver microsomes by liquid chromatography tandem mass spectrometry with in silico target peptide selection. *J Pharm Sci*. 2011;100:341–352.

Kaye J, Piryatinsky V, Birnberg T, et al. Laquinimod arrests experimental autoimmune encephalomyelitis by activating the aryl hydrocarbon receptor. *Proc Natl Acad Sci U S A*. 2016;113:E6145–E6152.

Kazmi F, Barbara JE, Yerino P, Parkinson A. A long-standing mystery solved: the formation of 3-hydroxydesloratadine is catalyzed by CYP2C8 but prior glucuronidation of desloratadine by UDP-glucuronosyltransferase 2B10 is an obligatory requirement. *Drug Metab Dispos*. 2015a;43:523–533.

Kazmi F, Smith B, Hvenegaard MG, et al. Identification of a novel carbamoyl glucuronide as a metabolism-dependent inhibitor of CYP2C8. *Drug Metab Rev*. 2010;42:A105.

Kazmi F, Yerino P, Barbara JE, Parkinson A. Further characterization of the metabolism of desloratadine and its cytochrome P450 and UDP-glucuronosyltransferase inhibition potential: identification of desloratadine as a relatively selective UGT2B10 inhibitor. *Drug Metab Dispos*. 2015b;43:1294–1302.

Kelly EJ, Nakano M, Rohatgi P, Yarov-Yarovoy V, Rettie AE. Finding homes for orphan cytochrome P450s: CYP4V2 and CYP4F22 in disease states. *Mol Interv*. 2011;11:124–132.

Kiang TK, Ensom MH, Chang TK. UDP-glucuronosyltransferases and clinical drug–drug interactions. *Pharmacol Ther*. 2005;106:97–132.

Kim HR, Lee GH, Cho EY, et al. Bax inhibitor 1 regulates ER-stress-induced ROS accumulation through the regulation of cytochrome P450 2E1. *J Cell Sci*. 2009;122:1126–1133.

Kim K-A, Kim M-J, Park J-Y, et al. Stereoselective metabolism of lansoprazole by human liver cytochrome P450 enzymes., *Drug Metab Dispos*. 2003;31:1227–1234.

Kim MS, Shen Z, Kochansky C, et al. Differences in the metabolism and pharmacokinetics of two structurally similar PPAR agonists in dogs: involvement of taurine conjugation. *Xenobiotica*. 2004;34:665–674.

Kim SH, Henry EC, Kim DK, et al. Novel compound 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-*O*-tolylazo-phenyl)-amide (CH-223191) prevents 2,3,7,8-TCDD-induced toxicity by antagonizing the aryl hydrocarbon receptor. *Mol Pharmacol*. 2006;69:1871–1878.

Kim SJ, Yoshikado T, Ieiri I, et al. Clarification of the mechanism of clopidogrel-mediated drug–drug interaction in a clinical cassette small-dose study and its prediction based on in vitro information. *Drug Metab Dispos*. 2016;44:1622–1632.

Kimura M, Higuchi S. Genetics of alcohol dependence. *Psychiatry Clin Neurosci*. 2011;65: 213–225.

Kinobe RT, Parkinson OT, Mitchell DJ, Gillam EMJ. P450 2C18 catalyzes the metabolic bioactivation of phenytoin. *Chem Res Toxicol*. 2005;18:1868–1875.

Kirby BJ, Unadkat JD. Impact of ignoring extraction ratio when predicting drug–drug interactions, fraction metabolized, and intestinal first-pass contribution. *Drug Metab Dispos*. 2010;38:1926–1933.

Kirkman SK, Zhang M-Y, Horwatt PM, Scatina J. Isolation and identification of bromfenac glucoside from rat bile., *Drug Metab Dispos*. 1998;26:720–723.

Kitada M, Kamataki T, Itahashi K, Rikihisa T, Kanakubo Y. P-450 HFLa, a form of cytochrome P-450 purified from human fetal livers, is the 16 alpha-hydroxylase of dehydroepiandrosterone 3-sulfate. *J Biol Chem*. 1987;262:13534–13537.

Klaassen CD, Aleksunes LM. Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol Rev*. 2010;62:1–96.

Klaassen CD, Boles JW. Sulfation and sulfotransferases 5: the importance of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in the regulation of sulfation. *FASEB J*. 1997;11:404–418.

Klaassen CD, Cui JY. Review: mechanisms of how the intestinal microbiota alters the effects of drugs and bile acids. *Drug Metab Dispos*. 2015;43:1505–1521.

Klaassen CD, Reisman SA. Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. *Toxicol Appl Pharmacol*. 2010;244:57–65.

Klaunig JE, Babich MA, Baetcke KP, et al. PPARalpha agonist-induced rodent tumors: modes of action and human relevance. *Crit Rev Toxicol*. 2003;33:655–780.

Klein K, Thomas M, Winter S, et al. PPARA: a novel genetic determinant of CYP3A4 in vitro and in vivo. *Clin Pharmacol Ther*. 2012;91:1044–1052.

Knight TR, Choudhuri S, Klaassen CD. Constitutive mRNA expression of various glutathione *S*-transferase isoforms in different tissues of mice. *Toxicol Sci*. 2007;100:513–524.

Knights KM, Sykes MJ, Miners JO. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. *Expert Opin Drug Metab Toxicol*. 2007;3:159–168.

Knockaert L, Descatoire V, Vadrot N, Fromenty B, Robin MA. Mitochondrial CYP2E1 is sufficient to mediate oxidative stress and cytotoxicity induced by ethanol and acetaminophen. *Toxicol In Vitro*. 2011a;25:475–484.

Knockaert L, Fromenty B, Robin MA. Mechanisms of mitochondrial targeting of cytochrome P450 2E1: physiopathological role in liver injury and obesity. *FEBS J*. 2011b;278:4252–4260.

Kobayashi K, Hashimoto M, Honkakoski P, Negishi M. Regulation of gene expression by CAR: an update. *Arch Toxicol*. 2015;89:1045–1055.

Koch RL, Goldman P. The anaerobic metabolism of metronidazole forms *N*-(2-hydroxyethyl)-oxamic acid. *J Pharmacol Exp Ther*. 1979;208:406–410.

Kochansky CJ, Xia Y-Q, Wang S, et al. Species differences in the elimination of a peroxisome proliferator-activated receptor agonist highlighted by oxidative metabolism of its acyl glucuronide. *Drug Metab Dispos*. 2005;33:1894–1904.

Kodama S, Koike C, Negishi M, Yamamoto Y. Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes. *Mol Cell Biol*. 2004;24: 7931–7940.

Kodama S, Negishi M. Phenobarbital confers its diverse effects by activating the orphan nuclear receptor car. *Drug Metab Rev*. 2006;38:75–87.

Kodama S, Negishi M. Sulfotransferase genes: regulation by nuclear receptors in response to xeno/endo-biotics. *Drug Metab Rev*. 2013;45:441–449.

Kodani SD, Hammock BD. The 2014 Bernard B. Brodie award lecture-epoxide hydrolases: drug metabolism to therapeutics for chronic pain. *Drug Metab Dispos*. 2015;43:788–802.

Koivusalo M, Baumann M, Uotila L. Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase. *FEBS Lett*. 1989;257:105–109.

Koren G, Cairns J, Chitayat D, Gaedigk A, Leeder SJ. Pharmacogenetics of morphine poisoning in a breastfed neonate of a codeine-prescribed mother. *Lancet*. 2006;368:704.

Kovalevsky A, Blumenthal DK, Cheng X, Taylor P, Radic Z. Limitations in current acetylcholinesterase structure-based design of oxime antidotes for organophosphate poisoning. *Ann N Y Acad Sci*. 2016;1378:41–49.

Kraemer MJ, Furukawa CT, Koup JR, et al. Altered theophylline clearance during an influenza B outbreak. *Pediatrics*. 1982;69:476–480.

Kroetz DL, Loiseau P, Guyot M, Levy RH. In vivo and in vitro correlation of microsomal epoxide hydrolase inhibition by progabide. *Clin Pharmacol Ther*. 1993;54:485–497.

Krueger S, VanDyke J, Williams D, Hines R. The role of flavin-containing monooxygenase (FMO) in the metabolism of tamoxifen and other tertiary amines. *Drug Metab Rev*. 2006;38:139–147.

Kubo T, Kim SR, Sai K, et al. Functional characterization of three naturally occurring single nucleotide polymorphisms in the CES2 gene encoding carboxylesterase 2 (HCE-2). *Drug Metab Dispos*. 2005;33:1482–1487.

Kudo M, Sasaki T, Ishikawa M, Hirasawa N, Hiratsuka M. Kinetics of 6-thioxanthine metabolism by allelic variants of xanthine oxidase. *Drug Metab Pharmacokinet*. 2010;25:361–366.

Kuhl H, Wiegratz I. Can 19-nortestosterone derivatives be aromatized in the liver of adult humans? Are there clinical implications? *Climacteric*. 2007;10:344–353.

Kuipers F, Bloks VW, Groen AK. Beyond intestinal soap—bile acids in metabolic control. *Nat Rev Endocrinol*. 2014;10:488–498.

Kumar S, Samuel K, Subramanian R, et al. Extrapolation of diclofenac clearance from in vitro microsomal metabolism data: role of acyl glucuronidation and sequential oxidative metabolism of the acyl glucuronide. *J Pharmacol Exp Ther*. 2002;303:969–978.

Kumarakulasingham M, Rooney PH, Dundas SR, et al. Cytochrome p450 profile of colorectal cancer: identification of markers of prognosis. *Clin Cancer Res*. 2005;11:3758–3765.

Kunutsor SK, Bakker SJ, James RW, Dullaart RP. Serum paraoxonase-1 activity and risk of incident cardiovascular disease: The PREVEND study and meta-analysis of prospective population studies. *Atherosclerosis*. 2016;245:143–154.

Kurian JR, Chin NA, Longlais BJ, Hayes KL, Trepanier LA. Reductive detoxification of arylhydroxylamine carcinogens by human NADH cytochrome b_5 reductase and cytochrome b_5. *Chem Res Toxicol*. 2006;19:1366–1373.

Kutty RK, Daniel RF, Ryan DE, Levin W, Maines MD. Rat liver cytochrome P-450b, P-420b, and P-420c are degraded to biliverdin by heme oxygenase. *Arch Biochem Biophys*. 1988;260:638–644.

La Du B. Human serum paraoxonase/arylesterase. In: Kalow W, ed. *Pharmacogenetics of Drug Metabolism*. New York: Pergamon; 1992.

Lafite P, Dijols S, Buisson D, et al. Design and synthesis of selective, high-affinity inhibitors of human cytochrome P450 2J2. *Bioorg Med Chem Lett*. 2006;16:2777–2780.

Lafite P, Dijols S, Zeldin DC, Dansette PM, Mansuy D. Selective, competitive and mechanism-based inhibitors of human cytochrome P450 2J2. *Arch Biochem Biophys*. 2007;464: 155–168.

Lai DY. Rodent carcinogenicity of peroxisome proliferators and issues on human relevance. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2004;22:37–55.

Lai L, Xu Z, Zhou J, Lee KD, Amidon GL. Molecular basis of prodrug activation by human valacyclovirase, an alpha-amino acid ester hydrolase. *J Biol Chem*. 2008;283:9318–9327.

Lai WG, Farah N, Moniz GA, Wong YN. A Baeyer-Villiger oxidation specifically catalyzed by human flavin-containing monooxygenase 5′. *Drug Metab Dispos*. 2011;39:61–70.

Lai XS, Yang LP, Li XT, et al. Human CYP2C8: structure, substrate specificity, inhibitor selectivity, inducers and polymorphisms. *Curr Drug Metab*. 2009;10:1009–1047.

Lake BG. Species differences in the hepatic effects of inducers of CYP2B and CYP4A subfamily forms: relationship to rodent liver tumour formation. *Xenobiotica*. 2009;39:582–596.

Lake BG, Price RJ, Osimitz TG. Mode of action analysis for pesticide-induced rodent liver tumours involving activation of the constitutive androstane receptor: relevance to human cancer risk. *Pest Manag Sci*. 2015;71:829–834.

Lake BG, Price RJ, Walters DG, et al. Studies on the metabolism of the thiofurans furfuryl mercaptan and 2-methyl-3-furanthiol in rat liver. *Food Chem Toxicol*. 2003;41:1761–1770.

Lammert C, Bjornsson E, Niklasson A, Chalasani N. Oral medications with significant hepatic metabolism at higher risk for hepatic adverse events. *Hepatology*. 2010;51:615–620.

Lammert C, Einarsson S, Saha C, et al. Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals. *Hepatology*. 2008;47:2003–2009.

Lappin GJ, Hardwick TD, Stow R, Pigott GH, Van Ravenzwaay B. Absorption, metabolism and excretion of 4-chloro-2-methylphenoxyacetic acid (MCPA) in rat and dog. *Xenobiotica*. 2002;32:153–163.

Largeron M. Amine oxidases of the quinoproteins family: their implication in the metabolic oxidation of xenobiotics. *Ann Pharm Fr*. 2011;69:53–61.

Lasser KE, Allen PD, Woolhandler SJ, et al. Timing of new black box warnings and withdrawals for prescription medications. *JAMA*. 2002;287:2215–2220.

Lawton MP, Cashman JR, Cresteil T, et al. A nomenclature for the mammalian flavin-containing monooxygenase gene family based on amino acid sequence identities. *Arch Biochem Biophys*. 1994;308:254–257.

Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA*. 1998;279:1200–1205.

Lecoeur S, Bonierbale E, Challine D, et al. Specificity of in vitro covalent binding of tienilic acid metabolites to human liver microsomes in relationship to the type of hepatotoxicity: comparison with two directly hepatotoxic drugs. *Chem Res Toxicol*. 1994;7:434–442.

Lee CA, Jones JP III, Katayama J, et al. Identifying a selective substrate and inhibitor pair for the evaluation of CYP2J2 activity. *Drug Metab Dispos*. 2012;40:943–951.

Lee CA, Neul D, Clouser-Roche A, et al. Identification of novel substrates for human cytochrome P450 2J2. *Drug Metab Dispos*. 2010a;38:347–356.

Lee CM, Chen SY, Lee YC, Huang CY, Chen YM. Benzo[*a*]pyrene and glycine *N*-methyltransferse interactions: gene expression profiles of the liver detoxification pathway. *Toxicol Appl Pharmacol*. 2006;214:126–135.

Lee GH, Kim HK, Chae SW, et al. Bax inhibitor-1 regulates endoplasmic reticulum stress-associated reactive oxygen species and heme oxygenase-1 expression. *J Biol Chem*. 2007;282:21618–21628.

Lee JM, Johnson JA. An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J Biochem Mol Biol*. 2004;37:139–143.

Lee SS, Jung HJ, Park JS, et al. Identification of a null allele of cytochrome P450 3A7: CYP3A7 polymorphism in a Korean population. *Mol Biol Rep*. 2010b;37:213–217.

Lefevre C, Bouadjar B, Ferrand V, et al. Mutations in a new cytochrome P450 gene in lamellar ichthyosis type 3. *Hum Mol Genet*. 2006;15:767–776.

Lehman-McKeeman LD, Caudill D, Vassallo JD, et al. Effects of musk xylene and musk ketone on rat hepatic cytochrome P450 enzymes. *Toxicology Letters*. 1999;111:105–115.

Levano S, Keller D, Schobinger E, Urwyler A, Girard T. Rapid and accurate detection of atypical and Kalow variants in the butyrylcholinesterase gene using denaturing high performance liquid chromatography. *Anesth Analg*. 2008;106:147–151, table of contents.

Levin W, Thomas PE, Oldfield N, Ryan DE. *N*-demethylation of *N*-nitrosodimethylamine catalyzed by purified rat hepatic microsomal cytochrome P-450: isozyme specificity and role of cytochrome b5′. *Arch Biochem Biophys*. 1986;248:158–165.

Levitt MD. Review article: lack of clinical significance of the interaction between H2-receptor antagonists and ethanol. *Aliment Pharmacol Ther*. 1993;7:131–138.

Levoin N, Blondeau C, Guillaume C, et al. Elucidation of the mechanism of inhibition of cyclooxygenases by acyl-coenzyme A and acylglucuronic conjugates of ketoprofen. *Biochem Pharmacol*. 2004;68:1957–1969.

Li A, Jiao X, Munier FL, et al. Bietti crystalline corneoretinal dystrophy is caused by mutations in the novel gene CYP4V2. *Am J Hum Genet*. 2004;74:817–826.

Li AP, Hartman NR, Lu C, Collins JM, Strong JM. Effects of cytochrome P450 inducers on 17a ethinyloestradiol (EE2) conjugation by primary human hepatocytes. *Br J Clin Pharmacol*. 1999;48:733–742.

Li B, Sedlacek M, Manoharan I, et al. Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem Pharmacol*. 2005;70:1673–1684.

Li C, Wu W, Cho KB, Shaik S. Oxidation of tertiary amines by cytochrome p450-kinetic isotope effect as a spin-state reactivity probe. *Chemistry*. 2009a;15:8492–8503.

Li D, Ma S, Ellis EM. Nrf2-mediated adaptive response to methyl glyoxal in HepG2 cells involves the induction of AKR7A2. *Chem Biol Interact*. 2015;234:366–371.

Li DN, Seidel A, Pritchard MP, Wolf CR, Friedberg T. Polymorphisms in P450 CYP1B1 affect the conversion of estradiol to the potentially carcinogenic metabolite 4-hydroxyestradiol. *Pharmacogenetics*. 2000;10:343–353.

Li H, Gu S, Cai X, et al. Ethnic related selection for an ADH Class I variant within East Asia. *PLoS One*. 2008;3:e1881.

Li L, Wei Y, Van Winkle L, et al. Generation and characterization of a Cyp2f2-null mouse and studies on the role of CYP2F2 in naphthalene-induced toxicity in the lung and nasal olfactory mucosa. *J Pharmacol Exp Ther*. 2011a;339:62–71.

Li TK. Pharmacogenetics of responses to alcohol and genes that influence alcohol drinking. *J Stud Alcohol*. 2000;61:5–12.
Li X, He Y, Ruiz CH, et al. Characterization of dasatinib and its structural analogs as CYP3A4 mechanism-based inactivators and the proposed bioactivation pathways. *Drug Metab Dispos*. 2009b;37:1242–1250.
Li X, Hu Z, Qu X,et al. Putative EPHX1 enzyme activity is related with risk of lung and upper aerodigestive tract cancers: a comprehensive meta-analysis. *PLoS One*. 2011b;6:e14749.
Li XQ, Hayes MA, Gronberg G, et al. Discovery of a novel microsomal epoxide hydrolase-catalyzed hydration of a spiro oxetane. *Drug Metab Dispos*. 2016;44:1341–1348.
Li Y, Yokoi T, Katsuki M, et al. In vivo activation of aflatoxin B1 in C57BL/6N mice carrying a human fetus-specific CYP3A7 gene. *Cancer Res*. 1997;57:641–645.
Libiad M, Sriraman A, Banerjee R. Polymorphic variants of human rhodanese exhibit differences in thermal stability and sulfur transfer kinetics. *J Biol Chem*. 2015;290:23579–23588.
Lieber CS. Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968–1998)—a review. *Alcohol Clin Exp Res*. 1999;23:991–1007.
Lieber CS. The discovery of the microsomal ethanol oxidizing system. *Drug Metab Rev*. 2004;36:511–529.
Liederer BM, Borchardt RT. Enzymes involved in the bioconversion of ester-based prodrugs. *J Pharm Sci*. 2006;95:1177–1195.
Lillibridge J, Kalhorn T, Slattery J. Metabolism of lisofylline and pentoxifylline in human liver microsomes and cytosol. *Drug Metab Dispos*. 1996a;24:1174–1179.
Lin YS, Dowling ALS, Quigley SD, et al. Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. *Mol Pharmacol*. 2002;62:162–172.
Lindberg RLP, Negishi M. Alteration of mouse cytochrome P450coh substrate specificity by mutation of a single amino-acid residue. *Nature*. 1989;339:632–634.
Lindsay J, Wang LL, Li Y, Zhou SF. Structure, function and polymorphism of human cytosolic sulfotransferases. *Curr Drug Metab*. 2008;9:99–105.
Link M, Hakala KS, Wsol V, Kostiainen R, Ketola RA. Metabolite profile of sibutramine in human urine: a liquid chromatography-electrospray ionization mass spectrometric study. *J Mass Spectrom*. 2006;41:1171–1178.
Liston HL, DeVane CL, Boulton DW, et al. Differential time course of cytochrome P450 2D6 enzyme inhibition by fluoxetine, sertraline, and paroxetine in healthy volunteers. *J Clin Psychopharmacol*. 2002;22:169–173.
Littler DR, Brown LJ, Breit SN, Perrakis A, Curmi PM. Structure of human CLIC3 at 2 A resolution. *Proteins*. 2010;78:1594–1600.
Liu C, Chen Z, Zhong K, et al. Human liver cytochrome P450 enzymes and microsomal thiol methyltransferase are involved in the stereoselective formation and methylation of the pharmacologically active metabolite of clopidogrel. *Drug Metab Dispos*. 2015a;43:1632–1641.
Liu C, Yang W, Pei D, et al. Genomewide approach validates thiopurine methyltransferase activity is a monogenic pharmacogenomic trait. *Clin Pharmacol Ther*. 2017a;101:373–381.
Liu K-H, Kim M-G, Lee D-J, et al. Characterization of ebastine, hydroxyebastine, and carebastine metabolism by human liver microsomes and expressed cytochrome P450 enzymes: major roles for CYP2J2 and CYP3A. *Drug Metab Dispos*. 2006a;34:1793–1797.
Liu S, Shi X, Tian X, et al. Effect of CYP3A4 *1G and CYP3A5 *3 polymorphisms on pharmacokinetics and pharmacodynamics of ticagrelor in healthy Chinese subjects. *Front Pharmacol*. 2017b;8:176.
Liu T, David SP, Tyndale RF, et al. Associations of CYP2A6 genotype with smoking behaviors in southern China. *Addiction*. 2011;106:985–994.
Liu YP, Xu HQ, Li M, et al. Association between thiopurine *S*-methyltransferase polymorphisms and azathioprine-induced adverse drug reactions in patients with autoimmune diseases: a meta-analysis. *PLoS One*. 2015b;10:e0144234.
Liu Y-J, Huang P-L, Chang Y-F, et al. GSTP1 genetic polymorphism is associated with a higher risk of DNA damage in pesticide-exposed fruit growers. *Cancer Epidemiol Biomarkers Prev*. 2006b;15:659–666.
Liu ZC, Uetrecht JP. Metabolism of ticlopidine by activated neutrophils: implications for ticlopidine-induced agranulocytosis. *Drug Metab Dispos*. 2000;28:726–730.
Li-Wan-Po A, Girard T, Farndon P, Cooley C, Lithgow J. Pharmacogenetics of CYP2C19: functional and clinical implications of a new variant CYP2C19*17. *Br J Clin Pharmacol*. 2010;69:222–230.
Lockhart CM, Nakano M, Rettie AE, Kelly EJ. Generation and characterization of a murine model of Bietti crystalline dystrophy. *Invest Ophthalmol Vis Sci*. 2014;55:5572–5581.
Lockley DJ, Howes D, Williams FM. Cutaneous metabolism of glycol ethers. *Arch Toxicol*. 2005;79:160–168.
Lockridge O. Genetic variants of human serum butyrylcholinesterase influence the metabolism of the muscle relaxant succinylcholine. In: Kalow W, ed. *Pharmacogenetics of Drug Metabolism*. New York: Pergamon; 1992.
Lohle M, Reichmann H. Controversies in neurology: why monoamine oxidase B inhibitors could be a good choice for the initial treatment of Parkinson's disease. *BMC Neurol*. 2011;11:112.
Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. *Cell*. 2006;125:411–414.
Long RM, Rickert DE. Metabolism and excretion of 2,6-dinitro [14C] toluene in vivo and in isolated perfused rat livers. *Drug Metab Dispos*. 1982;10:455–458.
Lu H, Cui W, Klaassen CD. Nrf2 protects against 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced oxidative injury and steatohepatitis. *Toxicol Appl Pharmacol*. 2011;256:122–135.
Lu Y, Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. *Free Radic Biol Med*. 2008;44:723–738.
Lunam CA, Hall PM, Cousins MJ. The pathology of halothane hepatotoxicity in a guinea-pig model: a comparison with human halothane hepatitis. *Br J Exp Pathol*. 1989;70:533–541.
Lundy PM, Hamilton MG, Sawyer TW, Mikler J. Comparative protective effects of HI-6 and MMB-4 against organophosphorous nerve agent poisoning. *Toxicology*. 2011;285:90–96.
Lurie Y, Loebstein R, Kurnik D, Almog S, Halkin H. Warfarin and vitamin K intake in the era of pharmacogenetics. *Br J Clin Pharmacol*. 2010;70:164–170.
Ma C, Gu L, Yang M, et al. rs1495741 as a tag single nucleotide polymorphism of *N*-acetyltransferase 2 acetylator phenotype associates bladder cancer risk and interacts with smoking: a systematic review and meta-analysis. *Medicine (Baltimore)*. 2016;95:e4417.
Ma Q, Lu AY. Origins of individual variability in P4501A induction. *Chem Res Toxicol*. 2003;16: 249–260.
Ma Q, Lu AY. CYP1A induction and human risk assessment: an evolving tale of in vitro and in vivo studies. *Drug Metab Dispos*. 2007;35:1009–1016.
Ma X, Idle JR, Krausz KW, Gonzalez FJ. Metabolism of melatonin by human cytochromes p450. *Drug Metab Dispos*. 2005;33:489–494.
Mackenzie PI, Walter BK, Burchell B, et al. Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenet Genomics*. 2005;15:677–685.
Madan A, Graham RA, Carroll KM, et al. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab Dispos*. 2003;31:421–431.
Madan A, Williams TD, Faiman MD. Glutathione- and glutathione-*S*-transferase-dependent oxidative desulfuration of the thione xenobiotic diethyldithiocarbamate methyl ester. *Mol Pharmacol*. 1994;46:1217–1225.
Maecker B, Sherr DH, Vonderheide RH, et al. The shared tumor-associated antigen cytochrome P450 1B1 is recognized by specific cytotoxic T cells. *Blood*. 2003;102:3287–3294.
Mahiout S, Linden J, Esteban J, et al. Toxicological characterisation of two novel selective aryl hydrocarbon receptor modulators in Sprague–Dawley rats. *Toxicol Appl Pharmacol*. 2017;326:54–65.
Makia NL, Goldstein JA. CYP2C8 is a novel target of peroxisome proliferator-activated receptor alpha in human liver. *Mol Pharmacol*. 2016;89:154–164.
Malatkova P, Maser E, Wsol V. Human carbonyl reductases. *Curr Drug Metab*. 2010;11:639–658.
Mani S, Dou W, Redinbo MR. PXR antagonists and implication in drug metabolism. *Drug Metab Rev*. 2013;45:60–72.
Mannervik B, Awasthi YC, Board PG, et al. Nomenclature for human glutathione transferases. *Biochem J*. 1992;282:305–306.
Mannervik B, Board PG, Hayes JD, Listowsky I, Pearson WR. Nomenclature for mammalian soluble glutathione transferases. *Methods Enzymol*. 2005;401:1–8.

Marchitti SA, Brocker C, Stagos D, Vasiliou V. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. *Expert Opin Drug Metab Toxicol*. 2008;4:697–720.

Marchitti SA, Deitrich RA, Vasiliou V. Neurotoxicity and metabolism of the catecholamine-derived 3,4-dihydroxyphenylacetaldehyde and 3, 4-dihydroxyphenylglycolaldehyde: the role of aldehyde dehydrogenase. *Pharmacol Rev*. 2007;59: 125–150.

Marchitti SA, Orlicky DJ, Brocker C, Vasiliou V. Aldehyde dehydrogenase 3B1 (ALDH3B1): immunohistochemical tissue distribution and cellular-specific localization in normal and cancerous human tissues. *J Histochem Cytochem*. 2010;58:765–783.

Marill J, Capron CC, Idres N, Chabot GG. Human cytochrome P450s involved in the metabolism of 9-*cis*- and 13-*cis*-retinoic acids. *Biochem Pharmacol*. 2002;63:933–943.

Marill J, Cresteil T, Lanotte M, Chabot GG. Identification of human cytochrome P450s involved in the formation of all-trans-retinoic acid principal metabolites. *Mol Pharmacol*. 2000;58:1341–1348.

Martinez Molina D, Eshaghi S, Nordlund P. Catalysis within the lipid bilayer-structure and mechanism of the MAPEG family of integral membrane proteins. *Curr Opin Struct Biol*. 2008;18:442–449.

Marzolini C, Gibbons S, Khoo S, Back D. Cobicistat versus ritonavir boosting and differences in the drug–drug interaction profiles with co-medications. *J Antimicrob Chemother*. 2016;71:1755–1758.

Masson P, Lockridge O. Butyrylcholinesterase for protection from organophosphorus poisons: catalytic complexities and hysteretic behavior. *Arch Biochem Biophys*. 2010;494:107–120.

Masukawa T, Sai M, Tochino Y. Methods for depleting brain glutathione. *Life Sci*. 1989;44:417–424.

Matsunaga T, Shintani S, Hara A. Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. *Drug Metab Pharmacokinet*. 2006;21:1–18.

Mazerska Z, Mroz A, Pawlowska M, Augustin E. The role of glucuronidation in drug resistance. *Pharmacol Ther*. 2016;159:35–55.

McClain RM. The significance of hepatic microsomal enzyme induction and altered thyroid function in rats: implications for thyroid gland neoplasia. *Toxicol Pathol*. 1989;17:294–306.

McCormack M, Alfirevic A, Bourgeois S, et al. HLA-A*3101 and carbamazepine-induced hypersensitivity reactions in Europeans. *N Engl J Med*. 2011;364:1134–1143.

McDonagh EM, Boukouvala S, Aklillu E, et al. PharmGKB summary: very important pharmacogene information for *N*-acetyltransferase 2. *Pharmacogenet Genomics*. 2014;24:409–425.

McDonald MG, Rieder MJ, Nakano M, Hsia CK, Rettie AE. CYP4F2 is a vitamin K1 oxidase: an explanation for altered warfarin dose in carriers of the V433M variant. *Mol Pharmacol*. 2009;75:1337–1346.

McGrath AP, Hilmer KM, Collyer CA, Dooley DM, Guss JM. A new crystal form of human diamine oxidase. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2010;66:137–142.

McIlwain CC, Townsend DM, Tew KD. Glutathione *S*-transferase polymorphisms: cancer incidence and therapy. *Oncogene*. 2006;25:1639–1648.

McLaughlin LA, Ronseaux S, Finn RD, Henderson CJ, Wolf CR. Deletion of microsomal cytochrome b5 profoundly affects hepatic and extrahepatic drug metabolism. *Mol Pharmacol*. 2010;78:269–278.

McLeod HL, Fang L, Luo X, Scott EP, Evans WE. Ethnic differences in erythrocyte catechol-*O*-methyltransferase activity in black and white Americans. *J Pharmacol Exp Ther*. 1994;270:26–29.

Meech R, Mackenzie PI. UGT3A: novel UDP-glycosyltransferases of the UGT superfamily. *Drug Metab Rev*. 2010;42:45–54.

Merali Z, Ross S, Pare G. The pharmacogenetics of carboxylesterases: CES1 and CES2 genetic variants and their clinical effect. *Drug Metabol Drug Interact*. 2014;29:143–151.

Metushi IG, Cai P, Zhu X, Nakagawa T, Uetrecht JP. A fresh look at the mechanism of isoniazid-induced hepatotoxicity. *Clin Pharmacol Ther*. 2011;89: 911–914.

Metushi IG, Sanders C, Lee WM, Uetrecht J; Group Acute Liver Study. Detection of anti-isoniazid and anti-cytochrome P450 antibodies in patients with isoniazid-induced liver failure. *Hepatology*. 2014;59: 1084–1093.

Metushi I, Uetrecht J, Phillips E. Mechanism of isoniazid-induced hepatotoxicity: then and now. *Br J Clin Pharmacol*. 2016;81:1030–1036.

Meulendijks D, Henricks LM, Sonke GS, et al. Clinical relevance of DPYD variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated toxicity: a systematic review and meta-analysis of individual patient data. *Lancet Oncol*. 2015;16:1639–1650.

Meyer SA, Jirtle RL. Old dance with a new partner: EGF receptor as the phenobarbital receptor mediating Cyp2B expression. *Sci Signal*. 2013;6:pe16.

Meyer UA. The molecular basis of genetic polymorphisms of drug metabolism. *J Pharm Pharmacol*. 1994;46:409–415.

Mezrich JD, Fechner JH, Zhang X, et al. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol*. 2010;185:3190–3198.

Miao J, Ling AV, Manthena PV, et al. Flavin-containing monooxygenase 3 as a potential player in diabetes-associated atherosclerosis. *Nat Commun*. 2015;6:6498.

Michaels S, Wang MZ. The revised human liver cytochrome P450 "Pie": absolute protein quantification of CYP4F and CYP3A enzymes using targeted quantitative proteomics. *Drug Metab Dispos*. 2014;42:1241–1251.

Michaud V, Frappier M, Dumas MC, Turgeon J. Metabolic activity and mRNA levels of human cardiac CYP450s involved in drug metabolism. *PLoS One*. 2010;5:e15666.

Michelakis ED, Sutendra G, Dromparis P, et al. Metabolic modulation of glioblastoma with dichloroacetate. *Sci Transl Med*. 2010;2:31ra34.

Mimori Y, Nakamura S, Kameyama M. Regional and subcellular distribution of cyanide metabolizing enzymes in the central nervous system. *J Neurochem*. 1984;43:540–545.

Miners JO, Knights KM, Houston JB, Mackenzie PI. In vitro–in vivo correlation for drugs and other compounds eliminated by glucuronidation in humans: pitfalls and promises. *Biochem Pharmacol*. 2006;71:1531–1539.

Miners JO, Mackenzie PI, Knights KM. The prediction of drug-glucuronidation parameters in humans: UDP-glucuronosyltransferase enzyme-selective substrate and inhibitor probes for reaction phenotyping and in vitro–in vivo extrapolation of drug clearance and drug–drug interaction potential. *Drug Metab Rev*. 2010;42:196–208.

Mirsalis JC, Butterworth BE. Induction of unscheduled DNA synthesis in rat hepatocytes following in vivo treatment with dinitrotoluene. *Carcinogenesis*. 1982;3:241–245.

Mitchell SC. Flavin mono-oxygenase (FMO)—the 'other' oxidase. *Curr Drug Metab*. 2008;9:280–284.

Mitchell SC. Xenobiotic-urea conjugates: chemical or biological? *Xenobiotica*. 2014;44:1055–1066.

Mitchell S, Steventon G, Waring R. Drug peptide conjugates in human urine? *Xenobiotica*. 2014;44:89–93.

Mitoma C, Posner HS, Leonard F. Aromatization of hexahydrobenzoic acid by mammalian liver mitochondria. *Biochim Biophys Acta*. 1958;27:156–160.

Miura M, Satoh S, Kagaya H, et al. Impact of the CYP3A4*1G polymorphism and its combination with CYP3A5 genotypes on tacrolimus pharmacokinetics in renal transplant patients. *Pharmacogenomics*. 2011;12:977–984.

Miura M, Satoh S, Tada H, Habuchi T, Suzuki T. Stereoselective metabolism of rabeprazole-thioether to rabeprazole by human liver microsomes. *Eur J Clin Pharmacol*. 2006;62:113–117.

Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML, Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res*. 1999;59:4356–4362.

Monks TJ, Anders MW, Dekant W, et al. Glutathione conjugate mediated toxicities. *Toxicol Appl Pharmacol*. 1990;106:1–19.

Moore RA, Derry S, Wiffen PJ, Straube S. Effects of food on pharmacokinetics of immediate release oral formulations of aspirin, dipyrone, paracetamol and NSAIDs—a systematic review. *Br J Clin Pharmacol*. 2015;80:381–388.

Moore TJ, Cohen MR, Furberg CD. Serious adverse drug events reported to the Food and Drug Administration, 1998–2005. *Arch Intern Med*. 2007;167:1752–1759.

Morar N, Bowcock AM, Harper JI, Cookson WO, Moffatt MF. Investigation of the chromosome 17q25 PSORS2 locus in atopic dermatitis. *J Invest Dermatol*. 2006;126:603–606.

Mordente A, Meucci E, Martorana GE, Giardina B, Minotti G. Human heart cytosolic reductases and anthracycline cardiotoxicity. *IUBMB Life*. 2001;52:83–88.

Moreau A, Vilarem MJ, Maurel P, Pascussi JM. Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. *Mol Pharm*. 2008;5:35–41.

Morel F, Aninat C. The glutathione transferase kappa family. *Drug Metab Rev*. 2011;43:281–291.

Morgan ET. Impact of infectious and inflammatory disease on cytochrome P450-mediated drug metabolism and pharmacokinetics. *Clin Pharmacol Ther*. 2009;85:434–438.

Morgan ET, Goralski KB, Piquette-Miller M, et al. Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos*. 2008;36:205–216.

Morgenstern R, Zhang J, Johansson K. Microsomal glutathione transferase 1: mechanism and functional roles. *Drug Metab Rev*. 2011;43: 300–306.

Morisseau C, Hammock BD. Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. *Annu Rev Pharmacol Toxicol*. 2005;45:311–333.

Morton NM, Beltram J, Carter RN, et al. Genetic identification of thiosulfate sulfurtransferase as an adipocyte-expressed antidiabetic target in mice selected for leanness. *Nat Med*. 2016;22:771–779.

Mukherjee B, Salavaggione OE, Pelleymounter LL, et al. Glutathione *S*-transferase omega 1 and omega 2 pharmacogenomics. *Drug Metab Dispos*. 2006;34:1237–1246.

Mukonzo JK, Bisaso RK, Ogwal-Okeng J, et al. CYP2B6 genotype-based efavirenz dose recommendations during rifampicin-based antituberculosis cotreatment for a sub-Saharan Africa population. *Pharmacogenomics*. 2016;17: 603–613.

Mukonzo JK, Nanzigu S, Waako P, et al. CYP2B6 genotype, but not rifampicin-based anti-TB cotreatments, explains variability in long-term efavirenz plasma exposure. *Pharmacogenomics*. 2014;15:1423–1435.

Mulder GJ. *Sulfation of Drugs and Related Compounds*. Boca Raton, FL: CRC; 1981.

Muller T. Catechol-*O*-methyltransferase inhibitors in Parkinson's disease. *Drugs*. 2015;75:157–174.

Murai T, Iwabuchi H, Ikeda T. Repeated glucuronidation at one hydroxyl group leads to structurally novel diglucuronides of steroid sex hormones. *Drug Metab Pharmacokinet*. 2005;20:282–293.

Murai T, Samata N, Iwabuchi H, Ikeda T. Human UDP-glucuronosyltransferase, UGT1A8, glucuronidates dihydrotestosterone to a monoglucuronide and further to a structurally novel diglucuronide. *Drug Metab Dispos*. 2006;34:1102–1108.

Murray IA, Krishnegowda G, DiNatale BC, et al. Development of a selective modulator of aryl hydrocarbon (Ah) receptor activity that exhibits anti-inflammatory properties. *Chem Res Toxicol*. 2010;23:955–966.

Mutlib AE, Chen H, Nemeth G, Gan LS, Christ DD. Liquid chromatography/mass spectrometry and high-field nuclear magnetic resonance characterization of novel mixed diconjugates of the non-nucleoside human immunodeficiency virus-1 reverse transcriptase inhibitor, efavirenz. *Drug Metab Dispos*. 1999;27:1045–1056.

Mutlib AE, Gerson RJ, Meunier PC, et al. The species-dependent metabolism of efavirenz produces a nephrotoxic glutathione conjugate in rats. *Toxicol Appl Pharmacol*. 2000;169:102–113.

Mutoh S, Osabe M, Inoue K, et al. Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3). *J Biol Chem*. 2009;284:34785–34792.

Mutoh S, Sobhany M, Moore R, et al. Phenobarbital indirectly activates the constitutive active androstane receptor (CAR) by inhibition of epidermal growth factor receptor signaling. *Sci Signal*. 2013;6:ra31.

Naesens M, Kuypers DR, Streit F, et al. Rifampin induces alterations in mycophenolic acid glucuronidation and elimination: implications for drug exposure in renal allograft recipients. *Clin Pharmacol Ther*. 2006;80:509–521.

Nagahara N, Nagano M, Ito T, Suzuki H. Redox regulation of mammalian 3-mercaptopyruvate sulfurtransferase. *Methods Enzymol*. 2015;554:229–254.

Nagar S, Remmel RP. Uridine diphosphoglucuronosyltransferase pharmacogenetics and cancer. *Oncogene*. 2006;25:1659–1672.

Nagar S, Blanchard R. Pharmacogenetics of uridine diphosphoglucuronosyltransferase (UGT) 1A family members and its role in patient response to irinotecan. *Drug Metab Rev*. 2006;38:393–409.

Nakajima M, Itoh M, Sakai H, et al. CYP2A13 expressed in human bladder metabolically activates 4-aminobiphenyl. *Int J Cancer*. 2006;119:2520–2526.

Nakajima T. Roles of sulfur metabolism and rhodanese in detoxification and anti-oxidative stress functions in the liver: responses to radiation exposure. *Med Sci Monit*. 2015;21:1721–1725.

Nakano H, Ogura K, Takahashi E, et al. Regioselective monosulfation and disulfation of the phytoestrogens daidzein and genistein by human liver sulfotransferases. *Drug Metab Pharmacokinet*. 2004;19:216–226.

Nakano K, Ohashi M, Harigaya S. The beta-glucosidation and beta-glucuronidation of pantothenic acid compared with *p*-nitrophenol in dog liver microsome. *Chem Pharm Bull (Tokyo)*. 1986;34:3949–3952.

Nakayama S, Atsumi R, Takakusa H, et al. A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab Dispos*. 2009;37:1970–1977.

Nakura H, Itoh S, Kusano H, et al. Evidence for the lack of hepatic *N*-acetyltransferase in suncus (*Suncus murinus*). *Biochem Pharmacol*. 1995;50:1165–1170.

NCBI. Available at: http://www.ncbi.nlm.nih.gov/projects/SNP. 2018.

Nebert DW, Shi Z, Galvez-Peralta M, Uno S, Dragin N. Oral benzo[*a*] pyrene: understanding pharmacokinetics, detoxication, and consequences—Cyp1 knockout mouse lines as a paradigm. *Mol Pharmacol*. 2013;84:304–313.

Nebert DW, Dalton TP. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nat Rev Cancer*. 2006;6:947–960.

Nebert DW, Dalton TP, Okey AB, Gonzalez FJ. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem*. 2004;279:23847–23850.

Negishi M. Phenobarbital meets phosphorylation of nuclear receptors. *Drug Metab Dispos*. 2017;45:532–539.

Negishi T, Kato Y, Ooneda O, et al. Effects of aryl hydrocarbon receptor signaling on the modulation of TH1/TH2 balance. *J Immunol*. 2005;175:7348–7356.

Nelson DR. Available at: http://drnelson.uthsc.edu/CytochromeP450.html. 2009a.

Nelson DR. The cytochrome p450 homepage. *Hum Genomics*. 2009b;4:59–65.

Neupert W, Brugger R, Euchenhofer C, Brune K, Geisslinger G. Effects of ibuprofen enantiomers and its coenzyme A thioesters on human prostaglandin endoperoxide synthases. *Br J Pharmacol*. 1997;122:487–492.

Neve EP, Kofeler H, Hendriks DF, et al. Expression and function of mARC: roles in lipogenesis and metabolic activation of ximelagatran. *PLoS One*. 2015;10:e0138487.

Newcomb M, Chandrasena RE. Highly reactive electrophilic oxidants in cytochrome P450 catalysis. *Biochem Biophys Res Commun*. 2005;338:394–403.

Newton DW, Kluza RB. pK_a values of medicinal compounds in pharmacy practice. *Drug Intell Clin Pharm*. 1978;12:546–554.

Ng DS, Lai TY, Ng TK, Pang CP. Genetics of Bietti crystalline dystrophy. *Asia Pac J Ophthalmol (Phila)*. 2016;5:245–252.

Nguyen T, Rushmore TH, Pickett CB. Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. Analysis of the antioxidant response element and its activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. *J Biol Chem*. 1994;269:13656–13662.

Nigg HN, Knaak JB. Blood cholinesterases as human biomarkers of organophosphorus pesticide exposure. *Rev Environ Contam Toxicol*. 2000;163:29–111.

Niinuma Y, Saito T, Takahashi M, et al. Functional characterization of 32 CYP2C9 allelic variants. *Pharmacogenomics J*. 2014;14:107–114.

Nirogi R, Palacharla RC, Mohammed AR, et al. Evaluation of metabolism dependent inhibition of CYP2B6 mediated bupropion hydroxylation in human liver microsomes by monoamine oxidase inhibitors and prediction of potential as perpetrators of drug interaction. *Chem Biol Interact*. 2015;230:9–20.

Nishida CR, Lee M, de Montellano PR. Efficient hypoxic activation of the anticancer agent AQ4N by CYP2S1 and CYP2W1. *Mol Pharmacol*. 2010;78:497–502.

Nishida CR, Ortiz de Montellano PR. Thermophilic cytochrome P450 enzymes. *Biochem Biophys Res Commun*. 2005;338:437–445.

Nishihara M, Sudo M, Kawaguchi N, et al. An unusual metabolic pathway of sipoglitazar, a novel antidiabetic agent: cytochrome P450-catalyzed oxidation of sipoglitazar acyl glucuronide. *Drug Metab Dispos*. 2012;40:249–258.

Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human ATP-binding cassette and solute carrier transporter superfamilies. *Drug Metab Pharmacokinet*. 2005;20:452–477.

Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human phase I metabolizing enzymes except for cytochrome P450 and phase II metabolizing enzymes. *Drug Metab Pharmacokinet*. 2006;21:357–374.

Nishimura M, Naito S, Yokoi T. Tissue-specific mRNA expression profiles of human nuclear receptor subfamilies. *Drug Metab Pharmacokinet*. 2004;19:135–149.

Nishimura M, Yaguti H, Yoshitsugu H, Naito S, Satoh T. Tissue distribution of mRNA expression of human cytochrome P450 isoforms assessed by high-sensitivity real-time reverse transcription PCR. *Yakugaku Zasshi*. 2003;123:369–375.

Nowell S, Falany CN. Pharmacogenetics of human cytosolic sulfotransferases. *Oncogene*. 2006;25:1673–1678.

O'Brien PJ. Peroxidases. *Chem Biol Interact*. 2000;129:113–139.

O'Byrne J, Hunt MC, Rai DK, Saeki M, Alexson SE. The human bile acid-CoA:amino acid *N*-acyltransferase functions in the conjugation of fatty acids to glycine. *J Biol Chem*. 2003;278: 34237–34244.

Oakley A. Glutathione transferases: a structural perspective. *Drug Metab Rev*. 2011;43:138–151.

Obach RS. Potent inhibition of human liver aldehyde oxidase by raloxifene. *Drug Metab Dispos*. 2004;32:89–97.

Obach RS, Cox LM, Tremaine LM. Sertraline is metabolized by multiple cytochrome P450 enzymes, monoamine oxidases, and glucuronyl transferases in human: an in vitro study. *Drug Metab Dispos*. 2005;33:262–270.

Obach RS, Kalgutkar AS, Soglia JR, Zhao SX. Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *Chem Res Toxicol*. 2008;21:1814–1822.

Obach RS, Reed-Hagen AE, Krueger SS, et al. Metabolism and disposition of varenicline, a selective alpha4beta2 acetylcholine receptor partial agonist, in vivo and in vitro. *Drug Metab Dispos*. 2006;34:121–130.

Obach RS, Ryder TF. Metabolism of ramelteon in human liver microsomes and correlation with the effect of fluvoxamine on ramelteon pharmacokinetics. *Drug Metab Dispos*. 2010;38:1381–1391.

Obach RS, Walker GS, Brodney MA. Biosynthesis of fluorinated analogs of drugs using human cytochrome P450 enzymes followed by deoxyfluorination and quantitative nuclear magnetic resonance spectroscopy to improve metabolic stability. *Drug Metab Dispos*. 2016;44:634–646.

Oda S, Fukami T, Yokoi T, Nakajima M. A comprehensive review of UDP-glucuronosyltransferase and esterases for drug development. *Drug Metab Pharmacokinet*. 2015;30:30–51.

Ogilvie BW, Parkinson A. Drugs as victims and perpetrators and the pharmacokinetic concept of maximum exposure. In: Lee P, Aizawa W, Gan L, Prakash C, Zhong D, eds. *Handbook of Metabolic Pathways of Xenobiotics*. Chichester, England: Wiley; 2014.

Ogilvie BW, Usuki E, Yerino P, Parkinson A. In vitro approaches for studying the inhibition of drug-metabolizing enzymes and identifying the drug-metabolizing enzymes responsible for the metabolism of drugs (reaction phenotyping) with emphasis on cytochrome P450. In: Rodrigues AD, ed. *Drug–Drug Interactions*. New York: Informa Healthcare USA; 2008.

Ogilvie BW, Yerino P, Kazmi F, et al. The proton pump inhibitor, omeprazole, but not lansoprazole or pantoprazole, is a metabolism-dependent inhibitor of CYP2C19: implications for coadministration with clopidogrel. *Drug Metab Dispos*. 2011;39(11):2020–2033.

Ogilvie BW, Zhang D, Li W, et al. Glucuronidation converts gemfibrozil to a potent, metabolism-dependent inhibitor of CYP2C8: implications for drug–drug interactions. *Drug Metab Dispos*. 2006;34:191–197.

Ogura K, Nishiyama T, Takubo H, et al. Suicidal inactivation of human dihydropyrimidine dehydrogenase by (E)-5-(2-bromovinyl)uracil derived from the antiviral, sorivudine. *Cancer Lett*. 1998;122:107–113.

Oguro A, Imaoka S. Lysophosphatidic acids are new substrates for the phosphatase domain of soluble epoxide hydrolase. *J Lipid Res*. 2012;53(3):505–512.

Oh ET, Park HJ. Implications of NQO1 in cancer therapy. *BMB Rep*. 2015;48:609–617.

Ohmori S, Fujiki N, Nakasa H, et al. Steroid hydroxylation by human fetal CYP3A7 and human NADPH-cytochrome P450 reductase coexpressed in insect cells using baculovirus. *Res Commun Mol Pathol Pharmacol*. 1998;100:15–28.

Ohtsuki S, Schaefer O, Kawakami H, et al. Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. *Drug Metab Dispos*. 2012;40:83–92.

Okamoto K, Matsumoto K, Hille R, et al. The crystal structure of xanthine oxidoreductase during catalysis: implications for reaction mechanism and enzyme inhibition. *Proc Natl Acad Sci U S A*. 2004;101:7931–7936.

Omiecinski CJ, Coslo DM, Chen T, Laurenzana EM, Peffer RC. Multispecies analyses of direct activators of the constitutive androstane receptor. *Toxicol Sci*. 2011a;123:550–562.

Omiecinski CJ, Vanden Heuvel JP, Perdew GH, Peters JM. Xenobiotic metabolism, disposition, and regulation by receptors: from biochemical phenomenon to predictors of major toxicities. *Toxicol Sci*. 2011b;120(suppl 1):S49–S75.

Omura T. Recollection of the early years of the research on cytochrome P450. *Proc Jpn Acad Ser B Phys Biol Sci*. 2011;87:617–640.

Opitz CA, Litzenburger UM, Sahm F, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature*. 2011;478:197–203.

Oppermann UC, Filling C, Jörnvall H. Forms and functions of human SDR enzymes. *Chem Biol Interact*. 2001;130-132:699–705.

Ortiz de Montellano PR. Mechanism and role of covalent heme binding in the CYP4 family of P450 enzymes and the mammalian peroxidases. *Drug Metab Rev*. 2008;40:405–426.

Osabe M, Negishi M. Active ERK1/2 protein interacts with the phosphorylated nuclear constitutive active/androstane receptor (CAR; NR1I3), repressing dephosphorylation and sequestering CAR in the cytoplasm. *J Biol Chem*. 2011;286:35763–35769.

Oshima M, Dinchuk JE, Kargman SL, et al. Suppression of intestinal polyposis in Apc[Delta]716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*. 1996;87:803–809.

Otto TC, Harsch CK, Yeung DT, et al. Dramatic differences in organophosphorus hydrolase activity between human and chimeric recombinant mammalian paraoxonase-1 enzymes. *Biochemistry*. 2009;48:10416–10422.

Pacher P, Nivorozhkin A, Szabo C. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. *Pharmacol Rev*. 2006;58:87–114.

Paine MF, Widmer WW, Hart HL, et al. A furanocoumarin-free grapefruit juice establishes furanocoumarins as the mediators of the grapefruit juice–felodipine interaction. *Am J Clin Nutr*. 2006;83:1097–1105.

Pal D, Mitra AK. MDR- and CYP3A4-mediated drug–herbal interactions. *Life Sci*. 2006;78:2131–2145.

Palm K, Stenberg P, Luthman K, Artursson P. Polar molecular surface properties predict the intestinal absorption of drugs in humans. *Pharm Res*. 1997;14:568–571.

Pang XY, Cheng J, Kim JH, et al. Expression and regulation of human fetal-specific CYP3A7 in mice. *Endocrinology*. 2012;153:1453–1463.

Pare G, Eriksson N, Lehr T, et al. Genetic determinants of dabigatran plasma levels and their relation to bleeding. *Circulation*. 2013;127:1404–1412.

Park BK, Boobis A, Clarke S, et al. Managing the challenge of chemically reactive metabolites in drug development. *Nat Rev Drug Discov*. 2011;10:292–306.

Park JE, Kim KB, Bae SK, Moon BS, Liu KH, Shin JG. Contribution of cytochrome P450 3A4 and 3A5 to the metabolism of atorvastatin. *Xenobiotica*. 2008;38:1240–1251.

Parker RS, Sontag TJ, Swanson JE, McCormick CC. Discovery, characterization, and significance of the cytochrome P450 omega-hydroxylase pathway of vitamin E catabolism. *Ann N Y Acad Sci*. 2004;1031:13–21.
Parkin DP, Vandenplas S, Botha FJ, et al. Trimodality of isoniazid elimination: phenotype and genotype in patients with tuberculosis. *Am J Respir Crit Care Med*. 1997;155:1717–1722.
Parkinson A, Hurwitz A. Omeprazole and the induction of human cytochrome P-450: a response to concerns about potential adverse effects. *Gastroenterology*. 1991;100:1157–1164.
Parkinson A, Kazmi F, Buckley DB, et al. System-dependent outcomes during the evaluation of drug candidates as inhibitors of cytochrome P450 (CYP) and uridine diphosphate glucuronosyltransferase (UGT) enzymes: human hepatocytes versus liver microsomes versus recombinant enzymes. *Drug Metab Pharmacokinet*. 2010;25:16–27.
Parkinson A, Kazmi F, Buckley DB, et al. An evaluation of the dilution method for identifying metabolism-dependent inhibitors of cytochrome p450 enzymes. *Drug Metab Dispos*. 2011;39:1370–1387.
Parkinson A, Leonard N, Draper A, Ogilvie BW. On the mechanism of hepatocarcinogenesis of benzodiazepines: evidence that diazepam and oxazepam are CYP2B inducers in rats, and both CYP2B and CYP4A inducers in mice. *Drug Metab Rev*. 2006;38:235–259.
Parkinson A, Mudra DR, Johnson C, Dwyer A, Carroll KM. The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. *Toxicol Appl Pharmacol*. 2004;199:193–209.
Parkinson OT, Kelly EJ, Bezabih E, Whittington D, Rettie AE. Bioactivation of 4-Ipomeanol by a CYP4B enzyme in bovine lung and inhibition by HET0016. *J Vet Pharmacol Ther*. 2012;35:402–405.
Pascual G, Glass CK. Nuclear receptors versus inflammation: mechanisms of transrepression. *Trends Endocrinol Metab*. 2006;17:321–327.
Patin E, Barreiro LB, Sabeti PC, et al. Deciphering the ancient and complex evolutionary history of human arylamine *N*-acetyltransferase genes. *Am J Hum Genet*. 2006;78:423–436.
Patki KC, von Moltke LL, Greenblatt DJ. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes P450: role of CYP3A4 and CYP3A5. *Drug Metab Dispos*. 2003;31:938–944.
Paulson GD, Caldwell J, Hutson DH, Menn JJ. *Xenobiotic Conjugation Chemistry*. Washington, DC: American Chemical Society; 1986.
Pearce RE, McIntyre CJ, Madan A, et al. Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Arch Biochem Biophys*. 1996a;331:145–169.
Pearce RE, Rodrigues AD, Goldstein JA, Parkinson A. Identification of the human P450 enzymes involved in lansoprazole metabolism. *J Pharmacol Exp Ther*. 1996b;277:805–816.
Penning T. Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr Rev*. 1997;18:281–305.
Penning TM. Human aldo-keto reductases and the metabolic activation of polycyclic aromatic hydrocarbons. *Chem Res Toxicol*. 2014;27:1901–1917.
Penning TM. The aldo-keto reductases (AKRs): overview. *Chem Biol Interact*. 2015;234:236–246.
Penning TM, Drury JE. Human aldo-keto reductases: function, gene regulation, and single nucleotide polymorphisms. *Arch Biochem Biophys*. 2007;464:241–250.
Petriello MC, Hoffman JB, Sunkara M, et al. Dioxin-like pollutants increase hepatic flavin containing monooxygenase (FMO3) expression to promote synthesis of the pro-atherogenic nutrient biomarker trimethylamine *N*-oxide from dietary precursors. *J Nutr Biochem*. 2016;33:145–153.
PharmVar. Available at: https://www.pharmvar.org/. 2018.
Phillips IR, Shephard EA. Flavin-containing monooxygenases: mutations, disease and drug response. *Trends Pharmacol Sci*. 2008;29:294–301.
Phillips IR, Shephard EA. Drug metabolism by flavin-containing monooxygenases of human and mouse. *Expert Opin Drug Metab Toxicol*. 2017;13:167–181.
Phuc NM, Wu Z, O Y, et al. LKY-047: first selective inhibitor of cytochrome P450 2J2. *Drug Metab Dispos*. 2017;45:765–769.
Piacentini S, Polimanti R, Porreca F, et al. GSTT1 and GSTM1 gene polymorphisms in European and African populations. *Mol Biol Rep*. 2011;38:1225–1230.
Picard N, Ratanasavanh D, Premaud A, Le Meur Y, Marquet P. Identification of the UDP-glucuronosyl transferase isoforms involved in mycophenolic acid phase II metabolism. *Drug Metab Dispos*. 2005;33:139–146.
Plaa GL. Chlorinated methanes and liver injury: highlights of the past 50 years. *Annu Rev Pharmacol Toxicol*. 2000;40:43–65.
Pohjanvirta R, Viluksela M, Tuomisto JT, et al. Physicochemical differences in the AH receptors of the most TCDD-susceptible and the most TCDD-resistant rat strains. *Toxicol Appl Pharmacol*. 1999;155:82–95.
Pohl HR, Scinicariello F. The impact of CYP2E1 genetic variability on risk assessment of VOC mixtures. *Regul Toxicol Pharmacol*. 2011;59:364–374.
Pohl LR, Kenna JG, Satoh H, Christ D, Martin JL. Neoantigens associated with halothane hepatitis. *Drug Metab Rev*. 1989;20:203–217.
Poland A, Glover E. Comparison of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a potent inducer of aryl hydrocarbon hydroxylase, with 3-methylcholanthrene. *Mol Pharmacol*. 1974;10:349–359.
Pondugula SR, Pavek P, Mani S. Pregnane X receptor and cancer: context-specificity is key. *Nucl Receptor Res*. 2016;3. pii: 101198.
Pozo OJ, Ibanez M, Sancho JV, et al. Mass spectrometric evaluation of mephedrone in vivo human metabolism: identification of phase I and phase II metabolites, including a novel succinyl conjugate. *Drug Metab Dispos*. 2015;43:248–257.
Price DA, Blagg J, Jones L, Greene N, Wager T. Physicochemical drug properties associated with in vivo toxicological outcomes: a review. *Expert Opin Drug Metab Toxicol*. 2009;5:921–931.
Probst MR, Beer M, Beer D, et al. Human liver arylacetamide deacetylase. Molecular cloning of a novel esterase involved in the metabolic activation of arylamine carcinogens with high sequence similarity to hormone-sensitive lipase. *J Biol Chem*. 1994;269:21650–21656.
Prochaska HJ, Talalay P. Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res*. 1988;48:4776–4782.
Proctor NJ, Tucker GT, Rostami-Hodjegan A. Predicting drug clearance from recombinantly expressed CYPs: intersystem extrapolation factors. *Xenobiotica*. 2004;34:151–178.
Prueksaritanont T, Li C, Tang C, et al. Rifampin induces the in vitro oxidative metabolism, but not the in vivo clearance of diclofenac in rhesus monkeys. *Drug Metab Dispos*. 2006;34(11):1806–1810.
Prueksaritanont T, Richards KM, Qiu Y, et al. Comparative effects of fibrates on drug metabolizing enzymes in human hepatocytes. *Pharm Res*. 2005;22:71–78.
Pryde DC, Dalvie D, Hu Q, et al. Aldehyde oxidase: an enzyme of emerging importance in drug discovery. *J Med Chem*. 2010;53:8441–8460.
Pyper SR, Viswakarma N, Yu S, Reddy JK. PPARalpha: energy combustion, hypolipidemia, inflammation and cancer. *Nucl Recept Signal*. 2010;8:e002.
Qiao E, Ji M, Wu J, et al. Expression of the PXR gene in various types of cancer and drug resistance. *Oncol Lett*. 2013;5:1093–1100.
Qiu XY, Jiao Z, Zhang M, et al. Association of MDR1, CYP3A4*18B, and CYP3A5*3 polymorphisms with cyclosporine pharmacokinetics in Chinese renal transplant recipients. *Eur J Clin Pharmacol*. 2008;64:1069–1084.
Quinn LP, Perren MJ, Brackenborough KT, et al. A beam-walking apparatus to assess behavioural impairments in MPTP-treated mice: pharmacological validation with R-(–)-deprenyl. *J Neurosci Methods*. 2007;164:43–49.
Quintana FJ, Murugaiyan G, Farez MF, et al. An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A*. 2010;107:20768–20773.
Quintana FJ, Sherr DH. Aryl hydrocarbon receptor control of adaptive immunity. *Pharmacol Rev*. 2013;65:1148–1161.
Racha JK, Rettie AE, Kunze KL. Mechanism-based inactivation of human cytochrome P450 1A2 by furafylline: detection of a 1:1 adduct to protein and evidence for the formation of a novel imidazomethide intermediate. *Biochemistry*. 1998;37:7407–7419.
Rachmilewitz D, Karmeli F, Schwartz LW, Simon PL. Effect of aminophenols (5-ASA and 4-ASA) on colonic interleukin-1 generation. *Gut*. 1992;33:929–932.
Radominska-Pandya A, Czernik PJ, Little JM, Battaglia E, Mackenzie PI. Structural and functional studies of UDP-glucuronosyltransferases. *Drug Metab Rev*. 1999;31:817–899.

Raeissi SD, Hidalgo IJ, Segura-Aguilar J, Artursson P. Interplay between CYP3A-mediated metabolism and polarized efflux of terfenadine and its metabolites in intestinal epithelial Caco-2 (TC7) cell monolayers. *Pharm Res*. 1999;16:625–632.

Rajagopalan KV. Xanthine oxidase and aldehyde oxidase. In Jakoby WB, ed. *Enzymatic Basis of Detoxication*. New York: Academic Press; 1980.

Ramchandani VA. Genetic aspects of alcohol metabolism. In: Watson RR, Preedy VR, eds. *Nutrition and Alcohol: Linking Nutrient Interactions and Dietary Intake*. Boca Raton, FL: CRC Press; 2004.

Ramirez J, Kim TW, Liu W, et al. A pharmacogenetic study of aldehyde oxidase I in patients treated with XK469. *Pharmacogenet Genomics*. 2014;24:129–132.

Ramkissoon A, Wells PG. Human prostaglandin H synthase (hPHS)-1 and hPHS-2 in amphetamine analog bioactivation, DNA oxidation, and cytotoxicity. *Toxicol Sci*. 2011;120:154–162.

Ramsay EE, Dilda PJ. Glutathione *S*-conjugates as prodrugs to target drug-resistant tumors. *Front Pharmacol*. 2014;5: 181.

Rannug A, Rannug U, Rosenkranz HS, et al. Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *J Biol Chem*. 1987;262:15422–15427.

Rao SI, Duffel MW. Inhibition of aryl sulfotransferase by carboxylic acids. *Drug Metab Dispos*. 1991;19:543–545.

Rasheva VI, Domingos PM. Cellular responses to endoplasmic reticulum stress and apoptosis. *Apoptosis*. 2009;14:996–1007.

Rasmussen BB, Brix TH, Kyvik KO, Brosen K. The interindividual differences in the 3-demthylation of caffeine alias CYP1A2 is determined by both genetic and environmental factors. *Pharmacogenetics*. 2002;12:473–478.

Rasmussen HB, Madsen MB, Hansen PR; Indices Consortium. Nomenclature for alleles of the human carboxylesterase 1 gene. *Pharmacogenet Genomics*. 2017;27:78–80.

Ratnayake JH, Hanna PE, Anders MW, Duggan DE. Sulfoxide reduction. In vitro reduction of sulindac by rat hepatic cytosolic enzymes. *Drug Metab Dispos*. 1981;9:85–87.

Reed DJ. Cellular defense mechanisms against reactive metabolites. In: Anders MW, ed. *Bioactivation of Foreign Compounds*. Orlando, FL: Academic Press; 1985.

Reed JR, Cawley GF, Backes WL. Inhibition of cytochrome P450 1A2-mediated metabolism and production of reactive oxygen species by heme oxygenase-1 in rat liver microsomes. *Drug Metab Lett*. 2011;5:6–16.

Reese MJ, Wurm RM, Muir KT, et al. An in vitro mechanistic study to elucidate the desipramine/bupropion clinical drug–drug interaction. *Drug Metab Dispos*. 2008;36:1198–1201.

Regan SL, Maggs JL, Hammond TG, et al. Acyl glucuronides: the good, the bad and the ugly. *Biopharm Drug Dispos*. 2010;31:367–395.

Reiss J, Hahnewald R. Molybdenum cofactor deficiency: mutations in GPHN, MOCS1, and MOCS2. *Hum Mutat*. 2010;32:10–18.

Reiss J, Johnson JL. Mutations in the molybdenum cofactor biosynthetic genes *MOCS1*, *MOCS2*, and *GEPH*. *Human Muta*. 2003;21:569–576.

Relling MV, Giacomini KM. Pharmacogenetics. In: Goodman LS, Gilman A, Brunton LL, Lazo JS, Parker KL, eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. New York: McGraw-Hill; 2006.

Rencurel F, Foretz M, Kaufmann MR, et al. Stimulation of AMP-activated protein kinase is essential for the induction of drug metabolizing enzymes by phenobarbital in human and mouse liver. *Mol Pharmacol*. 2006;70:1925–1934.

Rendic S, Guengerich FP. Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem Res Toxicol*. 2015;28:38–42.

Rettie AE, Fisher MB. Transformation enzymes: oxidative; non-P450. In: Woolf TF, ed. *Handbook of Drug Metabolism*. New York: Marcel Dekker; 1999.

Rettie AE, Jones JP. Clinical and toxicological relevance of CYP2C9: drug–drug interactions and pharmacogenetics. *Annu Rev Pharmacol Toxicol*. 2005;45:477–494.

Richardson TA, Klaassen CD. Role of UDP-glucuronosyltransferase (UGT) 2B2 in metabolism of triiodothyronine: effect of microsomal enzyme inducers in Sprague Dawley and UGT2B2-deficient Fischer 344 rats. *Toxicol Sci*. 2010;116:413–421.

Riches Z, Stanley EL, Bloomer JC, Coughtrie MW. Quantitative evaluation of the expression and activity of five major sulfotransferases (SULTs) in human tissues: the SULT "pie." *Drug Metab Dispos*. 2009;37:2255–2261.

Riedmaier S, Klein K, Hofmann U, et al. UDP-glucuronosyltransferase (UGT) polymorphisms affect atorvastatin lactonization in vitro and in vivo. *Clin Pharmacol Ther*. 2010;87:65–73.

Riedmaier S, Klein K, Winter S, et al. Paraoxonase (PON1 and PON3) polymorphisms: impact on liver expression and atorvastatin-lactone hydrolysis. *Front Pharmacol*. 2011;2:41.

Rittle J, Green MT. Cytochrome P450 compound I: capture, characterization, and C–H bond activation kinetics. *Science*. 2010;330:933–937.

Robert J, Le Morvan V, Smith D, Pourquier P, Bonnet J. Predicting drug response and toxicity based on gene polymorphisms. *Crit Rev Oncol Hematol*. 2005;54:171–196.

Roberts AB, Wallace BD, Venkatesh MK, Mani S, Redinbo MR. Molecular insights into microbial beta-glucuronidase inhibition to abrogate CPT-11 toxicity. *Mol Pharmacol*. 2013;84:208–217.

Roberts KM, Jones JP. Anilinic *N*-oxides support cytochrome P450-mediated *N*-dealkylation through hydrogen-atom transfer. *Chemistry*. 2010;16:8096–8107.

Rodrigues AD, Yang Z, Chen C, et al. Is celecoxib an inducer of cytochrome P450 3A4 in subjects carrying the CYP2C9*3 allele? *Clin Pharmacol Ther*. 2006;80:298–301; author reply 01-02.

Rodrigues AD. Comparison of levels of aldehyde oxidase with cytochrome P450 activities in human liver in vitro. *Biochem Pharmacol*. 1994;48:197–200.

Rogers SM, Back DJ, Stevenson PJ, Grimmer SF, Orme ML. Paracetamol interaction with oral contraceptive steroids: increased plasma concentrations of ethinyloestradiol. *Br J Clin Pharmacol*. 1987;23:721–725.

Rosemond MJ, Walsh J. Human carbonyl reduction pathways and a strategy for their study in vitro. *Drug Metab Rev*. 2004;36:335–361.

Rosen LS, Laxa B, Boulos L, et al. Phase 1 study of TLK286 (Telcyta) administered weekly in advanced malignancies. *Clin Cancer Res*. 2004;10:3689–3698.

Ross D. Functions and distribution of NQO1 in human bone marrow: potential clues to benzene toxicity. *Chem Biol Interact*. 2005;153-154:137–146.

Ross MK, Crow JA. Human carboxylesterases and their role in xenobiotic and endobiotic metabolism. *J Biochem Mol Toxicol*. 2007;21:187–196.

Rostami-Hodjegan A, Kroemer HK, Tucker GT. In-vivo indices of enzyme activity: the effect of renal impairment on the assessment of CYP2D6 activity. *Pharmacogenetics*. 1999;9:277–286.

Rostami-Hodjegan A, Lennard MS, Woods HF, Tucker GT. Meta-analysis of studies of the CYP2D6 polymorphism in relation to lung cancer and Parkinson's disease. *Pharmacogenetics*. 1998;8:227–238.

Roth A, Looser R, Kaufmann M, et al. Regulatory cross-talk between drug metabolism and lipid homeostasis: constitutive androstane receptor and pregnane X receptor increase Insig-1 expression. *Mol Pharmacol*. 2008;73:1282–1289.

Roth AD, Lee MY. Idiosyncratic drug-induced liver injury (IDILI): potential mechanisms and predictive assays. *Biomed Res Int*. 2017;2017:9176937.

Roth RA, Maiuri AR, Ganey PE. Idiosyncratic drug-induced liver injury: is drug-cytokine interaction the linchpin? *J Pharmacol Exp Ther*. 2017;360:461–470.

Rothhammer V, Borucki DM, Garcia Sanchez MI, et al. Dynamic regulation of serum aryl hydrocarbon receptor agonists in MS. *Neurol Neuroimmunol Neuroinflamm*. 2017;4:e359.

Rouse M, Singh NP, Nagarkatti PS, Nagarkatti M. Indoles mitigate the development of experimental autoimmune encephalomyelitis by induction of reciprocal differentiation of regulatory T cells and Th17 cells. *Br J Pharmacol*. 2013;169:1305–1321.

Rowland A, Knights KM, Mackenzie PI, Miners JO. Characterization of the binding of drugs to human intestinal fatty acid binding protein (IFABP): potential role of IFABP as an alternative to albumin for in vitro–in vivo extrapolation of drug kinetic parameters. *Drug Metab Dispos*. 2009;37:1395–1403.

Rowland A, Miners JO, Mackenzie PI. The UDP-glucuronosyltransferases: their role in drug metabolism and detoxification. *Int J Biochem Cell Biol*. 2013;45:1121–1132.

Ruell JA, Tsinman KL, Avdeef A. PAMPA—a drug absorption in vitro model. 5. Unstirred water layer in iso-pH mapping assays and pK_a(flux)–optimized design (pOD-PAMPA). *Eur J Pharm Sci*. 2003;20:393–402.

Rumack BH. Acetaminophen misconceptions. *Hepatology*. 2004;40:10–15.

Runge-Morris M, Kocarek TA. Regulation of sulfotransferases by xenobiotic receptors. *Curr Drug Metab*. 2005;6: 299–307.

Runge-Morris M, Kocarek TA, Falany CN. Regulation of the cytosolic sulfotransferases by nuclear receptors. *Drug Metab Rev*. 2013;45:15–33.

Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem*. 1991;266:11632–11639.

Rusyn I, Asakura S, Pachkowski B, et al. Expression of base excision DNA repair genes is a sensitive biomarker for in vivo detection of chemical-induced chronic oxidative stress: identification of the molecular source of radicals responsible for DNA damage by peroxisome proliferators. *Cancer Res*. 2004;64:1050–1057.

Ryan DE, Dixon R, Evans RH, et al. Rat hepatic cytochrome P-450 isozyme specificity for the metabolism of the steroid sulfate, 5 alpha-androstane-3 alpha, 17 beta-diol-3,17-disulfate. *Arch Biochem Biophys*. 1984;233:636–642.

Saarikoski ST, Rivera SP, Hankinson O, Husgafvel-Pursiainen K. CYP2S1: a short review. *Toxicol Appl Pharmacol*. 2005;207:62–69.

Saito K, Moore R, Negishi M. p38 Mitogen-activated protein kinase regulates nuclear receptor CAR that activates the CYP2B6 gene. *Drug Metab Dispos*. 2013;41:1170–1173.

Sakamoto A, Matsumaru T, Ishiguro N, et al. Reliability and robustness of simultaneous absolute quantification of drug transporters, cytochrome P450 enzymes, and UDP-glucuronosyltransferases in human liver tissue by multiplexed MRM/selected reaction monitoring mode tandem mass spectrometry with nano-liquid chromatography. *J Pharm Sci*. 2011;100:4037–4043.

Salman ED, Kadlubar SA, Falany CN. Expression and localization of cytosolic sulfotransferase (SULT) 1A1 and SULT1A3 in normal human brain. *Drug Metab Dispos*. 2009;37:706–709.

Sampayo R, Lavandera JV, Batlle A, Buzaleh AM. Sevoflurane: its action on heme metabolism and Phase I drug metabolizing system. *Cell Mol Biol (Noisy-le-grand)*. 2009;55:140–146.

Sanchez-Covarrubias L, Slosky LM, Thompson BJ, Davis TP, Ronaldson PT. Transporters at CNS barrier sites: obstacles or opportunities for drug delivery? *Curr Pharm Des*. 2014;20:1422–1449.

Sanderson LM, Degenhardt T, Koppen A, et al. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARalpha serves as a plasma free fatty acid sensor in liver. *Mol Cell Biol*. 2009;29:6257–6267.

Sandy J, Mushtaq A, Holton SJ, et al. Investigation of the catalytic triad of arylamine *N*-acetyltransferases: essential residues required for acetyl transfer to arylamines. *Biochem J*. 2005;390:115–123.

Sano M, Ernesto C, Thomas RG, et al. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. *N Engl J Med*. 1997;336:1216–1222.

Saruwatari J, Ishitsu T, Nakagawa K. Update on the genetic polymorphisms of drug-metabolizing enzymes in antiepileptic drug therapy. *Pharmaceuticals*. 2010;3:2709–2732.

Sato Y, Miyashita A, Iwatsubo T, Usui T. Simultaneous absolute protein quantification of carboxylesterases 1 and 2 in human liver tissue fractions using liquid chromatography-tandem mass spectrometry. *Drug Metab Dispos*. 2012;40:1389–1396.

Sato Y, Nagata M, Tetsuka K, et al. Optimized methods for targeted peptide-based quantification of human uridine 5´-diphosphate-glucuronosyltransferases in biological specimens using liquid chromatography-tandem mass spectrometry. *Drug Metab Dispos*. 2014;42:885–889.

Satoh T, Hosokawa M. The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxico*. 1998;38:257–288.

Satoh T, Hosokawa M. Structure, function and regulation of carboxylesterases. *Chem Biol Interact*. 2006;162:195–211.

Sau A, Pellizzari Tregno F, Valentino F, Federici G, Caccuri AM. Glutathione transferases and development of new principles to overcome drug resistance. *Arch Biochem Biophys*. 2010;500:116–122.

Saulter JY, Kurian JR, Trepanier LA, et al. Unusual dehydroxylation of antimicrobial amidoxime prodrugs by cytochrome b5 and NADH cytochrome b5 reductase. *Drug Metab Dispos*. 2005;33:1886–1893.

Saunders MP, Patterson AV, Harris AL, Stratford IJ. NADPH:cytochrome c (P450) reductase activates tirapazamine (SR4233) to restore hypoxic and oxic cytotoxicity in an aerobic resistant derivative of the A549 lung cancer cell line. *Br J Cancer*. 2000;82:651–656.

Savage RE, Tyler AN, Miao XS, Chan TC. Identification of a novel glucosylsulfate conjugate as a metabolite of 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione (ARQ 501, beta-lapachone) in mammals. *Drug Metab Dispos*. 2008;36:753–758.

Scheible H, Kraetzer F, Marx A, Johne A, Wimmer E. Metabolism of the MEK1/2 inhibitor pimasertib involves a novel conjugation with phosphoethanolamine in patients with solid tumors. *Drug Metab Dispos*. 2017;45:174–182.

Schmitt C, Kuhn B, Zhang X, Kivitz AJ, Grange S. Disease-drug–drug interaction involving tocilizumab and simvastatin in patients with rheumatoid arthritis. *Clin Pharmacol Ther*. 2011;89:735–740.

Schnekenburger M, Karius T, Diederich M. Regulation of epigenetic traits of the glutathione *S*-transferase P1 gene: from detoxification toward cancer prevention and diagnosis. *Front Pharmacol*. 2014;5:170.

Schofield PC, Robertson IG, Paxton JW. Inter-species variation in the metabolism and inhibition of *N*-[(2′-dimethylamino)ethyl]acridine-4-carboxamide (DACA) by aldehyde oxidase. *Biochem Pharmacol*. 2000;59:161–165.

Schrag ML, Cui D, Rushmore TH, et al. Sulfotransferase 1E1 is a low K_M isoform mediating the 3-*O*-sulfation of ethinyl estradiol. *Drug Metab Dispos*. 2004;32:1299–1303.

Schrag ML, Wienkers LC. Covalent alteration of the CYP3A4 active site: evidence for multiple substrate binding domains. *Arch Biochem Biophys*. 2001;391:49–55.

Schroth W, Goetz MP, Hamann U, et al. Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. *JAMA*. 2009;302:1429–1436.

Schugar RC, Brown JM. Emerging roles of flavin monooxygenase 3 in cholesterol metabolism and atherosclerosis. *Curr Opin Lipidol*. 2015;26:426–431.

Schuster I. Cytochromes P450 are essential players in the vitamin D signaling system. *Biochim Biophys Acta*. 2011;1814:186–199.

Scott F, Gonzalez Malagon SG, O'Brien BA, et al. Identification of flavin-containing monooxygenase 5 (FMO5) as a regulator of glucose homeostasis and a potential sensor of gut bacteria. *Drug Metab Dispos*. 2017;45(9):982–989.

Scott SA, Sangkuhl K, Gardner EE, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for cytochrome P450-2C19 (CYP2C19) genotype and clopidogrel therapy. *Clin Pharmacol Ther*. 2011;90:328–332.

SDR. Available at: http://www.sdr-enzymes.org. 2018.

Seitz J, Oneta C. Gastrointestinal alchohol dehydrogenase. *Nutr Rev*. 1998;56:52–60.

Senggunprai L, Yoshinari K, Yamazoe Y. Selective role of sulfotransferase 2A1 (SULT2A1) in the *N*-sulfoconjugation of quinolone drugs in humans. *Drug Metab Dispos*. 2009;37:1711–1717.

Seo YS, Keum B, Park S, et al. Gilbert's syndrome phenotypically expressed as Crigler–Najjar syndrome type II. *Scand J Gastroenterol*. 2007;42:540–541.

Seree E, Villard PH, Pascussi JM, et al. Evidence for a new human CYP1A1 regulation pathway involving PPAR-alpha and 2 PPRE sites. *Gastroenterology*. 2004;127:1436–1445.

Seto Y, Guengerich FP. Partitioning between *N*-dealkylation and *N*-oxygenation in the oxidation of *N*,*N*-dialkylarylamines catalyzed by cytochrome P450 2B1. *J Biol Chem*. 1993;268:9986–9997.

Shah YM, Morimura K, Yang Q, et al. Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation. *Mol Cell Biol*. 2007;27: 4238–4247.

Shakhnovich V, Vyhlidal C, Friesen C, et al. Decreased pregnane X receptor expression in children with active Crohn's disease. *Drug Metab Dispos*. 2016;44:1066–1069.

Shayiq RM, Roberts DW, Rothstein K, et al. Repeat exposure to incremental doses of acetaminophen provides protection against acetaminophen-induced lethality in mice: an explanation for high acetaminophen dosage in humans without hepatic injury. *Hepatology*. 1999;29:451–463.

Sheng X, Zhang H, Hollenberg PF, Newcomb M. Kinetic isotope effects in hydroxylation reactions effected by cytochrome P450 compounds I implicate multiple electrophilic oxidants for P450-catalyzed oxidations. *Biochemistry*. 2009;48:1620–1627.

Shephard EA, Chandan P, Stevanovic-Walker M, Edwards M, Phillips IR. Alternative promoters and repetitive DNA elements define the species-dependent tissue-specific expression of the FMO1 genes of human and mouse. *Biochem J*. 2007;406:491–499.

Shi D, Yang J, Yang D, et al. Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel. *J Pharmacol Exp Ther*. 2006;319:1477–1484.

Shield AJ, Murray TP, Cappello JY, Coggan M, Board PG. Polymorphisms in the human glutathione transferase Kappa (GSTK1) promoter alter gene expression. *Genomics*. 2010;95:299–305.

Shih DM, Wang Z, Lee R, et al. Flavin containing monooxygenase 3 exerts broad effects on glucose and lipid metabolism and atherosclerosis. *J Lipid Res*. 2015;56:22–37.

Shih J, Chen K, Tidd M. Monamine oxidase: from genes to behavior. *Annual Rev Neurosci*. 1999;22:197–217.

Shih P-S, Huang J-D. Pharmacokinetics of midazolam and 1′-hydroxymidazolam in Chinese with different CYP3A5 genotypes. *Drug Metab Dispos*. 2002;30:1491–1496.

Shimada T. Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. *Drug Metab Pharmacokinet*. 2006;21:257–276.

Shimizu M, Denton T, Kozono M, et al. Developmental variations in metabolic capacity of flavin-containing mono-oxygenase 3 in childhood. *Br J Clin Pharmacol*. 2011;71:585–591.

Shindo S, Numazawa S, Yoshida T. A physiological role of AMP-activated protein kinase in phenobarbital-mediated constitutive androstane receptor activation and CYP2B induction. *Biochem J*. 2007;401:735–741.

Shipkova M, Armstrong VW, Oellerich M, Wieland E. Acyl glucuronide drug metabolites: toxicological and analytical implications. *Ther Drug Monit*. 2003;25:1–16.

Shirasaka Y, Suzuki K, Nakanishi T, Tamai I. Intestinal absorption of HMG-CoA reductase inhibitor pravastatin mediated by organic anion transporting polypeptide. *Pharm Res*. 2010;27:2141–2149.

Shirasaka Y, Suzuki K, Shichiri M, Nakanishi T, Tamai I. Intestinal absorption of HMG-CoA reductase inhibitor pitavastatin mediated by organic anion transporting polypeptide and P-glycoprotein/multidrug resistance 1. *Drug Metab Pharmacokinet*. 2011;26:171–179.

Shirley MA, Guan X, Kaiser DG, Halstead GW, Baillie TA. Taurine conjugation of ibuprofen in humans and in rat liver in vitro. Relationship to metabolic chiral inversion. *J Pharmacol Exp Ther*. 1994;269:1166–1175.

Shitara Y, Maeda K, Ikejiri K, et al. Clinical significance of organic anion transporting polypeptides (OATPs) in drug disposition: their roles in hepatic clearance and intestinal absorption. *Biopharm Drug Dispos*. 2013;34:45–78.

Shrivas K, Mindaye ST, Getie-Kebtie M, Alterman MA. Mass spectrometry-based proteomic analysis of human liver cytochrome(s) P450. *Toxicol Appl Pharmacol*. 2013;267:125–136.

Sibbing D, Koch W, Gebhard D, et al. Cytochrome 2C19*17 allelic variant, platelet aggregation, bleeding events, and stent thrombosis in clopidogrel-treated patients with coronary stent placement. *Circulation*. 2010;121:512–518.

Siddens LK, Krueger SK, Henderson MC, Williams DE. Mammalian flavin-containing monooxygenase (FMO) as a source of hydrogen peroxide. *Biochem Pharmacol*. 2014;89:141–147.

Siegel D, Yan C, Ross D. NAD(P)H:quinone oxidoreductase 1 (NQO1) in the sensitivity and resistance to antitumor quinones. *Biochemical Pharmacology*. 2012;83(8):1033–1040.

Sifontis NM, Benedetti E, Vasquez EM. Clinically significant drug interaction between basiliximab and tacrolimus in renal transplant recipients. *Transplant Proc*. 2002;34:1730–1732.

Sim E, Walters K, Boukouvala S. Arylamine *N*-acetyltransferases: from structure to function. *Drug Metab Rev*. 2008;40:479–510.

Singh G, Driever PH, Sander JW. Cancer risk in people with epilepsy: the role of antiepileptic drugs. *Brain*. 2005;128:7–17.

Singh J, Petter RC, Baillie TA, Whitty A. The resurgence of covalent drugs. *Nat Rev Drug Discov*. 2011;10:307–317.

Sirot EJ, van der Velden JW, Rentsch K, Eap CB, Baumann P. Therapeutic drug monitoring and pharmacogenetic tests as tools in pharmacovigilance. *Drug Saf*. 2006;29:735–768.

Sit D, Perel JM, Luther JF, et al. Disposition of chiral and racemic fluoxetine and norfluoxetine across childbearing. *J Clin Psychopharmacol*. 2010;30:381–386.

Siu EC, Tyndale RF. Selegiline is a mechanism-based inactivator of CYP2A6 inhibiting nicotine metabolism in humans and mice. *J Pharmacol Exp Ther*. 2008;324:992–999.

Skarydova L, Wsol V. Human microsomal carbonyl reducing enzymes in the metabolism of xenobiotics: well-known and promising members of the SDR superfamily. *Drug Metab Rev*. 2012;44(2):173–191.

Sládek NE. Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. *J Biochem Mol Toxicol*. 2003;17:7–23.

Slitt AL, Cherrington NJ, Maher JM, Klaassen CD. Induction of multidrug resistance protein 3 in rat liver is associated with altered vectorial excretion of acetaminophen metabolites. *Drug Metab Dispos*. 2003;31:1176–1186.

Smirnov A, Comte C, Mager-Heckel AM, et al. Mitochondrial enzyme rhodanese is essential for 5 S ribosomal RNA import into human mitochondria. *J Biol Chem*. 2010;285:30792–30803.

Smith DA, Dalvie D. Why do metabolites circulate? *Xenobiotica*. 2012;42:107–126.

Smith KJ, Murray IA, Tanos R, et al. Identification of a high-affinity ligand that exhibits complete aryl hydrocarbon receptor antagonism. *J Pharmacol Exp Ther*. 2011;338:318–327.

Smith PA, Sorich MJ, Low LS, McKinnon RA, Miners JO. Towards integrated ADME prediction: past, present and future directions for modelling metabolism by UDP-glucuronosyltransferases. *J Mol Graph Model*. 2004;22:507–517.

Soldin OP, Chung SH, Mattison DR. Sex differences in drug disposition. *J Biomed Biotechnol*. 2011;2011:187103.

Song Y, Shan Z, Luo C, et al. Glutathione *S*-transferase T1 (GSTT1) null polymorphism, smoking, and their interaction in coronary heart disease: a comprehensive meta-analysis. *Heart Lung Circ*. 2017;26:362–370.

Sontag TJ, Parker RS. Influence of major structural features of tocopherols and tocotrienols on their omega-oxidation by tocopherol-omega-hydroxylase. *J Lipid Res*. 2007;48:1090–1098.

Sorenson RC, Primo-Parmo SL, Kuo C, et al. Reconsideration of the catalytic center and mechanism of mammalian paraoxonase/arylesterase. *Proc Natl Acad Sci U S A*. 1995;92:7187–7191.

Sorich MJ, Miners JO, McKinnon RA, Smith PA. Multiple pharmacophores for the investigation of human UDP-glucuronosyltransferase isoform substrate selectivity. *Mol Pharmacol*. 2004;65:301–308.

Sorich MJ, Smith PA, Miners JO, Mackenzie PI, McKinnon RA. Recent advances in the in silico modelling of UDP glucuronosyltransferase substrates. *Curr Drug Metab*. 2008;9:60–69.

Soucek P, Skjelbred CF, Svendsen M, et al. Single-track sequencing for genotyping of multiple SNPs in the *N*-acetyltransferase 1 (NAT1) gene. *BMC Biotechnol*. 2004;4:28.

Souto JC, Blanco-Vaca F, Soria JM, et al. A genomewide exploration suggests a new candidate gene at chromosome 11q23 as the major determinant of plasma homocysteine levels: results from the GAIT project. *Am J Hum Genet*. 2005;76:925–933.

Spiecker M, Liao JK. Cytochrome P450 epoxygenase CYP2J2 and the risk of coronary artery disease. *Trends Cardiovasc Med*. 2006;16:204–208.

Spina E, Avenoso A, Campo GM, Caputi AP, Perucca E. Phenobarbital induces the 2-hydroxylation of desipramine. *Ther Drug Monit*. 1996;18:60–64.

Sridar C, Kenaan C, Hollenberg PF. Inhibition of bupropion metabolism by selegiline: mechanism-based inactivation of human CYP2B6 and characterization of glutathione and peptide adducts. *Drug Metab Dispos*. 2012;40:2256–2266.

Staatz CE, Goodman LK, Tett SE. Effect of CYP3A and ABCB1 single nucleotide polymorphisms on the pharmacokinetics and pharmacodynamics of calcineurin inhibitors: Part I. *Clin Pharmacokinet*. 2010;49:141–175.

Stanley LA, Horsburgh BC, Ross J, Scheer N, Wolf CR. PXR and CAR: nuclear receptors which play a pivotal role in drug disposition and chemical toxicity. *Drug Metab Rev*. 2006;38:515–597.

Stark K, Wongsud B, Burman R, Oliw EH. Oxygenation of polyunsaturated long chain fatty acids by recombinant CYP4F8 and CYP4F12 and catalytic importance of Tyr-125 and Gly-328 of CYP4F8. *Arch Biochem Biophys*. 2005;441:174–181.

Stieger B, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology*. 2000;118:422–430.

Stierlin H, Faigle JW. Biotransformation of diclofenac sodium (Voltaren) in animals and in man. II. Quantitative determination of the unchanged drug and principal phenolic metabolites, in urine and bile. *Xenobiotica*. 1979;9:611–621.

Stierlin H, Faigle JW, Sallmann A, et al. Biotransformation of diclofenac sodium (Voltaren) in animals and in man. I. Isolation and identification of principal metabolites. *Xenobiotica*. 1979;9:601–610.

Stone AN, Mackenzie PI, Galetin A, Houston JB, Miners JO. Isoform selectivity and kinetics of morphine 3- and 6-glucuronidation by human UDP-glucuronosyltransferases: evidence for atypical glucuronidation kinetics by UGT2B7. *Drug Metab Dispos*. 2003;31:1086–1089.

Strange RC, Jones PW, Fryer AA. Glutathione *S*-transferase: genetics and role in toxicology. *Toxicology Lett*. 2000;112-113:357–363.

Strehlau J, Pape L, Offner G, Nashan B, Ehrich JH. Interleukin-2 receptor antibody-induced alterations of ciclosporin dose requirements in paediatric transplant recipients. *Lancet*. 2000;356:1327–1328.

Strelevitz TJ, Foti RS, Fisher MB. In vivo use of the P450 inactivator 1-aminobenzotriazole in the rat: varied dosing route to elucidate gut and liver contributions to first-pass and systemic clearance. *J Pharm Sci*. 2006;95:1334–1341.

Stresser DM, Perloff ES, Mason AK, et al. Selective time- and NADPH-dependent inhibition of human CYP2E1 by clomethiazole. *Drug Metab Dispos*. 2016;44:1424–1430.

Strickland KC, Krupenko NI, Dubard ME, et al. Enzymatic properties of ALDH1L2, a mitochondrial 10-formyltetrahydrofolate dehydrogenase. *Chem Biol Interact*. 2011;191:129–136.

Strolin-Benedetti M. FAD-dependent enzymes involved in the metabolic oxidation of xenobiotics. *Ann Pharm Fr*. 2011;69: 45–52.

Strolin Benedetti M, Allievi C, Cocchiara G, Pevarello P, Dostert P. Involvement of FAD-dependent polyamine oxidase in the metabolism of milacemide in the rat. *Xenobiotica*. 1992;22:191–197.

Strolin Benedetti M, Tipton KF, Whomsley R. Amine oxidases and monooxygenases in the in vivo metabolism of xenobiotic amines in humans: has the involvement of amine oxidases been neglected? *Fundam Clin Pharmacol*. 2007;21:467–480.

Subramanyam B, Woolf T, Castagnoli N Jr. Studies on the in vitro conversion of haloperidol to a potentially neurotoxic pyridinium metabolite. *Chem Res Toxicol*. 1991;4:123–128.

Sugiura M, Iwasaki K, Kato R. Reduction of tertiary amine *N*-oxides by liver microsomal cytochrome P-450. *Mol Pharmacol*. 1976;12:322–334.

Sugiura M, Kato R. Reduction of tertiary amine *N*-oxides by rat liver mitochondria. *J Pharmacol Exp Ther*. 1977;200:25–32.

Sun H, Ehlhardt WJ, Kulanthaivel P, et al. Dehydrogenation of indoline by cytochrome P450 enzymes: a novel "aromatase" process. *J Pharmacol Exp Ther*. 2007;322:843–851.

Sun H, Yost GS. Metabolic activation of a novel 3-substituted indole-containing TNF-alpha inhibitor: dehydrogenation and inactivation of CYP3A4. *Chem Res Toxicol*. 2008;21:374–385.

Sun H, Moore C, Dansette PM, et al. Dehydrogenation of the indoline-containing drug 4-chloro-*N*-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide (indapamide) by CYP3A4: correlation with in silico predictions. *Drug Metab Dispos*. 2009;37:672–684.

Sun H, Liu L, Pang KS. Increased estrogen sulfation of estradiol 17beta-D-glucuronide in metastatic tumor rat livers. *J Pharmacol Exp Ther*. 2006;319:818–831.

Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol*. 2011;13:184–190.

Tafazoli S, O'Brien PJ. Accelerated cytotoxic mechanism screening of hydralazine using an in vitro hepatocyte inflammatory cell peroxidase model. *Chem Res Toxicol*. 2008;21:904–910.

Tafazoli S, O'Brien PJ. Peroxidases: a role in the metabolism and side effects of drugs. *Drug Discov Today*. 2005;10:617–625.

Takahashi RH, Grigliatti TA, Reid RE, Riggs KW. The effect of allelic variation in aldo-keto reductase 1C2 on the in vitro metabolism of dihydrotestosterone. *J Pharmacol Exp Ther*. 2009;329:1032–1039.

Takahashi RH, Halladay JS, Siu M, et al. Novel mechanism of decyanation of GDC-0425 by cytochrome P450. *Drug Metab Dispos*. 2017;45:430–440.

Takeda S, Ishii Y, Iwanaga M, et al. Modulation of UDP-glucuronosyltransferase function by cytochrome P450: evidence for the alteration of UGT2B7-catalyzed glucuronidation of morphine by CYP3A4. *Mol Pharmacol*. 2005;67:665–672.

Takeno S, Sakai T. Involvement of the intestinal microflora in nitrazepam-induced teratogenicity in rats and its relationship to nitroreduction. *Teratology*. 1991;44:209–214.

Tamm R, Magi R, Tremmel R, et al. Polymorphic variation in TPMT is the principal determinant of TPMT phenotype: a meta-analysis of three genome-wide association studies. *Clin Pharmacol Ther*. 2017;101:684–695.

Tan BS, Tiong KH, Muruhadas A, et al. CYP2S1 and CYP2W1 mediate 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (GW-610, NSC 721648) sensitivity in breast and colorectal cancer cells. *Mol Cancer Ther*. 2011;10:1982–1992.

Tan IL, Lycklama a Nijeholt GJ, Polman CH, Ader HJ, Barkhof F. Linomide in the treatment of multiple sclerosis: MRI results from prematurely terminated phase-III trials. *Mult Scler*. 2000;6:99–104.

Tang BK, Carro-Ciampi G. A method for the study of *N*-glucosidation in vitro–amobarbital-*N*-glucoside formation in incubations with human liver. *Biochem Pharmacol*. 1980;29:2085–2088.

Tang C, Lin JH, Lu AY. Metabolism-based drug–drug interactions: what determines individual variability in cytochrome P450 induction? *Drug Metab Dispos*. 2005;33:603–613.

Tang C, Ma B. Glycosidation of an endothelin ETA receptor antagonist and diclofenac in human liver microsomes: aglycone-dependent UDP-sugar selectivity. *Drug Metab Dispos*. 2005;33:1796–1802.

Tang M, Mukundan M, Yang J, et al. Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterificated in the presence of ethyl alcohol. *J Pharmacol Exp Ther*. 2006;319:1467–1476.

Tang W, Lu AY. Metabolic bioactivation and drug-related adverse effects: current status and future directions from a pharmaceutical research perspective. *Drug Metab Rev*. 2010;42:225–249.

Tay-Sontheimer J, Shireman LM, Beyer RP, et al. Detection of an endogenous urinary biomarker associated with CYP2D6 activity using global metabolomics. *Pharmacogenomics*. 2014;15:1947–1962.

Temellini A, Franchi M, Giuliani L, Pacifici GM. Human liver sulphotransferase and UDP-glucuronosyltransferase: structure-activity relationship for phenolic substrates. *Xenobiotica*. 1991;21:171–177.

Terao M, Kurosaki M, Demontis S, Zanotta S, Garattini E. Isolation and characterization of the human aldehyde oxidase gene: conservation of intron/exon boundaries with the xanthine oxidoreductase gene indicates a common origin. *Biochem J*. 1998;332:383–393.

Testa B, Krämer SD. *The Biochemistry of Drug Metabolism: Principles, Redox Reactions, Hydrolyses*. Weinheim, Germany: Wiley-VCH; 2008.

Testa B, Krämer SD. *The Biochemistry of Drug Metabolism: Conjugations, Consequences of Metabolism, Influencing Factors*. Zurich, Switzerland: Wiley-VCH; 2010.

Teva. Available at: http://www.tevapharm.com/news/teva_and_active_biotech_announce_discontinuation_of_higher_doses_of_laquinimod_in_two_multiple_sclerosis_trials_01_16.aspx. 2016.

Tew KD, Townsend DM. Regulatory functions of glutathione *S*-transferase P1-1 unrelated to detoxification. *Drug Metab Rev*. 2011;43:179–193.

Thomas JA. "Black Box" warning labels and drug withdrawals. *Toxicol Appl Pharmacol*. 2002;183:81–82.

Thomas M, Burk O, Klumpp B, et al. Direct transcriptional regulation of human hepatic cytochrome P450 3A4 (CYP3A4) by peroxisome proliferator-activated receptor alpha (PPARalpha). *Mol Pharmacol*. 2013;83:709–718.

Thomas M, Winter S, Klumpp B, et al. Peroxisome proliferator-activated receptor alpha, PPARalpha, directly regulates transcription of cytochrome P450 CYP2C8. *Front Pharmacol*. 2015;6:261.

Thompson MA, Moon E, Kim UJ, et al. Human indolethylamine *N*-methyltransferase: cDNA cloning and expression, gene cloning, and chromosomal localization. *Genomics*. 1999;61:285–297.

Thomson RE, Bigley AL, Foster JR, et al. Tissue-specific expression and subcellular distribution of murine glutathione *S*-transferase class kappa. *J Histochem Cytochem*. 2004;52: 653–662.

Tijet N, Boutros PC, Moffat ID, et al. Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries. *Mol Pharmacol*. 2006;69:140–153.

Tinguely JN, Wermuth B. Identification of the reactive cysteine residue (Cys227) in human carbonyl reductase. *Eur J Biochem*. 1999;260:9–14.

Tirona RG, Kim RB. Nuclear receptors and drug disposition gene regulation. *J Pharm Sci*. 2005;94:1169–1186.

Tiwawech D, Srivatanakul P, Karalak A, Ishida T. Cytochrome P450 2A6 polymorphism in nasopharyngeal carcinoma. *Cancer Lett*. 2006;241:135–141.

Toide K, Terauchi Y, Fujii T, Yamazaki H, Kamataki T. Uridine diphosphate sugar-selective conjugation of an aldose reductase inhibitor (AS-3201) by UDP-glucuronosyltransferase 2B subfamily in human liver microsomes. *Biochem Pharmacol*. 2004;67:1269–1278.

Tolson AH, Wang H. Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv Drug Deliv Rev*. 2010;62:1238–1249.

Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell*. 1994;79:1147–1156.

Tornio A, Filppula AM, Kailari O, et al. Glucuronidation converts clopidogrel to a strong time-dependent inhibitor of CYP2C8: a phase II metabolite as a perpetrator of drug–drug interactions. *Clin Pharmacol Ther*. 2014;96:498–507.

Tornio A, Niemi M, Neuvonen M, et al. The effect of gemfibrozil on repaglinide pharmacokinetics persists for at least 12 h after the dose: evidence for mechanism-based inhibition of CYP2C8 in vivo. *Clin Pharmacol Ther*. 2008;84:403–411.

Toselli F, Fredenwall M, Svensson P, et al. Oxetane substrates of human microsomal epoxide hydrolas. *Drug Metab Dispos*. 2017;45:966–973.

Townsend DM, Tew KD. The role of glutathione-*S*-transferase in anti-cancer drug resistance. *Oncogene*. 2003;22:7369–7375.

Tracy TS, Chaudhry AS, Prasad B, et al. Interindividual variability in cytochrome P450-mediated drug metabolism. *Drug Metab Dispos*. 2016;44:343–351.

Travica S, Pors K, Loadman PM, et al. Colon cancer-specific cytochrome P450 2W1 converts duocarmycin analogues into potent tumor cytotoxins. *Clin Cancer Res*. 2013;19:2952–2961.

Tremaine LM, Stroh JG, Ronfeld RA. Characterization of a carbamic acid ester glucuronide of the secondary amine sertraline. *Drug Metab Dispos*. 1989;17:58–63.

Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. Cytosolic arylamine *N*-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. *Biochem Pharmacol*. 1997;54:73–80.

Tseng E, Walsky RL, Luzietti RA Jr, et al. Relative contributions of cytochrome CYP3A4 versus CYP3A5 for CYP3A-cleared drugs assessed in vitro using a CYP3A4-selective inactivator (CYP3cide). *Drug Metab Dispos*. 2014;42:1163–1173.

Tsuchiya Y, Nakajima M, Takagi S, Taniya T, Yokoi T. MicroRNA regulates the expression of human cytochrome P450 1B1. *Cancer Res*. 2006;66:9090–9098.

Tucker GT. Clinical implications of genetic polymorphism in drug metabolism. *J Pharm Pharmocol*. 1994;46:417–424.

Tucker GT, Houston JB, Huang SM. Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential—toward a consensus. *Pharm Res*. 2001;18:1071–1080.

Tukey RH, Strassburg C. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol*. 2000;40:581–616.

Turner ST, Schwartz GL, Chapman AB, Hall WD, Boerwinkle E. Antihypertensive pharmacogenetics: getting the right drug into the right patient. *J Hypertens*. 2001;19:1–11.

Uchaipichat V, Mackenzie PI, Elliot DJ, Miners JO. Selectivity of substrate (trifluoperazine) and inhibitor (amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfinpyrazone) "probes" for human UDP-glucuronosyl transferases. *Drug Metab Dispos*. 2006;34:449–456.

Uesugi M, Hosokawa M, Shinke H, et al. Influence of cytochrome P450 (CYP) 3A4*1G polymorphism on the pharmacokinetics of tacrolimus, probability of acute cellular rejection, and mRNA expression level of CYP3A5 rather than CYP3A4 in living-donor liver transplant patients. *Biol Pharm Bull*. 2013;36:1814–1821.

Uetrecht J. Prediction of a new drug's potential to cause idiosyncratic reactions. *Curr Opin Drug Discov Devel*. 2001;4:55–59.

Uetrecht J, Naisbitt DJ. Idiosyncratic adverse drug reactions: current concepts. *Pharmacol Rev*. 2013;65:779–808.

Ujiie S, Sasaki T, Mizugaki M, Ishikawa M, Hiratsuka M. Functional characterization of 23 allelic variants of thiopurine *S*-methyltransferase gene (TPMT*2–*24). *Pharmacogenet Genomics*. 2008;18:887–893.

University of Washington DIDB. *UW Metabolism and Transport Drug Interaction Database*. Available at: https://www.druginteractioninfo.org/. 2018.

Unwalla RJ, Cross JB, Salaniwal S, et al. Using a homology model of cytochrome P450 2D6 to predict substrate site of metabolism. *J Comput Aided Mol Des*. 2010;24:237–256.

Van Booven D, Marsh S, McLeod H, et al. Cytochrome P450 2C9-CYP2C9. *Pharmacogenet Genomics*. 2010;20:277–281.

van Kuilenburg A, Meinsma R, van Gennip A. Pyrimidine degradation defects and severe 5-fluorouracil toxicity. *Nucleosides Nucleotides Nucleic Acids*. 2004;23:1371–1375.

Van LM, Heydari A, Yang J, et al. The impact of experimental design on assessing mechanism-based inactivation of CYP2D6 by MDMA (Ecstasy). *J Psychopharmacol*. 2006;20:834–841.

van Raaij JA, Kaptein E, Visser TJ, van den Berg KJ. Increased glucuronidation of thyroid hormone in hexachlorobenzene-treated rats. *Biochem Pharmacol*. 1993;45:627–631.

Van Vleet TR, Liu H, Lee A, Blomme EAG. Acyl glucuronide metabolites: implications for drug safety assessment. *Toxicol Lett*. 2017;272:1–7.

Varaprath S, Salyers KL, Plotzke KP, Nanavati S. Identification of metabolites of octamethylcyclotetrasiloxane (D(4)) in rat urine. *Drug Metab Dispos*. 1999;27:1267–1273.

Varma MV, Rotter CJ, Chupka J, et al. pH-sensitive interaction of HMG-CoA reductase inhibitors (statins) with organic anion transporting polypeptide 2B1. *Mol Pharm*. 2011;8:1303–1313.

Vasiliou V, Pappa A, Estey T. Role of human aldehyde dehydrogenases in endobiotic and xenobiotic metabolism. *Drug Metab Rev*. 2004;36:279–299.

Vatsis KP, Weber WW, Bell DA, et al. Nomenclature for *N*-acetyltransferases. *Pharmacogenetics*. 1995;5:1–17.

Veeramah KR, Thomas MG, Weale ME, et al. The potentially deleterious functional variant flavin-containing monooxygenase 2*1 is at high frequency throughout sub-Saharan Africa. *Pharmacogenet Genomics*. 2008;18:877–886.

Velasquez MT, Ramezani A, Manal A, Raj DS. Trimethylamine *N*-Oxide: the good, the bad and the unknown. *Toxins (Basel)*. 2016;8(11):pii: E326.

Venkatakrishnan K, Obach RS. In vitro–in vivo extrapolation of CYP2D6 inactivation by paroxetine: prediction of nonstationary pharmacokinetics and drug interaction magnitude. *Drug Metab Dispos*. 2005;33:845–852.

Vijay N, Morris ME. Role of monocarboxylate transporters in drug delivery to the brain. *Curr Pharm Des*. 2014;20:1487–1498.

Visser TJ, Kaptein E, van Toor H, et al. Glucuronidation of thyroid hormone in rat liver: effects of in vivo treatment with microsomal enzyme inducers and in vitro assay conditions. *Endocrinology*. 1993;133:2177–2186.

Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. *Drug Discov Today*. 2010;15:40–56.

Wahl B, Reichmann D, Niks D, et al. Biochemical and spectroscopic characterization of the human mitochondrial amidoxime reducing components hmARC-1 and hmARC-2 suggests the existence of a new molybdenum enzyme family in eukaryotes. *J Biol Chem*. 2010;285:37847–37859.

Walgren JL, Mitchell MD, Thompson DC. Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit Rev Toxicol*. 2005;35: 325–361.

Wallace BD, Wang H, Lane KT, et al. Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. *Science*. 2010;330:831–835.
Walsh JS, Miwa GT. Bioactivation of drugs: risk and drug design. *Annu Rev Pharmacol Toxicol*. 2011;51:145–167.
Walsky RL, Astuccio AV, Obach RS. Evaluation of 227 drugs for in vitro inhibition of cytochrome P450 2B6. *J Clin Pharmacol*. 2006;46:1426–1438.
Walsky RL, Obach RS. A comparison of 2-phenyl-2-(1-piperidinyl)propane (ppp), 1,1′,1″-phosphinothioylidynetrisaziridine (thioTEPA), clopidogrel, and ticlopidine as selective inactivators of human cytochrome P450 2B6. *Drug Metab Dispos*. 2007;35:2053–2059.
Walton MI, Wolf CR, Workman P. The role of cytochrome P450 and cytochrome P450 reductase in the reductive bioactivation of the novel benzotriazine di-*N*-oxide hypoxic cytotoxin 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233, WIN 59075) by mouse liver. *Biochem Pharmacol*. 1992;44:251–259.
Wang C, Bammler TK, Guo Y, Kelly EJ, Eaton DL. Mu-Class GSTs are responsible for aflatoxin B1-8,9-epoxide-conjugating activity in the nonhuman primate *Macaca fascicularis* liver. *Toxicol Sci*. 2000;56:26–36.
Wang D, Guo Y, Wrighton SA, Cooke GE, Sadee W. Intronic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs. *Pharmacogenomics J*. 2011a;11: 274–286.
Wang D, Para MF, Koletar SL, Sadee W. Human *N*-acetyltransferase 1 *10 and *11 alleles increase protein expression through distinct mechanisms and associate with sulfamethoxazole-induced hypersensitivity. *Pharmacogenet Genomics*. 2011b;21:652–664.
Wang H, Tompkins LM. CYP2B6: new insights into a historically overlooked cytochrome P450 isozyme. *Curr Drug Metab*. 2008;9:598–610.
Wang J, Davis M, Li F, et al. A novel approach for predicting acyl glucuronide reactivity via Schiff base formation: development of rapidly formed peptide adducts for LC/MS/MS measurements. *Chem Res Toxicol*. 2004;17:1206–1216.
Wang L, Nguyen TV, McLaughlin RW, et al. Human thiopurine *S*-methyltransferase pharmacogenetics: variant allozyme misfolding and aggresome formation. *Proc Natl Acad Sci U S A*. 2005;102:9394–9399.
Wang L, Pelleymounter L, Weinshilboum R, et al. Very important pharmacogene summary: thiopurine *S*-methyltransferase. *Pharmacogenet Genomics*. 2010a;20:401–405.
Wang LQ, James MO. Inhibition of sulfotransferases by xenobiotics. *Curr Drug Metab*. 2006;7:83–104.
Wang MZ, Saulter JY, Usuki E, et al. CYP4F enzymes are the major enzymes in human liver microsomes that catalyze the *O*-demethylation of the antiparasitic prodrug DB289 [2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime]. *Drug Metab Dispos*. 2006;34:1985–1994.
Wang P, Pradhan K, Zhong XB, Ma X. Isoniazid metabolism and hepatotoxicity. *Acta Pharm Sin B*. 2016a;6:384–392.
Wang Q, Kurita H, Carreira V, et al. Ah receptor activation by dioxin disrupts activin, BMP, and WNT signals during the early differentiation of mouse embryonic stem cells and inhibits cardiomyocyte functions. *Toxicol Sci*. 2016b;149:346–357.
Wang TJ, Zhang F, Richards JB, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet*. 2010b;376:180–188.
Wang YH, Trucksis M, McElwee JJ, et al. UGT2B17 genetic polymorphisms dramatically affect the pharmacokinetics of MK-7246 in healthy subjects in a first-in-human study. *Clin Pharmacol Ther*. 2012;92:96–102.
Wang YX, Ulu A, Zhang LN, Hammock B. Soluble epoxide hydrolase in atherosclerosis. *Curr Atheroscler Rep*. 2010c;12:174–183.
Wardman P, Dennis MF, Everett SA, et al. Radicals from one-electron reduction of nitro compounds, aromatic *N*-oxides and quinones: the kinetic basis for hypoxia-selective, bioreductive drugs. *Biochem Soc Symp*. 1995;61:171–194.
Watanabe A, Fukami T, Takahashi S, et al. Arylacetamide deacetylase is a determinant enzyme for the difference in hydrolase activities of phenacetin and acetaminophen. *Drug Metab Dispos*. 2010;38:1532–1537.
Watkins PB. Noninvasive tests of CYP3A enzymes. *Pharmacogenetics*. 1994;4:171.
Watson JD, Prokopec SD, Smith AB, et al. TCDD dysregulation of 13 AHR-target genes in rat liver. *Toxicol Appl Pharmacol*. 2014;274:445–454.
Waxman DJ, Holloway MG. Sex differences in the expression of hepatic drug metabolizing enzymes. *Mol Pharmacol*. 2009;76:215–228.
Wei S, Liu Z, Zhao H, et al. A single nucleotide polymorphism in the alcohol dehydrogenase 7 gene (alanine to glycine substitution at amino acid 92) is associated with the risk of squamous cell carcinoma of the head and neck. *Cancer*. 2010;116:2984–2992.
Weil A, Martin P, Smith R, et al. Pharmacokinetics of vandetanib in subjects with renal or hepatic impairment. *Clin Pharmacokinet*. 2010;49:607–618.
Weinshilboum R. Methyltransferase pharmacogenetics. *Pharmacol Ther*. 1989;43:77–90.
Weinshilboum R. Sulfotransferase pharmacogenetics. In: Kalow W, ed. *Pharmacogenetics of Drug Metabolism*. New York: Pergamon; 1992a.
Weinshilboum RM. Pharmacogenomics: catechol *O*-methyltransferase to thiopurine *S*-methyltransferase. *Cell Mol Neurobiol*. 2006;26:539–561.
Weinshilboum R, Otterness D, Szumlanski C. Methylation pharmacogenetics: catechol *O*-methyltransferase, thiopurine methyltransferase, and histamine *N*-methyltransferase. *Annu Rev Pharmacol Toxicol*. 1999;39:19–52.
Weinshilboum RM. Methylation pharmacogenetics: thiopurine methyltransferase as a model system. *Xenobiotica*. 1992b;22:1055–1071.
Weinshilboum RM, Otterness DM, Aksoy IA, et al. Sulfation and sulfotransferases 1: sulfotransferase molecular biology: cDNAs and genes. *FASEB J*. 1997;11:3–14.
Wen B, Coe KJ, Rademacher P, et al. Comparison of in vitro bioactivation of flutamide and its cyano analogue: evidence for reductive activation by human NADPH:cytochrome P450 reductase. *Chem Res Toxicol*. 2008;21:2393–2406.
Wenning LA, Hanley WD, Brainard DM, et al. Effect of rifampin, a potent inducer of drug-metabolizing enzymes, on the pharmacokinetics of raltegravir. *Antimicrob Agents Chemother*. 2009;53:2852–2856.
Westlind-Johnsson A, Hermann R, Huennemeyer A, et al. Identification and characterization of CYP3A4*20, a novel rare CYP3A4 allele without functional activity. *Clin Pharmacol Ther*. 2006;79:339–349.
Westlind-Johnsson A, Malmebo S, Johansson A, et al. Comparative analysis of CYP3A expression in human liver suggests only a minor role for CYP3A5 in drug metabolism. *Drug Metab Dispos*. 2003;31:755–761.
Westlind A, Malmebo S, Johansson I, et al. Cloning and tissue distribution of a novel human cytochrome p450 of the CYP3A subfamily, CYP3A43. *Biochem Biophys Res Commun*. 2001;281:1349–1355.
Whalen R, Boyer TD. Human glutathione *S*-transferases. *Semin Liver Dis*. 1998;18:345–358.
Wheeler MA, Rothhammer V, Quintana FJ. Control of immune-mediated pathology via the aryl hydrocarbon receptor. *J Biol Chem*. 2017;292:12383–12389.
Whitcomb D, Block G. Association of acetominophen hepatoxicity with fasting and ethanol use. *JAMA*. 1994;272:1845–1850.
Whitfield JB, Pang D, Bucholz KK, et al. Monoamine oxidase: associations with alcohol dependence, smoking and other measures of psychopathology. *Psychol Med*. 2000;30:443–454.
Wielinga PR, Reid G, Challa EE, et al. Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. *Mol Pharmacol*. 2002;62:1321–1331.
Wilkinson GR, Guengerich FP, Branch RA. Genetic polymorphism of *S*-mephenytoin hydroxylation. *Pharmacol Ther*. 1989;43:53–76.
Williams AC, Cartwright LS, Ramsden DB. Parkinson's disease: the first common neurological disease due to auto-intoxication? *QJM*. 2005;98:215–226.
Williams GM, Iatropoulos MJ. Alteration of liver cell function and proliferation: differentiation between adaptation and toxicity. *Toxicol Pathol*. 2002;30:41–53.
Williams JA, Cook J, Hurst SI. A significant drug-metabolizing role for CYP3A5? *Drug Metab Dispos*. 2003b;31:1526–1530.
Williams JA, Hurst SI, Bauman J, et al. Reaction phenotyping in drug discovery: moving forward with confidence? *Curr Drug Metab*. 2003a;4:527–534.
Williams JA, Hyland R, Jones BC, et al. Drug–drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCI/AUC) ratios. *Drug Metabolism Dispos*. 2004;32:1201–1208.

Williams JA, Ring BJ, Cantrell VE, et al. Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos*. 2002;30:883–891.

Williams RT. *Detoxication Mechanisms: The Metabolism and Detoxication of Drugs, Toxic Substances, and Other Organic Compounds*. New York: Wiley; 1959.

Williams-Gray C, Goris A, Foltynie T, et al. No evidence for association between an MAOA functional polymorphism and susceptibility to Parkinson's disease. *J Neurol*. 2009;256:132–133.

Wincent E, Bengtsson J, Mohammadi Bardbori A, et al. Inhibition of cytochrome P4501-dependent clearance of the endogenous agonist FICZ as a mechanism for activation of the aryl hydrocarbon receptor. *Proc Natl Acad Sci U S A*. 2012;109:4479–4484.

Winter HR, Unadkat JD. Identification of cytochrome P450 and arylamine *N*-acetyltransferase isoforms involved in sulfadiazine metabolism. *Drug Metab Dispos*. 2005;33:969–976.

Wood AW, Swinney DC, Thomas PE, et al. Mechanism of androstenedione formation from testosterone and epitestosterone catalyzed by purified cytochrome P-450b. *J Biol Chem*. 1988;263:17322–17332.

Wood TC, Johnson KL, Naylor S, Weinshilboum RM. Cefazolin administration and 2-methyl-1,3,4-thiadiazole-5-thiol in human tissue: possible relationship to hypoprothrombinemia. *Drug Metab Dispos*. 2002;30:1123–1128.

Wood TC, Salavagionne OE, Mukherjee B, et al. Human arsenic methyltransferase (AS3MT) pharmacogenetics: gene resequencing and functional genomics studies. *J Biol Chem*. 2006;281:7364–7373.

Wu D, Li Y, Song G, et al. Crystal structure of human esterase D: a potential genetic marker of retinoblastoma. *FASEB J*. 2009;23:1441–1446.

Wu KC, Cui JY, Klaassen CD. Effect of graded Nrf2 activation on phase-I and -II drug metabolizing enzymes and transporters in mouse liver. *PLoS One*. 2012;7:e39006.

Wu WK, Sung JJ, Lee CW, Yu J, Cho CH. Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: an update on the molecular mechanisms. *Cancer Lett*. 2010;295:7–16.

Wu Z-L, Sohl CD, Shimada T, Guengerich FP. Recombinant enzymes overexpressed in bacteria show broad catalytic specificity of human cytochrome P450 2W1 and limited activity of human cytochrome P450 2S1. *Mol Pharmacol*. 2006;69:2007–2014.

Xiao Y, Guengerich FP. Metabolomic analysis and identification of a role for the orphan human cytochrome P450 2W1 in selective oxidation of lysophospholipids. *J Lipid Res*. 2012;53:1610–1617.

Xie W, Stribley JA, Chatonnet A, et al. Postnatal developmental delay and supersensitivity to organophosphate in gene-targeted mice lacking acetylcholinesterase. *J Pharmacol Exp Ther*. 2000;293:896–902.

Xu M, Ju W, Hao H, Wang G, Li P. Cytochrome P450 2J2: distribution, function, regulation, genetic polymorphisms and clinical significance. *Drug Metab Rev*. 2013;45:311–352.

Xu X, Zhang XA, Wang DW. The roles of CYP450 epoxygenases and metabolites, epoxyeicosatrienoic acids, in cardiovascular and malignant diseases. *Adv Drug Deliv Rev*. 2011;63:597–609.

Xu Y, Hashizume T, Shuhart MC, et al. Intestinal and hepatic CYP3A4 catalyze hydroxylation of 1alpha,25-dihydroxyvitamin D(3): implications for drug-induced osteomalacia. *Mol Pharmacol*. 2006;69:56–65.

Yamada R, Ymamoto K. Recent findings on genes associated with inflammatory disease. *Mutat Res*. 2005;573:136–151.

Yamano S, Tatsuno J, Gonzalez FJ. The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry*. 1990;29:1322–1329.

Yamazaki H, Johnson WW, Ueng YF, Shimada T, Guengerich FP. Lack of electron transfer from cytochrome b5 in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apo-cytochrome b5. *J Biol Chem*. 1996;271:27438–27444.

Yamazaki H, Shimada T, Martin MV, Guengerich FP. Stimulation of cytochrome P450 reactions by apo-cytochrome b5: evidence against transfer of heme from cytochrome P450 3A4 to apo-cytochrome b5 or heme oxygenase. *J Biol Chem*. 2001;276:30885–30891.

Yan B, Yang D, Brady M, Parkinson A. Rat kidney carboxylesterase. Cloning, sequencing, cellular localization, and relationship to rat liver hydrolase. *J Biol Chem*. 1994;269:29688–29696.

Yang H, Garzel B, Heyward S, et al. Metformin represses drug-induced expression of CYP2B6 by modulating the constitutive androstane receptor signaling. *Mol Pharmacol*. 2014;85:249–260.

Yang N, Sun R, Liao X, Aa J, Wang G. UDP-glucuronosyltransferases (UGTs) and their related metabolic cross-talk with internal homeostasis: a systematic review of UGT isoforms for precision medicine. *Pharmacol Res*. 2017;121:169–183.

Yang Q, Nagano T, Shah Y, et al. The PPAR alpha-humanized mouse: a model to investigate species differences in liver toxicity mediated by PPAR alpha. *Toxicol Sci*. 2008;101: 132–139.

Yang X, Gandhi YA, Duignan DB, Morris ME. Prediction of biliary excretion in rats and humans using molecular weight and quantitative structure–pharmacokinetic relationships. *AAPS J*. 2009;11:511–525.

Yang Y-H, Aloysius H, Inoyama D, Chen Y, Ju L-Q. Enzyme-mediated hydrolytic activation of prodrugs. *Acta Pharmaceutica Sinica B*. 2011;1:143–159.

Yao KS, Hageboutros A, Ford P, O'Dwyer PJ. Involvement of activator protein-1 and nuclear factor-kappa B transcription factors in the control of the DT-diaphorase expression induced by mitomycin C treatment. *Mol Pharmacol*. 1997;51:422–430.

Yeager RL, Reisman SA, Aleksunes LM, Klaassen CD. Introducing the "TCDD-inducible AhR-Nrf2 gene battery." *Toxicol Sci*. 2009;111:238–246.

Yeung CK, Fujioka Y, Hachad H, Levy RH, Isoherranen N. Are circulating metabolites important in drug–drug interactions? Quantitative analysis of risk prediction and inhibitory potency. *Clin Pharmacol Ther*. 2011;89:105–113.

Yin H, Bennett G, Jones JP. Mechanistic studies of uridine diphosphate glucuronosyltransferase. *Chem Biol Interact*. 1994;90:47–58.

Yoneyama M, Nishiyama N, Shuto M, et al. In vivo depletion of endogenous glutathione facilitates trimethyltin-induced neuronal damage in the dentate gyrus of mice by enhancing oxidative stress. *Neurochem Int*. 2008;52:761–769.

Yoshida A, Rzhetsky A, Hsu L, Chang C. Human aldehyde dehydrogenase gene family. *Eur J Biochem*. 1998;251:549–557.

Yraola F, Zorzano A, Albericio F, Royo M. Structure–activity relationships of SSAO/VAP-1 arylalkylamine-based substrates. *Chem Med Chem*. 2009;4:495–503.

Yu H, Balani SK, Chen W, et al. Contribution of metabolites to P450 inhibition-based drug–drug interactions: scholarship from the drug metabolism leadership group of the innovation and quality consortium metabolite group. *Drug Metab Dispos*. 2015;43:620–630.

Yu H, Tweedie D. A perspective on the contribution of metabolites to drug–drug interaction potential: the need to consider both circulating levels and inhibition potency. *Drug Metab Dispos*. 2013;41:536–540.

Yumibe N, Huie K, Chen KJ, et al. Identification of human liver cytochrome P450 enzymes that metabolize the nonsedating antihistamine loratadine. Formation of descarboethoxyloratadine by CYP3A4 and CYP2D6. *Biochem Pharmacol*. 1996;51:165–172.

Zamek-Gliszczynski MJ, Hoffmaster KA, Nezasa K, Tallman MN, Brouwer KL. Integration of hepatic drug transporters and phase II metabolizing enzymes: mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. *Eur J Pharm Sci*. 2006;27:447–486.

Zetterberg C, Maltais F, Laitinen L, et al. VX-509 (decernotinib)-mediated CYP3A time-dependent inhibition: an aldehyde oxidase metabolite as a perpetrator of drug–drug interactions. *Drug Metab Dispos*. 2016;44: 1286–1295.

Zhan P, Wang Q, Qian Q, Wei SZ, Yu LK. CYP1A1 MspI and exon7 gene polymorphisms and lung cancer risk: an updated meta-analysis and review. *J Exp Clin Cancer Res*. 2011;30:99.

Zhang D, Chando TJ, Everett DW, et al. In vitro inhibition of UDP glucuronosyltransferases by atazanavir and other HIV protease inhibitors and the relationship of this property to in vivo bilirubin glucuronidation. *Drug Metab Dispos*. 2005;33:1729–1739.

Zhang DG, Zhang C, Wang JX, et al. Obeticholic acid protects against carbon tetrachloride-induced acute liver injury and inflammation. *Toxicol Appl Pharmacol*. 2017;314:39–47.

Zhang H, Amunugama H, Ney S, Cooper N, Hollenberg PF. Mechanism-based inactivation of human cytochrome P450 2B6 by clopidogrel: involvement of both covalent modification of cysteinyl residue 475 and loss of heme. *Mol Pharmacol*. 2011a;80:839–847.

Zhang J, Cashman JR. Quantitative analysis of FMO gene mRNA levels in human tissues. *Drug Metab Dispos*. 2006;34:19–26.
Zhang L, Hanigan MH. Role of cysteine *S*-conjugate beta-lyase in the metabolism of cisplatin. *J Pharmacol Exp Ther*. 2003;306:988–994.
Zhang L, Zhang YD, Zhao P, Huang SM. Predicting drug–drug interactions: an FDA perspective. *AAPS J*. 2009;11:300–306.
Zhang W, Chang YZ, Kan QC, et al. CYP3A4*1G genetic polymorphism influences CYP3A activity and response to fentanyl in Chinese gynecologic patients. *Eur J Clin Pharmacol*. 2010;66:61–66.
Zhang Y, Talalay P, Cho C, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci U S A*. 1992;89:2399–2403.
Zhang Z, Tan M, Xie Z, et al. Identification of lysine succinylation as a new post-translational modification. *Nat Chem Biol*. 2011b;7:58–63.
Zhao Y, Wan D, Yang J, Hammock BD, Ortiz de Montellano PR. Catalytic activities of tumor-specific human cytochrome P450 CYP2W1 toward endogenous substrates. *Drug Metab Dispos*. 2016;44:771–780.
Zheng X, Deng J, Zhang T, et al. Potential anti-obesity effects of a long-acting cocaine hydrolase. *Chem Biol Interact*. 2016;259:99–103.
Zheng YM, Fisher MB, Yokotani N, Fujii-Kuriyama Y, Rettie AE. Identification of a meander region proline residue critical for heme binding to cytochrome P450: implications for the catalytic function of human CYP4B1. *Biochemistry*. 1998;37:12847–12851.
Zhi X, Chan EM, Edenberg HJ. Tissue-specific regulatory elements in the human alcohol dehydrogenase 6 gene. *DNA Cell Biol*. 2000;19:487–497.
Zhou C, Assem M, Tay JC, et al. Steroid and xenobiotic receptor and vitamin D receptor crosstalk mediates CYP24 expression and drug-induced osteomalacia. *J Clin Invest*. 2006a;116:1703–1712.
Zhou D, Guo J, Linnenbach AJ, Booth-Genthe CL, Grimm SW. Role of human UGT2B10 in *N*-glucuronidation of tricyclic antidepressants, amitriptyline, imipramine, clomipramine, and trimipramine. *Drug Metab Dispos*. 2010a;38:863–870.
Zhou H, Mascelli MA. Mechanisms of monoclonal antibody–drug interactions. *Annu Rev Pharmacol Toxicol*. 2011;51:359–372.
Zhou J, Zhai Y, Mu Y, et al. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem*. 2006b;281:15013–15020.
Zhou LY, Zuo XC, Chen K, et al. Significant impacts of CYP3A4*1G and CYP3A5*3 genetic polymorphisms on the pharmacokinetics of diltiazem and its main metabolites in Chinese adult kidney transplant patients. *J Clin Pharm Ther*. 2016;41:341–347.
Zhou SF. Polymorphism of human cytochrome P450 2D6 and its clinical significance: part I. *Clin Pharmacokinet*. 2009a;48:689–723.
Zhou SF. Polymorphism of human cytochrome P450 2D6 and its clinical significance: part II. *Clin Pharmacokinet*. 2009b;48:761–804.
Zhou SF, Liu JP, Chowbay B. Polymorphism of human cytochrome P450 enzymes and its clinical impact. *Drug Metab Rev*. 2009a;41:89–295.
Zhou SF, Wang B, Yang LP, Liu JP. Structure, function, regulation and polymorphism and the clinical significance of human cytochrome P450 1A2. *Drug Metab Rev*. 2010b;42:268–354.
Zhou SF, Zhou ZW, Yang LP, Cai JP. Substrates, inducers, inhibitors and structure-activity relationships of human cytochrome P450 2C9 and implications in drug development. *Curr Med Chem*. 2009b;16:3480–3675.
Zhou X, D'Agostino J, Li L, et al. Respective roles of CYP2A5 and CYP2F2 in the bioactivation of 3-methylindole in mouse olfactory mucosa and lung: studies using Cyp2a5-null and Cyp2f2-null mouse models. *Drug Metab Dispos*. 2012;40:642–647.
Zhu BT, Liehr JG. Inhibition of the catechol-*O*-methyltransferase-catalyzed *O*-methylation of 2- and 4-hydroxyestradiol by catecholamines: implications for the mechanism of estrogen-induced carcinogenesis. *Arch Biochem Biophys*. 1993;304:248–256.
Zhu HJ, Markowitz JS. Activation of the antiviral prodrug oseltamivir is impaired by two newly identified carboxylesterase 1 variants. *Drug Metab Dispos*. 2009;37:264–267.
Zhuo X, Cantone JL, Wang Y, et al. Phosphocholine conjugation: an unexpected in vivo conjugation pathway associated with hepatitis C ns5b inhibitors featuring a bicyclo[1.1.1]pentane. *Drug Metab Dispos*. 2016, Mar 9. pii: dmd.115.069062. [Epub ahead of print].
Ziegler DM. An overview of the mechanism, substrate specificities, and structure of FMOs. *Drug Metab Rev*. 2002;34: 503–511.
Ziegler DM. Recent studies on the structure and function of multisubstrate flavin-containing monooxygenases. *Annu Rev Pharmacol Toxicol*. 1993;33:179–199.
Zientek MA, Youdim K. Reaction phenotyping: advances in the experimental strategies used to characterize the contribution of drug-metabolizing enzymes. *Drug Metab Dispos*. 2015;43:163–181.
Zineh I, Beitelshees AL, Gaedigk A, et al.Pharmacokinetics and CYP2D6 genotypes do not predict metoprolol adverse events or efficacy in hypertension. *Clin Pharmacol Ther*. 2004;76:536–544.
Zollinger M, Gschwind HP, Jin Y, et al. Absorption and disposition of the sphingosine 1-phosphate receptor modulator fingolimod (FTY720) in healthy volunteers: a case of xenobiotic biotransformation following endogenous metabolic pathways. *Drug Metab Dispos*. 2011;39:199–207.
Zollinger M, Sayer C, Dannecker R, et al. The macrolide everolimus forms an unusual metabolite in animals and humans: identification of a phosphocholine ester. *Drug Metab Dispos*. 2008;36:1457–1460.
Zordoky BN, El-Kadi AO. Effect of cytochrome P450 polymorphism on arachidonic acid metabolism and their impact on cardiovascular diseases. *Pharmacol Ther*. 2010;125:446–463.

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chapter 7

Toxicokinetics

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INTRODUCTION

Toxicokinetics refers to the quantitative study of absorption, distribution, metabolism, and excretion (ADME) of chemicals in biota through measurement and modeling of their concentrations or amounts in biological matrices (e.g., blood, plasma, excreta, exhaled air, tissues) as a function of time. Experimental studies of toxicokinetics facilitate the characterization of the temporal profile of the concentration of chemicals and their metabolites in the target tissue or other biological matrices, reflective of the net effect of the rate and extent of absorption through one or more exposure routes, distribution to tissues and organs via systemic circulation as well as disposition by metabolism and excretion (Fig. 7-1). The experimental and quantitative approaches used today for characterizing toxicokinetics of xenobiotics were derived from such studies extensively conducted with drugs over the past decades (traditionally known as pharmacokinetics) (Gibaldi and Perrier, 1975, 1982; O'Flaherty, 1981; WHO, 1986; Rescigno, 1997; Tozer and Rowland, 2006). Toxicokinetic data and analyses constitute an essential part of systematic approaches to safety/risk evaluation of xenobiotics and other substances including therapeutic drugs.

A fundamental goal of toxicokinetics is to facilitate the determination of dose delivered to the organism in view of understanding systemic exposure to the toxicant. For a scientifically sound interpretation of toxicology studies and characterization of dose–response relationships, it is preferable to relate adverse responses to the dose delivered to the target organ (or to systemic circulation) in the form of

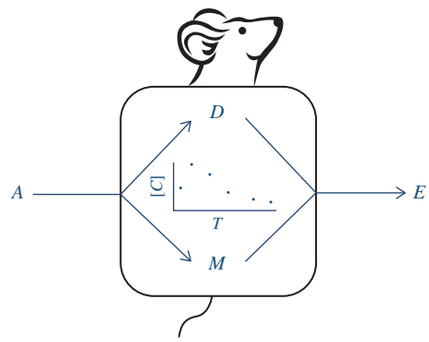

Figure 7-1. *Illustration of the key processes determining the time course of the concentration ([C] vs. T) of a toxic chemical in the target site (A: absorption from the dosing solution or the exposure medium; D: distribution in the blood stream and tissues; M: metabolism by hepatic and extrahepatic tissues; E: excretion).*

putative toxic moiety. In fact, the relationship between administered dose and adverse responses is better understood when the toxicokinetic data or models are used to translate the administered dose in terms of a relevant measure of internal dose (i.e., dose metrics) (U.S. EPA, 2006, 2014). Consistent with the current understanding of the mode of action of a particular chemical, the dose metrics reflect the intensity and level of a toxicologically active form of the chemical in the target or surrogate compartment during a specific period

Table 7-1

Considerations for Selecting a Dose Metric of Relevance to the Mode of Action of a Chemical

ASPECT	EXAMPLE
Intensity	Peak, average, or integral
Level	Concentration, amount, or rate of production
Moiety	Parent chemical, metabolites, or adducts
Time window	Instantaneous, daily, lifetime, or specific lifestage
Matrix	Blood, plasma, or target tissue (e.g., brain)

(Table 7-1). In this regard, the maximal blood concentration (C_{max}), the area under the blood concentration versus time curve (AUC) and the amount metabolized are the common dose metrics used in interpreting toxicity data. Fig. 7-2A presents a time course of the blood concentration of a chemical following its administration via the oral route. The C_{max} and AUC can be obtained following such time-course measurements of chemical concentrations in plasma, blood, or tissues in toxicokinetic studies. However, due to ethical and feasibility considerations, toxicokinetic data cannot always be obtained for all biological matrices, dose levels, time frames, exposure routes, and scenarios of interest in test animals and humans. Therefore, a pragmatic and scientifically sound approach would be to use the available toxicokinetic data to conduct a non-compartmental analysis or to develop mathematical models that may be empirical or mechanistic in nature. The models in toxicokinetics range from a simple, one-compartment model to complex multicompartment models, with some of them explicitly incorporating mechanistic determinants of chemical uptake, distribution, and disposition (O'Flaherty, 1981; Gibaldi and Perrier, 1982; Tozer and Rowland, 2006).

This chapter begins with an introduction to toxicokinetic data and concepts, followed by the fundamentals of classic and physiologic toxicokinetic models, as well as their applications in toxicology, risk assessment, and biomonitoring.

TOXICOKINETIC DATA

The interest in toxicokinetics arises from the need to interpret toxicity and biomonitoring studies on the basis of how much of a chemical was absorbed, biotransformed, and distributed to the target site or excreted out of the body at various time periods. The use of data on the fraction of the dose absorbed represents one level of refinement in relating to the onset and progression of adverse responses in a toxicology study. A more refined strategy would be to examine the internal dose of relevance to the mode of action of the chemical (e.g., maximal, average, or integrated concentration of the unbound chemical or its toxic metabolite in blood or tissue during a specific timeframe). In this regard, the whole blood and plasma are convenient sampling matrices that also reflect the net impact of ADME processes in the organism. Furthermore, these biological matrices, in many cases, can effectively reflect the extent of target tissue exposure as the cellular matrix equilibrates with the free chemical in the extracellular fluid. The C_{max} and AUC are the two dose metrics frequently obtained from quantification of chemical concentrations in whole blood or plasma samples in toxicokinetic studies.

Fig. 7-2A presents the blood kinetic data collected in a group of animals as part of the toxicological evaluation of a xenobiotic following a single oral dose of 100 mg/kg body weight. The toxicokinetic curve indicates an initial phase of increasing concentration determined by the rate of absorption, then a peak or plateau during which absorption rate equals elimination rate, followed by a phase dominated essentially by elimination. In this hypothetical example, the mean blood concentration of the parent chemical in the treatment group corresponded to 0, 1.32, 2.74, 3.37, 3.55, 2.89, 1.67, and 0.37 mg/L at pre-exposure, or at 2, 4, 6, 8, 10, 12, and 24 hours post-exposure, respectively. The $C_{max,blood}$ is 3.55 mg/L for this treatment group. Regarding AUC_{blood}, a common method of calculation involves summing the areas of trapezoids constituting the time-course curve (Gibaldi and Perrier, 1982; WHO, 1986). The trapezoidal method involves (i) averaging the blood concentrations found at two consecutive sampling times and (ii) multiplying it with the time interval between the two sampling periods (Figs. 7-2B and 7-2C). The lower panel in Fig. 7-2C illustrates the calculation of AUC for one trapezoid (4.56 (mg/L)·h), using the mean blood concentrations of 2.89 and 1.67 mg/L obtained at 10 and 12 hours post-dosing. For this particular dataset, AUC_{0-24h} (= 41.65 (mg/L)·h) corresponds to the sum of areas of the seven trapezoids, that is, 1.32, 4.06, 6.11, 6.92, 6.44, 4.56, and 12.24 (mg/L)·h. For determining the AUC until complete elimination of the substance (i.e., 24 hours to infinity), the area of the last trapezoid is additionally computed by dividing the last measured blood concentration by the rate of elimination (AUC_{last}/K_{el} where K_{el} = first-order elimination rate constant, h^{-1}). The trapezoidal rule can also be used to calculate the area under the first moment curve (i.e., [concentration × time] vs. [time]). In turn, the ratio of the area under the first moment curve (AUMC) to the AUC is indicative of the mean residence time (MRT), that is, the average time spent by drug or chemical molecules in the body (Rowland and Tozer, 2011).

The timing of sample collection and the time intervals between samples in toxicokinetic studies should be appropriate to enable reliable calculation of the dose metrics of interest. The time-course data collected should (i) capture the critical kinetic phases in the exposed animals, (ii) allow the estimation of fundamental toxicokinetic parameters, and (iii) permit, to the extent possible, the characterization of C_{max} and AUC. In practice, at least two time points are chosen for sampling in the early phase to characterize absorption and distribution phases, and at least three time points are chosen for sampling during the terminal elimination phase, with the collected samples representing change in concentration by at least a factor of two or preferably by an order of magnitude. Chemical-specific toxicokinetic properties, limit of detection of the analytical method along with statistical considerations can facilitate the determination of the number of samples, sampling time intervals, and volume of biological samples to be collected. The number and volume of samples drawn should be such that they do not compromise the normal physiological status of the animal (e.g., not drawing more than 10% of the blood volume during the AUC sampling interval (Cayen, 1995).

The accuracy of calculated dose metrics depends upon the number of data points collected in a toxicokinetic study whereas their magnitude depends upon the dose administered, exposure route, scenario (single or multiple doses), as well as the rates and extent of ADME influenced by factors such as age and pathophysiological state of the test subject or animal. When all required time-course data are not available or could not be collected in an animal or a human study to facilitate confident calculation of AUC or another metric of internal dose, the limited available data are subject to analysis or modeling to derive useful toxicokinetic parameters to gain a better characterization of the time course of the chemical in the biota. The choice of analysis or modeling method depends upon the underlying question(s) of the toxicological evaluation, the richness of the available data sets (toxicology, mode of action, and toxicokinetics), as well as the precision and predictive ability required for the end-use. These approaches, enabling the understanding of the net impact of ADME processes on the time-course behavior of

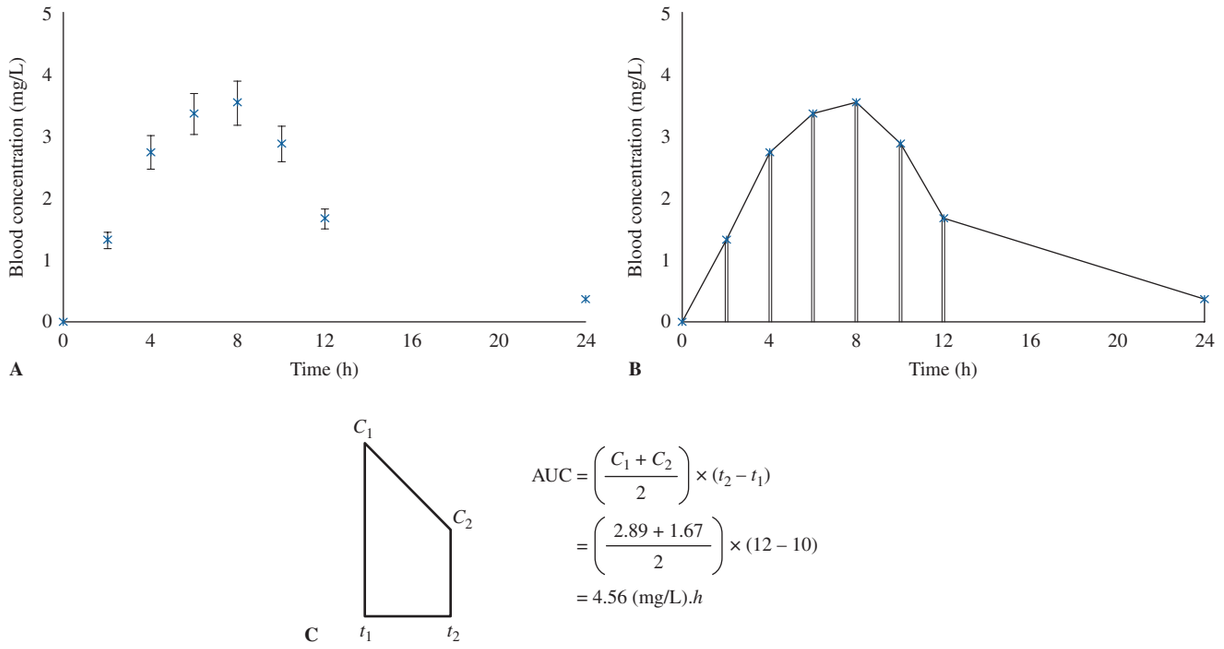

Figure 7-2. *Illustrations of (**A**) the measured blood concentration of a xenobiotic following a single oral dose, (**B**) the construction of trapezoids to calculate the area under the curve (AUC), and (**C**) the computation of the area under a single trapezoid corresponding to the blood concentrations (2.89 and 1.67 mg/L) at 10 and 12 hours post-dosing.*

xenobiotics, take into account the fundamental toxicokinetic concepts described below.

TOXICOKINETIC CONCEPTS

Absorption and Bioavailability

The rate and extent of absorption, or the entry of a chemical into the systemic circulation, depends upon the dose, exposure route, as well as the volume and type of exposure matrix or dosing vehicle. Whereas human exposure to environmental chemicals occurs through the dermal, inhalation, and oral routes, laboratory animal experiments might additionally involve administrations by the intravenous (IV), intraperitoneal (IP), or intramuscular (IM) routes (Turner et al., 2011). The rate and efficiency with which a given chemical crosses the biological membrane barrier determine how quickly and how much of it appears in the systemic circulation. Whereas absorption might be incomplete when exposure occurs via extravascular routes, the intravenous dosing assures full (100%) delivery of the dose into the systemic circulation, and can therefore be used effectively as a benchmark of absorption by non-IV routes. The extent of absorption of a toxicant can be experimentally determined by comparing the time course of plasma toxicant concentration after IV and extravascular dosing. The AUC associated with the extravascular dose in reference to the AUC for the intravenous route is called *bioavailability* (F). In single-dose toxicokinetic studies, F is determined by using different IV and non-IV doses according to the following equation, provided the toxicant does not display dose-dependent or saturable kinetics:

$$F = \frac{(\text{AUC}_{\text{non-IV}} \,/\, \text{Dose}_{\text{non-IV}})}{(\text{AUC}_{\text{IV}} \,/\, \text{Dose}_{\text{IV}})}, \tag{7-1}$$

where $\text{AUC}_{\text{non-IV}}$, AUC_{IV}, $\text{Dose}_{\text{non-IV}}$, and Dose_{IV} are the respective areas under the blood concentration versus time curves and doses for non-IV and IV administrations. Conceptually, the F value for a non-IV route (e.g., oral) would range between 0 and 1, with the value closer to 1 indicating better absorption and systemic availability of a chemical by that particular route of exposure. When $F < 1$, it indicates that less than 100% of the administered dose is able to reach the systemic circulation. For example, the systemic availability of an orally administered toxicant is governed by its absorption at the gastrointestinal barrier, metabolism within the intestinal mucosa, as well as metabolism and biliary excretion during its first passage through the liver. The chemical may be absorbed to a limited extent because of low aqueous solubility preventing its effective dissolution in the gastrointestinal fluid or low permeability across the brush-border membrane of the intestinal mucosa. Extensive degradation by metabolic enzymes residing at the intestinal mucosa and the liver may also minimize entry of the chemical in its intact form into the systemic circulation. Metabolism and excretion of the toxicant at the intestinal mucosa and the liver prior to its entry into the systemic circulation is called presystemic extraction or first-pass effect. The following equation accounts for the action of absorption and sequential first-pass extraction at the intestinal mucosa and the liver as determinants of the bioavailability (F) of a toxicant taken orally:

$$F = f_g \cdot (1 - E_m) \cdot (1 - E_h), \tag{7-2}$$

where f_g is the fraction of the applied dose that is released and absorbed across the mucosal barrier along the entire length of the gut, E_m is the extent of loss due to metabolism within the gastrointestinal mucosa, and E_h is the loss due to metabolism or biliary excretion during first-pass through the liver.

While the *extent* of absorption is important for calculating the total amount of the administrated dose that actually crossed the biologically barrier(s) to reach systemic circulation and tissues, the *rate* of absorption is critical to understand the time course of the plasma, blood, or tissue concentration of a chemical following dosing. For the oral route, in simple cases, it can be computed as the first-order absorption rate constant (K_a, h^{-1}) times the amount available for

absorption (mg). For illustrative purpose, let's assume that a xenobiotic possesses a K_a value of 0.1 h^{-1}. It would imply that the same fraction of the amount in gut is absorbed per unit time, regardless of the administered dose. In this particular case, 9.5% of the dose is absorbed after 1 hour, even though the rate constant is 0.1 h^{-1} (or 10 percent per hour), because the amount remaining in the gut continuously decreases with time. If the amount absorbed per unit time is constant (e.g., 10 mg/h) regardless of the magnitude of the dose administered (1, 10, or 100 g), then it is said to follow zero-order process. Dietary exposure to chemicals is frequently described as a zero-order (or constant) input process, because it results in a fairly constant input dose to the exposed animals. The zero-order description is also used to reflect the capacity-limitation of a system (e.g., transport system), that is, to represent a system working at its maximal capacity but not functioning proportionally after a certain dose. The concepts of proportionality, capacity-limitation, and saturation are applicable to all phases of toxicokinetics and are critical in interpreting toxicity data.

The impact of the rate of absorption on the resulting plasma concentration profile of a chemical is illustrated in Fig. 7-3 using three different values of K_a. As shown in Fig. 7-3, slowing the absorption rate of a toxicant leads to a decrease in the C_{max} and a delay in time to reach C_{max} (T_p) while maintaining the same extent of absorption or bioavailability (compare case 2 to case 1). The converse is true, that is, accelerating rate of absorption increases C_{max} and shortens T_p. The dependence of T_p and C_{max} on absorption rate has obvious implication in the speed of onset and maximum toxic effects following exposure to a chemical. Case 3 in Fig. 7-3 illustrates the peculiar situation when the absorption rate is so much slower than the elimination rate. In this case, the terminal rate of decline in plasma concentration reflects the absorption rate constant, instead of the elimination rate constant; that is, the washout of toxicant is rate-limited by slow absorption until the applied dose is completely absorbed or removed, beyond which time the toxicant remaining in the body will be cleared according to the elimination rate constant. This means that continual absorption of a chemical can affect the persistence of the toxic effect following an acute exposure, and that it is important to institute decontamination procedure quickly after overdose or accidental exposure to a toxicant. This is especially a consideration in occupational exposure via dermal absorption following skin contact with permeable industrial chemicals.

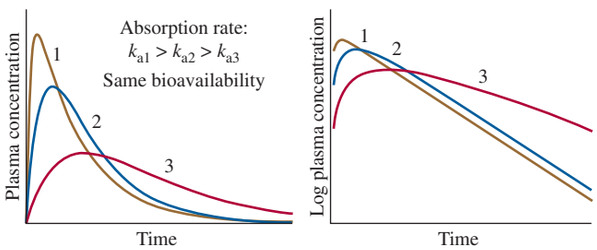

Figure 7-3. *Influence of absorption rate on the time to peak (T_p) and maximum plasma concentration (C_{max}) of a hypothetical toxicant.* The left panel illustrates the change in plasma concentration–time curves as the first-order absorption rate constant (k_a) decreases, while keeping the extent of absorption or bioavailability (F), hence the area-under-the-curve (AUC), constant. The right panel displays the same curves in a semilogarithmic plot. Time to peak plasma concentration shows a progressive delay as k_a decreases, along with a decrease in C_{max}. In cases 1 and 2, the terminal decline in plasma concentration is governed by elimination half-life; hence, the parallel decline in the semilogarithmic plot. In case 3, the absorption becomes so slow that decline in plasma concentration in the terminal phase reflects the absorption half-life, that is, washout of toxicant is rate-limited by absorption. Accordingly, the terminal decline is slower than in cases 1 and 2.

Volume of Distribution

The volume of distribution, V_d, corresponds to the apparent volume of a biological fluid in which a xenobiotic is diluted, and is expressed in units of volume (mL or L of blood, plasma, or plasma water). V_d would be approximately equal to the volume of blood if the chemical resided solely in blood and distributed uniformly among the blood components (plasma, erythrocytes). Hydrophilic substances exhibit smaller volumes of distribution, often close to 0.6 L/kg (or 60% of body weight) in adults, approximating the total body water in plasma (4%), interstitial fluid (21%), and intracellular fluid (35%). Those substances that are highly bound to plasma proteins and not showing any permeation in the blood capillaries or tissue membranes would result in V_d reflective of the extracellular fluid. Xenobiotics exhibiting a marked affinity for tissue or fat depots have large V_d (exceeding physiological fluid spaces and body weight) leading to low plasma concentrations. The mechanisms of tissue sequestration not only include partitioning of a toxicant into tissue lipids, but also involve binding to tissue proteins, concentrative uptake by active transporters, and trapping in specialized organelles (e.g., pH trapping of amine compounds in acidic lysosomes) (Assmus et al., 2017). In the case of xenobiotics that are extremely lipophilic, their V_d can be approximated to the lipid content of the body. For example, the V_d of DDT is about 17.5 L lipid (i.e., 25% of body weight of 70 kg) (Kirman et al., 2011). Conversely, the V_d in terms of L of whole blood can be calculated with the knowledge of tissue:blood partition coefficient (P_{tb}, in units of L blood/L tissue). The P_{tb} values reflect the relative distribution of a chemical between tissues and blood, at equilibrium. For example, $P_{tb} = 1$ for a chemical indicates that its concentration will be the same in the tissue and blood at equilibrium, whereas a P_{tb} value of 50 of a chemical in non-metabolizing tissue indicates that the concentration of this chemical will be 50 times greater in that tissue compared to the blood concentration at equilibrium. The P_{tb} along with the corresponding tissue volumes determines the extent of dilution of a chemical in the body. Thus, the sum total of blood volume and tissue volumes (expressed as blood-equivalent volume, i.e., $P_{tb} \times V_t$) would correspond to the V_d of chemicals (Poulin and Theil, 2002, 2009; Rodgers and Rowland, 2007):

$$V_d = V_b + \sum (V_t \cdot P_{tb}), \tag{7-3}$$

where V_b and V_t refer to the volumes of blood and tissue, respectively.

For example, in the case of a chemical that is uniformly distributed in the body with a P_{tb} of 1 (i.e., the concentration in the blood and soft tissues are identical at equilibrium), the total volume of distribution would be equal to 244 mL in the rat (Fig. 7-4 left panel; blood volume of 15 mL plus tissue volume of 229 mL). However, when the P_{tb} of a chemical is greater than 1 (e.g., overall P_{tb} for the soft tissues = 6.77 for *n*-hexane), the apparent distribution volume is greater than the actual physical volume of the animal (Fig. 7-4 right panel; tissue volume × tissue:blood partition coefficient = 229 mL tissue × 6.77 mL blood/mL tissue = 1551 mL blood-equivalent volume plus blood volume of 15 mL for a total V_d of 1566 mL in the rat). Accordingly, for 1,1,1,2-tetrachloroethane, a chemical with P_{tb} values of 2.1, 51.5, and 0.95 for the richly perfused tissues, adipose tissue, and rest of body (Gargas et al., 1989; Andersen et al., 1991), the computed V_d in the rat would be 1.18 L (Table 7-2) even though its body weight (i.e., physical volume) is only 0.25 kg. While V_d for environmental chemicals are often reported in units of L blood (or L blood per kg body weight), the V_d values for drugs are reported in units of L plasma or L plasma water, with or without normalization to body weight. V_d in units of

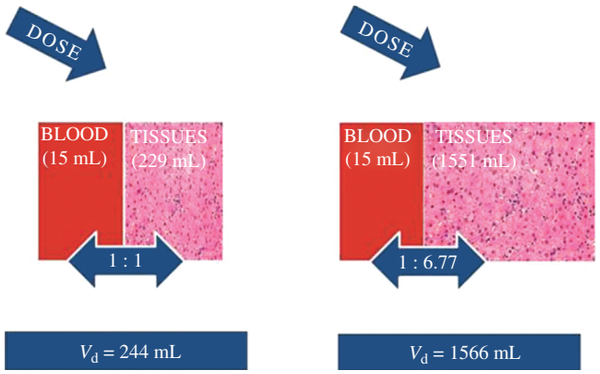

Figure 7-4. *Illustration of the physical and apparent volumes of distribution (V_d).* The left panel shows the physical volume of the rat corresponding to the sum of blood and tissue volumes in which a chemical is distributed equally throughout the body (V_d = 15 + 229 mL = 244 mL). The right panel shows the V_d for a lipophilic chemical equaling 1566 mL, as the sum of the volume of blood (15 mL) and the blood-equivalent volume of tissues calculated as the product of tissue:blood partition coefficient and the tissue volume (i.e., 6.77 × 229 mL = 1551 mL).

L of plasma is calculated by accounting for the interplay of binding to plasma and tissue proteins determining the free or unbound drug concentrations, as follows (Gibaldi and Perrier, 1982):

$$V_d = V_p + \sum\left(\frac{f_{up}}{f_{ut}}\right) \cdot V_t', \tag{7-4}$$

where f_{up} is the unbound and nonionized fraction of toxicant in plasma, f_{ut} is the effective unbound fraction in a tissue region, V_p corresponds to the volume of plasma, and V_t' represents the extravascular space plus erythrocyte volume. In this case, tissue:plasma partitioning is governed by the ratio f_{up}/f_{ut}. Thus, a drug that has high affinity for plasma proteins (e.g., albumin and/or α_1-acid glycoprotein) relative to tissue proteins has a restricted distribution volume; for example, the anticoagulant warfarin with a plasma-bound fraction of 0.995 or f_{up} of 0.005 (Table 7-3). On the contrary, a drug that has a high affinity for tissue proteins and lesser affinity for plasma proteins can have a very high V_d. For example, the tricyclic antidepressant nortriptyline has a good affinity for plasma proteins with a bound fraction of 0.92 or f_{up} of 0.08; however, binding of nortriptyline to tissues constituents is so much higher such that it has a V_d of 18 times the body weight in adult humans (Table 7-3).

Clearance

Clearance (CL) is the apparent volume of a physiological fluid (e.g., blood, plasma, plasma water) that is cleaned of the toxicant per unit time (i.e., mL/min or L/h). Thus, a CL value of 1 L blood/h is interpreted as 1 L of blood being completely cleared of a toxicant in systemic circulation per hour. Accordingly, high values of clearance indicate efficient and generally rapid removal of chemicals from the systemic circulation, whereas low clearance values indicate slow and less efficient removal of a toxicant from the body. A formal definition of total body clearance (CL_{total}) is the ratio of overall elimination rate of a toxicant divided by plasma concentration (i.e., amount eliminated per unit time/C_{plasma}); in effect then, CL_{total} expresses the overall efficiency of the elimination mechanisms operative in the body. Because this measure of elimination efficiency is with respect to blood or plasma toxicant concentration, it is also referred to as blood or plasma clearance.

CL_{total} of a xenobiotic equals the sum of all individual clearance processes occurring in parallel contributing to its removal from systemic circulation, as shown below (Gibaldi and Perrier, 1982; Rowland and Tozer, 2011):

$$CL_{total} = CL_{hepatic} + CL_{renal} + CL_{other}. \tag{7-5}$$

The integral measure of the blood concentration (AUC_0^∞) in relation to the systemic dose (i.e., $Dose_{IV}$, or Dose via extravascular routes adjusted for bioavailability) is useful in computing CL_{total} as follows:

$$CL_{total} = \frac{Dose_{iv}}{AUC_0^\infty} = \frac{F \cdot Dose_{oral}}{AUC_0^\infty}. \tag{7-6}$$

If the non-hepatic clearances are negligible for a chemical, then Eq. (7-5) becomes $CL_{total} = CL_{hepatic}$. Further, as per Eq. (7-6), the inverse relationship between CL and AUC would suggest that for a given dose, the chemical or the species with lower $CL_{hepatic}$ will exhibit higher AUC and hence greater systemic exposure to the parent chemical.

Table 7-2

Tissue:Blood Partition Coefficients (PCs) and Volume of Distribution (V_d) of Some Chemicals in the Rat

	TISSUE:BLOOD PCs			
SUBSTANCES	RICHLY PERFUSED TISSUES	ADIPOSE TISSUES	POORLY PERFUSED TISSUES	V_d (L)*
1,1,1,2-Tetrachloroethane	2.12	51.5	0.95	1.18
1,4-Dioxane	1.00	0.46	0.72	0.18
Acetone	0.60	0.31	0.55	0.14
Bromoform	1.06	20.8	0.58	0.53
Cyclohexane	5.67	169	0.74	3.36
Methyl chloride	1.40	5.47	0.39	0.22

**Volume of distribution (V_d) calculated as VB+ (VR × PRB) + (VF × PFB) + (VP × PPB); PRB, richly perfused tissues:blood PC (using data for liver); PFB, fat:blood PC; PPB, poorly perfused tissues:blood PC (using data for muscle); Blood volume, VB = 0.015 L; richly perfused tissue compartment volume, VR = 0.023 L; adipose tissue volume, VF = 0.018 L; poorly perfused tissue compartment volume VP = 0.188 L.*
SOURCES: Gargas et al. (1989); Reitz et al. (1990); da Silva et al. (1999); Clewell et al. (2001); Andersen et al. (1991).

Table 7-3

Volume of Distribution (V_d) and Unbound or Free Fraction in Plasma (f_{up}) for Several Drugs That Are of Clinical Toxicological Interest

CHEMICAL	V_d (L/kg)	f_{up}
Chloroquine	~200	~0.45
Nortriptyline	18	0.08
Oxycodone	2.0	0.55
Acetaminophen	0.95	<0.20
Phenytoin	0.64	0.11
Warfarin	0.14	0.005

SOURCE: Thummel et al. (2006).

Quantitative descriptions of $CL_{hepatic}$ have been developed by accounting for hepatic blood flow, unbound fraction of chemical at the site of metabolism, and intrinsic metabolic clearance (Gibaldi and Perrier, 1982; Wilkinson, 1987; Rowland and Tozer, 2011). If delivery of the toxicant to its intracellular site of removal is rate-limited by liver blood flow (Q_h) and the toxicant is assumed to have equal, ready access to all the hepatocytes within the liver (i.e., the so-called well-stirred model), then $CL_{hepatic}$ (CL_h) becomes:

$$CL_h = Q_h \cdot E_h = \frac{Q_h \cdot f_{ub} \cdot CL_{int}}{f_{ub} \cdot CL_{int} + Q_h}, \quad (7\text{-}7)$$

where E_h is the hepatic extraction ratio (fraction of the amount of chemical entering the liver that is subjected to elimination by metabolism) and $f_{ub}{\cdot}CL_{int}$ is the hepatic intrinsic clearance that embodies a combination of factors that determine the access of the toxicant to the enzymatic sites (e.g., plasma protein binding and sinusoidal membrane transport) as well as the biochemical efficiency of the metabolizing enzymes (e.g., V_{max}/K_M for an enzyme obeying Michaelis kinetics). CL_{int} would also embody canalicular transport activity if the toxicant is subject to biliary excretion. Eq. (7-7) dictates that CL_h of a toxicant from the blood is bounded by either liver blood flow (Q_h) or intrinsic clearance (i.e., $f_{ub}{\cdot}CL_{int}$). Note that when $f_{ub}{\cdot}CL_{int}$ is very much higher than Q_h, the E_h approaches unity (i.e., near-complete or very high extraction during each passage of toxicant through the hepatic sinusoid) and CL_h approaches Q_h. For example, trichloroethylene exhibits a high CL_{int} value of 15.6 L/h and E_h of 0.92 in the rat (Table 7-4), implying that liver blood flow rather than enzyme content would be the limiting factor of its hepatic clearance. This reflects the fundamental notion that hepatic clearance of a xenobiotic cannot exceed the hepatic blood flow rate (0.004, 0.02, and 1.6 L/min in mice, rats, and humans, respectively) (Travis and Hattemer-Frey, 1991) even if the intrinsic rate of metabolism in the liver is greater than the rate of hepatic blood flow. At the other extreme, when $f_{ub}{\cdot}CL_{int}$ is very much lower than Q_h, the E_h becomes quite small (i.e., low extraction) such that CL_h nearly equals $f_{ub}{\cdot}CL_{int}$. In this instance, CL_h would be limited by the enzyme capacity and not chemical delivery to liver, that is, even if hepatic blood flow brings more chemical than the metabolic capacity of the organ, the metabolism will only occur at the relatively inefficient rate characterized by its CL_{int} (e.g., CL_{int} = 0.02 L/h and E_h = 0.02 for 1,1,1-trichloroethane) (Table 7-4). The extent of hepatic extraction of drugs and xenobiotics is generally referred to as low, medium, or high when the value of E_h is below 0.3, between 0.3 and 0.7, or above 0.7, respectively. The emerging literature supports that the E_h not only reflects hepatic metabolism associated with the unbound chemical but also the albumin-facilitated uptake of certain substances (Poulin et al., 2016, 2017; Fukuchi et al., 2017).

The renal clearance depends upon glomerular filtration, tubular reabsorption, and tubular secretion as follows (Kenakin, 2009):

$$CL_{renal} = f_{ub} \cdot (GFR + CL_{secretion}) \cdot (1 - F_{reabsorbed}) \quad (7\text{-}8)$$

where f_{ub} = unbound fraction of the chemical in systemic circulation, GFR = glomerular filtration rate, and $F_{reabsorbed}$ = fraction reabsorbed. Physiologically, the filtration clearance depends upon GFR (1.79 mL/(min·kg)) while $CL_{secretion}$ is limited by the renal blood flow [17.14 mL/(min·kg)] similar to hepatic clearance (Kunze et al., 2014). Renal clearance can also be calculated by dividing the total amount eliminated in urine by the AUC_{blood}, or alternatively by dividing the urinary excretion rate by the blood concentration.

The pulmonary clearance corresponding to the volume of physiological fluid (e.g., blood) cleared of a toxicant by exhalation is calculated as the alveolar ventilation rate divided by blood:air partition coefficient (P_b). Table 7-4 lists the P_b and the $CL_{pulmonary}$ for some environmental chemicals in the rat. Accordingly, chemicals with a low P_b (e.g., carbon tetrachloride = 4.52) show high $CL_{pulmonary}$ (= 1.19 L/h in the rat), whereas the reverse is true for chemicals with high P_b value (e.g., bromochloromethane with a P_b of 41.5 exhibiting $CL_{pulmonary}$ of 0.13 L/h). The CL and V_d of a substance together determine the elimination constant and half-life, as discussed below.

Elimination Half-life

Elimination rate constant (K_{el}) represents the fraction of the amount of chemical in body that is removed from systemic circulation per unit time. This is analogous to the fraction of the volume of distribution that is subject to elimination by various clearance mechanisms (i.e., CL/V_d). For example, if the CL = 10 L/h and V_d = 100 L, then the K_{el} equals 0.1 h^{-1} implying that 10% of the V_d will be cleared of the chemical per hour. However, the actual amount eliminated in 1 hour would be slightly less given that the amount in the body is declining continuously (from time zero to 1 hour). Thus, in the case of first-order elimination kinetics, the fractional elimination is constant but the amount eliminated is proportional to the amount remaining. For example, if the K_{el} equals 0.3 h^{-1}, the amount eliminated is less than 30% compared to the expectation based on the starting amount in the body (Table 7-5). Because the percent eliminated over a given time period remains constant regardless of the dose, it is more intuitive and convenient to refer to an elimination half-life ($t_{1/2}$), that is, the time taken for the concentration or amount of a chemical (in a biological matrix such as blood or body) to be reduced by half. Thus, after one elimination half-life, the initial blood concentration [C_0] would decline by 50%, that is, [C_0] × 0.5. After the second half-life, the remaining concentration would diminish by 50% due to elimination processes, that is, (0.5 × 0.5 × [C_0]) = 0.25 × [C_0], for a total decline of C_0 by a factor of 0.75 or 75%. After three, four, five, six, and seven half-lives, the initial concentration C_0 would diminish by 87.5%, 93.75%, 96.9%, 98.5%, and 99.2%, respectively. Elimination half-life, $t_{1/2}$, thus reflects the rapidity of disappearance of a toxicant following discontinuation of exposure and it depends upon both V_d and CL as shown below:

$$t_{1/2} = \frac{0.693 \cdot V_d}{CL}. \quad (7\text{-}9)$$

Table 7-4

Hepatic Clearance (CL_h) and Pulmonary Clearance (CL_p) of Some Environmental Chemicals in the Rat

SUBSTANCES	V_{max} (μmol/h)	K_m μM	CL_{int} (L/h)*	CL_h (L/h)†	E‡	Blood:air PC	CL_p (L/h)§
1,1,1-Trichloroethane	0.9	43.1	0.02	0.02	0.02	5.67	0.93
Bromochloromethane	21.5	2.32	9.28	1.17	0.87	41.59	0.13
Carbon tetrachloride	0.9	1.63	0.56	0.40	0.29	4.52	1.19
Chloroform	21.6	2.09	10.3	1.19	0.88	20.8	0.26
Toluene	13.2	1.41	9.36	1.17	0.87	18.0	0.30
Trichloroethylene	29.6	1.9	15.6	1.24	0.92	21.9	0.25

*$CL_{int} = V_{max} / K_m$.
†Hepatic clearance $= (CL_{int} \times Ql) / (CL_{int} + Ql)$; Ql: blood flow to liver = 1.34 L/h.
‡Hepatic extraction ratio $(E) = CL_h / Ql$.
§Pulmonary clearance calculated as ratio of alveolar ventilation (5.38 L/h) to the blood:air partition coefficient of chemicals.
SOURCES: Gargas et al. (1986, 1990); Haddad et al. (1999); Tardif and Charest-Tardif (1999); Tardif et al. (1997).

Table 7-5

Elimination of a Toxicant That Follows First-Order Kinetics ($K_{el} = 0.3\ h^{-1}$) by 1 Hour After IV Administration at Four Different Dose Levels

DOSE (mg)	TOXICANT REMAINING (mg)	TOXICANT ELIMINATED (mg)	TOXICANT ELIMINATED (% of dose)
10	7.4	2.6	26
30	22	8	26
90	67	23	26
250	185	65	26

The above relationship among $t_{1/2}$, V_d, and CL emphasizes that care should be exercised in interpretation of data when relying upon $t_{1/2}$ as the sole representation of elimination of a chemical in toxicokinetic studies, since $t_{1/2}$ is influenced by both the V_d for the toxicant and the rate at which it is cleared from the blood. For a fixed V_d, the $t_{1/2}$ decreases as CL increases because the chemical is being removed from this fixed volume at a faster pace as clearance increases. Conversely, as V_d increases, $t_{1/2}$ increases for a fixed CL because the volume of fluid that must be cleared of chemical increases but the efficiency of clearance does not. In this regard, highly lipophilic persistent organic chemicals exhibit a large V_d and low CL, and thus $t_{1/2}$ is in the order of several years in human adipose tissues (e.g., 2,2′,4,4′,5,5′-hexabromo diphenyl ether: 11.7 years, DDT: 4.2 years) (Geyer et al., 2004; Kirman et al., 2011). It is important to note that because $t_{1/2}$ is a function of CL and V_d of a chemical, it is not an independent toxicokinetic parameter.

The classic and physiologic models of the toxicokinetics of chemicals grounded on the concepts reviewed above (i.e., F, V_d, and CL) are described in the following sections.

CLASSIC TOXICOKINETIC MODELS

Model Structure

Classic toxicokinetics involves empirical analysis of the time-course data of a chemical and/or its metabolite(s) to derive parameters characteristic of their behavior in the exposed organism. This type of analysis uses one or more compartments to capture the toxicokinetics of a chemical in the organism (Fig. 7-5). The simplest structure of the classic toxicokinetic model represents the body as a homogeneous one-compartment system. When the log [C] versus time plot of the toxicokinetic data yields a curve as shown in Fig. 7-6 (top panel vs. bottom panel), it would indicate that differential distribution and equilibration processes among the model compartments need to be accounted for. In such cases, more than one compartment is chosen to adequately model the toxicokinetics of the substance (Figs. 7-6 and 7-7). Basically, in classical toxicokinetic analysis, whether the model structure corresponds to one, two, three, or more compartments is determined by the time-course data. Even though the number and nature of the compartments may not have exact anatomical or physiological correspondence, the resulting model structure and mathematical representation for a chemical would consistently fit with the available empirical data on the kinetics in one or more biological matrices such as whole blood, plasma, urine, exhaled breath, or tissues.

Mathematical Representation

In one-compartment toxicokinetic model reflecting homogeneous distribution of a chemical in the body, the rate of change in the amount ($d\text{Amount}_{body}/dt$, mg/h) equals the difference between the amount absorbed ($\text{Dose}_{absorbed}$) per unit time and amount eliminated from the body ($\text{Amount}_{eliminated}$) per unit time:

$$\frac{d\text{Amount}_{body}}{dt} = \frac{\text{Dose}_{absorbed}}{\text{h}} - \frac{\text{Amount}_{eliminated}}{\text{h}}. \quad (7\text{-}10)$$

Notationally,

$$\frac{dA_{body}}{dt} = K_a D - K_{el} A_{body}, \quad (7\text{-}11)$$

where A_{body} = amount in the body (mg), D = dose administered (mg), K_a = first-order absorption rate constant (h^{-1}), and K_{el} = first-order elimination rate constant (h^{-1}, equal to CL/V_d).

For the simple scenario of a single IV dose, because there is no absorption component, Eq. (7-11) reduces to the following form for describing change in chemical concentration in blood (C_b) due to first-order elimination:

$$\frac{dC_b}{dt} = -K_{el} C_b. \quad (7\text{-}12)$$

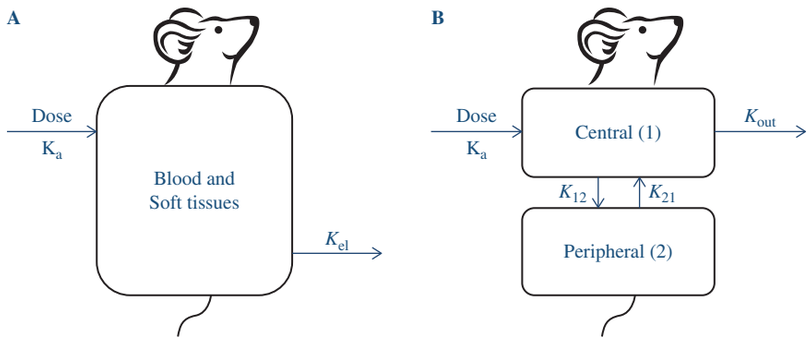

Figure 7-5. *Conceptual representations of compartmental toxicokinetic models.* Symbols for one-compartment model (**A**): K_a is the first-order absorption rate constant, and K_{el} is the first-order elimination rate constant. Symbols for two-compartment model (**B**): K_a is the first-order absorption rate constant into the central compartment (1), from the central compartment (1), K_{12} and K_{21} are the first-order rate constants for distribution between central (1) and peripheral (2) compartments whereas K_{out} is the first-order elimination rate constant from the central compartment.

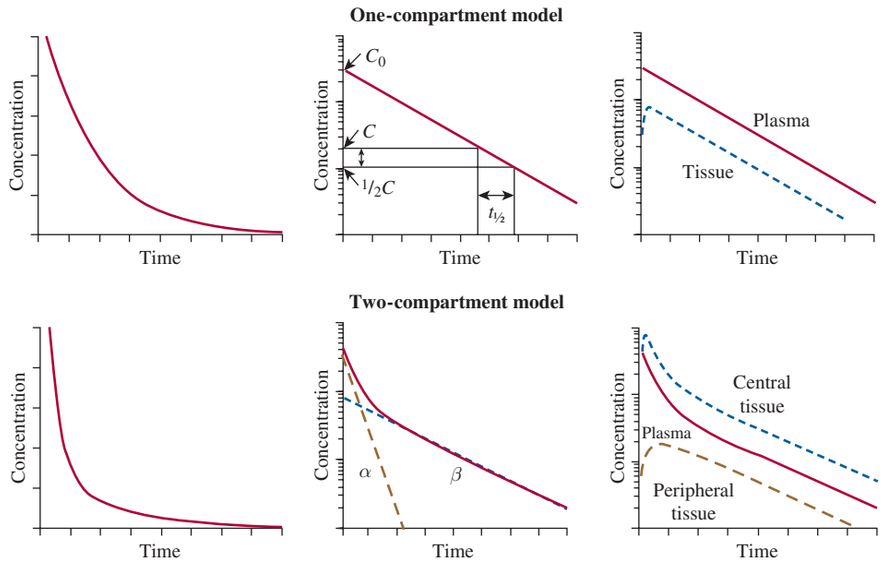

Figure 7-6. *Plasma concentration versus time curves of toxicants exhibiting kinetic behavior conforming to a one-compartment model (top row) and a two-compartment model (bottom row) following IV bolus injection. Left and middle panels show the plots on a rectilinear and semilogarithmic scale, respectively. Right panels illustrate the relationship between tissue (dash lines) and plasma (solid line) concentrations over time.* Note that tissue concentration can be higher, nearly the same, or lower than plasma concentration. Tissue concentration peaks almost immediately, and thereafter declines in parallel with plasma concentration. The right panel for the two-compartment model shows concentration–time profiles for typical tissues associated with the central (1) and peripheral (2) compartments as represented by short and long dash lines, respectively. For tissues associated with the central compartment, their concentrations decline in parallel with plasma. For tissues associated with peripheral compartment, toxicant concentration rises, while plasma concentration declines rapidly during the initial phase; it then reaches a peak and eventually declines in parallel with plasma in the terminal phase. Elimination rate constant K_{el} for one-compartment model and the terminal exponential rate constant β are determined from the slope of the log–linear concentration versus time curve. Half-life ($t_{1/2}$) is the time required for plasma toxicant concentration to decrease by one-half. C_0 is the concentration of a toxicant for a one-compartment model at $t = 0$ determined by extrapolating the log–linear concentration–time curve to the y-axis.

Upon integration based on the initial condition or time zero blood concentration ($C_{b,0}$), the equation to calculate chemical concentration in blood at any given time t ($C_{b,t}$) becomes:

$$\ln C_{b,t} - \ln C_{b,0} = -K_{el} \cdot t \tag{7-13}$$

or

$$C_{b,t} = C_{b,0} \cdot e^{-k_{el} \cdot t}. \tag{7-14}$$

Similarly, equations associated with a one-compartment model of single oral dose kinetics are presented in Table 7-6 (Gibaldi and Perrier, 1982).

When the toxicokinetic data indicate the existence of more than one characteristic tissue(s) or tissue group(s) with different equilibration and elimination profiles, a biexponential equation of the following form may be used to mathematically represent the kinetic behavior contained in the data:

$$C_t = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}, \tag{7-15}$$

where A and B are coefficients in units of toxicant concentration, α is the exponential constant for the distribution phase, and β is the exponential constant for the post-distribution or elimination phase.

In Eq. (7-15), the α constant corresponds to the slope of the residual log-linear plot and not the initial slope of the decline in

Table 7-6

Basic Equations of a One-Compartment Model for the Oral Route

DESCRIPTION	EQUATION
Rate of change in the amount of chemical in the body	$\frac{dA}{dt} = \text{In} - \text{Out}$
Rate of change expressed in terms of rates of absorption and elimination	$\frac{dA}{dt} = (K_a \cdot F \cdot D) - (K_{el} \cdot A_t)$
Concentration at any given time t (for $K_a > K_{el}$)	$C_t = \left(\frac{K_a \cdot F \cdot D}{V_d}\right)\left(\frac{e^{-K_{el}t}}{K_a - K_{el}}\right)$

Abbreviations: A, amount of chemical at any time t; C_t, concentration of chemical at any time t; D, oral dose administered; F, fraction of the oral dose absorbed; K_a, first-order absorption rate constant; K_{el}, first-order elimination constant; V_d, Volume of distribution.

the observed plasma toxicant concentration, that is, the initial rate of decline in plasma concentration approximates, but is not exactly equal to, the α rate constant (Fig. 7-6, bottom panel). It should be noted that distribution into and out of the tissues and elimination of the toxicant in the systemic circulation are occurring at all times, that is, elimination does occur during the "distribution" phase, and distribution between compartments is still ongoing during the "elimination" phase. As illustrated in Fig. 7-7, the dynamics of a bicompartment system is such that the governing factor, that is, be it intercompartmental distribution or elimination, for each of the phases depends on the relative magnitude of the rate constants for intercompartmental exchange and the elimination process. In the usual case, that of rapid distribution and relatively slow elimination (left panel of Fig. 7-7), the initial rapid phase indeed reflects extravascular distribution, and the later, slow phase reflects elimination after a dynamic equilibration between tissue and blood is attained. There are cases where distribution into some tissue group is much slower than elimination (right panel of Fig. 7-7). Then, the initial phase could largely reflect elimination, and the later phase is controlled by the slow redistribution of the toxicant from the tissues associated with the peripheral compartment to the central compartment, where it is eliminated (i.e., washout in the terminal phase is rate-limited by redistribution from the peripheral compartment).

The relative prominence of distributional phases in two-compartment models will often vary depending on the lipid solubility of the toxicant in each tissue grouping and any other sequestration and export mechanisms of a toxicant in particular tissues (e.g., tight binding to tissue proteins, active influx into or efflux out of the tissue cell types), as well as the competing influence of elimination from the visceral organs. For example, very rapid metabolism or excretion at the visceral organs would limit distribution into the slow or very slow tissue groupings. Also, there are times when equilibration rates of a toxicant into visceral organs overlaps with lean body tissues, such that the distribution kinetics of a toxicant into these two tissue groupings become indistinct with respect to the exponential decline of plasma concentration, in which case two instead of three tissue groupings may be observed. The concept of tissue groupings with respect to uptake or washout kinetics serves to justify the seemingly simplistic, yet pragmatic, mathematical description of extravascular distribution by the classic two- or three-compartment models.

Data Analysis and Parameter Estimation

Fig. 7-8A presents the toxicokinetic data, specifically blood concentration versus time, as measured in an experimental study following a single IV dose of 100 mg of a xenobiotic in the rat. Figs. 7-8B and 7-8C depict the same data following the application of $\log_{10}$ and natural log (ln) transformations. Given the behavior seen in these plots (i.e., a straight line in log transformed plots), a one-compartment model is fit to the concentration versus time-course data using Eq. (7-13). In this one-compartment model for a single IV dose, the initial concentration, C_0, corresponding to the

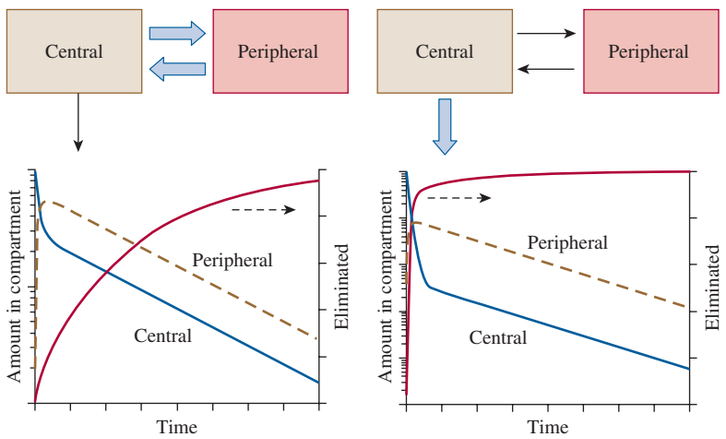

Figure 7-7. *Effects of interplay between kinetics of distribution and elimination processes on time course of exchange between peripheral (red) and central (blue) body compartments and removal from the body for toxicants whose disposition conforms to a two-compartment model.* Left panel depicts the more common scenario of rapid distribution between compartments relative to elimination, in which case elimination from the body occurs largely during the terminal phase when a dynamic equilibration between the central and peripheral compartment has been reached. Accordingly, half-life of the terminal phase is an appropriate measure of elimination. Right panel depicts the scenario of very slow distribution relative to elimination, in which case a substantial (>90% of dose) loss of toxicant occurs during the initial phase. The terminal phase reflects the slow redistribution of the toxicant sequestered in the peripheral site to the central site where it can be eliminated (i.e., washout is rate-limited by redistribution). Under this scenario, the initial phase reflects elimination kinetics, whereas the terminal phase reflects tissue distribution kinetics. (Adapted from Tozer and Rowland, 2006.)

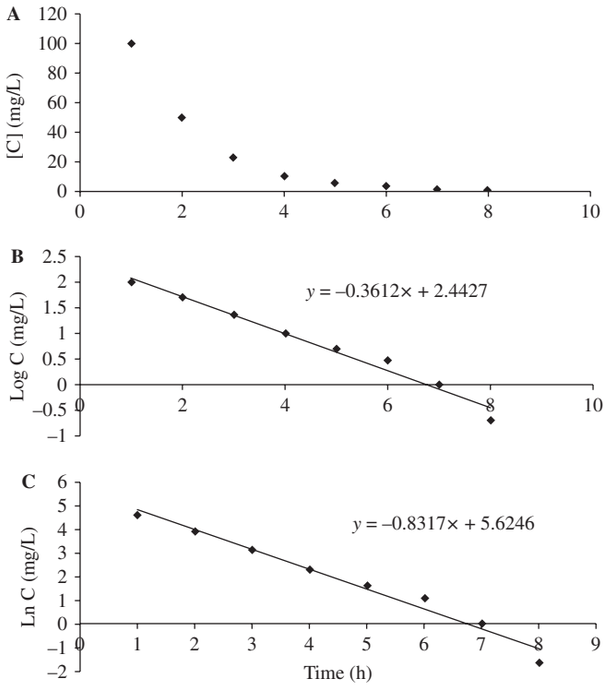

Figure 7-8. *Plots of the measured data (**A**) as well as the transformed data [log_{10} (**B**) and natural log (**C**)] on the blood concentration versus time following a single IV dose.*

intercept in Fig. 7-8C, equals the dose diluted in the apparent volume of distribution, V_d, as follows:

$$V_d = \frac{\text{Dose}}{C_0}. \tag{7-16}$$

Given that the dose in the present example equals 100 mg and the C_0 corresponding to the intercept is 5.62 (= 276 mg/L), the V_d equals 0.36 L. The K_{el} value equals the slope of the natural log transformation plot (Fig. 7-8C) corresponding to the concentration difference (i.e., ln C_t − ln C_0) over a time period (Δt). Thus, the K_{el} associated with this dataset equals 0.832 h^{-1} (Fig. 7-8C) or 0.3612 × 2.303 (Fig. 7-8B). The $t_{1/2}$ or the time taken for the initial concentration C_0 to become half its value (i.e., 0.5·C_0) can be calculated from the K_{el} value as follows:

$$t_{1/2} = \frac{\ln C_0 - (\ln C_0 + \ln 0.5)}{K_{el}} = \frac{0.693}{K_{el}}. \tag{7-17}$$

For the toxicant presented in Fig. 7-8, the $t_{1/2}$ value corresponds to 0.832 h. The toxicokinetic parameters discussed above, particularly K_{el} (or $t_{1/2}$) and V_d, can be used to calculate CL, which is an independent toxicokinetic determinant. For the chemical modeled after a single IV dose in Fig. 7-8, the CL equals 0.3 L/h (i.e., V_d of 0.36 L and K_{el} of 0.832 h^{-1}). The relationship among these parameters does not imply that CL is dependent on V_d or $t_{1/2}$; rather $t_{1/2}$ depends upon CL and V_d.

For a two-compartment model, CL is equal to V_d times β. In multicompartment models, V_d is calculated according to Eq. (7-18) based on the computation of area under the toxicant concentration–time curve:

$$V_d = \frac{\text{Dose}_{IV}}{\beta \cdot \text{AUC}_0^{\infty}}, \tag{7-18}$$

where Dose_{IV} is the IV dose or known amount of toxicant in the body at time zero, β is the elimination rate constant, and AUC_0^{∞} is the area under the toxicant concentration versus time curve from time zero to infinity. Because the concept of an overall apparent volume of distribution is strictly applicable to the terminal exponential phase, some investigators refer to it as V_β (for a two-compartment model) or V_z (for a general multicompartment model) (Gibaldi and Perrier, 1982). When Eq. (7-16), is applied to the situation of a multicompartment model, the resultant volume is the apparent volume of the central compartment and it corresponds to the proportionality constant relating the total amount of the toxicant in the central compartment to its concentration in plasma or whole blood.

When the empirical data on log [C] versus time exhibit behavior reflective of many compartments with differing characteristics, three, four, or more exponential terms may be required to obtain adequate fitting. Under these circumstances, the curve fitting can become a tedious exercise with the resulting model structure and parameters not being straightforward to interpret in physiological and biochemical terms. In such cases, it is preferable to opt for physiologically based models that can associate the observed kinetic behavior with identifiable compartments or tissues (e.g., liver, fat, kidney), as described in the next section.

PHYSIOLOGIC TOXICOKINETIC MODELS

The classic compartmental toxicokinetic models described in the previous section are developed by fitting empirical data on the concentration of parent chemical and/or metabolite(s) in the exposed organism. The parameter estimates obtained with these models are useful for toxicokinetic interpretations limited to the exposure duration, exposure route, dose levels, life stage, and species for which the experimental data were generated. In order to enhance the mechanistic basis of toxicokinetic analysis and to improve the application domain of the resulting parameter estimates, there is increasing interest in physiologically based pharmacokinetic (PBPK) models, also referred to as physiologically based toxicokinetic (PBTK) or biologically based biokinetic (BBBK) models.

The PBPK models describe the interrelationships among the key determinants of uptake and disposition to simulate the time-course behavior of xenobiotics and their metabolites in blood and various tissues. These models are essentially quantitative statements of the current state of knowledge concerning the mechanistic determinants of the toxicokinetics of a chemical in biota. The advantages of PBPK models in comparison with the classic compartmental models are that they permit: (i) the simulation of the concentration of chemicals and their metabolites not only in the blood but also in any other tissue of interest including the target organ, (ii) the evaluation of the nature and magnitude of the potential impact of physiological, physicochemical, and metabolic parameters on the toxicokinetics of chemicals, (iii) the prediction of the toxicokinetics of chemicals across species and populations using relevant input parameters, (iv) accounting for temporal change in physiological and metabolic parameters, complex dosing regimens, and saturable processes, and (v) the simulation of toxicokinetic datasets collected under various dosing situations (exposure routes, doses, and scenarios) with a single set of input parameters and equations. The impediments to the use of PBPK models relate to the fact that not only these models contain more parameters compared to the classic compartmental models but also the mathematics and programming required are not trivial. Furthermore, the range of values and distributions of input parameters are not always well defined for various chemicals and their metabolites in all species and pathophysiological states of interest. Nevertheless, PBPK models are conceptually

sound tools capable of providing insights into the toxicokinetics and tissue dose of toxicants beyond what classic compartmental models can provide because of the way in which they are constructed, parameterized, and implemented. The construction of the classic toxicokinetic model requires time-course data on the concentrations of chemicals in one or more biological matrices to begin with, whereas the PBPK modeling approach frequently uses a priori parameter estimates in mechanism-based equations to simulate the toxicokinetics of chemicals in biota. While the former approach determines the number, behavior, and volumes of the compartments based on the fitting exercise, the structure and complexity of a resulting PBPK model are determined by the critical determinants of toxicokinetics, available data sets, and intended end-use.

The practice of incorporating biological and physiological concepts into the analysis and interpretation of toxicokinetic data is not entirely new. The PBPK-type descriptions were developed for gaseous anesthetics as early as the 1920s and then applied to model the uptake and disposition of certain biopharmaceutics, antineoplastics, and volatile organic chemicals (Haggard, 1924; Teorell, 1937; Kety, 1951; Mapleson, 1963; Riggs, 1970; Bischoff et al., 1971; Dedrick et al., 1972; Fiserova-Bergerova, 1975). Since the publication of a PBPK for styrene by Ramsey and Andersen (1984), there has been increasing interest in the development of PBPK models for xenobiotics and drugs (Reddy et al., 2005; Krishnan and Andersen, 2010). Fig. 7-9 presents the key steps of PBPK modeling, with a specific end-use as the motivation for model development (e.g., chemical risk assessment) (WHO, 2010). Defining the scope and intended application of the PBPK model up-front is important to ensure that the model development process is concordant with the required level of precision and extent of evaluation. Overall, the construction of PBPK models begins with the development of a model structure, mathematical representation of key processes, and estimation of model parameters. The PBPK model equations and parameters are then written as a computer program, solved to provide toxicokinetic simulations, and compared with available data to guide further refinement or analysis (sensitivity, uncertainty, variability) in view of assessing the suitability of the model for the intended application.

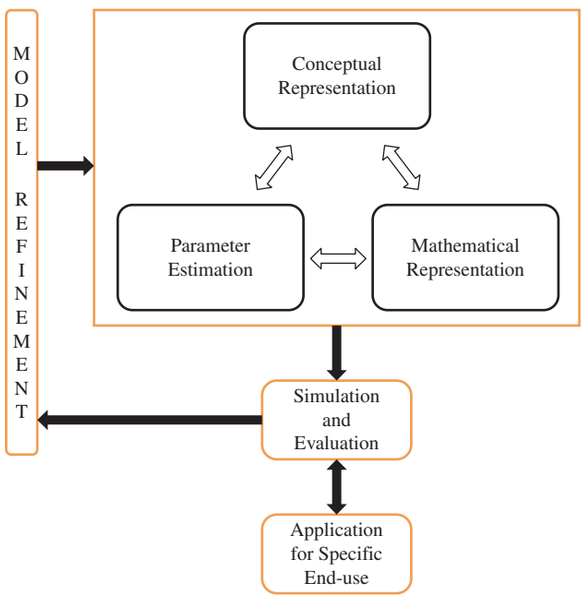

Figure 7-9. *Simplified schematic of the steps involved in the development of physiologically based toxicokinetic models.*

Model Structure

The structure of a PBPK model for a given chemical corresponds to a diagrammatic representation of key processes and pathways determining its toxicokinetics in the organism (i.e., species or individual). The PBPK model is represented as boxes and arrows, with the boxes corresponding to the organs (individual or groups) and the arrows reflecting the physiological flows and clearance processes. The art of developing a PBPK model structure requires striking a balance between the principles of parsimony (i.e., minimal but essential elements) and plausibility (i.e., reflective of physiological reality), while being consistent with the current state of knowledge on the toxicokinetics and mode of action of the chemical or drug under study. In other words, the complexity of PBPK models increases only when it is required to explain the behavior contained in the toxicokinetic data. In PBPK models, increments of complexity generally involve the addition of compartments with different time-constants (i.e., blood flow divided by the distribution volume specific to each tissue compartment) or mathematical representations of mechanisms to account for specific accumulation or disposition behavior of the chemical under study. The level of detail relates not only to the compartments (e.g., whole body, target tissue, or intracellular concentrations) but also to the chemical forms that are tracked within the model (e.g., parent chemical, metabolites, mixture components).

For a hydrophilic chemical whose concentrations across compartments are comparable, a one-compartment PBPK model might well be sufficient. However, for chemicals exhibiting different time-course profiles in various tissues, a multicompartmental PBPK description is required. Fig. 7-10 shows the structure of a four-compartmental PBPK model used for simulating the inhalation toxicokinetics of toluene, a lipophilic volatile organic chemical. In this particular case, the model compartments together represent ≈ 91% of body mass in adult rats. The remaining 9% (corresponding to the mass of the skeletal/structural components) is not included in the PBPK model because toluene does not exhibit significant affinity for, or accumulate in, skeletal and structural components. In this particular model, the "lung" compartment only represents the portal of entry, assuming no significant pulmonary metabolic activity toward toluene. The four compartments of this model corresponding to liver, fat, poorly perfused tissues, and richly perfused

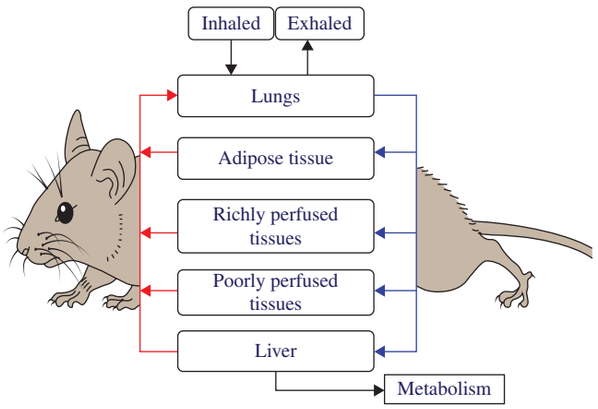

Figure 7-10. *Physiologically based toxicokinetic model for toluene, a low-molecular-weight volatile organic chemical.* The main route of its intake is via inhalation and absorption by the lungs. Transport of chemical throughout the body by blood is depicted by the arrows. Elimination of toluene as depicted by the model includes metabolism and exhalation. All compartments are perfusion-limited.

tissues together account for the mass balance of toluene entering the body and the mass balance of physiological flows in the model at any given time. The "fat" compartment in the PBPK model represents the adipose tissues in the body representing storage depot for toluene. The richly perfused tissue compartment comprises tissues such as adrenal, kidney, thyroid, brain, lung, heart, and testis whereas the poorly perfused tissue compartment represents muscle and skin, characterized by differences in the blood flow rate per gram tissue (Table 7-7). Whereas lumping of various tissues into a single compartment is ideal when the concentration versus time profiles is similar across the tissues, the splitting of compartments into component tissues are done often because of the need to simulate chemical concentrations in (i) target organ(s), (ii) organs with significant metabolism, binding, and excretion processes, (iii) sites with specific affinity or capacity for accumulation of the chemical under study (e.g., teeth, bone, fat), (iv) portals of entry into the body, or (v) those tissues that exhibit significant difference in time-constants due to physical activity or pathophysiological states.

Table 7-7

Examples of Tissues Often Lumped Together as a Compartment in PBPK Models

TISSUE GROUPS	EXAMPLES
POORLY PERFUSED TISSUES	Muscle
	Skin
RICHLY PERFUSED TISSUES	Adrenal
	Kidneys
	Thyroid
	Brain
	Lungs
	Heart
	Testis

Mathematical Representation

The fundamental equations used for characterizing xenobiotic concentrations in tissue compartments of PBPK models are given in Table 7-8. The following paragraphs provide sample descriptions of the computation of concentrations of neutral, nonionized chemicals in metabolizing and non-metabolizing organs as well as in systemic circulation (Himmelstein and Lutz, 1979; Gerlowski and Jain, 1983; Ramsey and Andersen, 1984; Rowland, 1985).

Computing Tissue Concentrations The rate of change in the amount of chemical in the tissue compartments (dA/dt) is proportional to the concentration difference ($C_{in} - C_{out}$) and the membrane permeability coefficient (k), as per Fick's first law as described below:

$$\frac{dA}{dt} = k \cdot (C_{in} - C_{out}). \tag{7-19}$$

Because the chemical input to the tissue (amount per unit time) equals the influx clearance (CL_{in}) times incoming concentration (C_{in}), whereas the chemical removal (amount per unit time) equals efflux clearance (CL_{out}) and the concentration leaving the compartment (C_{out}), the above mass balance differential equation (MBDE) can be rewritten as follows:

$$\frac{dA}{dt} = (CL_{in} \cdot C_{in}) - (CL_{out} \cdot C_{out}). \tag{7-20}$$

The concentration of free chemical leaving a tissue compartment, C_{out}, depends upon the amount of chemical in that particular compartment (A_t) and its distribution volume (i.e., tissue volume, V_t multiplied by tissue:blood partition coefficient, P_{tb}), such that the above equation becomes:

$$\frac{dA}{dt} = (CL_{in} \cdot C_{in}) - \left(CL_{out} \cdot \frac{A_t}{V_t \cdot P_{tb}}\right). \tag{7-21}$$

The clearance terms in the above equation depend upon the membrane permeability and tissue blood flow. Quantitatively, the influx

Table 7-8

Equations for Calculating the Rate of Change, Amount, and Concentration of Chemical in a Flow-Limited Compartment of a Physiologic Toxicological Model

DESCRIPTION OF THE EQUATION	MATHEMATICAL FORM
Rate of change in amount of chemical in tissue = Tissue blood flow rate × [Concentration in arterial blood – Concentration in venous blood leaving tissue]	$\frac{dA_{tissue}}{dt} = Q_{tissue} \times (C_a - C_{vt})$
Amount of chemical in tissue = [Rate of change in amount in tissue × Integration time interval] + Previous amount in tissue	$A_{tissue} = \left[\frac{dA_{tissue}}{dt} \times dt\right] + A_{tissue,t-1}$
Concentration of chemical in tissue = Amount in tissue/Tissue volume	$C_{tissue} = \frac{A_{tissue}}{V_{tissue}}$
Chemical concentration in venous blood leaving tissue = Concentration in tissue/Tissue:blood partition coefficient	$C_{vt} = \frac{C_{tissue}}{P_{tissue:blood}}$

Abbreviations: C_{tissue}, *concentration in tissue (mg/liter or mmol/liter);* Q_{tissue}, *blood flow rate (liters·h*$^{-1}$*);* V_{tissue}, *tissue volume (liters);* $P_{tissue:blood}$, *tissue:blood partition coefficient;* A_{tissue}, *amount in tissue (mg);* t, *current time;* $t-1$, *previous simulation time;* C_a, *arterial blood concentration;* C_{vt}, *venous blood leaving tissue.*
SOURCE: Based on Ramsey and Andersen (1984).

and efflux clearances can be expressed in terms of permeation area cross product (PA) and tissue blood flow rate (Q_t) as follows: PA $\times$ Q_t/(PA + Q_t). Accordingly, when the PA of a chemical is very large compared to Q_t, the latter becomes the limiting factor such that influx and efflux clearance values are equal to Q_t. In contrast, when PA << Q_t (i.e., membrane diffusion limiting the chemical movement into and out of a tissue compartment), then influx and efflux clearance values would equal PA (Gerlowski and Jain, 1983). In this regard, for small molecular weight compounds, blood flow rather than the membrane diffusion is the limited factor, such that the above MBDE can be rewritten as follows (Ramsey and Andersen, 1984):

$$\frac{dA}{dt} = Q_t(C_a - C_{vt}), \tag{7-22}$$

where C_a = concentration of chemicals in arterial blood entering the tissue compartment and C_{vt} = concentration of chemical in venous blood leaving the tissue

For high-molecular-weight compounds, the membrane diffusion rather than tissue blood flow is the limiting factor such that their flux through the cellular matrix and tissue blood needs to be accounted for separately (Table 7-9). If the diffusion of a chemical from tissue blood to cellular matrix is slow with respect to tissue blood flow, then both the extracellular and intracellular components are characterized (Fig. 7-11 and 7-12). However, when the tissue perfusion rate (i.e., blood flow) is very small compared to membrane diffusion, then the tissues are described as homogeneous, well-mixed compartments. Consequently, the rate of change in the amount of chemical in the tissue is described with a single equation for the whole tissue mass (Eq. 7-22), followed by the use of the output of this equation to calculate concentration in the tissue and venous blood leaving the tissue (Table 7-8).

For compartments exhibiting metabolic capacity, the rate of the amount cleared by metabolism (dA_{met}/dt) is also included such that the MBDE becomes:

$$\frac{dA}{dt} = Q_t(C_a - C_{vt}) - \frac{dA_{met}}{dt}. \tag{7-23}$$

The rate of metabolism in PBPK models is described as a first-order, second-order, or saturable process (Table 7-10). When the amount metabolized per unit time is directly proportional to the input variable (e.g., free concentration of chemical in liver), metabolism is described as a first-order process. The second-order equation is employed when the rate of metabolism depends on the available concentration of both the cofactor and the chemical. The saturable metabolism, often described as a Michaelis–Menten reaction, reflects first-order behavior at very low substrate concentrations and zero-order behavior at concentrations that are far excessive of the Michaelis parameter, K_m. All the metabolism descriptions presented in Table 7-10 are based on venous blood concentration (i.e., free concentration) of a xenobiotic, reflecting the "venous equilibration model" descriptions. Because the calculation of the rate of metabolism is integrated within Eq. (7-23), the blood flow limitation (i.e., perfusion limitation) of hepatic metabolism

Table 7-9

Equations Used for Describing Diffusion-Limited Uptake in Physiologic Toxicokinetic Models

SUBCOMPARTMENTS	EQUATIONS
Tissue blood	$\frac{dA_1}{dt} = Q_t \times (C_{in} - C_{out}) - [PA] \times (C_1 - C_2)$
Cellular matrix	$\frac{dA_2}{dt} = [PA] \times (C_1 - C_2)$

Abbreviations: C, concentration (mg/liter or mmol/liter); Q, flow rate (liters.h^{-1}); V, volume (liters); A, amount (mg); PA, permeability-area coefficient; C_1–C_2, concentration differential between tissue blood and cellular matrix.

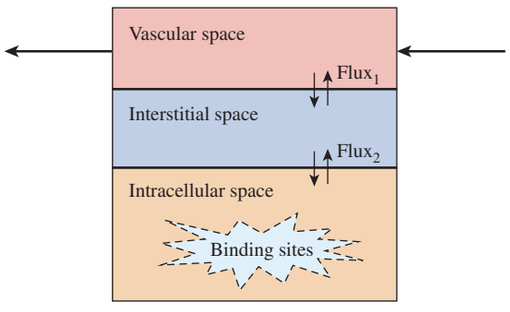

Figure 7-11. *Schematic representation of a lumped tissue compartment in a physiologically based toxicokinetic model.* The blood capillary and cell barriers separating the vascular, interstitial, and intracellular subcompartments are depicted in heavy black lines. The vascular and interstitial subcompartments are often combined into a single extracellular subcompartment.

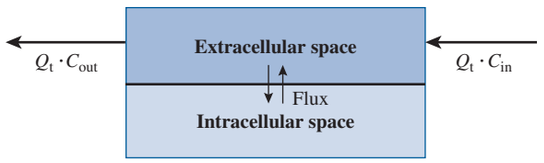

Figure 7-12. *Schematic representation of a tissue compartment that features membrane-limited uptake kinetics.* Perfusion of blood into and out of the extracellular compartment is depicted by longer arrows. Transmembrane transport (flux) from the extracellular to the intracellular subcompartment is depicted by shorter arrows. $\boldsymbol{Q}_t$ is blood flow, $\boldsymbol{C}_{in}$ is toxicant concentration entering the compartment, and $\boldsymbol{C}_{out}$ is toxicant concentration leaving the compartment.

Table 7-10

Examples of Equations Used for Calculating the Rate of the Amount of Chemical Metabolized (dA_{met}/dt) in Physiologic Toxicokinetic Models

REACTION TYPE	EQUATION
First-order process	$\frac{dA_{met}}{dt} = K_f \cdot C_{vt} \cdot V_t$
Second-order process	$\frac{dA_{met}}{dt} = K_s \cdot C_{vt} \cdot V_t \cdot C_{cf}$
Saturable process	$\frac{dA_{met}}{dt} = \frac{V_{max} \cdot C_{vt}}{K_m + C_{vt}}$

Abbreviations: C_{cf}, concentration of the cofactor; C_{vt}, concentration of chemical in venous blood leaving the tissue; K_f, first-order reaction rate; K_m, Michaelis constant; K_s, second-order reaction rate constant; V_{max}, maximal velocity; V_t, volume of metabolizing tissue.

is automatically accounted for. Alternatively, the blood flow to liver and hepatic extraction ratio can be used explicitly to describe metabolism in PBPK models (as $Q_h \cdot E_h \cdot C_a$, where Q_h = liver blood flow rate, E_h = hepatic extraction ratio, and C_a = arterial blood concentration), but the E_h may be dose-dependent when saturation occurs in the dose range of interest.

Analogous to the hepatic clearance, the MBDE can accommodate biliary and fecal clearances by taking into account the rate of bile flow, the rate of transfer into and reabsorption from the bile, as per the molecular weight of the chemical or its conjugated metabolite. Urinary excretion is modeled as a function of the rates of filtration, reabsorption, and secretion (Eq. 7-8). The amount of chemical filtered (dF/dt) and the rate of change in the concentration of chemical or its metabolite in the urine (dU/dt) are calculated as follows:

$$\frac{dF}{dt} = \text{GFR} \cdot C_v, \tag{7-24}$$

$$\frac{dU}{dt} = U_o \cdot C_u, \tag{7-25}$$

where GFR = glomerular filtration rate, U_o = urinary output per unit time, C_v = unbound blood concentration of xenobiotic, and C_u = xenobiotic concentration in urine. The amount of chemical secreted (or reabsorbed) then equals the difference between the amount in urine and the amount filtered (Eq. 7-8).

Computing Blood Concentrations The rate of change in the amount of chemical in blood compartment in PBPK models can be calculated conceptually as per Eq. (7-22). Alternatively, the mass conservation equations can be solved to compute blood concentrations, as done for volatile organic chemicals (Ramsey and Andersen, 1984). In this regard, chemical input to the lung compartment equals the amount inhaled ($Q_p \times C_{inh}$) plus the amount returning to lungs via mixed venous blood ($Q_c \times C_v$), whereas the output equals the amount leaving the lungs via arterial blood ($Q_c \times C_a$) plus the amount exhaled ($Q_p \times C_{alv}$), as depicted in Fig. 7-13. Notationally,

$$Q_p C_{inh} + Q_c C_v = Q_c C_a + Q_p C_{alv}, \tag{7-26}$$

where C_{inh} = inhaled concentration, C_v = mixed venous blood concentration, C_a = arterial concentration, C_{alv} = alveolar concentration, Q_p = alveolar ventilation rate, and Q_c = cardiac output.

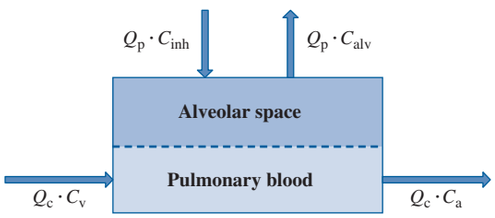

Figure 7-13. *Simple model for exchange of nonreactive volatile organic chemicals in the alveolar region of the respiratory tract.* Rapid exchange of toxicants in the simplified lung compartment between the alveolar gas (*blue*) and the pulmonary blood (*light blue*) maintains an equilibrium between them as symbolized by the dashed line. $\boldsymbol{Q_p}$ is alveolar ventilation (L/h); $\boldsymbol{Q_c}$ is cardiac output (L/h); $\boldsymbol{C_{inh}}$ is inhaled vapor concentration (mg/L); $\boldsymbol{C_a}$ is concentration of chemical in the arterial blood; $\boldsymbol{C_v}$ is concentration of chemical in the mixed venous blood. The equilibrium relationship between the chemical in the alveolar air ($\boldsymbol{C_{alv}}$) and the chemical in the arterial blood ($\boldsymbol{C_a}$) is determined by the blood:air partition coefficient $\boldsymbol{P_b}$, that is, $\boldsymbol{C_{alv}} = \boldsymbol{C_a}/\boldsymbol{P_b}$. (Based on Ramsey and Andersen, 1984.)

Given that lung equilibrates vapor between alveolar air and blood ($C_{alv} = C_a/P_b$), the above equation can be rewritten to compute arterial blood concentration as follows (Ramsey and Andersen, 1984):

$$C_a = \frac{Q_p C_{inh} + Q_c C_v}{\left(Q_c + \dfrac{Q_p}{P_b}\right)}. \tag{7-27}$$

The above equation simplifies to $C_a = C_v$ for highly nonvolatile chemicals (with a negligible value of C_{inh} and very large P_b) not metabolized in the lung compartment. In such situations, the conducting airways (i.e., nasal passages, larynx, trachea, bronchi, and bronchioles) are considered as inert tubes that carry the chemical to the pulmonary region, where diffusion occurs. When simple continuous ventilation-equilibration models are insufficient to describe the uptake behavior (e.g., regional uptake of highly soluble polar solvents), the adsorption and/or dissolution in the surface of the respiratory epithelium during inhalation as well as desorption during exhalation are taken into account along with the cyclic nature of respiratory exchange (Johanson, 1991).

The mixed venous blood concentration, C_v, entering the lung compartment results from the venous concentration of chemicals leaving the various tissue compartments following their equilibration as a function of the tissue:blood partition coefficients. The C_v is not a simple average of C_{vt} of the tissue compartments, as the fraction of cardiac output flowing into and out of the tissues is not identical across the compartments. Considering the fractional flow through the various compartments in the rat (e.g., liver = 0.25, fat = 0.09, slowly perfused tissues = 0.15, and richly perfused tissues = 0.51 [Tardif et al., 1997]), the C_v is calculated as the weighted average concentration as follows:

$$C_v = (0.25 \cdot C_{vl}) + (0.09 \cdot C_{vf}) + (0.15 \cdot C_{vs}) + (0.51 \cdot C_{vr}), \tag{7-28}$$

where subscripts vl, vf, vs, and vr correspond to venous blood leaving the liver, fat, slowly perfused tissue, and richly perfused tissue compartments.

Parameters: Significance and Estimation

The equations of the PBPK models contain a number of input parameters, which are conveniently grouped as physiological, physicochemical, or biochemical parameters including rate constants of reactions and clearance processes relevant to the chemical being modeled.

Physiological Parameters The physiological parameters consist of volumes and flows; the volumes are required for the tissues or tissue groups represented as boxes whereas flows are associated with the arrows in the model which include alveolar ventilation rate, cardiac output, and organ-specific blood flow rates. Tissue volumes along with their composition, relative to that of blood, determine the extent to which chemicals are diluted within each compartment. The greater the volume of tissue and lipid content, the larger the V_d of lipophilic xenobiotics, thus leading to lower blood concentrations. For example, the volume of adipose tissue represents about 19% to 25% of body weight in adult humans. This adipose volume along with the high P_{tb} for lipophilic organic chemicals (e.g., adipose tissue P_{tb} for DDT = 199) contributes significantly to the V_d (e.g., for DDT 70kg × 0.25 L/kg × 199 = 3483 L) (Haddad et al., 1999; Kirman et al., 2011), and as a source of prolonged internal exposure to such chemicals. In contrast, the tissue blood flow influences the rate at which a chemical in systemic circulation is delivered to a tissue. Tissues with smaller volumes and high perfusion rates will

attain maximal extent of accumulation for a given exposure situation (i.e., steady-state condition) faster compared to other tissues.

The ventilation rate in mammals, in general, is about 15 to 18 L/h/kg but it is not directly proportional to the body weight (BW) of mammals but to the body surface area or approximately to $BW^{0.7}$. Experimental measurements of breathing rates have been obtained with the use of a spirometer, plethysmograph, pneumotachograph, hotwire anemometer, or nonbreathing valves (Krishnan and Andersen, 2007). The cardiac output, a key determinant of the rate at which absorbed chemicals are distributed to and collected from all tissues, has been obtained from dye dilution curves using oximeters. The cardiac output is faster in smaller animals (e.g., mice) than in larger animals (e.g., humans), and again proportional to body surface area or $BW^{0.7}$. The cardiac output in mammals is often comparable to the ventilation rate such that the ratio of perfusion rate:ventilation rate is about 1 in resting individuals, but it can vary as a function of workload and physical activity.

While anthropometric data and breathing rates are often obtained in test animals/subjects, other physiological parameters required for PBPK modeling are obtained from existing literature or calculated on the basis of body weight for each species and life-stage (O'Flaherty, 1994; Brown et al., 1997; Corley et al., 2003; Price et al., 2003a, 2003b; Dewoskin and Thompson, 2008). Table 7-11 presents a summary of frequently used values of basic physiological parameters for adult rats, mice, and humans. Databases on animal and human physiological parameters for various age groups, pathophysiological states, and strains/races are still evolving. While the average value or the range of plausible values of physiological parameters is sufficient for routine model development, the statistical distributions of such parameters would be essential if the model is intended for use in estimating interindividual differences in tissue dosimetry.

Physicochemical Parameters The physicochemical parameters required for PBPK modeling are the partition coefficients, which represent the relative distribution of a nonionized chemical between two matrices at equilibrium. Of these, the tissue to blood partition coefficient (P_{tb}) is a key determinant of the volume of distribution specific to each tissue compartment of a PBPK model, whereas the blood:air partition coefficient (P_b) is a determinant of the pulmonary uptake and clearance of volatile organic chemicals. The pulmonary absorption of chemicals possessing a relatively high P_b is limited by alveolar ventilation rate, whereas that of chemicals with low P_b is limited by cardiac output. Experimentally, the values of P_b have been obtained in vitro by vial equilibration technique conducted at 37°C (Gargas et al., 1989). They can also be computed as the ratio of solubility of a chemical in blood and air as determined by the solubility in neutral lipid, phospholipid, and water components of the blood as follows (Poulin and Krishnan, 1996b):

$$P_b = P_{oa} \cdot (F_{nb} + 0.3F_{pb}) + P_{wa} \cdot (F_{wb} + 0.7F_{pb}), \tag{7-29}$$

where P_{oa} = *n*-octanol:air or vegetable oil:air partition coefficient, P_{wa} = water:air partition coefficient (inverse of the unitless Henry's law constant), F_{nb} = fraction of neutral lipids in blood, F_{pb} = fraction of phospholipids in blood (considered to behave as a mixture of neutral lipids [30%] and water [70%], based on the hydrophobicity characteristics of commercial lecithin [Poulin and Krishnan, 1995]), and F_{wb} = fraction of water in blood.

While this algorithm has worked well for the prediction of P_b of relatively hydrophilic organic chemicals (ketones, alcohols, acetates ester, and diethyl ether), it is insufficient for more lipophilic chemicals such as alkanes, halogenated alkanes, and aromatic hydrocarbons, given their affinity for, and binding to, the hydrophobic pockets in the hemoglobin. The apparent blood:air PC ($P_{b,app}$) for such chemicals has been successfully described with the P_b based on lipid and water solubility as well as binding calculated using the association constant (K_a), the concentration of haemoglobin (C_p), and the concentration of the free form of the chemical in erythrocytes ($C_{a_{free}}$) as shown below (Poulin et al., 1999):

$$P_{ba,app} = P_b \cdot \left(1 + \frac{K_a \cdot C_p}{(1 + K_a \cdot C_{a_{free}})}\right). \tag{7-30}$$

The tissue:blood or tissue:plasma partition coefficients can be estimated experimentally using in vivo pharmacokinetic data or in vitro techniques such as equilibrium dialysis and ultrafiltration (Krishnan and Andersen, 2007). Animal-alternative methods have been emerging, and have given rise to a variety of algorithms accounting for molecular features, physicochemical characteristics, or mechanisms relevant for a training set of chemicals (Table 7-12). In this regard, the simple mechanistic algorithms based on tissue and blood composition take into account the solubility of a xenobiotic in blood and tissues as the sum total of its solubility in neutral lipids, phospholipids, and water, as follows (Poulin and Krishnan, 1995, 1996a, 1996b):

$$P_{tb} = \frac{[P_{ow} \cdot (0.3F_{pt} + F_{nt})] + [0.7F_{pt} + F_{wt}]}{[P_{ow} \cdot (0.3F_{pb} + F_{nb})] + [0.7F_{pb} + F_{wb}]}, \tag{7-31}$$

where P_{tb} = tissue:blood partition coefficient, P_{ow} = *n*-octanol:water or vegetable:oil partition coefficient, F_{nt} = fraction of neutral lipids in the tissue, F_{pt} = fraction of phospholipids in tissue, and F_{wt} = fraction of water in tissue. Table 7-13 summarizes the data on the rat blood and tissue content of neutral lipids, phospholipids, and water.

In order to appreciate the mechanistic basis of P_{tb} using the above equation, let's examine highly lipophilic organic chemicals with $P_{ow} \geq 1000$ not exhibiting plasma protein binding. In this case, the second term in Eq. (7-31) (i.e., water solubility) is negligible compared to the first term (i.e., lipid solubility) in both the

Table 7-11
Physiological Parameters of Adult Mice, Rats, and Humans for PBPK Modeling

PHYSIOLOGICAL PARAMETERS	MOUSE	RAT	HUMAN
Cardiac output (Qc) (liters/min)	0.017	0.083	6.20
Alveolar ventilation (liters/min)	0.025	0.117	5.00
Tissue volumes (fraction of body weight)			
Liver	0.055	0.04	0.026
Fat	0.10	0.07	0.190
Organs	0.05	0.05	0.05
Muscle and skin	0.70	0.75	0.62
Tissue perfusion (fraction of cardiac output)			
Liver	0.25	0.25	0.26
Fat	0.09	0.09	0.05
Organs	0.51	0.51	0.44
Muscle and skin	0.15	0.15	0.25

SOURCE: Travis and Hattermer-Frey (1991).

Table 7-12

Tissue Composition Algorithms for Predicting Partition Coefficients for Physiologically Based Toxicokinetic Models

PARTITION COEFFICIENT	SUBSTANCES	REFERENCES
Tissue:blood partition coefficient	Volatile and nonvolatile organics	Poulin and Krishnan, 1995, 1996a, 1996b; Peyret et al., 2010
	Hydrophilic organics	Poulin and Krishnan, 1995, 1996a, 1996b; Haddad et al., 2000; Poulin and Theil, 2002; Peyret et al., 2010
Tissue:plasma water distribution coefficient	Bases	Poulin and Theil, 2002; Rodgers et al., 2005; Schmitt, 2008; Peyret et al., 2010
	Acid, neutrals, zwitterions	Poulin and Theil, 2002; Rodgers and Rowland, 2006; Schmitt, 2008; Peyret et al., 2010

Table 7-13

Average Lipid and Water Content (as %) of Rat Tissues and Blood*

TISSUE	NEUTRAL LIPIDS	PHOSPHOLIPIDS	WATER
Adipose	85.30	0.20	12
Blood	0.33	0.24	82
Liver	3.50	2.50	70
Muscle	0.90	1.00	74

**Data from Poulin et al. (1999).*

numerator and denominator, such that P_{tb} equals the ratio of the content of neutral lipid-equivalents (NLE, i.e., neutral lipids plus 30% of phospholipids) in tissues and blood. For example, in the case of organic chemicals with P_{ow} values ranging from 2738 to 67,200 (cyclohexane, *n*-pentane, methylcyclopentane, 2,2-dimethyl butane, *n*-hexane, 2-methyl pentane, *n*-heptane and 3-methyl hexane), the average human adipose tissue:blood ($P_{at:b}$) is nearly equal to 199, which is the ratio of the NLE content in adipose tissues and blood. Similarly, the reported human adipose tissue:serum partition coefficients of PCBs, PBBs, DDT, dieldrin, mirex, and hexachlorocyclohexane range from 140 to 336, whereas the average ratio of NLE content in human adipose tissue and serum equals 173 (Haddad et al., 2000). In contrast, the P_{tb} of highly hydrophilic chemicals equals the ratio of tissue to blood content of water-equivalents (WE, i.e., water plus 70% phospholipids). For example, in the case of alcohols, acetate esters and ketones with P_{ow} of ≤ 1, the human liver:blood partition coefficients range from 0.81 to 1.00, comparable to the ratio of WE in liver and blood (= 0.86). The ratio of the content of WE in muscle to blood equals 0.90 whereas that for human adipose tissue to blood is about 0.15 (Haddad et al., 2000).

Rate Constants and Biochemical Parameters Rate constants and biochemical parameters required for PBPK modeling include oral absorption rate constant, dermal permeability coefficient, membrane diffusion coefficient, maximal velocity for metabolism, Michaelis parameter, binding association constant, and urinary/biliary excretion rate. These parameters have often been determined on the basis of time-course data collected in vivo or in vitro (Krishnan and Andersen, 2007; Bessems et al., 2014). Typically, data analysis to estimate parameters is conducted using the portion of the time-course curve that is most sensitive to the dominant factor(s). Even though there have been examples of successful integration of in vitro data within PBPK models to simulate in vivo kinetics, the adequacy of in vitro–in vivo extrapolation is not well established for all test systems and processes (Terasaki et al., 1984; Farris et al., 1988; Frederick et al., 1992; Bale et al., 2014; Bessems et al., 2014).

In silico approaches applicable to xenobiotics are evolving based on the rich database for pharmaceutical substances regarding the determinants of oral absorption (i.e., lipophilicity, p*K*a, solubility, particle size, permeability, release kinetics, and dissolution kinetics), as well as mathematical models and algorithms to simulate the rate of drug absorption in humans. For the metabolism constants, there are increasing examples of scaling to in vivo of parameters obtained in vitro with microsomes, post-mitochondrial fractions preparations, and freshly isolated hepatocytes (Bale et al., 2014). Generally, the K_m values obtained in vitro have been used directly or by taking into account the difference in the composition of the in vitro and in vivo matrices, whereas the V_{max} obtained in vitro have been scaled to the whole organism based on the mass recovery of the particular fraction as exemplified by the following equation for the microsomal data:

$$V_{max(\text{in vivo})} = V_{max(\text{in vitro})} \cdot C_{protein} \cdot F_{tissue}, \qquad (7\text{-}32)$$

where $V_{max\ (\text{in vivo})}$ = maximal velocity of metabolism in vivo (mg/min/kg body weight), $V_{max\ (\text{in vitro})}$ = maximal velocity of metabolism in vitro (mg/min/mg microsomal protein), $C_{protein}$ = concentration of microsomal protein (mg/g tissue), and F_{tissue} = fractional volume of the metabolizing tissue (e.g., g liver/kg body weight). In the absence a priori estimates of numerical values of V_{max} and K_m, by specifying the range of plausible values of hepatic extraction in PBPK models (e.g., nearly zero as the lower limit and 100% extraction as the theoretically plausible upper limit), an envelope of the range of blood concentration–time profiles can be generated prior to in vivo experimentation (Fig. 7-14). In silico approaches for predicting metabolic rates and elimination half-lives of xenobiotics in experimental systems and biota are still evolving.

Model Simulation

The equations in a PBPK model are solved to obtain simulation of the toxicokinetics of xenobiotics for a given species, dose, and exposure scenario (Fig. 7-15). In order to solve the MBDE of each tissue compartment, the arterial (input) blood concentration needs to be known, which depends upon mixed venous concentration as well as the chemical input resulting from absorption. The mixed venous concentration in turn depends upon the chemical concentration in venous blood leaving the tissue compartments, such that the input and output of all compartments within the PBPK model are interconnected in a loop-type calculation. Fundamental to the

implementation of these calculations is the integration of the differential equations in the PBPK model. Thus, the temporal evolution of tissue concentration is computed from the amount of chemical in the tissue compartment, resulting from the integration of the differential equation for the rate of change in the amount of chemical in tissue (dA_t/dt) with the use of step-by-step procedures referred to as integration algorithms (e.g., Euler, Gear, Runge-Kutta routines, and predictor-corrector methods) (Rideout, 1991). In the simplest form, the integration algorithm takes into account incremental change in the dependent variable at the end of a simulation period (referred to as the integration interval) and adds it to the initial or starting value, to generate the new value of the dependent variable at the end of each time interval, as follows:

$$A_{t,t1} = A_{t,t0} + \left\{\frac{dA}{dt} \cdot \Delta t\right\}, \tag{7-33}$$

where Δt = integration interval (e.g., $t_1 - t_0$), $A_{t,t1}$ = final amount in the tissue after the integration interval, $A_{t,t0}$ = initial amount in tissue, and dA/dt = the rate of change in the amount of chemical in the tissue compartment during the time interval.

The numerical simulation models, such as the PBPK models, typically require the use of simulation software or packages. There are many commercially available software packages that have features required for rapid construction of PBPK models. Alternatively, spreadsheet programs such as Microsoft EXCEL® can be used to construct simple models and examine the temporal change in the chemical concentration in the various compartments (e.g., Haddad et al., 1996; Krishnan and Andersen, 2007).

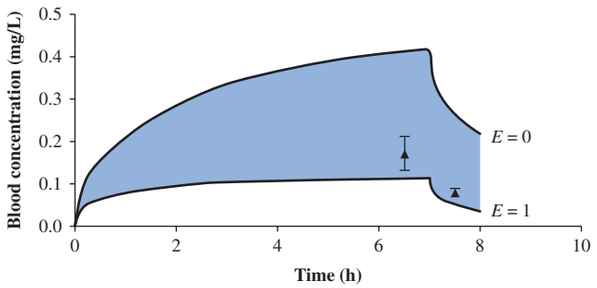

Figure 7-14. Physiologic toxicokinetic model simulations of the envelope of minimum and maximum venous blood concentration profiles of toluene following inhalation exposure, using hepatic extraction ratio (E) of zero (i.e., no metabolism in liver) and one (i.e., complete extraction in liver). The data points correspond to human data obtained from the published literature for the same exposure concentration and scenario.

Model Verification and Evaluation

Model verification focuses on whether the PBPK model is correctly coded (error-free) and the toxicokinetic simulations could be reproduced in a different programming software or computational platform. Model evaluation focuses on comparing the simulations (usually continuous) with experimental toxicokinetic data (usually discrete), and ascertaining whether the model with its structure and parameters is fit to provide output required for a specific end-use. Given that both the PBPK model simulations and experimental data are subject to uncertainty and variability, it is not realistic to expect an exact match between every single data point and the simulation output and therefore PBPK models providing predictions within a factor of 2 of the experimental data have generally been considered to be adequate. As such, the model evaluation focusing on fit-for-purpose and not simply on fit-to-data is becoming the practice. In this regard, WHO (2010) outlined a three-point approach to conduct evaluation of PBPK models with emphasis on the (i) biological basis, (ii) model performance, and (iii) reliability of the model output (i.e., predictions of dose metrics) for the intended end-use (Fig. 7-16). Accordingly, the consideration of biological basis focuses on whether the model structure and range of the input parameters used in model development are biologically plausible, without contradicting the fundamental physiological principles or the mass balance (Table 7-14, column 1). The aspect of model performance focuses on whether the model is able to adequately reproduce toxicokinetic data collected from more than one particular experiment using more than one dose and route of administration (Table 7-14, column 2). Finally, the consideration of reliability of dose metric predictions focuses on how well the PBPK model simulates the dose metrics of relevance to the end-use (e.g., AUCs for interspecies extrapolation in risk assessment), with particular emphasis on the results of uncertainty analysis and sensitivity analyses (Table 7-14, column 3). While the uncertainty analysis involves the evaluation

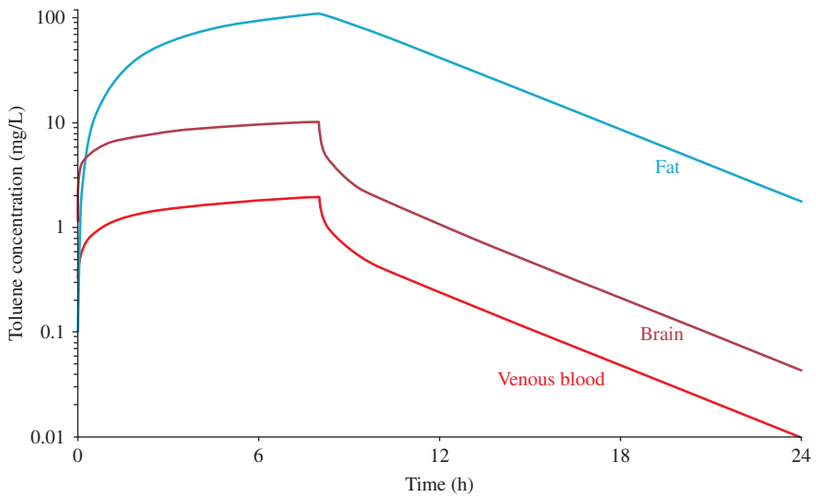

Figure 7-15. *Physiologic toxicokinetic model simulations of the time-course concentrations of toluene in fat, brain, and venous blood in humans exposed to 100 ppm for 8 hours.* (Based on Tardif et al., 1997.)

of the impact of the lack of precise knowledge of parameter values and model structure on toxicokinetic simulations, the sensitivity analysis is a quantitative evaluation of how input parameters influence the model output of dose metrics of relevance (e.g., AUC). As illustrated in Fig. 7-17, if the highly sensitive parameters are also the most uncertain or if the model simulations of dose metric are not sensitive to large variations of the values of some input parameters, then such results can be used to guide the refinement of the model and/or parameters before its intended use. However, if a small change in a parameter value (i.e., the magnitude of which is lower than that associated with measurement variability) only leads to changes in output that are smaller than, or comparable to, the variation in experimental measurement of the dose metric, then the PBPK model would be considered to be reliable. When conducting the uncertainty and sensitivity analyses, it is important to ensure that (i) the numerical values of physiological parameters remain within plausible limits, (ii) the sum of tissue volumes is within or equal to the body weight, (iii) the sum of tissue blood flows is

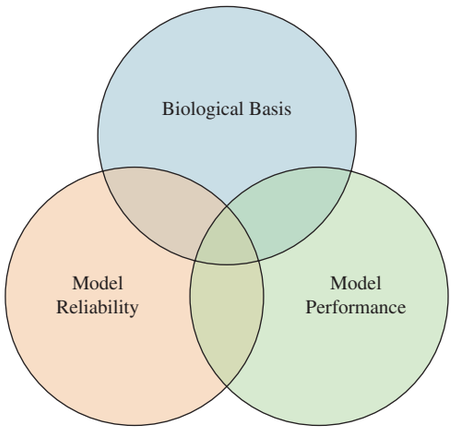

Figure 7-16. *Key aspects for characterizing the level of confidence in physiologic toxicokinetic models intended for specific end-use, as per WHO (2010).*

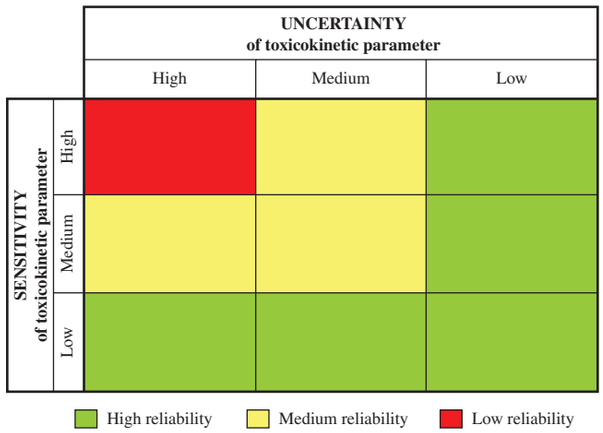

Figure 7-17. *Illustration of the role of sensitivity and uncertainty analyses in determining the reliability of the dose metric predictions, as per WHO (2010).* Sensitivity analysis results are presented as high (value of sensitivity ratio (SR) greater than or equal to 0.5), medium (SR greater than or equal to 0.2 but less than 0.5), or low (SR greater than or equal to 0.1 but less than 0.2). Uncertainty analysis results are summarized as high uncertainty (value could be a factor of 2 or higher), medium uncertainty (value could be a factor between 0.3 and 2), or low uncertainty (value could be a factor of 0.3 or lower). Uncertainty is defined as the ratio of the 95th percentile over the median value for the dose metric. A sensitivity ratio (SR) of 1 implies that a 1% change in input of a parameter value leads to a 1% change in dose metric prediction.

Table 7-14

Checklist for Characterizing the Level of Confidence in Physiologically Based Toxicokinetic (PBPK) Models

BIOLOGICAL BASIS OF PBPK MODELS	PERFORMANCE OF PBPK MODELS	RELIABILITY OF PBPK MODELS
• The sum total of the tissue blood flow rates equals the cardiac output specified within the model. • The sum total of the volumes of compartments equals the soft tissue volumes or body weight, as the case may be. • The values of tissue volumes and tissue blood flow rates (individual or group) specified in the model are within the documented range for the particular species and life stage. • The ventilation:perfusion ratio specified in the model is within physiological limits. • The allometric scaling of parameters, if applicable, is consistent with the known physiological principles. • The major sites/processes of absorption, distribution and clearance are included in the model. • The mathematical equations of ADME do not violate the known theoretical or biological principles. • The input parameters adequately reflect the characteristics of the subject, population, chemical, and exposure pathway.	• The PBPK model consistently reproduces the general trend of the toxicokinetic data (i.e., peaks, bumps, and valleys, saturation of metabolism). • The PBPK model predictions are within an acceptable level of correspondence with the experimental data (e.g., within a factor of two). • The PBPK model reproduces the chemical-specific toxicokinetic data under various experimental conditions or exposure scenarios.	• The PBPK model is capable of providing reliable predictions of the concentration–time course of the candidate dose metrics in the target organ or a suitable surrogate compartment (e.g., blood). • The data used for calibrating and/or evaluating the PBPK model are reliable. • The sensitivity of the output (dose metric) to change in numerical values of input parameters has been characterized for relevant human or animal exposures. • The uncertainty in PBPK model predictions of dose metric has been assessed for the relevant exposure conditions.

equal to the cardiac output, (iv) the mass balance is respected at all times (amount of chemical absorbed = amount in body + amount metabolized + amount of excreted), and (v) the covariant nature of parameters is taken into account, in conformity with the statistical and biological basis (e.g., the subject with the lowest breathing rate is not combined with the highest cardiac output).

In conclusion, the PBPK model is mechanistically based and more complex than the classical data-based toxicokinetic models but it is still a simplified representation of the biological system under study. The PBPK models, as other mechanistic models, only account for those determinants and processes that are hypothesized to be critical by the investigator(s). The complexity and number of compartments are thus not reflective of the accuracy and usefulness of a given PBPK model, but are considered to be critical for simulating the toxicokinetics of the chemical in a given experimental subject or species. Finally, the species, developmental stage, exposure route, and dose range corresponding to the toxicity study and the PBPK modeling study should be compared to select the appropriate model for use in interpreting a toxicity study.

TOXICOKINETIC TOPICS AND APPLICATIONS

The interpretation of toxicology studies has sometimes been conducted on the basis of administered dose or absorbed dose when such data were available. However, the dose to the target site is the more desired and relevant dose metric. In this regard, the toxicokinetic models are useful for estimating systemic exposure, in terms of the toxic form of a chemical (parent or metabolite) over a specific duration in blood or the target organ. Such a measure reflective of the dose to the target is critical not only for interpreting the toxicity data but also for conducting various extrapolations in the case of systemically acting chemicals (i.e., those chemicals that induce toxicity at target sites distant from the portals of entry by accessing them via systemic circulation). The following sections describe these topics and applications facilitated by toxicokinetic parameters and models.

Nonlinear Kinetics

The distribution and clearance of most chemicals follow first-order kinetics in the low-dose region. Accordingly, the K_{el}, V_d, CL, and $t_{1/2}$ are expected to be same in the low-dose range (i.e., no dose-dependent change in kinetic parameters occurs under first-order condition). In other terms, under first-order conditions, the dose metrics (e.g., C_{max}, AUC) will be nearly identical when compared on the basis of unit dose (e.g., per µg/kg/day) or exposure concentration (per ppm). A semilogarithmic plot of blood concentration versus time over a range of doses will show a set of parallel lines such that blood concentration at a given time or the AUC_0^∞ remain proportional to dose. Thus, a twofold change in dose (µg/kg/day) will result in a twofold change in blood concentration at a given time after dosing or in the value of AUC_0^∞ if first-order conditions prevail in the dose range. However, for some toxicants, as the dose increases, its V_d and/or CL may change, showing nonlinear or dose-dependent kinetics (Fig. 7-18). Enzyme-mediated metabolism, active transport processes, and protein binding have finite capacities and therefore can be saturated. For instance, most metabolic enzymes operate in accordance to Michaelis–Menten kinetics (Table 7-10), such that the rate of metabolism (amount per unit time) depends upon the unbound concentration of the substrate ([S]) as well as intrinsic clearance ($CL_{int} = \frac{V_{max}}{K_m + [S]}$), the latter representing the ability of the enzyme machinery to cleanse the medium in which the substrate is present (e.g., blood) (Gibaldi and Perrier, 1982; Rowland and Tozer, 2011). Accordingly, at very high substrate concentrations (i.e., [S] >> K_m), the rate of metabolism will be equal to V_{max} (i.e., the maximal capacity of the enzyme) and thus reflective of a zero-order process, that is, the rate of metabolism remains the same regardless of the substrate concentration when the latter is well above the K_m. In contrast, at very low substrate concentrations (i.e., when [S] is very small and negligible compared to K_m), the rate of metabolism is proportional to the concentration of the substrate (i.e., first-order process) because CL_{int} remains constant and equal to V_{max}/K_m. Taken together, it implies that as the dose increases and the unbound concentration of a toxicant at the site of metabolism approaches or exceeds the K_m (substrate concentration at one-half of V_{max}), the increase in metabolism rate becomes less than proportional to the dose and eventually approaches a maximum at very high doses. The model simulations shown in Fig. 7-19 indicate the nonlinear nature of blood AUC and C_{max} in the selected dose range for toluene. In such cases, the high dose to low dose extrapolation of tissue dosimetry should not be conducted assuming linearity; rather it should be performed using tools such as the PBPK models that account for the nonlinear mechanism and its determinants.

The transition from first-order to zero-order (or saturation) kinetics is important in toxicology because it can lead to prolonged persistence of a compound in the body after a single dose or an acute exposure, and excessive accumulation during repeated or chronic exposure. Some of the salient characteristics of nonlinear kinetics include the following: (i) the decline in the concentrations of the parent chemical in the body is not exponential, (ii) AUC_0^∞ and amount metabolized are not proportional to the dose, (iii) V_d, CL, K_{el} (or β), or $t_{1/2}$ change with increasing dose, (iv) the composition

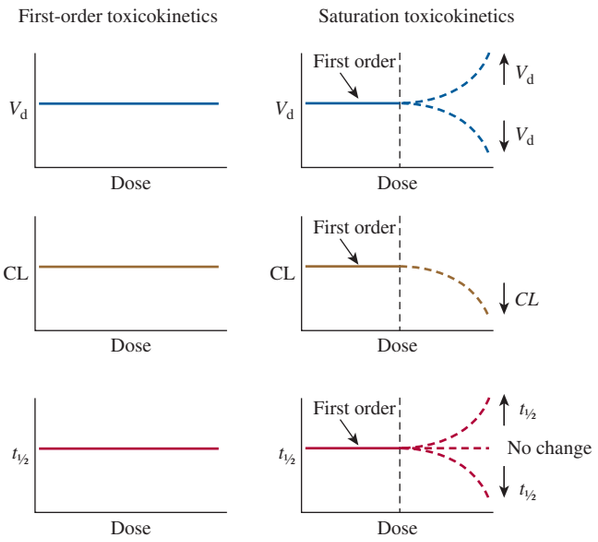

Figure 7-18. *Changes in V_d, CL, and $t_{1/2}$ following first-order toxicokinetics (left panels) and following saturable toxicokinetics (right panels).* Vertical dashed lines in the right panels represent point of departure from first-order to saturation toxicokinetics. Toxicokinetic parameters that follow first-order toxicokinetics are independent of dose. When plasma protein binding or elimination mechanisms are saturated with increasing dose, such parameter estimates become dose-dependent. V_d may increase, for example, when protein binding is saturated, allowing more free toxicant to distribute into extravascular sites. Conversely, V_d may decrease with increasing dose if tissue protein binding saturates. Then, toxicant may redistribute more freely back into plasma. When toxicant concentrations exceed the capacity for biotransformation by metabolic enzymes, overall clearance of the toxicant decreases. These changes may or may not have effects on $t_{1/2}$ depending upon the magnitude and direction of changes in both V_d and CL.

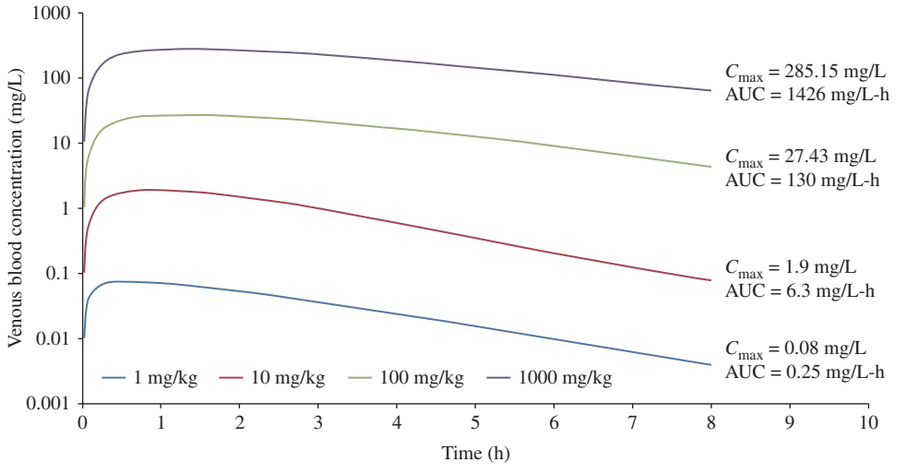

Figure 7-19. *Physiologic toxicokinetic model simulations of the venous blood concentration of toluene in humans administered a single oral dose of 1, 10, 100, and 1000 mg/kg.*

of excretory products changes quantitatively or qualitatively with the dose, and (v) dose–response curves depict a steeper change in response to an increasing dose, starting at the dose at which saturation effects become evident. In addition to the complication of dose-dependent kinetics, there are toxicants whose clearance might change over time (i.e., time-dependent kinetics). A common cause of time-dependent kinetics is autoinduction of metabolizing enzymes through activation of gene transcription by certain drugs and xenobiotics. The classic example of autoinduction is with the antiepileptic drug, carbamazepine. Daily administration of carbamazepine leads to a continual increase in clearance and shortening in $t_{1/2}$ over the first few weeks of therapy (Bertilsson et al., 1986).

PBPK models facilitate high-dose to low-dose extrapolation of tissue dosimetry by accounting for the mechanism(s) underlying the nonlinear kinetic behavior of chemicals (e.g., saturable metabolism, enzyme induction, enzyme inactivation, protein binding, and depletion of glutathione reserves) (Clewell and Andersen, 1987, 1994). Quantitative descriptions of such mechanisms are essential for any toxicokinetic model to facilitate extrapolation or interpolation of dose metrics from high dose to low dose. The capacity-limited hepatic metabolism leading to the nonlinear dose–response behavior is best illustrated with vinyl chloride (Slikker et al., 2004). Similarly, in the case of dichloromethane, the PBPK model facilitated high-dose to low-dose extrapolation of the potential dose metrics by accounting for the saturation of CYP2E1-mediated metabolism and disproportionate increase in glutathione *S*-transferase-mediated metabolism with increasing doses (Andersen et al., 1987, 1991). For the conduct of high-dose to low-dose extrapolation with PBPK models, frequently no change in the numerical values of input parameters would be required, with the exception of the administered dose or exposure concentration.

Single versus Repeated Dose Kinetics

Continuous or chronic exposure to a chemical can lead to its accumulation in the body depending upon its elimination half-life and interval between the doses. For example, lipophilic chemicals with large V_d and very small CL exhibit long half-lives and accumulate as the exposure continues. On the contrary, for a chemical with a very small $t_{1/2}$ compared to the time interval between doses, it is likely to be eliminated almost completely before the next dose such that there would be no significant accumulation. Let's consider a chemical with $t_{1/2}$ of 4 hours is administered to experimental animals once every 24 hours. In this case, the amount eliminated would be proportional to the body burden (i.e., first-order elimination kinetics) in the dose range of interest. Thus, the amount eliminated after one half-life or 4 hours = 50%, two half-lives = 75%, three half-lives = 87.5%, four half-lives = 93.75%, five half-lives = 96.875%, six half-lives = 98.438%, seven half-lives = 99.219%, and eight half-lives = 99.61%. If the second dose is administered at 4 hours, that is, after one half-life, then 50% of the chemical from the first dose will still be in the body to which the second dose adds leading to higher blood concentration than what was seen after a single dose. The narrower the interval between doses or the shorter the dosing interval compared to the $t_{1/2}$, the faster the attainment of steady state will be. Fig. 7-20 shows the same time to attain 50% steady state for various rates of intake; on the contrary, the steady-state blood concentration is strictly proportional to the intake rate. Typically, the rise in blood concentration is relatively rapid after dosing, as it is governed by the early (distribution) half-life, and becomes slower at later times when the terminal (elimination) half-life becomes dominant. Therefore, when the doses are given at certain intervals of time (and not in a continuous fashion), the fraction eliminated between two dosing intervals needs to be accounted for computing the dose metrics.

Let's consider that a single dose of 100 mg of a chemical (with an elimination rate constant K_{el} of 0.693 day^{-1}; elimination half-life of 1 day; and V_d = 10 L) is given to rats once per day. The initial blood concentration in this case would be 10 mg/L, which then becomes 5 mg/L after 1 day (i.e., 24 hours), and after the second dose it raises to 15 mg/L, which then becomes half-that-quantity after another day (i.e., 7.5 mg/L), and so on. The fraction eliminated during a given interval of time (τ) and the maximal and minimal concentrations attained during repeated doses given at intervals of τ can be calculated by accounting for the fraction eliminated during that time frame (i.e., $1 - e^{-K_{el} \cdot \tau}$). In the example presented in Fig. 7-21, for chemical A with K_{el} = 0.0007 h^{-1}, the concentrations increase markedly after each additional dose. If the K_{el} is increased to 0.01 h^{-1}, then 21% is eliminated between doses as shown in Fig. 7-21 for chemical B. On the contrary, if the elimination is rapid, then it is likely that the "between-dose" accumulation could be negligible (Fig. 7-21, chemical C). Therefore, the knowledge of the fraction eliminated during the time interval between doses, and the extent to which C_{max} and C_{min} fluctuate during a dosing interval, figure among key considerations in designing toxicity studies.

At a fixed level of continuous exposure, the toxicant in the body reaches a steady state when the intake rate equals its elimination rate such that the body burden stays constant, as illustrated by the conceptual PBPK model depicted in Fig. 7-22. Steady-state concentration of a toxicant in blood (C_{ss}) is related to the intake rate (mg/kg/day) and clearance (L/kg/day) as follows:

$$C_{ss} = \frac{\text{Intake Rate}}{\text{CL}}. \qquad (7\text{-}34)$$

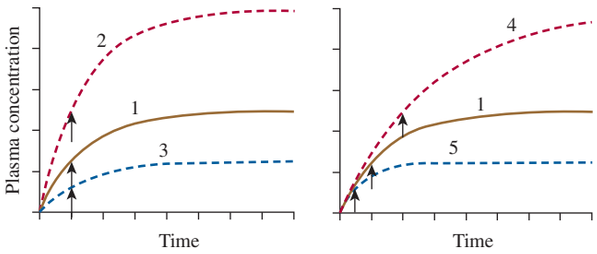

Figure 7-20. *Accumulation of plasma toxicant concentration over time during constant, continuous exposure as a function of exposure level (left panel) and elimination half-life (right panel).* These simulations are based on a one-compartment model at a constant apparent volume of distribution. Case 1 serves as the reference with an elimination half-life set equal to one arbitrary time unit. In the left panel, which illustrates accumulation of toxicant as a function of exposure level, exposure level is raised by twofold in case 2 and lowered by 50% in case 3. The changes in eventual steady-state concentration are proportional to the changes in exposure level, that is, increased by twofold in case 2 and decreased by 50% in case 3. During continuous exposure, 50% of steady state is achieved in one half-life. Near plateau or steady state (>90%) is reached after four half-lives. Since the elimination half-life is constant across cases 1 to 3 in the left panel, the time it takes to attain 50% of steady-state concentration (see arrows) is the same. Right panel illustrates the influence of elimination half-life and clearance on accumulation at a fixed constant rate of exposure. Case 4 represents a 50% decrease in clearance and a corresponding twofold increase in elimination half-life compared to case 1. Case 5 represents a twofold increase in clearance and a corresponding 50% decrease in elimination half-life. Changes in both the time to attain steady state and the steady-state concentration are evident. In case 4, the steady-state concentration increased by twofold as a result of a 50% reduction in clearance, and the time to achieve 50% of steady state increased by twofold as a result of the prolonged elimination half-life. In case 5, the steady-state concentration is reduced by 50%, while the time to reach 50% steady state is shortened by 50%.

The daily dose in the above equation should account for the route-specific bioavailability (F) and the CL in turn can be calculated as $V_d.K_{el}$. When the target steady-state blood or plasma concentration is fixed (e.g., in case of therapeutic application), the product of CL and C_{ss} equals the maintenance dose rate. The C_{ss} as derived in Table 7-15 is reflective of the AUC averaged over the time interval τ, whereas the product of the average C_{ss} and V_d equals the average body burden at steady state.

The compartmental models including the PBPK models described in the preceding sections are useful in the estimation of blood and tissue concentrations associated with a single dose or repeated doses given by various routes, and this can frequently be accomplished using a single set of parameters. In other instances, re-estimation or adjustment of parameter values might be necessary to accommodate the observed or anticipated change in input parameters with time (e.g., change in V_d due to body fat content, change in CL_{int} due to enzyme induction). Fundamentally, the rate of change in the amount of chemical in non-metabolizing tissues in PBPK models becomes equal to zero at steady state, such that the tissue concentrations remain stable during repeated exposures. The steady-state blood concentration obtained with the PBPK model will be the same as that obtained using Eq. (7-34) (Andersen, 1981; Gibaldi and Perrier, 1982). PBPK models also have been used to predict the kinetic behavior and tissue dosimetry of chemicals during unusual exposure scenarios (e.g., adjusted threshold limit values during extended hours of work, physical activity/work load, pregnancy and lactation, or coexposure to chemicals) (Clewell and Andersen, 1987; Krishnan et al., 2002; Corley et al., 2003).

Metabolite Kinetics

Metabolism of a parent chemical or drug can result in bioactivation or detoxication, with the resulting products subjected to further metabolism to more active or inactive moieties. When the toxicity is attributed to the biotransformation product(s), the formation and subsequent disposition kinetics of a toxic metabolite are of interest not only from the perspective of toxicology but also biological monitoring. As the parent chemical is transformed into a metabolite, the blood concentration of the metabolite rises depending upon its reactivity, V_d, and CL. Once formed, the metabolite might exhibit reactivity/stability, be subjected to further biotransformation reactions to form other metabolites, or undergo excretion via the kidneys

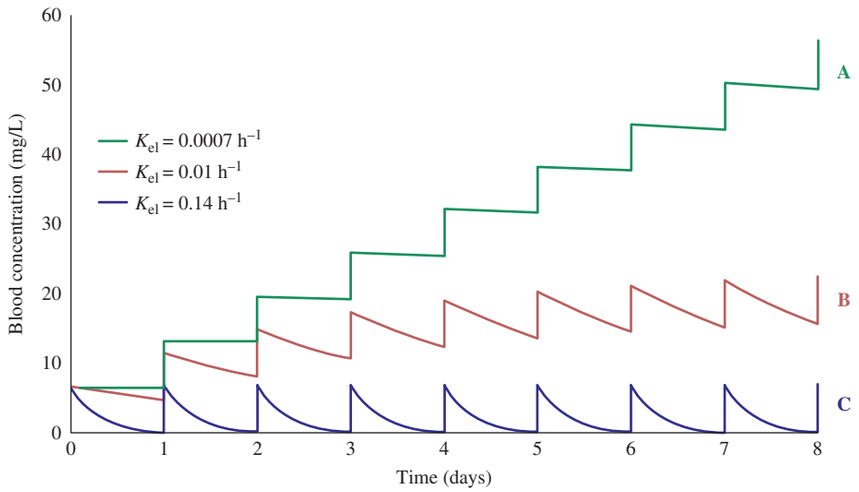

Figure 7-21. *Time-course plots of the blood concentrations of xenobiotics A, B, and C administered once a day.* The first-order elimination rate constants are 0.0007 h^{-1}, 0.01 h^{-1}, and 0.14 h^{-1}, respectively for A, B, and C.

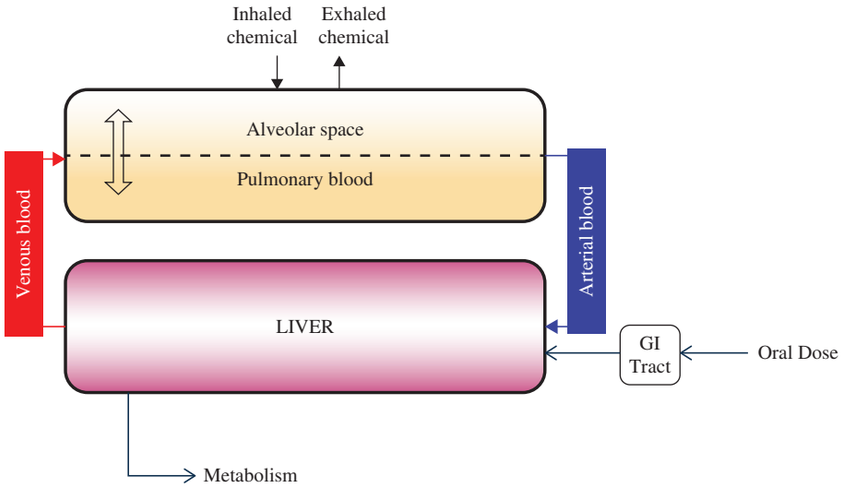

Figure 7-22. *Physiologic toxicokinetic model of the steady-state kinetics of a chemical administered by the oral and inhalation route.*

Table 7-15

Set of Equations to Understand Steady-State Kinetics

DESCRIPTION	EQUATION
Rate of change in the amount of a chemical in the body	$\frac{dA}{dt} = \text{Dose rate} - (A_t \cdot K_{el})$
Rate of change at steady state	$\text{Dose rate} - (A_t \cdot K_{el}) = 0$
Amount in body at steady state	$A_{ss} = \frac{\text{Dose rate}}{K_{el}}$
Blood concentration at steady state	$C_{ss} = \frac{\text{Dose rate}}{V_d \cdot K_{el}}$
Blood concentration at steady state in relation to clearance	$C_{ss} = \frac{\text{Dose rate}}{\text{CL}}$

Abbreviations: A_{ss}, amount in the body at steady state; CL, clearance; C_{ss}, blood concentration of chemical at steady state; Dose rate, absorbed dose per unit time; K_{el}, first-order elimination constant; V_d, Volume of distribution.

or bile; hence at some point in time, the blood concentration of the metabolite exhibits a peak and falls thereafter. Fig. 7-23 illustrates the blood concentration–time course of a primary metabolite in relation to its parent compound under contrasting scenarios. The left panel shows the case when the elimination rate constant of the metabolite is much greater than the overall elimination rate constant of the parent compound. Here, the terminal decline of the metabolite parallels that of the parent compound; the metabolite is cleared as quickly as it is formed or its washout is rate-limited by conversion from the parent compound. The right panel shows the opposite case when the elimination rate constant of the metabolite is much lower than the overall elimination rate constant of the parent compound. The slower terminal decline of the metabolite compared to the parent compound simply reflects a longer elimination half-life of the metabolite. It should also be noted that AUC_0^∞ of the metabolite relative to the parent compound is dependent on the partial clearance of the parent chemical to the metabolite and clearance of the derived metabolite. A biologically active metabolite assumes toxicological significance when it is cleared much less efficiently than the parent compound and/or exhibits accessibility and reactivity in the target site.

Cross-Route Dose Extrapolation

The comparison or extrapolation of toxicity data across exposure routes can be conducted for systemically acting chemicals based on administered dose or more appropriately based on toxicokinetic considerations. Cross-route comparisons or extrapolations conducted on the assumption of 100% bioavailability of administered dose (mg/kg/day) fail to take into account the significance and impact of route-specific first-pass effect, as well as the differences in rates and extent of absorption between exposure routes that may impact the dose metric of relevance to the mode of action (Pauluhn, 2003). The data on route-specific bioavailable fraction are therefore useful for the conduct of route-to-route extrapolation of toxicokinetically equivalent dose of chemicals. For example, the bioavailable dose for each exposure route can be calculated by multiplying the administered dose with the route-specific bioavailability (F_{route}). The extrapolation of equivalent dose between two exposure routes (e.g., oral to dermal route in units of mg/kg/day) is then conducted on the basis of the ratio of the route-specific bioavailable fraction (F_{route1}/F_{route2}) as follows (IGHRC, 2005):

$$\text{Dose}_{route2} = \text{Dose}_{route1} \frac{F_{route1}}{F_{route2}}. \quad (7\text{-}35)$$

A more scientifically sound approach would involve the use of PBPK models, by accounting for the species- and route-specific rate and magnitude of absorption as well as first-pass effect to facilitate the conduct of route to route extrapolation on the basis of equivalent tissue dose of toxic moiety (Fig. 7-24; Clewell and Andersen, 1987, 1994; Gerrity et al., 1990). For example, a 4-hour inhalation exposure to 83.4 ppm chloroform in the rat is equal to an oral dose of 1 mg/kg, as determined with PBPK models on the basis of equivalent AUC_{blood} (U.S. EPA, 2006). When the dose metric based on parent compound is the basis of cross-route equivalence, it can be computed as being equal to the bioavailable dose divided by the total clearance (Valcke and Krishnan, 2013, and references cited therein), for chronic exposure situations leading to steady state. Accordingly,

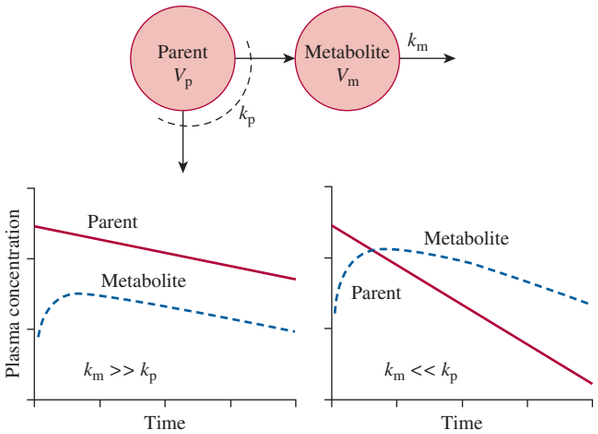

Figure 7-23. *Plasma concentration–time course of a primary metabolite and its parent compound under contrasting scenarios: when elimination of the metabolite is much more rapid than its formation ($k_m >> k_p$ lower left panel) and when elimination of the metabolite is much slower than its formation ($k_m << k_p$, lower right panel).* Semilogarithmic plots are shown to compare the slope of the terminal decline of parent compound and its metabolite. The top panel shows the model for conversion of the parent compound to a single metabolite. Note that the elimination rate constant for the parent compound (k_p) includes both the rate constants for metabolism and extra-metabolic routes of elimination. k_m is the elimination rate constant for the derived metabolite. When $k_m >> k_p$, the terminal decline of the metabolite parallels that of the parent compound, that is, metabolite washout is rate-limited by its formation. When $k_m << k_p$, the terminal decline of metabolite concentration is much slower than that of parent compound, that is, metabolite washout is rate-limited by its elimination.

given that the total clearance (sum of renal, hepatic, and pulmonary clearances, expressed as L/day) is the same regardless of the exposure route, the extrapolation of oral dose (mg/kg/day) to inhalation concentration (mg/m^3) can be conducted as follows:

$$\text{Dose}_{\text{inh}} = \frac{\text{Dose}_{\text{oral}} \times \text{BW} \times F_{\text{oral}}}{Q_{\text{alv}}}, \tag{7-36}$$

where BW = body weight, F_{oral} = bioavailable fraction for the oral route (accounting for first pass effect and absorption by the oral route) associated with the relevant dose (e.g., NOAEL), and Q_{alv} = alveolar ventilation rate (m^3/day).

Interspecies Dose Extrapolation

The extrapolation of toxicologically equivalent dose from one species to another (typically from test animal species to humans) has been historically addressed with the use of a factor of 10 (Dourson et al., 1996). WHO (2005), based on work by Renwick (1993, 1999), subdivided it into toxicokinetic and toxicodynamic components underlying interspecies differences and interindividual variability (Fig. 7-25). The default value of the toxicokinetic component is equal to interspecies ratio of BW to a fractional power (0.67 to 0.75), consistent with the observations that several physiological parameters (GFR, breathing rate, cardiac output) and volume of extracellular water are more closely related to body surface rather than body weight of mammals. It is considered that such allometric dose adjustments (e.g., $\text{Dose}_{\text{human}}(\text{mg/kg/d}) = \text{Dose}_{\text{animal}}(\text{mg/kg/d})\left(\frac{\text{BW}_{\text{Animal}}}{\text{BW}_{\text{Human}}}\right)^{0.33}$) not only reflect interspecies differences in body surface area and physiological flow rates but also provide toxicokinetically equivalent

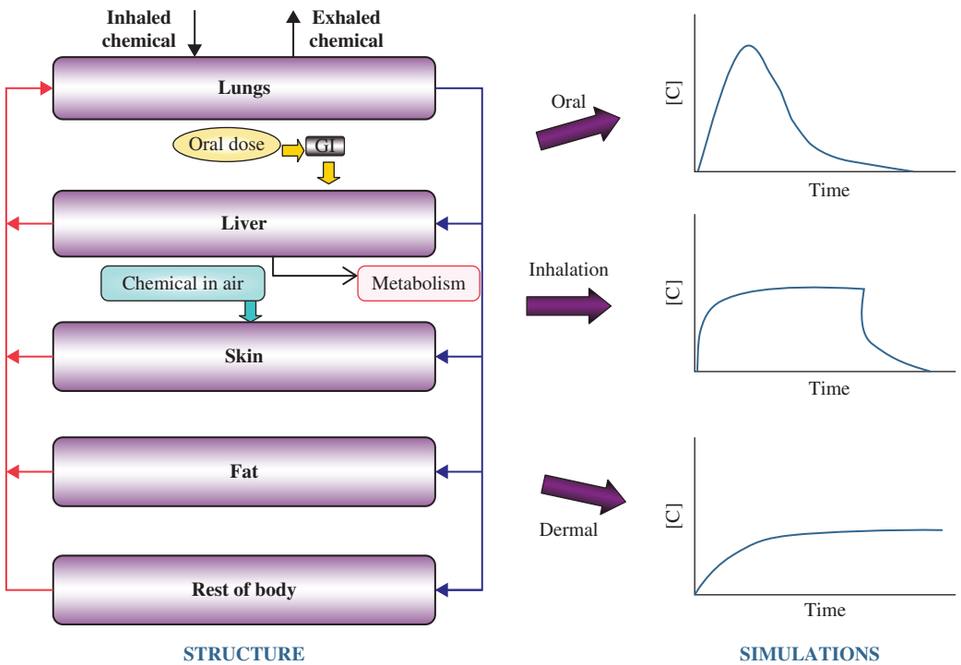

Figure 7-24. *Illustration of the use of physiologic toxicokinetic model to simulate the blood concentration [C] as a function of time for three routes of exposure: oral, inhalation, and dermal.* For simulating the route-specific toxicokinetics of the chemical, only the route-specific input parameters are specified along with the chemical concentration in the corresponding exposure medium.

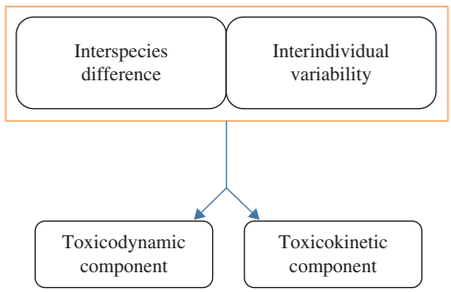

Figure 7-25. *Components of interspecies difference and interindividual variability in response associated with exposure to toxicants.* As per WHO (2005), the toxicokinetic and toxicodynamic components of rat to human differences are given values of 4 and 2.5, respectively, for a total combined (multiplicative) factor of 10. In consideration of interindividual variability in responses from a risk assessment perspective, WHO (2005) suggested default values of 3.16 each for the toxicokinetic and toxicodynamic components, which in turn can be refined with chemical-specific data.

doses across species as long as the parent chemical is the potential toxic moiety and AUC_{blood} is the appropriate measure of dose to target tissue. Such a generic approach for extrapolating doses across species, particularly for the chronic oral exposure leading to steady-state condition, will hold when CL of a xenobiotic is proportional to the body surface area in both animals and humans. Otherwise, it is more relevant to conduct interspecies extrapolation on the basis of chemical-specific data on the dose metrics of relevance to the mode of action (AUC, C_{max}) or the associated toxicokinetic parameter (CL). Thus, the chemical-specific animal to human kinetic adjustment factor (AK_{AF}) is calculated as the ratio of clearance ($AK_{AF} = \frac{CL_{Animal}}{CL_{Human}}$) or the ratio of dose metrics ($AK_{AF} = \frac{AUC_{Human}}{AUC_{Animal}}$) (WHO, 2005). In this regard, the PBPK models are useful for computing AK_{AF} based on the simulation of alternative dose metrics for the dose levels and exposure scenarios of relevance to a toxicological evaluation or risk assessment (Fig. 7-26).

The application of PBPK models for interspecies extrapolation of the toxicokinetics and internal dose of xenobiotics is performed in several steps. The initial step involves the development of a PBPK model in the test animal species and comparison of toxicokinetic simulations with experimental data. Following evaluation of the biological basis, model performance and reliability of dose metric simulations, the physiological parameters of the PBPK model are scaled to humans and the chemical-specific parameters for the species of interest (e.g., humans) are replaced. Whereas the adult physiological parameters vary coherently across species, the rates and affinities for various metabolic reactions across species are not generalizable. For several xenobiotics showing high hepatic extraction ratios in both animals and humans, the scaling of V_{max} to the 3/4th power of body weight, while keeping the K_m species-invariant, has proved to be a useful first approximation. The confidence will be greater if the metabolic parameters are obtained in vitro using human tissues/components for incorporation within PBPK models or following a parallelogram approach to make use of in vivo parameter estimates in rodents as well as in vitro estimates in both rodents and humans (Reitz et al., 1988; Lipscomb et al., 1998). Qualitative differences, if any, between species (e.g., rat to fish) can also be factored into the PBPK models in addition to quantitative differences in mechanistic determinants of toxicokinetics for the conduct of interspecies extrapolation.

Interindividual Kinetic Variability

The toxicokinetic parameters (e.g., CL, $t_{1/2}$) and dose metrics (e.g., C_{max}, AUC) for a xenobiotic may vary among individuals and between various subgroups in the human population. The interindividual variability in toxicokinetics, as characterized by the human kinetic adjustment factor (HK_{AF}), is represented by the ratio between the upper percentile value (i.e., 95th, 99th) and its central tendency value in the entire population of a toxicokinetic parameter or dose metric of relevance for a given chemical (Meek et al., 2003; WHO, 2005; Valcke and Krishnan, 2014). Alternatively, the HK_{AF} is computed as the ratio between an upper percentile value in a selected sensitive subpopulation and the central tendency value for the general population (i.e., $\frac{CL_{Median}}{CL_{Subpopulation}} = \frac{AUC_{Subpopulation}}{AUC_{Median}}$) (Fig. 7-27). In addition to the knowledge of population distribution of chemical-specific toxicokinetic parameters or dose metrics, an understanding of the mode of action and putative toxic moiety of a chemical is fundamental to the derivation of HK_{AF}. The population distribution of dose metrics can also be obtained with PBPK models by accounting for the population distribution of physiological parameters, metabolizing enzymes (including polymorphism), and rates of toxicokinetic processes. By using inputs sampled from statistical distributions for each PBPK model parameter, the Monte Carlo approach facilitates the simulation of distributions of the output, that is, dose metric in a population. Using the PBPK predictions of dose metric corresponding to the 95th percentile and 50th percentile for unimodel, normal distribution, the magnitude of HK_{AF} has been computed (Naumann et al., 2001). Alternatively, subject-specific PBPK models can be constructed to evaluate the variability of dose metrics as a function of gender, age, and lifestage (birth, pregnancy, lactation) or physiological state (obesity) by accounting for quantitative changes in physiological parameters, tissue composition, and metabolic activity (O'Flaherty 1994; Corley et al., 2003; Price et al., 2003a, 2003b; Nong et al., 2006; Valcke and Krishnan, 2013).

Kinetic Interactions During Mixed Exposures

Toxicokinetic interactions during mixed exposures result when one chemical alters the mechanistic determinant of another chemical at the level of absorption (e.g., alteration of ventilation rate, skin permeability coefficient), distribution (e.g., competition for binding to hemoglobin, albumin, metallothionein, alteration of the concentrations of binding proteins, alteration of tissue blood flow), metabolism (e.g., enzyme induction or inhibition), or excretion (e.g., GFR) (Krishnan and Brodeur, 1994). Such interactions result in an increase or decrease in the internal dose per unit administered dose during mixed exposures in comparison with the individual chemical exposures. For example, during mixed exposures, xenobiotics may compete directly with each other for an enzymatic binding site (competitive inhibition), bind directly to the enzyme-xenobiotic complex but not to the free enzyme (uncompetitive inhibition), or do both of these (noncompetitive inhibition) (Segal, 1975). In such cases, toxicokinetic analysis should account for the nonlinearity arising from not only the saturable metabolism of individual mixture components but also the impact of the metabolic inhibition as a function of the concentration and affinity of one mixture component (substrate) in comparison with the free concentration and potency of another mixture component (inhibitor). When induction of metabolizing enzymes occurs, the toxicokinetic analysis would indicate that the impact is more pronounced in cases where hepatic

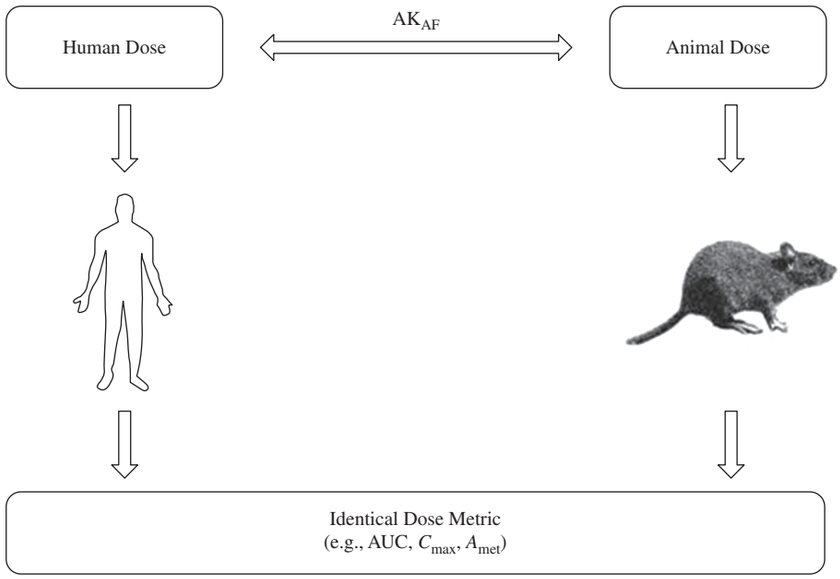

Figure 7-26. *Interspecies extrapolation in toxicokinetics.* The animal to human kinetic adjustment factor (AK_{AF}) reflects the difference in the external (or administered) dose producing the same level of the dose metric in the test animals and humans. (Adapted from U.S. EPA, 2014.)

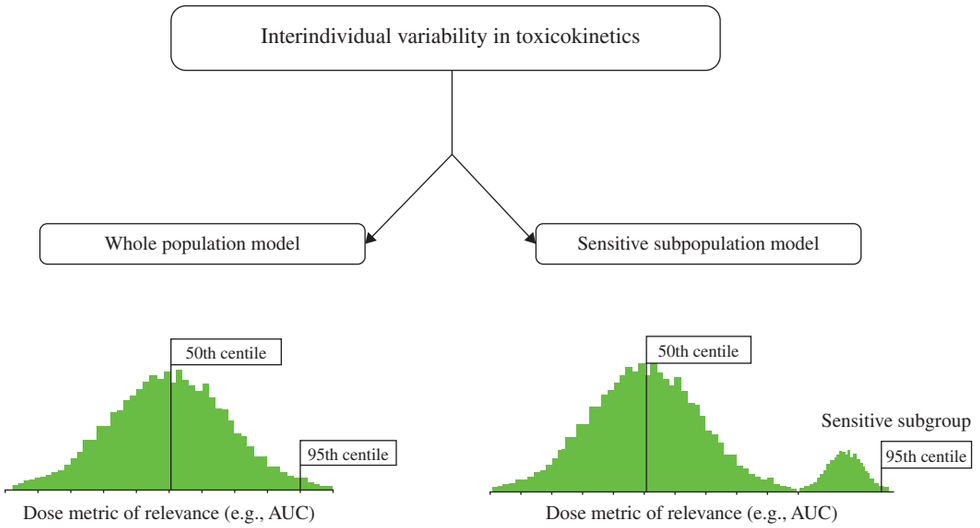

Figure 7-27. *Illustration of the use of toxicokinetic data (e.g., area under the concentration vs. time curve [AUC]) to characterize the magnitude of interindividual variability, in consideration of the simulated or measured values in the whole population (left panel) or a sensitive subgroup within a population (right panel).*

metabolism is capacity-limited (e.g., high doses, xenobiotics exhibiting low hepatic extraction ratio).

Most experimental studies and modeling tools have historically dealt with binary mixtures, due to their inability to account for and interpret the higher order interactions among components. The PBPK model framework allows the tracking of changes in mechanistic determinants induced by each of the mixture components to simulate the impact on the toxicokinetics in more complex mixtures. Tardif et al. (1997) conducted PBPK modeling of mixtures by interconnecting individual chemical models mechanistically at the site of interaction (e.g., two mixture components A and B competing for metabolizing enzymes in liver) (Fig. 7-28). In this approach, when a third chemical C is added to the binary mixture model, the PBPK model not only simulates the new binary interactions involving C (i.e., C-A, C-B), but also the modulatory effect of C on the existing binary interaction between A and B because all components of the mixture are linked. Based on this analogy, PBPK models facilitate prediction of the impact of the addition of another chemical D to the ternary mixture, and so forth because every new chemical becomes an integral part of the existing network of binary interactions (Tardif et al., 1997; Krishnan et al., 2002). Conceptually then, with increasing complexity of mixtures, the inhibition constant for binary interactions is not modified during mixed exposures; rather the free concentration of the inhibitors is

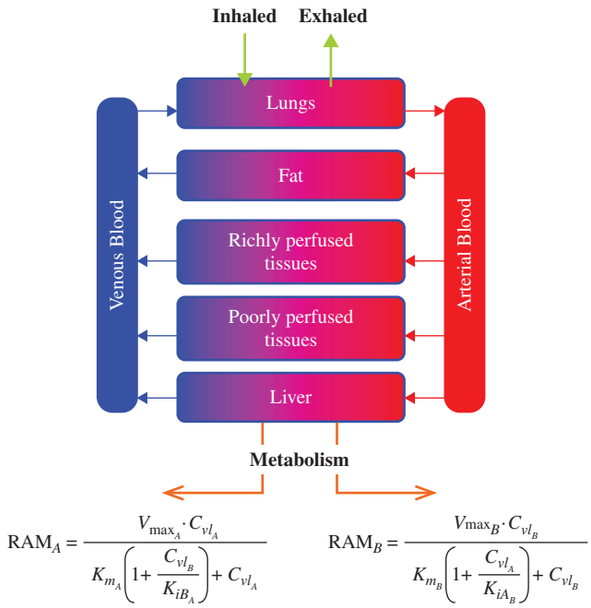

Figure 7-28. *Physiologic toxicokinetic model of inhalation exposure of workers to two volatile organic chemicals.* The two chemicals (A and B) circulate simultaneously and interact with each other in the liver compartment. Chemical A and chemical B compete and inhibit each other's metabolism by the hepatic CYP2E1. RAM is the rate of the amount metabolized, V_{max} is the maximal velocity of metabolism, K_i is inhibition constant, K_m is the Michelis affinity parameter, and C_{vl} is the free concentration of the chemical at the site of metabolism.

altered according to the potency and number of the other inhibitors as well as their concentrations in systemic circulation.

Interpretation of Human Biomonitoring Data

Human biomonitoring or biological monitoring refers to the systematic sampling of body fluids (e.g., blood, urine, exhaled air), and at times body tissues, for the purpose of estimating an individual's internal dose from exposure to chemicals in the workplace or in the general environment (Paustenbach and Galbraith, 2006; Manno and Viau, 2010). Biomonitoring accounts for all exposure sources and routes (oral, dermal, and inhalation), individual differences in toxicant absorption and disposition, and critical personal or lifestyle variables, such as body size and composition, workload that affects pulmonary ventilation, or cigarette smoking that could affect the metabolic status of an individual. Although biomonitoring provides a measure of internal exposure, in the final analysis the data obtained must be relatable to the health guidance values, point of departure underlying such values or health risks. For each chemical of concern, it is useful to establish a reference biomonitoring value below which a reasonable assurance of safety exists. In this regard, the tools for interpreting biomonitoring data make use of forward dosimetry or reverse dosimetry approaches (Clewell et al., 2008; Gurusankar et al., 2017). Forward dosimetry refers to the use of toxicokinetic data and models to develop concentrations of exposure biomarkers corresponding to the existing health guidance values (e.g., occupational exposure limit, reference dose, tolerable daily intake) or point of departure in a dose–response curve (e.g., NOAEL). Well-known examples of the use of the concept of forward dosimetry in human biomonitoring include the development of (i) biological exposure indices (BEIs) by the American Conference of Governmental Industrial Hygienists (ACGIH), (ii) biological tolerance values (BATs) and biological guidance values (BLW) by the German MAK Commission, as well as (iii) biological monitoring equivalents associated with exposure guidance values or the underlying point of departure values (BE, BE_{POD}) for environmental chemicals (Hays et al., 2007; Angerer et al., 2011; Gurusankar et al., 2017). Reverse dosimetry, in contrast, involves the use of toxicokinetic parameters and models to reconstruct or estimate exposure levels associated with the measured concentrations in biomarkers in biological matrices (urine, blood, plasma, exhaled air).

Forward Dosimetry In order to compare the human biomonitoring data for environmental chemicals with reference values for the general population, the forward dosimetry approaches are useful in converting the exposure guidance value or the point of departure (POD such as NOAEL) underlying the guidance values in terms of biomarker concentrations (Aylward et al., 2013; St-Amand et al., 2014). For example, the $BE_{POD,}$ corresponding to steady-state plasma concentration for triclosan (3 mg/L) was derived from the human-equivalent POD (POD_{HED}) of 3 mg/kg/day from a study in baboons and CL = 0.98 L/kg/day as follows:

$$BE_{POD}(\text{mg/L plasma}) = \frac{POD_{HED}}{CL} \tag{7-37}$$

where POD_{HED} = POD (30 mg/kg/day) divided by animal-human uncertainty factor of 10.

Following the application of the interindividual uncertainty factor of 10, plasma BE of 0.3 mg/L was obtained (Krishnan et al., 2010a, 2010b).

A forward dosimetry approach for developing urinary BE_{POD}, under chronic exposure conditions leading to steady state, involves multiplying the ingested dose with a factor representing the urinary excretion fraction (Aylward and Hays, 2008; Krishnan et al., 2010a, 2010b). On this basis, the steady-state urinary biomarker concentrations (mg/L urine and mg/g creatinine) associated with the human-equivalent PODs (BE_{POD}) are developed as follows:

$$BE_{POD}(\text{mg/L Urine}) = \frac{[POD_{HED}(\text{mg/kg/day}) \times BW(\text{kg}) \times F_{UE}]}{\text{Urine Volume (L/day)}}, \tag{7-38}$$

$$BE_{POD}(\text{mg/g Creatinine}) = \frac{[POD_{HED}(\text{mg/kg/day}) \times BW(\text{kg}) \times F_{UE}]}{\text{Creatinine (g/day)}}, \tag{7-39}$$

where BW = body weight and F_{UE} = fraction of the ingested daily dose excreted via urine

By dividing the BE_{POD} obtained with Eqs. (7-38) and (7-39) by the interindividual variability factor of 10, the BE (in units of mg/L urine and mg/g creatinine) is obtained. Thus, for bisphenol A, the urinary BE_{POD} and BE of 10 mg/L and 1 mg/L were derived from POD_{HED} of 0.25 mg/kg/day (based on rat NOAEL of 25 mg/kg/day) and F_{UE} value of 1 (i.e., 100% of the ingested dose excreted in urine) (Krishnan et al., 2010b). Similarly, for triclosan the urinary BE_{POD} and BE equal 64 mg/L and 6.4 mg/L, given that the POD_{HED} = 3 mg/kg/day (based on baboon NOAEL of 30 mg/kg/day) and the F_{UE} = 0.54 (Krishnan et al., 2010a). The BE and BE_{POD} values derived using forward dosimetry approaches inherently reflect the underlying limitations and uncertainties associated with the exposure guidance value and the toxicokinetic database used in their derivation (Hays et al., 2007).

Reverse Dosimetry Reverse dosimetry approaches use the toxicokinetic parameters or models to convert the biomarker concentration into the exposure dose. The sampling time relative to the exposure pattern would be a straightforward matter if exposure is continuous and constant, in which case blood, urine, or other samples can be collected at any time after steady state is reached. In reality, exposure is often episodic or intermittent, and in occupational settings exposure is dictated by the work shift. Systematically timed sampling is hardly feasible in a community population exposure study, where sampling is typically random, and exposure comes from diffuse sources and may not occur with any definable regularity. The only approach to overcome such shortcoming is to obtain samples at different times of a day to provide diurnal coverage, which introduces obvious logistical and cost challenges in a population surveillance program. It is not often appreciated that the observed level of a determinant for a chemical exposure is a function of not only current exposure, but also what happened some time before (Droz et al., 1991). This is particularly so when exposure is continuous or the interval between intermittent exposures is on the order of the chemical's elimination half-life (e.g., an elimination $t_{1/2}$ of 24 hours or longer when exposure reoccurs on a daily basis). In other words, the elimination half-life of a biomarker or index compound dictates how far back in exposure history it reports. A corollary to this principle is the fact that short half-life biomarkers reflect more of the within day or day-to-day fluctuation in exposure, whereas daily fluctuation in exposure is dampened with long half-life biomarkers. Although the former cases may be viewed as undesirable because of variability in the measurement, it offers a better reflection of the oscillation in internal dose and particularly the transient peak exposures. The latter cases provide a less variable, time-averaged measure of exposure.

Reverse dosimetry has been conducted using deterministic or probabilistic toxicokinetic models. The simple deterministic approaches are based on either assumption of steady-state kinetics or urinary mass-balance calculation. More sophisticated approaches use the classic compartmental and physiological models described in the previous sections to perform the back-calculations for various scenarios, routes, and populations (Clewell et al., 2008; Georgopoulos et al., 2009).

An example of the application of reverse dosimetry at steady-state condition involves the calculation of the daily dose absorbed (mg/kg/day) from measured blood concentration at steady state (mg/L) as well as K_{el} (day^{-1}) and V_d (L blood/kg) as follows (NRC, 2006):

$$D_{abs} = K_{el} \times V_d \times C_{b,ss}. \tag{7-40}$$

Similarly, back-calculation of daily dose to which exposure has occurred can be conducted as follows, based on measured urinary concentration:

$$\text{Daily intake(mg/kg/day)} = \frac{C_{urine} \times V_{urine}}{\text{Body weight} \times F_{UE}}. \tag{7-41}$$

It is relevant to note that the urinary excretion rate (dU_B/dt) of a biomarker is related to its concentration in blood or plasma concentration (C) by renal clearance (Cl_r) as follows:

$$\frac{dU_B}{dt} = Cl_r \cdot C. \tag{7-42}$$

In the above equation, dU_B/dt represents an average urinary excretion over a relatively short collection interval; C is the blood or plasma concentration at the mid-time point. The differential form of Eq. (7-42) can be integrated over an extended collection period, say 24 hours, to yield a relationship between the total amount excreted in the collection period ($U_{B,24}$) and the corresponding area under the blood or plasma concentration–time curve (AUC_{0-24}, an integral measure of exposure):

$$U_{B,24h} = Cl_r \cdot AUC_{0-24}. \tag{7-43}$$

Hence, either excretion rate or cumulative excretion over a defined collection period of a urinary biomarker is the most appropriate index secondary to blood concentration measurement. Urinary excretion rate is in turn related to pooled urine concentration ($C_{UB,\Delta t}$) and urinary flow rate ($V_{\Delta t}/\Delta t$).

$$\frac{dU_B}{dt} = C_{UB,\Delta t} \cdot \left(\frac{V_{\Delta t}}{\Delta t}\right). \tag{7-44}$$

Urinary concentration as measured from spot collection offers convenience and flexibility; however, it has the distinct disadvantage of being influenced by urine flow rate, which varies greatly from time to time depending on the hydration state of the individual. Several methods have been proposed to adjust for variation in urine flow rate. Urinary creatinine is most often used to normalize urinary concentration data. The following equation shows one such normalization (e.g., Garde et al., 2004):

$$U_{B,24} = \left(\frac{C_{UB}}{C_{UCr}}\right) \cdot U_{Cr,24}, \tag{7-45}$$

where C_{UB} and C_{UCr} are the respective biomarker and creatinine concentration in the spot urine collection, and $U_{Cr,24}$ is the daily urinary output of creatinine. This method assumes that blood concentration of biomarker is reasonably constant throughout the day (e.g., during stable, continuous exposure or for a chemical with very long elimination half-life). The daily urinary output of creatinine is assumed to be constant between individuals; alternately, it can also be adjusted according to individual variables that are known to affect creatinine production such as body mass, age, and sex (Boeniger et al., 1993).

An alternate, more commonly adopted normalization is to simply express the amount of biomarker in the spot urine collection per mmol of creatinine. This simple normalization is valid for spot urine collection if we assume creatinine excretion is constant throughout the day and reproducible from individual to individual, which are often not true. It is not surprising that validity of normalizing urinary biomarker to per mmol creatinine has been questioned (Boeniger et al., 1993). Other normalization methods including adjustment to a standard urine flow rate, specific gravity, or osmolality have also been proposed (Hill et al., 1988; Boeniger et al., 1993; Gurusankar et al., 2017).

CONCLUSIONS

Toxicokinetic data and models are central to the selection of doses and species for toxicity testing, interpretation of dose–response relationships, and efficient design of experiments for focused and reduced animal use. The toxicokinetic principles are also key to the design and interpretation of human biological monitoring studies as established in occupational health research for many decades and more recently in screening level risk assessment of environmental pollutants. The emerging toxicity testing and risk assessment paradigms as well as the extensive ongoing human biomonitoring programs will require toxicokinetic data and tools for a large number of chemicals. In this regard, increasing focus on the development of high throughput (HTP) approaches to derive toxicokinetic

parameters and PBPK models for new chemicals, mixtures, and particles is required. So far, there have been few successful attempts to integrate in vitro data and quantitative structure-property relationships for partition coefficients within the PBPK framework to simulate the inhalation toxicokinetics of chemicals (Peyret and Krishnan, 2011; Bessems et al., 2014). Further, there has been successful application of the HTP data on plasma protein binding (equilibrium dialysis) and intrinsic clearance (cryopreserved hepatocytes) to generate steady-state concentrations for the oral route, to facilitate the conversion of HTP toxicity study results into daily doses in humans (Wetmore et al., 2013; Wetmore, 2015). The emerging toxicity testing and risk assessment paradigms require HTP in vitro and in silico approaches that have broad capability and application domains in terms of chemical space, exposure route, and toxicokinetic parameters. In addition, advances in the development of physiologically based cellular toxicokinetic models will facilitate simulations of dose metrics at macro and micro levels to better understand the relationship among the concentrations of chemicals causing perturbations in HTP screening assays, levels of chemicals and their metabolites found in population level biomonitoring studies, as well as the human exposure guidance values.

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REFERENCES

Andersen ME. A physiologically based toxicokinetic description of the metabolism of inhaled gases and vapors: analysis at steady state. *Toxicol Appl Pharmacol*. 1981;60:509–526.

Andersen ME, Clewell HJIII, Gargas ML. Physiologically-based pharmacokinetic modeling with dichloromethane, its metabolite carbon monoxide and blood carboxyhemoglobin in rats and humans. *Toxicol Appl Pharmacol*. 1991;108:14–27.

Andersen ME, Clewell HJIII, Gargas ML, et al. Physiologically-based pharmacokinetics and risk assessment process for methylene chloride. *Toxicol Appl Pharmacol*. 1987;87:185–205.

Angerer J, Aylward LL, Hays SM, et al. Human biomonitoring assessment values: approaches and data requirements. *Int J Hyg Environ Health*. 2011;214:348–360.

Assmus F, Houston BJ, Galetin A. Incorporation of lysosomal sequestration in the mechanistic model for prediction of tissue distribution of basic drugs. *Eur J Pharm Sci*. 2017;109:419–430.

Aylward LL, Hays SM. Biomonitoring equivalents (BEs) dossier for 2,4-dichloroephenoxyacetic acid (2,4-D) (CAS No. 94-75-7). *Regul Toxicol Pharmacol*. 2008;51:S37–S48.

Aylward LL, Kirman CR, Schoeny R, et al. Evaluation of biomonitoring data from the CDC National Exposure Report in a risk assessment context: perspectives across chemicals. *Environ Health Perspect*. 2013;121:287–294.

Bale AS, Kenyon E, Flynn TJ, et al. Correlating in vitro data to in vivo findings for risk assessment. *ALTEX*. 2014;31(1):79–90.

Bessems JG, Loizou G, Krishnan K, et al. PBTK modelling platforms and parameter estimation tools to enable animal-free risk assessment: recommendations from a joint EPAA–EURL ECVAM ADME workshop. *Regul Toxicol Pharmacol*. 2014;68(1):119–139.

Bertilsson L, Tomson T, Tybring G. Pharmacokinetics: time-dependent changes—autoinduction of carbamazepine epoxidation. *J Clin Pharmacol*. 1986;26:459–462.

Bischoff KB, Dedrick RL, Zaharko DS, Longstreth JA. Methotrexate pharmacokinetics. *J Pharm Sci*. 1971;60:1128–1133.

Boeniger MF, Lowry LL, Rosenberg J. Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments—a review. *Am Ind Hyg Assoc J*. 1993;54:615–627.

Brown RP, Delp MD, Lindstedt SL, et al. Physiological parameter values for physiologically based pharmacokinetic models. *Toxicol Ind Health*. 1997;13:407–484.

Cayen MN. Considerations in the design of toxicokinetic programs. *Toxicol Pathol*. 1995;23(2):148–157.

Clewell HJ III, Andersen ME. Physiologically-based pharmacokinetic modeling and bioactivation of xenobiotics. *Toxicol Ind Health*. 1994;10:1–24.

Clewell HJ III, Andersen ME. Dose, species and route extrapolations using physiologically based pharmacokinetic modeling. In: *Drinking Water and Health*. Vol. 10. Washington, DC: National Academy of Sciences;1987: 159–184.

Clewell HJ III, Gentry PR, Gearhart JM, et al. Development of a physiologically based pharmacokinetic model of isopropanol and its metabolite acetone. *Toxicol Sci*. 2001;63(2):160–172.

Clewell HJ, Tan YM, Campbell JL, et al. Quantitative interpretation of human biomonitoring data. *Toxicol Appl Pharmacol*. 2008;231: 122–133.

Corley RA, Mast TJ, Carney EW, et al. Evaluation of physiologically based models of pregnancy and lactation for their application in children's health risk assessments. *Crit Rev Toxicol*. 2003;33:137–211.

da Silva LM, Charest-Tardif G, Krishnan K, et al. Influence of oral administration of a quaternary mixture of trihalomethanes on their blood kinetics in the rat. *Toxicol Lett*. 1999;106:49–57.

Dedrick RL, Forrester DD, Ho DH. In vitro-in vivo correlation of drug metabolism–deamination of 1-beta-D-arabinofuranosylcytosine. *Biochem Pharmacol*. 1972;21(1):1–16.

Dewoskin RS, Thompson CM. Renal clearance parameters for PBPK model analysis of early lifestage differences in the disposition of environmental toxicants. *Regul Toxicol Pharmacol*. 2008;51(1):66–86.

Dourson ML, Felter SP, Robinson D. Evolution of science-based uncertainty factors in noncancer risk assessment. *Regul Toxicol Pharmacol*. 1996;24(2 pt 1):108–120.

Droz PO, Berode M, Wu NN. Evaluation of concomitant biological and air monitoring results. *Appl Occup Environ Hyg*. 1991;6:465–474.

Farris FF, Dedrick RL, King FG. Cisplatin pharmacokinetics: applications of physiological model. *Toxicol Lett*. 1988;43:117–137.

Fiserova-Bergerova V. Mathematical modeling of inhalation exposure. *J Combust Toxicol*. 1975;32:201–210.

Frederick CB, Potter DW, Chang-Mateu MI, et al. A physiologically based pharmacokinetic and pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate and its implications for risk assessment. *Toxicol Appl Pharmacol*. 1992;114:246–260.

Fukuchi Y, Toshimoto K, Mori T, et al. Analysis of nonlinear pharmacokinetics of a highly albumin-bound compound: contribution of albumin-mediated hepatic uptake mechanism. *J Pharm Sci*. 2017;106:2704–2714.

Garde H, Hansen A, Kristensen J, et al. Comparison of uncertainties related to standardization of urine samples with volume and creatinine correction. *Ann Occup Hyg*. 2004;48:171–179.

Gargas ML, Andersen ME, Clewell HJIIIrd. A physiologically based simulation approach for determining metabolic constants from gas uptake data. *Toxicol Appl Pharmacol*. 1986;86:341–352.

Gargas ML, Burgess RJ, Voisard DE, et al. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. *Toxicol Appl Pharmacol*. 1989;98:87–99.

Gargas ML, Clewell HJ, Andersen ME. Gas uptake inhalation techniques and the rates of metabolism of chloromethanes, chloroethanes and chloroethylenes in the rat. *Inhal Toxicol*. 1990;2:295–319.

Georgopoulos PG, Sasso AF, Isukapalli SS, et al. Reconstructing population exposures to environmental chemicals from biomarkers: challenges and opportunities. *J Expo Sci Environ Epidemiol*. 2009;19:149–171.

Gerlowski LE, Jain RK. Physiologically based pharmacokinetic modeling: principles and applications. *J Pharm Sci*. 1983;72:1103–1127.

Gerrity TR, Henry CJ, Birnbaum L. *Principles of Route-to-Route Extrapolation for Risk Assessment*. New York: Elsevier; 1990.

Geyer HJ, Schramm K-W, Darnerud PO, et al. Terminal elimination half-lives of the brominated flame retardants TBBPA, HBCD, and lower brominated PBDEs in humans. *Organohalogen Compounds*. 2004;66:3820–3825.

Gibaldi M, Perrier D. Toxicokinetics. In: Swarbrick J, ed. *Drugs and Pharmaceutical Sciences*. New York: Marcel Dekker Inc.; 1975.

Gibaldi M, Perrier D. *Pharmacokinetics*. 2nd ed. New York: Marcel Dekker; 1982.

Gurusankar R, Yenugadhati N, Krishnan K, et al. The role of human biological monitoring in health risk assessment. *Int J Risk Assess Manage*. 2017;20:136–197.

Haddad S, Pelekis M, Krishnan K. A methodology for solving physiologically based pharmacomodels without the use of simulation softwares. *Toxicol Lett*. 1996;85:113–126.

Haddad S, Tardif R, Charest-Tardif G, Krishnan K. Physiological modeling of the toxicokinetic interactions in a quaternary mixture of aromatic hydrocarbons. *Toxicol Appl Pharmacol*. 1999;161:249–257.

Haddad S, Poulin P, Krishnan K. Relative lipid content as the sole mechanistic determinant of the adipose tissue:blood partition coefficients of highly lipophilic organic chemicals. *Chemosphere*. 2000;40:839–843.

Haggard HW. The absorption, distribution, and elimination of ethyl ether: II. Analysis of the mechanism of the absorption and elimination of such a gas or vapor as ethyl ether. *J Biol Chem*. 1924;49:753–770.

Hays SM, Becker RA, Leung HW, et al. Biomonitoring equivalents: a screening approach for interpreting biomonitoring results from a public health risk perspective. *Regul Toxicol Pharmacol*. 2007;47:96–109.

Hill RH, Guillemin M, Droz PO. Sample collection. In: Kneip TJ, Crable JV, eds. *Methods in Biological Monitoring*. Washington, DC: American Public Health Association; 1988:37–63, Chap 6.

Himmelstein KJ, Lutz RJ. A review of the applications of physiologically based pharmacokinetic modeling. *J Pharmacokinet Biopharm*. 1979;7:127–145.

IGHRC. *Guidelines on route-to-route extrapolation of toxicity data when assessing health risks of chemicals, Draft v6.0*. Medical Research Council Institute for Environment and Health, University of Leicester, Leicester, 2005:59.

Johanson G. Modelling of respiratory exchange of polar solvents. *Ann Occup Hyg*. 1991;35:323–339.

Kenakin TP. *A Pharmacology Primer: Theory, Applications and Methods*. 3rd ed. Amsterdam: Elsevier; 2009:179–238.

Kety SS. The theory and applications of the exchange of inert gas at the lungs and tissues. *Pharmacol Rev*. 1951;3:1–41.

Kirman CR, Aylward LL, Hays SM, et al. Biomonitoring equivalents for DDT/DDE. *Regul Toxicol Pharmacol*. 2011;60:172–180.

Krishnan K, Andersen ME. Physiologically based pharmacokinetic and toxicokinetic modeling. In: Hayes AW, ed. *Principles and Methods of Toxicology*. Philadelphia, PA: Taylor & Francis; 2007:233–291.

Krishnan K, Andersen ME, eds. *Quantitative Modeling in Toxicology*. Chichester, UK: John Wiley Publishers; 2010.

Krishnan K, Brodeur J. Toxic interactions among environmental pollutants: corroborating laboratory observations with human experience. *Environ Health Perspect*. 1994;102(suppl 9):11–17.

Krishnan K, Haddad S, Beliveau M, et al. Physiological modeling and extrapolation of pharmacokinetic interactions from binary to more complex chemical mixtures. *Environ Health Perspect*. 2002;110(suppl 6):989–994.

Krishnan K, Gagne M, Nong A, et al. Biomonitoring equivalents for triclosan. *Regul Toxicol Pharmacol*. 2010a;58:10–17.

Krishnan K, Gagne M, Nong A, et al. Biomonitoring equivalents for bisphenol A (BPA). *Regul Toxicol Pharmacol*. 2010b;58:18–24.

Kunze A, Huwyler J, Poller B, et al. In vitro–in vivo extrapolation method to predict human renal clearance of drugs. *J Pharm Sci*. 2014;103:994–1001.

Lipscomb JC, Fisher JW, Confer PD, et al. In vitro to in vivo extrapolation for trichloroethylene metabolism in humans. *Toxicol Appl Pharmacol*. 1998;152:376–387.

Manno M, Viau C. Biomonitoring for occupational health risk assessment (BOHRA). *Toxicol Lett*. 2010;192:3–16.

Mapleson WW. An electric analogue for uptake and exchange of inert gases and other agents. *J Appl Physiol*. 1963;18:197–204.

Meek B, Renwick A, Sonich-Mullin C, International Programme on Chemical Safety. Practical application of kinetic data in risk assessment—an IPCS initiative. *Toxicol Lett*. 2003;138:151–160.

National Research Council. *Human Biomonitoring for Environmental Chemicals*. Washington, DC: National Academies Press; 2006.

Naumann BD, Silverman KC, Dixit R, Faria EC, Sargent EV. Case studies of categorical data-derived adjustment factors. *Human Ecol Risk Assess*. 2001;7(1):61–105.

Nong A, McCarver DG, Hines RN, Krishnan K. Modeling interchild differences in pharmacokinetics on the basis of subject-specific data on physiology and hepatic CYP2E1 levels: a case study with toluene. *Toxicol Appl Pharmacol*. 2006;214:78–87.

O'Flaherty EJ. *Toxicant and Drugs: Kinetics and Dynamics*. New York: John Wiley; 1981.

O'Flaherty EJ. Physiologic changes during growth and development. *Environ Health Perspect*. 1994;102(suppl 11):103–106.

Pauluhn J. Issues of dosimetry in inhalation toxicity. *Toxicol Lett*. 2003;140-141:229–238.

Paustenbach D, Galbraith D. Biomonitoring and biomarkers: exposure assessment will never be the same. *Environ Health Perspect*. 2006;114:1143–1149.

Peyret T, Poulin P, Krishnan K. A unified algorithm for predicting partition coefficients for PBPK modeling of drugs and environmental chemicals. *Toxicol Appl Pharmacol*. 2010;249:197–207.

Peyret T, Krishnan K. QSARs for PBPK modelling of environmental contaminants. *SAR QSAR Environ Res*. 2011;22:129–169.

Poulin P, Bteich M, Haddad S. Supplemental analysis of the prediction of hepatic clearance of binary mixtures of Bisphenol A and Naproxen determined in an isolated perfused rat liver model to promote the understanding of potential albumin-facilitated hepatic uptake. *J Pharm Sci*. 2017;106:3207–3214.

Poulin P, Burczynsky FJ, Haddad S. The role of extracellular binding proteins in the cellular uptake of drugs: impact on quantitative in vitro-to-in vivo extrapolations of toxicity and efficacy in physiologically based pharmacokinetic-pharmacodynamic research. *J Pharm Sci*. 2016;105:497–508.

Poulin P, Beliveau M, Krishnan K. Mechanistic animal-replacement approaches for predicting pharmacokinetics of organic chemicals. In: Salem H, Katz SA, eds. *Animal Alternatives in Toxicology: Recent Advances*. Totowa, NY: Humana Press; 1999:115–139.

Poulin P, Krishnan K. A biologically-based algorithm for predicting human tissue: blood partition coefficients of organic chemicals. *Hum Exp Toxicol*. 1995;14:273–280.

Poulin P, Krishnan K. A tissue composition-based algorithm for predicting tissue:air partition coefficients of organic chemicals. *Toxicol Appl Pharmacol*. 1996a;136:126–130.

Poulin P, Krishnan K. A mechanistic algorithm for predicting blood:air partition coefficients of organic chemicals with the consideration of reversible binding in hemoglobin. *Toxicol Appl Pharmacol*. 1996b;136:131–137.

Poulin P, Theil FP. Prediction of pharmacokinetics prior to in vivo studies. 1. Mechanism-based prediction of volume of distribution. *J Pharm Sci*. 2002;91:129–156.

Poulin P, Theil FP. Development of a novel method for predicting human volume of distribution at steady-state of basic drugs and comparative assessment with existing methods. *J Pharm Sci*. 2009;98:4941–4961.

Price K, Haddad S, Krishnan K. Physiological modeling of age-specific changes in the pharmacokinetics of organic chemicals in children. *J Toxicol Environ Health*. 2003a;66(A):417–433.

Price PS, Conolly RB, Chaisson K, et al. Modeling interindividual variation in physiological factors used in PBPK models of humans. *Crit Rev Toxicol*. 2003b;33:469–503.

Ramsey JC, Andersen ME. A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol Appl Pharmacol*. 1984;73:159–175.

Reddy MB, Yang RSH, Clewell HJIII, Andersen ME. *Physiologically Based Pharmacokinetic Modeling: Science and Application*. Hoboken, NJ: Wiley & Sons; 2005:420.

Reitz RH, Mandrela AL, Park CN, et al. Incorporation of in vitro enzyme data into the physiologically-based pharmacokinetic (PBPK) model for methylene chloride: implications for risk assessment. *Toxicol Lett*. 1988;43:97–116.

Reitz RH, McCroskey PS, Park CN, et al. Development of a physiologically based pharmacokinetic model for risk assessment with 1,4-dioxane. *Toxicol Appl Pharmacol*. 1990;105(1):37–54.

Renwick AG. Data safety factors for the evaluation of food additives and environmental contaminants. *Food Additives Contam*. 1993;10:275–305.

Renwick AG. Subdivision of uncertainty factors to allow for toxicokinetics and toxicodynamics. *Human Ecol Risk Assess*. 1999;5(5)1035–1050.

Rescigno A. Fundamental concepts in pharmacokinetics. *Pharmacol Res*. 1997;35:363–390.

Rideout VC. *Mathematical and Computer Modeling of Physiological Systems*. New York: Prentice-Hall; 1991.

Riggs DS. *The Mathematical Approach to Physiological Problems: A Critical Treatise*. Cambridge, MA: MIT Press; 1970.

Rodgers T, Leahy D, Rowland M. Physiologically based pharmacokinetic modeling 1: predicting the tissue distribution of moderate-to-strong bases. *J Pharm Sci*. 2005;94:1259–1276.

Rodgers T, Rowland M. Physiologically based pharmacokinetic modelling 2: predicting the tissue distribution of acids, very weak bases, neutrals and zwitterions. *J Pharm Sci*. 2006;95:1238–1257.

Rodgers T, Rowland M. Mechanistic approaches to volume of distribution predictions: understanding the processes. *Pharm Res*. 2007;24:918–933.

Rowland M. Physiologic pharmacokinetic models and interanimal species scaling. *Pharmacol Ther*. 1985;29:49–68.

Rowland M, Tozer TN. *Clinical Pharmacokinetics and Pharmacodynamics–Concepts and Applications*. Philadelphia, PA: Wolters Kluwer/Lippincott, Williams & Wilkins; 2011.

Schmitt W. General approach for the calculation of tissue to plasma partition coefficients. *Toxicol In Vitro*. 2008;22:457–467.

Segal IH. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. New York: Wiley Interscience; 1975:957.

Slikker W, Andersen ME, Bogdanffy MS, et al. Dose-dependent transitions in mechanisms of toxicity. *Toxicol Appl Pharmacol*. 2004;201(3):203–225.

St-Amand A, Werry K, Aylward LL, et al. Screening of population level biomonitoring data from the Canadian Health Measures Survey in a risk-based context. *Toxicol Lett*. 2014;231:126–134.

Tardif R, Charest-Tardif G. The importance of measured end-points in demonstrating the occurrence of interactions: a case study with methylchloroform and m-xylene. *Toxicol Sci*. 1999;49(2):312–317.

Tardif R, Charest-Tardif G, Brodeur J, et al. Physiologically based pharmacokinetic modeling of a ternary mixture of alkyl benzenes in rats and humans. *Toxicol Appl Pharmacol*. 1997;144:120–134.

Teorell T. Kinetics of distribution of substances administered to the body: I. The extravascular modes of administration. *Arch Int Pharmacodyn Ther*. 1937;57:205–225.

Terasaki T, Iga T, Sugiyama Y, et al. Nuclear binding as a determinant of tissue distribution of adriamycin, daunomycin, adriamycinol, daunorubicinol and actinomycin D. *J Pharmacobiodyn*. 1984;7:269–277.

Thummel KE, Shen DD, Isoherranen N, et al. Appendix II. Design and optimization of dosage regimens: pharmacokinetic data. In: Brunton LL, Parker KL, Buxton IOL, Blumenthal DK, eds. *Goodman & Gilman's: The Pharmacological Basis of Therapeutics*. 11th ed. New York: The McGraw-Hill Companies; 2006.

Tozer NT, Rowland M. *Introduction to Pharmacokinetics and Pharmacodynamics: The Quantitative Basis of Drug Therapy*. Philadelphia, PA: Lippincott Williams & Wilkins; 2006.

Travis CC, Hattemer-Frey HA. Physiological pharmacokinetic models. In: Krewski D, Franklin C, eds. *Statistics in Toxicology*. New York: Gordon and Breach; 1991:170.

Turner PV, Brabb T, Pekow C, et al. Administration of substances to laboratory animals: routes of administration and factors to consider. *J Am Assoc Lab Anim Sci*. 2011;50:600–613.

U.S. Environmental Protection Agency (EPA). Approaches for the application of physiologically based pharmacokinetic (PBPK) models and supporting data in risk assessment. National Center for Environmental Assessment, Washington, DC; EPA/600/R-05/043F. 2006.

U.S. EPA (Environmental Protection Agency). Guidance for applying quantitative data to develop data-derived extrapolation factors for interspecies and intraspecies extrapolation. Risk Assessment Forum, Office of the Science Advisor, Washington, DC; EPA/100/R-14/002. 2014.

Valcke M, Krishnan K. Assessing the impact of child/adult differences in hepatic first pass effect on the human kinetic adjustment factor. *Reg Toxicol Pharmacol*. 2013;65(1):126–134.

Valcke M, Krishnan K. Characterization of the human kinetic adjustment factor for the health risk assessment of environmental contaminants. *J Appl Toxicol*. 2014;34:227–240.

Wetmore BA. Quantitative in vitro-to-in vivo extrapolation in a high-throughput environment. *Toxicology*. 2015;332:94–101.

Wetmore BA, Wambaugh JF, Ferguson SS, et al. Relative impact of incorporating pharmacokinetics on predicting in vivo hazard and mode of action from high-throughput in vitro toxicity assays. *Toxicol Sci*. 2013;132:327–346.

World Health Organization (WHO). *Principles of Toxicokinetic Studies. Environmental Health Criteria 57*. Geneva: WHO; 1986.

World Health Organization (WHO). Chemical-specific adjustment factors for interspecies differences and human variability: guidance document for use of data in dose/concentration-response assessment. International Program on Chemical Safety (IPCS) Harmonization Project Document No. 2. 2005.

World Health Organization (WHO). Characterization and application of physiologically based pharmacokinetic models in risk assessment. International Program on Chemical Safety (IPCS) Harmonization Project Document No. 9. 2010.

Wilkinson GR. Clearance approaches in pharmacology. *Pharmacol Rev*. 1987;39:1–47.

Unit II

Non-Organ-Directed Toxicity

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chapter 8

Chemical Carcinogenesis

James E. Klaunig and Zemin Wang

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Chemical Carcinogenesis in Humans

Evaluation of Carcinogenicity in Humans

Summary

Acknowledgments

References

OVERVIEW

Cancer is a disease characterized by genomic mutation, modified gene expression, cell proliferation, and aberrant cell growth. It ranks as one of the leading causes of death in the world. In the United States, cancer ranks as the second leading cause of death, with over 1.68 million new cases of cancer diagnosed and more than 0.6 million Americans dying from cancer annually (Siegel et al., 2016). Multiple causes of cancer have been established including infectious agents, radiation, and chemicals. Estimates suggest that 70% to 90% of all human cancers have a linkage to environmental, dietary, and behavioral factors (Fig. 8-1). Although our understanding of the biology of the progression from a normal cell to a malignant one has advanced considerably in the past several decades, many aspects of the cause, prevention, and treatment of human cancer and in particular the influence of lifestyle remain to be completely resolved.

Definitions

An understanding of the cellular and molecular aspects of the cancer process requires an understanding of the scientific terms involved in defining and describing the pathology of neoplasia (Table 8-1). Neoplasia is defined as new growth or autonomous growth of tissue. A neoplastic lesion is referred to as a neoplasm. A neoplasm can be either benign or malignant. Both types of lesions are induced by chemical carcinogens. Benign neoplasms (e.g., adenomas) are lesions characterized by expansive growth, frequently exhibiting slow rates of proliferation that do not invade

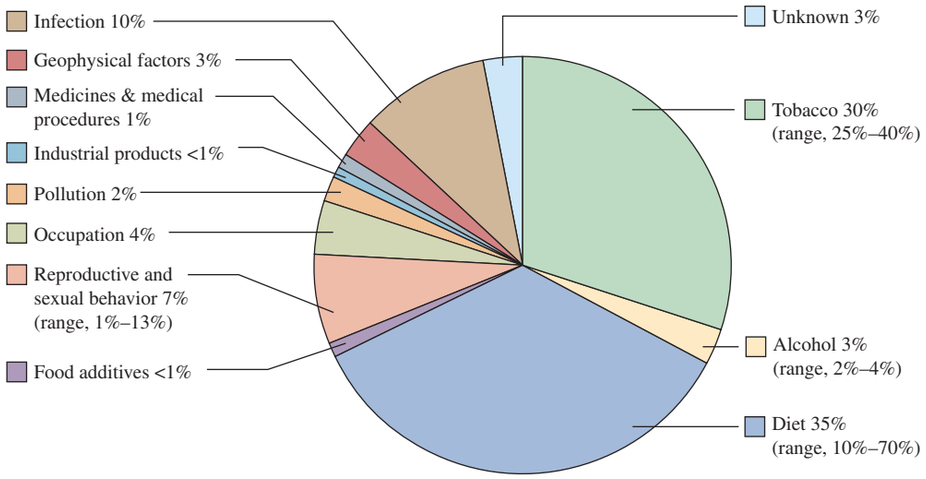


Figure 8-1. *Proportions of human cancer deaths attributed to various factors.* (Reproduced with permission from [no authors listed] Harvard reports on cancer prevention: causes of human cancer. Center for Cancer Prevention Harvard School of Public Health. *Cancer Causes and Control.* 1996;7 (Suppl 1): S3–S4, 1996.)

Table 8-1
Terminology

Neoplasia	New growth or autonomous growth of tissue
Neoplasm	The lesion resulting from the neoplasia
Benign	Lesions characterized by expansive growth, frequently exhibiting slow rates of proliferation that do not invade surrounding tissues
Malignant	Lesions demonstrating invasive growth, capable of metastases to other tissues and organs
Metastases	Secondary growths derived from a primary malignant neoplasm
Tumor	Lesion characterized by swelling or increase in size, may or may not be neoplastic
Cancer	Malignant neoplasm
Carcinogen	A physical or chemical agent that causes or induces neoplasia
Genotoxic	Carcinogens that interact with DNA resulting in mutation
Non-genotoxic	Carcinogens that modify gene expression but do not damage DNA

surrounding tissue or other organs. Benign neoplasms can impair and damage the normal function of an organ through its growth by impeding blood flow. A malignant neoplasm (e.g., a carcinoma) demonstrates invasive growth characteristics, capable of spreading not only through the organ of origin but also via metastasis to other organs. Metastases are secondary growths derived from the cells of the primary malignant neoplasm.

The term tumor is a general term that is frequently used synonymous with a neoplastic lesion and describes a grossly visible lesion. Further histopathological examination is required to confirm exact type, grade, and stage of the neoplasm. In classifying neoplasms, the nomenclature reflects both the tissue or cell of origin, and the characteristics of the type of tissue involved (Table 8-2). For benign neoplasms, the tissue of origin is frequently followed by the suffix "oma"; for example, a benign fibrous neoplasm would be termed fibroma, and a benign glandular epithelium termed an adenoma. Malignant neoplasms from epithelial origin are called carcinomas (from which the term cancer has evolved) while those derived from mesenchymal origin are referred to as sarcoma. Thus, a malignant neoplasm of fibrous tissue would be a fibrosarcoma while that derived from bone would be an osteosarcoma. Similarly, a malignant neoplasm from the liver would be a hepatocellular carcinoma while that derived from skin squamous epithelium is referred to as a squamous cell carcinoma. Preneoplastic lesions have also been observed in a number of target organs of both animal models and humans, and reflect an early reversible lesion in neoplasm progression. The characterization and study of preneoplastic cells have led to further understanding of the process of cancer formation.

The term cancer describes a subset of neoplasia that represents malignant neoplasms. A carcinogen is an agent, chemical or physical or biological (viruses), that causes or induces a cancer, although it is often also used to describe an agent that produces benign neoplastic lesions in rodent bioassays. In regulatory science this has been further defined as an agent whose administration to previously untreated animals leads to a statistically significant increased incidence of neoplasia of one of more histogenetic types as compared with the incidence seen in untreated control animals (Pitot, 1986). Thus, the induction of either or both benign and malignant neoplasms by a chemical or physical agent is included in the definition of a carcinogen. Carcinogens can be chemicals, viruses, hormones, radiation, or solid materials. Carcinogens either produce new neoplastic growth in a tissue or organ or increase the incidence and/or multiplicity of background spontaneous neoplastic formation in the target tissue.

Table 8-2
Neoplasm Nomenclature

TISSUE OF ORIGIN	BENIGN NEOPLASM	MALIGNANT NEOPLASM
Connective tissue		
Bone	Osteoma	Osteosarcoma
Fibrous	Fibroma	Fibrosarcoma
Fat lipid	Lipoma	Liposarcoma
Blood cells and related cells		
Hematopoietic cells		Leukemias
Lymphoid tissue		Lymphomas
Muscle		
Smooth	Leiomyoma	Leiomyosarcoma
Striated	Rhabdomyoma	Rhabdomyosarcoma
Endothelium	Hemangioma	Angiosarcoma
Mesothelium		Mesothelioma
Epithelial		
Squamous	Squamous cell papilloma	Squamous cell or carcinoma
Respiratory	Bronchial adenoma	Bronchogenic carcinoma
Renal epithelium	Renal tubular adenoma	Renal cell carcinoma
Liver cells	Liver cell adenoma	Hepatocellular carcinoma
Urinary epithelium	Transitional cell papilloma	Transitional cell carcinoma
Testicular epithelium		Seminoma
Melanocytes		Malignant melanoma

Table 8-3
Features of Genotoxic and Non-genotoxic Carcinogens

Genotoxic carcinogens
- Mutagenic
- Can be complete carcinogens
- Tumorigenicity is dose responsive
- No theoretical threshold

Non-genotoxic carcinogens
- Nonmutagenic
- Threshold, reversible
- Tumorigenicity is dose responsive
- May function at tumor promotion stage
- No direct DNA damage
- Species, strain, tissue specificity

The process of carcinogenesis is a multistage and multistep process involving modification and mutation to genes that regulate important normal cellular processes. An extensive literature base exists to describe the changes that cells undergo during the formation of neoplasia. Characteristics of cancers and the biological processes involved have been extensively reviewed (Hanahan and Weinberg, 2011). For the purpose of regulatory classification, carcinogens have frequently been divided simplistically into two major categories based on their general mode of action: genotoxic and non-genotoxic. Genotoxic carcinogens are those agents that interact with DNA to damage or change its structure. They are frequently mutagenic in a dose-responsive manner, and for regulatory purposes they are classified as exhibiting low-dose linear patterns, which approximates a straight line at very low doses without a threshold and a lack of reversibility of effect upon removal of the agent.

Non-genotoxic carcinogens are the agents that do not directly interact with nuclear DNA. Non-genotoxic carcinogens may change gene expression, modify normal cell function, bind to or modify cellular receptors, and increase the number of cells in the target tissue. These agents work through epigenetic mechanisms that modify DNA and proteins associated with DNA which alter the gene expression or the cellular phenotype without modifying or directly damaging its structure. Non-genotoxic chemical carcinogens create a situation in a cell or tissue that makes it more susceptible to DNA damage from other sources (natural or xenobiotic), which in turn may increase the rate of mutagenesis and thus carcinogenesis. Non-genotoxic agents for regulatory purposes exhibit a nonlinear, threshold, and dose–response pattern in their carcinogenicity. Common features of genotoxic and non-genotoxic carcinogens are shown in Table 8-3.

For regulatory purposes, several attempts have been made to characterize chemical carcinogens based on cellular and molecular pathways that are perturbed following agent exposure. Smith and associates have compiled a list of 10 key effects (Table 8-4) of carcinogens in an effort to use established mode of action data to help assess the potential carcinogenicity of untested agents (Smith et al., 2016). This builds on the mode of action framework (Sonich-Mullin et al., 2001; Cohen et al., 2003; Boobis et al., 2006). As with all approaches to characterizing, identifying, and evaluating mechanistic data when assessing whether an agent is a potential human carcinogen, it is important to consider dose–response and adverse versus adaptive changes from the experimental data. In addition, one should be cognizant of the interaction and multiplicity of pathways in the cancer process. For example increased inflammation results in oxidative stress and damage that can in turn induce epigenetic changes including increased cell number.

HISTORICAL BACKGROUND

A historical foundation for the linkage of the induction of cancer in humans and animal models by chemicals has been well documented (Table 8-5). Several excellent reviews of the historical background of carcinogenesis and cancer research have been published (Shimkin, 1980; Diamandopoulos, 1996; Creech, 2000; Harvey, 2011). Studies over the last three centuries on chemically induced cancer are marked initially by epidemiological observations followed by experimental studies involving animal carcinogenesis models.

In 1775, Percival Pott described a linkage between the increased occurrence of scrotal and nasal cancer among chimney sweeps and their occupation. Pott concluded that chimney soot was the causative agent for cancer induction in these individuals (Pott, 1775). Other investigators also recognized an association between exposure

Table 8-4

Key Characteristics of Carcinogens

CHARACTERISTIC	EXAMPLES OF RELEVANT EVIDENCE
1. Is electrophilic or can be metabolically activated	Parent compound or metabolite with an electrophilic structure (e.g., epoxide, quinone), formation of DNA and protein adducts
2. Is genotoxic	DNA damage (DNA strand breaks, DNA–protein cross-links, unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (e.g., chromosome aberrations, micronuclei)
3. Alters DNA repair or causes genomic instability	Alterations of DNA replication or repair (e.g., topoisomerase II, base-excision or double-strand break repair)
4. Induces epigenetic alterations	DNA methylation, histone modification, microRNA expression
5. Induces oxidative stress	Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g., DNA, lipids)
6. Induces chronic inflammation	Elevated white blood cells, myeloperoxidase activity, altered cytokine, and/or chemokine production
7. Is immunosuppressive	Decreased immunosurveillance, immune system dysfunction
8. Modulates receptor-mediated effects	Receptor in/activation (e.g., ER, PPAR, AhR) or modulation of endogenous ligands (including hormones)
9. Causes immortalization	Inhibition of senescence, cell transformation
10. Alters cell proliferation, cell death, or nutrient supply	Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis

Abbreviations: AhR, aryl hydrocarbon receptor; ER, estrogen receptor; PPAR, peroxisome proliferator–activated receptor.
Any of the 10 characteristics in this table could interact with any other (e.g., oxidative stress, DNA damage, and chronic inflammation), which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone.
SOURCE: Adapted from Smith MT et al. Environ Health Perspect. 2016;124(6):713–721.

to chemicals and the induction of human cancer. Thiersch (1875) described a relationship between sunlight exposure and skin cancer in humans. A linkage between an increased incidence of lung cancer and uranium mining was detected (Harting and Hesse, 1879). Butlin, in a follow-up of the Potts' observations, noted that scrotal cancer in chimney sweeps in the European continent was relatively rare on the continent compared to that seen in England (Butlin, 1892). He attributed this difference to better hygiene practices by the European sweeps as well as to the use of younger boys in England, suggesting that age of exposure and the duration of exposure influence the formation of the cancer. In 1895, Rehn reported a linkage between the manufacturer of aniline dyes and the induction of bladder cancer in dye workers (Rehn, 1895). Due to the increase in demand for synthetic dyes in the 19th century, production of the aniline-based dyes increased, as did the development of skin and bladder cancer in exposed workers. The specific chemicals in the dyes related to the cause of skin and bladder cancer were later determined to be the aromatic compounds 2-naphthylamine and benzidine (Hueper et al., 1938). Exposure of workers to metals, such as chromium (Alwens and Jonas, 1938) and asbestos (Wood and Gloyne, 1934), was shown to be associated with an increased incidence of lung cancers in workers. Tobacco use and its linkage to lung cancer provide an excellent case study on the linkage between epidemiology and experimental carcinogenesis. As early as 1912, Adler noted an association between smokers and lung cancer (Spiro and Silvestri, 2005). Similarly, Lickint reported on the linkage between tobacco smoking and lung cancer in the 1920s and also associated the higher rate of lung cancer seen in males with tobacco smoking (Proctor, 2001). Subsequent investigations led to the observation by Doll and colleagues, in a longitudinal epidemiology study, that there was a direct cause and effect of smoking to lung cancer (Doll et al., 2004).

Based on the epidemiological observations in humans, experimental investigations starting in the first half of the 20th century were performed to understand the biological mechanisms, nature of these chemical mixtures, and individual chemical compounds in the mixtures using animal models. Initial studies by Yamagiwa and co-workers examined the linkage between exposure to coal tar and its derivatives on cancer induction in humans by painting with coal tar on rabbit ears, which resulted in the formation of skin cancer (Yamagiwa and Ichikawa, 1915). Subsequently, Kennaway and Hieger purified dibenz(*a*,*h*)anthracene from this coal tar extract, and determined that it was at least one of the components of the coal tar mixture that was responsible for inducing skin cancer (Kennaway and Hieger, 1930). Cook et al. (1933) similarly extracted another polyaromatic hydrocarbon (PAH), benzo(*a*)pyrene, from the coal tar and showed this to also function as a carcinogen in rodents. In similar approaches with aromatic amines, Hueper and colleagues reported that 2-naphthylamine induced bladder tumors in dogs (Hueper et al., 1938), and Yoshida (1933) and Kinosita (1936) found that feeding aminoazotoluene or aminoazobenzene to rodents induced liver cancer. James and Elizabeth Miller (and their students) in the last half of the 20th century established the role of chemical structure and metabolism in the "activation" of carcinogens to a macromolecular interactive form (Miller, 1951; Miller and Miller, 1981; Miller et al., 1983). Studies with the aromatic amine compounds in animal models correlated with the human epidemiological studies that had revealed an association between bladder cancer in humans and exposure to aniline dyes in

Table 8-5

Historical Events in Chemically Induced Cancer

DATE	INVESTIGATOR(S)	CAUSATIVE AGENT
1775	Pott	Soot and chimney sweeps
1822	Ayrton	Arsenic containing metal
1875	Thiersch	Sunlight
1876	Manourriez	Coal tar
1879	Harting and Hesse	Lung cancer and uranium
1892	Butlin	Soot and chimney sweeps
1895	Rehn	Manufacture of aniline dyes
1902	Frieben	X-rays
1915	Davis	Pipe smokers and betel nut chewers
1915	Yamagiwa, Ichikawa, and Tsusui	Induction of skin cancer in rabbits and mice by coal tar
1920	Leitch and Seguina	Radium radiation
1928	Delore and Bergamo	Benzene
1930	Kennaway and Hieger	Tumor induction by dibenz(*a*,*h*)anthracene
1932	Stephens	Nickel
1932	Alwens	Chromium compounds
1933	Cook, Hewett, and Hieger	Isolation of the carcinogen benzo[*a*]pyrene from coal tar
1936	Yoshida and Kinosita	Induction of liver cancer in rats by *o*-aminoazotoluene
1934	Wood and Gloyne	Arsenicals, beryllium, and asbestos
1934	Neitzel	Mineral oil mists and radiation
1936	Kawahata	Coal tar fumes
1938	Hueper, Wiley, and Wolfe	Induction of urinary cancer in dogs by 2-naphthylamine
1941	Berenblum, Rous, MacKenzie, and Kidd	Initiation and promotion stages in skin carcinogenesis with benzo[*a*]pyrene
1951	Miller and Miller	Carcinogen binding to cellular macromolecule
1956	Doll and Hill	Lung cancer and other causes of death in relation to smoking

the occupational setting, and helped to define the specific chemicals in the dyes responsible for the cancer induction. Studies by investigators such as Harris and colleagues over the last 30 years have utilized the laboratory to examine the environmentally induced cancers reported in epidemiological finding, especially in the area of lung cancer and tobacco smoking (Greenblatt et al., 1994). These epidemiological and experimental studies have shown a clear correlation between the induction of cancer in humans and rodents by chemical exposure.

An important historical event in the development of carcinogenesis testing was the development of a short-term bacterial-based mutagenesis bioassay in the early 1970s by Ames and colleagues. This Salmonella-based assay has become a stalwart in the early detection of mutagenic compounds that have the potential to be carcinogenic. A recent review of the historical aspects of the development and use of the Salmonella assay has been published (Claxton et al., 2010). The success of the Ames Salmonella Assay spurred the use of in vitro assays for mutagenicity in bacteria and mammalian cells as predictors of potential rodent and human carcinogens (Tennant et al., 1987), culminating in the current genetic toxicity test battery (Eastmond et al., 2009). As we approach the third decade of the 21st century, the need to further develop high-throughput and shortened bioassays for carcinogen detection (cell or molecular biology based) has been identified (National Research Council (US) Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology, 2007).

Projects within and supported by the US Federal government have been developed to address this need. These programs include the Toxicology Testing in the 21st Century (Tox 21) which is a collaboration between the National Institute of Health and the USEPA and the USEPA's Toxcast program. The latter using in vitro screening approaches coupled with computational support to help prioritize possible toxic chemicals for additional investigation. Both the Tox 21 and the Toxcast programs are new approaches to toxicity risk assessment (including cancer risk) that are founded in our current understanding of the molecular and cellular cancer-dependent pathways.

MULTISTAGE CARCINOGENESIS

Extensive experimental studies with animal models and pathological evaluation of human cancers have demonstrated that the carcinogenesis process involves a series of definable stages and steps (Shih et al., 2001; Osada and Takahashi, 2002). Operationally, three defined stages, initiation, promotion, and progression have been identified in rodent studies (initially in skin and liver tumors models) (Fig. 8-2). These steps follow a temporal sequence of events

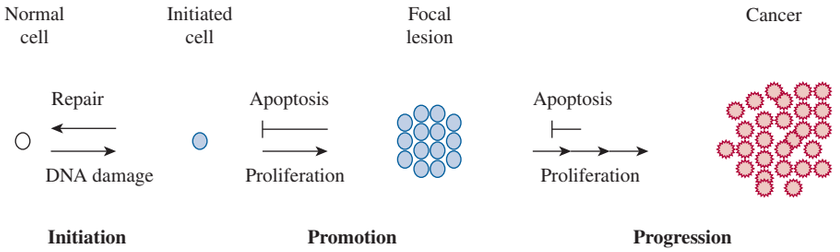


Figure 8-2. *Multistage model carcinogenesis.*

demonstrable by histopathology and observed in a wide variety of target tissues. The defining characteristics of each of these stages are outlined in Table 8-6. It is important to remember that the defining of these three stages is used to help characterize the multistage nature of chemically induce tumors. Once a neoplasm is formed, additional intracellular and extracellular changes occur in the process of the development of a malignant cancer (Goyette et al., 1992; Vogelstein and Kinzler, 2004).

Initiation

The first stage of the cancer process involves initiation, a process that is defined as a stable, heritable change. This stage is a relatively rapid, irreversible process that results in a carcinogen-induced mutational event. Chemical and physical agents that function at this stage are referred to as initiators or initiating agents. Initiating agents lead to genetic changes including mutations and deletions. Chemical carcinogens that covalently bind to DNA and form adducts that result in mutations are initiating agents. Included among chemicals classified as initiating carcinogens are compounds such as polycyclic hydrocarbons and nitrosamines, biological agents, certain viruses, and physical agents such as x-rays and ultraviolet (UV) light. Most chemical carcinogens that function at the initiation stage of the cancer process are indirect-acting genotoxic compounds that require metabolic activation in the target cell to produce the DNA-damaging event. For these compounds, the chemical must be taken into the target site and metabolized (in the case of an indirect genotoxic carcinogen). The ultimate form of the carcinogen is then able to bind to nuclear DNA, resulting in adduct formation. The initiating event becomes "fixed" when the DNA adducts or other damage to DNA is not correctly repaired or is incompletely repaired prior to DNA synthesis. This event can lead to inappropriate base pairing and formation of a mutation.

Initiation by itself does not appear to be sufficient for neoplastic formation. Once initiated cells are formed, their fate has multiple potential outcomes: (1) the initiated cell can remain in a static nondividing state through influences by growth control either via normal surrounding cells or through endocrine influence; (2) the initiated cell may possess mutations incompatible with viability or normal function and be deleted through apoptotic mechanisms; or (3) the cell, through stimuli such as intrinsic factors or from chemical exposure, may undergo cell division resulting in the growth in the proliferation of the initiated cell. Besides the production of an initiated cell through carcinogen binding and misrepair, additional evidence has come forth showing that induction of continual stress, resulting in continual cell proliferation, can also produce new mutated, initiated cells (Matthews et al., 2009; Johnstone and Baylin, 2010). Therefore, modification of cell signaling and gene expression (while usually ascribed to the tumor promotion process and as an epigenic pathway [see below]) may indirectly through sustained cell proliferation also result in the formation of mutations (Lewandowska and Bartoszek, 2011). In some instances, typically following relatively high doses and/or repeated exposure to the genotoxic carcinogen, a chemical carcinogen may function as a complete carcinogen, that is, it is capable of progressing through all stages of the cancer process.

Table 8-6

Characteristics of the Stages of Carcinogenesis Process

Initiation
DNA modification
Mutation
Genotoxic
One cell division necessary to lock-in mutation
Modification is not enough to produce cancer
Nonreversible
Single treatment can induce mutation
Promotion
No direct DNA modification
Non-genotoxic
No direct mutation
Multiple cell divisions necessary
Clonal expansion of the initiated cell population
Increase in cell proliferation or decrease in cell death (apoptosis)
Reversible
Multiple treatments (prolonged treatment) necessary
Threshold
Progression
DNA modification
Genotoxic event
Mutation, chromosome disarrangement
Changes from preneoplasia to neoplasia benign/malignant
Irreversible
Number of treatments needed with compound unknown (may require only single treatment)

Promotion

The second stage of the carcinogenesis process (the promotion stage) involves the selective clonal expansion of initiated cells to produce a preneoplastic lesion. Exogenous and endogenous agents

that function at this stage are frequently referred to as tumor promoters. Compounds that function at the promotion stage are not mutagenic and generally are not able to induce tumors by themselves. They act though mechanisms that involve changes in gene expression that in turn result in sustained cell number in the target tissue either through increases in cell proliferation and mitogenesis and/or the inhibition of apoptosis. This stage involves the modulation of gene expression through receptor or non–receptor-mediated processes. Non-genotoxic carcinogens frequently function at the tumor promotion stage. The growth of preneoplastic lesions requires repeated applications or continuous exposure to tumor-promoting compounds. Although initial exposure to tumor promoters may result in an increase in cell proliferation and/or DNA synthesis in normal cells in an organ, this is usually a transient effect. With repeated applications of the chemical only initiated cells continue to clonally expand and divide into a focal lesion (Fig. 8-2). Tumor promotion is a dose-dependent and reversible process whereby removal of the promotional agent, the focal cells cease proliferation and through apoptosis the focal lesion may return back to a small group of initiated cells. In addition, agents that function at the promotion stage demonstrate a dose-dependent threshold for their effects. Below a certain dose or frequency of application the chemical is unable to induce cell growth. Carcinogens that function at the tumor promotion stage in general are organ specific. For example, phenobarbital functions at the tumor promotion stage selectively in the liver but will not promote tumorigenesis in the skin or most other tissues.

Progression

A third stage of the chemical carcinogenesis process, progression, involves the conversion of the preneoplastic lesions to a neoplasm. In this stage, additional genotoxic events occur resulting in additional DNA damage including chromosomal damage such as aberrations and translocations. In the neoplastic state, cells accumulate mutations and epigenetic changes that release normal growth control of the cells and cause them to outgrow the surrounding normal cells. The tumor microenvironment is an important component of this process and the presence of "normal" cells and stroma within the lesion is critical for the neoplastic cells to survive and propagate. During the progression stage, the clonal nature of the neoplastic lesion is typically lost with a polyclonal appearance of cells within the lesion. Agents that impact on the progression stage are usually genotoxic agents although the acquisition of additional mutagenic events may occur through the continual stimulation of cell proliferation. These events result in the transfer from preneoplastic, clonally derived cell populations into neoplastic cell populations. By definition, the progression stage is an irreversible stage in that neoplasm formation, whether benign or malignant, occurs. With the formation of neoplasia, an autonomous growth and/or lack of growth control is achieved. Spontaneous progression can occur from spontaneous karyotypic changes that occur in mitotically active initiated cells during promotion. An accumulation of nonrandom chromosomal aberrations and karyotypic instability are hallmarks of progression. As such, chemicals that function at the progression stage of carcinogenesis many times are clastogenic and are capable of causing chromosomal abnormalities.

Complete carcinogens have the ability to function at all three stages of carcinogenesis and hence have genotoxic properties. A number of rodent and in vitro models are available to test for carcinogenicity and to study the multistep mechanisms involved in chemical carcinogenesis. These models are described below.

MECHANISMS OF ACTION OF CHEMICAL CARCINOGENS

The formation of a neoplasm is a multistage, multistep process that involves the ultimate release of the neoplastic cells from normal growth control processes and creating a tumor microenvironment. In two reviews, Hanahan and Weinberg (2000, 2011) defined the major characteristics of a neoplasm as it progresses into a malignant state. The authors have proposed eight "hallmarks of cancer" that involve modifications to the homeostasis of normal cells imparting the growth and metastatic properties seen in the development of a neoplastic cell. The eight properties include (1) sustaining cell proliferation, (2) resisting cell death (apoptosis), (3) inducing angiogenesis, (4) enabling replicative immortality, (5) activating invasion and metastasis, (6) evading growth suppressors, (7) reprogramming of energy metabolism, and (8) evading immune destruction (Table 8-7). An important concept from this review is the fact that tumors are not just a collection of clonal neoplastic cells but a complex tissue with multiple cell populations that interact with one another and function as a unique tissue. This tumor microenvironment involves the recruitment of normal stromal and inflammatory cells that contribute to the growth of the development of the neoplasm.

Although our understanding of the cancer process has expanded exponentially in the past several decades as illustrated by the "hallmarks-of-cancer" concept, in its minimalist form two major processes are needed with regard to the induction of neoplasia by chemicals: a mutational event and a selective proliferation of the mutated cell to form a neoplasm. Based on this supposition, chemicals that induce cancer have been classified into one of two broad categories (as noted above) genotoxic (DNA-reactive) agents and non-genotoxic (epigenetic) agents. This is admittedly a very simplistic approach to the complexities of the cancer process (as illustrated by the Hanahan and Weinberg's review [2011]); however, for the purposes of regulatory evaluation and cancer risk assessment this categorization has for the most part continued.

Genotoxic/DNA-Reactive Compounds

Genotoxic compounds interact with nuclear DNA of a target cell producing unrepaired DNA damage that is inherited in subsequent daughter cells. Experimental and epidemiological observations made in the middle of the 20th century identified a number of chemicals that could cause cancer in humans or experimental animals. Coal tar carcinogens including benzo(*a*)pyrene, pesticides such as 2-acetylaminofluorine, and azo dyes such as

Table 8-7

Hallmarks of Cancer

Eight properties of neoplastic cells that impart and contribute to their ability to independently grow and eventually metastasize

1. Sustaining cell proliferation
2. Resisting cell death (apoptosis)
3. Inducing angiogenesis
4. Enabling replicative immortality
5. Activating invasion and metastasis
6. Evading growth suppressors
7. Reprogramming of energy metabolism
8. Evading immune destruction

SOURCE: Data from Hanahan and Weinberg (2011).

Table 8-8

Examples of Genotoxic Carcinogens

A. Direct-acting carcinogens
- Nitrogen or sulfur mustards
- Propane sulfone
- Methyl methane sulfonate
- Ethyleneimine
- B-Propiolactone
- 1,2,3,4-Diepoxybutane
- Dimethyl sulfate
- Bis-(Chloromethyl) ether
- Dimethylcarbamyl chloride

B. Chemicals requiring activation (indirect-acting carcinogens)
- Polycyclic aromatic hydrocarbons and heterocyclic aromatics
- Aromatic amines
- *N*-Nitrosoamines
- Azo dyes
- Hydrazines
- Cycasin
- Safrole
- Chlorinated hydrocarbons
- Aflatoxin
- Mycotoxin
- Pyrrolizidine alkaloids
- Bracken fern
- Carbamates

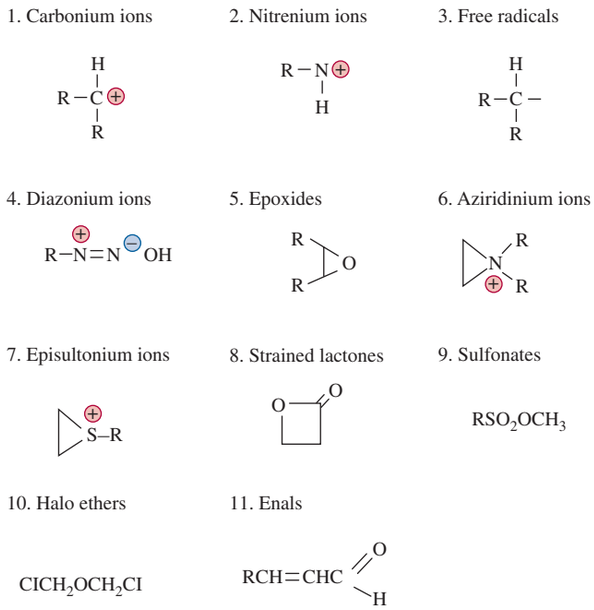


Figure 8-3. *Structures of reactive electrophiles.*

diaminobenzamide were among the first chemical carcinogens to be studied. DNA-reactive carcinogens can be further subdivided according to whether they are active in their parent form (i.e., direct-acting: chemicals that can directly bind to DNA without being metabolized) and those that require metabolic activation (i.e., indirect-acting carcinogens: compounds that require metabolism in order to react with DNA). Examples of direct-acting and indirect-acting carcinogens are listed in Table 8-8.

Direct-Acting (Activation-Independent) Carcinogens A class of carcinogens that do not require metabolic activation or modification to induce cancer are termed direct-acting or activation-independent carcinogens. These chemicals are also defined as ultimate carcinogens. Examination of the chemical structure of these chemicals reveals that they are highly reactive electrophilic molecules that can interact with and bind to nucleophiles such as cellular macromolecules including DNA. Some common electrophilic species in these groups are shown in Fig. 8-3. Generally, these chemicals containing these moieties frequently cause tumor formation at the site of chemical exposure.

Direct-acting carcinogens include epoxides, imines, alkyl and sulfate esters, and mustard gases (Fox and Scott, 1980; Sontag, 1981). Direct-acting electrophilic carcinogenic chemicals typically test positive in the Ames test without additional bioactivation with a liver metabolic fraction. The relative carcinogenic strength of direct-acting carcinogens depends in part on the relative rates of interaction between the chemical and genomic DNA, as well as competing reactions with the chemical and other cellular nucleophiles. The relative carcinogenic activity of direct-acting carcinogens is dependent upon such competing reactions and also on detoxification reactions. Chemical stability, transport, and membrane permeability determine the carcinogenic activity of the chemical. Direct-acting carcinogens are typically carcinogenic at multiple sites and in all species examined. A number of direct-acting alkylating chemicals, including a number of chemotherapeutic drugs, are carcinogenic in humans (Marselos and Vainio, 1991).

Indirect-Acting Genotoxic Carcinogens An important discovery in the understanding of chemical carcinogenesis came from the investigations of the Millers who established that many carcinogens are not intrinsically carcinogenic, but require metabolic activation to be carcinogenic. They demonstrated that azo dyes covalently bind to proteins in liver, leading to the conclusion that carcinogens may bind to proteins that are critical for cell growth control (Miller and Miller, 1947). Work with benzo(*a*)pyrene showed covalent binding of benzo(*a*)pyrene or the metabolites of benzo(*a*)pyrene in rodents (Miller, 1951). Subsequent investigations with other indirect-acting genotoxic carcinogens by other investigators confirmed that metabolism of the parent compound was necessary to produce a metabolite (activation) that was able to interact with DNA (Miller, 1970).

It has since been shown that the majority of DNA-reactive carcinogens are found as parent compounds, or procarcinogens. Procarcinogens are stable chemicals that require subsequent metabolism to be carcinogenic (Miller and Miller, 1981; Weisburger and Williams, 1981; Conney, 1982; Miller et al., 1983). The terms procarcinogen, proximate carcinogen, and ultimate carcinogen have been coined to define the parent compound (procarcinogen) and its metabolite form, either intermediate (proximate carcinogen) or final (ultimate carcinogen) that reacts with DNA (Fig. 8-4). The ultimate form of the carcinogen is most likely the chemical species that results in mutation and neoplastic transformation. The ultimate form of certain carcinogenic chemicals is not known, whereas for other chemicals there may be more than one ultimate carcinogenic metabolite depending on the metabolic pathway followed. It is important to note that besides activation of the procarcinogen to a DNA-reactive form, detoxification pathways may also occur inactivating the carcinogen.

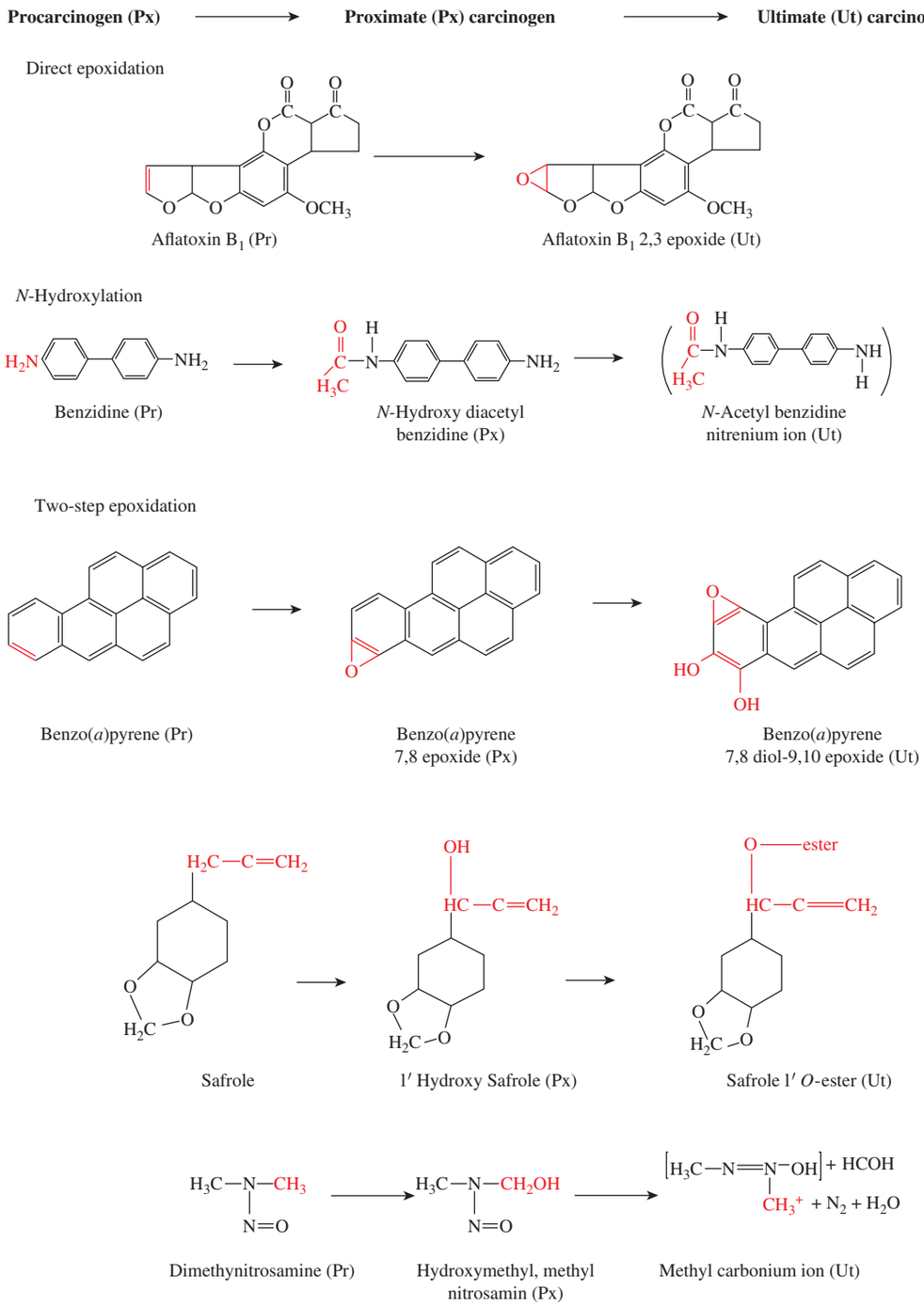


Figure 8-4. *Structures of representative indirect-acting carcinogens and their metabolic derivatives, the proximate (Px) and ultimate (Ut) carcinogenic form result from the metabolism of the procarcinogenic form (Pr).*

Indirect-acting genotoxic carcinogens usually produce their neoplastic effects, not at the site of exposure (as seen with direct-acting genotoxic carcinogens) but at the target tissue where the metabolic activation of the chemical occurs. Indirect-acting genotoxic carcinogens include the polycyclic PAHs, nitrosamines, aromatic amines, and aflatoxin B1. Fig. 8-4 shows the parent (procarcinogen) and metabolites for several representative indirect-acting genotoxic carcinogens.

Mutagenesis

The reaction of a carcinogen with genomic DNA either directly or indirectly may result in DNA adduct formation or DNA damage, and frequently produces a mutation. Several mechanisms of mutagenesis are known to occur. Modification of DNA by electrophilic carcinogens can lead to a number of products. These events are dependent upon when in the cell cycle the adducts are formed, where the adducts are formed, and the type of repair process used in response to the damage.

Transitions are a substitution of one pyrimidine by the other or one purine by the other (changes within a chemical class), whereas a transversion occurs when a purine is replaced by a pyrimidine or a pyrimidine is replaced by a purine (changes across a chemical class). Carcinogens can induce transitions and transversions several ways. In one scenario, when adducts (or apurinic or apyrimidinic sites) are encountered by the DNA replication processes, they may be misread. The polymerase may preferentially insert an adenine (A) in response to a noninformative site. Thus, the daughter strand of an A, C, G, or T adduct will have an adenine (A) and this change is fixed (mutation) and resistant to subsequent DNA repair. A second scenario, a shift in the reading frame (resulting in a frame shift mutation) may also result from carcinogen–DNA adducts formation. Most frame shift mutations are deletions and occur more frequently when the carcinogen–DNA adduct is formed on a nucleotide. In a third scenario, DNA strand breaks can also result from carcinogen–DNA adducts. These may arise either as a result of excision repair mechanisms that are incomplete during DNA replication or via direct alkylation of the phosphodiester backbone leading to backbone cleavage. Strand scission can lead to double-strand breaks, recombination, or loss of heterozygosity. It is worth noting that epigenetic changes such as altered DNA methylation, aberrant histone modifications, and dysregulated noncoding RNA expression play an important role in certain genotoxic chemicals induced carcinogenesis (Baccarelli and Bollati, 2009; Pogribny et al., 2011; Chappell et al., 2016).

Damage by Alkylating Electrophiles

As noted above, most chemical carcinogens require metabolic activation to exert a carcinogenic effect. The ultimate carcinogenic forms of these chemicals are frequently strong electrophiles (Fig. 8-3) that can readily form covalent adducts with nucleophilic targets. Because of their unpaired electrons, S:, O:, and N: atoms are nucleophilic targets of many electrophiles. The extent of adducts formed is limited by the structure of DNA where bulky electrophilic chemicals can bind, and size of the ultimate carcinogenic form. In general, the stronger electrophiles display a greater range of nucleophilic targets (i.e., they can attack weak and strong nucleophiles), whereas weak electrophiles are only capable of alkylating strong nucleophiles (e.g., S: atoms in amino acids). In addition, the metabolic capability of a target cell will dictate the extent and types of electrophiles generated from the procarcinogenic parent.

An important and abundant source of nucleophiles is contained not only in the DNA bases, but also in the phosphodiester backbone (Fig. 8-5). Although carcinogen–DNA adducts may be formed at all sites in DNA, the most common sites of alkylation include the N^7 of guanine, the N^3 of adenine, the N^1 of adenine, the N^3 of guanine, and the O^6 of guanine. Alkylations of phosphate may also occur at a high frequency. Selective examples of carcinogen interactions with proteins and nucleic acids are shown in Fig. 8-6. Different electrophilic carcinogens will often display different preferences for nucleophilic sites in DNA and different spectra of damage. Dimethylnitrosamine and diethylnitrosamine, for example, are metabolized by P450 oxidation to yield a methyl carbonium ion (CH_3^+) and an ethyl carbonium ion ($CH_3CH_3^+$), respectively. Despite the structural similarities of the ultimate electrophiles, they display significant differences in alkylation profiles (Pegg, 1984). The relative proportions of methylated bases present in DNA following reaction with carcinogen-methylating chemicals are shown in Table 8-9 (Pegg, 1984). The predominant adduct formed following exposure to methylating chemicals such as methylmethane sulfonate is 7-methylguanine. In contrast, ethylating agents produce adducts predominately in the phosphate backbone of DNA. The carcinogenic potential of the type of adducts formed is often debated; some believe that O^6-alkylguanine is the most

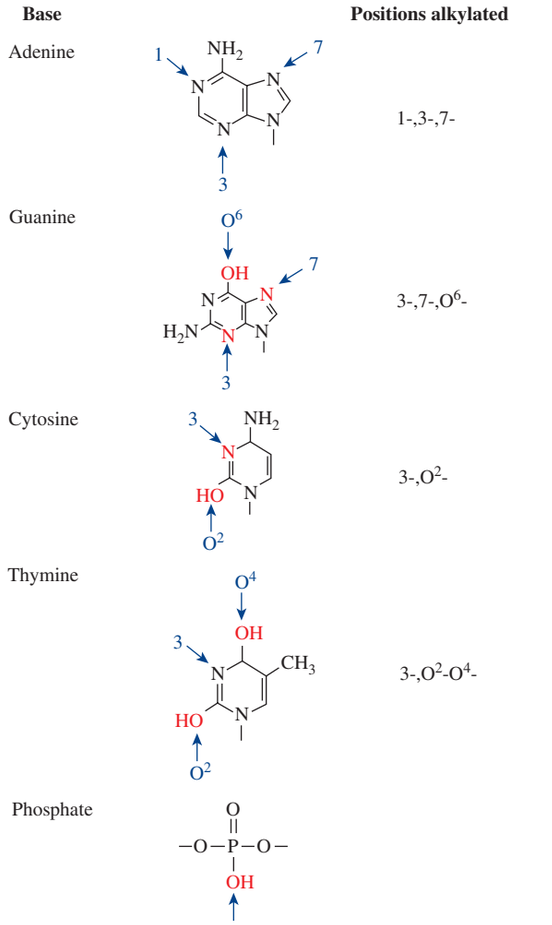


Figure 8-5. *Examples of cellular nucleophiles and sites of possible adduct formation.*

carcinogenic adduct, whereas others report that O^4-alkylthymine is more important in the carcinogenic process due to its persistence relative to other adducts (Pegg, 1984; Swenberg et al., 1984).

Another common modification to DNA is the hydroxylation of DNA bases. Oxidative DNA adducts have been identified in all four DNA bases (Fig. 8-7); however, 8-hydroxyguanine is among the most prevalent oxidative DNA adduct and an established biomarker of oxidative stress (Floyd, 1990; Valavanidis et al., 2009; Klaunig et al., 2011). The source of oxidative DNA damage is typically formed from free radical reactions that occur endogenously in the cell or from exogenous sources (Floyd, 1990; Ames and Shigenaga, 1993; Klaunig et al., 2011). Although a relatively large amount of oxidative DNA adducts have been proposed to be formed per day, repair mechanisms exist that maintain the cellular level low and keep endogenous mutations to a relatively low level. The role of oxidative damage and oxidative stress is discussed in greater detail later in this chapter.

Although a large number of adducts can be formed following exposure to chemicals, whether a particular DNA adduct will result in mutation and participate in the carcinogenesis process is dependent in part on the persistence of the adduct through the process of DNA replication, which is also in part dependent upon DNA repair.

DNA Repair

Following the formation of a carcinogen–DNA adduct, the persistence of the adduct is a major determinant of the outcome. This persistence depends on the ability of the cell to repair the altered DNA.

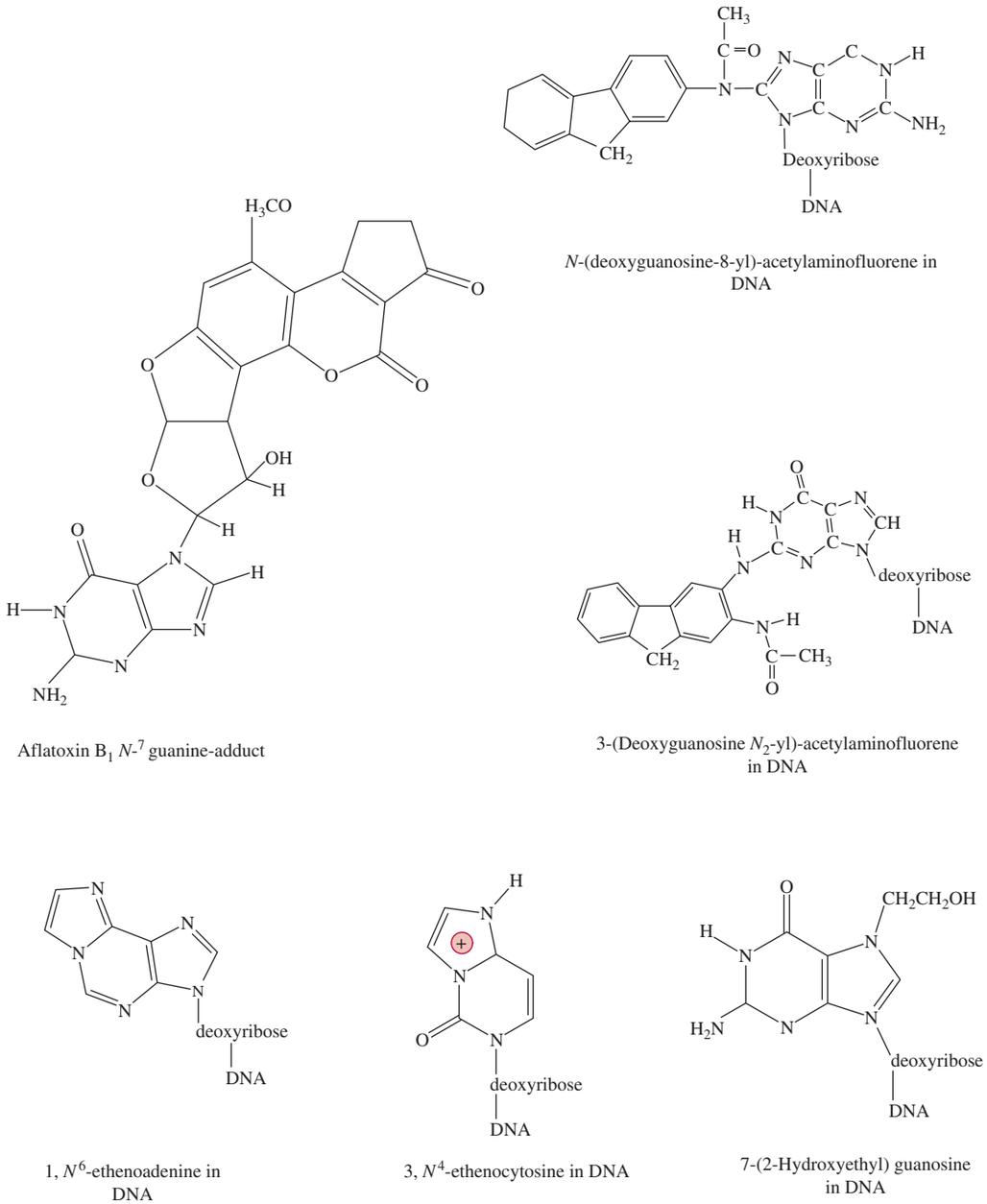


Figure 8-6. *Select structures of protein- and nucleic acid–bound forms of certain chemical carcinogens.*

The detection of unique DNA adducts has proven to be important in understanding the mechanism of action of specific carcinogens and has been correlated with carcinogenesis (Goth and Rajewsky, 1974; Kadlubar et al., 1981; Becker and Shank, 1985; Swenberg et al., 1985; Bailey et al., 1996). However, the presence of a DNA adduct is not sufficient for the carcinogenesis process to proceed. The relative rates or persistence of particular DNA adducts may be an important determinant of carcinogenicity, for example, O^4-ethylthymine is relatively stable in DNA whereas O^6-ethylguanine does not persist in DNA after continuous exposure to diethylnitrosamine (Swenberg et al., 1984). The persistence of DNA adducts of *trans*-4-aminostilbene does not correlate with organ carcinogenicity and/or tissue susceptibility. Although the liver and kidney exhibited the greatest burden and persistence of the adduct and the Zymbals gland showed the least amount of DNA adducts, the latter tissue was more susceptible to carcinogenesis by this chemical (Neumann, 1983). As such, differences in susceptibility to carcinogenesis are likely the result of a number of factors, including DNA replication within a tissue and repair of a DNA adduct.

The quantification of covalent DNA adducts in tissues has been used to demonstrate exposure of humans to carcinogenic chemicals and to assess the relative risk from exposure to carcinogenic chemicals. For example, DNA adducts of carcinogenic PAHs have been demonstrated at relatively high levels in tissues and blood of smokers and foundry workers compared with nonexposed individuals (Perera et al., 1991). In addition, DNA adducts of aflatoxin B1 were seen in samples of human placenta and cord blood in individuals in Taiwan, an area that demonstrates a high incidence of liver cancer (Hsieh and Hsieh, 1993). The presence of macromolecular carcinogen adducts may be important to their mechanism of carcinogenicity,

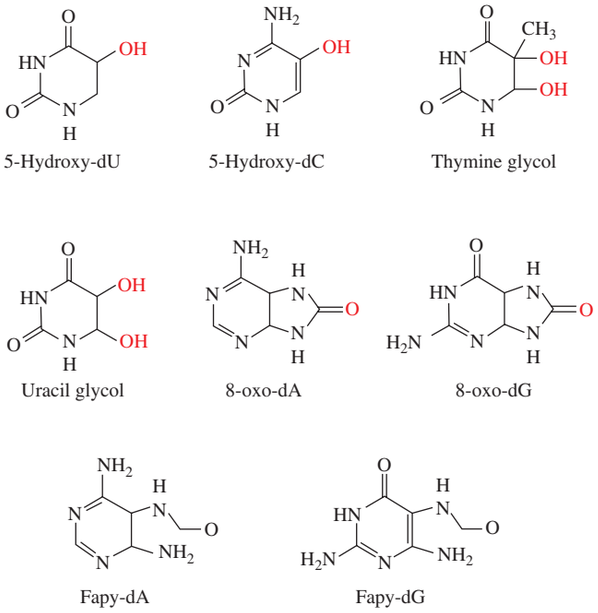


Figure 8-7. *Structures of selected oxidative bases.*

Table 8-9

Relative Proportions of Methylated Bases Present in DNA After Reaction with Carcinogenic Alkylating Agents

	PERCENT OF TOTAL ALKYLATION BY	
	DIMETHYLNITROSAMINE, *N*-METHYL-*N*-NITROSOUREA, OR 1,2-DIMETHYLHYDAZINE	DIETHYLNITROSAMINE OR *N*-ETHYL-*N*-NITROSOUREA
1-Alkyladenine	0.7	0.3
3-Alkyladenine	8	4
7-Alkyladenine	1.5	0.4
3-Alkylguanine	0.8	0.6
7-Alkylguanine	68	12
O^6-Alkylguanine	7.5	8
3-Alkylcytosine	0.5	0.2
O^2-Alkylcytosine	0.1	3
3-Alkylthymine	0.3	0.8
O^2-Alkylthymine	0.1	7
O^4-Alkylthymine	0.1–0.7	1–4
Alkylphosphates	12	53

the presence and persistence of the adducts is only one factor in the process of cancer development.

Experimental and epidemiological evidence indicates that the development of cancer following exposure to chemical carcinogens is a relatively rare event. This can be explained by the ability of a cell to recognize and repair DNA. The DNA region containing the adduct is removed and a new patch of DNA is synthesized, using the opposite intact strand as a template. The new DNA segment is then spliced into the DNA molecule in place of the defective one. To be effective in restoring a cell to normal, repair of DNA must occur prior to cell division; if repair is not complete prior to replication, the presence of the adducts can give rise to mispairing of bases and other genetic effects such as rearrangements and translocations of DNA segments. Thus, a chemical that alters the repair process or the rate of cell division can itself affect the frequency of mutation and neoplastic transformation.

DNA Repair Mechanisms

Although cells possess mechanisms to repair many types of DNA damage, these are not always completely effective, and residual DNA damage can lead to the insertion of an incorrect base during DNA replication, followed by transcription and translation of the mutated templates, ultimately leading to the synthesis of altered protein. Mutations in an oncogene, tumor-suppressor gene or gene that controls the cell cycle, can result in a clonal cell population with a proliferative or survival advantage. The development of a tumor requires many such events, occurring over a long period of time, and for this reason human cancer induction often takes place within the context of chronic exposure to chemical carcinogens.

A number of structural alterations may occur in DNA as a result of interaction with reactive chemicals or radiation, and the more frequent types of damage are depicted in Fig. 8-8. The reaction of chemical species with DNA can produce adducts within the bases, sugar, and phosphate backbone of DNA. In addition, bifunctional alkylating chemicals (such as mustards) may cause DNA cross-linking between two opposing bases. Other structural changes such as pyrimidine dimer formation are specific for exposure to UV light whereas double-stranded breaks in DNA are more commonly associated with ionizing radiation. A variety of mechanisms have evolved to effectively remediate and repair DNA damage. The more common types of DNA repair mechanisms seen in mammalians are listed in Table 8-10.

In addition to the proofreading activity of DNA polymerases that can correct miscopied bases during replication, cells have several mechanisms for repairing DNA damage. Repair of DNA damage does not always occur prior to cell replication, and repair of DNA damage by some chemicals is relatively inefficient. As such, exposure to chemicals can increase the probability of acquiring mutations that ultimately lead to cancer development.

Mismatch Repair of Single-Base Mispairs Spontaneous mutations may occur through normal cellular DNA replication mistakes. Many spontaneous mutations are point mutations, a change in a single base pair in the DNA sequence, or a small insertion or deletion or a frame shift mutation of some modest size. The issue for mismatch repair is determining which is the normal DNA and which is the damaged DNA strand, and, therefore, repairing the mutated strand such that the correct base pairs are restored. Depurination is a fairly common occurrence and spontaneous event in mammals, and results in the formation of apurinic sites. If these lesions are left unrepaired, mutations are generated during DNA replication as DNA synthetic machinery is unable to determine the appropriate base with which to pair. All mammalian cells possess apurinic endonucleases that function to cut DNA near apurinic sites. The cut is then extended by exonucleases, and the resulting gap repaired by DNA polymerase and ligase.

Excision Repair DNA regions containing chemically modified bases, or DNA chemical adducts, are typically repaired by

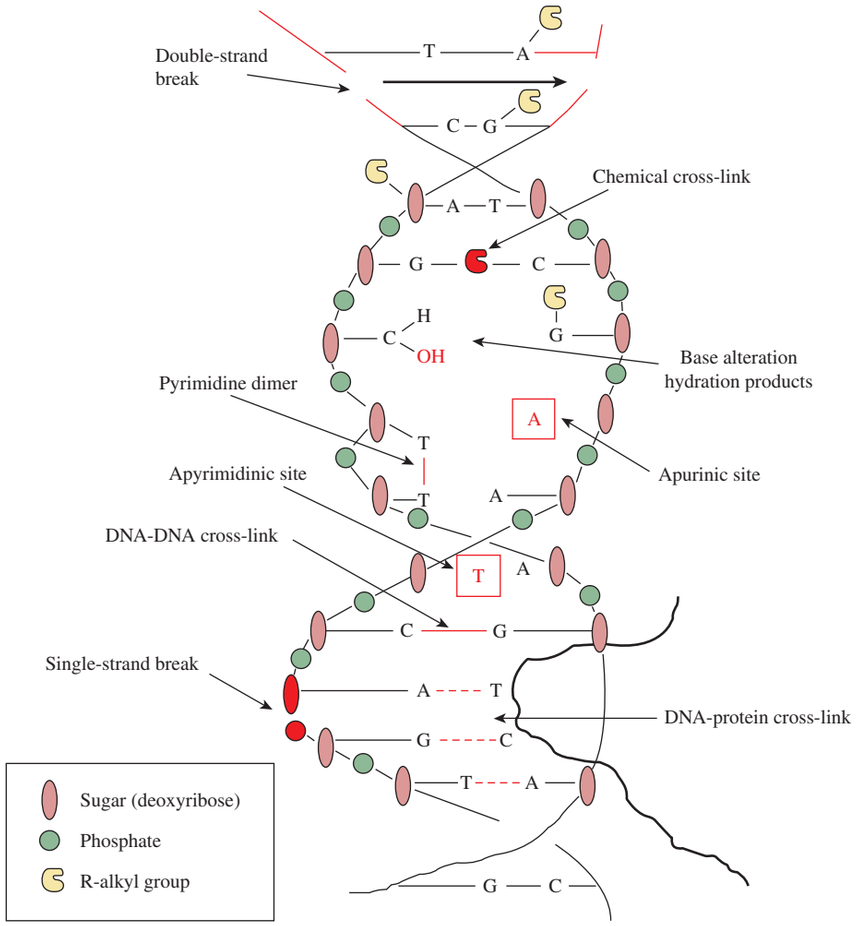


Figure 8-8. *Schematic representation of chemical and radiation-induced lesions in DNA.*

Table 8-10

DNA Repair Pathways

1. Direct reversal of DNA damage
2. Excision repair systems
   - Base excision repair (BER)
   - Nucleotide excision repair (NER)
   - Mismatch repair (MMR)
3. Double-strand break repair
   - Homologous recombination (HR)
   - Nonhomologous end-jointing (NHEJ)

excision repair processes. DNA adducts cause a distortion in the normal shape of DNA. Proteins that slide along the surface of a double-stranded DNA molecule recognize the irregularities in the shape of the double helix, and affect the repair of the lesion. DNA lesions that are repaired by excision repair processes include thymine–thymine dimers, produced following exposure to UV light, dimers that interfere with both replication and transcription of DNA. The repair of DNA regions containing bases altered by the attachment of large chemical adducts such as benzo(*a*)pyrene are also effectively repaired by excision repair processes (Fig. 8-9A, B).

Homologous Recombination and Nonhomologous End-Joining Repair of DNA A cell that has double-strand breaks can be repaired by joining the free DNA ends. The joining of broken ends from different chromosomes, however, will lead to the translocation of DNA pieces from one chromosome to another, translocations that have the potential to enable abnormal cell growth by placing a proto-oncogene next to, and, therefore, under the control of another gene promoter. Double-strand breaks can be caused by ionizing radiation and drugs such as anticancer drugs. Double-strand breaks are correctly repaired only when the free ends of DNA rejoin exactly. The repair of double-stranded DNA is therefore confounded by the absence of single-stranded regions that can signal the correct base pairing during the rejoining process. Homologous recombination is one of two mechanisms responsible for the repair of double-strand breaks (San Filippo et al., 2008). In this process, the double-strand break on one chromosome is repaired using the information on the homologous, intact chromosome. A double-strand break of DNA that occurs in the S and G_2 phases is predominantly repaired by homologous recombination, whereas double-strand breaks formed during G_1 phase are mainly repaired via nonhomologous end joining (Branzei and Foiani, 2008).

The predominant mechanism for double-stranded DNA repair in multicellular organisms is nonhomologous repair and involves the rejoining the ends of the two DNA molecules (Lieber, 2010). Although this process yields a continuous double-stranded molecule, several base pairs are lost at the joining point. This type of deletion may produce a possibly mutagenic coding change.

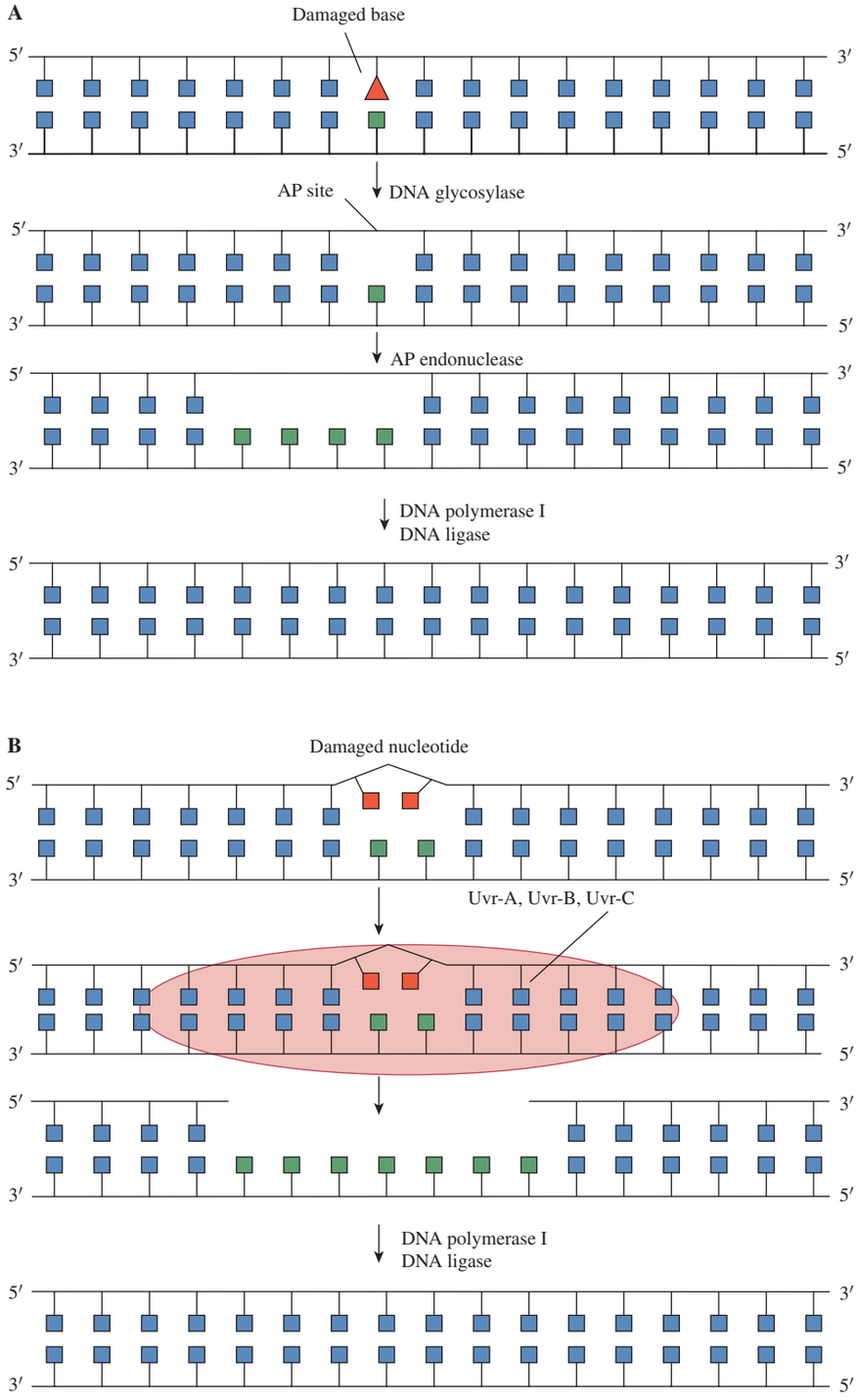


Figure 8-9. *(**A**) DNA repair by base excision. (**B**) DNA repair by nucleotide excision.*

Classes of Genotoxic Carcinogens

Polyaromatic Hydrocarbons PAHs are found at high levels in charcoal-broiled foods, cigarette smoke, and in diesel exhaust. Representative chemicals belonging to this class are shown in Fig. 8-10A. A recent review of the causes of human cancer (*120 human carcinogens*) also lists those PAHs linked to human cancer by the International Agency for Research in Cancer (IARC monographs [Vol. 100 A–F]). Benzo(*a*)pyrene is a representative polycyclic hydrocarbon that has been extensively studied. The metabolism and pathways that lead to tumor formation have been characterized through the work of a number of laboratories (Conney, 1982). The ultimate carcinogen is a diol epoxide of benzo(*a*)pyrene, formed following three separate enzymatic reactions (Sims et al.,

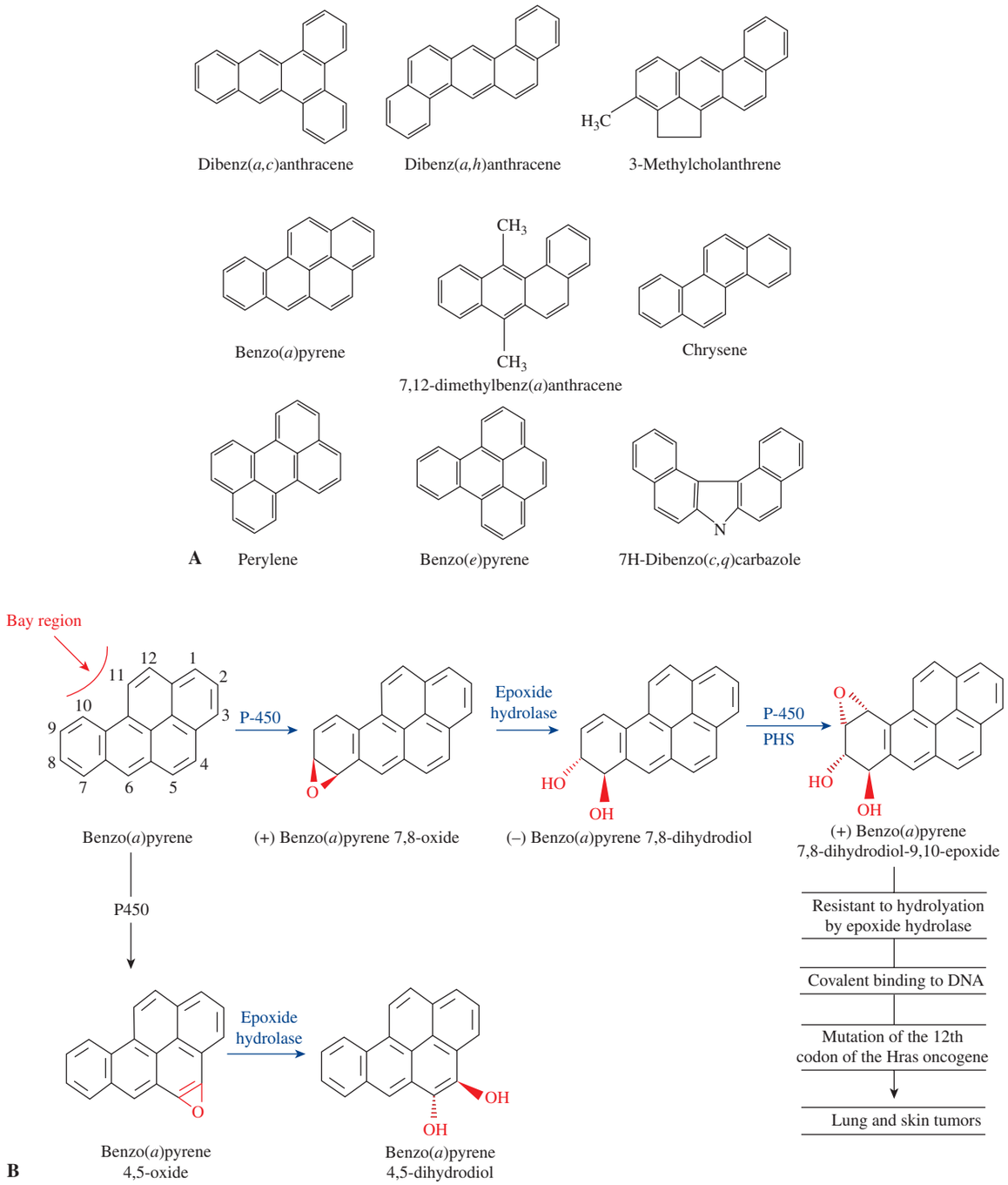


Figure 8-10. *(**A**) Chemical structures of selected carcinogenic polycyclic hydrocarbons. (**B**) Role of epoxide hydrolase in the inactivation of benzo[a]pyrene 4,5-oxide and in the conversion of benzo[a]pyrene to its tumorigenic bay region diolepoxide.*

1974). Benzo[*a*]pyrene is first oxidized by cytochrome P4501A1 to form benzo[*a*]pyrene 7,8-oxide, further metabolized by epoxide hydrolase, yielding the 7,8-dihydrodiol. And then further metabolism by cytochrome P4501A1 to yield the ultimate carcinogen, the 7,8-dihydrodiol-9,10-epoxide (Yang et al., 1976; Lowe and Silverman, 1994; Schwarz et al., 2001). The Bay region or K region of the benzo(*a*)pyrene molecule is the site of metabolic targeting and DNA interaction (Fig. 8-10B). The importance of the Bay region (K region) of the benzo(*a*)pyrene molecule was demonstrated from the understanding of the metabolism of the benzo(*a*)pyrene to its ultimate DNA-reactive form. Similar regions (to the Bay region) have been identified in other carcinogenic polycyclic hydrocarbons and used to access and predict carcinogenic potential of other PAHs. Evidence has emerged that besides the P450 metabolism pathway, PAHs can also be metabolized through a peroxidase pathway and the aldo–keto reductase (AKR) pathway. The latter of which has been attributed to

cancer risk in humans exposed to coal burning smoke in China (Lan et al., 2004). Oxidative damage-related genes AKR1C3 and oxoguanine glycosylase 2 (OGG2) also appear to modulate risks for lung cancer due to exposure to PAH-rich coal combustion emissions.

Alkylating Agents Alkylating agents represent an important class of chemical carcinogens. Although some alkylating chemicals are direct-acting genotoxic chemicals, many require metabolic activation to produce electrophilic metabolites that can react with DNA. Alkylating agents can be classified into several groups including the direct-acting alkylalkanesulfonates (methyl- and ethyl methanesulfonate) and nitrosamides (*N*-methyl-*N*-nitrosourea, *N*-ethyl-*N*-nitrosourea, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine), and the indirect-acting nitrosamides (dimethyl- and diethylnitrosamines) (Fig. 8-11). The alkylating compounds (or in the case of diethylnitrosamine and dimethylnitrosamine and their metabolites) readily react with DNA at more than 12 sites. The N^7 position of guanine and the N^3 position of adenine are the most reactive sites for alkylating chemicals to bind to DNA. The reactions of DNA methylation occur more readily and thus exhibit >20 more adducts formed compared with ethylation reactions. However, ethylation reactions have a greater affinity for oxygen centers, an event that appears to correlate with the mutagenicity and carcinogenicity of these compounds. Nitrosamines were initially used as solvents in chemistry. Their toxic effects were identified when workers using the nitrosamine solvents developed jaundice and liver damage. Subsequent studies in animal models revealed that dimethylnitrosamine and, eventually, diethylnitrosamine were highly hepatotoxic and hepatocarcinogenic.

Other alkylating chemicals including the nitrogen mustards (e.g., chlorambucil, cyclophosphamide) have been used in cancer chemotherapy. They produce DNA adducts as well as induce the formation of DNA strand breaks. The alkylation of DNA by nitrogen mustards requires the formation of highly reactive *N*-alkylazirdinium ions (Fig. 8-12). Nitrogen mustards can produce a wide spectrum of mutations including base pair substitutions

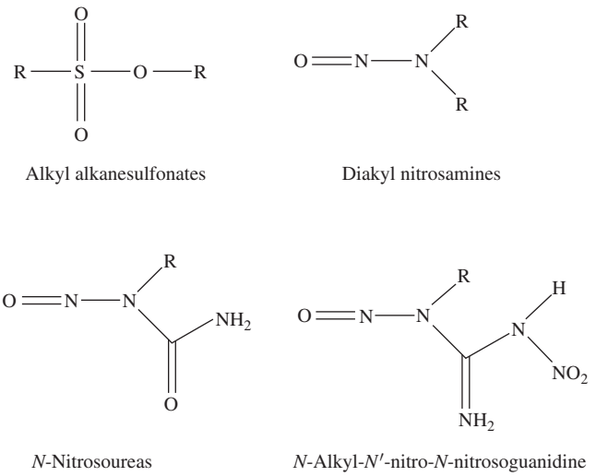
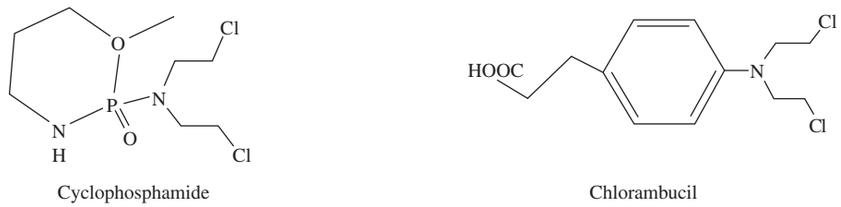


Figure 8-11. *Structures of representative methylating and ethylating agents.*



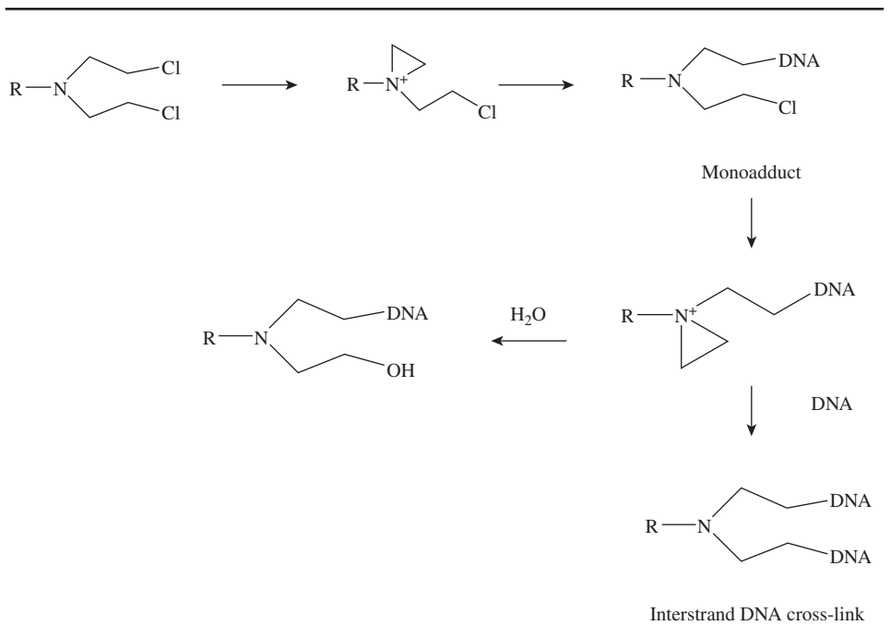
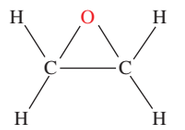


Figure 8-12. *Nitrogen mustards and proposed mechanism for the reactions of nitrogen mustards with DNA.*

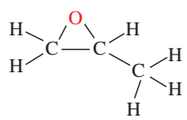
(AT and GC) and deletions. In addition, nitrogen mustards are potent clastogens causing chromosomal aberrations and sister chromatid exchanges (SCEs), predominantly in GC-rich regions.

Ethylene oxide and propylene oxide are other examples of mutagenic and carcinogenic alkylating agents (Fig. 8-13). Ethylene oxide is a direct-acting alkylating carcinogen in rodents and perhaps of human concern (Hogstedt et al., 1986). Ethylene oxide is mutagenic in short-term in vitro assays and produces chromosomal aberrations and SCEs in eukaryotic cells (Ehrenberg and Hussain, 1981). Propylene oxide is also a mutagenic rodent carcinogen inducing nasal tumors in rodents following inhalation exposure (NTP, 1985). Alkylation of DNA by ethylene and propylene oxide occurs predominantly at the N^7 position of guanine, yielding 7-(2-hydroxyethyl)guanine and 7-(2-hydroxypropyl)guanine adducts, respectively. These adducts represent the major adducts formed following either in vitro or in vivo exposure (Walker et al., 1992). In addition, both ethylene oxide and propylene oxide can form chemically stable adducts with hemoglobin, which can be used as a biomarker for exposure (Kolman et al., 2002).

Vinyl chloride is another known rodent and human carcinogen, producing angiosarcomas in the liver, increased hepatocellular carcinoma and tumors in the lung and hematopoietic system in humans (Doll, 1988; Bosetti et al., 2003; IARC, 2008). Limited evidence also suggests that vinyl chloride exposure results in brain tumors. Vinyl chloride is mutagenic and is metabolized by cytochrome P450 to form chloroethylene oxide, which rearranges nonenzymatically to produce chloroacetaldehyde, both of which can alkylate DNA. Vinyl chloride and metabolites form several DNA adducts including 7-(2′-oxoethyl)guanine, N^2, 3-etheneoguanine, 3,N^4-ethenocytosine, and 1,N^6-ethenoadenine (Guengerich, 1994). Vinyl chloride also induces an increased rate of unscheduled DNA synthesis, sister chromatid exchange, micronuclei formation, and chromosomal aberrations (IARC, 2008).



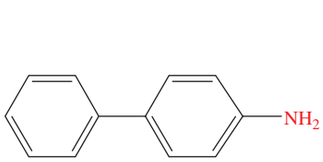
Ethylene oxide



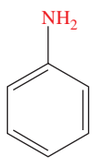
Propylene oxide

Figure 8-13. *Chemical structures for ethylene and propylene oxide.*

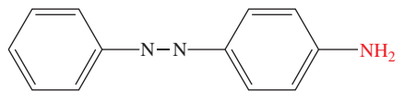
Aromatic Amines and Amides Aromatic amines and amides encompass a class of chemicals with varied structures (aromatic amines, e.g., aniline/azo dyes, 2-natphylamine, benzidine, 2-acetylaminofluofene) (Fig. 8-14). Because of their use in the dye industry and other industrial processes, their carcinogen risk to humans was realized in the late 19th century. Exposure to these chemicals still occurs through cigarette smoke and environmental sources, even though proper industrial hygiene processes have considerably reduced the human exposure to aromatic amines and amides in the workplace. The aromatic amines undergo both phase I and phase II metabolism. Phase I reactions occur mainly by cytochrome P450-mediated reactions, yielding hydroxylated metabolites that are often associated with adduct formation in proteins and DNA, and produce liver and bladder carcinogenicity (Miller et al., 1964). In addition, azo dyes can be cleaved by skin and gastrointestinal microflora into mutagenic and carcinogenic subunits (Platzek et al., 1999;



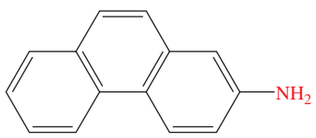
4-Aminobiphenyl (ABP)



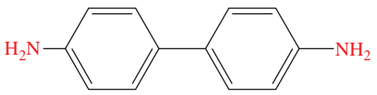
Aniline



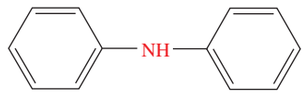
4-Aminoazobenzene



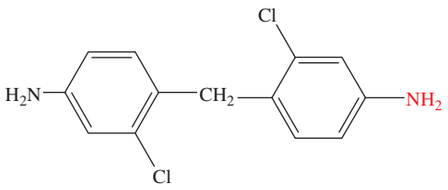
2-Aminophenanthrene



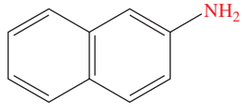
Benzidine



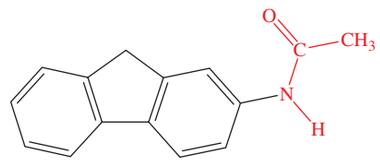
Diphenylamine



4,4′-Methylene-*bis*-2-chloroaniline



2-Naphthylamine



2-Acetylaminofluorene (2-AAF)

Figure 8-14. *Chemical structures of selected carcinogenic aromatic amines.*

Chung, 2016). Metabolism of 2-acetylaminofluorene (AAF) results in the formation of *N*-hydroxy-AAF, which is a metabolite responsible for the liver tumorigenicity. Similarly, 1-naphthylamine exhibits carcinogenic activity only in test systems capable of producing the *N*-hydroxy metabolite of naphthylamine. Aromatic amines are capable of forming adducts with several DNA bases. Both heterocyclic amines (HCAs) and PAHs are formed when muscle meat, including beef, pork, fish, and poultry, is cooked using high-temperature methods, such as pan frying or grilling directly over an open flame. HCAs are also formed from fried foods, specifically fried meats (Turesky, 2007). Mutagenic activity of cooked meats at high temperature has been reported using the Ames Salmonella mutagenesis assay. In particular, an increase in HCAs (specifically MeIQx(2-amino-3,8-dimethylimidazo[4,5-*b*]quinoxaline), DiMeIQx (2-amino-3,4,8-dimethylimidazo [4,5-*f*]quinoxaline), and IQ (2-amino-3-methylimidazo [4,5-*f*]quinolone)) was found with increasing frying temperatures and cooking duration (Knize et al., 1995). In support of these experimental studies, epidemiology linkages between consumption of high-temperature cooked foods and human cancer have been reported (Cross and Sinha, 2004; Rohrmann et al., 2007; Abid et al., 2014).

Classes and Mode of Action of Non-genotoxic (Epigenetic) Carcinogens

A number of chemicals that produce tumors in experimental animals following chronic treatment appear to act via mechanisms not involving direct binding, damage, or interaction of the chemical or its metabolites with DNA (Williams and Whysner, 1996). Based on the lack of genotoxicity, yet their ability to induce tumors in rodent models, these chemicals have been labeled non-genotoxic carcinogens. The organ and tissue targets induced by non-genotoxic carcinogens are many times in tissues where a significant incidence of background, spontaneous tumors is seen in the animal model. Prolonged exposure to relatively high levels of chemicals is usually necessary for the production of tumors by this mechanism. In addition, with non-genotoxic carcinogens, tumors are not theoretically expected to occur at exposures below a threshold at which relevant cellular effects are not observed. In contrast to DNA-reactive genotoxic effects, non–DNA-reactive mechanisms may be unique to the rodent species used for testing. Certain chemical carcinogens have been well studied and provide examples for the use of mechanistic information in risk assessment. Further, the biochemical modes of action for non–DNA-reactive carcinogens are diverse. Examples include chemicals that function via sustained cytotoxicity, receptor-mediated (e.g., CAR, peroxisome proliferator–activated receptor alpha [PPARα], aryl hydrocarbon receptor [AhR]) effects, hormonal perturbation, as well as the induction of oxidative stress and modulation of methylation status (Table 8-11). Each of these potential mechanisms is discussed in greater detail in the following sections.

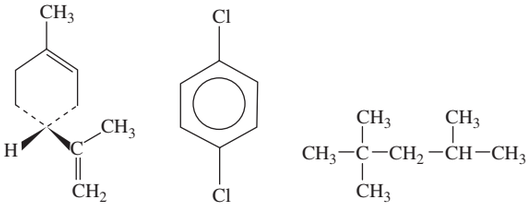
Table 8-11

Proposed Modes of Action for Selected Non-genotoxic Chemical Carcinogens

MODE OF ACTOIN	EXAMPLE
Cytotoxicity	Chloroform Melamine
α2u-Globulin binding	D-Limonene 1,4-Dichlorobenzene
Receptor-mediated	
CAR	Phenobarbital Toxaphene
PPARa	Trichloroethylene Perchloroethylene Diethylhexylphthalate Fibrates (e.g., clofibrate)
AhR	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Polychlorinated biphenyls (PCBs) Polybrominated biphenyls (PBBs)
Hormonal	Biogenic amines Steroid and peptide hormones Diethylstilbestrol (DES) Phytoestrogens (Bisphenol-A) Tamoxifen Phenobarbital
Altered methylation	Phenobarbital Choline deficiency Diethanolamine
Immunosuppression	Atrazine Bisphenol A Phthalates
Oxidative stress inducers	Ethanol TCDD Lindane Dieldrin Acrylonitrile

Cytotoxicity Cytotoxicity and consequent regenerative hyperplasia is well-documented mode of action for a variety of non–DNA-reactive chemical carcinogens (Dietrich and Swenberg, 1991). Chloroform-induced liver and kidney tumors and melamine-induced bladder tumors are classic examples of chemical carcinogens that are classified as functioning via a cytolethal mode of action (Bull et al., 1986; Butterworth, 1990; Larson et al., 1994; Pereira, 1994; Andersen et al., 1998). Chemicals that function through this mechanism produce sustained cell death, often related to metabolism of the chemical, that is accompanied by persistent regenerative growth, resulting in the potential for the acquisition of "spontaneous" DNA mutations and allowing mutated cells to accumulate and proliferate. This process then gives rise to preneoplastic focal lesions that upon further expansion can lead to tumor formation. Chloroform has been shown to induce mouse liver tumors only at doses of compound that produce liver necrosis, thus demonstrating an association between necrosis with compensatory hyperplasia and the resulting tumorigenicity, and also supports that a threshold for the induction of tumors is likely at doses that do not produce toxicity (Beddowes et al., 2003). It is important to note that the induction of cytotoxicity may be observed with many carcinogens both genotoxic and non-genotoxic when high toxic exposures occur. Thus, the induction of cytotoxicity with compensatory hyperplasia may contribute to the observed tumorigenicity of many carcinogenic chemicals at high-dose levels.

α2u-Globulin-Binding Drugs The carcinogens D-limonene, 1,4-dichlorobenzene, and trimethylpentane (Fig. 8-15) as well



D-Limonene 1,4-Dichlorobenzene 2,2,4-Trimethylpentane C_8H_{18}

Figure 8-15. *Examples of selective α2u-globulin-binding chemicals.*

unleaded gasoline induce renal tumors selectively in the male rat, and provide excellent examples of the species, sex, and tissue specificity of non–DNA-reactive carcinogens. The mechanism for the species and sex specificity is related to the ability of these compounds to bind to α2u-globulin, a protein synthesized by the male rat liver at the onset of puberty, as the mechanism of tumorigenesis. α2u-Globulin is filtered through the glomerulus, and only partially excreted (~50%) in the urine. The reabsorbed fraction accumulates in lysosomes of the P2 segment of the proximal tubules, where it is hydrolyzed to amino acids (Melnick et al., 1996). Chemicals with the ability to bind to α2u-globulin decrease the rate of digestion of α2u-globulin and result in the accumulation in the lysosomes, dysfunction of this organelle, and subsequent release of digestive enzymes and cell necrosis. The greater loss of tubule cells leads to increased cell proliferation in the P2 segment, which may be responsible for the tumor development and malignant transformation (Dietrich and Swenberg, 1991) (See Chap. 14 for additional details).

Receptor-Mediated

CAR Receptor-Mediated (Phenobarbital-Like Carcinogens)

Phenobarbital is a commonly studied non–DNA-reactive compound that is known to cause tumors by a non-genotoxic mechanism involving liver hyperplasia (Williams and Whysner, 1996). One feature seen following phenobarbital exposure is the induction of P450 enzymes, particularly Cyp2b (Nims and Lubet, 1996). Chemicals that functioned as P450 inducers in a manner similar to phenobarbital were classified as phenobarbital-like or P450-inducing carcinogens. Because a number of diverse chemicals are known to induce various members of the P450 system (e.g., dieldrin, ethanol, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]), the specificity of this effect to the carcinogenesis has been questioned. Recent evidence has shown that the induction of Cyp2b is mediated by activation of the constitutive androstane receptor (CAR), a member of the nuclear receptor family (Honkakoski et al., 1998; Ueda et al., 2002; Kodama et al., 2004). CAR-null mice show no induction of Cyp2b following phenobarbital exposure (Wei et al., 2000; Wang et al., 2015, 2017). Other phenobarbital responses that are critical for tumor formation include increased cell proliferation, inhibition of apoptosis, inhibition of gap junctional communication, hypertrophy, and development of preneoplastic focal lesions in the liver (Whysner et al., 1996; Elcombe et al., 2014), effects that have all been shown to be CAR-dependent (Wei et al., 2000; Kodama et al., 2004; Elcombe et al., 2014). CAR can be activated by both direct ligand binding and ligand-independent (indirect) mechanisms. Phenobarbital activates CAR indirectly by inhibiting epidermal growth factor receptor (EGFR) signaling (Mutoh et al., 2013). Phenobarbital binds to EGFR that inhibits the binding of EGF and prevents the activation of EGFR; disruption of EGFR signaling leading to dephosphorylation of receptor for activated C kinase 1, which further recruits PP2A (protein phosphatase 2A) and dephosphorylates CAR, releasing CAR into nucleus, where CAR forms a dimer with RXR and binds to PBREMs, resulting in increased gene expression (Fig. 8-16) (see Chapter 6 for additional details on CAR induction of CYP).

Peroxisome Proliferator–Activated Receptor α

A wide array of chemicals are capable of increasing the number and volume of peroxisomes in the cytoplasm of cells. These chemicals, termed peroxisome proliferators, include lipid-lowering fibrate drugs (e.g., ciprofibrate, clofibrate), herbicides (e.g., 2,4-dichlorophenoxyacetic acid), chlorinated solvents (e.g., trichloroethylene and perchloroethylene), perfluorinated compounds (e.g., perfluorooctane sulfonate and perfluorooctanoic acid), plasticizers (e.g., diethylhexylphthalate and other phthalates), and natural products. In addition, many of the chemicals within this group produce liver enlargement and hepatocellular carcinoma in rats and mice through non–DNA-reactive mechanisms (Lake, 1995; Reddy and Rao, 1997). Two additional tumor types are also associated with exposure to peroxisome-proliferating compounds: Leydig cell tumors and pancreatic acinar cell tumors in rats (Kennedy et al., 2004). Studies conducted either in vivo or in vitro in primary hepatocyte cultures have shown important interspecies differences in the hepatic peroxisome proliferation responses to chemicals. The rat and the mouse were clearly revealed as responsive species, whereas primates and the guinea pig proved to be nonresponders. The Syrian hamster exhibits an intermediate response (Bentley et al., 1993; Lake, 1995). The mechanism(s) involved in peroxisome proliferation and tumorigenesis went unrecognized for years. The currently accepted mode of action for this class of chemicals involves the agonist binding and/or activating the nuclear receptor, the PPARα. Largely through the use of PPARα knockout mice, it has been demonstrated that the activation of PPARα by agonists is needed for these chemicals to induce peroxisome proliferation and tumorigenesis in rodents (Issemann and Green, 1990; Lee et al., 1995; Peters et al., 1998, reviewed in Klaunig et al., 2003 and Corton et al., 2014). PPARα is highly expressed in cells that have active fatty acid oxidation capacity (e.g., hepatocytes, cardiomyocytes, enterocytes). It is well documented that PPARα plays a central role in lipid metabolism and acts as a transcription factor to modulate gene expression following ligand activation. This latter effect arises through the heterodimerization of PPARα and RXRα, which results in binding to response elements (PPREs) and subsequent modulation of target gene transcription (Fig. 8-17) (see Chap. 6). Following this event is the induction of cell proliferation and suppression of apoptosis (Marsman et al., 1992; James and Roberts, 1996; Burkhardt et al., 2001). Both of these events would be expected to affect tumor development as these effects would enhance the rate of fixation of DNA damage in the genome, leading to changes in gene expression, such as the silencing of tumor-suppressor genes or increased expression of oncogenes, or suppress apoptosis that may normally remove DNA-damaged, potentially tumorigenic, cells. Because humans are exposed to a number of these chemicals, the relevance of this mode of action to humans has been evaluated (Klaunig et al., 2003; Corton et al., 2014). Although the same events would be expected to occur in exposed humans, several species differences have been noted including a lack of induction of cell proliferation in nonhuman primates (Pugh et al., 2000), and the finding that PPARα in human liver is less than 10 percent that observed in rat or mouse liver (Palmer et al., 1998; Tugwood et al., 1998). Based on these kinetic and dynamic differences between species, it has been

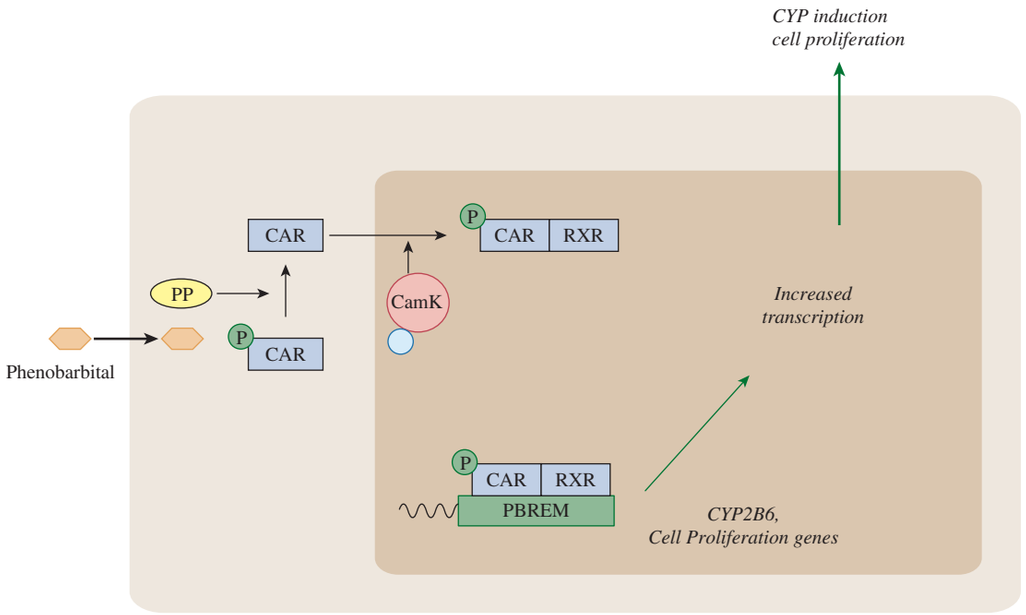


Figure 8-16. *Proposed mechanism for the involvement of the constitutive androstane receptor (CAR) in phenobarbital-induced gene expression changes.* EGFR, epithelial growth factor receptor; PP2A, protein phosphatase 2A; RXR, retinoic acid receptor; PBREM, phenobarbital response element. Phenobarbital activates CAR indirectly by inhibiting EGFR. Following dephosphorylation of CAR by protein phosphatase 2A, CAR translocates to nucleus and forms a dimer with RXR and binds to PBREMs, resulting in increased gene expression.

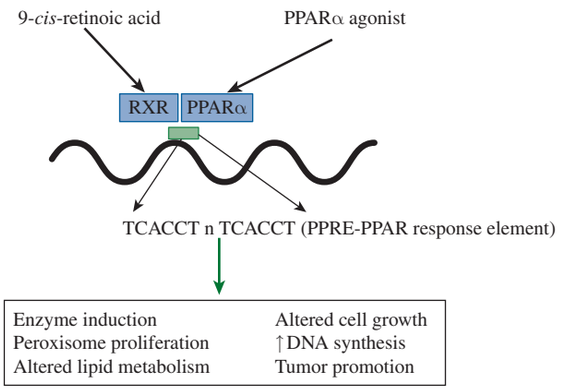
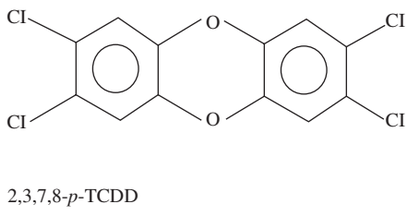


Figure 8-17. *Mechanism for altered gene expression by peroxisome proliferator activated receptor α (PPARα) agonist binding.* Following agonist binding to PPARα, the receptor dimerizes with the retinoic acid receptor (RXR). This complex then binds to PPREs, resulting in enhanced gene transcription.



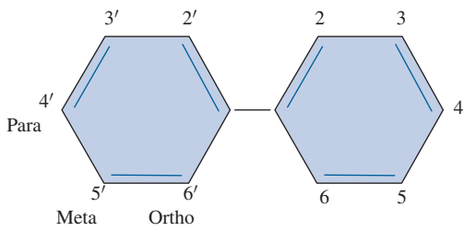


Figure 8-18. *Selective examples of aryl hydrocarbon receptor-binding chemicals.* Prototypical structure for PCBs or PBBs. PCBs and PBBs exhibit varying degrees of chlorination or bromination at positions 2-6 and 2′-6′ of the biphenyl molecule.

concluded that tumors are not likely to occur in humans by PPARα agonists (Klaunig et al., 2003; Corton et al., 2014).

Aryl Hydrocarbon Receptor Activators of the AhR including TCDD and selected polychlorinated- and brominated-biphenyl (PCBs and PBBs) compounds (Fig. 8-18) have been linked to tumor promotion in rodent livers (Pitot et al., 1982; IARC, 1997a, 1997b, 2010). The tumor response has been determined to be AhR-dependent (Knutson and Poland, 1982; Budinsky et al., 2014) (Fig. 8-19). Upon ligand binding to the AhR, the ligand bound AhR translocates to the nucleus, dimerizes with the Ah receptor nuclear translocator (ARNT), and binds to aryl hydrocarbon response elements (AREs also known as dioxin response elements [DRE] and xenobiotic response elements [XRE]) (for review see Nebert et al., 2000) (see Chap. 6). AhR-ARNT–dependent genes include cytochrome P450 family members, NAD(P)H:quinone oxidoreductase, a cytosolic aldehyde dehydrogenase 3, a UDP-glucuronosyltransferase, and a glutathione transferase (Nebert et al., 2000), genes that are involved in metabolic activation as well as detoxification of chemicals. It has been hypothesized that there are additional AhR-ARNT–dependent genes (Nebert et al., 2000). AhR knockout mice have a diminished response to tumor induction by AhR ligand, dibenzo(a,l)pyrene (Nakatsuru et al., 2004), and conversely, constitutively overexpressed AhR resulted in an

increased incidence of liver tumors (Moennikes et al., 2004), supporting the theory that agonist binding to AhR is a required event for tumor induction by this class of chemicals. As seen with other hepatic tumor-promoting chemicals, TCDD also inhibits apoptosis in hepatic foci (Stinchcombe et al., 1995).

Hormonal Mode of Action Hormonally active chemicals include steroids and peptide hormones that produce tissue-specific changes through interaction with a receptor. A number of non–DNA-reactive chemicals induce neoplasia through receptor-mediated mechanisms, and/or perturbation of hormonal balance. Trophic hormones are known to induce cell proliferation at their target organs. This action may lead to the development of tumors when the mechanisms of hormonal control are disrupted and some or other hormone shows persistently increased levels. Several well-studied examples include the induction of ovarian neoplasms via decreased estradiol and increased luteinizing hormone (LH) levels (Capen et al., 1995), and the induction of thyroid tumors in rats by phenobarbital-type P450 inducers (which modulate T3 and T4) (McClain, 1995; Whysner et al., 1996).

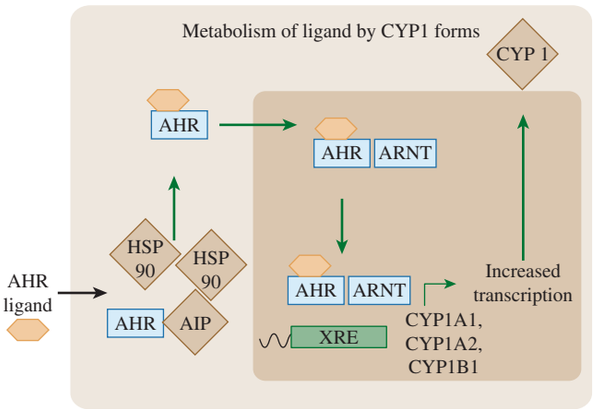
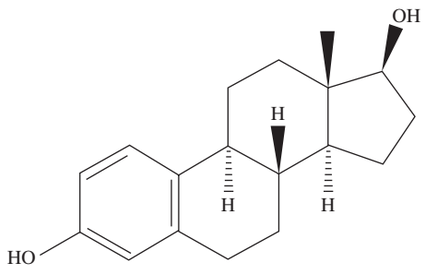


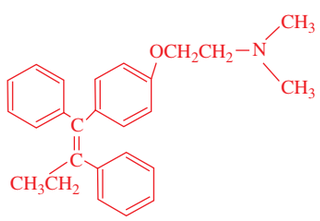
Figure 8-19. *Proposed mechanism of aryl hydrocarbon receptor (AhR) mediated gene expression.* ARNT, AhR nuclear transporter; XRE, xenobiotic response element. Non-ligand bound AhR is maintained in the cytoplasm via association with chaperone proteins (e.g., AIP, Hsp90). Following ligand binding, chaperone proteins dissociate and AhR translocates to the nucleus where it binds with ARNT. The heterodimer binds to XREs resulting in an increase in gene transcription.

Estrogenic Agents Estrogen and estrogen-mimic chemicals can induce tumors in estrogen-dependent tissue. Selective estrogenic chemicals (agonists and antagonists) are shown in Fig. 8-20. Oral administration of 17β-estradiol to female mice increases the incidence of mammary tumors (Welsch et al., 1977; Highman et al., 1978) whereas subcutaneous administration of estradiol to young female mice produced tumors of the cervix and vagina (Pan and Gardner, 1948). Evidence that estrogenic chemicals are carcinogenic to humans comes from epidemiological data on breast and ovarian cancer, which indicates that individuals with higher circulating estrogen levels and those with exposure to the potent estrogenic chemical diethylstilbestrol (DES) are at increased risk for cancer development. DES was first shown to induce mammary tumors in male mice following subcutaneous administration of the hormone. DES has been causally associated to the higher incidence of adenocarcinomas of the vagina and cervix in daughters of women treated with the hormone during pregnancy (Herbst and Scully, 1970; Herbst et al., 1972; Noller et al., 1972). The mechanism of action for DES is believed to function through its ability to induce aneuploidy (Li et al., 1997; Tsutsui et al., 1997). The effects of steroidal chemicals on the cell cycle (Sutherland et al., 1995) and on microtubule assembly (Metzler et al., 1996) may be important in the aneuploidy (aneuploidy is the presence of an abnormal number of chromosomes in a cell) inducing effects of some hormonal chemicals (Li et al., 1996).

Chronic exogenous administration of hormonally active chemicals including synthetic estrogens and anabolic steroids can

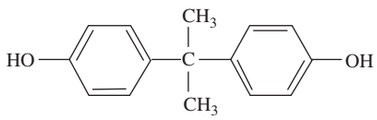


17β-estradiol
(Agonist)



Tamoxifen

Tamoxifen (Antagonist)



Bisphenol A
(Agonist)

Figure 8-20. *Chemical examples of estrogenic agonists and antagonists.*

increase hepatic adenoma incidence in rats (Li et al., 1992) and in humans (IARC, 1997a, 1997b, 2007, 2012). Women of childbearing age showed sensitivity to hepatic adenoma formation, which was exacerbated by the use of oral contraceptives containing high concentrations of estrogenic compounds. These adenomas regress upon hormone cessation (Edmondson et al., 1977) and can progress with continued administration (Christopherson et al., 1978). Chronic administration (>8 years) is required to detect this increased liver tumor risk from oral contraceptives (Tavani et al., 1993). It is important to note that the liver tumor effects seen with the early introduced oral contraceptives were related to the high dose of estrogen compounds in the formulation.

Estrogenic substances in plants (phytoestrogens) have been identified. These include genistein, daidzein, glycitein, equol, and their metabolites found in soy products and various lignan derivatives. In addition, a number of environmental nonsteroidal synthetic compounds demonstrate apparent estrogenic activity (e.g., nonylphenol, bisphenol-A, chlorinated hydrocarbons; Soto et al., 1997). The potential for these chemicals to induce cancer in humans remains a highly debated topic.

Species and tissue specificity in response to receptor-mediated carcinogenesis is often observed. As an example, tamoxifen is antiestrogenic in the chick oviduct, estrogenic in the mouse uterus with acute administration, but antiestrogenic with chronic administration in the same tissue, whereas it is estrogenic in the rat uterus. In contrast estrogens have also been shown to act as a protective chemical in prostate cancer. Although the differences observed may be in part due to tissue and species differences in coactivator and corepressor levels and availability, other pharmacokinetic and pharmacodynamic properties are likely to participate in the response (Carthew et al., 1995). To further exemplify the complexity of the role of estrogen in cancer development, estrogens have also been reported to function in a protective manner in androgen-dependent prostate cancer (Bosland, 2005).

Induction of ovarian tumors in mice by dietary administration of nitrofurantoin, an antibiotic used to treat urinary tract infections is an example of a tumorigenic effect secondary to drug-induced hormonal disturbance. Nitrofurantoin treatment resulted in ovarian atrophy with absence of Graafian follicles and sterility. The reduction of follicles induced a reduction of sex steroid hormones by the ovary, resulting in increased production of gonadotrophins, notably LH. This was due to the decreased negative feedback on the hypothalamus–pituitary axis by estrogens. Persistent stimulation by LH of the murine ovary cells resulted in development of tumors (Capen et al., 1995). A similar tumorigenic effect on the ovary occurs with dopamine agonists. The induction of hypoprolactinemia changes the number of LH receptors and leads to enhanced sensitivity to LH and a higher stimulation by LH similar to nitrofurantoin.

Thyroid Hormone A reduction of thyroid hormone concentrations (T4 and/or T3) and increased thyroid-stimulating hormone (TSH) have been shown to induce neoplasia in the rodent thyroid. TSH increases proliferative activity in the thyroid. After chronic treatment chemical-induced increase in TSH leads to follicular cell hypertrophy, hyperplasia, and eventually neoplasia. Inducers of metabolic enzymes in the rat liver, for example with phenobarbital (Hood et al., 1999), results in increased thyroid hormone metabolism and leads to an increase in TSH levels (Fig. 8-21). It is this latter event that is associated with the development of thyroid tumors in rodents. Recent evidence suggests that the enhancement of metabolism and excretion of thyroid hormones by xenobiotics (via induction of phase II enzymes in particular UDP-glucuronosyltransferase) is a consequence of CAR, PXR, AhR, and/or PPARα activation (Shelby and Klaassen, 2006; Yanagiba et al., 2009). Qualitatively, thyroid gland function in rodents is much more susceptible to disturbances of thyroid hormone levels than in humans. In contrast to humans, rodents lack the thyroid-binding globulin, which is the predominant plasma protein (at least in humans) responsible for thyroxin binding and transport (McClain, 1995). A relatively higher level of functional activity is present in rat thyroid compared to humans.

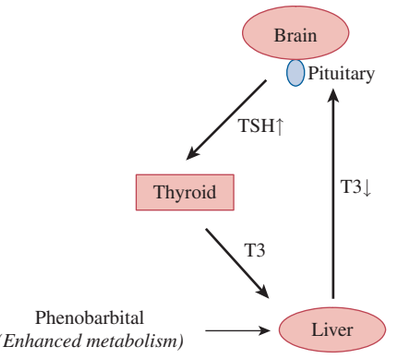


Figure 8-21. *Mechanism for phenobarbital-induced thyroid tumors.* Through alteration of gene expression, phenobarbital enhances metabolism of thyroid hormone (T3) resulting in enhanced TSH production. Increased TSH then leads to increased cell proliferation in the thyroid.

DNA Methylation and Carcinogenesis Post-DNA synthetic methylation of the five position on cytosine (5-methylcytosine; 5mC) is a naturally occurring modification to DNA in higher eukaryotes that influences gene expression (Holliday, 1990) (Fig. 8-22). Methylation of DNA occurs through an enzymatic reaction catalyzed by DNA methyltransferases (DNMTs). Under normal conditions, DNA is methylated symmetrically on both strands. Immediately following DNA replication, the newly synthesized double-stranded DNA contains hemimethylated sites that signal for DNA maintenance methylases to transfer methyl groups from *S*-adenosylmethionine to cytosine residues on the new DNA strand (Hergersberg, 1991). The degree of methylation within a gene inversely correlates with the expression of that gene; hypermethylation of genes is associated with gene silencing, whereas hypomethylation results in an enhanced expression of genes. Several chemical carcinogens are known to modify DNA methylation, methyltransferase activity, and chromosomal structure. During carcinogenesis, both hypomethylation and hypermethylation of the genome have been observed (Counts and Goodman, 1995; Robertson and Jones, 2000; Luczak and Jagodzinski, 2006). Alterations in methylation of promoter regions may mimic the effect of mutations of various tumor suppressor genes or proto-oncogenes, resulting in carcinogenesis. Increased methylation of CpG islands have been observed in bladder cancer and tumor-suppressor genes such as the retinoblastoma gene, $p16^{ink4a}$, and $p14^{ARF}$ have been reported to be hypermethylated in tumors (Stirzaker et al., 1997; Belinsky et al., 1998; Myöhänen et al., 1998; Salem et al., 2000; Esteller et al., 2001). Hypomethylation has been associated with increased mutation rates. Most metastatic neoplasms in humans have significantly lower 5MeC than normal tissue (Gama-Sosa et al., 1983); furthermore, many oncogenes are hypomethylated and their expression amplified.

Choline and methionine, from dietary sources, are a common source of methyl groups used in methylation reactions. Rats exposed to choline and/or methionine-deficient diets displayed hepatocellular proliferation and neoplasia (Abanobi et al., 1982; Wainfan and Poirier, 1992) (Table 8-12). In rodents fed choline or

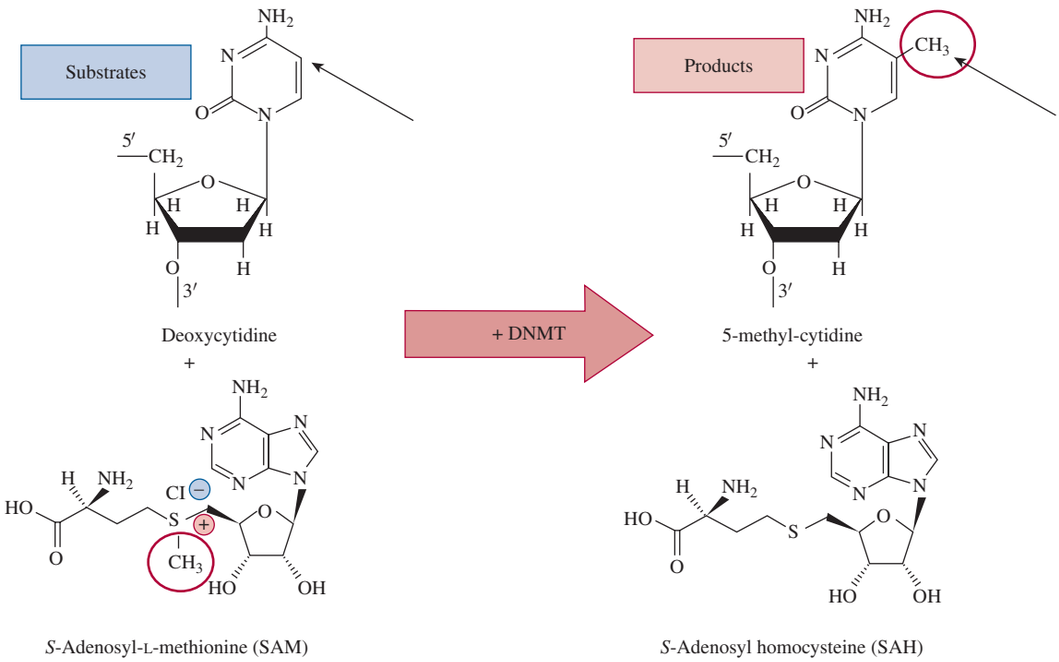


Figure 8-22. *Substrates and products involved in DNA methylation.* Methylation of DNA occurs through an enzymatic reaction catalyzed by DNA methyltransferases (DNMTs). DNA is methylated at the five-position of cytosine and requires the cofactor *S*-adenosylmethionine.

Table 8-12

Evidence for Choline Depletion in Hepatocarcinogenesis

CHOLINE	METHIONINE	FOLATE	HEPATOCELLULAR CARCINOMA (%)	DURATION (MONTHS)	REFERENCES
–	+	+	100	12	Nakae et al. (1992)
–	+	+	73	12	Chandar and Lombardi (1988)
–	Low	–	100	15	Henning et al. (1997)
–	–	+	51	13	Ghoshal and Farber (1984)

the hepatic neoplasia is thought to arise from hypomethylation of *c-myc*, *c-fos*, and *c-H-ras* proto-oncogenes, due to the decreased availability of *S*-adenosylmethionine (Newberne et al., 1982; Wainfan and Poirier, 1992). Chemicals such as diethanolamine result in hepatic neoplasia, in part, via a mechanism involving choline depletion, altered methylation, and modulation of gene expression (Kamendulis and Klaunig, 2005; Bachman et al., 2006). Reactive oxygen species have also been shown to modify DNA methylation resulting in changes in DNA methylation profiles through interfering with the ability of methyltransferases to interact with DNA, leading to hypomethylation of CpG sites (Weitzman et al., 1994; Turk et al., 1995). Also, the presence of 8-OHdG in DNA can lead to hypomethylation of DNA because this adduct, if present in CpCpGpGp sequences, will inhibit the methylation of adjacent C residues. Thus, oxidative DNA damage may be an important contributor to the carcinogenesis process brought about by the loss of DNA methylation, allowing the expression of normally quiescent genes. Also, the abnormal methylation pattern observed in cells transformed by chemical oxidants may contribute to an overall aberrant gene expression and promote the tumor process.

Methylation of deoxycytidine residues is a well-studied DNA adduct. This reaction occurs by the transfer of a methyl group from *S*-adenosylmethionine by DNA methyltransferases (Holliday, 1990). Methylation of DNA results in heritable expression or repression of genes, with hypomethylation associated with active transcription of genes, whereas hypermethylated genes tend to be rarely transcribed (Pogribny and Beland, 2013). Chemical carcinogens may inhibit DNA methylation by several mechanisms including forming covalent adducts, single-strand breaks in the DNA, alteration of methionine pools, and inactivation of the DNA methyltransferase responsible for methylation (Riggs and Jones, 1983). The importance of DNA methylation in chemical carcinogenesis is discussed further later in this chapter.

MicroRNA and Chemical Carcinogenesis MicroRNAs (miRNAs) are small noncoding RNAs, usually consist of 21 to 25 nucleotides, that typically regulate gene expression through repression or degradation of targeted messenger RNAs (mRNAs), controlling genes involved in multiple cellular processes. Dysregulation of mRNAs has been implicated in the development (initiation), promotion, and progression of cancer (Lee and Dutta, 2009;

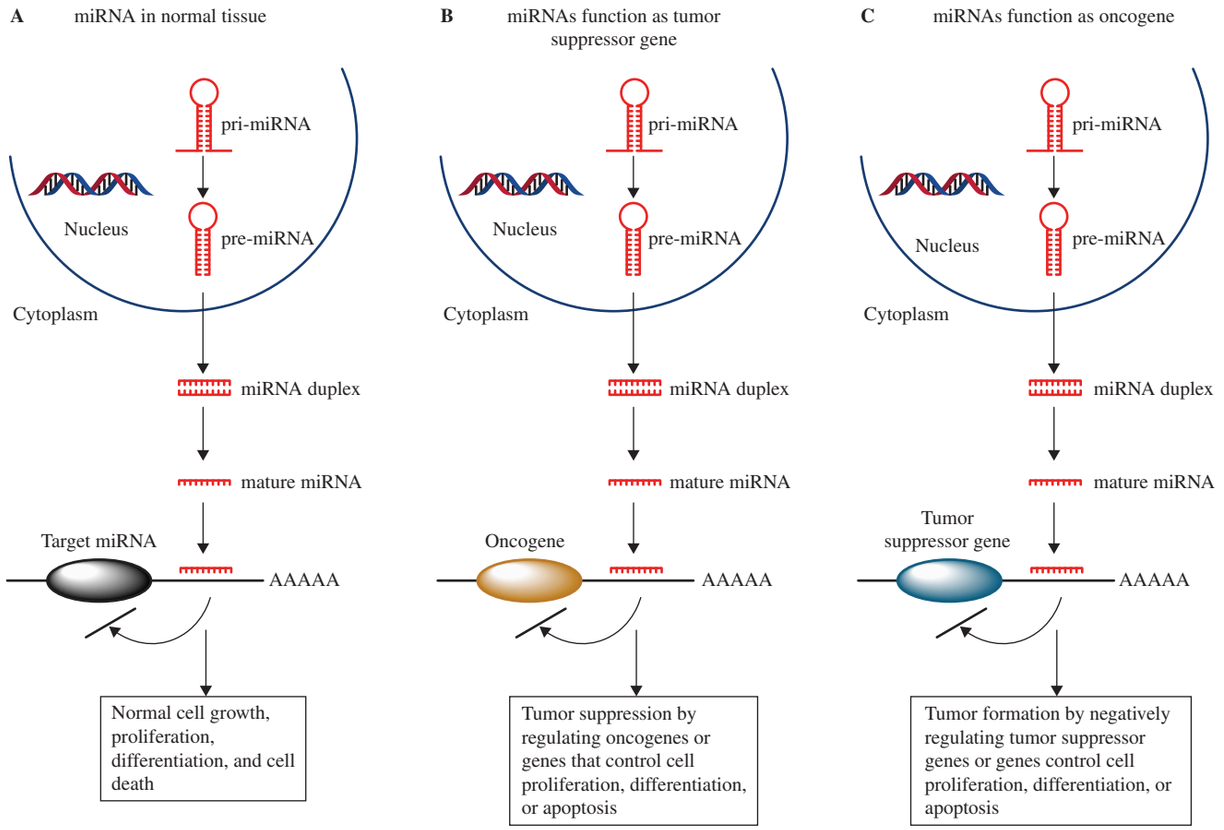


Figure 8-23. *Role of microRNA in carcinogenesis.*

Chen, 2010). Emerging evidence has demonstrated the involvement of miRNA deregulation in the early stages of lung, liver, and breast carcinogenesis induced by chemical carcinogens, suggesting their major role as contributors to the pathogenesis of cancer (Pogribny, 2009; Pogribny et al., 2016). Many miRNAs function as antitumor agents (or tumor suppressor genes), others behave as oncogenes, commonly known as oncomiRNAs (Esquela-Kerscher and Slack, 2006) (Fig. 8-23). Some well-defined oncomiRNA are miR-17, miR-19, miR-21, and miR-155, whose upregulation have been linked to environmental carcinogens exposure (Izzotti and Pulliero, 2014). Thus, the carcinogenic effect of a carcinogen can be dependent on the balance between these two groups of miRNAs. The alterations in miRNAs expression following exposure to carcinogens are usually tissue and chemical specific. Phenobarbital and bis(2-ethylhexyl) phthalate treatment of rats for 90 days produced a persistent, progressive, and highly correlated induction of the miR-200a/200b/429 and miR-96/182 clusters in liver tissue (Koufaris et al., 2012, 2013). The miRNA profiles and miRNAs specific to carcinogen exposure also have the potential to be used as indicators of the carcinogenic process, biomarkers for carcinogen exposure, and for the identification of potential chemical carcinogens.

Immunosuppression and Carcinogenesis Cancer immune surveillance is considered to be an essential host protection mechanism to inhibit carcinogenesis by identifying and removing early preneoplastic cells from the body and to maintain cellular homeostasis (Swann and Smyth, 2007; Vesely et al., 2011; Corthay, 2014). An association between the development of cancer and immunosuppression has long been recognized (Penn and Starzl, 1972). The initial evidence for the role of immunosuppression in cancer induction resulted from the observation that transplant patients receiving immunosuppression therapy developed tumors (Penn et al., 1969, 1971; Penn and Starzl, 1970). Solid-organ transplant recipients are at a three- to fivefold increased risk of developing de novo cancers compared with the general population (Billups et al., 2015). Evading immune recognition and destruction is one of the hallmarks of cancer identified by Hanahan and Weinberg (2011). Immune suppression has also been proposed as a possible mode of action of non-genotoxic carcinogens (USEPA, 2005; Hattis et al., 2009).

It has long been appreciated that inflammation secondary to chronic infection or irritation is a predisposal to cancer induction (Colotta et al., 2009; Hanahan and Weinberg, 2011). Prolonged inflammation can destroy immune cells leading to immune suppression. This mechanism of immunosuppression has been suggested to be involved in the carcinogenesis of environmental immune disruptors such as bisphenol A, atrazine, phthalates, and PBDEs (Thompson et al., 2015).

Oxidative Stress and Chemical Carcinogenesis Experimental evidence has shown that increases in reactive oxygen in the cell, through either physiological modification or chemical carcinogen exposure, contribute to the carcinogenesis processes (Vuillaume, 1987; Trush and Kensler, 1991; Witz, 1991; Guyton and Kensler, 1993). Reactive oxygen species encompass a series of reactive compounds including the superoxide anion ($^{\bullet}O_2^-$), hydroperoxyl radical ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$), all derived through the reduction of molecular oxygen

Table 8-13

Pathways for Intercellular Oxidant Generation

Generation of reactive oxygen species via reduction of molecular oxygen

$O_2 + e^- \rightarrow O_2^{\cdot-}$ (superoxide anion)

$O_2^{\cdot-} + H_2O \rightarrow HO_2^{\cdot}$ (hydroperoxyl radical)

$HO_2^{\cdot} + e^- + H \rightarrow H_2O_2$ (hydrogen peroxide)

$H_2O_2 + e^- \rightarrow OH^- + {}^{\cdot}OH$ (hydroxyl radical)

A series of oxygen radicals are produced by the reduction of molecular oxygen. Of the radicals produced, the hydroxyl radical, hydroperoxyl radical, and the superoxide anion are sufficiently reactive and may interact with biomolecules.

Table 8-14

Reactive Oxygen Species Generation and Removal in the Cell

Cellular oxidants

Endogenous	Exogenous
Mitochondria $O_2^{\cdot-}$, H_2O_2, ${}^{\cdot}OH$	Redox cycling compounds $O_2^{\cdot-}$
Cytochrome P450 $O_2^{\cdot-}$, H_2O_2	Metals (Fenton reaction) $H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\cdot}OH + Fe^{3+}$
Macrophage/ Inflammatory cells $O_2^{\cdot-}$, NO, H_2O_2, OCl^-	Radiation ${}^{\cdot}OH$
Peroxisomes H_2O_2	

Cellular antioxidants

Enzymatic	Nonenzymatic
Superoxide dismutase	Vitamin E
Catalase	Glutathione
Glutathione peroxidase	Vitamin C
Glutaredoxin	Catechins
Thioredoxin	

Oxidants > Antioxidants → Oxidative Damage (DNA, RNA, Lipid, Protein)

Oxidants can be produced via both endogenous and exogenous sources. Antioxidants function to maintain the cellular redox balancing. However, excess production of oxidants and/or inadequate supplies of antioxidants result in damage to cellular biomolecules and may impact on neoplastic development.

(Table 8-13) (see Chap. 3). Oxygen radicals can be produced from both endogenous and exogenous sources and are typically counterbalanced by antioxidants (Table 8-14). Antioxidant defenses are both enzymatic (e.g., superoxide dismutase, glutathione peroxidase, and catalase) and nonenzymatic (e.g., vitamin E, vitamin C, β-carotene, and glutathione) (Betteridge, 2000; Abuja and Albertini, 2001); importantly, many of these antioxidants are provided through dietary intake (Clarkson and Thompson, 2000).

Endogenous sources of reactive oxygen species include oxidative phosphorylation, P450 metabolism, peroxisomes, and inflammatory cell activation (Table 8-14). Within the mitochondria, a small percentage of oxygen is converted into the superoxide anion via 1-electron reduction of molecular oxygen. Superoxide can be dismutated by superoxide dismutase to yield hydrogen peroxide (Barber and Harris, 1994). In the presence of partially reduced metal ions, hydrogen peroxide is converted into the highly reactive hydroxyl radical through Fenton and Haber–Weiss reactions (Betteridge, 2000). Neutrophils, eosinophils, and macrophages are another intracellular source of reactive oxygen species. Activated macrophages, through a "respiratory burst," elicit a rapid increase in oxygen uptake that gives rise to a variety of reactive oxygen species including superoxide anion, hydrogen peroxide, and nitric oxide. The release of cytokines and reactive oxygen intermediates from activated Kupffer cells (the resident macrophage of the liver) has been implicated in hepatotoxicological and hepato-carcinogenic events (Rose et al., 1999; Rusyn et al., 1999). Recent studies show that activation of the Kupffer cell may function at the promotion stage of carcinogenesis. Reactive oxygen species can also be produced by cytochrome P450-mediated mechanisms including (1) redox cycling in the presence of molecular oxygen, (2) peroxidase-catalyzed single electron drug oxidations, and (3) "futile cycling" of cytochromes P450 (Parke, 1994; Parke and Sapota, 1996). Ethanol, phenobarbital, and a number of chlorinated and nonchlorinated compounds such as dieldrin, TCDD, and lindane are among the xenobiotics shown to increase reactive oxygen species through P450-mediated mechanisms (Eksrom and Ingleman-Sundberg, 1989; Rice et al., 1994; Klaunig et al., 1997). PPARα activators (e.g., clofibrate, phthalate esters) induce cytochrome P450 (CYP4A) and increase the formation of peroxisomes. As such, an increase in H_2O_2 production often accompanies exposure to chemicals that stimulate the number and activity of peroxisomes (Rao and Reddy, 1991; Wade et al., 1992). A number of agents that induce cancer also induce reactive oxygen species formation, oxidative stress, and frequently oxidative damage to the cell (Rice-Evans and Burdon, 1993; Klaunig et al., 1997, 2011).

Oxidative DNA Damage and Carcinogenesis Reactive oxygen species left unbalanced by antioxidants can result in damage to cellular macromolecules. Reactive oxygen species can produce single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links (von Sonntag, 1987; Dizdaroglu, 1992; Demple and Harrison, 1994). Persistent DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, events that are potentially involved in carcinogenesis. Oxidation of guanine at the C8 position results in the formation of 8-hydroxydeoxyguanosine (8-OHdG; see Fig. 8-7). The 8-OHdG adduct is mutagenic in bacterial and mammalian cells, produces dose-related increases in cellular transformation, and causes G → T transversions that are commonly observed in mutated oncogenes and tumor-suppressor genes (Shibutani et al., 1991; Moriya, 1993; Hussain and Haris, 1998; Zhang et al., 2000). During DNA replication, 8-OHdG in the nucleotide pool can incorporate into the DNA template strand opposite dC or dA resulting in A:T to C:G transversions (Cheng et al., 1992; Demple and Harrison, 1994). Oxidative damage to mitochondrial DNA and mutations in mitochondrial DNA have been identified in a number of cancers (Schumacher et al., 1973; Horton et al.,

1996; Cavalli and Liang, 1998; Tamura et al., 1999; Chatterjee et al., 2006). Compared to nuclear DNA, the mitochondrial genome is relatively susceptible to oxidative base damage due to (1) close proximity to the electron transport system, a major source of reactive oxygen species (Barber and Harris, 1994); (2) mitochondrial DNA is not protected by histones; and (3) DNA repair capacity is limited in the mitochondria (Bohr and Dianov, 1999; Sawyer and Van Houten, 1999). Although many pathways exist that enable the formation of oxidative DNA damage, mammalian cells also possess specific repair pathways for the remediation of oxidative DNA damage (Tchou and Grollman, 1993; Fortini et al., 1999).

Aside from oxidized nucleic acids, oxygen radicals can also damage cellular biomembranes resulting in lipid peroxidation. Peroxidation of biomembranes generates a variety of products including reactive electrophiles, such as epoxides and aldehydes, and malondialdehyde (MDA) (Janero, 1990). MDA is a highly reactive aldehyde and exhibits reactivity toward nucleophiles, and can form MDA–MDA dimers. Both MDA and the MDA–MDA dimers are mutagenic in bacterial assays and the mouse lymphoma assay (Riggins and Marnett, 2001).

Oxidative Stress and Cell Growth Regulation Reactive oxygen species production and oxidative stress can affect both cell proliferation and apoptosis (Cerutti, 1985; Burdon, 1995; Slater et al., 1995). H_2O_2 and superoxide anion can induce cell proliferation in several mammalian cell types (D'Souza et al., 1993). Conversely, high concentrations of reactive oxygen species can initiate apoptosis (Dypbukt et al., 1994). Low levels of reactive oxygen species influence signal transduction pathways and alter gene expression (Fiorani et al., 1995). Many xenobiotics, by increasing cellular levels of oxidants, alter gene expression through activation of signaling pathways including cAMP-mediated cascades, calcium-calmodulin pathways, and transcription factors such as AP-1, Nrf2, Hif1, and NF-κB as well as signaling through mitogen-activated protein (MAP) kinases (extracellular signal-regulated kinases [ERK], c-Jun N-terminal kinases [JNK], and the p38 kinases) (Klaassen and Reisman, 2010; Kerr, 1992; Muller et al., 1997; Timblin et al., 1997; Klaunig et al., 2011). Activation of these signaling cascades by reactive oxygen species induced by chemical carcinogens ultimately leads to altered gene expression for a number of genes including those affecting proliferation, differentiation, and apoptosis (Hollander and Fornace, 1989; Amstad et al., 1992; Pinkus, 1993; Zawaski, 1993; Brown et al., 1998; Chang and Karin, 2001; Martindale and Holbrook, 2002) (Fig. 8-24). Activation of NF-κB, a ubiquitously expressed transcription factor, is regulated, in part, by reactive oxygen species and the cellular redox status. NF-κB activation has been observed following a variety of extracellular stimuli including exposure to chemical carcinogens and physical agents that induce oxidative stress (Beauerle et al., 1988; Li and Karin, 1998; Schulze-Oshoff et al., 1998; Pahl, 1999; Rusyn et al., 1999; Nebreda and Porrai, 2000; Glauert et al., 2001; Molina et al., 2001) (Fig. 8-25).

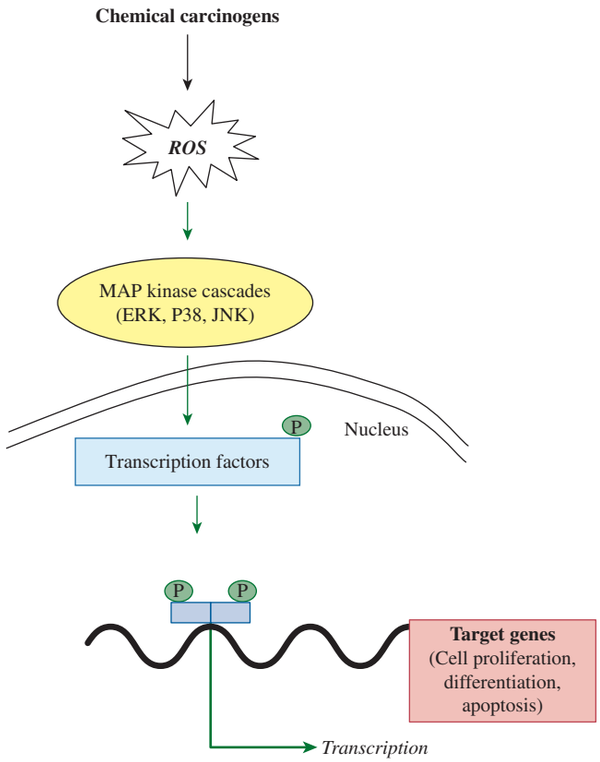


Figure 8-24. *Role of reactive oxygen species (ROS) on altered gene expression.* Many chemical carcinogens increase ROS and activate signaling cascades, such as the mitogen activated (MAP) kinase family. These serene/threonine kinases activate transcription factors, leading to DNA binding and gene expression changes.

Gap Junctional Intercellular Communication and Carcinogenesis

Cells within an organism communicate in a variety of ways including through gap junctions, which are aggregates of connexin proteins that form a conduit between two adjacent cells (Loewenstein, 1981) (Fig. 8-26). Gap junctional intercellular communication appears to play an important role in the regulation of cell growth and cell death, in part, through the ability to exchange small molecules (<1 kDa) between cells through gap junctions. Loss of gap junctions or decreased cell-to-cell communication is seen during liver regeneration following partial hepatectomy, a situation in which gap junction expression remains decreased until liver mass and lobular conformation are restored. Aberrant growth control is an essential feature of cancer cells, and because the absence or reduction in cell-to-cell communication has been observed between cancer cells, between cancer and normal cells, and in transformed cells, it has been speculated that altered gap junctional cell communication is involved in carcinogenesis (Trosko and Ruch, 1998). If cell communication is blocked between tumor and normal cells, the exchange of growth inhibitory signals from normal cells would be prevented from acting on initiated cells, thus allowing the potential for unregulated growth and clonal expansion of initiated cell populations. This would therefore allow for the acquisition of additional genetic changes that may lead to neoplastic transformation (Klaunig and Ruch, 1990).

Intercellular communication is also decreased by growth factor administration and following exposure to a variety of tumor-promoting compounds (e.g., phenobarbital, phthalates, dieldrin, phorbol esters). Tumor-promoting chemicals inhibit gap junctional intercellular communication in a number of cell types following exposure both in vivo and in vitro (Klaunig and Ruch, 1990; Klaunig et al., 1990a). The ability of a tumor-promoting compound to block cell-to-cell communication in cultured cells correlates with its ability to induce rodent tumors (Klaunig and Ruch, 1987). In addition, the inhibition of gap junctional intercellular communication by tumor promoters appears to be tissue- and species-specific; they produce inhibition in target organs and sensitive species, whereas they do

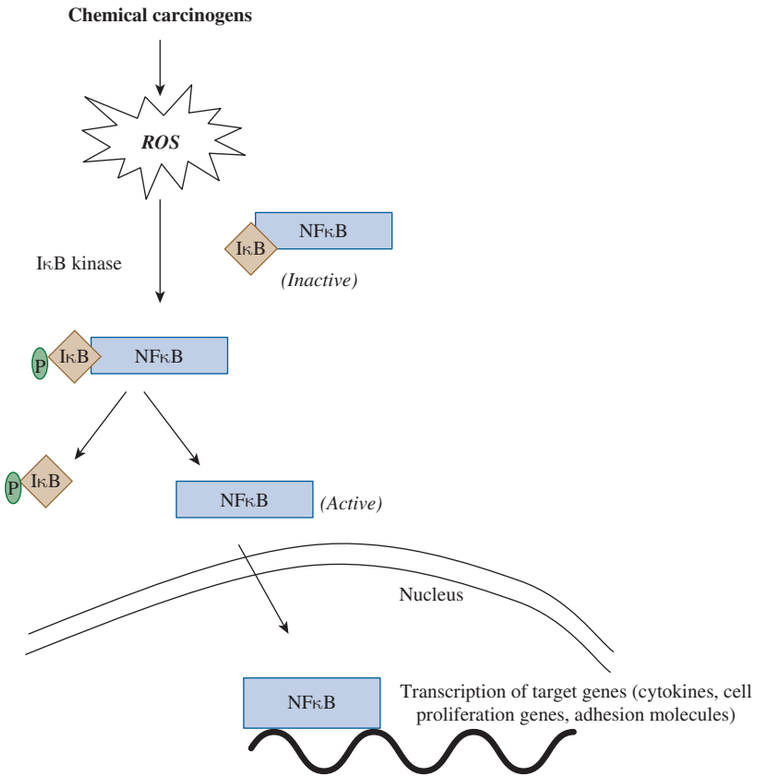


Figure 8-25. *Interaction between reactive oxygen species (ROS) and NFκB-induced gene expression.* A number of chemicals that increase ROS have been shown to activate NFκB. It is proposed that ROS enhance the dissociation of IκB from the NFκB/IκB complex leading to active NFκB. NFκB can then translocate into the nucleus where it binds DNA and increases gene transcription.

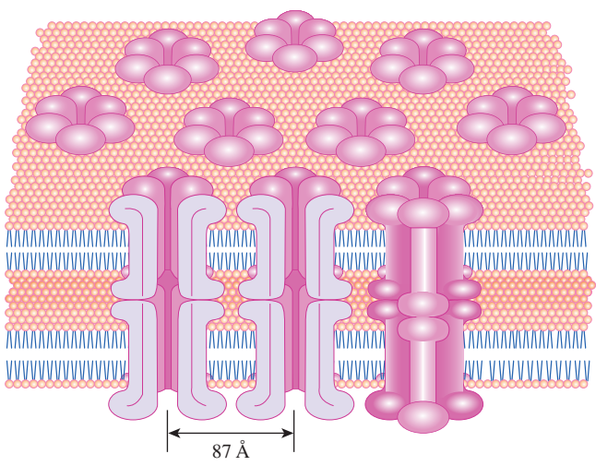


Figure 8-26. *Diagram of a gap junction traversing between two adjacent cells.* Connexons made up of six connexin proteins form the conduit between the two cells. (Reproduced with permission from Feldman RS, Quenzer LF. *Fundamentals of Neuropsychopharmacology*. Strauer Associates, Inc.; 1984.)

not inhibit cell-to-cell communication in nontarget tissues for carcinogenicity (Klaunig and Ruch, 1987). The mechanisms by which tumor promoters inhibit intercellular communication are not clear but may be due to decreased synthesis and/or enhanced degradation of gap junction proteins in the cell, reducing the number of channels in cell membranes, or other changes that modulate gap junction function and/or protein expression (Sugie et al., 1987; Klaunig and Ruch, 1990; Klaunig, 1991). Alternately, transient inhibition of gap junctional communication is under second messenger control (calcium and cAMP for example) (Klaunig and Ruch, 1990).

Inorganic Carcinogens

Metals Several metals exhibit carcinogenicity in experimental animals and/or exposed humans. Table 8-15 provides a listing of some common metals and their corresponding carcinogenicity in animals and humans. A recent IARC review has examined in detail inorganic compounds deemed human carcinogens (IARC, 2012). Additional details on selected compounds are provided below. As with all carcinogenic compounds, appreciation for the dose-response characteristics of the compound is important in understanding the relative risk to humans. (Chapter 23 provides additional information on toxicity and carcinogenicity of metals.)

Arsenic Arsenic is a naturally occurring metal. Arsenic is found in food, water, soil, and air. Arsenic is absorbed by plants and concentrated in the plant. Some seafood also contains arsenic. As such, human exposure to arsenic can occur from arsenic-containing water, soil, food, and air (on dust particles). Most exposure to inorganic arsenic occurs through arsenic-containing drinking water and to a lesser extent through food, while most organic arsenic exposure is through consuming seafood. Arsenic compounds are poorly mutagenic in both bacterial and mammalian cell assays (Lofroth and Ames, 1978). Metallic arsenic, arsenic trioxide, sodium arsenite, sodium arsenate, potassium arsenite,

Table 8-15

Carcinogenicity of Metals

	ANIMAL			HUMAN	
METAL	SPECIES	TUMOR SITE	TUMOR TYPE	EXPOSURE	TUMOR TYPE
Arsenic	Mice, dogs, rats	None observed	None observed	Cu refinery As pesticides Chemical plants Drinking water (oral)	Pulmonary carcinoma Lymphoma, leukemia Dermal carcinoma Hepatic angiosarcoma
Beryllium	Mice, rats, monkeys	Bone Lung	Osteosarcoma Carcinoma	None observed	None observed
Cadmium	Mice, rats, chickens	Injection site Testes	Sarcoma Teratoma	Cd refinery	Pulmonary carcinoma
Chromium	Mice, rats, rabbits	Injection site Lung	Sarcoma Carcinoma	Cr refinery Chrome plating Chromate pigments	Pulmonary carcinoma Gastrointestinal carcinoma
Cobalt	Rats, rabbits	Injection site	Sarcoma	None observed	None observed
Iron	Hamsters, mice, rats, rabbits	Injection site	Sarcoma	None observed	None observed
Lead	Mice, rats	Kidney	Carcinoma	None observed	None observed
Nickel	Mice, rats, cats, hamsters, rabbits guinea pigs	Injection site Lung Kidney	Carcinoma Carcinoma Carcinoma	Ni refinery	Pulmonary carcinoma Nasolaryngeal carcinoma Gastric and renal carcinoma Sarcoma (?)
Titanium	Rats	Injection site	Sarcoma	None observed	None observed
Zinc	Chickens, rats, hamsters	Testes Testes	Carcinoma Teratoma	None observed	None observed

lead arsenate, calcium arsenate, and pesticide mixtures containing arsenic have been tested for carcinogenicity in experimental animals (IARC, 1980, 1987, 2004, 2012). Based on the overall findings in animals, there is sufficient evidence in experimental animals for carcinogenicity of inorganic arsenic compounds. In addition, arsenic and inorganic arsenic compounds have been deemed to be carcinogenic to humans. Epidemiological studies of humans exposed to arsenic compounds demonstrated that exposure to inorganic arsenic compounds increases the risk of cancer in the skin, lung, digestive tract, liver, bladder, kidney, as well as lymphatic and hematopoietic systems (IARC, 1973, 1980, 2012; Martinez et al., 2011). Several epidemiological studies have reported dose–response relationships between arsenic in drinking water and several types of cancer, including bladder, kidney, lung, and skin (Cantor, 1997; Ferreccio et al., 2000). The mechanisms for cancer formation are unclear but are thought to possibly involve the induction of oxidative stress, altered cell signaling, modulation of apoptosis, and/or altered cell cycle (Harris and Shi, 2003; Quian et al., 2003; Hughes and Kitchin, 2006). The latency period in humans of arsenic-related carcinogenesis is considered to be 30 to 50 years. The first signs of chronic exposure, frequently seen in water supplies contaminated with arsenic, are skin pigmentation, depigmentation, hyperkeratosis of palms and soles, and skin lesions. A unique peripheral vascular disease associated with chronic arsenic exposure is black foot disease, starting with numbness and ulceration of extremities and ending in gangrene and spontaneous amputations (Chen et al., 1988). Dimethylarsinic acid and monomethylarsonic acid are possibly carcinogenic to humans. Arsenobetaine and other organic arsenic compounds are not well metabolized in humans and thus are not carcinogenic to humans (IARC, 1987, 2012).

Beryllium Most exposure of humans to beryllium occurs in the workplace, by inhalation of beryllium dust or fumes from the burning of coal or fuel oil and in tobacco smoke, by the ingestion of many fruits and vegetables and water, or through natural occurrence in soils. Mutagenicity tests for beryllium and its salts are either negative or weakly positive (Gordon and Bowser, 2003; IARC, 2012). Mechanistically, beryllium salts bind to nucleoproteins and inhibit enzymes involved in DNA synthesis, resulting in infidelity of DNA synthesis and also induce gene mutations in cultured cells (Leonard and Lauwerys, 1987). Studies in animal models have consistently reported increases in lung tumors in rodents and nonhuman primates exposed to beryllium or beryllium compounds (IARC, 1993; Finch et al., 1996; NTP, 1999). Beryllium metal and several beryllium compounds (e.g., beryllium-aluminum alloy, beryl ore, beryllium chloride, beryllium hydroxide, beryllium sulfate tetrahydrate, and beryllium oxide) induced lung tumors in rats. Beryllium oxide and beryllium sulfate produced lung cancer (anaplastic carcinoma) in monkeys after intrabronchial implantation or inhalation. In rabbits, osteosarcomas were reported after exposure to beryllium metal, beryllium carbonate, beryllium oxide, beryllium phosphate, beryllium silicate, or zinc beryllium silicate (IARC, 1993).

Beryllium and beryllium compounds have been classified as human carcinogens (Group 1) based on animal studies and evidence of carcinogenicity in humans (IARC, 1993, 2012; OSHA, 2017). Epidemiological studies indicate an increased risk of lung cancer in occupational groups exposed to beryllium or beryllium compounds (Steenland and Ward, 1991; Ward et al., 1992; Sanderson et al., 2001; Schubauer-Berigan et al., 2008). An association with lung cancer has consistently been observed in occupational populations exposed to beryllium or beryllium compounds. Acute beryllium pneumonitis, a marker for exposure to beryllium, has been shown to be associated with higher lung cancer rates (Steenland and Ward, 1991). In contrast, others have failed to observe a direct relationship between beryllium and human cancer risk (Boffetta et al., 2012, 2016).

Cadmium Cadmium exposure to humans is predominantly through consumption of food and drinking water, inhalation of cadmium-containing particles from ambient air or cigarette smoke, or ingestion of contaminated soil and dust. Animal studies have shown that cadmium and cadmium compounds induce tumor formation at various sites in multiple species of experimental animals, following multiple exposure routes, including the induction of prostate tumors in rats, testicular tumors in rats and mice, lymphoma in mice, adrenal gland tumors in hamsters and mice, and lung and liver tumors in mice (IARC, 1993; Waalkes et al., 1994, 1999). It has been suggested that ionic cadmium, or compounds that release ionic cadmium, results in genetic damage, and thus represents the carcinogenic species. Increased frequencies of chromosomal aberrations (changes in chromosome structure or number) have been observed in lymphocytes of workers occupationally exposed to cadmium. Many studies of cultured mammalian cells have shown that cadmium compounds cause genetic damage, including gene mutations, DNA strand breaks, chromosomal damage, cell transformation, and disrupted DNA repair (IARC, 1993).

Cadmium and cadmium compounds have been classified as known human carcinogens based on evidence of carcinogenicity in humans, including epidemiological and mechanistic information that indicate a causal relationship between exposure to cadmium and cadmium compounds and human cancer (9th RoC, 2000). Epidemiological studies of cadmium workers found that exposure to various cadmium compounds increased the incidence or risk of death from lung cancer (IARC, 1993, 2012). Follow-up analysis of some of these cohorts has confirmed that cadmium exposure is associated with elevated lung cancer risk under some industrial circumstances (Sorahan et al., 1995; Sorahan and Lancashire, 1997; Garcia-Esquinas et al., 2014). Some epidemiological evidence has also suggested an association between cadmium exposure and prostate cancer (Shigematsu et al., 1982; van der Gulden et al., 1995), kidney cancer (Mandel et al., 1995), bladder cancer (Siemiatycki et al., 1994), pancreatic cancer (Kriegel et al., 2006; Luckett et al., 2012; Garcia-Esquinas et al., 2014; Chen et al., 2015), breast cancer, and endometrial cancer (Cho et al., 2013).

Chromium Human exposure to chromium (VI) compounds can occur through inhalation of ambient air, ingestion of water, or dermal contact with products that contain chromium (VI) compounds, such as pressure-treated wood. Chromium has multiple oxidation states—from −2 to +6; however, the most common forms are the trivalent (III) and hexavalent (VI) forms. With regard to carcinogenicity, chromium III does not exhibit carcinogenicity in laboratory animals whereas chromium VI has been shown to be positive for genotoxicity and carcinogenicity in a variety of bioassays (Langard, 1988; IARC, 1990). Chromium VI compounds cause genetic damage including gene mutations and DNA damage in bacteria. Several chromium VI compounds also caused mutations in yeast and insects. Several chromium VI compounds caused genetic damage in cultured human and other animal cells and in experimental animals exposed in vivo, including sister chromatid exchange (SCE), chromosomal aberrations, and cell transformation. Chromosomal aberrations, SCE, and aneuploidy were observed in workers exposed to chromium VI compounds (IARC, 1990). Chromium VI (calcium chromate, chromium trioxide, sodium dichromate, lead chromates, strontium chromate, or zinc chromates) exposure of rats following inhalation, intrabronchial, intrapleural, intratracheal, intramuscular, or subcutaneous administration resulted in benign lung tumors and malignant lung tumors were observed in rats in a number of studies. In mice, calcium chromate caused benign lung tumors and chromium trioxide caused malignant lung tumors. Exposure of hamsters, guinea pigs, and rabbits to chromium (VI) compounds by intratracheal instillation did not cause lung tumors (IARC, 1980, 1990). Although the mechanisms of action for chromium VI carcinogenicity remain unresolved, it has been proposed that chromium VI may induce lung tumor following inhalation exposure via direct interaction with DNA causing mutations (mutagenic mode of action; McCarroll et al., 2010) or induction of oxidative stress following reduction to chromium III (non-mutagenic mode of action; Proctor et al., 2014).

Hexavalent chromium (chromium VI) compounds have been classified by IARC as known human carcinogens based on data from animal studies and human epidemiological studies (IARC 1990, 2012). Human epidemiological studies have consistently reported increased risks of lung cancer among chromate workers. Chromate workers are exposed to a variety of chromium compounds, including chromium (VI) and trivalent (III) species. In addition, an increased risk of a rare cancer of the sinonasal cavity was observed in these workers (IARC, 1990). Some studies suggested that exposure to chromium among workers such as chromium-exposed arc welders, chromate pigment workers, chrome platers, and chromium tanning workers may also be associated with stomach cancer (Welling et al., 2015), leukemia, and bone cancer (Costa, 1997).

Nickel Nickel is present in air, water, food, and consumer products; therefore, people can be exposed to nickel through food, nickel alloys, and nickel-plated materials, such as coins, steel, and jewelry. Occupational exposure to nickel occurs mainly through inhalation of dust particles and fumes or through dermal contact. Nickel workers can also ingest nickel-containing dusts. Many studies in cultured rodent and human cells have shown that a variety of nickel compounds, including both soluble and insoluble forms of nickel, exhibit genotoxicity, producing DNA strand breaks, mutations, chromosomal damage, cell transformation, increasing oxidative reactive species, and modulation of DNA repair. Soluble nickel salts can be complete carcinogens and/or initiators of carcinogenesis (Kasprzak et al., 1990; Diwan et al., 1992). In rats and mice, inhalation or intratracheal instillation of nickel subsulfide or nickel oxide produced dose-related increases of benign and malignant lung tumors (IARC, 1990, 2012; NTP, 1996). Inhalation of nickel compounds also caused malignant and benign pheochromocytoma (NTP, 1996), and adrenal cortex tumors in rats (Oller et al., 2008). Short-term intraperitoneal exposure during gestation to soluble nickel salt induced malignant pituitary tumors in the offspring. Additionally, exposure to nickel acetate through the placenta followed by exposure of the offspring to barbital (a known tumor promoter) produces tumors of the kidney (renal cortical and pelvic tumors) (Diwan et al., 1992). In adult rats, injection of soluble nickel salts followed by exposure to a promoting carcinogen

resulted in kidney cancer (renal cortical adenocarcinomas) that frequently metastasized to the lung, liver, and spleen (Kasprzak et al., 1990). The carcinogenic properties of metallic nickel are thought to be due to ionic nickel, which can slowly dissolve in the body from nickel compounds.

Nickel compounds are classified as known human carcinogens (11th RoC) based on animal data and sufficient evidence of carcinogenicity from human studies. The IARC (1990, 2012) evaluation of nickel and nickel compounds concluded that nickel compounds are carcinogenic to humans based on sufficient evidence for the carcinogenicity of nickel compounds in the nickel refining industry and very strong evidence of carcinogenicity of a variety of nickel compounds in experimental studies in rodents. Several cohort studies of workers exposed to various nickel compounds showed an elevated risk of death from lung and nasal cancers (IARC, 1990, 2012). An excess risk of lung and nasal cancer was seen in nickel refinery workers exposed primarily to soluble nickel compounds (Andersen et al., 1996; Grimsrud et al., 2002, 2003, 2005; Pavela et al., 2017). In addition, a recent meta-analysis revealed that high serum nickel levels were associated with increased risk of breast cancer (Yu and Zhang, 2017), while high soil levels of nickel were correlated with high prevalence of esophageal and oral cancer (Lee et al., 2016; Pavela et al., 2017).

Lead Lead is a naturally occurring metal that can be found in air, soil, and water. Humans can be exposed to lead via use of fossil fuels including past use of leaded gasoline, some types of industrial facilities, and past use of lead-based paint in homes. Children are more sensitive to the damaging effects of lead than adults. They can be exposed to lead eating and drinking food or water containing lead, or inhaling lead dust from lead-based paint or lead-contaminated soil or from playing with toys with lead paint. Lead compounds do not appear to cause genetic damage directly, but may do so through several indirect mechanisms, including inhibition of DNA synthesis and repair, oxidative damage, and interaction with DNA-binding proteins and tumor-suppressor proteins (NTP, 2003). Lead has exhibited conflicting results concerning its genotoxicity; it does not cause mutations in bacteria, but does cause chromosomal aberrations in vitro and in vivo, and causes DNA damage in vivo and in cell-free systems, whereas in mammalian systems, conflicting results have been observed. Lead also inhibits the activity of DNA and RNA polymerase in cell-free systems and in mammalian cell cultures. Conflicting results were observed for SCE and micronucleus formation in mammalian test systems.

In studies with laboratory animals, carcinogenicity has been observed for soluble (lead acetate and lead subacetate) and insoluble (lead phosphate, lead chromate) inorganic lead compounds as well as for tetraethyl lead (an organic lead compound), following exposure via oral intake or injection, and in offspring exposed via the placenta or lactation. Although kidney tumors (including adenomas, carcinomas, and adenocarcinomas) were most frequently associated with lead exposure, tumors of the brain, hematopoietic system, and lung were reported in some studies (IARC, 1980, 1987, 2006; Waalkes et al., 1995). Lead also appears to function as a tumor promoter, leading to increased incidence in kidney tumors initiated by *N*-ethyl-*N*-hydroxyethylnitrosamine and *N*-(4′-fluoro-4-biphenyl)acetamide (IARC, 1980, 1987, 2006). The mechanisms by which lead causes cancer are not understood.

Lead and lead compounds are classified as reasonably anticipated to be human carcinogens based on limited evidence from studies in humans and sufficient evidence from studies in experimental animals by NTP (11th RoC, 2004) and Group 2A carcinogen by IARC (2006). Lead exposure has been associated with increased risk of lung, stomach, and bladder cancer in diverse human populations (Fu and Boffetta, 1995; Steenland and Boffetta, 2000; NTP, 2003; IARC, 2006). Epidemiological studies link lead exposure to increased risk for lung and stomach cancer. However, most studies of lead exposure and cancer reviewed had limitations, including poor exposure assessment and failure to control for confounding factors.

Modifiers of Chemical Carcinogenic Effects

Genetic and environmental factors have a significant impact on the way in which individuals and/or organisms respond to carcinogen exposure. As with most genes, enzymes that metabolize carcinogens are expressed in a tissue-specific manner. Within tissues, the enzymatic profile can vary with cell type or display differential localization within cells. Further, carcinogen metabolizing enzymes are differentially expressed among species. These differences may represent an underlying factor explaining the differential responses to chemical carcinogens across species.

The expression of many carcinogen metabolizing enzymes is highly inducible by foreign chemicals. Compounds such as benzo(*a*)pyrene induce P450 isozymes CYP1A1 and 1A2, enzymes that display catalytic activity toward a number of polycyclic aromatics. A number of other enzymes, for example, GST isozymes, uridine diphosphate glucuronyltransferases (UDPGTs) are also induced by PAHs, thus the net result is enhanced metabolism of PAHs. Numerous other chemical carcinogens are potent inducers of drug metabolizing enzymes; examples include chemicals such as phenobarbital, dichlorodiphenyltrichloroethane (DDT), ethanol, 17β-estradiol, PPARα agonists, each of these results in the upregulation of a distinct set of phase I and phase II enzymes (see Chap. 6). Some carcinogens also produce inhibitory effects on drug metabolism enzymes, and could either increase the half-life of a chemical carcinogen or prevent or delay the formation of active metabolites of carcinogens.

Polymorphisms in Carcinogen Metabolism and DNA Repair

Genetic polymorphisms arise from human genetic variability. A genetic polymorphism is when a gene has more than one allele. In assessing variability in the human genome project it was found that base variations occurred at approximately once in every 1000 base pairs. Therefore, there may be over one million genetic variations between any two individuals. A single nucleotide polymorphism (SNP) is a variant in DNA sequence found in greater than 1% of the population (Fig. 8-27). Thus, by definition, changes in DNA sequence goes from mutation to polymorphism when a unique genotype is seen in over 1% of the population. Over 3 million candidate SNPs have been identified to date with up to 10 million being estimated to be present within the human genome. In carcinogenesis, genetic polymorphisms may account for the susceptibility of some individuals to certain cancers. In particular, SNPs of genes involved in carcinogen metabolism (activation and detoxification) and DNA repair have received considerable attention. A number of polymorphisms have been described in carcinogen metabolizing enzymes, with certain alleles linked to altered risk of selective cancers (Boddy and Ratain, 1997; Naoe et al., 2000). Glutathione-*S*-transferases (GSTs), which are involved in detoxification of many chemical carcinogens and also in the remediation of oxidative stress, are highly polymorphic in humans. Polymorphisms of the GSTM1 phenotype have been related to bladder, gastric, and lung cancer in humans (Tsuchida and Sato, 1992; Welfare et al., 1999). Individuals expressing the GSTM1 null genotype have a 1.5-fold higher risk for bladder cancer and 1.7-fold increase risk for gastric

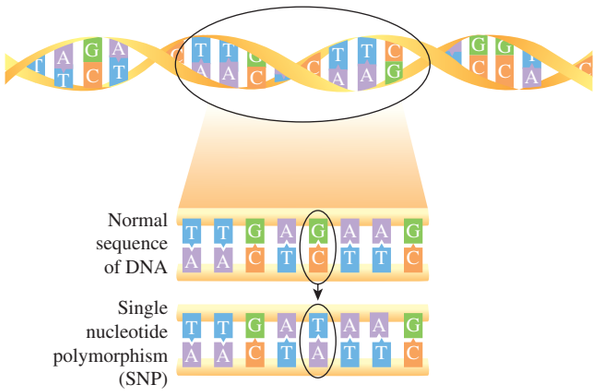


Figure 8-27. *Example of a single nucleotide polymorphism in DNA.* Single nucleotide polymorphisms are instances in which a single nucleotide is changed within a DNA sequence.

cancer compared to individuals expressing the genotype (Risch et al., 2001; Ates et al., 2005). The GSTM1 isoform is of particular important in carcinogenesis because this isoform exhibits the highest reactivity toward epoxides. GSTT1 isoform null individuals show a threefold increase risk in breast cancer, a 1.6-fold increase in gastric cancer, and a fourfold increase in liver cancer compared to individuals having the genotype (Bosch et al., 2006).

Similarly in women (nonsmokers) with lung cancer, a higher level of PAH DNA adducts have been detected. Higher DNA adduct levels are also found to be associated with the expression of the cytochrome P4501A1 (*CYP1A1*) gene, which bioactivates PAHs (Han et al., 2003). An increase in benzo(*a*)pyrene DNA adduct levels correlate with increased expression of the CYP1A1. DNA repair genes, in particular oxidative DNA repair, OGG1, involved in repair of 8-OHdG–induced damage, have been linked to an increase in lung cancer in individuals with lower OGG1 expression (Goode et al., 2002; Klaunig et al., 2011).

In the case of prostate cancer, studies have suggested roles for a number of candidate SNPs. Recent evidence suggests that the presence of a genetic variant that is more prevalent in African American men that is less effective of clearing reactive oxygen species. Examples of candidate genes that likely contribute to prostate cancer carcinogenesis include the androgen receptor gene, steroid synthesis, and metabolism genes (*CYP17*, *CYP1B1*, *CYP3A4*, *CYP3A5*, *CYP3A43*, *SD5A2*, *HSD3B1*, *HSD3B2*, *CYP19*), transmembrane drug efflux gene (*ABCG2*), and reactive oxygen species metabolizing genes (*MnSOD*, *APE1*, *XRCC1*, *GSTP1*; Dong, 2005). Individually, polymorphisms in each of these genes have been associated with an increased risk of prostate cancer.

The importance of SNPs, cancer susceptibility, and cancer risk are illustrated in Table 8-16. If exposure to a chemical carcinogen is low and the genetic susceptibility for genes related to the carcinogenic activity is low, then the risk for cancer will also be low. If exposure to a chemical carcinogen is high but the genetic susceptibility is low, then the risk for cancer development is likely to be low. However, if the genetic susceptibility is high, then exposure to a chemical carcinogen will result in a higher risk for cancer development.

Proto-Oncogenes and Tumor-Suppressor Genes

Proto-oncogenes and tumor-suppressor genes play a key role in cancer induction. These genes encode a wide array of proteins that function to control cell growth and proliferation. Common characteristics of oncogenes and tumor-suppressor genes are shown in Table 8-17. Mutations in both oncogenes and tumor-suppressor genes contribute to the progressive development of human cancers. Accumulated damage to multiple oncogenes and/or tumor-suppressor genes can result in altered cell proliferation, differentiation, and/or survival of cancer cells (Croce, 2008; Huff, 2011).

Table 8-16

Carcinogen Exposure and Cancer Risk in Humans

EXPOSURE	SUSCEPTIBILITY (GENOTYPE)	RESULT (DISEASE)
↓	↓	Low risk
↑	↓	Low risk
↓	↑	Moderate risk
↑	↑	High risk

Table 8-17

Characteristics of Proto-Oncogenes, Cellular Oncogenes, and Tumor Suppressor Genes

PROTO-ONCOGENES	ONCOGENES	TUMOR SUPPRESSOR GENES
Dominant	Dominant	Recessive
Broad tissue specificity for cancer development	Broad tissue specificity for cancer development	Considerable tissue specificity for cancer development
Germline inheritance rarely involved in cancer development	Germline inheritance rarely involved in cancer development	Germline inheritance frequently involved in cancer development
Analogous to certain viral oncogenes	No known analogues in oncogenic viruses	No known analogues in oncogenic viruses
Somatic mutations activated during all stages of carcinogenesis	Somatic mutations activated during all stages of carcinogenesis	Germline mutations may initiate, but mutation to neoplasia occurs only during progression stage

Retroviruses Evidence that specific genes (oncogenes) could cause cancer first came from studies showing sarcomas could be produced in healthy chickens by injecting them with cell-free extracts derived from chicken tumors (Rous, 1911). The transforming agent in the filtrate eventually was shown to be an RNA-type tumor virus, the *Rous sarcoma virus* (RSV) that is capable of transforming a normal cell and produce sarcomas within weeks of injection (Temin and Rubin, 1958). The genome of RSV and other retroviruses consists of two identical copies of mRNA, which is

then reverse transcribed into DNA and incorporated into the host-cell genome. Oncogenic transforming viruses such as RSV contain the v-*src* gene, a gene required for cancer induction by these viruses. It was later demonstrated that normal cells from chickens and other species contain a gene closely related to v-*src* in RSV; the normal "proto-oncogene" commonly is distinguished from the viral gene by the prefix "c" (c-*src*; Oppermann et al., 1979). This discovery showed that cancer may be induced by the action of normal, or nearly normal, genes.

DNA Viruses Six major classes of DNA tumor viruses have been identified: simian virus 40 (SV40), polyoma virus, hepatitis B virus, papilloma viruses, adenoviruses, herpes viruses, and poxviruses. Unlike retroviral oncogenes, which are derived from normal cellular genes and have no function for the virus, the known oncogenes of DNA viruses are integral parts of the viral genome required for viral replication. SV40 or polyoma viruses are not capable of inducing tumors in their natural host species (monkey and mice, respectively). Infection by small DNA viruses is lethal to most non-host animal cells; however, a small proportion integrates the viral DNA into the host-cell genome. The cells that survive infection become permanently transformed due to the presence of one or more oncogene in the viral DNA. For example, SV40 codes for the large T antigen, which alone is capable of inducing transformation (Fried and Prives, 1986). Both SV40 and polyoma viruses use the host-cell's DNA synthetic apparatus for their own replication. Papilloma viruses can infect and cause tumors in rabbits, cows, and humans (Lancaster and Olson, 1982). Of the human papilloma viruses, types 6, 10, and 11 are associated with genital warts, whereas types 16, 18, 31, and 33 are associated with human cervical cancers (Vousden, 1989). Herpes viruses are complex and are capable of producing tumors in frogs, chickens, monkeys, and humans (Rapp, 1974). Included among this class is the Epstein–Barr virus, a causative agent in the development of Burkett B-cell lymphomas and in nasopharyngeal carcinoma (de-Thé et al., 1978), and is mediated by genes encoding *EBNA-1, EBNA-2*, and *latent membrane protein* (Fahreus et al., 1990). Adenoviruses affect host and non-host cells differently; in host cells, infection causes lysis whereas in non-host cells, infection results in transformation via *E1A* and *E1B* genes (Pettersson and Roberts, 1986). Hepatitis B viruses specifically infect the liver of ducks, woodchucks, and squirrels, and are strongly associated with liver cancer development in humans; infected individuals exhibit a >100-fold risk for cancer development (Synder et al., 1982). Hepatitis B gene X is thus far the main gene implicated in the transformation process (Kim et al., 1991). Poxviruses (e.g., Yaba monkey virus, Shope fibroma virus) replicate in the cytoplasm of infected cells and produce benign tumors (Niven et al., 1961).

Proto-Oncogenes An oncogene is a gene encoding a protein that is capable of transforming cells in culture or inducing cancer in animals. Of the known oncogenes, the majority appear to have been derived from normal genes (i.e., proto-oncogenes), and are involved in cell signaling cascades (Table 8-18). Because most proto-oncogenes are essential for maintaining viability, they are highly conserved evolutionarily. It has been clearly demonstrated that altered expression of these genes results in unregulated control of cell growth. Activation of proto-oncogenes arises through mutational events occurring within proto-oncogenes (Alitalo and Schwab, 1986; Bos, 1989). It has been recognized that a number of chemical carcinogens are capable of inducing mutations in proto-oncogenes (Balmain and Pragnell, 1983; Hoenerhoff et al., 2009).

Table 8-18
Examples of Oncogenes and Cancer Association

	ONCOGENE	NEOPLASM
Tyrosine kinases	EGFR	Squamous cell carcinoma
	PDGF	Lung carcinoma
		Astrocytoma
	v-fms	Sarcoma
	v-kit	Sarcoma
	v-ros	Sarcoma
	v-fgr	Sarcoma
	v-fps	Sarcoma
	Src	Colon carcinoma
	Neu	Breast carcinoma
		Neuroblastoma
	Ret	Thyroid carcinoma
	Trk	Colon carcinoma
Serine/threonine kinases	v-raf	Sarcoma
	v-mos	Sarcoma
G proteins	H-Ras	Colon and lung carcinoma
	K-Ras	Melanoma, acute myeloid leukemia (AML), and thyroid carcinoma
	N-Ras	Carcinoma and melanoma
	NF-1	Neurofibromas
Nuclear proteins	c-Myc	CML, Burkitt lymphoma
	N-Myc	AML, breast and lung carcinoma
	L-Myc	Neuroblastoma and lung carcinoma
	v-myb	Lung carcinoma
	v-jun	Sarcoma
	v-fos	Osteosarcoma
	v-rel	Leukemia
	v-ets	Myeloblastosis
	v-erbA	Erythroblastosis

Several oncogene products are associated with growth factors. The *sis* oncogene encodes for platelet-derived growth factor (PDGF), which consists of type A and B chains. Active PDGF can consist of homo- or heterodimers of the A and B chains. Only cells that express the PDGF receptor are susceptible to transformation by the *sis* oncogene (Dooliter et al., 1983). Fibroblast growth factor (FGF), epidermal growth factor (EGF), and the *wnt* family also have oncogenes that encode for their production (Burgess et al., 1989; Nusse and Varmus, 1992). Enhanced expression of these growth factor genes leads to transformation of cultured cells. In addition, lung carcinomas and astrocytomas in humans express both PDGF and its receptor.

In order for the growth factors listed above to induce cell growth, the cell must also express the appropriate growth factor receptors. Ligand binding to receptors in many cases results in the activation of signaling cascades, of which a number have protein kinase activity. Protein kinases can phosphorylate at tyrosine, serine, or threonine residues. Several oncogene products have been

identified that have tyrosine kinase activity (e.g., *src, fps, fgr, fms, kit,* and *ros*; Collett and Erikson, 1978). Growth factor receptors including EGF, PDGF, insulin, insulin-like growth factor, and FGF have tyrosine activity (Wells, 1999). An extensive number of protein kinases are serine or threonine kinases, some of which can be activated in tumor cells and lead to transformation. The protein kinase C family consists of serine/threonine kinases (Nishizuka, 1988). This family of proteins participates in the inositol–phospholipid second messenger pathway and can be activated by calcium and diacylglycerol, and phorbol ester tumor promoters. *Raf* and *mos* oncogenes are other examples of serine threonine kinases that interact with mitogen activated kinase pathways, leading to enhance the expression of "immediate-early" genes such as Myc, and Fos/Jun heterodimers, and ultimately lead to enhanced cell growth (Su and Karin, 1996).

Guanine nucleotide binding proteins is a subset of the guanosine-5′-triphosphate (GTP) superfamily. The primary oncogenic members of this family are the *ras* genes (e.g., *Ha-*, *Ki-*, and *n-ras*), which were initially found in murine retroviruses, and are frequently activated in human tumors and in chemically induced tumors in rodent models (McCormick, 1994). The three ras forms differ by only 20 amino acids and have a conserved cytosine-186, which is a site for posttranslational modification by a farnesyl, isoprenyl group, a modification that is needed for membrane localization (Hancock et al., 1989). Mutations in *ras* affect the association of the Ras with GTP, and because Ras is active when GTP is bound, constitutive Ras activation is often seen with mutated forms of *ras*. Many mutation sites are associated with activation of *ras* and include codons 12, 13, 61, 116, 117, 119, and 146 (de Vos et al., 1988). Although a dominant gain-of-function mutation in the *ras* gene converts it into an oncogene, a recessive loss-of-function mutation in the *NF1* gene also leads to constitutive Ras activation. *NF1* encodes a GTPase-activating enzyme (GAP) that accelerates hydrolysis of GTP and the conversion of active GTP-bound Ras to inactive GDP-bound Ras. The loss of GAP leads to sustained Ras activation of downstream signal transduction proteins. Individuals with neurofibromatosis have inherited a single mutant *NF1* allele; subsequent somatic mutation in the other allele leads to formation of neurofibromas.

In addition to acting at the receptor level, some oncogene products are nuclear transcription factors and thus cause alteration of gene expression. One of the first to be identified was the erbA oncogene product, which is an altered form of the thyroid hormone receptor (Damm et al., 1989). This altered form is unable to bind thyroid hormone and allows the oncogene protein to act in a dominant-negative manner, functioning as a constitutive repressor of normal expression of genes regulated by thyroid hormone. Another family of oncogenes encode for the AP-1 transcription factor, a complex consisting of *jun* and *fos*, as well as Jun family members with homology to c-jun (Jun B and Jun D) (Chiu et al., 1988; Vogt, 2001). Phorbol esters and several other tumor-promoting compounds are able to rapidly induce gene expression of both *fos* and *jun*. Activation of both *fos* and *jun* results in the constitutive expression of the activator protein 1 (AP-1) transcription factor and sustained stimulation of cell growth signals. The *myc* family (*c-myc, N-myc*, and *L-myc*) encode for transcription factors that are found activated in a number of tumor types (Marcu et al., 1992).

Tumor-Suppressor Genes

Retinoblastoma (Rb) Gene In contrast to oncogenes, the proteins encoded by most tumor-suppressor genes act as inhibitors of cell proliferation or cell survival (Table 8-19). The prototype tumor-suppressor gene, Rb, was identified by studies of inheritance of retinoblastoma. Loss or mutational inactivation of Rb contributes to the development of a wide variety of human cancers. In its unphosphorylated form, Rb binds to the E2 factor (*E2F*) transcription factors preventing E2F-mediated transcriptional activation of a number of genes whose products are required for DNA synthesis. In addition, the Rb–E2F complex acts as a transcriptional repressor for many of these same genes (Chellappan et al., 1991). During the early G_1 phase of the cell cycle, Rb is an unphosphorylated state, but becomes phosphorylated during late G_1, causing dissociation from E2F—a process that allows E2F to induce synthesis of DNA replication enzymes, resulting in a commitment into the cell cycle. Rb phosphorylation is initiated by an active cyclin-dependent kinase 4 (Cdk4)–cyclin D complex and is completed by other cyclin-dependent kinases (Sherr, 1993; Weinberg, 1995). Most tumors contain an oncogenic mutation of one of the genes in this pathway such that cells enter the S phase of the cell cycle in the absence of the proper extracellular growth signals that regulate Cdk activity. In addition, Rb is bound and inactivated by the E1A protein of the adenoviruses (Whyte et al., 1988) and also by the large T antigen of SV40 and the E7 protein of the papilloma virus, suggesting that the elimination of Rb function may be an important mechanism in tumor development by viruses and/or mutations induced by chemical carcinogens.

Table 8-19

Examples of Tumor Suppressor Genes and Cancer Association

TUMOR SUPPRESSOR	DISORDER	NEOPLASM
Rb1	Retinoblastoma	Small-cell lung carcinoma
p53	Li–Fraumeni syndrome	Breast, colon, lung cancers
BRCA1	Unknown	Breast carcinoma
WT-1	Wilms tumor	Lung cancer
p16	Unknown	Melanoma

p53 Gene The p53 protein is a tumor-suppressor gene that is essential for the checkpoint control and arrests the cell cycle in cells with damaged DNA in G_1. Cells with functional p53 arrest in G_1 when exposed to DNA-damaging agents such as irradiation, whereas cells lacking functional p53 are unable to block the cell cycle. p53 is activated by a wide array of stressors including UV light, γ irradiation, heat, and several carcinogens. DNA damage by γ irradiation or by other stresses leads to the activation of certain kinases, including ataxia telangiectasia mutated (ATM), which is encoded by the gene mutated in ataxia telangiectasia and a DNA-dependent protein kinase. Phosphorylation of p53 by these and other kinases results in stabilization of p53 and an increase in cell content of this protein (Finlay et al., 1989). The active form of p53 is a tetramer of four identical subunits. A missense point mutation in one *p53* of the two alleles in a cell can abrogate almost all p53 activity because virtually all the oligomers will contain at least one defective subunit and such oligomers cannot function as a transcription factor. Oncogenic *p53* mutations thus act as "dominant negatives," in contrast to tumor-suppressor genes such as Rb.

Virtually, all p53 mutations abolish their ability to bind to specific DNA sequences and activate gene expression (El-Deiry et al., 1992). The transcription of cyclin-kinase inhibitor p21, which binds to and inhibits mammalian G_1 Cdk–cyclin complexes, is induced by p53. As a result, cells with damaged DNA are arrested in G_1 until the damage is repaired and the levels of p53 and p21 fall; the cells then can progress into the S phase. In most cells, accumulation of p53 also leads to induction of proteins that promote apoptosis, and, therefore, would prevent proliferation of cells that are likely to accumulate multiple mutations. When the p53 checkpoint control does not operate properly, damaged DNA can replicate, producing mutations and DNA rearrangements that contribute to the development of transformed cells. Proteins that interact with and regulate p53 are also altered in many human tumors. The gene encoding one such protein, MDM2 (murine double minute 2 homolog), is amplified in many sarcomas and other human tumors that maintain functional p53 (Leach et al., 1993). Under normal conditions, MDM2 binds the N-terminus of p53, repressing the ability of p53 to activate transcription of p21 and genes mediating p53 degradation. Thus, MDM2 normally inhibits the ability of p53 to block at G_1 or induce apoptosis. Enhanced MDM2 levels in tumor cells therefore results in decreased functional p53 and prevents p53 from growth arrest in response to irradiation or chemical carcinogens. For example, benzo(*a*)pyrene produces inactivating mutations at codons 175, 248, and 273 of the p53 gene in cultured cells; these same positions are mutational hot spots in human lung cancer (Hollstein et al., 1991). Aflatoxin exposure induces a G to T transversion at codon 249 of p53, and methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) can alter methylation of DNA leading to inactivation of p53 (Bressac et al., 1991).

BRCA1 Gene Genetic analysis of breast tumors has revealed a hereditary predisposition for breast cancer linked to BRCA1 (breast cancer gene 1), a tumor-suppressor gene. Mutation of a single BRCA1 allele results in a 60% probability of developing breast cancer by age 50. In families transmitting the mutant BRCA1 allele, the normal allele is often absent in tumors. A number of investigators have shown that germ line mutations lead to loss of function of the BRCA1 gene, perhaps by acting as a transcription factor through binding at a zinc finger domain. However, no mutations have been observed in sporadic breast cancers (Futreal et al., 1994), suggesting that BRCA1 gene silencing may occur through nonmutational mechanisms.

Wilms Tumor Gene (WT1) Wilms tumor occurs in the developing kidney at a rate of approximately one per 10,000 children. The WT1 gene is considered to be responsible for tumor development and is thought to function as a transcription factor (Hastie, 1994). The DNA-binding region of the WT1 gene contains four zinc finger domains, and binds a consensus sequence shared with EGR1; however, WT1 acts as a transcriptional repressor at this site (Rauscher et al., 1990). Similar to p53, WT1 mutant alleles act in a dominant-negative manner.

p16 Gene The group of proteins that function as cyclin-kinase inhibitors play an important role in cell cycle regulation. Mutations, especially deletions of the p16 gene, that inactivate the ability of p16 to inhibit cyclin D–dependent kinase activity are common in several human cancers including a high percentage of melanomas (Kamb et al., 1994). Loss of p16 would mimic cyclin D1 overexpression, leading to Rb hyperphosphorylation and release of active E2F transcription factor. Thus, p16 normally acts as a tumor suppressor. As with the BRCA1 gene, relatively few mutations have been found in this gene, and some researchers have speculated that epigenetic mechanisms such as gene silencing by DNA methylation may occur during tumorigenesis (Merlo et al., 1995).

Hormesis, Dose Response, and Carcinogenesis

Hormesis is defined as a dose–response curve in which a U, J, or inverted U-shaped dose–response is observed, with low-dose exposures often resulting in beneficial rather than harmful effects (Calabrese, 2002) (Fig. 8-28). One of the first reports establishing a hormetic response was observed with ionizing radiation, in which it was hypothesized to be due to adaptation to background radiation exposure, as well as enhanced metabolism and detoxification (Calabrese, 2002; Parsons, 2003). Numerous examples exist that provide evidence for U- and J-shape dose relationships to different biomarkers of carcinogenesis, both for initiating and promoting carcinogens. For example, low-dose styrene treatment resulted in a decrease of chromosomal aberrations (Camurri et al., 1983). A reduction in DNA strand breaks in human keratinocytes was seen following low-dose exposure to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, whereas high doses were greatly elevated compared to controls (Kleczkowska and Althaus, 1996). Several chronic bioassays for carcinogenicity in rats and mice have demonstrated a negative correlation between proliferative hepatocellular lesions and lymphomas at low- and medium-dose levels (Young and Gries, 1984). TCDD at hepatocarcinogenic doses resulted in a dose-dependent reduction in mammary and uterine tumors (Kociba et al., 1978). TCDD also reduces tumor incidence after exposure to low doses of radiation and selenium (Nordberg and Andersen, 1981; Pollycove and Feinendegen, 2001). Evidence for a U-shaped dose–response curve has also been demonstrated for phenobarbital (Kitano et al., 1998; Ito et al., 2003; Kinoshita et al., 2003). These investigators have shown that phenobarbital promoted the growth of diethylnitrosamine-induced hepatic lesions at doses between 60 and 500 ppm, whereas doses between 1 and 7.5 ppm decreased lesion growth, hepatocellular carcinoma, and tumor multiplicity. Other examples include DDT (Sukata et al., 2002; Kushida et al., 2005) and α-benzene hexachloride (Puatanachokchai et al., 2006).

Adaptive mechanisms have been proposed to explain the hormetic effects observed by chemical carcinogens. When experimental animals are exposed to chemicals, the initial response is an adaptive response to maintain homeostasis (Calabrese, 2002; Kakehashi et al., 2013). Adaptive responses usually involve actions of the chemical on cellular signaling pathways that lead to changes in gene expression, resulting in enhanced detoxification and excretion of the chemical as well as preserving the cell cycle and programmed cell death. It has been proposed that following

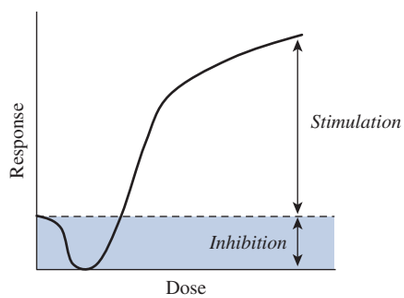


Figure 8-28. *Hormesis curve.*

very low doses of chemicals, the upregulation of these mechanisms overcompensates for cell injury such that a reduction in tumor promotion and/or tumor development is seen, and would explain the U- or J-shaped response curves obtained following carcinogen exposure.

A common feature of chemical carcinogens for which hormetic effects have been proposed is the formation of reactive oxygen species and the induction of cytochrome P450 isoenzymes. Inhibition of carcinogenesis by low levels of phenobarbital has been postulated to involve the suppression of 8-OHdG generation and cellular proliferation within areas of glutathione-*S*-transferase pi (GST-P) positive foci, possibly related to enhanced expression of the gene encoding the enzyme oxoguanine glycosylase one (Ogg1), which is responsible for the repair of 8-OHdG lesions (Kinoshita et al., 2003; Fukushima et al., 2005). Another explanation for the suppressive effect of low-level phenobarbital on the development of preneoplastic lesions involves the stimulation of hepatic drug metabolizing enzymes, which detoxify carcinogens (Pitot et al., 1987). In a low-dose phenobarbital study, cDNA microarray analysis resulted in a suppression of mRNA expression of MAP kinase p38, JNK1, 2, and other intracellular kinases (Kinoshita et al., 2003). Thus, suppression of signal transduction modulators appears to be involved in the inhibitory effect of phenobarbital on cell proliferation.

Chemoprevention

The study of chemicals that prevent, inhibit, or slow down the process of cancer is referred to as chemoprevention. This is in contrast to chemotherapy where drugs are used to target an already formed neoplasm with the purpose of selectively killing the cancer cells. A number of chemicals, including drugs, antioxidants, foodstuffs, and vitamins have been found to inhibit or retard components of the cancer process (Table 8-20) in both in vitro and in vivo models (Stoner et al., 1997). Cancer chemoprevention involves the administration or inclusion in the diet of natural or synthetic chemicals in an attempt to prevent, halt, or reverse the process of carcinogenesis. An assumption in chemoprevention is that treating early stages of the malignant process will halt or delay the progression to neoplasia. Cancer chemopreventive chemicals may function at one or more of the stages of the carcinogenesis process, functioning via one or several mechanisms. These include inhibition of carcinogen formation, blocking agents, and/or as suppressing agents (Table 8-20). Blocking agents serve to prevent the metabolic activation of genotoxic or non-genotoxic carcinogens by either inhibiting their metabolism or enhancing the detoxification mechanisms. In the case of reactive oxygen species generation by a carcinogen, antioxidants, such as, *N*-acetylcysteine, or vitamins E, A, and C serve as blocking agents by trapping the oxidative species before it can induce cellular damage. The drug oltipraz induces phase II

Table 8-20

Classes of Chemopreventive Agents

INHIBITORS OF CARCINOGEN FORMATION	EXAMPLES
Reductive acids	Ascorbic acid
Phenols	Caffeic acid, ferulic acid, gallic acid
Sulfhydryl compounds	*N*-Acetylcysteine
Amino acids	Praline, thioproline
Blocking Agents	
Inhibition of cytochrome P450	Dithiocarbamates, ellagic acid, diallyl sulfide, isothiocyanates
Induction of cytochrome P450	Indole-3-carbinol, β-naphthoflavone
Induction of phase II enzymes	
Glutathione *S*-transferase	Allyl sulfides, dithiolethiones, isothiocyanates
UDP-glucuronyltransferase	Polyphenols
Glutathione peroxidase	Selenium
Scavenge electrophiles	Ellagic acid, *N*-acetylcysteine
Scavenge free radicals	Sodium thiosulfate, polyphenols, vitamin E
Increase overall levels of DNA repair	Vanillin
Increase poly(ADP-ribosyl) transferase	*N*-Acetylcysteine
Suppress error-prone DNA repair	Protease inhibitors
Suppressing Agents	
Inhibit polyamine metabolism	DRv10, polyphenols, substituted putrescines
Induce terminal cell differentiation	Calcium, retinoids, vitamin D_3
Modulate signal transduction	Glycyrrhetinic acid, nonsteroidal anti-inflammatory drugs (NSAIDs), polyphenols, retinoids
Modulate hormonal/growth factor activity	
Inhibit oncogene activity	NSAIDs, retinoids, tamoxifen
Promote intracellular communication	Genistein, NSAIDs, monoterpenes
Restore immune response	Carotenoids, polyphenols, retinoids
Induce apoptosis	NSAIDs, selenium, vitamin E
Correct DNA methylation imbalances	Butyric acid, genistein, selenium, sulindac sulfone, retinoids
Inhibit basement membrane degradation	Folic acid, choline, methionine
Inhibit arachidonic acid metabolism	Protease inhibitors
	Glycyrrhetinic acid, *N*-acetylcysteine, NSAIDs, polyphenols

enzymes; in particular, glutathione-*S*-transferase by activating the transcription factor Nrf2, and has been shown to reduce the carcinogenicity of aflatoxin B1 by enhancing its detoxification (Jacobson et al., 1997). Nrf2 is a transcription factor that positively regulates the basal and inducible expression of a number of cellular protective genes. (Klaassen and Reisman, 2010). Activation of Nrf2 has been shown to increase the expression of genes involved in the detoxification of electrophiles and oxidative stress (Reisman et al. 2009a, b). Chemopreventive chemicals increase tissue resistance function on the target tissue, usually an early preneoplastic lesion by increasing tissue maturation and/or decreasing cell proliferation. Suppressing chemicals induce tissue differentiation, may counteract oncogenes, enhance tumor-suppressor gene activities, inhibit proliferation of premalignant cells, or modify the effect of the carcinogen on the target tissue. Retinoic acid is a classic agent that functions by inducing target tissue differentiation. Similarly, viral vectors containing wild-type p53 have been shown to negate the effects of tumor growth driven via p53 mutation. Extensive experimental evidence exists showing the inhibition or retarding of the cancer process by chemopreventive chemicals. These studies have been performed in animal models and cell systems. Although the human results of chemoprevention interventions are less convincing than the experimental models, considerable epidemiological evidence demonstrates an inverse relationship of some nutrient factors with cancer risks.

ASSESSING CARCINOGENICITY OF CHEMICALS

A number of in vivo and in vitro experimental systems are available to assess the potential carcinogenicity of chemicals. The types of tests available to identify chemicals with carcinogenic potential can be classified into general categories, based on the duration required to conduct the test (Table 8-21). Short-term tests are typically of the duration of days to a few weeks, intermediate-term tests last from weeks up to a year, whereas chronic long-term tests usually encompass 6 months to 2 years exposure to a chemical. These bioassays use bacterial and mammalian targets.

Short-Term Tests for Mutagenicity

Short-term tests for mutagenicity were developed to identify potentially carcinogenic chemicals based on their ability to induce mutations in DNA either in vivo or in vitro. A variety of in vivo and in vitro short-term tests are available to test the potential carcinogenicity of a chemical (Table 8-22). The majority of these tests quantify the mutagenicity of chemicals as a surrogate for carcinogenicity. Therefore, although they are usually very predictive of indirect acting and direct acting (if a metabolic source is provided), these tests routinely fail to detect non-genotoxic carcinogens.

Table 8-21

General Methods for Identification of Potential Carcinogens

METHOD	TIME FRAME
Short term	
Transformation in cell culture	Several weeks
Transformation in cell culture	1–3 months
Medium term	
Qualitative and quantitative analysis of preneoplasia	2–8 months
Long term	
Chronic bioassay in animals	18–24 months

In Vitro Gene Mutation Assays The most widely used short-term test is the Ames assay (Ames et al., 1975; Mortelmans and Zeiger, 2000). The relative simplicity and low cost of the test make it a valuable screening tool for mutagenic carcinogens. *Salmonella typhimurium* strains, deficient in DNA repair and unable to synthesize histidine, are used. In the presence of a mutagenic chemical, the defective histidine gene can be mutated back to a functional state (*back mutation*), resulting in a restoration of bacterial growth in medium lacking histidine. The mutant colonies, which can make histidine, are referred to as "revertants." The Ames test in basic form can detect direct but not indirect genotoxic carcinogens. With the inclusion of a metabolic source, specifically the 9000 g supernatant (S9) of a rat liver homogenate to promote metabolic conversion of the chemical, the Ames test can also detect indirect-acting genotoxic carcinogens. Fig. 8-29 describes the standard method used for performing the Ames assay.

Genetically unique strains of the *S. typhimurium* bacterium have been developed for determining specific mutational targets. Strains TA100 and TA1535 are able to detect point mutations whereas strains TA98, TA1537, and TA1538 are able to detect frame shift mutations. Chemicals are typically tested at several concentrations (usually five or more) and the mutation frequency (number of revertants) is calculated. Activation-independent (e.g., sodium azide, methyl methanesulfonate) and activation-dependent (e.g., 2-aminoanthracene) positive controls are included in each assay.

The mouse lymphoma assay is a mutagenicity assay used to determine whether a chemical is capable of inducing mutation in eukaryotic cells. Typically, mouse lymphoma L5178Y cells are used, and the ability of the cell cultures to acquire resistance to trifluorothymidine (the result of forward mutation at the thymidine kinase [TK] locus) is quantified. Another mammalian cell mutation assay, the Chinese hamster ovary (CHO) test, is also commonly used to assess the potential mutagenicity of chemicals. This assay uses the hypoxanthine–guanine phosphoribosyltransferase (HGPRT) gene as the endpoint. The basis for these assays is shown in Fig. 8-30. Cells are treated with the test chemical and then placed into suspension with selective medium for replication and fixation of induced mutations. Cells are then plated for colony growth, and after several days, colony numbers and colony size are recorded. The number of mutant colonies is a measure of the ability of the test chemical to induce a genetic change at the TK or HGPRT loci in these transformed cells.

As with the Ames assay, these assays are frequently performed in the presence of an exogenous metabolic source (e.g., irradiated epithelial cell feeder layer). Because not all carcinogens are mutagens and/or directly damage DNA, the concordance with the chronic in vivo bioassay for these mutagenicity assays is relatively low. For a historical perspective on the utilization of the Ames/Salmonella assay, a recent review by Claxton et al. (2010) provides more information on the use of the assay and the role of metabolic activation and mutagenicity testing with respect to genotoxic carcinogens.

In Vivo Gene Mutation Assays For the assessment of the carcinogenic potential of chemicals in vivo genotoxicity tests are important complement to in vitro mutagenicity tests. The in vivo tests have advantages over the in vitro test systems in that they

Table 8-22

Short-Term Tests for Mutagenicity

TEST	ENDPOINT	REFERENCES
Gene mutation assays in vitro		
Prokaryote mutagenesis in vitro (Ames test)	Back or forth mutations in specific bacterial strains	Maron and Ames (1983)
Mouse lymphoma thymidine kinase (TK)	Mutations in TK	Majeska and Matheson (1990)
Chinese hamster ovary (CHO) and V79 hypoxanthine guanine phosphoribosyltranferase (HGPRT)	Mutations in HGPRT	Li et al. (1987)
Gene mutation assays in vivo		
Dominant lethal assay	Death of fertilized egg in mammalian implanted species	Bateman (1973) Lockhart et al. (1992)
Sperm abnormality induction	Microscopically abnormal sperm	Wyrobek and Bruce (1975)
Pig-a assay	Mutations in *GPI* gene	Gollapudi et al. (2015)
Mutation induction in transgenes in vivo		
LacZ mouse	Mutations in *LacZ* gene	Myhr (1991)
LacI mouse	Mutations in *LacI* gene	cf. Mirsalis et al. (1994)
LacI rat	Mutations in *LacI* gene	de Boer et al. (1996)
rpsL mouse	Mutations in *rpsL* gene	Gondo et al. (1996)
Chromosomal alterations in vivo		
Heritable translocation test (mice)	Translocations induced in germ cells	Generoso et al. (1980)
Rat bone marrow clastogenesis in vivo	Chromosomal aberrations in bone marrow cells in vivo	Ito et al. (1994)
Micronucleus test	Appearance of micronuclei in bone marrow cells in vivo	Tinwell and Ashby (1994) Heddle et al. (1983)
Chromosomal alterations in vitro		
Mitotic recombination, mitotic crossing over, or mitotic gene conversion in yeast	Conversion of heterozygous alleles to homozygous state	Wintersberger and Klein (1988)
Induced chromosomal aberrations in cell lines	Visible alterations in karyotype	Galloway et al. (1985)
Sister chromatid exchange	Visible exchange of differentially labeled sister chromatids	Latt (1981)
Primary DNA damage		
DNA repair in vivo or in vitro	Unscheduled DNA synthesis and/or DNA strand breaks	Furihata and Matsushima (1987)
Rodent liver: unscheduled DNA synthesis induction	Unscheduled DNA synthesis in rodent cells in vivo and/or in vitro	Kennelly (1995), Steinmetz et al. (1988)

take into account whole animal processes such as absorption, tissue distribution, metabolism, and excretion of chemicals and their metabolites. The commonly used in vivo models include transgenic rodent mutation assay systems based on the genes of the *lac* operon, MutaMouse (Gossen et al., 1989), Big Blue (Kohler et al., 1991a, 1991b), and the recently developed Pig-a gene mutation assay (Gollapudi et al., 2015). Some details of the *lac* operon are shown in Fig. 8-31).

The MutaMouse is a transgenic mouse in which a vector prepared from bacteriophage λDNA (λgt10) has been stably inserted into the genome and exists within every cell. Within the λDNA sequence, the bacterial gene *lacZ*, which encodes for β-galactosidase, was inserted at the single *Eco*RI restriction site. The entire vector contains ~47,000 bp, of which the *lacZ* gene comprises 3126 bp. The construction and analysis of MutaMouse have been previously described (Gossen et al., 1989). The Big Blue transgenic mouse was developed using a 47.6-kb λshuttle vector containing the bacterial *lacI* gene (1080 bp), which encodes the repressor protein of the *lacZ* gene. As with the MutaMouse, this vector is stably inserted into the genome and exists in every cell. Details on the Big Blue system are described elsewhere (Kohler et al., 1991a, 1991b).

To detect mutations, following exposure of mice to test chemicals, mutations are analyzed in high molecular DNA isolated from the tissue under investigation. The next step is to isolate a viable λphage for the analysis of mutations in the *lacZ* or *lacI* genes from the mouse. The infectious λparticles are detected as plaques that form on a bacterial (*Escherichia coli* strain) lawn grown on an agar surface. In the case of MutaMouse a positive selection system is used that involves the scoring of clear plaques on titration plates to determine the total number of plaque forming units and on selection plates to determine the number of mutants (Gossen and Vijg, 1993). For Big Blue, a nonselectable color screening assay is used, scoring blue mutant plaques among the nonmutant clear plaques

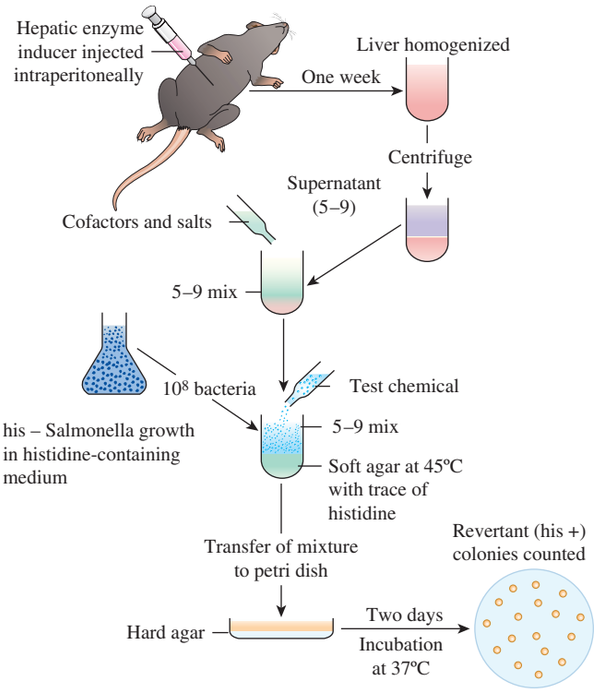


Figure 8-29. *Scheme of the Ames test for mutagenesis of chemicals in Salmonella bacterial strains.*

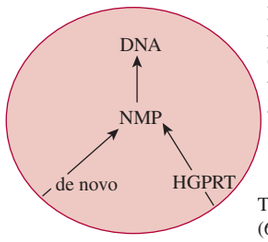
(Kohler et al., 1991a). In each case the ratio of mutants to the total population provides a mutation frequency for each chemical and each organ tested. As with the other short-term tests that have been discussed, it is unlikely that in vivo genotoxicity test systems will identify non-genotoxic/non–DNA-reactive compounds.

The Pig-a gene mutation assay is a relative new in vivo assay primarily performed in rats, based on the X-linked Pig-a gene (phosphatidylinositol *N*-acetylglucosaminyltransferase, subunit A), which is involved in the production of glycosylphosphatidylinositol (GPI) anchor proteins on the cell surface (Kinoshita et al., 1997). Loss of GPI anchored surface markers can be used as a surrogate for Pig-a gene mutation. The use of Pig-a as a positive in vivo somatic mutation reporter gene was initially proposed based on the studies of patients with paroxysmal nocturnal hemoglobinuria (Takahashi et al., 1993; Takeda et al., 1993; Boccuni et al., 2000). Assays of Pig-a gene mutation in vivo were developed thereafter in rodents and have undergone intensive international validation (Miura et al., 2008a, 2008b; Dertinger et al., 2011; Kimoto et al., 2016). Currently, the assay is optimized for measuring the Pig-a mutant phenotype in peripheral blood erythrocytes including reticulocytes (RETs) and red blood cells (RBC) or rats primarily by quantification of CD59-negative RETs and RBCs (the RET^{CD59-} and RBC^{CD59-} respectively) (Fig. 8-32). The typical recommended study design is 28-day repeated dose subchronic toxicity study in male rats consisting of five to six doses (including a concurrent vehicle control), with blood sample collected at the end of treatment and analyzed (at least 1 to 5 million erythrocytes)

Mammallian cell line used for gene mutation studies

Mouse lymphoma L5178Y	TK, HGPRT
CHO	HGPRT
V79 hamster cells	HGPRT

Selection of forward mutations by loss of HPGRT + phenotype in V79 or Chinese hamster ovary cells.



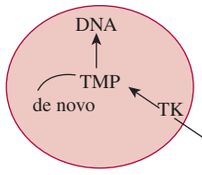
Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is an X-linked gene that confers resistance to purine analogues such as 6-thioguanine.

Toxic analogue (6-thioguanine)

Performed in the presence of an activation system or hepatic feeder layer

Selection of mutagen-induced TK –/– phenotype in TK +/– in mouse lymphoma assay (MOLY) in L5178Y cells. This assay detects forward mutations at the TK locus.

Treat TK (+/–) cells with test compound. Fluoruracil is converted to a toxin, killing the cells. If mutation in TK occurs, FU is not metabolized to a toxin and colonies grow.



Thymidine kinase (TK) confers resistance to pyrimidine analogues. It is a somatic gene.

Toxic analogue (bromodeoxyuridine)

Figure 8-30. *Outline of chemically induced mutation in mouse cell lines with TK or HGPRT as the target gene.*

with flow cytometer (Gollapudi et al., 2015). The Pig-a gene is highly conserved in humans, rats, mice, and monkeys, therefore it allows comparison of mutation induction across multiple species. In addition, it can be easily integrated into standard toxicity studies as well as existing genotoxicity endpoints such as chromosome aberrations, micronucleus, and DNA damage (comet) assays (Stankowski et al., 2011, 2015) to provide additional data for toxicity assessment.

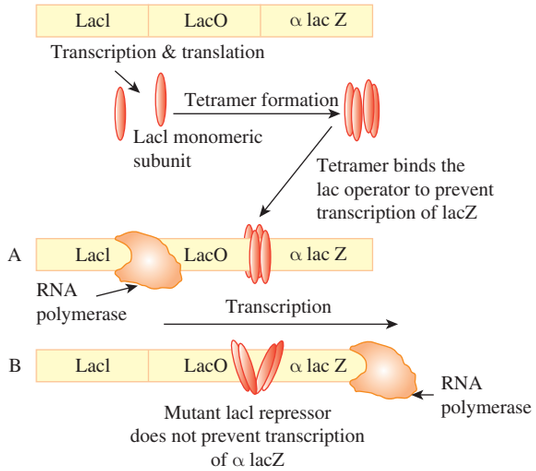


Figure 8-31. *Schematic representation of the lac operon in E. coli.*

Chromosomal Alterations Chromosomal alterations are quite common in malignant neoplasms, as such the detection of chromosomal abnormalities by test chemicals is considered an excellent test for the assessment of carcinogenic potential. Both in vivo and in vitro assays are available to assess chromosomal alterations. In mammalian cell lines, most of the test systems used the same lines as used in the mutation assay. To assess induction of chromosomal alterations, cells are harvested in their first mitotic division after the initiation of chemical exposure. Cells are stained with Giemsa, and scored for completeness of karyotype (21 ± 2 chromosomes). The classes of aberrations recorded include breaks and terminal deletions, rearrangements and translocations, as well as despiralized chromosomes, and cells containing 10 or more aberrations.

Sister chromatid exchanges (SCEs) are a measure of DNA damage and increased levels of DNA damage, events that are associated with mutation induction and cancer. During metaphase, sister chromatids, each encompassing a complete copy of one chromosome, are bound together through specific protein interactions. SCEs are a reflection of an interchange of DNA between different chromatids at homologous loci within a replicating chromosome (Latt et al., 1981). Differential staining of chromatids is therefore used to assess SCEs. Typically, 5-bromodeoxyuridine (BrdU) is

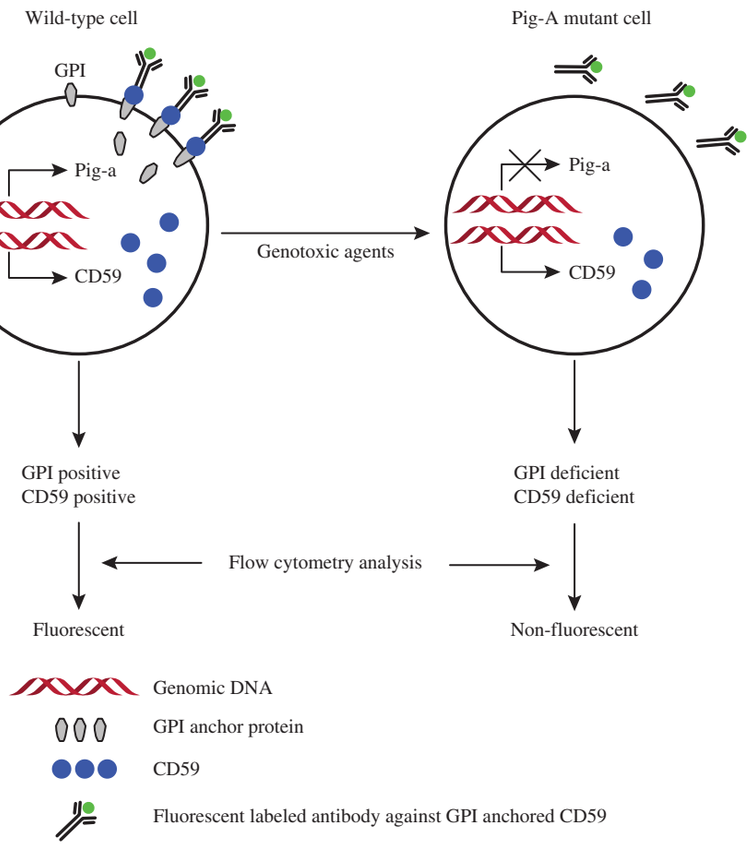


Figure 8-32. *Principle of the rat erythrocyte Pig-a assay.*

added along with the test chemical for one cell replication followed by replacement of the medium with medium containing BrdU and colcemid, without test chemical. Cells are then harvested, stained and second-division metaphase cells scored to determine the frequency of SCE/cell for each dose level.

In vivo analysis of chromosomal alterations has been described since the 1980s (Sasaki, 1980). Disruption of the DNA replication process or damage to chromosomes by chemicals can alter the genetic material distributed to either of the two daughter nuclei. When this occurs, the genetic material that is not incorporated into a new nucleus may form its own "micronucleus," which is clearly visible with a microscope. For this assay, animals are exposed to chemicals and the frequency of micronucleated cells is determined at some specified time after treatment. Micronucleus tests must be performed on cells that are dividing, most typically in cells from bone marrow samples (Heddle et al., 1983). As with other short-term tests, the micronucleus test provides complementary information to gene mutation data but neither test enhances the ability to detect genotoxic carcinogens, nor is capable of detecting non–DNA-reactive carcinogens. However, this assay does provide information on the ability of a chemical to disrupt mammalian chromosome structure and function.

DNA Damage Primary DNA damage represents possible premutational events that can be detected, either directly or indirectly, by a number of techniques using mammalian cells in culture or using rodent tissue. Unscheduled DNA synthesis (UDS) is a commonly used assay that measures the ability of a test article to induce DNA lesions by quantifying the increase in DNA repair. Among the available techniques is the measurement of DNA strand breaks both in vivo and in vitro including the gamma gamma-H2AX assay (Miyamae et al., 1997; Kuo and Yang, 2008). Although a wide array of assays are available to assess DNA damage, the comet assay, or single cell gel electrophoresis, has become one of the standard methods for assessing DNA damage and repair at the individual cell level. Since its introduction in 1988, the alkaline comet assay has been modified to increase the utility of this technique for detecting various forms of DNA damage (e.g., single- and double-strand breaks, oxidative DNA base damage, and DNA–DNA/DNA–protein/DNA–Drug cross-linking) and DNA repair in virtually any eukaryotic cell (Singh et al., 1988). Through the use of lesion-specific endonucleases, the levels of UV-induced pyrimidine dimers, oxidized bases, and alkylation damage can be assessed in biological samples (Miyamae et al., 1997, 1998; Collins, 2004; Pu et al., 2015). The in vivo version of the comet assay has recently been implemented in regulatory genotoxicity testing of pharmaceuticals and chemicals with inclusion into the ICH S2R21 guidance (EMA, 2008) and adoption in the Organization for Economic Co-operation and Development (OECD) Test Guideline (TG) 489 (OECD, 2014).

Short-Term Tests: Transformation Assays A variety of in vitro test methods including cell transformation assays have been developed to assess the carcinogenic potential of chemicals (Creton et al., 2012). The C3H/10T½ cell line has been widely used for the transformation assay. It was originally derived from fibroblasts taken from the prostate of a C3H mouse embryo. The cells are approximately tetraploid but the chromosome number in the cells varies widely. As such, these cells are chromosomally abnormal and have already passed through some of the stages that might be involved in the production of a cancerous cell. Upon plating these cells, they will stop growing when their density is sufficiently high (contact growth inhibition). However, the contact inhibition can fail, resulting in cell piling forming a transformed colony. Therefore, following exposure to xenobiotics, this assay assesses carcinogenic potential based on the percentage of colonies that are transformed (Reznikoff et al., 1973).

The most frequently used endpoint for cell transformation is morphological transformation of mammalian cell fibroblasts in culture. Transformation assays using BALB3T3 and Syrian hamster embryo (SHE) cells are available for the assessment of the carcinogenic potential of chemicals. BALB/3T3 transformation assay, however, has not gained full acceptance as a carcinogenic screening assay mainly due to issues regarding its relatively low sensitivity, low reproducibility, and relatively long test period (Schechtman, 1985; Sakai and Sato, 1989). The SHE cell assay, a diploid cell transformation assay, measures carcinogenic potential of xenobiotics by assessing transformed colonies based on morphological criterion. The SHE cell assay has several features that make it an appealing method to evaluate the carcinogenic potential of xenobiotics: (1) the assay has a >85% concordance with the two-year rodent bioassay; (2) the stages involved in the clonal transformation of SHE cells (e.g., morphological transformation, immortalization, and tumorigenicity) closely resemble those associated with the classic defined stages of carcinogenesis (e.g., initiation, promotion, and progression); and (3) non-genotoxic/non–DNA-reactive carcinogens elicit a positive response on morphological transformation in the SHE cell assay (Barrett and Lamb, 1985; LeBoeuf et al., 1990; Isfort et al., 1994, 1996). The SHE assay offers an alternative, regulatory approved, means to check the validity of the positive result before embarking on potentially unnecessary and expensive chronic or subchronic testing protocols.

Chronic Testing for Carcinogenicity

The majority of in vivo carcinogenicity testing is performed in rodent models. The administration of chemicals in the diet, often for extended periods, for assessment of their safety and/or toxicity began in the 1930s (Sasaki and Yoshida, 1935). Animal testing today remains a standard approach for determining the potential carcinogenic activity of xenobiotics. In addition to the lifetime exposure rodent models, organ-specific model systems, multistage models, and transgenic models are being developed and used in carcinogen testing (Table 8-23).

Chronic (Two Year) Bioassay Two-year studies in laboratory rodents remain the primary method by which chemicals or physical agents are identified as having the potential to be hazardous to humans. The most common rodents used are the rat and mouse. Typically the bioassays are conducted over the lifespan of the rodents (two years). Historically, selective rodent strains have been used in the chronic bioassay; however, each strain has both advantages and disadvantages. For example, the NTP typically uses Fisher 344 (F344) rats and B6C3F1 mice. The F344 rat has a high incidence of testicular tumors and leukemias whereas the B6C3F1 mouse is associated with a high background of liver tumors (Table 8-24).

In the chronic bioassay, two or three dose levels of a test chemical and a vehicle control are administered to 50 males and 50 females (mice and rats), beginning at 8 weeks of age, continuing throughout their lifespan. The route of administration can be via oral (gavage), dietary (mixed in feed), or inhalation (via inhalation chambers) exposure. Typically a number of short-term in vivo tests are conducted prior to the chronic bioassay to determine

Table 8-23

Animal Models of Neoplastic Development

	ENDPOINT	REFERENCES
Chronic 2-year bioassay	Tumors in all organs	Sontag (1977)
Tissue-specific bioassays		
Liver, mouse	Hepatomas	Carmichael et al. (1997)
Lung, mouse	Pulmonary adenomas	Shimkin and Stoner (1975)
Brain, rat	Gliomas	Kroh (1987)
Mammary gland, rat/mouse	Adenomas and carcinomas	Dunnick et al. (1995)
Medium-term bioassays		
Ito model	Hepatic adenomas and carcinomas	Ito et al. (1989)
Newborn mouse	Neoplasms in liver, lung, lymphoid organs	Fujii (1991)
Multistage models of neoplastic Development		
Bladder, rat	Papillomas/carcinomas	Hicks (1980)
Colon, rat	Aberrant crypt polyp	Sutherland and Bird (1994)
Epidermis, mouse	Papillomas	DiGiovanni (1992)
Liver, rat	Altered hepatic foci	Pitot (1997)
Transgenic mice		
Knockout of p53 tumor suppressor gene ($p53^{def}$)	Tumors in heterozygous animals having normal phenotype	Donehower (1996)
v-Ha-ras with zetaglobin promoter; tandem insertion on chromosome 11 (TG AC)	Induced transgene expression in skin leads to papilloma development	Spalding et al. (1993)

acute toxicity profiles, appropriate route of administration, and the maximum tolerated dose (MTD). MTD is defined for regulatory purposes as the highest daily dose of a chemical that does not cause overt toxicity in a 90-day study in laboratory mice or rats. Generally, the MTD is used to set the high dose in the chronic study. The use of the MTD as the upper dose level has been questioned by many investigators, as it is recognized that the doses selected represent those that are considered unrealistically high for human exposure. Pharmacokinetics and metabolism at high dose are frequently unrepresentative of those at lower doses; in addition, a general relationship between toxicity and carcinogenicity cannot be drawn for all classes of chemicals. During the study, food consumption and bodyweight gain should be monitored and the animals observed clinically on a regular basis; at necropsy, the tumor number, location, and diagnosis for each animal is thoroughly assessed.

Organ-Specific Bioassays and Multistage Animal Models Many tissue-specific bioassays have been developed with the underlying goal being to produce a sensitive and reliable assay that could be conducted in a time frame shorter in duration than the two-year chronic bioassay. These assays are commonly used to detect carcinogenic activity of chemicals in various target organs (Weisburger and Williams, 1984). Of the many models available, three models, the liver, skin, and lung models are more widely used.

Carcinogenicity Testing in the Liver The liver represents a major target organ for chemical carcinogens. It has been estimated that nearly half of the chemicals tested in the two-year chronic bioassay by the NTP showed an increased incidence of liver cancer. The rodent liver has been used as an animal model for carcinogenesis since the 1930s. Early pioneering work by Peraino and Pitot as well as Farber showed the multistaged process that occurs in the liver. The multistage nature of carcinogenesis is paralleled in the animal models; the system is characterized by well-defined changes including the formation of initiated cells by genotoxic chemicals that then progress to preneoplastic focal lesions, which subsequently convert into neoplasms. The use of preneoplastic lesions as endpoints in carcinogenicity testing may shorten experiment from two years to a few

Table 8-24

Spontaneous Tumor Incidence (Combined Benign and Malignant) in Selected Sites of the Two Species, B6C3F1 Mice and F344 Rats, Used in the NCI/NTP Bioassay

	B6C3F1 MICE		F344 RATS	
SITE	MALE	FEMALE	MALE	FEMALE
Liver				
Adenoma	10.3	4.0	3.4	3.0
Carcinoma	21.3	4.1	0.8	0.2
Pituitary	0.7	8.3	24.7	47.5
Adrenal	3.8	1.0	19.4	8.0
Thyroid	1.3	2.1	10.7	9.3
Hematopoietic	12.7	27.2	30.1	18.9
Mammary gland	0	1.9	2.5	26.1
Lung	17.1	7.5	2.4	1.2

months. Several rodent liver focus assays have been developed to assess the ability of a chemical to induce liver cancer and study the mechanisms involved in tumor development (Williams, 1982; Bannasch, 1986a, 1986b). Liver carcinogenesis assays have been developed to study and distinguish chemicals that affect the initiation or promotion stage of hepatocarcinogenesis. During the assay for initiating activity of a chemical, the test substance is given prior to exposure to a promoting chemical. Although a single initiating dose can result in the induction of focal lesions, exposure over a several week period is often used to increase the sensitivity of the model (Williams, 1982; Parnell et al., 1988). Phenobarbital is a commonly used tumor promoter; however, a wide range of chemicals have also been used as promoting agents (Solt and Farber, 1976; Oesterle and Deml, 1988). To assess the promoting activity of a chemical, the liver is initiated with a genotoxic chemical, often diethylnitrosamine. The test chemical is then administered for a duration of weeks to several months, and chemicals with promoting activity may stimulate the proliferation of initiated cells or may inhibit the proliferation of the surrounding putatively normal cells. The dose of the initiating carcinogen should represent a dose that will not induce liver tumors during the course of the experiment.

Another approach was developed in Japan by Ito and co-workers (Ogiso et al., 1990; Ito et al., 1994; Shirai et al., 1999). The "Ito assay" requires 8 weeks to perform. Rats are initiated with a single dose of diethylnitrosamine, followed by a 2-week recovery period, after which the rats are exposed to the test compound for 8 weeks. After 1 week of exposure to the test substance, the animals are given a two-third partial hepatectomy. The control group receives the same initiation and partial hepatectomy, but is not exposed to the test chemical. Hepatic focal lesions, while individually are clonal in nature, express a number of phenotypic alterations in various enzyme markers. The endpoint used in the Ito assay is the formation of liver lesions that stain for GST-P, a marker that stains many focal lesions in the rat. Using this assay, these investigators demonstrated a significant correlation between the results obtained using this assay and medium- and long-term study results (Ogiso et al., 1990). This group has also modified the original procedure to enable the detection of promoting chemicals. In this protocol, carcinogens are given over a 4-week period to initiate the formation of focal lesions, after which, test chemicals are administered for an additional 24- to 36-week period (Ito et al., 1997). In this manner, the ability of the test chemical to promote the growth of preneoplastic lesions can be assessed. The newborn mouse model originally described by Shubik and co-workers (Pietra et al., 1959) has also been used as a model for hepatocarcinogenesis (Vesselinovitch et al., 1985; Fujii, 1991). In this model, a single dose of diethylnitrosamine is administered to infant mice to initiate focal lesions. This step is then followed by exposure to test chemicals for several weeks to assess their potential to promote focal lesion development in the liver (Weghorst and Klaunig, 1989; Weghorst et al., 1989; Klaunig et al., 1990)

Identification of hepatic foci in H&E-stained sections is regarded as the most reliable approach for the diagnosis and quantification of preneoplastic liver lesions in rodents. Preneoplastic lesions are obligatory precursor lesions that can lead to liver tumors and will progress to benign and malignant liver cell tumors without further chemical exposure, and are used as endpoints in carcinogenicity testing (Pitot et al., 1987; Pereira and Herren-Freund, 1988; Ito et al., 1989; Maronpot et al., 1989). In addition to the sensitive detection of these preneoplastic lesions in conventional H&E staining, a number of histochemically detectable phenotypic alterations have been used for their quantification; however, these markers are only useful in the rat model, as focal lesions in mice do not exhibit these same phenotypic markers (Klaunig, 1993; Kolaja et al., 1995, 1996a, 1996b).

Carcinogenicity Testing in the Skin The mouse skin model is one of the most extensively studied and used models for understanding multistage carcinogenesis. This model of carcinogenesis is an assay that has been used to dissect mechanisms of carcinogenesis and is also purported to be a useful intermediate-term cancer bioassay. The skin was the target organ of the first experimental induction of chemical carcinogenesis (Yamagiwa and Ichikawa, 1915). The early studies by Friedwald and Rous (1944) and Berenblub and Shubik (1947) introduced the two-stage concept of carcinogenesis in the skin (Fig. 8-33). This model exploits many of the unique properties of mouse skin, one major being that the development of neoplasia can be followed visually. In addition, the number and relative size of papillomas and carcinomas can be quantified as the tumors progress.

Both initiating and promoting activities of chemical carcinogens can be assessed using this model. In the promotion assay, a number of chemical carcinogens have been used to initiate cells in mouse skin, including urethane, UV light, benzo(*a*)pyrene, and dimethylbenzanthracene, with the latter being of more common usage. The requirement for all initiating chemicals is to induce a genotoxic event that upon failure to repair DNA damage results in the formation of a mutated cell. Grossly, initiated cells of the skin appear identical to normal skin. Initiation in skin is frequently linked with the mutation of the *CHr* gene. Because the terminally differentiated cells in the skin are no longer capable of undergoing cell division, only initiated cells retain their proliferative capacity and thus represent the cell populations that give rise to tumors. To assess promotion by a chemical, an initiating chemical is applied first and is followed by the administration of a test substance for several weeks on the shaved skin of mice (Slaga, 1984). The promotion of initiated keratinocytes is commonly assessed using the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is routinely included as a positive control in this assay. The current hypothesis is that during the initiation stage the expansion or of initiated cells occurs as a result of inflammation and hyperplasia from either TPA or through mechanical wound healing mechanisms. Upon repeated application of tumor promoters, selective

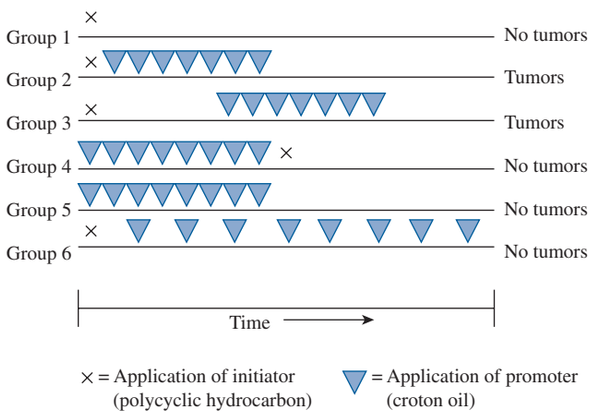


Figure 8-33. *Experiments demonstrating the initiation and promotion phases of carcinogenesis in mice. Group 2: application of promoter repeated at twice-weekly intervals for several months. Group 3: application of promoter delayed for several months and then applied twice weekly. Group 6: promoter applied at monthly intervals.*

clonal expansion of initiated keratinocytes occurs, resulting in skin papillomas, which over time can progress to carcinomas. In the standard two-stage protocol for mouse skin, malignant progression is relatively rare with approximately 5% of the papillomas progressing to the carcinoma stage. For the detection of initiating activity, the test substance is applied to skin prior to promotion with phorbol esters. Several mouse models are available, including hairless mice and SENCAR mice, both of which have enhanced sensitivity to the induction of skin cancer (Brown and Balmain, 1995; Sundberg et al., 1997).

Carcinogenicity Testing in the Lung Strain A mice are genetically susceptible to the development of lung tumors, with lung tumors being observed in control animals as early as 3 to 4 weeks of age with a steady increase to nearly 100% by 24 months of age (Shimkin and Stoner, 1975). Chemically induced tumors appear to be derived from hyperplastic lesions that progress to adenoma, carcinoma within adenoma, and ultimately to carcinomas (Stoner et al., 1993). In this model, carcinogenicity is typically assessed as an acceleration of tumor development rather than an increase in tumor incidence. The protocol currently used is that the chemical is administered for a period of 8 weeks, after which the animals remain on test for 4 additional months without chemical exposure. The strain A mouse lung tumor assay is sensitive to particular classes of chemicals, such as PAHs, nitrosamines, nitrosoureas, carbamates, aflatoxin, certain metals, and hydrazines (Stoner and Shimkin, 1985; Maronpot et al., 1986; Stoner, 1991).

Carcinogenicity Testing in Other Organs Test systems to examine the ability of a chemical to promote neoplastic development at organ sites other than liver, skin, and lung have also been developed. The available systems include animal models directed at examining carcinogenicity in the kidney, bladder, pancreas, stomach, colon, small intestine, and oral cavity. These models vary in the initiating carcinogen used, and frequency, duration, and site of application, as well as duration of promoting chemical exposure. Table 8-25 provides an overview of the animal models available for these target organs.

Transgenic Animals in Carcinogenicity Assessment

Due to the development of animal models with genetic alterations that invoke a susceptibility to carcinogenesis by chemicals, the use of transgenic and knockout animals in carcinogenicity assessment is gaining more popularity. The common models that have been used include the Tg.AC and rasH2 transgenic mice, and $p53^{+/-}$ and $XPA^{-/-}$ knockout mice (Gulezian et al., 2000; Pritchard et al., 2003; Boverhof, 2011; Eastmond et al., 2013). The feasibility of using transgenic animal models as an alternative to the two year chronic bioassay was evaluated in a review by the Health and Environmental Sciences Institute (HESI) branch of the International Life Sciences Institute (ILSI). In this assessment, 21 chemicals were evaluated, encompassing genotoxic, nongenotoxic, and noncarcinogenic chemicals. The conclusions drawn from the scientific review suggested that these models appear to have usefulness as screening models for assessment of chemical carcinogenicity; however, they do not provide definitive proof of potential human carcinogenicity. Further the scientific panel suggested that these models could be used in place of the mouse two-year bioassay (Blaauboer et al., 1998; Cohen et al., 2001). Coupled with information on genotoxicity, particularly DNA reactivity, structure–activity relationships, results from other bioassays, and the results of other mechanistic investigations including toxicokinetics, metabolism, and mechanistic information, these alternate mouse models for carcinogenicity appear to be useful models for assessing the carcinogenicity of chemicals. In general, replacement of the two-year rodent bioassay by transgenic mouse assay has not been as successful as with anticipated (Boverhof, 2011). However, as models for studying the roles of specific pathways and mechanisms in the carcinogenesis process, these models remain an excellent resource.

New Approaches A major concern for the correct evaluation of the safety of chemicals is the need to obtain reliable and pertinent scientific information on which to develop proper risk evaluation and assessment. Our current bioassays approaches have been criticized as being too time consuming and not pertinent to human health. Research during the last decade of the 20th century and the turn of the 21st century have resulted in a dramatic increase in our knowledge of the cellular and molecular pathways that contribute to the induction and prevention of cancer. This coupled with technological advances in high-throughput assays, and computational science has raised the question if the current approaches for carcinogenesis evaluation of chemicals (and mixtures) should be reevaluated. Following a NRC report in 2007 (Toxicity Testing in the 21st Century: A Vision and a Strategy), several approaches have begun utilizing high-throughput and computational approaches to evaluate the effects of chemicals on biological processes and pathways that are important in toxicity (including cancer). These approaches are directed to using cells, cell lines and components of cells, as well as in silico computer models. Significant progress has been made on several of these recently launched approaches including the USEPA Toxcast program, the NTP/NIEHS lead Tox 21 program (tox21 is a consortium of NIH, FDA, USEPA), the NIH-NCATS-FDA-sponsored microphysiological systems, and the EU sponsored SEURAT-1 (Knudsen et al., 2015). In particular, the last decade has witnessed a dynamic growth in the development of in silico methods and their application in predicting chemical toxicity including genotoxicity and carcinogenesis (examples can be found in Benfenati et al., 2009; Golbamaki and Benfenati, 2016; Raies and Bajic, 2016; and Frenzel et al., 2017).

CHEMICAL CARCINOGENESIS IN HUMANS

Recently, the IARC (Vol 100A-F) has reported a review of chemically induced cancer in humans. This body has identified and classified 120 agents as carcinogenic in humans. This review is divided into six major categories of carcinogens deemed to be human carcinogens by the IARC. These include pharmaceuticals; biological agents; metals, particles, and fibers; radiation; personal habits and household exposures; and chemical agents and related occupations. A number of factors have been implicated in the induction of cancer in humans. Infectious agents, lifestyle, medical treatments, environmental, and occupational exposure account either directly or indirectly to the majority of cancers seen in humans. Of these, the component that contributes the most to human cancer induction and progression is lifestyle: tobacco use, alcohol use, and poor diet (Table 8-26). Tobacco usage either through smoking tobacco, chewing tobacco, or tobacco snuff-type products is estimated be responsible for 25% to 40% of all human cancers. In particular, a strong correlation between tobacco usage and mouth, larynx, lung, esophageal, and bladder cancer exists. It has been estimated (Doll and Peto, 1981) that 85% to 90% of

Table 8-25

Target Organ Models for Studying Chemically Induced Carcinogenesis

ORGAN	SPECIES	INITIATING CARCINOGEN	INITIATOR DURATION	CARCINOGEN (PROMOTER) DURATION	REFERENCES
Kidney	Rat	*N*-Ethyl-*N*-hydroxyethylnitrosamine	Single exposure	20 weeks	Hiasa et al. (1991)
Bladder	Rat	*N*-Nitrosobutyl (4-hydroxybutyl)amine	4 weeks	32 weeks	Fukushima et al. (1983)
Pancreas	Rat, Syrian hamsters	*N*-nitrosobis(2-oxopropyl) amine, *N*-nitroso (2-hydroxypropyl) (2-oxopropyl)amine	Single exposure		Longnecker et al. (1984, 1985)
Stomach (forestomach)	Rat	Benzo(*a*)pyrene	1–2 times/week, 4 weeks	40 weeks	Silva et al. (1995)
Stomach (glandular)	Rat	*N*-Methyl-*N*′-nitro-*N*-nitronitrosoguanidine	Single exposure	40 weeks	Takahashi et al. (1986)
Colon	Rat	Azoxymethane	2 exposures	12 weeks	Yamashita et al. (1995)
Small intestine	Rat, mice	1,2-Dimethylhydrazine *N*-Ethyl-*N*′-nitro-*N*-nitrosoguanidine	2–9 weeks 4 weeks	16–20 weeks	Lindenschmidt et al. (1987), Jagadeesan et al. (1994)
Oral cavity (lip, oral, nasal)	Rat	4-Nitroquinoline *N*-oxide	4 weeks		Johansson et al. (1989)
Oral cavity (tongue)	Rat	4-Nitroquinoline *N*-oxide	8 weeks		Tanaka et al. (1998)
Buccal cells (squamous cell carcinoma)	Syrian hamster	—	—	Repeated application 10–16 weeks	Solt et al. (1987)
Buccal cells (squamous cell carcinoma)	Syrian hamster	Dimethylbenz[*a*]anthracene	Single exposure	45 weeks	Gimenez-Conti and Slaga (1993)

Table 8-26

Carcinogenic Factors Associated with Lifestyle

CHEMICAL(S)	NEOPLASM(S)
Alcohol beverage	Esophagus, liver, oropharynx, and larynx
Aflatoxins	Liver
Betel chewing	Mouth
Dietary intake (fat, protein, calories)	Breast, colon, endometrium, gallbladder
Tobacco smoking	Mouth, pharynx, larynx, lung, esophagus, bladder

all lung cancer cases in the United States are a direct result of tobacco use. The induction of pancreatic cancer also appears to have a linkage to tobacco use. Alcohol consumption also contributes anywhere from 2% to 4% of cancers of the esophagus, liver, and larynx.

Poor diets whether high-fat, low-protein, high-calories or diets lacking in needed antioxidants and minerals account for anywhere from 10% to 70% of human cancers. Diet contaminated by molds such as *Aspergillus flavis* (which produces aflatoxin B1) have been linked epidemiologically to a higher incidence of liver cancer. It also appears that aflatoxin B1 exposure coupled with hepatitis B virus infection produces an increased incidence of liver cancer compared to aflatoxin B1 or hepatitis B exposure individually. Mold-contaminated foodstuffs have also been shown to produce nitroso compounds.

There is substantial evidence that overnutrition either through excess calories and/or high-fat diets contribute to a number of human cancers (Doll and Peto, 1981). In particular, high-fat and high-calorie diets have been linked to breast, colon, and gall bladder and liver cancer in humans. Diets poor in antioxidants and/or vitamins such as vitamin A and vitamin E probably also contribute to the onset of cancer. The method of cooking may also influence the production of carcinogens produced in the cooking process. Carcinogenic heterocyclic amines (HCAs) and PAHs are formed during broiling and grilling of meat. Acrylamide, a suspected human carcinogen, has been found in fried foods at low concentrations.

Table 8-27

Occupational Human Carcinogens

AGENT	INDUSTRIAL PROCESS	NEOPLASMS
Asbestos	Construction, asbestos mining	Peritoneum, bronchus
Arsenic	Mining and smelting	Skin, bronchus, liver
Alkylating agents (mechlorethamine hydrochloride and bis[chloromethyl] ether)	Chemical manufacturing	Bronchus
Benzene	Chemical manufacturing	Bone marrow
Benzidine, β-naphthylamine	Dye and textile	Urinary bladder
Chromium and chromates	Tanning, pigment making	Nasal sinus, bronchus
Nickel	Nickel refining	Nasal sinus, bronchus
Polynuclear aromatic hydrocarbons	Steel making, roofing, chimney cleaning	Skin, scrotum, bronchus
Vinyl chloride monomer	Chemical manufacturing	Liver
Wood dust	Cabinet making	Nasal sinus
Beryllium	Aircraft manufacturing, electronics	Bronchus
Cadmium	Smelting	Bronchus
Ethylene oxide	Production of hospital supplies	Bone marrow
Formaldehyde	Plastic, textile, and chemical	Nasal sinus, bronchus
Polychlorinated biphenyls	Electrical-equipment production and maintenance	Liver

A number of occupations have been associated with the development of specific cancers (Table 8-27). As noted earlier, the linkage between chimney sweeps who as young boys in England were exposed to PAHs through the constant exposure to soot developed scrotal cancer. The linkage between occupational exposure to asbestos and the development of bronchiogenic carcinoma and as well as malignant mesothelioma has been clearly established. The appearance of bronchiogenic carcinoma was much higher in shipyard workers who were exposed to both asbestos and cigarette smoking. Muscat and Wynder (1995) noted no association between cigarette smoking and mesothelioma formation. Similarly, asbestos exposure by itself (without smoking) does not seem to increase the risk of bronchiogenic carcinoma. Aromatic amines used in the chemical and dye industries have been shown to produce or induce bladder cancer in humans. Prolonged high exposure to benzene in an occupational setting has been linked to the formation of acute myelogenous leukemia in humans.

Table 8-28

Human Carcinogenic Chemicals Associated with Medical Therapy and Diagnosis

CHEMICAL OR DRUG	ASSOCIATED NEOPLASMS
Alkylating agents (cyclophosphamide, melphalan)	Bladder, leukemia
Azathioprine	Lymphoma, reticulum cell sarcoma, skin, Kaposi sarcoma
Chloramphenicol	Leukemia
Diethylstilbestrol	Vagina (clear cell carcinoma)
Estrogens	Liver cell adenoma, endometrium, skin
Phenacetin	Renal pelvis (carcinoma)
Phenytoin	Lymphoma, neuroblastoma
Thorotrast	Liver (angiosarcoma)

A number of drugs and medical diagnostic tools have also been linked to the induction of human cancer (Table 8-28). Anticancer drugs such as the alkylating agent cyclophosphamide have been associated with bladder tumors and leukemia in patients receiving these treatments. The administration of the synthetic estrogenic compound diethylstilbestrol to pregnant women in order to improve embryo implantation and prevent spontaneous abortion has been shown to result in the formation of clear cell carcinomas of the vagina in the female offspring of mothers treated with diethylstilbestrol during pregnancy. The use of oral contraceptives containing synthetic estrogens as their major or only component has been implicated in the induction of liver cell adenomas. In addition, an association exists between prolonged use of estrogenic oral contraceptives and an increase incidence of premenopausal breast cancer. Androgenic steroids and synthetic testosterone compounds have been implicated in hepatocellular carcinoma induction. Therapeutic immunosuppression given to transplant patients or arising secondary to selective diseases such as AIDS result in an increase in a variety of different neoplasms. These results further support the role of the immune system in identifying and removing early preneoplastic cells from the body. In addition, the previously used diagnostic tracer Thorotrast has been sufficiently linked to the formation of hemangiosarcomas.

EVALUATION OF CARCINOGENICITY IN HUMANS

The assessment and designation of a chemical or a mixture of chemicals as carcinogenic in humans is evaluated by various agencies worldwide. The evaluation usually encompasses epidemiological, experimental animal, and in vitro data utilizing assays as described earlier in this chapter. One of the first schemes for the classification of an agent's carcinogenicity was devised by the IARC (Table 8-29). The IARC approach assigns the chemical or mixture to one of five groupings based upon strength of evidence

Table 8-29

IARC Classification of the Evaluation of Carcinogenicity for Human Beings

GROUP	EVIDENCE
1. Agent is carcinogenic to humans	Human data strong Animal data strong
2A. Agent is probably carcinogenic to humans	Human epidemiology data suggestive Animal data positive
2B. Agent is possibly carcinogenic to humans	Human epidemiology data weak Animal data positive
3. Agent is not classifiable as to carcinogenicity to humans	Human and animal data inadequate
4. Agent is probably not carcinogenic to humans	Human and animal data negative

for the chemical's possible, probable, or definite carcinogenicity to humans. In Group 1 classification, the chemical or mixture is classified as definitely carcinogenic to humans. The second grouping is Group 2A in which the chemical is probably carcinogenic to humans. In Group 2B, the chemical is classified as possibly carcinogenic to humans. Group 3, the agent is not classifiable. And in the last group, Group 4, the agent is not carcinogenic to humans. The IARC produces a series of monographs that describe the methodology for the evaluation of specific chemicals and the rationale for their final classification. Currently, 120 chemicals or mixtures or exposure circumstances have been classified by IARC as falling in Group 1, which shows sufficient evidence for carcinogenicity to humans.

Similar classifications exist for the USEPA, the Food & Drug Administration, and the European Community (EC). The classification of chemicals with regard to human carcinogenicity can many times be very difficult in particular, when animal data and/or epidemiological data in humans are inconclusive or confounded.

New USEPA Guidelines for Cancer Risk Assessment (2005) uses descriptors for defining the relative carcinogenic risk to humans (Table 8-30). These descriptors include carcinogenic to humans, likely to be carcinogenic to humans, suggestive evidence of carcinogenic potential, inadequate information to assess carcinogenic potential, and not likely to be carcinogenic in humans. The EPA Guidelines take into account the understanding of the mode of carcinogenic action and associated key precursor events needed for the cancer to form (Table 8-31). Central to the USEPA Guidelines for Cancer Risk Assessment is the utilization of the mode of action framework to define the key events in rodents, and assessment of whether those same key events and mode of action can occur in humans (Table 8-32). This approach is similar to that has been developed by the International Program on Chemical Safety and by panels in the International Life Sciences Institute. Similar to the mode of action analysis, adverse outcome pathway (AOP) approach was recently launched by OECD, executed in collaboration with USEPA, Joint Research Center of European Commission, the US Army Corps of Engineers, to support chemical risk assessment based on mechanistic reasoning. An AOP is a conceptual framework that describes existing knowledge (in vitro data, animal toxicity studies, and in silico computational systems) concerning the linkage

Table 8-30

USEPA Cancer Guidelines Descriptors

Carcinogenic to humans:
– Strong evidence of human carcinogenicity, including convincing epidemiological evidence of a causal association between human exposure and cancer
– The mode(s) of carcinogenic action and associated key precursor events have been identified in animals, and there is strong evidence that the key precursor events in animals are anticipated to occur in humans

Likely to be carcinogenic to humans:
– Weight of the evidence is adequate to demonstrate carcinogenic potential to an agent in animal experiments in more than one species, sex, strain, site, or exposure route, with or without evidence of carcinogenicity in humans

Suggestive evidence of carcinogenic potential:
– The weight of evidence is suggestive of carcinogenicity; a concern for potential carcinogenic effects in humans is raised, but the data are judged not sufficient for a stronger conclusion

Inadequate information to assess carcinogenic potential:
– Available data are judged inadequate for applying one of the other descriptors

Not likely to be carcinogenic to humans:
– This descriptor is appropriate when the available data are considered robust, there is no basis for human hazard concern, evidence in both humans and animals that the agent is not carcinogenic

Table 8-31

USEPA Mode of Action Definitions

Mode of action: Key events and processes, starting with the interaction of an agent with a cell, through functional and anatomical changes, resulting in cancer or other health endpoints

Key event: Empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element

Table 8-32

USEPA Mode of Action Framework

Mode of action criteria
Summary description of the hypothesized mode of action
Identification of key events
Strength, consistency, specificity of association
Dose–response concordance
Temporal relationship
Biological plausibility and coherence
Consideration of the possibility of other MOAs
Is the mode of action sufficiently supported in the test animals?
Is the mode of action relevant to humans?
Which populations or life stages can be particularly susceptible to the mode of action?

between a chemical interacting with a biological process, a direct molecular initiating event (MIE), and an adverse human or environmental health outcome (AO), by capturing the sequential chain of causally linked key events (KEs) at different levels of biological organization (Fig. 8-34). KEs are both measurable and essential for the AO, and the evidence supporting the causal linkages between individual KEs, termed Key Event Relationships (KERs), should be based on both biological plausibility and empirical data. A number of AOPs have been developed or currently under development that can be found on AOP Wiki (http://aopwiki.org/) as well as the AOP Knowledge Base (http://aopkb.org/).

The current listing of chemicals, occupations, or behaviors deemed to be carcinogenic in humans by IARC and the NTP are noted in Tables 8-33 to 8-34, respectively.

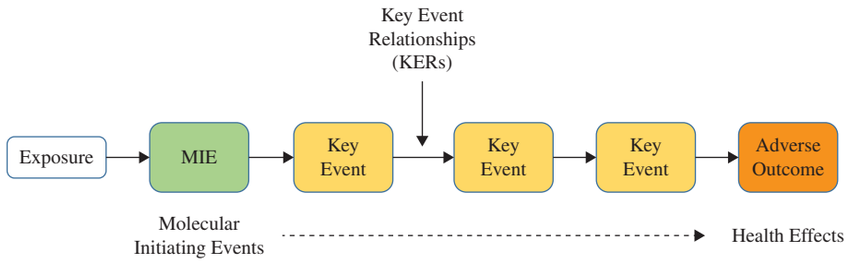


Figure 8-34. *Adverse outcome pathway.*

Table 8-33

IARC Known Human Carcinogens (Updated Until 2017)

AGENT	GROUP	VOLUME	YEAR
Formaldehyde	1	Sup 7, 62, 88, 100F	2012
Cyclophosphamide	1	26, Sup 7, 100A	2012
Benzo[*a*]pyrene	1	Sup 7, 92, 100F	2012
Thiotepa	1	Sup 7, 50, 100A	2012
Busulfan	1	4, Sup 7, 100A	2012
Diethylstilbestrol	1	21, Sup 7, 100A	2012
Lindane (see also Hexachlorocyclohexanes)	1	113	In prep.
Phenacetin	1	24, Sup 7, 100A	2012
Ethanol in alcoholic beverages	1	96, 100E	2012
Benzene	1	29, Sup 7, 100F	2012
Vinyl chloride	1	Sup 7, 97, 100F	2012
Acetaldehyde associated with consumption of alcoholic beverages	1	100E	2012
Ethylene oxide	1	Sup 7, 60, 97, 100F	2012
1,2-Dichloropropane	1	41, Sup 7, 71, 110	2016 online
Trichloroethylene	1	Sup 7, 63, 106	2014
Pentachlorophenol (see also Polychlorophenols)	1	53, 71, 117	In prep.
2-Naphthylamine	1	4, Sup 7, 99, 100F	2012
4-Aminobiphenyl	1	1, Sup 7, 99, 100F	2012
Benzidine	1	29, Sup 7, 99, 100F	2012
orth-Toluidine	1	Sup 7, 77, 99, 100F	2012
4,4'-Methylenebis(2-chloroaniline) (MOCA)	1	Sup 7, 57, 99, 100F	2012
1,3-Butadiene	1	Sup 7, 54, 71, 97, 100F	2012
Melphalan	1	9, Sup 7, 100A	2012

(*Continued*)

Table 8-33

IARC Known Human Carcinogens (Updated Until 2017) (Continued)

AGENT	GROUP	VOLUME	YEAR
Methoxsalen (8-methoxypsoralen) plus ultraviolet A radiation	1	24, Sup 7, 100A	2012
Treosulfan	1	26, Sup 7, 100A	2012
Chlorambucil	1	26, Sup 7, 100A	2012
Aristolochic acid	1	82, 100A	2012
Aristolochic acid, plants containing	1	82, 100A	2012
Azathioprine	1	26, Sup 7, 100A	2012
Chlornaphazine	1	4, Sup 7, 100A	2012
Sulfur mustard	1	9, Sup 7, 100F	2012
Bis(chloromethyl)ether; chloromethyl methyl ether (technical-grade)	1	4, Sup 7, 100F	2012
Asbestos (all forms, including actinolite, amosite, anthophyllite, chrysotile, crocidolite, tremolite)	1	14, Sup 7, 100C	2012
Polychlorinated biphenyls	1	18, Sup 7, 107	2016
Aflatoxins	1	56, 82, 100F, Sup 7	2012
2,3,7,8-Tetrachlorodibenzo-*para*-dioxin	1	Sup 7, 69, 100F	2012
Plutonium	1	78, 100D	2012
Thorium-232 and its decay products	1	78, 100D	2012
Arsenic and inorganic arsenic compounds	1	23, Sup 7, 100C	2012
Beryllium and beryllium compounds	1	Sup 7, 58, 100C	2012
Cadmium and cadmium compounds	1	58, 100C	2012
Coal-tar distillation	1	92, 100F	2012
Radon-222 and its decay products	1	43, 78, 100D	2012
Tamoxifen	1	66, 100A	2012
Radium-224 and its decay products	1	78, 100D	2012
Semustine [1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea, Methyl-CCNU]	1	Sup 7, 100A	2012
Radium-226 and its decay products	1	78, 100D	2012
Phosphorus-32, as phosphate	1	78, 100D	2012
Silica dust, crystalline, in the form of quartz or cristobalite	1	Sup 7, 68, 100C	2012
Radium-228 and its decay products	1	78, 100D	2012
N′-Nitrosonornicotine (NNN) and 4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)	1	Sup 7, 89, 100E	2012
Chromium (VI) compounds	1	Sup 7, 49, 100C	2012
Etoposide	1	76, 100A	2012
Etoposide in combination with cisplatin and bleomycin	1	76, 100A	2012
2,3,4,7,8-Pentachlorodibenzofuran	1	100F	2012
3,4,5,3′,4′-Pentachlorobiphenyl (PCB-126)	1	100F	2012
Cyclosporine	1	50, 100A	2012
Coal-tar pitch	1	35, Sup 7, 100F	2012
Erionite	1	42, Sup 7, 100C	2012
Shale oils	1	35, Sup 7, 100F	2012

Table 8-33

IARC Known Human Carcinogens (Updated Until 2017) (Continued)

AGENT	GROUP	VOLUME	YEAR
Acheson process, occupational exposure associated with	1	111	In prep.
Acid mists, strong inorganic	1	54, 100F	2012
Alcoholic beverages	1	44, 96, 100E	2012
Aluminum production	1	34, Sup 7, 92, 100F	2012
Areca nut	1	85, 100E	2012
Auramine production	1	Sup 7, 99, 100F	2012
Benzidine, dyes metabolized to	1	99, 100F	2012
Betel quid with tobacco	1	Sup 7, 85, 100E	2012
Betel quid without tobacco	1	Sup 7, 85, 100E	2012
Clonorchis sinensis (infection with)	1	61, 100B	2012
Coal gasification	1	Sup 7, 92, 100F	2012
Coal, indoor emissions from household combustion of	1	95, 100E	2012
Coke production	1	Sup 7, 92, 100F	2012
Engine exhaust, diesel	1	46, 105	2013
Epstein–Barr virus	1	70, 100B	2012
Estrogen therapy, postmenopausal	1	72, 100A	2012
Estrogen–progestogen menopausal therapy (combined)	1	72, 91, 100A	2012
Estrogen-progestogen oral contraceptives (combined)	1	72, 91, 100A	2012
Fission products, including strontium-90	1	100D	2012
Fluoro-edenite fibrous amphibole	1	111	In prep.
Haematite mining (underground)	1	1, Sup 7, 100D	2012
Helicobacter pylori (infection with)	1	61, 100B	2012
Hepatitis B virus (chronic infection with)	1	59, 100B	2012
Hepatitis C virus (chronic infection with)	1	59, 100B	2012
Human immunodeficiency virus type 1 (infection with)	1	67, 100B	2012
Human papillomavirus types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	1	64, 90, 100B	2012
Human T-cell lymphotropic virus type I	1	67, 100B	2012
Ionizing radiation (all types)	1	100D	2012
Iron and steel founding (occupational exposure during)	1	34, Sup 7, 100F	2012
Isopropyl alcohol manufacture using strong acids	1	Sup 7, 100F	2012
Kaposi sarcoma herpesvirus	1	70, 100B	2012
Leather dust	1	100C	2012
Magenta production	1	Sup 7, 57, 99, 100F	2012
Mineral oils, untreated or mildly treated	1	33, Sup 7, 100F	2012
MOPP and other combined chemotherapy including alkylating agents	1	Sup 7, 100A	2012
Neutron radiation	1	75, 100D	2012
Nickel compounds	1	Sup 7, 49, 100C	2012
Opisthorchis viverrini (infection with)	1	61, 100B	2012

(*Continued*)

Table 8-33

IARC Known Human Carcinogens (Updated Until 2017) (Continued)

AGENT	GROUP	VOLUME	YEAR
Outdoor air pollution	1	109	2016
Outdoor air pollution, particulate matter in	1	109	2016
Painter (occupational exposure as a)	1	47, 98, 100F	2012
Phenacetin, analgesic mixtures containing	1	Sup 7, 100A	2012
Polychlorinated biphenyls, dioxin-like	1	107	2016
Processed meat (consumption of)	1	114	In prep.
Radioiodines, including iodine-131	1	78, 100D	2012
Radionuclides, alpha-particle-emitting, internally deposited	1	78, 100D	2012
Radionuclides, beta-particle-emitting, internally deposited	1	78, 100D	2012
Rubber manufacturing industry	1	28, Sup 7, 100F	2012
Salted fish, Chinese-style	1	56, 100E	2012
Schistosoma haematobium (infection with)	1	61, 100B	2012
Solar radiation	1	55, 100D	2012
Soot (as found in occupational exposure of chimney sweeps)	1	35, Sup 7, 92, 100F	2012
Tobacco smoke, second-hand	1	83, 100E	2012
Tobacco smoking	1	83, 100E	2012
Tobacco, smokeless	1	Sup 7, 89, 100E	2012
Ultraviolet radiation (wavelengths 100–400 nm, encompassing UVA, UVB, and UVC)	1	55, 100D*, 118#	In prep.
Ultraviolet-emitting tanning devices	1	100D	2012
Welding fumes	1	49, 100D*, 118#	In prep.
Wood dust	1	62, 100C	2012
X- and gamma-radiation	1	75, 100D	2012

Refer to IARC Web site for latest information: http://monographs.iarc.fr/ENG/Classification/latest_classif.php.

Table 8-34

NTP Known to Be Human Carcinogens (14th RoC)

CASRN	NAME OR SYNONYM	LISTING IN THE 14TH ROC
1402-68-2	Aflatoxins	*Known*
NA	Alcoholic Beverage Consumption	*Known*
92-67-1	4-Aminobiphenyl	*Known*
NA	Analgesic Mixtures Containing Phenacetin (*see* Phenacetin and Analgesic Mixtures containing Phenacetin)	*Known*
NA	Aristolochic Acids	*Known*
7440-38-2	Arsenic (*see* Arsenic and Inorganic Arsenic Compounds)	*Known*
NA	Arsenic Compounds, Inorganic	*Known*
1332-21-4	Asbestos	*Known*
446-86-6	Azathioprine	*Known*
71-43-2	Benzene	*Known*
92-87-5	Benzidine (*see* Benzidine and Dyes Metabolized to Benzidine)	*Known*

Table 8-34

NTP Known to Be Human Carcinogens (14th RoC) (Continued)

CASRN	NAME OR SYNONYM	LISTING IN THE 14TH ROC
7440-41-7	Beryllium (*see* Beryllium and Beryllium Compounds)	*Known*
NA	Beryllium Compounds (*see* Beryllium and Beryllium Compounds)	*Known*
542-88-1	Bis(chloromethyl) Ether (*see* Bis(chloromethyl) Ether and Technical-Grade Chloromethyl Methyl Ether)	*Known*
106-99-0	1,3-Butadiene	*Known*
55-98-1	1,4-Butanediol Dimethanesulfonate	*Known*
7440-43-9	Cadmium (*see* Cadmium and Cadmium Compounds)	*Known*
NA	Cadmium Compounds (*see* Cadmium and Cadmium Compounds)	*Known*
305-03-3	Chlorambucil	*Known*
13909-09-6	1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (*see* Nitrosourea Chemotherapeutic Agents)	*Known*
107-30-2	Chloromethyl Methyl Ether (*see* Bis(chloromethyl) Ether and Technical-Grade Chloromethyl Methyl Ether)	*Known*
18540-29-9	Chromium (VI) (*see* Chromium Hexavalent Compounds)	*Known*
8007-45-2	Coal Tar (*see* Coal Tars and Coal-Tar Pitches)	*Known*
NA	Coal-Tar Pitches (*see* Coal Tars and Coal-Tar Pitches)	*Known*
NA	Coke Oven Emissions	*Known*
50-18-0	Cyclophosphamide	*Known*
59865-13-3	Cyclosporin A	*Known*
56-53-1	Diethylstilbestrol	*Known*
NA	Dyes Metabolized to Benzidine (*see* Benzidine and Dyes Metabolized to Benzidine)	*Known*
NA	Environmental Tobacco Smoke (*see* Tobacco-Related Exposures)	*Known*
NA	Epstein–Barr Virus	*Known*
66733-21-9	Erionite	*Known*
NA	Estrogens, Steroidal	*Known*
75-21-8	Ethylene Oxide	*Known*
50-00-0	Formaldehyde	*Known*
NA	Hepatitis B Virus	*Known*
NA	Hepatitis C Virus	*Known*
NA	Human Immunodeficiency Virus Type 1	*Known*
NA	Human Papillomaviruses: Some Genital Mucosal Type	*Known*
NA	Human T-Cell Lymphotropic Virus Type 1	*Known*
NA	Kaposi Sarcoma-Associated Herpesvirus	*Known*
148-82-3	Melphalan	*Known*
NA	Merkel Cell Polyomavirus	*Known*
298-81-7	Methoxsalen (*see* Methoxsalen with Ultraviolet A Therapy)	*Known*
NA	Methoxsalen with Ultraviolet A Therapy	*Known*
NA	Mineral Oils: Untreated and Mildly Treated	*Known*
505-60-2	Mustard Gas	*Known*
91-59-8	2-Naphthylamine	*Known*
NA	Neutrons (*see* Ionizing Radiation)	*Known*
NA	Nickel Compounds (*see* Nickel Compounds and Metallic Nickel)	*Known*
95-53-4	*o*-Toluidine (*see o*-Toluidine and Its Hydrochloride)	*Known*
10043-92-2	Radon (*see* Ionizing Radiation)	*Known*

Table 8-34

NTP Known to Be Human Carcinogens (14th RoC) (Continued)

CASRN	NAME OR SYNONYM	LISTING IN THE 14TH ROC
NA	Silica, Crystalline (Respirable Size)	*Known*
NA	Smokeless Tobacco (*see* Tobacco-Related Exposures)	*Known*
NA	Solar Radiation (*see* Ultraviolet-Radiation-Related Exposures)	*Known*
NA	Soots	*Known*
NA	Strong Inorganic Acid Mists Containing Sulfuric Acid	*Known*
7664-93-9	Sulfuric Acid (*see* Strong Inorganic Acid Mists Containing Sulfuric Acid)	*Known*
NA	Sunlamps and Sunbeds, Exposure to (*see* Ultraviolet-Radiation-Related Exposures)	*Known*
10540-29-1	Tamoxifen	*Known*
1746-01-6	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	*Known*
52-24-4	Thiotepa	*Known*
1314-20-1	Thorium Dioxide (*see* Ionizing Radiation)	*Known*
NA	Tobacco Smoking (*see* Tobacco-Related Exposures)	*Known*
79-01-6	Trichloroethylene	*Known*
NA	UV Radiation, Broad Spectrum (*see* Ultraviolet-Radiation-Related Exposures)	*Known*
75-01-4	Vinyl Chloride (*see* Vinyl Halides [Selected])	*Known*
NA	Wood Dust	*Known*
NA	X-Radiation and Gamma Radiation (*see* Ionizing Radiation)	*Known*

Refer to the NTP Web site for more detailed information: https://ntp.niehs.nih.gov/pubhealth/roc/index-1.html.

SUMMARY

The induction of cancer by chemicals is well established in animal models as well as in humans. Linkages between chemicals found in human lifestyle, occupational exposure, and environmental exposure provides strong evidence for the induction or contribution to environmental occupational lifestyle carcinogens to human cancer. Cancer is a multistage process in its most reductive form involves initial mutational events followed by changes in gene expression leading to the selected clonal proliferation of the precancerous cell. Neoplasia appears to exhibit multiple characteristics including increase selective lesion growth (through sustained cell proliferation and /or resistance to apoptosis), the induction of angiogenesis, enabling replicative immortality, activation of factors that influence invasion and metastasis, evasion of normal growth suppression, modulation of energy metabolism, and the avoidance of attack by the immune system. The multistage nature and characteristics of the process have been extensively examined with regard to molecular, cellular, tissue, and organ events.

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REFERENCES

Abanobi SE, Lombardi B, Shinozuka H. Stimulation of DNA synthesis and cell proliferation in the liver of rats fed a choline-devoid diet and their suppression by phenobarbital. *Cancer Res*. 1982;41:412–415.

Abid Z, Cross AJ, Sinha R. Meat, dairy, and cancer. *Am J Clin Nutr*. 2014;100(suppl 1):386S–393SS.

Abuja PM, Albertini R. Methods for monitoring oxidative stress, lipid peroxidation, and oxidation resistance of lipoproteins. *Clin Chim Acta*. 2001;306:1–17.

Alitalo K, Schwab M. Oncogene amplification in tumor cells. *Adv Cancer Res*. 1986;47:235–281.

Alwens W, Jonas W. Chrome lung cancer. *Acta Unio Internationalis contra Cancrum*. 1938;3:1–114.

Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. *Mutat Res*. 1975;31:347–364.

Ames BN, Shigenaga MK. Oxidants are a major contributor to cancer and aging. In: Halliwell B, Aruoma O, eds. *DNA and Free Radicals*. London: Ellis Horwood Ltd; 1993:1–15.

Amstad PA, Krupitza G, Cerutti PA. Mechanisms of c-fos induction by active oxygen. *Cancer Res*. 1992;52:3952–3960.

Andersen A, Berge SR, Engeland A, Norseth T. Exposure to nickel compounds and smoking in relation to incidence of lung and nasal cancer among nickel refinery workers. *Occup Environ Med*. 1996;53(10):708–713.

Andersen M, Brusick D, Cohen S, et al. U.S. Environmental Protection Agency's revised cancer guidelines for carcinogen risk assessment. *Toxicol Appl Pharmacol*. 1998;153:133–136.

Ates NA, Tamer L, Ates C, et al. Glutathione S-transferase M1, T1, P1 genotypes and risk for development of colorectal cancer. *Biochem Genet*. 2005;43(3–4):149–163.

Baccarelli A, Bollati V. Epigenetics and environmental chemicals. *Curr Opin Pediatr*. 2009;21(2):243–251.

Bachman AN, Kamendulis LM, Goodman JI. Diethanolamine and phenobarbital produce an altered pattern of methylation in GC-rich regions of DNA in B6C3F1 mouse hepatocytes similar to that resulting from choline deficiency. *Toxicol Sci*. 2006;90:317–325.

Bailey EA, Iyer RS, Stone MP, Harris TM, Essigmann JM. Mutational properties of the primary aflatoxin B1-DNA adduct. *Proc Natl Acad Sci U S A*. 1996;93(4):1535–1539.

Balmain A, Pragnell IB. Mouse skin carcinomas induced in vivo by chemical carcinogens have a transforming Harvey-ras oncogene. *Nature*. 1983;303:72–74.

Bannasch P. Preneoplastic lesions as end points in carcinogenicity testing I. Hepatic preneoplasia. *Carcinogenesis*. 1986a;7(5):689–695.

Bannasch P. Preneoplastic lesions as end points in carcinogenicity testing. II. Preneoplasia in various non-hepatic tissues. *Carcinogenesis*. 1986b;7(6):849–852.

Barber DA, Harris SR. Oxygen free radicals and antioxidants: a review. *Am Pharm*. 1994;NS34:26–35.

Barrett JC, Lamb PW. Tests with the Syrian hamster embryo cell transformation assay. In: Ashbey J, DeSerres FJ, Draper M, eds. *Progress in Mutation Research-Evaluation of Short Term Tests for Carcinogens*. Vol. 5. New York: Elsevier; 1985:623–628.

Bateman AJ. The dominant lethal assay in the mouse. *Agents Actions*. 1973;3(2):73–76.

Beauerle PA, Lenardo M, Pierce JW, Baltimore D. Phorbol-ester-induced activation of the NFkB transcription factor involved dissociation of an apparently cytoplasmic Nf-$_{K}$B/Inhibitor complex. *Cold Springs Harb Symp Quant Biol*. 1988;53:789–798.

Becker RA, Shank RC. Kinetics of formation and persistence of ethylguanines in DNA of rats and hamsters treated with diethylnitrosamine. *Cancer Res*. 1985;45:2076–2084.

Beddowes EJ, Faux SP, Chipman JK. Chloroform, carbon tetrachloride and glutathione depletion induce secondary genotoxicity in liver cells via oxidative stress. *Toxicology*. 2003 May 3;187(2–3):101–115.

Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci U S A*. 1998;95:11891–11896.

Benfenati E, Benigni R, Demarini DM, et al. Predictive models for carcinogenicity and mutagenicity: frameworks, state-of-the-art, and perspectives. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2009;27(2):57–90.

Bentley P, Calder I, Elcombe C, Grasso P, Stringer D, Wiegand HJ. Hepatic peroxisome proliferation in rodents and its significance for humans. *Food Chem Toxicol*. 1993;31:857–907.

Berenblub J, Shubik P. A new quantitative approach to the study of the stages of chemical carcinogenesis in the mouse's skin. *Br J Cancer*. 1947;1:389–391.

Betteridge DJ. What is oxidative stress? *Metabolism*. 2000;49:3–8.

Billups K, Neal J, Salyer J. Immunosuppressant-driven de novo malignant neoplasms after solid-organ transplant. *Prog Transplant*. 2015;25(2):182–188.

Blaauboer BJ, Balls M, Barratt M, et al. 13th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): alternative testing methodologies and conceptual issues. *Environ Health Perspect*. 1998;106(suppl 2):413–418.

Boccuni P, Del Vecchio L, Di Noto R, Rotoli B. Glycosyl phosphatidylinositol (GPI)-anchored molecules and the pathogenesis of paroxysmal nocturnal hemoglobinuria. *Crit Rev Oncol Hematol*. 2000;33(1):25–43.

Boddy AV, Ratain MJ. Pharmacogenetics in cancer etiology and chemotherapy. *Clin Cancer Res*. 1997;3:1025–1030.

Boffetta P, Fordyce TA, Mandel JS. A mortality study of beryllium workers. *Cancer Med*. 2016;5(12):3596–3605.

Boffetta P, Fryzek JP, Mandel JS. Occupational exposure to beryllium and cancer risk: a review of the epidemiologic evidence. *Crit Rev Toxicol*. 2012;42(2):107–118.

Bohr VA, Dianov GL. Oxidative DNA damage processing in nuclear and mitochondrial DNA. *Biochimie*. 1999;81:155–160.

Boobis AR, Cohen SM, Dellarco V, et al. IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit Rev Toxicol*. 2006;36(10):781–792.

Bos JL. Ras oncogene in human cancer: a review. *Cancer Res*. 1989;49:4682–4689.

Bosch TM, Meijerman I, Beijnen JH, Schellens JHM. Genetic polymorphisms of drug metabolising enzymes and drug transporters in relation to cancer risk. *Curr Ther Rev*. 2006;2(2):137–155.

Bosetti C, La Vecchia C, Lipworth L, McLaughlin JK. Occupational exposure to vinyl chloride and cancer risk: a review of the epidemiologic literature. *Eur J Cancer Prev*. 2003;12(5):427–430.

Bosland MC. The role of estrogens in prostate carcinogenesis: a rationale for chemoprevention. *Rev Urol*. 2005;7(suppl 3):S4–S10.

Boverhof DR. Transgenic animal models in toxicology: historical perspectives and future outlook. *Toxicol Sci*. 2011;121:207–233.

Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol*. 2008;9(4):297–308.

Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature*. 1991;350:429–431.

Brown JR, Nigh E, Lee RJ, et al. Fos family members induce cell cycle entry by activating Cyclin D1. *Mol Cell Biol*. 1998;18:55609–55619.

Brown K, Balmain A. Transgenic mice and squamous multistage skin carcinogenesis. *Cancer Metastasis Rev*. 1995;14:113–124.

Bull RJ, Brown JM, Meierhenry EA, Jorgenson TA, Robinson M, Stober JA. Enhancement of the hepatotoxicity of chloroform in B6C3F1 mice by corn oil: implications for chloroform carcinogenesis. *Environ Health Perspect*. 1986;69:49–58.

Budinsky RA, Schrenk D, Simon T, et al. Mode of action and dose-response framework analysis for receptor-mediated toxicity: the aryl hydrocarbon receptor as a case study. *Crit Rev Toxicol*. 2014;44(1):83–119.

Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic Biol Med*. 1995;18:775–794.

Burgess WH, Maciag T, Jerome H. The heparin-binding (fibroblast) growth factor family of proteins. *Annu Rev Biochem*. 1989;58:575–606.

Burkhardt S, Mellert W, Reinacher M, Bahnenmann R. Zonal evaluation of proliferation and apoptosis in mice reveals new mechanistic data for PB, WY 14,643 and CH. *Toxicologist*. 2001;60:286.

Butlin HJ. Three lectures on cancer of the scrotum in chimney sweeps and others. I. Secondary cancer without primary cancer. II. Why foreign sweeps do not suffer from scrotal cancer. III. Tar and paraffin cancer. *Br Med J*. 1892;1:1341–1346, 2:1–6, 66–71.

Butterworth BE. Consideration of both genotoxic and nongenotoxic mechanisms in predicting carcinogenic potential. *Mutat Res*. 1990;239:117–132.

Calabrese EJ. Hormesis: changing view of the dose–response, a personal account of the history and current status. *Mutat Res*. 2002;511:181–189.

Camurri L, Codeluppi S, Pedroni C, Scarduelli L. Chromosomal aberrations and sister-chromatid exchanges in workers exposed to styrene. *Mutat Res*. 1983;119:361–369.

Cantor KP. Drinking water and cancer. *Cancer Causes Control*. 1997;8(3):292–308.

Capen CC, Dayan AD, Green S. Receptor-mediated mechanisms in carcinogenesis: an overview. *Mutat Res*. 1995;333(1–2):215–224.

Carmichael NG, Enzmann H, Pate I, Waechter F. The significance of mouse liver tumor formation for carcinogenic risk assessment: results and conclusions from a survey of ten years of testing by the agrochemical industry. *Environ Health Perspect*. 1997;105(11):1196–1203.

Carthew P, Martin EA, White IN, et al. Tamoxifen induces short-term cumulative DNA damage and liver tumors in rats: promotion by phenobarbital. *Cancer Res*. 1995;55(3):544–547.

Cavalli LR, Liang BD. Mutagenesis, tumorigenicity, and apoptosis: are the mitochondria involved? *Mutat Res*. 1998;398:19–26.

Cerutti PA. Prooxidant states and tumor promotion. *Science*. 1985;227:375–381.

Chandar N, Lombardi B. Liver cell proliferation and incidence of hepatocellular carcinomas in rats fed consecutively a choline-devoid and a choline supplemented diet. *Carcinogenesis*. 1988;9(2):259–263.

Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature*. 2001;410:37–40.

Chappell G, Pogribny IP, Guyton KZ, Rusyn I. Epigenetic alterations induced by genotoxic occupational and environmental human chemical carcinogens: a systematic literature review. *Mutat Res Rev Mutat Res*. 2016;768:27–45.

Chatterjee A, Mambo E, Sidransky D. Mitochondrial DNA mutations in human cancer. *Oncogene*. 2006;25(34):4663–4674.

Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR. The E2F transcription factor is a cellular target for the RB protein. *Cell*. 1991;65:1053–1061.

Chen C, Xun P, Nishijo M, Sekikawa A, He K. Cadmium exposure and risk of pancreatic cancer: a meta-analysis of prospective cohort studies and case-control studies among individuals without occupational exposure history. *Environ Sci Pollut Res Int*. 2015;22(22):17465–17474.

Chen CJ, Wu MM, Lee SS, et al. Atherogenicity and carcinogenicity of high-arsenic artesian well water. Multiple risk factors and related malignant neoplasms of blackfoot disease. *Arteriosclerosis*. 1988;8:452–460.

Chen T. The role of MicroRNA in chemical carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2010;28(2):89–124.

Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanosine, an abundant form of oxidative DNA damage, causes G → T and A → C substitutions. *J Biol Chem*. 1992;267:166–172.

Chiu R, Boyle J, Meek J, et al. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell*. 1988;54: 541–552.

Cho YA, Kim J, Woo HD, Kang M. Dietary cadmium intake and the risk of cancer: a meta-analysis. *PLoS One*. 2013;8(9):e75087.

Christopherson W, Mays E, Barrows G. Hepatocellular carcinoma in young women on oral contraceptives. *Lancet*. 1978;2:38.

Chung KT. Azo dyes and human health: a review. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2016;34(4):233–261.

Clarkson PM, Thompson HS. Antioxidants: what role do they play in physical activity and health? *Am J Clin Nutr*. 2000;72:637–646.

Claxton LD, Umbuzeiro GdeA, DeMarini DM. The Salmonella mutagenicity assay: the stethoscope of genetic toxicology for the 21st century. *Environ Health Perspect*. 2010;118:1515–1522.

Cohen SM, Meek ME, Klaunig JE, Patton DE, Fenner-Crisp PA. The human relevance of information on carcinogenic modes of action: overview. *Crit Rev Toxicol*. 2003;33(6):581–589.

Cohen SM, Robinson D, MacDonald J. Alternative models for carcinogenicity testing. *Toxicol Sci*. 2001;64:14–19.

Collett MS, Erikson RL. Protein kinase activity associated with the avian sarcoma virus src gene product. *Proc Natl Acad Sci USA*. 1978;75:2021–2024.

Collins AR. The Comet Assay for DNA damage and repair: principals, applications, and limitations. *Mol Biotech*. 2004;26:249–261.

Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*. 2009;30(7):1073–1081.

Conney AH. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons. *Cancer Res*. 1982;42:4875–4917.

Cook JW, Hewett CL, Hieger I. The isolation of a cancer-producing hydrocarbon from coal tar. *J Chem Soc*. 1933;24:395–405.

Corthay A. Does the immune system naturally protect against cancer? *Front Immunol*. 2014;5:197.

Corton JC, Cunningham ML, Hummer BT, et al. Mode of action framework analysis for receptor-mediated toxicity: the peroxisome proliferator-activated receptor alpha (PPARalpha) as a case study. *Crit Rev Toxicol*. 2014;44(1):1–49.

Costa M. Toxicity and carcinogenicity of Cr(VI) in animal models and humans. *Crit Rev Toxicol*. 1997;27(5):431–442.

Counts JL, Goodman JI. Alterations in DNA methylation may play a variety of roles in carcinogenesis. *Cell*. 1995;83:13–15.

Creech HJ. A brief history of the American Association for Cancer Research, Inc.: 1907–1995. In: *Directory of the American Association for Cancer Research*. Philadelphia, PA: American Association for Cancer Research; 2000:viii–xi.

Creton S, Aardema MJ, Carmichael PL, et al. Cell transformation assays for prediction of carcinogenic potential: state of the science and future research needs. *Mutagenesis*. 2012;27(1):93–101.

Croce CM. Molecular origins of cancer: oncogenes and cancer. *N Engl J Med*. 2008;358:502–511.

Cross AJ, Sinha R. Formation and biochemistry of carcinogenic heterocyclic aromatic amines in cooked meats and meat-related mutagens/carcinogens in the etiology of colorectal cancer. *Environ Mol Mutagen*. 2004;44(1):44–55.

D'Souza RJ, Philips EM, Jones PW, Strange RC, Aber GM. Interactions of hydrogen peroxide with interleukin-6 and platelet-derived growth factor in determining mesangial cell growth: effect of repeated oxidant stress. *Clin Sci*. 1993;86:747–751.

Damm K, Thompson CC, Evans RM. Proteinencoded by v-erbA functions as a thyroid-hormone receptor antagonist. *Nature*. 1989;339: 593–597.

de Boer JG, Mirsalis JC, Provost GS, Tindall KR, Glickman BW. Spectrum of mutations in kidney, stomach, and liver from lacI transgenic mice recovered after treatment with tris(2,3-dibromopropyl)phosphate. *Environ Mol Mutagen*. 1996;28(4):418–423.

de Vos AM, Tong L, Milburn MV, et al. Three dimensional structure of an oncogene protein: catalytic domain of human C-H-ras p21. *Science*. 1988;239:888–893.

Demple B, Harrison L. Repair of oxidative damage to DNA. *Annu Rev Biochem*. 1994;63:915–948.

de-Thé G, Geser A, Day NE, et al. Epidemionological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. *Nature*. 1978;274:756–761.

Dertinger SD, Phonethepswath S, Weller P, et al. International Pig-a gene mutation assay trial: evaluation of transferability across 14 laboratories. *Environ Mol Mutagen*. 2011;52(9):690–698.

Diamandopoulos GT. Cancer: an historical perspective. *Anticancer Res*. 1996;16(4A):1595–1602.

Dietrich DR, Swenberg JA. Preneoplastic lesions in rodent kidney induced spontaneously or by non-genotoxic agents: predictive nature and comparison to lesions induced by genotoxic carcinogens. *Mutat Res*. 1991;248:239–260.

DiGiovanni J. Multistage carcinogenesis in mouse skin. *Pharmacol Ther*. 1992;54(1):63–128.

Diwan BA, Kasprzak KS, Rice JM. Transplacental carcinogenic effects of nickel(II) acetate in the renal cortex, renal pelvis and adenohypophysis in F344/NCr rats. *Carcinogenesis*. 1992;13(8):1351–1357.

Dizdaroglu M. Oxidative damage to DNA in mammalian chromatin. *Mutat Res*. 1992;275:331–342.

Doll R. Effects of exposure to vinyl chloride. An assessment of evidence. *Scand J Work Environ Health*. 1988;14:61–78.

Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst*. 1981;66(6):1191–1308.

Doll R, Peto R, Boreham J, Sutherland I. Mortality in relation to smoking: 50 years' observations on male British doctors. *BMJ*. 2004;328(7455):1519.

Donehower LA. The p53-deficient mouse: a model for basic and applied cancer studies. *Semin Cancer Biol*. 1996;7(5):269–278.

Dong J-T. Prevalent mutations in prostate cancer. *J Cell Biochem*. 2005;97(3):433–447.

Dooliter RF, Hunkapiller MW, Hood LE, et al. Simian sarcoma virus oncogene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science*. 1983;221:275–277.

Dunnick JK, Elwell MR, Huff J, Barrett JC. Chemically induced mammary gland cancer in the National Toxicology Program's carcinogenesis bioassay. *Carcinogenesis*. 1995;16(2):173–179.

Dypbukt JM, Ankarcrona M, Burkitt M, et al. Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. *J Biol Chem*. 1994;269:30533–30560.

Eastmond DA, Hartwig A, Anderson D, et al. Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme. *Mutagenesis*. 2009;24(4):341–349.

Eastmond DA, Vulimiri SV, French JE, Sonawane B. The use of genetically modified mice in cancer risk assessment: Challenges and limitations. *Crit Rev Toxicol*. 2013;43(8):611–631.

Edmondson H, Reynolds T, Henderson B, Benton B. Regression of liver cell adenomas associated with oral contraceptives. *Ann Intern Med*. 1977;86:180–182.

Ehrenberg L, Hussain S. Genetic toxicity of some important epoxides. *Mutat Res*. 1981;86:1–113.

Elcombe CR, Peffer RC, Wolf DC, et al. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: a case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. *Crit Rev Toxicol*. 2014;44(1):64–82.

Eksrom G, Ingleman-Sundberg M. Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol inducible cytochromes P-450. *Biochem Pharmacol*. 1989;38: 1313–1319.

El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. *Nat Genet*. 1992;1:45–49.
EMA. European Medicines Agency. Note for ICH guideline S2(R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use (EMA/CHMP/ICH/126642/2008) (June 2012). 2008.
Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6(4):259–269.
Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res*. 2001;61:3225–3229.
Fahreus R, Rymo L, Rhim JS, et al. Morphological transformation of human keratinocytes expressing the LMP gene of Epstein-Barr virus. *Nature*. 1990;345:447–449.
Ferreccio C, González C, Milosavjlevic V, Marshall G, Sancha AM, Smith AH. Lung cancer and arsenic concentrations in drinking water in Chile. *Epidemiology*. 2000;11(6):673–679.
Finch GL, Hoover MD, Hahn FF, et al. Animal models of beryllium-induced lung disease. *Environ Health Perspect*. 1996;104(suppl 5):973–979.
Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. *Cell*. 1989;57:1083–1093.
Fiorani M, Cantoni O, Tasinto A, Boscoboinik D, Azzi A. Hydrogen peroxide- and fetal bovine serum-induced DNA synthesis in vascular smooth muscle cells; positive and negative regulation by protein kinase C isoforms. *Biochem Biophys Acta*. 1995;1269:98–104.
Floyd RA. 8-Hydoxy-2′-deoxyguanosine in carcinogenesis. *Carcinogenesis*. 1990;11:1447–1450.
Fortini P, Parlanti E, Sidorkina OM, Laval J, Dogliotti E. The type of DNA glycosylase determines the base excision repair pathway in mammalian cells. *J Biol Chem*. 1999;274:230–236.
Fox M, Scott D. The genetic toxicology of nitrogen and sulphur mustard. *Mutat Res*. 1980;75(2):131–168.
Frenzel F, Buhrke T, Wenzel I, Andrack J, Hielscher J, Lampen A. Use of in silico models for prioritization of heat-induced food contaminants in mutagenicity and carcinogenicity testing. *Arch Toxicol*. 2017;91(9):3157–3174.
Fried M, Prives C. In: Botchan M, Grodzicker T, Sharp PA, eds. *Cancer Cells 4: DNA Tumor Viruses*. New York: Cold Spring Harbor; 1986:1–16.
Friedwald WF, Rous P. Initiating and promoting elements in tumor production: analysis of effects of tar, benzpyrene and methylcholanthrene on rabbit skin. *J Exp Med*. 1944;80:101–126.
Fu H, Boffetta P. Cancer and occupational exposure to inorganic Lead compounds: a meta-analysis of published data. *Occup Environ Med*. 1995;52(2):73–81.
Fujii K. Evaluation of the newborn mouse model for chemical tumorigenesis. *Carcinogenesis*. 1991;12(8):1409–1415.
Fukushima S, Imaida K, Sakata T, Okamura T, Shibata M, Ito N. Promoting effects of sodium L-ascorbate on two-stage urinary bladder carcinogenesis in rats. *Cancer Res*. 1983;43(9):4454–4457.
Fukushima S, Kinoshita A, Puatanachokchai R, Kushida M, Wanibuchi H, Morimura K. Hormesis and dose-response-mediated mechanisms in carcinogenesis: evidence for a threshold in carcinogenicity of non-genotoxic carcinogens. *Carcinogenesis*. 2005;26(11):1835–1845.
Furihata C, Matsushima T. Use of in vivo/in vitro unscheduled DNA synthesis for identification of organ-specific carcinogens. *Crit Rev Toxicol*. 1987;17(3):245–277.
Futreal PA, Liu Q, Shattuck-Eidens D, et al. BRCA1 mutations in primary breast and ovarian carcinomas. *Science*. 1994;266(5182):120–122.
Galloway SM, Bloom AD, Resnick M, et al. Development of a standard protocol for in vitro cytogenetic testing with Chinese hamster ovary cells: comparison of results for 22 compounds in two laboratories. *Environ Mutagen*. 1985;7(1):1–51.
Gama-Sosa MA, Slagel VA, Trewyn RW, et al. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res*. 1983;11: 6883–6894.
Garcia-Esquinas E, Pollan M, Tellez-Plaza M, et al. Cadmium exposure and cancer mortality in a prospective cohort: the strong heart study. *Environ Health Perspect*. 2014;122(4):363–370.
Generoso WM, Cain KT, Krishna M, Sheu CW, Gryder RM. Heritable translocation and dominant-lethal mutation induction with ethylene oxide in mice. *Mutat Res*. 1980;73(1):133–142.
Ghoshal AK, Farber E. The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. *Carcinogenesis*. 1984;5(10):1367–1370.
Gimenez-Conti IB, Slaga TJ. The hamster cheek pouch carcinogenesis model. *J Cell Biochem*. 1993;5283–5290.
Glauert HP, Robertson LW, Silberhorn EM. PCBs and tumor promotion. In: Robertson LW, Hansen LG, eds. *PCBs: Recent Advances in Environmental Toxicology and Health Effects*. Lexington, KY: University Press of Kentucky; 2001:355–371.
Golbamaki A, Benfenati E. In Silico Methods for Carcinogenicity Assessment. *Methods Mol Biol*. 2016;1425:107–119.
Gollapudi BB, Lynch AM, Heflich RH, et al. The in vivo Pig-a assay: a report of the International Workshop on Genotoxicity Testing (IWGT) Workgroup. *Mutat Res Genet Toxicol Environ Mutagen*. 2015;783:23–35.
Gondo Y, Shioyama Y, Nakao K, Katsuki M. A novel positive detection system of in vivo mutations in rpsL (strA) transgenic mice. *Mutat Res*. 1996;360(1):1–14.
Goode EL, Ulrich CM, Potter JD. Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiol Biomarkers Prev*. 2002;11:1513–1530.
Gordon T, Bowser D. Beryllium: genotoxicity and carcinogenicity. *Mutat Res*. 2003;533(1–2):99–105.
Gossen JA, de Leeuw WJF, Tan CHT, et al. Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations in vivo. *Proc Natl Acad Sci U S A*. 1989;86:7971–7975.
Gossen JA, Vijg J. A selective system for lacZ$^-$ phage using a galactose-sensitive *E. coli* host. *Biotechniques*. 1993;14:326–330.
Goth R, Rajewsky MF. Persistence of O6-ethylguanine in rat-brain DNA: correlation with nervous system-specific carcinogenesis by ethylnitrosourea. *Proc Natl Acad Sci U S A*. 1974;71(3):639–643.
Goyette MC, Cho K, Fasching CL, et al. Progression of colorectal cancer is associated with multiple tumor suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect via chromosome transfer. *Mol Cell Biol*. 1992;12(3):1387–1395.
Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*. 1994;54(18):4855–4878.
Grimsrud TK, Berge SR, Haldorsen T, Andersen A. Exposure to different forms of nickel and risk of lung cancer. *Am J Epidemiol*. 2002;156(12):1123–1132.
Grimsrud TK, Berge SR, Haldorsen T, Andersen A. Can lung cancer risk among nickel refinery workers be explained by occupational exposures other than nickel? *Epidemiology*. 2005;16(2):146–154.
Grimsrud TK, Berge SR, Martinsen JI, Andersen A. Lung cancer incidence among Norwegian nickel-refinery workers 1953-2000. *J Environ Monit*. 2003;5(2):190–197.
Guengerich FP. Mechanisms of formation of DNA adducts from ethylene dihalides, vinyl halides, and arylamines. *Drug Metab Rev*. 1994;26:47–66.
Gulezian D, Jacobson-Kram D, McCullough CB, et al. Use of transgenic animals for carcinogenicity testing: considerations for risk assessment. *Toxicol Pathol*. 2000;28(3):482–499.
Guyton KZ, Kensler TW. Oxidative mechanisms in carcinogenesis. *Br Med Bul*. 1993;49:523–544.
Han W, Pentecost BT, Spivack SD. Functional evaluation of novel single nucleotide polymorphisms and haplotypes in the promoter regions of *CYP1B1* and *CYP1A1* genes. *Mol Carcinogen*. 2003;37(3):158–169.
Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57–70.
Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646–674.
Hancock JF, Magee AI, Childs JE, et al. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell*. 1989;57:1167–1177.
Harris GK, Shi X. Signaling by carcinogenic metals and metal-induced reactive oxygen species. *Mutat Res*. 2003;533:183–200.
Harting FH, Hesse W. Der lungenkrebs, die Bergkrankheit in den Schneeberger gruben. *Vjschr Gerichtl Med Offentl Gesundheitswesen*. 1879;31:102–132, 313–337.
Harvey RG. Historical overview of chemical carcinogenesis. *Curr Cancer Res*. 2011:1–26.
Hastie ND. The genetics of Wilms' tumor—a case of disrupted development. *Annu Rev Genet*. 1994;28:523–558.
Hattis D, Chu M, Rahmioglu N, et al. A preliminary operational classification system for nonmutagenic modes of action for carcinogenesis. *Crit Rev Toxicol*. 2009;39(2):97–138.

Heddle JA, Hite M, Kirkhart B, et al. The induction of micronuclei as a measure of genotoxicity. A report of the US Environmental Protection Agency GENE-TOX program. *Mutat Res*. 1983;123:61–118.

Herbst AL, Kurman RJ, Scully RE, Poskanzer DC. Clear-cell adenocarcinoma of the genital tract in young females: registry report. *N Engl J Med*. 1972;287:1259–1264.

Henning SM, Swendseid ME, Coulson WF. Male rats fed methyl-and folate-deficient diets with or without niacin develop hepatic carcinomas associated with decreased tissue NAD concentrations and altered poly (ADP-ribose) polymerase activity. *J Nutr*. 1997;127:30–36.

Herbst AL, Scully RE. Adenocarcinoma of the vagina in adolescence: a report of 7 cases including 6 clear-cell carcinomas (so-called mesonephromas). *Cancer*. 1970;25:745–757.

Hergersberg M. Biological aspects of cytosine methylation in eukaryotic cells. *Experientia*. 1991;47:1171–1185.

Hiasa Y, Konishi N, Nakaoka S, et al. Possible application to medium-term organ bioassays for renal carcinogenesis modifiers in rats treated with N-ethyl-N-hydroxyethylnitrosamine and unilateral nephrectomy. *Jpn J Cancer Res*. 1991;82(12):1385–1390.

Hicks RM. Multistage carcinogenesis in the urinary bladder. *Br Med Bull*. 1980;36(1):39–46.

Highman B, Norvell MJ, Shellenberger TE. Pathological changes in female C3H mice continuously fed diets containing diethylstilbestrol or 17beta-estradiol. *J Environ Pathol Toxicol*. 1978;1:1–30.

Hoenerhoff MJ, Hong HH, Ton TV, Lahousse SA, Sills RC. A review of the molecular mechanisms of chemically induced neoplasia in rat and mouse models in National Toxicology Program bioassays and their relevance to human cancer. *Toxicol Pathol*. 2009;37(7):835–848.

Hogstedt C, Aringer L, Gustavsson A. Epidemiological support for ethylene oxide as a cancer-causing agent. *JAMA*. 1986;255:1575–1578.

Hollander MC, Fornace AJ Jr. Induction of fos RNA by DNA-damaging agents. *Cancer Res*. 1989;49:1687–1692.

Holliday R. Mechanisms for the control of gene activity during development. *Biol Rev*. 1990;65:431–471.

Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science*. 1991;253(5015):49–53.

Honkakoski P, Zelko I, Sueyoshi T, Negishi M. The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol*. 1998;18:5652–5658.

Hood A, Liu J, Klaassen CD. Effects of phenobarbital, pregnenolone-16α-carbonitrile, and propylthiouracil on thyroid follicular cell proliferation. *Toxicol Sci*. 1999;50:45–53.

Horton TM, Petros JA, Heddi A, et al. Novel mitochondrial DNA deletion found in renal cell carcinoma. *Genes Chromosomes Cancer*. 1996;15:95–101.

Hsieh LL, Hsieh TT. Detection of aflatoxin B1-DNA adducts in human placenta and cord blood. *Cancer Res*. 1993;53:1278–1280.

Hueper WC, Wiley FH, Wolfe HD. Experimental production of bladder tumors in dogs by administration of beta-naphthylamine. *J Ind Hyg Toxicol*. 1938;20:46–84.

Huff V. Wilms' tumours: about tumour suppressor genes, an oncogene and a chameleon gene. *Nat Rev Cancer*. 2011;11:111–121.

Hughes MF, Kitchin KT. Arsenic, oxidative stress and carcinogenesis. In: Singh KK, ed. *Oxidative Stress Disease and Cancer*. London: Imperial College Press; 2006.

Hussain SP, Haris CC. Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res*. 1998;58:4023–4037.

IARC. Some Inorganic and organometallic compounds. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*. Vol 2. Lyon, France: IARC; 1973:181.

IARC. Some metals and metallic compounds. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*. Vol 23. Lyon, France: International Agency for Research on Cancer; 1980:438.

IARC. Overall evaluations of carcinogenicity. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*. Suppl 7. Lyon, France: International Agency for Research on Cancer; 1987:440.

IARC. Chromium, nickel and welding. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*. Vol 49. Lyon, France: International Agency for Research on Cancer; 1990:677.

IARC. Beryllium, cadmium, mercury and exposures in the glass manufacturing industry. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*. Vol 58. Lyon, France: International Agency for Research on Cancer; 1993:444.

IARC. Polychlorinated dibenzo-para-dioxins and polychlorinated dibenzofurans. *IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans*. Vol 69. Lyon, France: IARC; 1997a.

IARC. Polychlorinated dibenzofurans. *Monographs on the Evaluation of Carcinogenic Risks to Humans*. Vol 69. Lyon, France: IARC; 1997b.

IARC. Working Group on the Evaluation of Carcinogenic Risks to Humans. Some drinking-water disinfectants and contaminants, including arsenic. Monographs on chloramine, chloral and chloral hydrate, dichloroacetic acid, trichloroacetic acid and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone. *IARC Monogr Eval Carcinog Risks Hum*. 2004;84:269–477.

IARC. Working Group on the Evaluation of Carcinogenic Risks to Humans. Inorganic and organic lead compounds. *IARC Monogr Eval Carcinog Risks Hum*. 2006;87:1–471.

IARC. Working Group on the Evaluation of Carcinogenic Risks to Humans. Combined estrogen-progestogen contraceptives and combined estrogen-progestogen menopausal therapy. *IARC Monogr Eval Carcinog Risks Hum*. 2007;91:1–528.

IARC. Working Group on the Evaluation of Carcinogenic Risks to Humans. IARC monographs on the evaluation of carcinogenic risks to humans. Volume 97. 1,3-butadiene, ethylene oxide and vinyl halides (vinyl fluoride, vinyl chloride and vinyl bromide). *IARC Monogr Eval Carcinog Risks Hum*. 2008;97:3–471.

IARC. Working Group on the Evaluation of Carcinogenic Risks to Humans. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. *IARC Monogr Eval Carcinog Risks Hum*. 2010;92:1–853.

IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 100C. A Review of Human Carcinogens: Arsenic, Metals, Fibres, and Dusts*. IARC; 2012.

Isfort RJ, Cody DB, Doersen CJ, Kerckaert GA, LeBoeuf RA. Alterations in cellular differentiation, mitogenesis, cytoskeleton and growth characteristics during Syrian hamster embryo cell multistep in vitro transformation. *Int J Cancer*. 1994;59:114–125.

Isfort RJ, Kerckaert GA, LeBoeuf RA. Comparison of the standard and reduced pH Syrian hamster embryo (SHE) cell in vivo transformation assays in predicting the carcinogenic potential of chemicals. *Mutat Res*. 1996;356:11–63.

Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*. 1990;347:645–650.

Ito N, Hasegawa R, Imaida K, et al. Medium-term rat liver bioassay for rapid detection of hepatocarcinogenic substances. *J Toxicol Pathol*. 1997;10:1–11.

Ito N, Hasegawa R, Imaida K, Takahashi S, Shirai T. Medium-term rat liver bioassay for rapid detection of carcinogens and modifiers of hepatocarcinogenesis. *Drug Metab Rev*. 1994;26:431–442.

Ito N, Imaida K, Hasegawa R, Tsuda H. Rapid bioassay methods for carcinogens and modifiers of hepatocarcinogenesis. *Crit Rev Toxicol*. 1989;19:386–415.

Ito N, Tamano S, Shirai T. A medium-term rat liver bioassay for rapid in vivo detection of carcinogenic potential of chemicals. *Cancer Sci*. 2003:94:3–8.

Izzotti A, Pulliero A. The effects of environmental chemical carcinogens on the microRNA machinery. *Int J Hyg Environ Health*. 2014;217(6):601–627.

Jacobson LP, Zhang BC, Zhu YR, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: study design and clinical outcomes. *Cancer Epidemiol Biomarkers Prev*. 1997;6(4):257–265.

Jagadeesan V, Rao JN, Sesikeran B. Effect of iron deficiency on DMH-induced gastrointestinal tract tumours and occurrence of hepatocyte abnormalities in Fischer rats. *Nutr Cancer*. 1994;22:285–291.

James NH, Roberts RA. Species differences in response to peroxisome proliferators correlate in vitro with induction of DNA synthesis rather than suppression of apoptosis. *Carcinogenesis*. 1996;17:1623–1632.

Janero DR. Malondialdehyde and thiobarbituric acid reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med*. 1990;9:515–540.

Johansson SL, Hirsch JM, Larsson PA, Saidi J, Osterdahl BG. Snuff-induced carcinogenesis: effect of snuff in rats initiated with 4-nitroquinoline N-oxide. *Cancer Res*. 1989;49(11):3063–3069.

Johnstone SE, Baylin SB. Stress and the epigenetic landscape: a link to the pathobiology of human diseases? *Nat Rev*. 2010;11:806.
Kadlubar FF, Unruh LE, Flammang TJ, Sparks D, Mitchum RK, Mulder GJ. Alteration of urinary levels of the carcinogen, *N*-hydroxy-2-naphthylamine, and its *N*-glucuronide in the rat by control of urinary pH, inhibition of metabolic sulfation, and changes in biliary excretion. *Chem Biol Interact*. 1981;33:129–147.
Kakehashi A, Wei M, Fukushima S, Wanibuchi H. Oxidative stress in the carcinogenicity of chemical carcinogens. *Cancers (Basel)*. 2013;5(4):1332–1354.
Kamb A, Gruis NA, Weaver-Feldhaus J, et al. A cell cycle regulator potentially involved in genesis of many tumor types. *Science*. 1994;264(5151):436–440.
Kasprzak KS, Diwan BA, Konishi N, Misra M, Rice JM. Initiation by nickel acetate and promotion by sodium barbital of renal cortical epithelial tumors in male F344 rats. *Carcinogenesis*. 1990;11(4): 647–652.
Kennaway EL, Hieger I. Carcinogenic substances and their fluorescence spectra. *Br Med J*. 1930;1:1044–1046.
Kennedy GLJr, Butenhoff JL, Olsen GW, et al. The toxicology of perfluorooctanoate. *Crit Rev Toxicol*. 2004;34(4):351–384.
Kennelly JC. Design and interpretation of rat liver UDS assays. *Mutagenesis*. 1995;10(3):215–221.
Kerr LD. Signal transduction: the nuclear target. *Curr Opin Cell Biol*. 1992;4:496–501.
Kim CM, Koike K, Saito I, et al. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature*. 1991;351:317–320.
Kimoto T, Horibata K, Miura D, et al. The PIGRET assay, a method for measuring Pig-a gene mutation in reticulocytes, is reliable as a short-term in vivo genotoxicity test: Summary of the MMS/JEMS-collaborative study across 16 laboratories using 24 chemicals. *Mutat Res*. 2016;811:3–15.
Kinoshita A, Wanibuchi H, Morimura K, et al. Phenobarbital at low dose exerts hormesis in rat hepatocarcinogenesis by reducing oxidative DNA damage, altering cell proliferation, apoptosis and gene expression. *Carcinogenesis*. 2003;24:1389–1399.
Kinosita R. Researches on the carcinogenesis of the various chemical substances. *Gann*. 1936;30:423–426.
Kinoshita T, Ohishi K, Takeda J. GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. *J Biochem*. 1997;122(2):251–257.
Kitano M, Ichihara T, Matsuda T, et al. Presence of a threshold for promoting effects of phenobarbital on diethylnitrosamine-induced hepatic foci in the rat. *Carcinogenesis*. 1998;19:1475–1480.
Klaassen, CD and Reisman, SA. Nrf2 the Rescue: Effects of the Antioxidative/Electrophilic Response on the Liver. *Toxicol Appl Pharmacol*. 2010; 244(1):57–65.
Klaunig JE. Alterations in intercellular communication during the stage of promotion. *Proc Soc Exp Biol Med*. 1991;198(2):688–692.
Klaunig JE. Selective induction of DNA synthesis in mouse preneoplastic and neoplastic hepatic lesions after exposure to phenobarbital. *Environ Health Perspect*. 1993;101(Suppl 5):235–239.
Klaunig JE, Babich MA, Baetcke KP, et al. PPARα agonist-induced rodent tumors: modes of action and human relevance. *Crit Rev Toxicol*. 2003;33:655–780.
Klaunig JE, Ruch RJ. Strain and species effects on the inhibition of hepatocyte intercellular communication by liver tumor promoters. *Cancer Lett*. 1987;36:161–168.
Klaunig JE, Ruch RJ. Role of inhibition of intercellular communication in carcinogenesis. *Lab Invest*. 1990;62(2):135–146.
Klaunig JE, Ruch RJ, Weghorst CM, Hampton JA. Role of inhibition of intercellular communication in hepatic tumor promotion. *In Vitro Toxicol*. 1990a;3(1):91–107.
Klaunig JE, Weghorst TR, Weghorst CM. Liver tumor promoting ability of corn oil gavage in B6C3F1 male mice. *Cancer Lett*. 1990b;50(3):215–219.
Klaunig JE, Wang Z, Pu X, Zhou S. Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol Appl Pharmacol*. 2011;254:86–99.
Klaunig JE, Xu Y, Bachowski S, Jiang J. Free-radical oxygen-induced changes in chemical carcinogenesis. In: Wallace KB, ed. *Free Radical Toxicology*. London: Taylor & Francis; 1997:375–400.
Kleczkowska HE, Althaus FR. Response of human keratinocytes to extremely low concentrations of N-methyl-N'-nitro-N-nitrosoguanidine. *Mutat Res*. 1996;367:151–159.
Knize MG, Dolbeare FA, Cunningham PL, Felton JS. Mutagenic activity and heterocyclic amine content of the human diet. *Princess Takamatsu Symp*. 1995;23:30–38.
Knudsen TB, Keller DA, Sander M, et al. FutureTox II: in vitro data and in silico models for predictive toxicology. *Toxicol Sci*. 2015;143(2):256–267.
Knutson JC, Poland A. Response of murine epidermis to 2,3,7,8-tetrachlorodibenzo-p-dioxin: interaction of the Ah and hr loci. *Cell*. 1982;30:225–234.
Kociba RJ, Keyes DG, Beyer JE, et al. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. *Toxicol Appl Pharmacol*. 1978;46:279–303.
Kodama S, Koike C, Negishi M, Yamamoto Y. Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes. *Mol Cell Biol*. 2004;24:7931–7940.
Kohler SW, Provost GS, Fieck A, et al. Spectra of spontaneous and mutagen-induced mutations in the lacI gene in transgenic mice. *Proc Natl Acad Sci U S A*. 1991a;88:7958–7962.
Kohler SW, Provost GS, Fieck A, et al. Spectra of spontaneous and induced mutations using a lambda ZAP/lacI shuttle vector. *Environ Mol Mutagen*. 1991b;18:316–321.
Kolaja KL, Stevenson DE, Walborg EFJr, Klaunig JE. The effect of dieldrin and phenobarbital on preneoplastic hepatic lesion growth in male F344 rat and B6C3F1 mouse. *Prog Clin Biol Res*. 1995;391:409–423.
Kolaja KL, Stevenson DE, Walborg EFJr, Klaunig JE. Reversibility of Promoter Induced Hepatic Focal Lesion Growth in Mice. *Carcinogenesis*. 1996a;17(7):1403–1409.
Kolaja KL, Stevenson DE, Walborg EFJr, Klaunig JE. Selective dieldrin promotion of hepatic focal lesions in mice. *Carcinogenesis*. 1996b; 17(6):1243–1250.
Kolman A, Chovanec M, Osterman-Golkar S. Genotoxic effects of ethylene oxide, propylene oxide and epichlorohydrin in humans: update review (1990-2001). *Mutat Res*. 2002;512(2–3):173–194.
Koufaris C, Wright J, Currie RA, Gooderham NJ. Hepatic microRNA profiles offer predictive and mechanistic insights after exposure to genotoxic and epigenetic hepatocarcinogens. *Toxicol Sci*. 2012;128(2):532–543.
Koufaris C, Wright J, Osborne M, Currie RA, Gooderham NJ. Time and dose-dependent effects of phenobarbital on the rat liver miRNAome. *Toxicology*. 2013;314(2–3):247–253.
Kriegel AM, Soliman AS, Zhang Q, et al. Serum cadmium levels in pancreatic cancer patients from the East Nile Delta region of Egypt. *Environ Health Perspect*. 2006;114(1):113–119.
Kroh H. Periphery of ethylnitrosourea-induced spinal gliomas in rats with special reference to the vascular structure. *Acta Neuropathol*. 1987;73(1):92–98.
Kushida M, Sukata T, Uwagawa S, et al. Low dose DDT inhibition of hepatocarcinogenesis initiated by diethylnitrosamine in male rats: possible mechanisms. *Toxicol Appl Pharmacol*. 2005;208(3):285–294.
Kuo LJ, Yang LX. Gamma-H2AX—a novel biomarker for DNA double-strand breaks. *In Vivo*. 2008;22(3):305–309.
Lake BG. Mechanisms of hepatocarcinogenicity of peroxisome-proliferating drugs and chemicals. *Annu Rev Pharmacol Toxicol*. 1995;35:483–507.
Lan Q, Zhang L, Li G, et al. Hematotoxicity in workers exposed to low levels of benzene. *Science*. 2004;306;1774–1776.
Lancaster WD, Olson C. Animal papilloma-viruses. *Microbiol Rev*. 1982;46:191–207.
Langard S. Chromium carcinogenicity: a review of experimental animal data. *Sci Total Environ*. 1988;71:341–350.
Larson JL, Wolf DC, Butterworth BE. Induced cytolethality and regenerative cell proliferation in the livers and kidneys of male B6C3F1 mice given chloroform by gavage. *Fundam Appl Toxicol*. 1994;23:537–543.
Latt SA. Sister chromatid exchange formation. *Annu Rev Genet*. 1981;15:11–55.
Latt SA, Allen J, Bloom SE, et al. Sister-chromatid exchanges: a report of the GENE-TOX program. *Mutat Res*. 1981;87(1):17–62.
Leach FS, Tokino T, Meltzer P, et al. Mutation and MDM2 amplification in human soft tissue sarcomas. *Cancer Res*. 1993;53(10 suppl):2231–2234.
LeBoeuf RA, Kerckaert GA, Aardema MJ, Gibson DP. Multistage neoplastic transformation of Syrian hamster embryo cells cultured at pH 6.70. *Cancer Res*. 1990;50:3722–3729.
Lee CP, Lee YH, Lian IB, Su CC. Increased prevalence of esophageal cancer in areas with high levels of nickel in farm soils. *J Cancer*. 2016;7(12):1724–1730.

Lee SS, Pineau T, Drago J, et al. Targeted disruption of the alpha isoform of the peroxisome proliferators-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol*. 1995;15:3012–3022.

Lee YS, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol*. 2009;4:199–227.

Leonard A, Lauwerys R. Mutagenicity, carcinogenicity and teratogenicity of beryllium. *Mutat Res*. 1987;186:35–42.

Lewandowska J, Bartoszek A. DNA methylation in cancer development, diagnosis and therapy—multiple opportunities for genotoxic agents to act as methylome disruptors or remediators. *Mutagenesis*. 2011;26:475–487.

Li AP, Carver JH, Choy WN, et al. A guide for the performance of the Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl-transferase gene mutation assay. *Mutation Res*. 1987;189:135–141.

Li J, Kirkman H, Li S. Synthetic estrogens and liver cancer: risk analysis of animal and human data. In: Li J, Nandi S, Li S, eds. *Hormonal Carcinogenesis*. New York: Springer-Verlag; 1992:217–224.

Li N, Karin M. Ionizing radiation and short wavelength UV activate NF-$_{\kappa}$B through two distinct mechanisms. *Proc Natl Acad Sci USA*. 1998;95:13012–13017.

Li R, Yerganian G, Duesberg P, et al. Aneuploidy correlated 100% with chemical transformation of Chinese hamster cells. *Proc Natl Acad Sci USA*. 1997;94:14506–14511.

Li S, Hou X, Li J. Estrogen carcinogenesis: a sequential, epi-genotoxic, multistage process. In: Li J, Nandi S, Li S, eds. *Hormonal Carcinogenesis*. New York: Springer-Verlag; 1996:200–208.

Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem*. 2010;79:181–211.

Lindenschmidt RC, Tryka AF, Witschi H. Modification of gastrointestinal tumour development in rats by dietary butylatedhydroxytoluene. *Fundam Appl Toxicol*. 1987;8(4):474–481.

Lockhart AM, Piegorsch WW, Bishop JB. Assessing overdispersion and dose-response in the male dominant lethal assay. *Mutat Res*. 1992;272(1):35–58.

Loewenstein WR. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol Rev*. 1981;61:829–913.

Lofroth G, Ames BN. Mutagenicity of inorganic compounds in *Salmonella typhimurium*: arsenic, chromium and selenium. *Mutat Res*. 1978;53: 65–66.

Longnecker DS, Roebuck BD, Kuhlmann ET. Enhancement of pancreatic carcinogenesis by a dietary unsaturated fat in rats treated with saline or N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine. *J Natl Cancer Inst*. 1985;74(1):219–222.

Longnecker DS, Wiebkin P, Schaeffer BK, Roebuck BD. Experimental carcinogenesis in the pancreas. *Int Rev Exp Pathol*. 1984;26:177–229.

Lowe JP, Silverman BD. Predicting the carcinogenicity of polycyclic aromatic hydrocarbons. *J Am Chem Soc*. 1994;106:5955–5958.

Luckett BG, Su LJ, Rood JC, Fontham ET. Cadmium exposure and pancreatic cancer in south Louisiana. *J Environ Public Health*. 2012;2012:180186.

Luczak MW, Jagodzinski PP. The role of DNA methylation in cancer development. *Folia Histochem Cytobiol*. 2006;44(3):143–154.

Mandel JS, McLaughlin JK, Schlehofer B, et al. International renal-cell cancer study. IV. Occupation. *Int J Cancer*. 1995;61(5): 601–605.

Majeska JB, Matheson DW. Development of an optimal S9 activation mixture for the L5178Y TK+/− mouse lymphoma mutation assay. *Environ Mol Mutagen*. 1990;16(4):311–319.

Marcu KB, Bossone SA, Patel AJ. Myc function and regulation. *Annu Rev Biochem*. 1992;61:809–860.

Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. *Mutat Res*. 1983;113(3–4):173–215.

Maronpot RR, Pitot HC, Peraino C. Use of rat liver altered focus models for testing chemicals that have completed two-year carcinogenicity studies. *Toxicol Pathol*. 1989;17:651–662.

Maronpot RR, Shimkin MB, Witschi HP, Smith I, Cline JM. Strain A mouse pulmonary tumor test results for chemicals previously tested in National Cancer Institute carcinogenicity tests. *J Natl Cancer Inst*. 1986;76:1101–1112.

Marselos M, Vainio H. Carcinogenic properties of pharmaceutical agents evaluated in the IARC Monographs programme. *Carcinogenesis*. 1991;12:1751–1766.

Marsman DS, Goldsworthy TL, Popp JA. Contrasting hepatocytic peroxisome proliferation, lipofuscin accumulation and cell turnover for the hepatocarcinogens WY 14,643-14,643 and clofibric acid. *Carcinogenesis*. 1992;3:1011–1017.

Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol*. 2002;192:1–15.

Martinez VD, Vucic EA, Becker-Santos DD, Gil L, Lam WL. Arsenic exposure and the induction of human cancers. *J Toxicol*. 2011;2011:431287.

Matthews LA, Crea F, Farrar WL. Epigenetic gene regulation in stem cells and correlation to cancer. *Differentiation*. 2009;78:1–17.

McCarroll N, Keshava N, Chen J, Akerman G, Kligerman A, Rinde E. An evaluation of the mode of action framework for mutagenic carcinogens case study II: chromium (VI). *Environ Mol Mutagen*. 2010;51(2):89–111.

McClain RM. Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. *Mutat Res*. 1995;333:131–142.

McCormick F. Activators and effectors of ras p21 proteins. *Curr Opin Genet Dev*. 1994;4:71–76.

Melnick RL, Kohn MC, Portier CJ. Implications for risk assessment of suggested nongenotoxic mechanisms of chemical carcinogenesis. *Environ Health Perspect*. 1996;104(S1):123–133.

Merlo A, Herman JG, Mao L, et al. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med*. 1995;1:686–692.

Metzler M, Pfeiffer E, Schuler M, Rosenberg B. Effects of estrogens on microtubule assembly: significance for aneuploidy. In: Li J, Li S, Gustafsson J-A, Nandi S, Seakely L, eds. *Hormonal Carcinogenesis II*. New York: Springer-Verlag; 1996:193–199.

Miller EC. Studies on the formation of protein-bound derivatives of 3,4-benzpyrene in the epidermal fraction of mouse skin. *Cancer Res*. 1951;11;100–108.

Miller EC, Miller JA. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. *Cancer*. 1981;47(10):2327–2345.

Miller EC, Miller JA. The presence and significance of bound amino azodyes in the livers of rats fed p-dimethylaminoazobenzene. *Cancer Res*. 1947;7:468–480.

Miller EC, Miller JA, Enomoto M. The comparative carcinogenicities of 2-acetylaminofluorene and its N-hydroxy metabolite in mice, hamsters, and guinea pigs. *Cancer Res*. 1964;24:2018–2031.

Miller EC, Swanson AB, Phillips DH, Fletcher TL, Liem A, Miller JA. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res*. 1983;43:1124–1134.

Miller JA. Carcinogenesis by chemicals: an overview—G. H. A. Clowes memorial lecture. *Cancer Res*. 1970;3:559–576.

Mirsalis JC, Monforte JA, Winegar RA. Transgenic animal models for measuring mutations in vivo. *Crit Rev Toxicol*. 1994;24(3):255–280.

Miura D, Dobrovolsky VN, Kasahara Y, Katsuura Y, Heflich RH. Development of an in vivo gene mutation assay using the endogenous Pig-A gene: I. Flow cytometric detection of CD59-negative peripheral red blood cells and CD48-negative spleen T-cells from the rat. *Environ Mol Mutagen*. 2008a;49(8):614–621.

Miura D, Dobrovolsky VN, Mittelstaedt RA, Kasahara Y, Katsuura Y, Heflich RH. Development of an in vivo gene mutation assay using the endogenous Pig-A gene: II. Selection of Pig-A mutant rat spleen T-cells with proaerolysin and sequencing Pig-A cDNA from the mutants. *Environ Mol Mutagen*. 2008b;49(8):622–630.

Miyamae Y, Iwasaki K, Kinae N, et al. Detection of DNA lesions induced by chemical mutagens by the single cell gel electrophoresis (Comet) assay: relationship between DNA migration and alkaline conditions. *Mutat Res*. 1997;393:107–113.

Miyamae Y, Zaizen K, Ohara K, Mine Y, Sasaki YF. Detection of DNA lesions induced by chemical mutagens by the single cell electrophoresis (Comet) assay. 1. Relationship between the onset of DNA damage and the characteristics of mutagens. *Mutat Res*. 1998;415(3):229–235.

Moennikes O, Loeppen S, Buchmann A, et al. Constitutively active dioxin/aryl hydrocarbon receptor promotes hepatocarcinogenesis in mice. *Cancer Res*. 2004;64(14):4707–4710.

Molina EP, Klatt P, Vasquez J, et al. Glutathionylation of the p50 subunit of NF-$_{\kappa}$B: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry*. 2001;40:14134–14142.

Moriya M. Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted G:C → T:A transversions in simian kidney cells. *Proc Natl Acad Sci U S A*. 1993;90:1122–1126.

Mortelmans K, Zeiger E. The Ames salmonella microsome mutagenicity assay. *Mutat Res*. 2000;455:29–60.

Muller JM, Krauss B, Kaltschmidt C, Baeuerle PA, Rupec RA. Hypoxia induces c-fos transcription via mitogen-activated protein kinase-dependent pathway. *J Biol Chem*. 1997;272:23435–23439.

Muscat JE, Wynder EL. Diesel engine exhaust and lung cancer: an unproven association. *Environ Health Perspect*. 1995;103(9):812–818.

Mutoh S, Sobhany M, Moore R, et al. Phenobarbital indirectly activates the constitutive active androstane receptor (CAR) by inhibition of epidermal growth factor receptor signaling. *Sci Signal*. 2013;6(274):ra31.

Myhr BC. Validation studies with Muta Mouse: a transgenic mouse model for detecting mutations in vivo. *Environ Mol Mutagen*. 1991;18(4): 308–315.

Myöhänen SK, Baylin SB, Herman JG. Hypermethylation can selectively silence individual $p16^{ink4a}$ alleles in neoplasia. *Cancer Res*. 1998;58:591–593.

Nakae D, Yoshiji H, Mizumoto Y, et al. High incidence of hepatocellular carcinomas induced by a choline deficient L-amino acid defined diet in rats. *Cancer Res*. 1992;52(18):5042–5045.

Nakatsuru Y, Wakabayashi K, Fuji-Kuriyama T, Kusuma K, Ide F. Dibenzo[A,L]pyrene-induced genotoxic and carcinogenic responses are dramatically suppressed in aryl hydrocarbon receptor-deficient mice. *Int J Cancer*. 2004;112(2):179–183.

Naoe T, Takeyama K, Yokozawa T, et al. Analysis of genetic polymorphism in *NQO1, GST-M1, GST-T1,* and *CYP3A4* in 469 Japanese patients with therapy-related leukemia/myelodysplastic syndrome and *de novo* acute myeloid leukemia. *Clin Cancer Res*. 2000;6:4091–4095.

National Research Council (US) Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology. *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment*. Washington, DC: National Academies Press; 2007.

Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP. Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem Pharmacol*. 2000;59:65–85.

Nebreda AR, Porrai A. P38 MAP kinases: beyond the stress response. *Trends Biochem Sci*. 2000;25:257–260.

Neumann HG. Role of extent and persistence of DNA modifications in chemical carcinogenesis by aromatic amines. *Recent Results Cancer Res*. 1983;84:77–89.

Newberne PM, deCanargo JLV, Clark AJ. Choline deficiency, partial hepatectomy, and liver tumors in rats and mice. *Toxicol Pathol*. 1982;10:95–106.

Nims RW, Lubet RA. The CYP2B subfamily. In: Ioannides C, ed. *Cytochromes P450: Metabolic and Toxicological Aspects*. Boca Raton, FL: CRC Press; 1996:135–160.

Nishizuka Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*. 1988;334:661–665.

Niven JSF, Armstrong JA, Andrews CH, et al. Subcutaneous "growths" in monkeys produced by a poxvirus. *J Pathol Bacteriol*. 1961; 81:1–14.

Noller KL, Decker DG, Lanier AP, Kurkland LT. Clear-cell adenocarcinoma of the cervix after maternal treatment with synthetic estrogens. *Mayo Clin Proc*. 1972;47:629–630.

Nordberg GF, Andersen O. Metal interactions in carcinogenesis: enhancement, inhibition. *Environ Health Perspect*. 1981;40:65–81.

National Toxicology Program. *Toxicology and Carcinogenesis Studies of Propylene Oxide (CAS No. 75-569) in F344/N Rats and B6C3F1 Mice (Inhalation Studies), TR-267*. Research Triangle Park, NC: National Toxicology Program; 1985.

National Toxicology Program. *Toxicology and Carcinogenesis Studies of Nickel Subsulfide (CAS No. 12035-72-2) in F344 Rats and B6C3F1 Mice (Inhalation Studies). Technical Report Series No 453*. Research Triangle Park, NC: National Toxicology Program; 1996:365.

National Toxicology Program. Report on Carcinogens Background Document for Lead and Lead Compounds. National Toxicology Program. 2003. Available at: http://ntp.niehs.nih.gov/ntp/newhomeroc/roc11/Lead-Public.pdf.

Nusse R, Varmus HE. Wnt genes. *Cell*. 1992;69:1073–1087.

OECD. Guidelines for Testing of Chemicals; Guideline no. 489: In Vivo Mammalian Alkaline Comet Assay. Adopted, September 26, 2014.

Oesterle D, Deml E. Lack of initiating and promoting activity of thiourea in rat liver foci bioassay. *Cancer Letters*. 1988;41:245–249.

Ogiso T, Tatematsu M, Tamano S, Hasegawa R, Ito N. Correlation between medium-term liver bioassay system data and results of long-term testing in rats. *Carcinogenesis*. 1990;11:561–566.

Oller AR, Kirkpatrick DT, Radovsky A, Bates HK. Inhalation carcinogenicity study with nickel metal powder in Wistar rats. *Toxicol Appl Pharmacol*. 2008;233(2):262–275.

Oppermann H, Levinson AD, Varmus HE, Levintow L, Bishop JM. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (src). *Proc Natl Acad Sci U S A*. 1979;76:1804–1808.

Osada H, Takahashi T. Genetic alterations of multiple tumor suppressors and oncogenes in the carcinogenesis and progression of lung cancer. *Oncogene*. 2002;21:7421–7434.

OSHA. Occupational Safety Health Administration, Department of Labor. Occupational Exposure to Beryllium. Final rule. *Fed Regist*. 2017;82(5):2470–2757.

Pahl HL. Activators and target genes of Rel/NF-$_{\kappa}$B transcription factors. *Oncogene*. 1999;18:6853–6866.

Palmer CN, Hsu MH, Griffin KJ, Raucy JL, Johnson EF. Peroxisome proliferator activated receptor-alpha expression in human liver. *Mol Pharmacol*. 1998;53:14–22.

Pan SC, Gardner WU. Carcinomas of the uterine cervix and vagina in estrogen- and androgen-treated hybrid mice. *Cancer Res*. 1948;8:337–341.

Parke DV. The cytochromes P450 and mechanisms of chemical carcinogenesis. *Environ Health Perspect*. 1994;102:852–853.

Parke DV, Sapota A. Chemical toxicity and reactive oxygen species. *Int J Occup Med Environ Health*. 1996;9:331–340.

Parnell MJ, Exon JH, Koller LD. Assessment of hepatic initiation-promotion properties of trichloroacetic acid. *Arch Environ Contam Toxicol*. 1988;17:429–436.

Parsons PA. Energy, stress and the invalid linear no-threshold premise: a generalization illustrated by ionizing radiation. *Biogerontology*. 2003;4:227–231

Pavela M, Uitti J, Pukkala E. Cancer incidence among copper smelting and nickel refining workers in Finland. *Am J Ind Med*. 2017;60(1): 87–95.

Pegg AE. Methylation of the O6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer Invest*. 1984;2(3):223–231.

Penn I, Halgrimson CG, Starzl TE. De novo malignant tumors in organ transplant recipients. *Transplant Proc*. 1971;3(1):773–778.

Penn I, Hammond W, Brettschneider L, Starzl TE. Malignant lymphomas in transplantation patients. *Transplant Proc*. 1969;1(1):106–112.

Penn I, Starzl TE. Malignant lymphomas in transplantation patients: a review of the world experience. *Int Z Klin Pharmakol Ther Toxikol*. 1970;3(1):49–54.

Penn I, Starzl TE. Immunosuppression and neoplasia. *Behring Inst Mitt*. 1972;51:204–208.

Pereira MA. Route of administration determines whether chloroform enhances or inhibits cell proliferation in the liver of B6C3F1 mice. *Fundam Appl Toxicol*. 1994;23:87–92.

Pereira MA, Herren-Freund SL Liver initiation assay, the rat liver foci bioassay of carcinogens and noncarcinogens. Evaluation of short-term assays for carcinogens. In: Ashby J, de Serres FJ, Shelby MD, Margolin BH, Ishidate M, Becking GC, eds. *Evaluation of Short-term Tests for Carcinogens, Report of the International Programme on Chemical Safety's Collaborative Study on In Vivo Assays*. England: Cambridge University Press; 1988:1.325–1.328.

Perera F, Mayer J, Santella RM, et al. Biological markers in risk assessment for environmental carcinogens. *Environ Health Perspect*. 1991;90: 247–254.

Peters JM, Aoyama T, Cattley RC, Nobumitsu U, Hashimoto T, Gonzalez FJ. Role of peroxisome proliferator-activated receptor alpha in altered cell cycle regulation in mouse liver. *Carcinogenesis*. 1998;19:1989–1994.

Pettersson U, Roberts RJ. Adenovirus gene expression and replication. A historic review. In: Botchan M, Grodzicker T, Sharp PA, eds. *Cancer Cells 4: DNA Tumor Viruses*. New York: Cold Spring Harbor; 1986:37–57.

Pietra G, Spencer K, Shubik P. Response of newly born mice to a chemical carcinogen. *Nature*. 1959;183:1689.

Pinkus R. Phenobarbital induction of AP-1 binding activity mediate activation of glutathione S-transferase and quinine reductase gene expression. *Biochem J*. 1993;290:637–640.

Pitot HC. *Fundamentals of Oncology*. New York: Marcel Dekker, Inc; 1986.

Pitot HC. Multistage carcinogenesis. In: Bertino JR, ed. *Encyclopedia of Cancer*. Vol II. New York: Academic Press; 1997:1108–1119.

Pitot HC, Goldsworthy T, Moran S, Sirica J. Properties of incomplete carcinogens and promoters in hepatocarcinogenesis. *Carcinogen Compr Surv*. 1982;7:85–98.

Pitot HC, Goldsworthy TL, Moran S, et al. A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose–response relationships to altered hepatic foci. *Carcinogenesis*. 1987;8:1491–1499.

Platzek T, Lang C, Grohmann G, Gi US, Baltes W. Formation of a carcinogenic aromatic amine from an azo dye by human skin bacteria in vitro. *Hum Exp Toxicol*. 1999;18(9):552–559.

Pogribny IP. MicroRNA dysregulation during chemical carcinogenesis. *Epigenomics*. 2009;1(2):281–290.

Pogribny IP, Beland FA. DNA methylome alterations in chemical carcinogenesis. *Cancer Lett*. 2013;334(1):39–45.

Pogribny IP, Beland FA, Rusyn I. The role of microRNAs in the development and progression of chemical-associated cancers. *Toxicol Appl Pharmacol*. 2016;312:3–10.

Pogribny IP, Muskhelishvili L, Tryndyak VP, Beland FA. The role of epigenetic events in genotoxic hepatocarcinogenesis induced by 2-acetylaminofluorene. *Mutat Res*. 2011;722(2):106–113.

Pollycove M, Feinendegen LE. Biologic responses to low doses of ionizing radiation: Detriment versus hormesis. Part 2. Dose responses of organisms. *J Nucl Med*. 2001;42:26N–32N, 37N.

Pott P. The chirurgical works. In: Hawes WC, Collins R, eds. *Chirurgical Observations Relative to the Cataract, the Polypus of the Nose, the Cancer of the Scrotum, the Different Kinds of Ruptures, and the Mortification of the Toes and Feet*. Ch. III. T.J. Carnegy: London: 1775:60–68.

Pritchard JB, French JE, Davis BJ, Haseman JK. The role of transgenic mouse models in carcinogen identification. *Environ Health Perspect*. 2003;111(4):444–454.

Proctor DM, Suh M, Campleman SL, Thompson CM. Assessment of the mode of action for hexavalent chromium-induced lung cancer following inhalation exposures. *Toxicology*. 2014;325:160–179.

Proctor RN. Commentary: Schairer and Schoniger's forgotten tobacco epidemiology and the Nazi quest for racial purity. *Int J Epidemiol*. 2001;30(1):31–34.

Pu X, Wang Z, Klaunig JE. Alkaline comet assay for assessing DNA damage in individual cells. *Curr Protoc Toxicol*. 2015;65:3.12.1–11.

Puatanachokchai R, Morimura K, Wanibuchi H, et al. Alpha-benzene hexachloride exerts hormesis in preneoplastic lesion formation of rat hepatocarcinogenesis with the possible role for hepatic detoxifying enzymes. *Cancer Lett*. 2006;240(1):102–113.

Pugh G Jr, Isenberg JS, Kamendulis LM, et al. Effects of diisononyl phthalate, di-2-ethylhexyl phthalate, and clofibrate in cynomolgus monkeys. *Toxicol Sci*. 2000;56:181–188.

Quian Y, Luo J, Leonard SS, et al. Hydrogen peroxide formation and actin filament reorganization by Cdc42 are essential for ethanol-induced in vitro angiogenesis. *J Biol Chem*. 2003;278:16189–16197.

Raies AB, Bajic VB. In silico toxicology: computational methods for the prediction of chemical toxicity. *Wiley Interdiscip Rev Comput Mol Sci*. 2016;6(2):147–172.

Rao MS, Reddy JK. An overview of peroxisome proliferator-induced hepatocarcinogenesis. *Environ Health Perspect*. 1991;93:205–209.

Rapp F. Herpesviruses and cancer. *Adv Cancer Res*. 1974;19:265–302.

Rauscher FJ III, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilms tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science*. 1990;250:1259–1262.

Reddy JK, Rao MS. Malignant tumors in rats fed nafenopin, a hepatic peroxisome proliferator. *J Natl Cancer Inst*. 1997;59:1645–1650.

Reisman SA, Yeager RL, Yamamoto M, Klaassen CD. Increased Nrf2 activation in livers from Keap1-knockdown mice increases expression of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. *Toxicol Sci*. 2009;108(1):35–47.

Reisman SA, Yeager RL, Yamamoto M, Klaassen CD. Nrf2 activation enhances biliary excretion of sulfobromophthalein by inducing glutathione-S-transferase activity. *Toxicol Sci*. 2009b;109(1):24–30.

Rehn L. Blasengeschwülste bei Fuchsin-arbeitern. *Arch Klin Chir*. 1895;50:588–600.

9th RoC. Report on Carcinogens 9th Edition. National Toxicology Program. Research Triangle Park, NC; 2000.

Reznikoff CA, Brankow DW, Heidelberger C. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res*. 1973;33:3231–3238.

Rice JM, Diwan BA, Hu H, Ward JM, Nims RW, Lubet RA. Enhancement of hepatocarcinogenesis and induction of specific cytochromes P450-dependent monooxygenase activities by the barbiturates allobarbital, aprobarbital, pentobarbital, secobarbital, and 5-phenyl- and 5-ethylbarbituric acids. *Carcinogenesis*. 1994;15:395–402.

Rice-Evans C, Burdon R. Free radical-lipid interactions and their pathological consequences. *Prog Lipid Res*. 1993;32:71–110.

Riggins JN, Marnett LJ. Mutagenicity of the malondialdehyde oligerimerization products 2-(3′oxo-1′-propeneyl)-malondialdehyde and 2,4-dyhydroxymethylene-3-(2, 2-dimethoxyethyl)-gluteraldehyde in Salmonella. *Mutat Res*. 2001;497:153–157.

Riggs AD, Jones PA. 5-Methylcytosine, gene regulation and cancer. *Adv Cancer Res*. 1983;40:1–30.

Risch A, Wikman H, Thiel S, et al. Glutathione-S-transferase M1, M3, T1 and P1 polymorphisms and susceptibility to non-small-cell lung cancer subtypes and hamartomas. *Pharmacogenetics*. 2001;1(9):757–764.

Robertson KD, Jones PA. DNA methylation: past, present and future directions. *Carcinogenesis*. 2000;21(3):461–467.

Rohrmann S, Platz EA, Kavanaugh CJ, Thuita L, Hoffman SC, Helzlsouer KJ. Meat and dairy consumption and subsequent risk of prostate cancer in a US cohort study. *Cancer Causes Control*. 2007;18(1):41–50.

Rose ML, Rivera CA, Bradford BU, Graves LM, Cattley RC, Thurman RG. Kupffer cell oxidant production is central to the mechanism of peroxisome proliferators. *Carcinogenesis*. 1999;20:27–33.

Rous P. A sarcoma of the foul transmissible by an agent separable from the tumor cells. *J Exp Med*. 1911;13(4):397–411.

Rusyn I, Bradham CA, Cohn L, Schoonhoven R, Swenberg JA, Thurman RG. Corn oil rapidly activates nuclear factor-kB in hepatic Kupffer cells by oxidant-dependant mechanisms. *Carcinogenesis*. 1999;20:2095–2100.

Sakai A, Sato M. Improvement of carcinogen identification in BALB/3T3 cell transformation by the application of a 2-stage method. *Mutat Res*. 1989;214:289–296.

Salem C, Liang G, Tsai YC, et al. Progressive increases in de novo methylation of CpG islands in bladder cancer. *Cancer Res*. 2000;60: 2473–2476.

San Filippo J, Sung P, Klein H. Mechanism of eukaryotic homologous recombination. *Annu Rev Biochem*. 2008;77:229–257.

Sanderson WT, Ward EM, Steenland K, Petersen MR. Lung cancer case-control study of beryllium workers. *Am J Ind Med*. 2001;39(2):133–144.

Sasaki MS. Chromosome aberration formation and sister chromatid exchange in relation to DNA repair in human cells. In: Generoso WM, Shelby MD, De Serres FJ, eds. *DNA Repair and Mutagenesis in Eukaryotes*. New York, NY: Plenum Press; 1980:285–313.

Sasaki T, Yoshida T. Experimentelle Erzeugung des Lebercarcinoms durch Fütterung mit o-Amidoazotoluol. *Virchows Archiv, Pathologische Anatomie*. 1935;295:175–200.

Sawyer DE, Van Houten B. Repair of DNA damage in mitochondria. *Mutat Res*. 1999;434:161–176.

Schechtman LM. BALB/3T3 cell transformation: protocols, problems and improvements. *IARC Sci Publ*. 1985;67:165–184.

Schubauer-Berigan MK, Deddens JA, Steenland K, Sanderson WT, Petersen MR. Adjustment for temporal confounders in a reanalysis of a case-control study of beryllium and lung cancer. *Occup Environ Med*. 2008;65(6):379–383.

Schulze-Oshoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. *Eur J Biochem*. 1998;254:439–459.

Schumacher HR, Szelkely LE, Patel SB, Fisher DR. Mitochondria: a clue to oncogenesis? *Lancet*. 1973;2:327.
Schwarz D, Kisselev P, Cascorbi I, Schunck WH, Roots I. Differential metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol by human CYP1A1 variants. *Carcinogenesis*. 2001;22(3):453–459.
Shelby MK, Klaassen CD. Induction of rat UDP-glucuronosyltransferases in liver and duodenum by microsomal enzyme inducers that activate various transcriptional pathways. *Drug Metab Dispos*. 2006;34(10):1772–1778.
Sherr CJ. Mammalian G_1 cyclins. *Cell*. 1993;73:1059–1065.
Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidative-damage base 8-oxo-dG. *Nature*. 1991;349:431–434.
Shigematsu IS, Kitamaru J, Takeuchi M, et al. A retrospective mortality study on cadmium exposed populations in Japan. In: Wilson D, Volpe RA, eds. *Third International Cadmium Conference*. Cadmium Association/ Cadmium Council; 1982.
Shih IM, Zhou W, Goodman SN, Lengauer C, Kinzler KW, Vogelstein B. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. *Cancer Res*. 2001;61:818–822.
Shimkin M. *Some Classics of Experimental Oncology, 50 Selections, 1775–1965*. US Department of Health and Human Services; 1980.
Shimkin MB, Stoner GD. Lung tumors in mice: application to carcinogenesis bioassay. *Adv Cancer Res*. 1975;21:1–58.
Shirai T, Hirose M, Ito N. Medium-term bioassays in rats for rapid detection of the carcinogenic potential of chemicals. *IARC Scientific Publ*. 1999;146:251–272.
Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin*. 2016;66(1):7–30.
Siemiatycki J, Dewar R, Nadon L, Gerin M. Occupational risk factors for bladder cancer: results from a case-control study in Montreal, Quebec, Canada. *Am J Epidemiol*. 1994;140(12):1061–1080.
Silva RA, Muñoz SE, Guzmán CA, Eynard AR. Effects of dietary n-3, n-6 and n-9 polyunsaturated fatty acids on benzo(a)pyrene-induced forestomachtumorigenesis in C57BL6J mice. *Prostaglandins Leukot Essent Fatty Acids*. 1995;53(4):273–277.
Sims P, Grover PL, Swaisland A, Pal K, Hewer A. Metabolic activation of benzo[a]pyrene proceeds by a diol-epoxide. *Nature*. 1974;252:326–328.
Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988;175(1):184–191.
Slaga TJ. Multistage skin carcinogenesis, a useful model for the study of the chemoprevention of cancer. *Acta Pharmacol Toxicol*. 1984;55:107–124.
Slater AF, Stefan C, Nobel I, van den Dobbelsteen DJ, Orrenius S. Signalling mechanisms and oxidative stress in apoptosis. *Toxicol Lett*. 1995;82–83:149–153.
Smith MT, Guyton KZ, Gibbons CF, et al. Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogens. *Environ Health Perspect*. 2016;124(6):713–721.
Solt D, Farber E. New principle for the analysis of chemical carcinogenesis. *Nature*. 1976;263:701–703.
Solt DB, Polverini PJ, Calderon L. Carcinogenic response of hamster buccal pouch epithelium to 4 polycyclic aromatic hydrocarbons. *J Oral Pathol Med*. 1987;6:294–302.
Sonich-Mullin C, Fielder R, Wiltse J, et al. IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul Toxicol Pharmacol*. 2001;34(2):146–152.
Sontag J. Carcinogenicity of substituted-benzenediamines (phenylenediamines). in rats and mice. *J Natl Cancer Inst*. 1981;66:591–602.
Sontag JM. Aspects in carcinogen bioassay. In: Hiatt HH, Watson JD, Winstein JA, eds. *Origins of Human Cancer*. New York: Cold Spring Harbor; 1977:1327–1338.
Sorahan T, Lancashire RJ. Lung cancer mortality in a cohort of workers employed at a cadmium recovery plant in the United States: an analysis with detailed job histories. *Occup Environ Med*. 1997;54(3):194–201.
Sorahan T, Lister A, Gilthorpe MS, Harrington JM. Mortality of copper cadmium alloy workers with special reference to lung cancer and non-malignant diseases of the respiratory system, 1946–92. *Occup Environ Med*. 1995;52(12):804–812.
Soto AM, Fernandez MF, Luizzi MF, et al. Developing a marker of exposure to xenoestrogen mixtures in human serum. *Environ Health Perspect*. 1997;105:647–654.
Spalding JW, Momma J, Elwell MR, Tennant RW. Chemically induced skin carcinogenesis in a transgenic mouse line (TG.AC) carrying a v-Ha-ras gene. *Carcinogenesis*. 1993;14(7):1335–1341.
Spiro SG, Silvestri GA. One hundred years of lung cancer. *Am J Respir Crit Care Med*. 2005;172(5):523.
Stankowski LF Jr, Aardema MJ, Lawlor TE, et al. Integration of Pig-a, micronucleus, chromosome aberration and comet assay endpoints in a 28-day rodent toxicity study with urethane. *Mutagenesis*. 2015;30(3):335–342.
Stankowski LF Jr, Roberts DJ, Chen H, et al. Integration of Pig-a, micronucleus, chromosome aberration, and Comet assay endpoints in a 28-day rodent toxicity study with 4-nitroquinoline-1-oxide. *Environ Mol Mutagen*. 2011;52(9):738–747.
Steenland K, Boffetta P. Lead and cancer in humans: where are we now? *Am J Ind Med*. 2000;38(3):295–299.
Steenland K, Ward E. Lung cancer incidence among patients with beryllium disease: a cohort mortality study. *J Natl Cancer Inst*. 1991;83(19):1380–1385.
Steinmetz KL, Green CE, Bakke JP, Spak DK, Mirsalis JC. Induction of unscheduled DNA synthesis in primary cultures of rat, mouse, hamster, monkey, and human hepatocytes. *Mutat Res*. 1988;206(1):91–102.
Stinchcombe S, Buchmann A, Bock KW, Schwarz M. Inhibition of apoptosis during 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated tumour promotion in rat liver. *Carcinogenesis*. 1995;16(6):1271–1275.
Stirzaker C, Millar DS, Paul CL, et al. Extensive DNA methylation spanning the RB promoter in retinoblastoma tumors. *Cancer Res*. 1997;57:2229–2237.
Stoner GD. Lung tumors in strain A mice as a bioassay for carcinogenicity of environmental chemicals. *Exp Lung Res*. 1991;17:405–423.
Stoner GD, Adam-Rodwell G, Morse MA. Lung tumors in strain A mice: application for studies in cancer chemoprevention. *J Cell Biochem*. 1993;17F:95–103.
Stoner GD, Morse MA, Kelloff GJ. Perspectives in cancer chemoprevention. *Environ Health Perspect*. 1997;105(suppl 4):945–954.
Stoner GD, Shimkin MB. Lung tumors in strain A mice for carcinogenesis bioassays. In: Milman H, Weisburger EK, eds. *Handbook of Carcinogen Testing*. Park Rigde, NJ: Noyes Publications; 1985:179–214.
Su B, Karin M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol*. 1996;8:402–411.
Sugie S, Mori H, Takahashi M. Effect of in vivo exposure to the liver tumor promoters phenobarbital or DDT on the gap junctions of rat hepatocytes: a quantitative freeze-fracture analysis. *Carcinogenesis*. 1987;8:45–51.
Sukata T, Uwagawa S, Ozaki K, et al. Detailed low-dose study of 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane carcinogenesis suggests the possibility of a hormetic effect. *Int J Cancer*. 2002;99(1):112–118.
Sundberg JP, Sundberg BA, Beamer WG. Comparison of chemical carcinogen skin tumor induction efficacy in inbred, mutant, and hybrid strains of mice: morphologic variations of induced tumors and absence of a papilloma virus. *Mol Carcinog*. 1997;20:19–32.
Sutherland LA, Bird RP. The effect of chenodeoxycholic acid on the development of aberrant crypt foci in the rat colon. *Cancer Lett*. 1994;76(2–3):101–107.
Sutherland RL, Hamilton J, Sweeney KJ, Watts CK, Musgrove EA. Steroidal regulation of cell cycle progression. *Ciba Found Symp*. 1995;191:218–228.
Swann JB, Smyth MJ. Immune surveillance of tumors. *J Clin Invest*. 2007;117(5):1137–1146.
Swenberg JA, Dyroff MC, Bedell MA, et al. O^4-Ethyldeoxythymidine, but not O^6-ethyldeoxyguanosine, accumulates in hepatocyte DNA of rats exposed continuously to diethylnitrosamine. *Proc Nat Acad Sci USA*. 1984;81(6):1692–1695.
Swenberg JA, Richardson FC, Boucheron JA, Dyroff MC. Relationships between DNA adduct formation and carcinogenesis. *Environ Health Perspect*. 1985;62:177–183.
Synder RL, Tyler G, Summers J. Chronic hepatitis and hepatocellular carcinoma associated with woodchuck hepatitis virus. *Am J Pathol*. 1982;107:422–425.
Takahashi M, Hasegawa R, Furukawa F, Toyoda K, Sato H, Hayashi Y. Effects of ethanol, potassium metabisulfite, formaldehyde and hydrogen peroxide on gastric carcinogenesis in rats after initiation with N-methyl-N′-nitro-N-nitrosoguanidine. *Jpn J Cancer Res*. 1986;77(2):118–124.

Takahashi M, Takeda J, Hirose S, et al. Deficient biosynthesis of N-acetylglucosaminyl-phosphatidylinositol, the first intermediate of glycosyl phosphatidylinositol anchor biosynthesis, in cell lines established from patients with paroxysmal nocturnal hemoglobinuria. *J Exp Med*. 1993;177(2):517–521.

Takeda J, Miyata T, Kawagoe K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-a gene in paroxysmal nocturnal hemoglobinuria. *Cell*. 1993;73(4):703–711.

Tamura G, Nishizuka S, Maesawa C, et al. Mutations in mitochondrial control region DNA in gastric tumors of Japanese patients. *Eur J Cancer*. 1999;35:316–319.

Tanaka T, Kawabata K, Kakumoto M, et al. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by citrus auraptene in rats. *Carcinogenesis*. 1998;19:425–431.

Tavani A, Negri E, Parazzini F, Franceschi S, La Vecchia C. Female hormone utilisation and risk of hepatocellular carcinoma. *Br J Cancer*. 1993;67(3):635–637.

Tchou J, Grollman AP. Repair of DNA containing the oxidatively damaged base, 8-oxoguanine. *Mutat Res*. 1993;299:277–287.

Temin HM, Rubin H. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. *Virology*. 1958;6:669–688.

Tennant RW, Margolin BH, Shelby MD, et al. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. *Science*. 1987;236(4804):933–941.

Thiersch K. Der Epithelialkrebs, Namentliehlder Ausseren Haut. Leipzig, Germany; 1875.

Thompson PA, Khatami M, Baglole CJ, et al. Environmental immune disruptors, inflammation and cancer risk. *Carcinogenesis*. 2015;36(suppl 1): S232–S253.

Tinwell H, Ashby J. Comparative activity of human carcinogens and NTP rodent carcinogens in the mouse bone marrow micronucleus assay: an integrative approach to genetic toxicity data assessment. *Environ Health Perspect*. 1994;102(9):758–762.

Timblin CR, Janssen YMW, Mossman BT. Free-radical-mediated alterations of gene expression by xenobiotics. In: Wallace KB, ed. *Free Radical Toxicology*. London: Taylor & Francis; 1997:325–349.

Trosko JE, Ruch R. Cell-cell communication and carcinogenesis. *Front Biosci*. 1998;3:208–236.

Trush MA, Kensler TW. An overview of the relationship between oxidative stress and chemical carcinogenesis. *Free Radic Biol Med*. 1991;10:201–209.

Tsuchida S, Sato K. Glutathione transferases and cancer. *Crit Rev Biochem Mol Biol*. 1992;27:337–384.

Tsutsui T, Taguchi S, Tanaka Y, Barrett JC. 17beta-estradiol, diethylstilbestrol, tamoxifen, toremifene, and ICI 164384 induce morphological transformation and aneuploidy in cultured Syrian hamster cells. *Int J Cancer*. 1997;70:188–193.

Tugwood JD, Holden PR, James NH, Prince RA, Roberts RA. A PPAR alpha cDNA cloned from guinea pig liver encodes a protein with similar properties to the mouse PPAR alpha: implications for species differences in response to peroxisome proliferators. *Arch Toxicol*. 1998;72:169–177.

Turesky RJ. Formation and biochemistry of carcinogenic heterocyclic aromatic amines in cooked meats. *Toxicol Lett*. 2007;168(3):219–227.

Turk PW, Laayoun A, Smith SS, Weitzman SA. DNA adduct 8-hydroxy-2′-deoxyguanosine (8-hydroxy-guanosine) affects function of human DNA methyltransferase. *Carcinogenesis*. 1995;16:1253–1256.

Ueda A, Hamadeh HK, Webb HK, et al. Diverse roles of the nuclear orphan receptor car in regulating hepatic genes in response to phenobarbital. *Mol Pharmacol*. 2002;61:1–6.

USEPA. Guidelines for Carcinogen Risk Assessment. EPA/630/P-03/001F, Mar 2005. 2005.

Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2′ -deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2009;27(2):120–139.

van der Gulden JW, Kolk JJ, Verbeek AL. Work environment and prostate cancer risk. *Prostate*. 1995;27(5):250–257.

Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol*. 2011;29:235–271.

Vesselinovitch SD, Hacker HJ, Bannasch P. Histochemical characterization of focal hepatic lesions induced by single diethylnitrosamine treatment in infant mice. *Cancer Res*. 1985;45:2774–2780.

Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med*. 2004;10(8):789–799.

Vogt PK. Jun, the oncoprotein. *Oncogene*. 2001;20:2365–2377.

von Sonntag C. New aspects in the free-radical chemistry of pyrimidine nucleobases. *Free Radic Res Commun*. 1987;2:217–224.

Vousden KH. Human papillomavirus and cervical carcinoma. *Cancer Cells*. 1989;1:43–50.

Vuillaume M. Reduced oxygen species, mutation, induction and cancer initiation. *Mutat Res*. 1987;186:43–72.

Waalkes MP, Anver MR, Diwan BA. Chronic toxic and carcinogenic effects of oral cadmium in the Noble (NBL/Cr) rat: induction of neoplastic and proliferative lesions of the adrenal, kidney, prostate, and testes. *J Toxicol Environ Health*. 1999;58(4):199–214.

Waalkes MP, Diwan BA, Ward JM, Devor DE, Goyer RA. Renal tubular tumors and atypical hyperplasias in B6C3F1 mice exposed to lead acetate during gestation and lactation occur with minimal chronic nephropathy. *Cancer Res*. 1995;55(22):5265–5271.

Waalkes MP, Rehm S, Sass B, Kovatch R, Ward JM. Chronic carcinogenic and toxic effects of a single subcutaneous dose of cadmium in male NFS and C57 mice and male Syrian hamsters. *Toxic Subst J*. 1994;13:15–28.

Wade N, Marsman DS, Popp JA. Dose related effects of hepatocarcinogen Wy 14,263 on peroxisomes and cell replication. *Fundam Appl Toxicol*. 1992;18:149–154.

Wainfan E, Poirier LA. Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res*. 1992;52(suppl):S2071–S2077.

Walker VE, Fennell TR, Upton PB, et al. Molecular dosimetry of ethylene oxide: formation and persistence of 7-(2-hydroxyethyl) guanine in DNA following repeated exposures of rats and rice. *Cancer Res*. 1992;52:5328–5334.

Wang Z, Li X, Wu Q, Lamb JCt, Klaunig JE. Toxaphene-induced mouse liver tumorigenesis is mediated by the constitutive androstane receptor. *J Appl Toxicol*. 2017;37(8):967–975.

Wang Z, Neal BH, Lamb JC, Klaunig JE. Mechanistic Investigation of Toxaphene Induced Mouse Liver Tumors. *Toxicol Sci*. 2015;147(2):549–561.

Ward E, Okun A, Ruder A, Fingerhut M, Steenland K. A mortality study of workers at seven beryllium processing plants. *Am J Ind Med*. 1992;22(6):885–904.

Weghorst CM, Klaunig JE. Phenobarbital promotion in diethylnitrosamine-initiated infant B6C3F1 mice: influence of gender. *Carcinogenesis*. 1989;10(3):609–612.

Weghorst CM, Periera MA, Klaunig JE. Strain differences in hepatic tumor promotion by phenobarbital in diethylnitrosamine- and dimethylnitrosamine-initiated infant male mice. *Carcinogenesis*. 1989;10(8): 1409–1412.

Wei P, Zhang J, Egan-Hafley M, Liang S, Moore DD. The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature*. 2000;407:920–923.

Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995;81:323–330.

Weisburger JH, Williams GM. Metabolism of chemical carcinogens. In: Becker FF, ed. *Cancer: A Comprehensive Treatise*. 2nd ed. New York: Plenum; 1981:241–333.

Weisburger JH, Williams GM. Bioassay of carcinogens: in vitro and in vivo tests. In: Searle CE, ed. *Chemical Carcinogens*. Vol 2. Washington, DC: American Chemical Society; 1984:1323–1373.

Weitzman SA, Turk PW, Milkowski DH, Kozlowski K. Free radical adducts induce alterations in DNA cytosine methylation. *Proc Natl Acad Sci U S A*. 1994;91:1261–1264.

Welfare M, Monesola Adeokun A, Bassendine MF, Daly AK. Polymorphisms in GSTP1, GSTM1, and GSTT1 and susceptibility to colorectal cancer. *Cancer Epidemiol Biomarker Prev*. 1999;8:289–292.

Welling R, Beaumont JJ, Petersen SJ, Alexeeff GV, Steinmaus C. Chromium VI and stomach cancer: a meta-analysis of the current epidemiological evidence. *Occup Environ Med*. 2015;72(2):151–159.

Wells A. EGF receptor. *Int J Biochem Cell Biol*. 1999;31:637–643.

Welsch CW, Adams C, Lambrecht LK, Hassett CC, Brooks CL. 17b-Oestradiol and enovid mammary tumorigenesis in C3H/HeJ female mice: Counteraction by concurrent 2-bromo-a-ergocryptine. *Br J Cancer Res*. 1977;52:1–17.

Whysner J, Ross PM, Williams GM. Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation, and tumor promotion. *Pharmacol Ther*. 1996;71:153–191.

Whyte P, Buchkovich FJ, Horowitz JM, et al. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature*. 1988;334:124–129.

Wintersberger U, Klein F. Yeast-mating-type switching: a model system for the study of genome rearrangements induced by carcinogens. *Ann N Y Acad Sci*. 1988;534:513–520.

Williams GM. In vivo and in vitro markers of liver cell transformation. In: Smith GJ, Stewart BW, eds. *In Vitro Epithelial Cell Differentiation and Neoplasia*. Cancer Forum; 1982;6:166–172.

Williams GM, Whysner J. Epigenetic carcinogens: evaluation and risk assessment. *Exp Toxicol Pathol*. 1996;48:189–195.

Witz G. Active oxygen species as factors in multistage carcinogenesis. *Proc Soc Exp Biol Med*. 1991;198:675–682.

Wood WB, Gloyne SR. Pulmonary asbestosis. A review of one hundred cases. *Lancet*. 1934;2:1383–1385.

Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice. *Proc Natl Acad Sci U S A*. 1975;72(11):4425–4429.

Yamagiwa K, Ichikawa K. Experimentelle Studie über die Pathogenese der Epithelialgeschwülste. *Mitt Med Fak Kaiserl Univ Tokio*. 1915; 15:295–344.

Yamashita N, Minamoto T, Ochia A, Onda M, Esumi H. Frequent and characteristic K-ras activation and absence of p53 protein accumulation in aberrant crypt foci of colon. *Gastroenterology*. 1995;108:434–440.

Yanagiba Y, Ito Y, Kamijima M, Gonzalez FJ, Nakajima T. Octachlorostyrene induces cytochrome P450, UDP-glucuronosyltransferase, and sulfotransferase via the aryl hydrocarbon receptor and constitutive androstane receptor. *Toxicol Sci*. 2009;111(1):19–26.

Yang SK, McCourt DW, Roller PP, Gelboin HV. Enzymatic conversion of benzo[a]pyrene leading predominantly to the diol-epoxide r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene through a single enantiomer of r-7,t-8-dihydroxy-7,8-dihydrobenzo[a]pyrene. *Proc Nat Acad Sci USA*. 1976;73(8):2594–2598.

Yoshida T. über die serienweise Verfolgung der Veränderungen der Leber der experimentellen Hepatomerzeugung durch o-aminoazotoluol. *Trans Jap Path Soc*. 1933;23:636–638.

Young SS, Gries CL. Exploration of the negative correlation between proliferative hepatocellular lesions and lymphoma in rats and mice—establishment and implications. *Fundam Appl Toxicol*. 1984;4:632–640.

Yu M, Zhang J. Serum and hair nickel levels and breast cancer: systematic review and meta-analysis. *Biol Trace Elem Res*. 2017;179(1):32–37.

Zawaski K. Evidence for enhanced expression of c-fos, c-jun, and the CA^{2+}-activated neutral protease in rat liver following carbon tetrachloride administration. *Biochem Biophys Res Commun*. 1993;197:585–590.

Zhang H, Kamendulis LM, Xu Y, Klaunig JE. The role of 8-hydroxy-2′-deoxyguanosine in morphological transformation of Syrian hamster embryo (SHE) Cells. *Toxicol Sci*. 2000;56:303–312.

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chapter 9

Genetic Toxicology

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Acknowledgments

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GENETIC TOXICOLOGY DEFINITION

Genetic toxicology is the study of genetic damage to the hereditary material that results in genetic alterations. Included in this broad definition are chemical and physical agents that cause mutagenicity, or transmissible genetic alterations, and genotoxicity. In contrast to mutagenicity, genotoxicity covers a broader spectrum of endpoints, including DNA damage such as DNA strand breaks and DNA adduct biomarkers (both pro-mutagenic and non-mutagenic), unscheduled DNA synthesis (UDS), and sister chromatid exchanges (SCEs); these are all measures of genotoxicity, as opposed to mutagenicity because they are not themselves transmissible from cell to cell or generation to generation. Genotoxicity also encompasses the mechanisms by which DNA damage occurs and the cellular responses to that damage. All of these effects can be assessed directly by measuring the interaction of agents with DNA or more indirectly through the assessment of DNA repair or the production of gene mutations or chromosome alterations. Standardized assay outcomes that detect mutations in populations of cells and/or organisms have been applied to systematic hazard classifications of chemicals under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Under GHS, the chemical hazards associated with germ cell mutagenicity are assigned to one of two hazard categories: (1) substances known to induce heritable mutations (subcategory 1A) or regarded as if they induce heritable mutations (subcategory 1B) in the germ cells of humans; and (2) substances which cause concern that they may induce heritable mutations in the germ cells of humans (UN, 2015).

It is worth mentioning that in the last decades, there has been an increased emphasis on the role of epigenetic changes that control gene expression through DNA methylation and histone modifications in the production of altered phenotypes (Watson and Goodman, 2002; LeBaron et al., 2010; Dearfield et al., 2017). Although these epigenetic changes can be transmitted between generations, these are also fluid and adaptive, are not mutations by definition as they do not involve changes in DNA sequence, and have

not been observed to lead to amended point-of-departure values for risk assessment (Hamilton, 2011; Alyea et al., 2014).

In this chapter, we will discuss the history of the development of the field of genetic toxicology, the cellular pathways that counteract DNA damage on a daily basis, the use of genetic toxicology data in cancer and genetic risk assessments, the mechanisms underlying genetic toxicology assays, the assays that can be used for detecting genotoxic endpoints, the use of the same assays for better understanding mechanisms of mutagenesis, and new methods for the assessment of genetic alterations. This field of toxicology is evolving rapidly, and a review of its past and present state will set the stage to allow for a consideration of what are likely next major landmarks.

HISTORY OF GENETIC TOXICOLOGY

The field of genetic toxicology can be considered to have its roots in the pioneering work of H.J. Muller (1927), who showed that x-rays could induce mutations in the fruit fly, *Drosophila*. In his studies, he showed not only that radiation exposure could increase the overall frequencies of mutations but also that the types of mutations induced were exactly the same in effect, or phenotype, as those observed in the absence of radiation exposure. Thus, the induced mutagenic responses should be assessed in relation to background mutations. As a conclusion to this study of radiation-induced mutations, Muller predicted the utility of mutagenesis studies not only for the study of mutations themselves but also for gene mapping approaches.

Karl Sax (1938) built upon Muller's original studies of radiation-induced mutations by showing that x-rays could also induce structural alterations to chromosomes in *Tradescantia* pollen grains. Sax and his colleagues, notably in the absence of knowledge of DNA structure and chromosomal organization, showed that at least two critical lesions in a nuclear target are required for the production of an exchange within (intrachromosome) or between (interchromosome) chromosomes. We know now that the lesions identified by Sax are DNA double-strand breaks, base damages, or multiply damaged sites that are clusters of damages within one or two helical turns of DNA (reviewed by Ward, 1988). In addition, Sax and colleagues (Sax, 1939; Sax and Luippold, 1952) showed that the yield of chromosome aberrations was reduced if the total dose of x-rays was delivered over extended periods of time or split into two fractions separated by several hours. These observations led to the concept of restitution of radiation-induced damage, which was later recognized as involving specific DNA repair processes (see below).

Consideration of the genetic effects of exogenous agents on cells was expanded to include chemicals in 1946, when Charlotte Auerbach and colleagues reported that mustard gas could induce mutations in *Drosophila* and that these mutations were phenotypically similar to those induced by x-rays (Auerbach and Robson, 1946). Thus, the field of chemical mutagenesis was initiated to run in parallel with studies of radiation mutagenesis. These original studies of Auerbach (actually conducted in 1941) are placed in a historical and biological perspective by the delightful review of Geoffrey Beale (1993).

Although the scientific value of the analysis of mutations in *Drosophila* was clear, there was an impression that the extrapolation to predict similar effects in human populations was too wide a step. Thus, a research effort of great magnitude was initiated to attempt to assess radiation-induced mutations in mice. This effort resulted in the publication by William Russell (1951) of data on x-ray–induced mutations using a mouse-specific-locus mutation assay. These data clearly showed that the type of results obtained with *Drosophila* could be replicated in a mammalian system. The mouse tester strain developed for the specific-locus assay has recessive mutations at seven loci coding for visible mutations, such as coat color, eye color, and ear shape. This homozygous recessive tester strain can be used for identifying recessive mutations induced in wild type genes at the same loci in mice treated with radiation or chemical mutagens. It was noteworthy that the mutation rate for x-ray–induced mutations in germ cells was similar in mouse and *Drosophila*. Subsequent studies by Liane Russell and colleagues showed that chemicals could induce mutations at the same seven loci (Russell et al., 1981).

Over the next 20 years, genetic toxicologists investigated the induction of mutations and chromosomal alterations in somatic and germ cells, largely following exposures to radiation, but increasingly using chemical mutagens as well. The ability to grow cells in vitro, either as primary cultures or as transformed cell lines, enhanced these quantitative studies. The in vitro culture of human lymphocytes, stimulated to re-enter the cell cycle by phytohemagglutinin, greatly expanded the information on the assessment of chromosomal alterations in human cells (Hsu, 1979). It also became feasible to use cytogenetic alterations in human lymphocytes as a biodosimeter for assessing human exposures to ionizing radiations (Bender and Gooch, 1962).

Two events during the 1970s served to expand the utility of mutagenicity data into the realm of risk assessment. The Millers and their colleagues (Miller and Miller, 1977) showed that chemical carcinogens could react to form stable, covalent derivatives with DNA, RNA, and proteins both in vitro and in vivo. In addition, they reported that these derivatives could require the biotransformation of the parent chemical to form reactive metabolites. This metabolism is required for some chemicals to become mutagens and carcinogens. Metabolic capability is endogenous in vivo, but most in vitro cell lines do not express this capacity. To overcome this for in vitro mutagenicity studies, Heinrich Malling and colleagues developed an exogenous metabolizing system based on a rodent liver homogenate (S9) (Malling and Frantz, 1973; Malling, 2004). Although this exogenous metabolism system has had utility, it does have drawbacks related to species and tissue specificity and loss of cellular compartmentalization. The development of transgenic cell lines containing P450 genes has overcome this drawback to some extent (Sawada and Kamataki, 1998; Crespi and Miller, 1999).

The second development in the 1970s that changed the field of genetic toxicology was the development by Bruce Ames (Ames et al., 1975) of a simple, inexpensive mutation assay with the bacterium *Salmonella typhimurium*. This assay can be used to detect chemically induced reverse mutations in several histidine mutant strains and can include the exogenous metabolizing S9 system described above. The Ames assay, as it is generally called, has been expanded and modified to enhance its performance as discussed below (under section "Gene Mutations in Prokaryotes"). The assay has been used extensively, especially for hazard identification, as part of the cancer risk assessment process. This use was based on the assumption that carcinogens were mutagens and that cancer required mutation induction. This latter dogma proved to be somewhat inhibitory to the field of genetic toxicology because it provided a framework that was too rigid. Nonetheless, over the decade of the mid-1970s to mid-1980s somewhere on the order of 200 short-term genotoxicity and mutagenicity assays were developed for screening potentially carcinogenic chemicals. The screens included mutation induction, chromosomal damage, DNA damage, DNA repair, and cell killing or other genotoxic activities.

Several international collaborative studies were organized to establish the sensitivity and specificity of a select group of assays

as well as to assess interlaboratory variation (IPCS, 1988). Most assays were able to detect carcinogens or noncarcinogens with an efficiency of about 70% as compared with the outcome of two-year cancer bioassays (Preston, 1999a). The concordance between standard battery of in vitro genotoxicity assays and cancer bioassays was addressed to some extent by Tennant et al. (1987), who compared the effectiveness of four individual and well-characterized short-term assays to identify carcinogens. Again, this battery predicted about 70% of known carcinogens with the concordance of each individual assay was approximately 60%. The authors concluded that no battery of tests improved substantially on the overall performance of the *Salmonella* assay. Subsequently, the lack of a tight correlation between carcinogenicity and mutagenicity (and the converse, non-carcinogenicity and non-mutagenicity) was found to be due to the fact that some chemicals were not directly mutagenic, but instead induced changes necessary for tumor development indirectly by, for example, clonally expanding preexisting mutant cells (i.e., tumor promotion), inflammation, or through the production of reactive oxygen species. Such chemicals are referred to as nongenotoxic to contrast them with genotoxic mechanism of action. In the context of the mechanism of their mutagenicity, it is preferable to distinguish between DNA-reactivity and its correlate non-DNA-reactivity. Emphasis has recently been placed on identifying mechanisms whereby not directly mutagenic chemicals can be involved in tumor production. Those identified include cytotoxicity with regenerative cell proliferation, mitogenicity, receptor-mediated processes, changes in methylation status, immunosuppression, and alterations in cell–cell communication.

In the last two decades, the field of genetic toxicology has moved away from the short-term assay approach for assessing carcinogenicity to a much more mechanistic approach, fueled by the advances in molecular biology, analytical techniques, and statistical methodologies. In addition, genetic toxicology as a field has made significant advances from a primarily qualitative assessment of hazard without consideration of the dose to a quantitative examination of the dose–response to facilitate risk assessment (Gollapudi et al., 2013; Johnson et al., 2014; Gollapudi, 2017). The ability to manipulate and characterize DNA, RNA, and proteins and to understand basic cellular processes and how they can be perturbed has advanced enormously over this period. Knowing how to take advantage of these technical developments is paramount. This chapter addresses these changes in approach to genetic toxicology: the assays for qualitative and quantitative assessment of cellular changes induced by chemical and physical agents, the underlying molecular mechanisms for these changes, and how such information can be incorporated into cancer and genetic risk assessments.

HEALTH IMPACT OF GENETIC ALTERATIONS

Mutations are permanent, transmissible alterations in DNA sequences that result in changes to the amount or structure of genetic material in the cell. Both small (i.e., one or few base-pair changes of a single gene) and large scale (i.e., changes affecting multiple genes) DNA alterations can lead to mutations. Mutagenicity refers to induction of mutations and the substances that induce mutations are referred to as mutagens. Clastogenicity refers to the process of inducing chromosomal breaks, and clastogens are the substances that induce chromosomal breakage. Clastogenicity can lead to chromosomal deletions and rearrangements and these events can lead to mutations provided that they are not lethal to the cell harboring them. Substances that induce numerical changes in chromosome complement of a cell through loss or gain of whole chromosome(s) are referred to as aneugen and these substances induce aneugenicity through changes in the ploidy (i.e., aneuploidy) of the normal chromosome complement of the cell. The importance of mutations and chromosomal alterations for human health is evident from their roles in genetic disorders, including birth defects and cancer. Mutations in both germ cells and somatic cells need to be considered when an overall risk resulting from mutations is assessed. However, mutations are also the underlying mechanism of variability and adaptation, the basis for evolution.

Somatic Cells

Somatic mutations arise in the genomes of all normal dividing and transformed cells as a result of endogenous and exogenous processes and stressors (Greenman et al., 2007). An association between mutation and cancer has long been evident, such as through the correlation between the mutagenicity and carcinogenicity of chemicals, especially in biological systems that have the requisite metabolic activation capabilities. Moreover, human chromosome instability syndromes and DNA repair deficiencies are associated with increased cancer risk (Friedberg, 1985). Recent evidence shows that an average cancer genome contains about 10^3 to 10^4 point mutations, 10 to 10^2 small insertions or deletions, and 1 to 10 large-scale chromosome rearrangements (including copy-number alterations) (Zhang and Pellman, 2015). Most somatic mutations are likely to be "passengers" that do not contribute to oncogenesis (Greenman et al., 2007). Overall, approximately 13,000 unique mutations from 5000 genes and ~340,000 tumors have been catalogued with the new next-generation sequencing (NGS) techniques (Bell, 2010). However, cancers are specifically driven by the accumulation of "driver" mutations in a subset of consensus "cancer genes" that confer growth advantage to cells; more than 410 mutated genes implicated in cancer development have been identified (Futreal et al., 2004; Greenman et al., 2007; Stratton et al., 2009; Bell, 2010; Pleasance et al., 2010). "Cancer genes" exhibit attributes that confer advantages with the capacity to generate their own mitogenic signals, the evasion of apoptosis, the resistance of exogenous growth-inhibitory signals, reprogramming of energy metabolism, evading immune destruction, cellular proliferation without limits, and acquisition of angiogenic, invasive and metastatic properties (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011); these are classified into 12 signaling pathways that regulate three core cellular processes: cell fate, cell survival, and genome maintenance (Vogelstein et al., 2013). In turn, these characteristics and dysregulated pathways allow cancer pathogenesis through genomic instability and mutability that drive tumor progression, and elevated inflammatory responses that are tumor-promoting (Hanahan and Weinberg, 2011).

The advances in the NGS technologies have enabled the comprehensive characterization of somatic mutations in large number of tumor samples, allowing for new insight and revealing unanticipated complexities in the patterns of somatic alterations in cancers (Watson et al., 2013). These advances challenge the previously held belief of a linear process driven by serially acquired new mutations, including base pair substitutions, small insertions and deletions of bases, chromosomal rearrangements, and gains and losses in gene copy number (Vogelstein and Kinzler, 1993). In a study of 210 diverse human cancers, more than 1000 somatic base pair mutations have been identified along with the evidence for "driver" mutations in approximately 120 genes that contribute to the development of the studied cancers (Greenman et al., 2007). New NGS evidence suggests that most point mutations (including small insertion or

deletion events) in cancer genomes are passengers that do not contribute to cancer development, and their patterns reflect the underlying mutational processes (Zhang and Pellman, 2015).

New NGS data show that cancer genomes have histopathologically distinct cancer subtypes of the same organ site and often have divergent underlying genomic alterations (Watson et al., 2013). The recent landmark, large-scale cancer genomics analysis, applied to almost five million somatic mutations identified in 7042 cancer samples, has provided insight into the global signatures of mutational processes in human cancer (Alexandrov et al., 2013). Each of the cancer types had at least two active mutational signatures, while some cancers, including liver and uterus, had up to six distinct mutational signatures; mutational signatures include substitutions, indels, dinucleotide substitutions, *kataegis*, and strand bias. The analysis revealed 27 distinct mutational signatures; 22 of these mutational signatures were validated and etiology was proposed for 11 of the 22 validated mutational signatures. Two major mutational signatures were associated with age of patient at cancer diagnosis and exhibited clear features of C-to-T transitions at CpG sites; these major somatic mutations were hypothesized to reflect mutations due to normal cellular processes such as deamination of 5-methylcytosine of CpG sites and DNA replication errors. These background mutations were proposed to account for the majority of somatic mutations prior to neoplastic development (Alexandrov et al., 2013). Two mutational processes were associated with the activity of endogenous APOBEC family of deaminases; these exhibit predominantly C-to-T and C-to-G mutations at TpC sites and have been observed in 17% of all examined cancer samples. Distinct mutational signatures were associated with BRCA1/2 mutations in breast and pancreatic cancers; DNA mismatch repair defects in colorectal cancers; hypermutation of the immunoglobulin genes in chronic lymphocytic leukemia; and recurring polymerase ε mutations in uterine and colorectal cancers among others. Mutational signatures were also confirmed for ultraviolet light exposure as to C-to-T transitions at CpC or TpC sites, and for "tobacco smoking signature" as C-to-A transversions.

Cancer cytogenetics have revealed chromosomal alterations, including deletions, translocations, inversions, and amplifications, and these changes have been implicated in many human leukemias and lymphomas as well as in some solid tumors (Rabbitts, 1994; Zhang et al., 2010). Also, somatic mutations in the noncoding gene promoter regions have been recognized as genetic alterations in multiple cancer types (Piraino and Furney, 2016). Cancer genomes often harbor multiple large-scale chromosomal alterations including extensive or whole-chromosome aneuploidy, telomere-bound copy-number alterations, chromosomal rearrangements, and complex karyotypes within the same region of chromosomes (Zhang and Pellman, 2015). Compared with the analysis of point mutations, the analysis of chromosomal alterations is very preliminary, but suggests that chromosomal breakage occurs frequently at centromeres and is enriched either in early-replicating, gene-rich regions or late-replicating, gene-poor regions, possibly reflecting different underlying mechanisms (Zhang and Pellman, 2015). On the phenotypic level, the large-scale copy-number alterations and chromosomal rearrangements can cause varying changes in fitness and are tumor type–dependent. Some aneuploidies are recurrent in certain tumor types and almost certainly under positive selection (Gordon et al., 2012).

Initial evidence that mutation plays a role in cancer has come from molecular studies of acquired somatic mutations in oncogenes, or positive growth regulators, and inactivation of tumor-suppressor genes. Oncogenes are genes that stimulate the transformation of normal cells into cancer cells (Bishop, 1991). They originate when genes called proto-oncogenes, involved in normal cellular growth and development, are genetically altered. Normal regulation of cellular proliferation requires a balance between factors that promote growth and those that restrict it. Mutational alteration of proto-oncogenes can lead to overexpression of their growth-stimulating activity, whereas mutations that inactivate tumor-suppressor genes, which normally restrain cellular proliferation, free cells from their inhibitory influence (Hanahan and Weinberg, 2000, 2011).

The action of oncogenes is genetically dominant in that a single active oncogene is expressed, even though its normal allele is present in the same cell. Proto-oncogenes can be converted into active oncogenes by point mutations or chromosomal alterations. Base pair substitutions in *ras* proto-oncogenes are found in many human tumors (Bishop, 1991; Barrett, 1993; Croce, 2008). Among chromosomal alterations that activate proto-oncogenes, translocations are especially prevalent (Rabbitts, 1994, Croce, 2008; Zhang et al., 2010). For example, Burkitt lymphoma involves a translocation between the long arm of chromosome 8, which is the site of the *c-MYC* oncogene, and chromosome 14 (about 90% of cases), 22, or 2. A translocation can activate a proto-oncogene by moving it to a new chromosomal location, typically the site of a T-cell receptor or immunoglobulin gene, where its expression is enhanced. A similar translocation-based mechanism also applies to various other hematopoietic cancers. Alternatively, the translocation may join two genes, resulting in a protein fusion that contributes to cancer development. Fusions have been implicated in other hematopoietic cancers and some solid tumors (Rabbitts, 1994; Croce, 2008; Zhang et al., 2010). Like translocations, other chromosomal alterations can activate proto-oncogenes, and genetic amplification of oncogenes can magnify their expression (Bishop, 1991; Croce, 2008).

Mutational inactivation or deletion of tumor-suppressor genes has been implicated in many cancers. Unlike oncogenes, the cancer-causing alleles that arise from tumor-suppressor genes are typically recessive in that they are not expressed when they are heterozygous (Evans and Prosser, 1992). However, several genetic mechanisms, including mutation, deletion, chromosome loss, and mitotic recombination, can cause loss of heterozygosity (LOH), in which the normal allele is inactivated or eliminated creating a locus with no normal gene function. LOH leads to the expression of the recessive cancer gene in a formerly heterozygous cell (Cavenee et al., 1983; Turner et al., 2003; Reliene et al., 2007). The inactivation of tumor-suppressor genes has been associated with various cancers, including those of the eye, kidney, colon, brain, breast, lung, and bladder (Fearon and Vogelstein, 1990; Marshall, 1991). Mutations in a tumor-suppressor gene called *P53*, located on chromosome 17, occur in many different human cancers, and molecular characterization of *P53* mutations has linked specific human cancers to mutagen exposures (Harris, 1993; Aguilar et al., 1994; Royds and Iacopetta, 2006).

In the simplest model for the action of tumor-suppressor genes, two events are considered to be required for the development of retinoblastoma, a tumor of the eye, because both normal alleles must be inactivated or lost (Knudson, 1997). In sporadic forms of the cancer without family history of disease, the two genetic events occur independently, but in familial cases, such as familial retinoblastoma, the first mutation is inherited, leaving the need for only a single additional event for expression. The strong predisposition to cancer in the inherited disease stems from the high likelihood that a LOH will occur by mutation, recombination, or aneuploidy in at least one or a few cells in the development of the affected organ. The simple model involving two events and a single pair of alleles cannot explain all observations concerning tumor-suppressor genes because many cancers involve more than one tumor-suppressor

gene. For example, the childhood kidney tumor called Wilms tumor can be caused by damage in at least three different genes (Marshall, 1991), and colorectal carcinomas are often found to have lost not only the wild-type *P53* tumor-suppressor gene but also other tumor-suppressor genes (Fearon and Vogelstein, 1990; Stoler et al., 1999). Moreover, a single mutation in a tumor-suppressor gene, even though not fully expressed, may contribute to carcinogenesis. For example, a single *P53* mutation in a developing colorectal tumor may confer a growth advantage that contributes to the development of the disease (Venkatachalam et al., 1998). Subsequent LOH will increase the growth advantage as the tumor progresses from benign to malignant (Fearon and Vogelstein, 1990). In this regard (mutation and selection), carcinogenesis has been likened to an evolutionary process, with genomic instability providing the substrate and with growth advantage as the selection pressure (Gatenby and Vincent, 2003; Fischer et al., 2004).

Many cancers involve both activation of oncogenes and inactivation of tumor-suppressor genes (Fearon and Vogelstein, 1990; Bishop, 1991; Croce, 2008). The observation of multiple genetic changes supports the view that cancer results from an accumulation of genetic alterations and that carcinogenesis is a multistep process (Kinzler and Vogelstein, 1996; Hahn et al., 1999; Stoler et al., 1999; Croce, 2008). At least three stages have been defined in carcinogenesis: initiation, promotion, and progression (Barrett, 1993). *Initiation* involves the induction of a genetic alteration, such as the mutational activation of a *ras* proto-oncogene either spontaneously or by a mutagen. It is an irreversible step that starts the process toward cancer. *Promotion* involves cellular proliferation in an initiated cell population. Promotion can lead to the development of benign tumors such as papillomas. Agents called promoters stimulate this process. While some mutagens can also act as tumor promoters, most promoters are not mutagens. Promotion is a reversible process and generally requires the continuous exposure to the promoter. *Progression* involves the accumulation of additional irreversible genetic changes and it is marked by increasing genetic instability and malignancy. More recent studies are beginning to change this view, leading to the concept of acquired capabilities (Hanahan and Weinberg, 2000). In their *Hallmarks of Cancer*, Hanahan and Weinberg (2000) describe a set of six acquired characteristics that are essential for the formation of all tumors irrespective of tumor type and species. These characteristics are broadly described as follows: self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. It seems probable that there is no specific order for obtaining these characteristics. Hanahan and Weinberg have revisited their hallmarks concept, and, based on progress in the field over the past decade, they have added two emerging hallmarks of potential generality to their existing six (Hanahan and Weinberg, 2011). These are reprogramming of energy metabolism and evading immune destruction. In addition, they describe genome instability and inflammation as general features that underlie their now eight hallmarks (Hanahan and Weinberg, 2011).

Genomic instability is a feature of all cancers, with the great majority having a large number of chromosomal and gene mutations and aneuploidies. Gene mutations, chromosome aberrations, and aneuploidy are all implicated in the development of cancer. Mutagens and clastogens contribute to carcinogenesis as initiators. Their role does not have to be restricted to initiation, however, in that mutagens, clastogens, and aneugens may also contribute to the multiple genetic alterations that characterize progression or the development of acquired capabilities. Other agents that contribute to carcinogenesis, such as promoters, need not be mutagens. However, the role of mutations is critical, and analyzing mutations and mutagenic effects is essential for understanding and predicting chemical carcinogenesis. Currently, there is a debate on the fraction of cancers arising from random mutations during normal cell division versus those mutations contributed by environmental and lifestyle factors, with some authors proposing that nearly two-thirds of cancer-causing mutations occur spontaneously during cell proliferation (Tomasetti and Vogelstein, 2017).

Germ Cells

The relevance of gene mutations to health is evident from the many disorders that are inherited as simple Mendelian characteristics (Mohrenweiser, 1991). About 1.3% of newborns suffer from autosomal dominant (1%), autosomal recessive (0.25%), or sex-linked (0.05%) genetic diseases (Sankaranarayanan, 1998; Elespuru and Sankaranarayanan, 2007; National Research Council [NRC], 2007a, 2007b). Molecular analysis of the mutations responsible for Mendelian diseases has revealed that almost half of these mutations are base pair substitutions; of the remainder, most are small deletions (Sankaranarayanan, 1998; Elespuru and Sankaranarayanan, 2007). In contrast to somatic mutations, common structural variants in the germ cells have been determined with NGS methods to be deletions, tandem duplications, transpositions, inversions, and insertions that affect only a small region of the genome and are nonoverlapping (Zhang and Pellman, 2015).

Many genetic disorders (such as cystic fibrosis, phenylketonuria, Tay-Sachs disease) are caused by the expression of recessive mutations. These mutations are mainly inherited from previous generations and are expressed when an individual inherits the mutant gene from both parents. New mutations make a larger contribution to the incidence of dominant diseases than to that of recessive diseases because only a single dominant mutation is required for expression. Thus, new dominant mutations are expressed in the first generation. If a dominant disorder is severe, its transmission between generations is unlikely because of reduced fitness. For dominants with a mild effect, reduced penetrance, or a late age of onset, the contribution from previous generations is undoubtedly larger than it is for mutations with severe early expression. Estimating the proportion of all Mendelian genetic diseases that can be ascribed to new mutations is not straightforward; a rough estimate is 20% (Shelby, 1994).

Besides causing diseases that exhibit Mendelian inheritance, gene mutations undoubtedly contribute to human disease through the genetic component of disorders with a complex etiology (Sankaranarayanan et al., 1999; Sankaranarayanan, 2006). Some 3% (United Nations Scientific Committee on the Effects of Atomic Radiation [UNSCEAR], 2001) or 5% to 6% (Sankaranarayanan, 1998) of infants are affected by congenital abnormalities; if one includes multifactorial disorders that often have a late onset, such as heart disease, hypertension, and diabetes, the proportion of the population affected increases to more than 60% (Sankaranarayanan, 1998, 2006; UNSCEAR, 2001). Such frequencies are necessarily approximate because of differences among surveys in the reporting and classification of disorders. A higher prevalence would be found if less severe disorders were included in the tabulation. Nevertheless, such estimates provide a sense of the large impact of genetic disease.

Sensitive cytogenetic methods have led to the discovery of minor variations in chromosome structure that have no apparent effect. On the contrary, other relatively minor structural chromosome aberrations cause fetal death or serious abnormalities. Aneuploidy (gain or loss of one or more chromosomes) also

contributes to fetal deaths and causes disorders such as Down syndrome. About four infants per 1000 live births have syndromes associated with chromosomal abnormalities, including translocations and aneuploidy. The majority of these syndromes (about 85%) result from trisomies (Griffin, 1996; Nagaishi et al., 2004; NRC, 2007a, 2007b). Most of the adverse effects of chromosomal abnormalities occur prenatally. It has been estimated that 5% of all recognized pregnancies involve chromosomal abnormalities, as do about 6% of infant deaths and 30% of all spontaneous embryonic and fetal deaths (Mohrenweiser, 1991; Nagaishi et al., 2004). Among the abnormalities that have been observed, aneuploidy is the most common, followed by polyploidy. Structural aberrations constitute about 5% of the total. Unlike gene mutations, many of which are inherited from the previous generation, about 85% of the chromosomal anomalies observed in newborns arise de novo in the germ cells of the parents (Mohrenweiser, 1991). The frequency of aneuploidy assessed directly in human sperm is 3% to 4%; about 0.4% are sex chromosome aneuploidies (Martin et al., 1991, 1996). The frequency of aneuploidy in human oocytes is about 18% (Martin et al., 1991).

As the field of oncology incorporates NGS approaches into tumor and germline analyses, comparisons of tumor to the germline genomes may identify variants associated with susceptibility to cancer as well as other hereditary diseases (Stadler et al., 2014). These new advances in NGS, together with identification of genetic sequence variants associated with common diseases, have the potential to shift the paradigm of personalized medicine, providing estimates of personal disease risk and the germline genetic risk assessment to more precisely guide medical management (Abul-Husn et al., 2014). For example, meta-analysis of somatic and germline DNA sequence biomarker variants of esophageal cancer has identified a number of germline polymorphism markers in *TP53* and nucleotide excitation DNA repair pathways associated with survival and recurrence following treatment of esophageal cancer (Findlay et al., 2015).

CANCER AND GENETIC RISK ASSESSMENTS

Cancer Risk Assessment

Genotoxicity and mutagenicity testing is a prerequisite to the assessment of carcinogenic and hereditary risks associated with chemical exposures (Moolenaar, 1994; EPA, 1996, 2005; ECHA, 2016; Eastmond, 2017). Harmonized strategies for mutagenicity testing for chemical risk assessment have been developed (Ashby et al., 1996; Cimino, 2006; Eastmond et al., 2009; MacGregor et al., 2015a). Chemicals are screened in a battery of mutagenicity and genotoxicity assays to identify carcinogenic agents with a mutagenic mode of action (MOA). Mutagenic carcinogens share common characteristics and typically produce positive gene mutation and chromosomal alteration in multiple in vitro and in vivo test systems (EPA, 2005). Mutagenic carcinogens are also active at multi-organ level, in different species, as well as at low-dose exposures across multiple doses (Tennant and Spalding, 1996). An adverse outcome pathway for mutagenic liver carcinogens was recently proposed involving a number of essential key events (KEs) (Becker et al., 2017; Moore et al., 2018). The sequence of KEs leading to cancer is initiated with a formation of pro-mutagenic DNA adduct/lesion. Following insufficient or misrepair of the adduct, a KE of an early mutation in critical gene(s) is expected that is to be followed by a KE of cellular proliferation and clonal expansion leading to an adverse outcome of hepatocellular carcinoma. However, it is important to remember that a substance that has induced tumors in animal bioassays is not necessarily acting via a mutagenic MOA (Dearfield and Moore, 2005). Moreover, accumulated evidence has demonstrated that somatic mutations are brought about by the combination of genetic effects and cellular proliferation either by (1) increasing genetic damage so there are more errors produced during DNA replication; or (2) increasing the rate of DNA replication that increases the likelihood that spontaneous errors in DNA become fixed in daughter cells (Ames and Gold, 1990; Cohen and Ellwein, 1991; Cohen et al., 1991; Cohen and Arnold 2011; Tomasetti and Vogelstein, 2015; Tomasetti et al., 2017). Thus, for carcinogen risk assessment, it is important to understand impacts not just on genotoxicity, but also on cellular proliferation rates, and to distinguish substances that are DNA-reactive from substances that act by mechanisms increasing cellular proliferation. For substances that are not DNA-reactive, mechanisms that can increase cell proliferation include receptor-mediated responses (e.g., enzyme induction CAR, PXR, AhR) and non-receptor mediated MOA (e.g., cytotoxicity leading to regeneration) (Becker et al., 2017). These MOAs can also be organized into adverse outcome pathway frameworks that provide a basis for research, regulatory toxicology testing and risk assessment, among other applications such as in eventual regulatory decision-making.

When assessing carcinogenic risks to humans, evaluation of complex genotoxicity databases should involve a weight-of-evidence approach in which studies are critically evaluated based on their quality, reproducibility and consistency, significance of genetic alternations, phylogenic relevance to humans, type (in vivo vs. in vitro), and relevance of the route of chemical administration (Eastmond, 2017). Using these guidelines, the most weight is allocated to high-quality in vivo studies of gene and chromosome mutations in humans and mammals exposed via a physiologically relevant oral, dermal, or inhalation routes (Eastmond, 2017). Following evaluation and assessment of the chemical dataset, the weight-of-evidence approach is used to reach conclusions about chemical's genotoxicity potential.

The formalized process for conducting a cancer risk assessment has many variations based on national requirements and regulations. Historically in the United States, the default has been linear extrapolation from the rodent bioassay tumor data to exposure levels consistent with human environmental or occupational exposures (NRC, 1983, 1994; EPA, 1986). The assessment of risk has required the application of a series of default approaches for extrapolation, for example, from laboratory animals to humans, from high to low exposures, from intermittent to chronic lifetime exposures, and from route to route of exposure. Subsequently, approaches based on mechanistic data were published in *Guidelines for Carcinogen Risk Assessment* to inform the risk assessment process, particularly for dose–response assessment and risk characterization at low environmental exposures (EPA, 2005). These United States Environmental Protection Agency's (EPA) cancer risk assessment guidelines provide a framework that utilizes MOA approach as the tool to organize the set of key events (KEs) required for a chemical to produce a tumor (Sonich-Mullin et al., 2001; EPA, 2005; Preston and Williams, 2005). The EPA guidelines invoke a linear extrapolation and dose–response models in case of mutagenic MOA and as a default in case of an absence of sufficient MOA information. MOA is a biologically plausible series of chemical-specific key events starting with exposure and proceeding through the interaction of an agent with a cell, subsequent physiological, tissue, or organ changes, and resulting in an adverse effect or outcome (Sonich-Mullin et al., 2001; Dellarco and Fenner-Crisp, 2012). Furthermore, this approach is more consistent with frameworks developed around a MOA/human relevance concepts (MOA framework) by the

World Health Organization/International Programme on Chemical Safety (IPCS) (Boobis et al., 2006; Meek et al., 2014a, 2014b) and International Life Sciences Institute (ILSI) (Meek et al., 2003).

The IPCS MOA framework has been now widely adopted around the world into regulatory guidance (European Food Safety Authority, European Chemicals Agency, U.S. EPA, Joint FAO/WHO Meeting on Pesticide Residues), and hazard characterization/risk assessment, although variability in its use and conclusions has led to different interpretations of the sufficiency of evidence in support of hypothesized and alternative MOAs (Dourson et al., 2013; Becker et al., 2017). The MOA framework is an analytical approach where different lines of evidence are organized, linked, and integrated at different levels of biological organization into a more efficient, hypothesis-driven approach to safety evaluation that informs a human health risk assessments of chemical exposures (Dellarco and Fenner-Crisp, 2012; Meek et al., 2014a; Becker et al., 2017). The MOA framework has been extended to enable the evaluation for species concordance of effects in the context of environmental levels of exposure (Meek et al., 2014b).

The MOA framework continuously evolves to increase transparency and defensibility of decisions as well as systematic analysis of a weight-of-evidence supporting of all postulated chemical MOAs (Becker et al., 2017). The qualitative analysis in support of a MOA includes the scoping of potential MOAs and consideration of qualitative comparative weight-of-evidence of the supporting data. The latest addition to the framework involves quantitative weight-of-evidence confidence scoring based on the evolved Bradford Hill considerations (Meek et al., 2014a, 2014b; Becker et al., 2015, 2017); this latest methodology includes evaluation of the positive, negative, or contradictory studies as well as strength of such evidence/counter-evidence that facilitates identification of the best supported MOA. The evolved Bradford Hill considerations for causality evaluate (1) biological concordance; (2) essentiality of key events; (3) dose–response concordance, temporal concordance, and incidence concordance of empirical observation; (4) consistency among different biological contexts; and (5) analogy (consistency across structurally significant chemicals) to promote better common understanding, consistency in use as well as confidence in particular MOA (Meek et al., 2014b). Lines of evidence are evaluated and integrated to elucidate whether a substance acts to induce cancer primarily via DNA reactivity or whether it acts by mechanisms that increase cell proliferation (e.g., receptor-mediated or cytotoxicity leading to regeneration). Once an animal MOA and its KEs are established, qualitative and quantitative comparisons between experimental animals and humans are made to determine whether the MOA is likely operative in humans and, if so, whether it can result in a more refined hazard and dose–response assessment (Dellarco and Fenner-Crisp, 2012; Meek et al., 2014a, 2014b).

Genetic Risk Assessment

Historically, genetic risk assessment has been interpreted in a qualitative, dichotomous "yes" or "no" manner without analysis of dose-response relationships. It has been recognized that these qualitative outcomes of in vitro genotoxicity tests do not adequately predict the outcomes of rodent carcinogenicity bioassays or in vivo genotoxicity assays. The traditional "parallelogram approach" has involved estimation of the frequency of genetic alterations in human germ cells by using rodent germ cell and somatic cell data (Brewen and Preston, 1974; Sobels, 1982; Brusick, 1994; Preston et al., 1995; UNSCEAR, 2001; Eastmond, 2012, 2017).

New in vitro and in vivo data have demonstrated the existence of nonlinear/bilinear dose responses for genotoxic effects, defined as a dose–response curve with a slope not significantly different from zero gradient below the estimated threshold or Break Point Dose (BPD); within the BPD region, no significant difference in mutant frequency between the spontaneous background of control and the low-dose exposure region following treatment with DNA-reactive agents (Klapacz et al., 2016; White and Johnson, 2016; Wills et al., 2016). Recent international multi-sector efforts have been undertaken to improve human health risk assessment for DNA-reactive chemicals utilizing a quantitative dose–response relationships from in vitro and in vivo genetic toxicology data sets to support risk characterization and risk management (Gollapudi et al., 2013; Johnson et al., 2014; MacGregor et al., 2015a, 2015b; White and Johnson, 2016).

Under the general framework for risk assessment with DNA-reactive chemicals, the identification of key elements and factors should take place such as: (1) hazard assessment; (2) assessment of exposure to genotoxicants; (3) methods for dose and effect assessment; (4) risk characterization strategies for genotoxic environmental chemicals; and (5) monitoring of environmental genotoxicant levels (Lewtas et al., 1994; Favor et al., 1995). Under this framework presented in Fig. 9-1, the risk assessment can be applicable to most situations; furthermore, the risk characterization measures require implementation by the regulatory agencies as well as monitoring of environmental exposure situations by risk managers (NAS, 1983; Lewtas et al., 1994; Favor et al., 1995; MacGregor et al., 2015a).

This framework focuses on minimization of the genotoxic risk and protecting against the risk of developing diseases (MacGregor et al., 2015a). A number of quantitative approaches for analyzing dose–response curves for genotoxic effects and deriving point-of-departure (PoD) metrics have been evaluated that are based on the research data; these PoD metrics are: the no-observed-genotoxic-effect-level (NOGEL) or the highest dose tested for which there is no significant increase in genotoxic effect compared to control; the threshold effect level (BPD or Td) or the dose at which the slope changes from zero (horizontal) to positive, with its standard error forming the confidence bounds (90% CI); the benchmark dose (BMD_{10}) or the dose that produces 10% increase over the fitted background; BMD_{1SD} (the change in response corresponding to one standard deviation) or 10% excess risk for individuals below and above the 2nd and 98th percentiles, respectively; and slope-transition-dose (STD) or the lowest dose for which the lower bound of the 95% confidence interval of the slope exceeds zero (Gollapudi et al., 2013; Johnson et al., 2014).

The PoD descriptors were determined for a number of chemicals, including the data-rich, prototypical S_N1 and S_N2-type alkylating agents (Gocke and Muller, 2009; Gollapudi et al., 2013; Cao et al., 2014; Johnson et al., 2014; Ji et al., 2016). NOGEL, $BMDL_{10}$, and $BMDL_{1SD}$ PoD metrics can be readily calculated from most gene mutation and chromosomal damage studies and the $BMDL_{10}$ values were often lower than the other PoDs (Johnson et al., 2014; MacGregor et al., 2015a). Analysis of obtained PoD values showed that these different approaches are complementary with respect to their advantages and limitations; have values within the same order of magnitude; and demonstrate more variability for the in vivo assays. The $BMDL_{10}$ is considered a satisfactory PoD matrix for the analysis of genotoxic effects, as it typically yields a good fit to the data, generally provides the most conservative value, is flexible, and can be readily applied to a wide range of datasets. It should be noted that many DNA-reactive chemicals have been demonstrated to exhibit nonlinear/bilinear dose responses for genotoxic effects, such as formaldehyde, vinyl acetate, and propylene oxide (Swenberg et al., 1983; Albertini and Sweeney, 2007; Albertini, 2013).

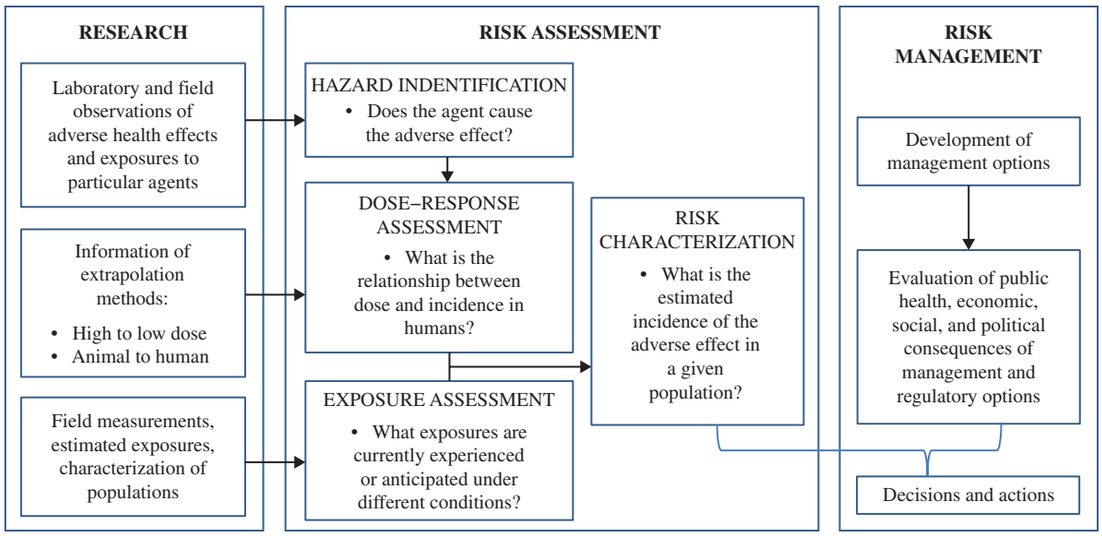

Figure 9-1. *Elements of risk assessment and risk management processes of the quantitative risk assessment framework for DNA-reactive agents.* (Reproduced with permission from MacGregor et al., 2015a.)

Once the genotoxic PoD values are obtained from in vivo test systems or target tissues with relevance to human health, appropriate extrapolation method(s) and uncertainty/assessment/safety factors need to be applied for the risk assessment. The choice of appropriate methods and assessment factors depends on knowledge of the mechanisms behind the dose–response relationships, the critical MOA information for DNA-reactive agents, and the entire weigh-of-evidence of the toxicological dataset (MacGregor et al., 2015b). The mechanistic information used by authoritative bodies in their risk assessment has included: critical involvement of non-DNA targets; contribution of DNA repair mechanisms; exceeded detoxification capacity; disruption of enzymes involved in DNA synthesis or replication; chemical reactivity or properties unlikely to occur in vivo; inadequate uptake or toxicokinetics limiting distribution to target; mutational spectrum in tumor genes similar to those in untreated animals; structural similarities to similar threshold-acting chemicals; secondary or indirect origin of the observed damage; and species and tumor-specific non-genotoxic MOA (MacGregor et al., 2015b). Parameters and safety factors that need to be evaluated in order to minimize the uncertainty in the assessment include: (a) species differences and allometric scaling; (b) differences in absorption, distribution, metabolism, and pharmacokinetics; (c) differences in duration of exposure; (d) severity of toxicity endpoint; (e) variability among individuals; and (f) uncertainty in PoD (MacGregor et al., 2015b). When data that permit assessment of these parameters/factors are not available, default adjustment factors of up to 10,000-fold can be applied to extrapolate to the acceptable human exposure levels. Knowledge of exposure, metabolism, and pharmacokinetics can help refine this extrapolation and reduce the assessment factor values by replacement with the experimentally determined parameters. Knowledge of a particular DNA adduct formed, its stability, its pro-mutagenic potential, its miscoding properties, the efficiency and fidelity of DNA repair in a particular tissue or cell type, and the replication rates of tissue or cell types can be used as a surrogate marker of a biologically effective dose (MacGregor et al., 2015b).

Overall, the derived genotoxic PoD values can be combined with appropriate assessment factors for the purpose of conducting the quantitative risk assessment for DNA-reactive agents. This approach will facilitate the derivation of reference doses below which there are no additional significant risk to human health as well as margin of exposure values useful for evaluating human health risk and regulatory decision making.

FORMATION OF DNA DAMAGE AND DNA DAMAGE RESPONSE PATHWAYS

DNA Damage

Preservation of genome integrity is central to cellular survival and successful propagation of hereditary material, yet all organisms sustain a certain number of background mutations as a result of limited DNA chemical stability, numerous stressors, cellular processes, and interactions with the environment (Lindahl, 1993). Endogenous and exogenous chemicals produce a wide array of DNA lesions such as covalent DNA base adducts, cross-links between DNA bases or between DNA bases and proteins, and single- and double-strand breaks. DNA replication, transcription, or erroneous incorporation of ribonucleotides into DNA can also be a source of spontaneous mutation and genome instability (Echols and Goodman, 1991; Datta and Jinks-Robertson, 1995; McElhinny et al., 2010). Ubiquitously present ultraviolet radiation, ionizing radiation, and exogenous hydrocarbons also present threats to genomic stability (Fig. 9-2).

In order to maintain the genome integrity and to assure accurate duplication of genomic information by DNA and RNA polymerases, this damage must be faithfully repaired and/or addressed by posttranslational activation of cell cycle networks and transcriptional networks for apoptosis/cell death (Guerard et al., 2015; Thomas et al., 2015; Klapacz et al., 2016). The understanding of the cellular mechanisms acting within the background mutation regions of the genotoxic dose–response curve can facilitate better risk characterization from exposures to DNA-reactive chemicals, the determination of points of departure (PoDs) metrics for genotoxic effects that minimize genotoxic risk and protect against disease development (Gollapudi et al., 2013; Johnson et al., 2014; MacGregor et al., 2015a, 2015b; Klapacz et al., 2016). The aim of this section is to introduce the topic of DNA damage because such damage is the molecular initiating event for the formation of genetic alterations

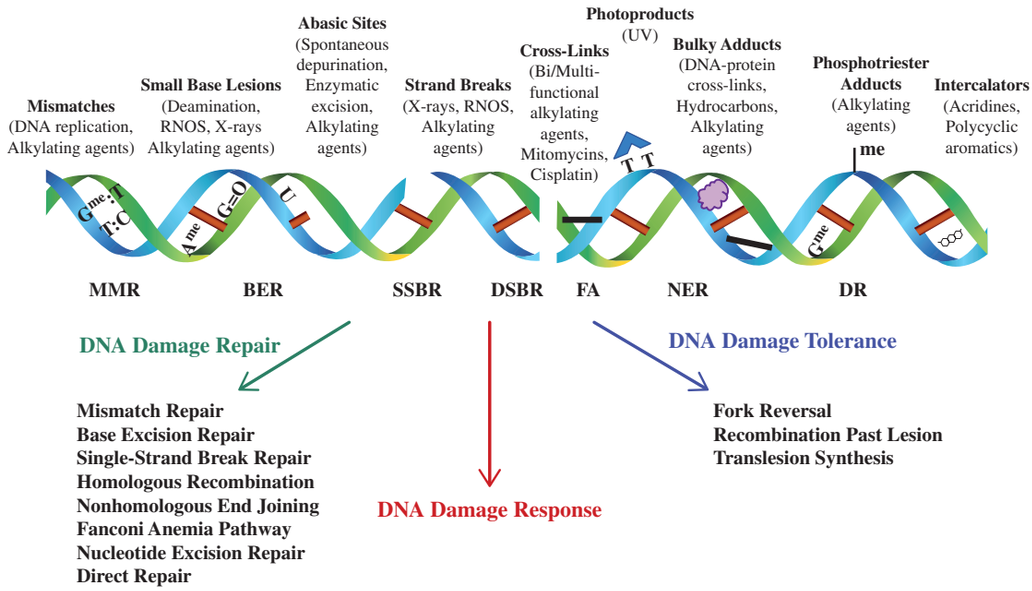

Figure 9-2. *Cellular mechanisms of DNA repair and damage tolerance to spontaneous and induced DNA damage.*

and genotoxicity in general. For an expressed mutation, several key events must occur downstream from the initial DNA adduct formation, including insufficient adduct repair, DNA replication, and cell division (Pottenger and Gollapudi, 2010). Much greater detail can be found in recent reviews that are referenced within this section.

Background DNA Damage Genomes sustain a continuous damage load due to numerous stressors, cellular metabolism, and limited chemical stability of DNA (Lindahl, 1993, 2000). The electrophilic by-products of endogenous metabolism include reactive oxygen species formed as by-products of cellular oxygen consumption (such as superoxide $O_2^{\cdot}$, hydroxyl free radicals $^{\cdot}OH$, and hydrogen peroxide), nitric oxide and its metabolites causing nitrosative stress and pro-inflammatory responses, lipid peroxidation by-products, aldehydes, and alkylators. Endogenous processes produce oxidized and alkylated bases, single- and double-strand breaks, interstrand cross-links, hydrolytic and enzymatic deamination of cytosine and 5-methylcytosine leading to uracil and thymine, respectively. In the absence of any significant exogenous exposures, the steady-state background DNA damage load was estimated at ≥50,000 lesions per cell (Swenberg et al., 2011). The spontaneous daily damage load includes ~30,000 noninstructional and pro-mutagenic abasic sites resulting from spontaneous purine losses or enzymatic adduct/base cleavage, alkylated (*N7*-methyl-dG) and oxidized (8-oxo-dG) bases, formaldehyde adducts, acrolein-dG adducts, and various etheno-purines (Lindahl, 1993; Swenberg et al., 2011). This significant and ubiquitous background of pro-mutagenic DNA background damage is similar to that caused by exogenous DNA-reactive agents and is likely causative for the normal range of background mutations (Swenberg et al., 2011; Sharma et al., 2014).

The process of DNA replication itself is error-prone as one in about 10^6 replicated bases can be mis-inserted by replicative polymerases, or the polymerase can slip at repetitive sequences resulting in as small insertion–deletion loops (IDLs). However, the frequency of mis-inserted bases in replication is not the sole determinant of the spontaneous mutation frequency. Living cells reduce the rate of polymerase error appreciably through error recognition and repair processes (Johnson, 2010). A high-fidelity polymerase may make one error in a million bases while copying roughly 300 base pairs per second, but polymerase proofreading and mismatch correction then lower the error rate as much as 1000-fold (Johnson, 2010). The evolutionarily conserved mismatch repair pathway further corrects base pair mismatches as well as IDLs that escape the proofreading activity of DNA polymerases (Modrich and Lahue, 1996; Schofield and Hsieh, 2003); mismatch repair contributes about 1000-fold to the overall DNA replication fidelity (Jaworski et al., 1995; Jiricny, 2006). The result is a low spontaneous mutation rate of the DNA replication process. A recent study based on the sequencing of 19 *Escherichia coli* genomes in a 40,000-generation experiment estimated the rate of spontaneous base pair substitutions to be 8.9×10^{-11} per base pair per generation (Wielgoss et al., 2011). Finally, high levels of transcription in all organisms stimulate a wide variety of mutation types that reflect multiple molecular mechanisms (Jinks-Robertson and Bhagwat, 2014).

Ionizing Radiation Electromagnetic waves that carry enough energy to ionize or remove electrons from an atom are defined as ionizing radiation (IR). Major exposure sources of general populations to IR include background waves, nuclear medicine, occupational exposure, industrial and consumer sources (NCRP, 2008). X-rays, γ-rays, neutrons, and α and β particles are types of IR that can produce radiochemical DNA damage either by direct or indirect mechanisms causing single- and double-strand breaks, abasic sites and a broad range of oxidative base damages from indirect creation of free radicals (Goodhead, 1994; Wallace, 1994; Ward, 1994; Cadet et al., 2011). The signature of IR exposure is the induction of DNA clustered damaged sites, defined as two or more lesions within one to two helical turns of DNA (Eccles et al., 2011). Clustered damage can either be associated with double-strand breaks, oxidized base lesions or abasic sites or with single-strand breaks, oxidized base lesions, abasic sites. These multiple lesions can be formed in DNA from the same radiation energy deposition event (Blaisdell et al., 2001). The relative proportions of these different classes of DNA damage vary with type of radiation. For example, single-strand breaks and base damages predominate with x-rays, for

which ionization density is sparse, whereas increased frequencies of single- and double-strand breaks occur as a result of exposure to α particles for which ionization is dense. The 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) base damage is considered as a biomarker for IR exposure and radiosensitivity (Erhola et al., 1997; Roszkowski and Olinski, 2012). Other base lesions such as thymine glycols, hypoxanthine, 1,N^6-ethenoadenine, or 3,N^4-ethenocytosine were also quantified following IR exposure (Le et al., 1998; Olipitz et al., 2012). It has been demonstrated that the modified histone γ-H2AX can be used as a sensitive biomarker of IR exposure following formation of DNA double-strand breaks (Nakamura et al., 2006; Mah et al., 2010; Shee et al., 2013). The alkaline comet assay is also routinely used to measure DNA strand break damage following patient exposure to IR (Alapetite et al., 1999; Dusinska and Collins, 2008; Borrego-Soto et al., 2015).

Low-dose ionizing radiation is known to induce radioadaptive responses in vitro as well as in mice in vivo. The importance of dose-rate as the critical determinant of a genotoxic and adverse outcome has been demonstrated and presumably reflects saturation of the DNA repair and damage response capabilities at high-dose exposures leading to adverse outcomes including mutation. No accumulation of DNA damage such as base lesions and micronuclei, sequence rearrangements, and gene expression of several radiation-sensitive genes following low-dose subacute exposure to 10.5 cGy γ-radiation was seen in mice (Olipitz et al., 2012). This IR dose corresponds to approximately 400-fold increase over background dose, inducing formation of additional ~400 base lesions per cell. Meanwhile, when the same total amount of radiation was delivered in one acute dose, DNA double-strand breaks and DNA stress responses were detected.

Ultraviolet Light Ultraviolet (UV) light is a type of electromagnetic nonionizing radiation with the wavelength range shorter than that of the violet end of the visible spectrum, but longer than that of x-rays. Our bodies use UV light to make vitamin D, but DNA is certainly one of its key ubiquitous targets; high UV exposure causes skin burns and can even lead to cancer. UV radiation induces two of the most predominant and cytotoxic DNA lesions, cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) as well as their Dewar valence isomers. These lesions and biological responses to these lesions in various organisms have been well characterized and are described below (Friedberg et al., 1995). Organisms have developed a number of repair and tolerance mechanisms to counteract UV-induced DNA damage (Sinha and Häder, 2002). Photoreactivation with the help of the enzyme photolyase is one of the most important and frequently occurring repair mechanisms in a variety of microorganisms. Excision repair plays an important role in eukaryotic DNA repair of UV-induced damage. In addition, mechanisms such as mutagenic repair, dimer translesion bypass, homologous recombination repair, cell-cycle checkpoints, apoptosis, and certain alternative repair pathways are also operative in various organisms to counteract consequences of UV exposures.

Chemical Agents The major classes of DNA-reactive chemicals include polycyclic aromatic hydrocarbons, arylamines and nitroaryl hydrocarbons, aflatoxins, small alkylators, oxidating, reducing and deaminating chemicals, halogenated hydrocarbons, DNA-DNA and DNA-protein cross-linkers, strand breakers, and DNA intercalators; these are presented in Figure 9-2. Chemicals can produce DNA alterations either directly by DNA-reactive mechanisms of adduct formation or indirectly by intercalation of polycyclic, aromatic, and planar chemicals between the base pairs (Heflich, 1991). The conversion of DNA adducts or other structural modifications into gene and chromosomal mutations by errors of DNA replication on the damaged template, or occasionally by errors of DNA repair, can lead to cells being initiated along the key events pathway to the adverse outcome of cancer.

Many electrophilic chemicals react with nucleophilic sites within DNA, forming covalent addition products called biomarker DNA adducts, or some other DNA structural modifications. The DNA bases involved and the positions on DNA bases can be specific for a given chemical; base pairing can protect certain base positions from the adduction. Such specificity of DNA damage can result in a spectrum of mutations that is chemical-specific (Dogliotti et al., 1998; Jarabek et al., 2009). Some alkylated bases can mispair, causing mutations when DNA is replicated, other adducts are cytotoxic, while others can be both mutagenic and cytotoxic; importantly the most common alkylating adduct at *N7* position of dG is not mutagenic. Ubiquitous alkyl adducts at the *N3* and *N7* positions of purines destabilize the *N*-glycosidic bond between the base and sugar backbone making these modified bases more susceptible to depurination into abasic sites (Wyatt and Pittman, 2006; Swenberg et al., 2011). Abasic sites are noninstructional and pro-mutagenic as the insertion of incorrect bases across from the abasic sites can cause mutations (Laval et al., 1990). The most abundant alkyl adduct produced by alkylating agents is at the *N7* position of guanine, a site that has the highest negative electrostatic potential in DNA (Pullman and Pullman, 1981; Beranek, 1990). However, *N7* C1-C3 alkyl guanine adducts are not considered to be cytotoxic or mutagenic as they do not block DNA replication and are well tolerated in the cells (Boiteux and Laval, 1983; Larson et al., 1985; Boysen et al., 2009; Philippin et al., 2014).

Bulky DNA adducts that distort DNA double helix, such as cisplatin, metabolites of benzo(*a*)pyrene, or *N*-2-acetylaminofluorene, are recognized and repaired by excision repair in a similar way to UV damages (Gillet and Scharer, 2006). These bulky adducts impede progress of DNA and RNA polymerases, causing replication fork collapse or stalled transcription, respectively. If unrepaired, these lesions can be bypassed during DNA replication in an error-prone or error-free manner by translesion synthesis (TLS).

Indirect intercalators such as acridine compounds (9-aminoacridine, ethidium bromide, etc.) and other planar molecules such as daunomycin, doxorubicin, and thalidomide in repetitive DNA sequences have been associated with the induction of frameshift mutations in which one or two base pairs have been gained or lost (Ferguson and Denny, 1990; Hoffmann et al., 2003).

DNA Repair and Tolerance Pathways

The thousands of the steady-state background DNA lesions are readily repaired in cells by an integrated network of DNA repair pathways that includes direct repair (DR), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), nonhomologous end-joining of double-strand breaks (NHEJ), homologous repair pathway (HR), and Fanconi Anemia (FA) pathway; these are presented in Figure 9-2. Among these repair activities, base excision repair is considered one of the most active, handling thousands of abasic sites, alkylated, oxidized and deaminated bases, single-strand breaks and nicks per day. These pathways also have overlapping repair substrates; for example, the most predominant abasic sites are also processed by homologous recombination between two similar or identical DNA sequences, nucleotide excision repair, or translesion synthesis that allows the DNA replication past unrepaired DNA lesions (Boiteux and Guillet 2004; Covo et al., 2004; Adar et al., 2009). The basic principles underlying DNA repair processes involve damage recognition, followed by either direct reversal of the damage (sealing

of strand breaks or removal of alkyl adducts from DNA bases) or excision of damage that involves sequential steps of repair DNA synthesis, and ligation of strands. DNA repair is usually error-free, but there may be rare events where the misrepair will result in genotoxic outcomes (Wiktor-Brown et al., 2008; Sukup-Jackson et al., 2014; Klapacz et al., 2016). DNA repair influences the genotoxic outcome, the dose–response of mutation and chromosome damage following exposure to DNA-reactive agents at all exposure levels (Kirsch-Volders et al., 2000; Parry et al., 2000; Guerard et al., 2015; Thomas et al., 2015).

In contrast, if the sustained damage is extensive, the cell can undergo apoptosis (programmed cell death), effectively avoiding it becoming a mutant cell (Evan and Littlewood, 1998). If the damage is less severe, it can be repaired by a range of processes that are a part of generalized and complex cellular response networks; these processes can return the damaged DNA to its undamaged state by error-free post-replication damage avoidance pathway utilizing HR, or to an improved, but still altered, state involving the Radiation Sensitivity Proteins RAD6 and RAD18 protein complex responsible for Proliferating Cell Nuclear Antigen (PCNA) modification and activation of translesion synthesis (TLS) (Hoege et al., 2002; McIntyre et al., 2006). During TLS, a family of prokaryotic and eukaryotic TLS polymerases, such as the human Y-family polymerases eta, iota, kappa, and Rev1, can bypass lesions that otherwise would block replication by the normal processive DNA polymerases (Rattray and Strathern, 2003; Prakash et al., 2005). These conserved polymerases have the ability to bypass specific DNA lesions or groups of lesions. The result of the lesion bypass can be an insertion of incorrect dNTP or a correct one depending on the induced lesion and the particular bypass polymerase.

This chapter presents a brief outline of the major classes of DNA repair and tolerance pathways (Fig. 9-3); much greater detail can be found in the reviews provided for each section and general reviews by Van Houten and Albertini (1995), Friedberg (2000), Krokan et al. (2000), Wood et al. (2005), Bansbach and Cortez (2011), Chatterjee and Siede (2013), and Coleman and Huang (2016).

Base Excision Repair (BER) The base excision repair (BER) pathway is one of the most active DNA repair pathways in cells operating at all cell cycle stages. BER repairs non-bulky oxidative, deaminated, and alkylated modifications to bases or the sugar phosphate backbone as well as abasic sites both in the nucleus and mitochondria (Chatterjee and Walker, 2017). BER involves five key enzymatic steps (Fig. 9-3A) to remove the initial DNA lesion and restore the original sequence, starting with (i) lesion recognition and excision of damaged base or inappropriate base by *N*-glycosylase; (ii) incision of the phosphodiester backbone at the resulting abasic site by apurinic/apyrimidinic (AP) endonuclease; (iii) termini clean-up to permit repair synthesis and/or nick ligation; (iv) gap-filling to replace the excised nucleotide by DNA polymerase; and (v) sealing of the final nick by DNA ligase enzymes.

BER is initiated by a class of lesion-specific enzymes called DNA glycosylases that recognize and cleave the damaged base to an abasic site (Lindahl, 1976; Demple and Harrison, 1994; Seeberg et al., 1995; Wood, 1996; McCullough et al., 1999; Sung and Demple, 2006); at least 11 different DNA glycosylases have been identified. Glycosylases are known to share overlapping substrates; for example, 8-oxoguanine glycosylase (OGG1), nei endonuclease VIII-like NEIL1, and NEIL3 glycosylases can all process 8-oxo-dG. DNA glycosylases initiate BER by either short-patch (one nucleotide) or long-patch (often 6 to 13 nucleotides) BER. The abasic sites formed by DNA glycosylases are recognized and processed by AP endonucleases, such as APE1 or Apn1 to create incision of the phosphodiester backbone (Wood et al., 2001). DNA synthesis to fill the repair patch is completed by DNA polymerases, such as Pol β for short-patch repair, and processive Pol δ or Pol ε polymerases for long-patch repair. Two additional strand incision repair pathways that feed into long-patch BER have been characterized: nucleotide incision repair (NIR) where AP endonuclease directly incises 5′ to oxidized and alkylated pyrimidines (Ischenko and Saparbaev, 2002); and ribonucleotide excision repair (RER) initiated by the RNase H2 complex that incises the strand 5′ to the misincorporated ribonucleotide (Sparks et al., 2012). Finally, repair of direct or indirect single stranded DNA breaks is undertaken by the single-strand break repair (SSBR) pathway that feeds into BER. SSBR is divided into four different processes beginning with detection of a break, followed by DNA end processing, gap-filling, and finally ligation of the backbone (Fan and Wilson 2005). The importance of the BER pathway is highlighted by the fact that deficiencies in the core BER enzymes are incompatible with life and result in embryonic lethality and cell death (Xanthoudakis et al., 1996; Tebbs et al., 1999; Fung and Demple, 2005). Defects in BER enzymes lead to reduced

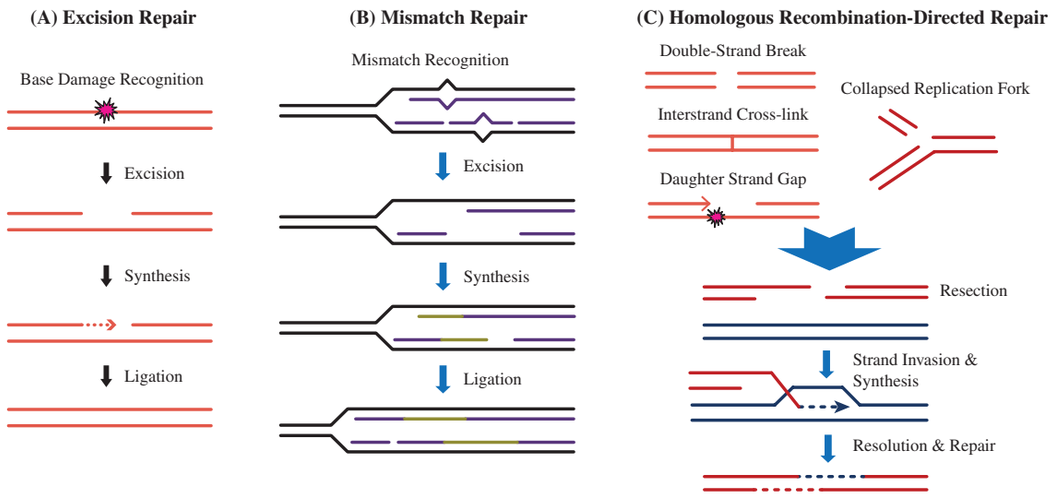

Figure 9-3. *Schematics of basic steps of key DNA repair pathways.* (**A**) Excision repair; (**B**) mismatch repair; (**C**) homologous recombination-directed DNA repair.

cell survival, elevated mutation rates, DNA-damaging agent hypersensitivities, and altered cellular responsiveness to relevant chemotherapeutics, such as alkylators (e.g., temozolomide), nucleoside analogs (e.g., 5-fluorouracil), and ionizing radiation (Kim and Wilson, 2012).

Nucleotide Excision Repair (NER) The nucleotide excision repair (NER) pathway is a highly conserved pathway that recognizes and removes bulky DNA lesions that distort DNA double helix and impede progress of DNA and RNA polymerases, causing replication fork collapse or stalled transcription, respectively (Lommel et al., 1995; Gillet and Scharer, 2006; Jiang and Sancar, 2006). Substrates for NER include UV-induced lesions; cisplatin and hydrocarbon base adducts; chemicals in tobacco smoke, aflatoxin B1, endogenous lipid peroxidation products; and ROS-induced base modifications (cyclopurines). NER uses about 30 proteins to remove the damage-containing 25 to 30 base pairs long oligonucleotide from DNA (Reardon and Sancar, 2005; Reed, 2011). The basic four steps of NER involve: (i) damage recognition and incision on both sides of the lesion, (ii) excision, (iii) repair synthesis, and (iv) ligation of the nicks (Fig. 9-3A). If unrepaired, these lesions can be bypassed in an error-prone manner by TLS polymerases. The characterization of these steps has been facilitated by the use of rodent mutant cell lines and cells from individuals with syndromes characterized by the UV-hypersensitivity, neurodevelopmental abnormalities, and skin cancers (Broughton et al., 2001). The defects in NER pathway are genetically linked to a group of autosomal recessive human diseases such as xeroderma pigmentosum (XP), Cockayne syndrome (CS), and photosensitive form of trichothiodystrophy. The cardinal feature of such patients is an extremely high incidence of UV-related cancers.

NER consists of a general pathway termed global genomic repair (GGR) that removes lesions from the entire genome and a specialized pathway referred to as transcription-coupled repair (TCR). These pathways differ in the mechanism of damage recognition, but share the same mechanism for removal and repair of the damage. In GGR, the xeroderma pigmentosum group C-complementing, or XPC complex, is the initial damage recognition factor that recognizes the damage-induced structural change in DNA, binds to the strand opposite the adduct, mediates the recruitment of transcription factor II human (TFIIH) complex, and initiates excision repair (Maillard et al., 2007; Min and Pavletich, 2007; Scharer, 2007). In TCR, stalled RNA polymerase II (RNAP) serves as the damage recognition step (Mellon and Hanawalt, 1989). In both pathways, TFIIH complex, containing XP type B and D, XPB (3′-5′) and XPD (5′-3′) helicases respectively, promotes the opening of DNA duplex around the adduct (Coin et al., 2007; Sugasawa et al., 2009; Mathieu et al., 2010). XPF-ERCC1 (for XP type F and excision repair cross complementation group 1) and XP type G (XPG) endonucleases are then responsible for dual incision 5′ and 3′ to the DNA damage, respectively (Evans et al., 1997). After the dual incision event and removal of the damage-containing oligonucleotide fragment, DNA Pols δ, ε, or κ carry out gap-filling synthesis in cooperation with replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) (Tang and Chu, 2002; Mocquet et al., 2008; Ogi et al., 2010). Finally, the nick is sealed in by ligase I or III complexes (Aboussekhra et al., 1995; Araújo et al., 2000; Moser et al., 2007; Mocquet et al., 2008).

Double-Strand Break Repair Cell survival and genome integrity are seriously compromised by the presence of broken chromosomes. Despite these consequences, double-strand breaks are exploited by cells to control a number of biological processes such as initiation of V(D)J recombination during antibody diversification, recombination of genetic material during meiosis, and decatenation of DNA molecules by topoisomerase II (Helleday et al., 2007). When double-strand breaks occur, the ends of the DNA are mostly not directly ligatable. To cope with the damage, all organisms have evolved several double-strand break repair mechanisms, of which NHEJ and HR are the most prominent (Shinohara and Ogawa, 1995; Critchlow and Jackson, 1998; Haber, 2000; Sonoda et al., 2006; Mladenov and Iliakis, 2011). Alternative error-prone double-strand break repair pathways, namely alternative end joining (alt-EJ) and single-strand annealing (SSA), have been recognized to compete for repair with classical HR (Fig. 9-3C) (Decottignies, 2013; Ceccaldi et al., 2015). These pathways are largely similar across a broad range of species from yeast to humans, although the most frequently utilized one differ among species. Unrepaired double-strand breaks activate DNA damage repair (DDR) networks that allow either recognition of the break, activation of cellular checkpoints and DNA repair, or induction of apoptosis (Misteli and Soutoglou, 2009). Chromatin plays a crucial role in the regulation of DDR and double-strand break repair (Lemaître and Soutoglou, 2014) with phosphorylated histone H2AX (γH2AX) serving as an early response following double-strand break formation (Rogakou et al., 1998, 1999). Formed γH2AX/MCD1 complex then either recruits p53 binding protein 1 (53BP1) to direct repair toward NHEJ, or BRCA1 to direct repair toward HR. MRN complex and ATM participate in both HR and NHEJ (Paull and Gellert, 1998, 2000). Recent evidence also suggests that small non-protein-coding RNAs are generated at the sites of DNA damage and control DDR network responses (d'Adda di Fagagna, 2014).

Homologous Recombination (HR) Eukaryotes undergo homologous recombination (HR) between similar or identical DNA sequences as part of their normal activities both in germ cells (meiotic recombination) and somatic cells (mitotic recombination) (Zheng et al., 2011). In eukaryotes, the process has been characterized most extensively for yeast, and these processes have been largely conserved in higher eukaryotes (Johnson et al., 1999; Krejci et al., 2003; West, 2003; Cahill et al., 2006). HR is the principal mode of double-strand break repair in the S and G_2 phases of the cell cycle and is facilitated by the proximity of a sister chromatid with essentially the identical sequence as the broken chromosome (Fig. 9-3C). HR has been proposed to be initiated by the recognition of double-strand break by the Mre11-Rad50-Nbs1 (MRN) complex that acts as a break sensor and recruits ataxia-telangiectasia mutated (ATM) kinase to the site of damage (Stracker and Petrini, 2011). The initiating step involves the processing of the DNA ends immediately adjacent to the double-strand break and the production of a 3′-single-stranded tail by exonucleases or helicase activity. This is followed by the location of regions of homology, strand invasion with D-loop formation, and branch migration whereby the single-stranded tail invades an undamaged homologous DNA molecule and Holliday junction DNA complex is formed; Holliday junction is cross-shaped structure that forms when two double-stranded DNA molecules become separated into four strands in order to exchange segments of genetic information. DNA synthesis and the resolution of the Holliday junction by its cleavage produce two DNA molecules (with or without a structural crossover). Additional models have also been proposed (Haber, 2000; Jasin and Rothstein, 2013). A detailed description of the specific enzymes known to be involved can be found in Shinohara and Ogawa (1995), Cahill et al. (2006), Helleday et al. (2007), Hiom (2010), and Jasin and Rothstein (2013).

Nonhomologous End-Joining (NHEJ) Classical nonhomologous end-joining (NHEJ) pathway joins two broken DNA ends in close spatial proximity to each other by direct re-ligation (Lieber

et al., 2003). NHEJ does not require extensive sequence homology or genetic recombination and the repair is influenced by regions of microhomology of to 14 base pairs. NHEJ is the major double-strand break repair pathway in higher eukaryotes (Burma et al, 2006). NHEJ can occur throughout cell cycle, but it predominates in the G_0/G_1 and G_2 phases (Lieber, 2010). NHEJ is also an integral part of the V(D)J recombination, generating the vast antibody diversity and T-cell receptor proteins, while ensuring genomic stability (Malu et al., 2012).

NHEJ entails three main steps: recognition of the two-ended strand breaks; processing to remove non-ligatable termini; and joining of the two ends. To initiate NHEJ, the Ku70/Ku80 heterodimer binds directly to the two broken ends, and recruits the DNA-dependent protein kinase DNA-PKcs to facilitate the alignment of the broken ends (Mimori et al., 1986; Cary et al., 1997; Ramsden and Gellert, 1998). DNA-PKcs activates DNA endonucleases that trim the 3′ and 5′ single-stranded overhangs at the DNA ends to reveal complementary nucleotide stretches (Ma et al., 2002; Neal and Meek, 2011). This processing often results in nucleotide loss. In instances where short nucleotide gaps remain after microhomology-mediated annealing, DNA Pols μ and λ fill in the small gap segments (Mahajan et al., 2002; Fan and Wu, 2004). Finally, ligase IV complex stimulates end joining to complete the repair process (Ahnesorg et al., 2006).

Fanconi Anemia (FA) Pathway Fanconi anemia (FA) DNA excision repair pathway is involved in the repair of interstrand cross-links (ICLs) produced by cross-linking chemicals such as cisplatin, diepoxybutane, and mitomycin C (Coleman and Huang, 2016). ICLs can interfere with cellular processes requiring separation of the two DNA strands, such as DNA replication and transcription. Unrepaired ICLs can lead to mutations, chromosome breakage, and mitotic catastrophe. In addition to recognition and repair of ICLs, the complex FA pathway orchestrates cooperation of other DNA repair processes to complete ICLs repair process, such as HR, NER, and TLS. Individuals with FA protein defects exhibit hypersensitivity to cross-linking agents, developmental defects, bone marrow failure, and cancer predisposition.

Mismatch Repair (MMR) The principal function of mismatch repair (MMR) system is the maintenance of genome integrity by correcting base pair mismatches and IDLs that escape the proofreading activity of DNA polymerases, and mismatches that occur after HR events (Modrich and Lahue, 1996; Jiricny, 1998; Schofield and Hsieh, 2003; Jun et al., 2006). MMR also responds to certain forms of DNA damage caused by alkylating agents, cisplatin, ROS, and UV radiation where it targets base pair mismatches following base mis-incorporation opposite DNA lesion (Earley and Crouse, 1998; Colussi et al., 2002; Mazurek et al., 2002; Stojic et al., 2004). In this role of DNA damage surveillance, MMR proteins activate cell cycle checkpoints and apoptosis through interaction with signaling kinases such as ATM (or ataxia-telangiectasia mutated), ATR (or ataxia telangiectasia and Rad3 related), and checkpoint kinases CHK1, and CHK2 (Wang and Qin, 2003; Stojic et al., 2004; Yoshioka et al., 2006; Liu et al., 2010). Individuals with defective MMR exhibit mutator phenotype (or increase in the mutation rate); microsatellite (or repetitive DNA sequence motifs) instability at mono-, di-, and trinucleotide repeats resulting in length changes of these motifs; and predisposition to hereditary Lynch cancer syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC), at an early age (Ionov et al., 1993; Shibata et al., 1994; Kolodner, 1995; Lynch and Lynch, 2000).

The principal steps of prokaryotic and eukaryotic MMR are damage recognition and binding to the mismatch, stabilizing of the binding by the addition of other proteins, cutting the DNA at a distance from the mismatch, excision past the mismatch, resynthesis, and ligation (Fig. 9-3B). Organisms differ in their strategies to discriminate the parental template strand from the newly synthesized daughter strand. These mechanisms are understood in Gram-negative bacteria such as *E. coli* that utilize the DNA methylation at the N^6 position of adenine in 5′-GATC-3′ sequences to direct the MMR-dependent strand excision to the newly synthesized, unmethylated DNA strand (Putnam, 2016). In higher eukaryotes the strand discrimination mechanisms are not completely understood; however, MMR is associated with eukaryotic replication machinery, suggesting that this interaction facilitates strand discrimination (Modrich, 1997; Mastrocola and Heinen, 2010; Pluciennik et al., 2010). Data implicate PCNA (the "clamp") together with replication factor C (RFC) complex ("clamp loader") in early steps of eukaryotic MMR, as well as in gap repair synthesis. Following mismatch recognition, the mutS homolog (MSH) heterodimers convert to ATP-dependent sliding clamps and recruit mutL homolog (MLH) heterodimers that together coordinate daughter strand excision. MutLα complex is a latent endonuclease activated by MutSα, RFC, and PCNA in a mismatch- and ATP-dependent manner (Kadyrov et al., 2006). DNA daughter segment flanked by two-strand breaks is then removed by exonuclease 1 and finally replaced by the processive DNA synthesis machinery (Kadyrov et al., 2006).

Direct Repair (DR) The error-free direct repair (DR) mechanism is defined as the elimination of DNA and RNA damage using chemical reversion that does not require a nucleotide template, breaking of the phosphodiester backbone, or DNA synthesis (Nay and O'Connor, 2013). There are two distinct DR mechanisms in the majority of organisms: photoreactivation of UV-induced pyrimidine dimers by photolyases, and the removal of the alkyl groups from DNA and RNA by methyltransferases and dioxygenases.

Photolyases have been identified in various organisms, but were lost early in the mammalian evolution and are not present in humans (Gerkema et al., 2013). DNA photolyases repair the UV-A- and UV-B-induced major pyrimidine dimers in DNA and RNA (Kim and Sancar, 1993). Photolyases bind to DNA containing a pyrimidine dimer in a light-independent reaction and absorb a photon to initiate the splitting of the dimer into two pyrimidines (Rupert et al., 1958; Rupert, 1961; Sancar, 1994).

Methylguanine DNA Methyltransferase (MGMT) O^6-Alkylguanines are major cytotoxic and pro-mutagenic adducts produced by a variety of endogenous and exogenous alkylating chemicals (Loechler et al., 1984; Goldmacher et al., 1986; Beranek, 1990). If the cells enter S phase prior to O^6-methylguanine (O^6-MeG) repair, processive DNA polymerases commonly mispair O^6-MeG with dT resulting in O^6-MeG:T mismatch and fixation of a G:C-to-A:T transition mutation in the second round of replication (Fig. 9-4) (Ito et al., 1994). The ubiquitous methylguanine DNA methyltransferases (MGMT) remove alkyl from O^6 position of dG. The human MGMT protein is constitutively expressed, housekeeping gene that is expressed in all tissues (Pieper, 1997). In all organisms, the alkyl group (such as methyl, ethyl, propyl, butyl, benzyl, and 2-chloroethyl) is transferred via a one-step reaction to the repair protein by covalent binding to a highly conserved, active site cysteine directly restoring the DNA base and inactivating the protein (Tano et al., 1990; Grombacher et al., 1996; Margison et al., 2003; Parkinson et al., 2008). This is referred to as a "suicide mechanism" where the protein is inactivated and targeted for elimination by proteasome (Srivenugopal et al., 1996). A clear link between the levels of MGMT repair protein and the existence of thresholds for *HPRT* gene mutation induction was demonstrated in human-derived

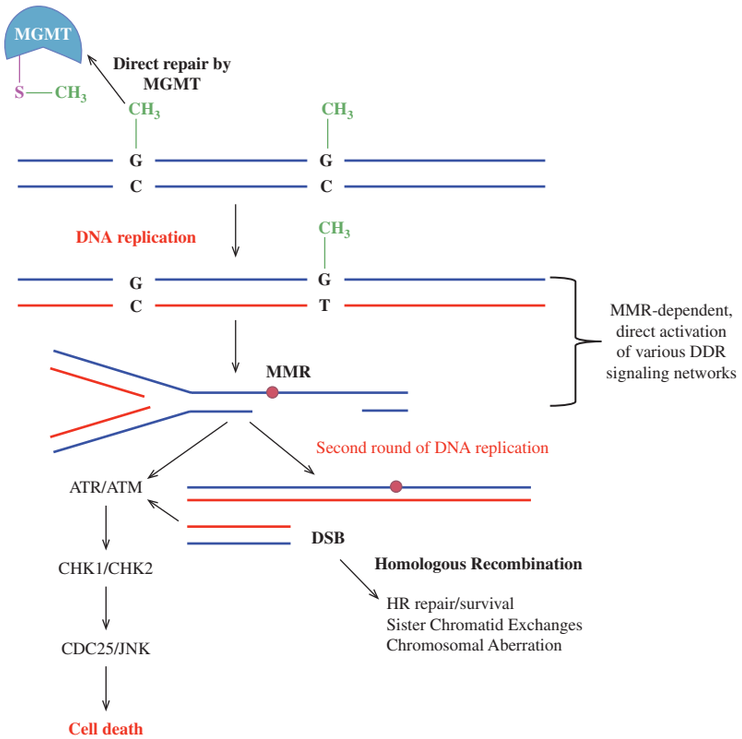

Figure 9-4. *Interactions of DNA repair and damage response pathways in the processing and repair of O^6-MeG adducts in DNA.* MGMT repair protein directly repairs O^6-MeG adducts. If unrepaired, O^6-MeG preferably mispairs with T during DNA replication leading to G:C-to-A:T transitions. Alternatively, O^6-MeG lesion induces apoptosis via the mismatch repair-dependent pathway. O^6-MeG/mismatch repair-dependent DNA damage response includes multi-DNA damage response pathway, multi-time scale signaling network activation led by early ATM, H2AX, ATR-CHK1 kinases, and p53 phosphorylation, then followed by late phosphorylation of ATM-CHK2 and JNK kinase, as well as dramatic increases in p53 levels and p53 transcriptional target. Sister chromatid exchanges and chromosomal aberrations are induced by O^6-MeG lesions via the mismatch repair-dependent pathway in the second cell cycle. The gaps and nicks present during this phase can form double-strand breaks that are handled by homologous recombination. With loss of mismatch repair activity, cells become "methylation-tolerant" accumulating mutations and escaping cell death in the presence of unrepaired O^6-MeG. (Reproduced with permission from Klapacz et al., 2016.)

AHH-1 cells (Thomas et al., 2013). When levels of MGMT are not adequate or depleted, the MMR pathway serves as a backup repair pathway by eliminating cells with O^6-MeG bases and preventing mutation (Fig. 9-4) (Duckett et al., 1996; Kaina et al., 1997; Cejka et al., 2004). O^6-alkylguanine adducts can also be repaired by NER pathway (Taira et al., 2013). Biochemical studies have revealed that O^6-MeG can be bypassed by TLS polymerases Pol η, Pol κ, and Pol ξ (Ito et al., 1994).

Alkbh Fe(II)/α-Ketoglutarate-Dependent Dioxygenases Direct repair of *N3*-cytosine and *N1*-adenine alkyl adducts in DNA and RNA has been associated with the action of alkylation protein B (AlkB) family of dioxygenases (Kataoka et al., 1983; Delaney et al., 2005; Frick et al., 2007). The AlkB enzyme was first discovered in *E. coli* and a number of its homologues (ALKBH) have been identified in humans (Aravind and Koonin, 2001; Kurowski et al., 2003). DNA adducts that are repaired by these enzymes interfere with base-pairing, block DNA replication and transcription, and trigger cell cycle checkpoints and apoptosis (Kataoka et al., 1983; Kurowski et al., 2003; Westbye et al., 2008). Dealkylation reaction catalyzed by AlkB and ALKBH occurs via transformation of α-ketoglutarate into succinate and restoration of the undamaged base (Trewick et al., 2002; Lee et al., 2005); removal of methyl adducts releases formaldehyde, removal of ethyl adducts releases acetaldehyde, whereas removal of etheno adducts releases glyoxal (Trewick et al., 2002; Delaney and Essigmann, 2004). *E. coli* AlkB mutants and Alkbh2- and Alkbh3-deficient mouse embryonic fibroblasts exhibit increased sensitivity to S_N2-type alkylating chemicals and increased mutant frequency (Delaney and Essigmann, 2004; Ringvoll et al., 2006; Nieminuszczy et al., 2009).

Translesion Synthesis (TLS) Translesion synthesis (TLS) is carried out by 11 highly conserved and specialized DNA polymerases that are lesion- and species-specific (Prakash et al., 2005). TLS involves a temporary switch from the replicative, high-fidelity polymerases to specialized, error-prone polymerases that help the cells bypass and tolerate the unrepaired damage (Moldovan et al., 2007). Common characteristics of TLS polymerases are large active sites that accommodate the damage, fewer contacts with DNA, low processivity, and lack of 3′–5′ exonuclease activity. For example, yeast and human homologues of TLS Pol η replicate past TT dimers in an error-free manner; however, these incorrectly insert dG across from thymidine in 6-4 pyrimidine dimer photoproducts causing T-to-C mutations (Masutani et al., 2000; Johnson et al., 2001). XPV (xeroderma pigmentosum variant) patients have demonstrated congenital lack of this Pol η that leads to UV-hypersensitivity and predisposition to cutaneous malignancies (Tung et al., 1996; Masutani et al., 1999; Kannouche et al., 2004). Two TLS models have been proposed: the polymerase switch model to accommodate lesion bypass, and single-strand gap-filling model that typically falls outside of S phase of cell cycle (Chatterjee and Walker, 2017). After nucleotides are incorporated across from the damaged bases, the aberrant primer termini are typically extended by Pol ζ.

DNA Damage Response (DDR) DNA damage response (DDR) networks effectively remove thousands of background DNA load and maintain a very low level of spontaneous mutagenesis (Chatterjee and Siede, 2013). DNA repair enzymes and signaling networks are regulated by DDR kinases including ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-Related), and DNA-PKcs (DNA-dependent protein kinases) at the sites of DNA damage via posttranslational protein modification cascades. These kinases sense the damage together with their mediator proteins, then pass the damage signal onto the transducer checkpoint kinases CHK1 and CHK2 (Bartek et al., 2001; Chen and Sanchez, 2004; Tanaka, 2010), followed by direct or indirect mediators of cell cycle progression such as p53, cell division cycle 25 CDC25A, B, or C phosphatases (Falck et al., 2001; Zhao et al., 2002; Sørensen et al., 2003). Activation of the p53 checkpoint provides the cell with a more prolonged G_1 cell cycle arrest (Bartek and Lukas, 2001). The p53 protein is phosphorylated directly by ATM and ATR as well as by CHK1 and CHK2 to stabilize and increase the level of cellular p53 (Shiloh, 2003). As most mutations in DNA occur when a cell with unrepaired damage enters S phase of the cell cycle, DDR checkpoints arrest the progression of cell cycle to allow additional time for excision repair of the damage, or for DNA damage avoidance, or for TLS. Should the checkpoints fail to prevent polymerase stalling, or if the damage is too extensive, cells can undergo apoptosis to eliminate heavily damaged or dysregulated cells.

As cells continually respond to endogenous DNA damage, the adaptive and homeostatic responses have been shown to also be utilized following exposures to DNA-reactive agents at all exposure doses (Noonan et al., 2012; Tentner et al., 2012; Li et al., 2014; Zhang et al., 2015). The fast-acting posttranslational networks, involving signaling ATM, ATR, p38, CHK2, and p53 cascades, are the main contributors to the avoidance of genotoxic outcomes at endogenous and at low exogenous DNA damage load of various classes (Zhang et al., 2014, 2015). However, the transcriptional up-regulation of genes occurred at higher concentrations of bilinear dose–response curves with clear increases in micronuclei formation (Seidel et al., 2004; Zhang et al., 2014, 2015). ATM signaling kinase and p53 tumor suppressor are key players in DDR, but cells can utilize parallel DDR signaling pathways that are independent of ATM and p53 cascades, underscoring the complexity and interconnectedness of biological responses to DNA damage (Hickman and Samson, 1999; Debiak et al., 2004; Noonan et al., 2012).

Taken together, the probability that DNA damage can be converted into a genetic alteration is influenced by the dose–response relationships of various lesion classes, activated pathways, the complex biological interactions between these pathways, the rate of the damage repair, and the fidelity and completeness of the repair. The mechanisms of induction of gene mutations and chromosome alterations discussed in the following sections build upon the assessment of the probability and the mechanistic knowledge of repair versus mis-repair versus non-repair of the different DNA repair and DDR mechanisms.

MECHANISMS OF INDUCTION OF GENETIC ALTERATIONS

Formation of Gene Mutations

Somatic Cells Mutations result in changes to the structure of a gene (Lodish et al., 2000). Gene mutations are considered to be small DNA sequence changes confined to a single gene such as point mutations, which involve alterations in a single base pair that affect the function of only one gene, and small deletions/additions in any DNA region. Larger genomic changes are considered below, under the section "Formation of Chromosomal Alterations." Changes in a single base pair may produce one of three types of mutation: *missense mutation,* which results in a protein with one amino acid substituted for another; *nonsense mutation,* in which a stop codon replaces an amino acid codon, leading to premature termination of translation; and *frameshift mutation,* which causes a change in the reading frame by an addition or deletion of one or a few base pairs (not in multiples of three) in protein-coding regions, leading to pre-mature termination of the protein (Ripley; 1991; Lodish et al., 2000). Base substitutions can be further subdivided as transitions where the change is purine for purine or pyrimidine for pyrimidine, and transversions where the change is purine for pyrimidine and vice versa. Historically, nonsense mutations where the stop codon in the transcribed RNA is UAA are termed as *ochre* mutations, those with UAG codon are called *amber* mutations, and those with UGA are referred to as *opal* mutations. For the discussion of the mechanism of induction of gene mutations and chromosomal alterations, it is necessary to distinguish substances by their general mode of action (MOA). Chemicals that can produce genetic alterations with similar effectiveness in all stages of the cell cycle are called radiomimetic because they act like radiation; the radiomimetic group includes bleomycin, streptonigrin, neocarzinostatin, and 8-ethoxycaffeine. Mutations caused by such chemicals tend to be deletions caused by DNA strand breaks ranging in size from a few bases to multilocus events (Thacker, 1992). As discussed previously, DNA strand breaks are rapidly repaired by multiple repair processes before DNA replication takes place. Therefore, mutations induced by ionizing radiation tend to be the result of errors of DNA repair (Preston, 1992). In contrast, chemicals that produce genetic alterations far more effectively in the S phase of the cell cycle are described as nonradiomimetic. The great majority of chemicals are nonradiomimetic.

Gene mutations can arise in the absence of specific exogenous exposures to radiation and chemicals. The great majority of spontaneous mutations arise from polymerase slippage, DNA biosynthetic errors, and replication errors of an altered template; spontaneous altered template can be a result of oxidative and alkylated damage, or the deamination of bases especially cytosine in DNA/5-methylcytosine at CpG sites resulting in uracil or thymine, respectively, eventually leading to G:C-to-A:T transitions after two rounds of DNA replication (Shinde et al., 2003; Jinks-Robertson and Bhagwat, 2014).

Gene mutations induced by a majority of chemicals and non-ionizing radiations are base substitutions, frameshifts, and small deletions produced by errors of DNA replication on a damaged template. The relative mutation frequency of an adduct will be the outcome of kinetics between its repair and DNA replication, that is, in general terms, the more repair that takes place prior to replication, the lower the mutation frequency for a given amount of induced DNA damage. Furthermore, the nature of an unrepaired adduct dictates its miscoding properties expressed by DNA polymerases during the S phase (Jarabek et al., 2009). Not all DNA adducts are miscoding; small alkyl adducts frequently found at the *N7* position of guanine are not mutagenic and do not block DNA replication progress (Beranek, 1990; Boysen et al., 2009; Swenberg et al., 2011; Philippin et al., 2014). Activation of S phase at a G_1/S cell cycle checkpoints through signaling cascades involving kinases such as ATM, ATR, CHEK1, CHK2, and p53 is an important modulator of these kinetics, as more repair can take place prior to the cell starting/re-starting DNA replication (Mercer, 1998; Giglia-Mari et al., 2011). Finally, error-prone translesion synthesis past

pro-mutagenic lesions can lead to mutations (Fix et al., 2008; Sale, 2013).

Germ Cells Mutations in germ cells are changes to the DNA sequence of sperm or egg that can be inherited by the offspring (Yauk et al., 2015). The mechanism of production of gene mutations in germ cells is basically the same as in somatic cells. It was estimated that a child inherits an average of one de novo mutation per 100×10^6 parental nucleotides (Roach et al., 2010; Conrad et al., 2011). An important consideration for assessing gene mutations induced by chemicals in germ cells is the relationship between exposure and the timing of DNA replication. In the mammalian spermatogonial stem cell, only a small fraction of the 8-day or longer cell cycle is being occupied by the S phase; thus, the probability of DNA repair taking place prior to DNA replication is high. However, for considerations of the genetic risk, the spermatogonial stem cell is present throughout the reproductive lifetime of an individual; each time it divides, it produces a differentiating spermatogonium and a stem cell that can accumulate genetic damage. Differentiating spermatogonia undergo a series of mitotic divisions before entering meiosis, followed by a series of morphological transformation to produce spermatozoa (Clermont, 1972); the late spermatid/spermatozoa stages become DNA repair deficient (Marchetti and Wyrobek, 2005; Olsen et al., 2005). Postmeiotic germ cells are particularly sensitive to mutation induction by nonradiomimetic chemicals, especially following acute exposures (Russell, 2004). The fairly short duration of this stage (approximately 21 days in the mouse) means that their contribution to genetic risk following chronic exposures is quite small. The DNA damage incurred during late spermatid/spermatozoa stages can be repaired post-fertilization using the egg's repair proteins (Marchetti et al., 2007). In contrast, oocyte is resistant to the induction of gene mutations by nonradiomimetic chemicals for which DNA repair is the mode of formation of mutations, and DNA repair occurs in oocytes as there is no further S phase until the zygote (Brewen and Preston, 1982).

The mechanistic aspects of the production of heritable gene mutations in male premeiotic germ cells has recently been considered for monofunctional alkylating chemicals and presented as an adverse outcome pathway (AOP) (Yauk et al., 2015). In general, AOPs describe a simplified series of key events (KEs) along the biological organization of organisms that lead to an adverse health or population effects (Villeneuve et al., 2014a, 2014b). An AOP links a molecular initiating event (MIE) to an adverse outcome (AO) through intermediate KEs. The tailored Bradford-Hill considerations for key event relationships (KERs) and evidence evaluation of the essentiality of KEs to AO are systematically assessed under this framework. The AOP for alkylation of DNA in male premeiotic germ cells leading to heritable mutations is presented in Fig. 9-5. The MIE for this AOP has been designated as the alkylation of DNA in male premeiotic germ cells. This MIE is followed by the KE1: insufficient or incorrect DNA repair in male premeiotic germ cells, and KE2: increased mutations in male premeiotic germ cells. Mutations that do not impair spermatogenesis persist and eventually are present in mature sperm can be measured with in vivo transgenic rodent mutation (OECD TG 488, 2013) assay or mutations in simple tandem repeat DNA sequences (Yauk, 2004). Eventually, these series of KEs lead to an adverse outcome (AO) of increased heritable mutations in the offspring. Inherited mutations in the offspring can lead to embryonic or fetal death, or be transmitted to the offspring and cause phenotypic outcomes; thus, heritable mutation is an important regulatory endpoint used by the agencies around the world for risk assessment and regulatory purposes (Vinken, 2013; Yauk et al., 2015). The overall AOP is generally accepted as dogma with strong essentiality of its KEs and KERs as well as strong biological plausibility without notable inconsistencies and uncertainties.

Formation of Chromosomal Alterations

Somatic Cells

Structural Chromosome Aberrations Large-scale changes in chromosome structure can affect the functioning of numerous genes, resulting in major phenotypic consequences (Lodish et al., 2000). These chromosomal mutations, or abnormalities, can involve deletion or insertion of several contiguous genes, inversion of genes on a chromosome, or the exchange of large segments of DNA between nonhomologous chromosomes. Chromosome aberrations induced by ionizing radiations are generally formed by errors of DNA repair, whereas those produced by nonradiomimetic chemicals are generally formed by errors of DNA replication on a damaged DNA template.

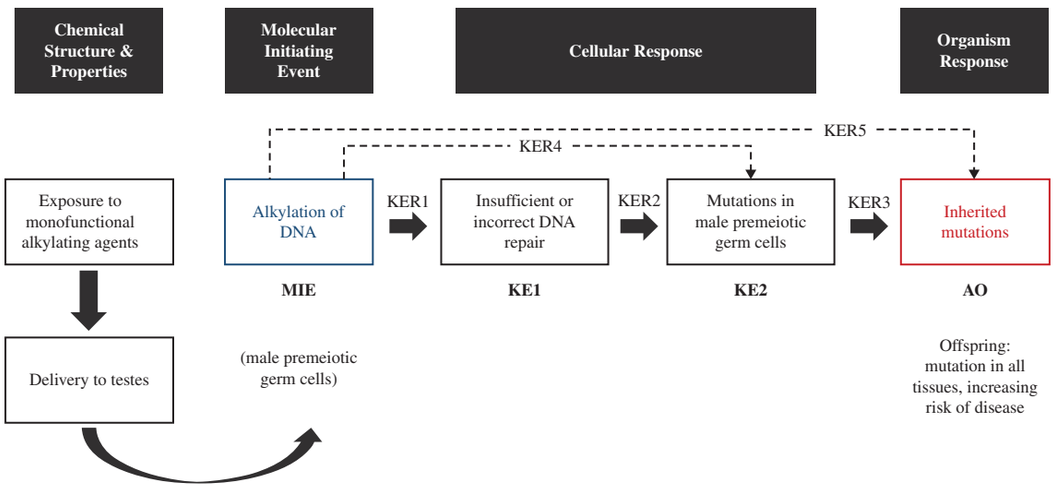

Figure 9-5. *Flow diagram of the alkylation of DNA in male premeiotic germ cells leading to heritable mutations AOP depicting the MIE, KEs, AO, and KERs.* (Reproduced with permission from Yauk et al., 2015.)

The DNA repair errors that lead to the formation of chromosome aberrations following ionizing radiation (and radiomimetic chemical) exposure arise from misligation of double-strand breaks or interaction of coincidentally repairing regions during excision repair of damaged bases. The overall kinetics and fidelity of DNA repair influence the sensitivity of cells to the induction of chromosomal aberrations produced by misrepair. The broad outcomes of misrepair are that incorrect rejoining of chromosomal pieces during repair leads to chromosomal exchanges within (e.g., inversions and interstitial deletions) and between (e.g., dicentrics and reciprocal translocations) chromosomes. Failure to rejoin double-strand breaks or to complete the repair of other types of DNA damage leads to terminal deletions.

Acentric fragments arise from interstitial deletions, terminal deletions, and the formation of dicentric chromosomes and rings. The failure to incorporate an acentric fragment into a daughter nucleus at anaphase/telophase, or the failure of a whole chromosome to segregate at anaphase to the cellular poles, can result in the formation of a membrane-bound micronucleus that resides in the cytoplasm.

Errors of DNA replication on a damaged template can lead to a variety of chromosomal alterations. The majority of these involve deletion or exchange of individual chromatids (chromatid-type aberrations). Thus, nonradiomimetic chemicals induce only chromatid-type aberrations, whereas radiation and radiomimetic chemicals induce chromatid-type aberrations in the S and G_2 phases of the cell cycle, but chromosome-type aberrations affecting both chromatids in G_1. The reason for this latter observation is that the G_1 (or G_0) chromosome behaves as a single DNA molecule and aberrations formed in it will be replicated in the S phase and will involve both chromatids. This distinction is important for considerations of outcome of the aberrations and the probability of an effect on cells because for chromatid-type aberrations, one chromatid remains intact and genetically unaltered, in contrast to chromosome-type aberrations in which both chromatids are damaged (Preston et al., 1995).

Numerical Chromosome Changes Numerical changes (e.g., monosomies, trisomies, and ploidy changes) can arise from errors in chromosomal segregation. The complexity of the control and the mechanics of the mitotic process means that alteration of various cellular components can result in failure to segregate the sister chromatids to separate daughter cells or in failure to segregate a chromosome to either pole (Bickel and Orr-Weaver, 1996; Preston, 1996; Hunt, 2006). The mechanisms underlying chromosomal loss are pertinent to those involved in the formation of micronuclei. Aneuploidy is a known cause of human disease and represents a major cause of infertility, pregnancy failure, and serious genetic disorders in the offspring.

A limited set of chemicals has been demonstrated to cause aneuploidy through interaction with components of the structures that facilitate chromosome movement (Preston, 1996; Aardema et al., 1998). These include benomyl, griseofulvin, nocodazole, colchicine, colecemid, vinblastine, and paclitaxel. These chemicals affect tubulin polymerization or spindle microtubule stability. A group of small molecule drugs that are targeted to inhibit Aurora kinases, a family of serine/threonine kinases important for proper attachment of microtubules at spindle assembly during cell division, have been shown to induce aneuploidy (Gollapudi et al., 2014; Bavetsias and Linardopoulos, 2015). To date, other mechanisms of aneuploidy induction by chemicals have not been firmly identified.

Germ Cells The formation of chromosomal alterations in germ cells is basically the same as that for somatic cells, namely, via misrepair for ionizing radiations and radiomimetic chemicals for treatments in G_1 and G_2, and by errors of replication for all radiations and chemicals for DNA damage present during the S phase.

The types of aberrations formed in germ cells are the same as those formed in somatic cells (e.g., deletions, inversions, translocations), although their appearance in diplotene/diakinesis of meiosis I, where analysis is frequently conducted, is rather different because of the homologous chromosome pairing that takes place in meiotic cells (Léonard, 1973). The specific segregation of chromosomes during meiosis influences the probability of recovery of an aberration, particularly a reciprocal translocation, in the offspring of a treated parent (Preston et al., 1995).

An adverse outcome pathway (AOP) for chemical binding to tubulin in oocytes leading to aneuploid offspring has been developed and published (Marchetti et al., 2016). Colchicine, a prototypical chemical that binds to tubulin and causes microtubule depolymerization has been used to develop the AOP. In this AOP specific to female germ cells exposed during the periovulation period, chemicals that bind to tubulin (MIE) cause microtubule depolymerization (KE1) resulting in spindle disorganization (KE2) followed by altered chromosome alignment and segregation (KE3) and the generation of aneuploid oocytes (KE4), ultimately leading to an increased incidence of aneuploidy syndromes in the offspring (AO). The available evidence suggests that the MIE and KEs are conserved across species and would be relevant for human oocytes. The development of AOPs related to mutagenicity in germ cells is expected to aid the identification of potential hazards to germ cell genomic integrity and support regulatory efforts to protect population health (Marchetti et al., 2016).

ASSAYS FOR DETECTING GENETIC ALTERATIONS

Introduction to Assay Design

Genetic toxicology assays are used to identify germ cell mutagens, somatic cell mutagens, and potential carcinogens. These assays can detect diverse kinds of genetic alterations (e.g., gene mutations, chromosome aberrations, and aneuploidy) that are relevant to the production of adverse human health outcomes. Over the last three decades, hundreds of chemicals and complex mixtures have been evaluated for genotoxic effects. Genetic toxicology assays serve two interrelated but distinct purposes in the toxicologic evaluation of chemicals: (1) identifying mutagens for purposes of hazard identification and (2) characterizing dose–response relationships and mutagenic mechanisms, both of which contribute to an understanding of genetic and carcinogenic risks.

A common experience when surveying the genetic toxicology literature is encountering a bewildering array of assays in viruses, bacteria, fungi, cultured mammalian cells, plants, insects, and mammals. More than 200 assays for mutagens have been proposed, and useful information has been obtained from many of them. Although most genetic toxicology testing and evaluation relies on relatively few assays, data from relatively obscure assays can sometimes contribute to a judgment about the genetic activity of a compound.

Table 9-1 lists key assays that have a prominent place in genetic toxicology. Table 9-2 is a more comprehensive list that provides literature citations to many of the assays that one might encounter in the genetic toxicology literature. Even this extensive table is not exhaustive, in that it emphasizes methods in applied genetic toxicology and not those assays whose use has been largely restricted to studies of mutational mechanisms. The commonly used assays rely on phenotypic effects as indicators of gene mutations or small deletions and on cytological methods for observing gross chromosomal damage. Detailed information on assay design, testing data, controls, sample sizes, and other factors in effective testing is found in the references cited.

Table 9-1

Principal Assays in Genetic Toxicology

BATTERY	ASSAY TYPE	ASSAY DESCRIPTION
Current strategies for genotoxicity testing in vitro	Bacterial reverse mutation test (Ames test) for gene mutations	At least five amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* are used to detect point mutations (base-pair substitution, addition, or deletion of one or a few DNA base pairs) that restore the functional capability of the bacteria to synthesize an essential amino acid.
	Chromosomal damage and aneuploidy in mammalian cell cultures	In vitro mammalian chromosomal aberration test in somatic cells. A micronucleus assay detecting both clastogenicity and aneugenicity.
	Gene mutations in mammalian cell cultures	Forward mutation assay at *tk* locus of mouse lymphoma L5178Y cells or at the *hprt* locus of cell lines derived from Chinese hamster or human lymphoblastoid TK6 cells.
Current strategies for genotoxicity testing in vivo	An assay for the assessment of cytogenetic damage in rodent bone marrow cells	A micronucleus assay is preferred since it detects both clastogenicity and aneugenicity. A mammalian in vivo chromosome aberration test detects structural chromosome aberrations in rodent bone marrow cells.
	Gene-mutation assays in rodent somatic cells	Transgenic *lacI*, *lacZ*, *cII* loci of Big Blue®, Muta™ Mouse, etc., detect mutations in vivo in any tissue of choice.
	Genotoxicity assay for detection of DNA damage and repair	In vivo comet assay (single cell gel electrophoresis assay) recommended for follow-up testing of positive in vitro findings. Also useful as a tool for the evaluation of local genotoxicity, especially for organs/cell types which cannot easily be evaluated with other standard tests.
Other genetic toxicity assays	Assays for gene mutations	Gene-mutation assays in rodent somatic cells, e.g., *Pig-a*.
		Saccharomyces cerevisiae Gene Mutation Assay (OECD guideline deleted).
		Drosophila sex-linked recessive lethal assay (OECD guideline deleted).
	Genotoxicity assays	Sister chromatid exchange assay detects reciprocal exchanges of DNA segments between two sister chromatids of a duplicating chromosome.
		Unscheduled DNA synthesis test with mammalian liver cells detects DNA synthesis after excision repair of damaged DNA.
		Mitotic recombination assay in *Saccharomyces cerevisiae* (OECD guideline deleted).
	Mammalian germ cell assays	Mouse specific-locus tests.
		Cytogenetic analysis and heritable translocation assays.
		DNA damage and repair in rodent germ cells.
		Mutation analysis in germ cells of transgenic rodents.
		Mutation analysis in tandem-repeat loci in mice.

OECD guideline deletion means that no new testing shall be initiated using any deleted Test Guideline, but the results generated shall continue to be accepted in the regulatory settings and for weight-of-evidence assessments.

Some assays for gene mutations detect forward mutations whereas others detect reversion. Forward mutations, such as those detected in the *thymidine kinase* (*tk*) gene in the widely used assay in mouse lymphoma cells (Clements, 2000; Wang et al., 2009; Schisler et al., 2013), are genetic alterations in a wild type gene that are detected by a change in phenotype caused by the alteration or loss of gene function. In contrast, reversion mutations are those that restore gene function in a mutant, such as the histidine revertants detected in the Ames assay in *Salmonella* (Ames et al., 1975; Mortelmans and Zeiger, 2000; Claxton et al., 2010). Thus, a back mutation or reversion that restores gene function in a mutant brings about a return to the wild type phenotype. In principle, forward-mutation assays should respond to a broad spectrum of mutagens because any mutation that interferes with gene expression should confer the detectable phenotype. A reversion assay might be expected to have a more restricted mutational response because only mutations that correct or compensate for the specific mutation in a particular mutant will be detected. In fact, some reversion assays respond to a broader spectrum of mutational changes than one might expect because mutations at a site other than that of the original mutation, either within the test gene or in a different gene (i.e., a suppressor mutation), can sometimes confer the selected phenotype. Both forward mutation assays and reversion assays are used extensively in genetic toxicology.

The simplest gene mutation assays rely on selection techniques to detect mutations. A selection technique is a means of imposing experimental conditions under which only cells or organisms that have undergone mutation can grow. For example, only cells that have a mutation in the *tk* gene can grow in medium containing the inhibitory chemical trifluorothymidine (Seifried et al., 2006; Wang et al., 2009; Schisler et al., 2013). Selection techniques greatly facilitate the identification of rare cells that have experienced mutation among the many cells that have not. Forward mutations (Clements, 2000; Vlasakova et al., 2005) and reversions (Josephy, 2000; Mortelmans and Riccio, 2000; Mortelmans and Zeiger, 2000; Kamber et al., 2009) can both be detected by selection techniques in microorganisms and cultured mammalian cells. Due to their speed, low cost, and ease of detecting events that occur at low frequency (i.e., mutation), assays in microorganisms and cell cultures have figured prominently in genetic toxicology.

Studying mutagenesis in intact animals requires assays of more complex design than the simple selection methods used in

Table 9-2

Overview of Genetic Toxicology Assays

ASSAYS	SELECTED LITERATURE CITATIONS
I. Prediction of genotoxicity	
A. Interpretation of chemical structure	
Structural alerts to genotoxicity	Ashby (1994), Ashby and Tennant (1991), Ashby and Paton (1993)
B. In silico predictive models	
Computational and structural programs: MCASE, TOPKAT, DEREK	Snyder et al. (2004), Snyder and Smith (2005), Snyder (2009), Mahadevan et al. (2011)
Quantitative structure–activity relationship (QSAR) modeling	Votano et al. (2004), Snyder and Smith (2005), Mahadevan et al. (2011)
II. DNA damage and repair assays	
A. Direct detection of DNA damage:	
Alkaline elution assays for DNA strand breakage in hepatocytes	Storer et al. (1996), Gealy et al. (2007)
Comet assay (single-cell gel electrophoresis) for DNA strand breakage	Fairbairn et al. (1995), Singh (2000), Tice et al. (2000), Collins (2004), Olive (2009)
CometChip	Weingeist et al. (2013), Ge et al. (2014)
Nonmammalian comets in ecotoxicology	Cotelle and Férard (1999), Lee and Steinert (2003), Jha (2004)
Assays for chemical adducts in DNA	Phillips et al. (2000), Farmer and Singh (2008), Himmelstein et al. (2009)
B. DNA repair, recombination, and genotoxic stress responses as indicators of damage:	
Differential killing of repair-deficient and wild type bacteria	Takigami et al. (2002)
Induction of the bacterial SOS system	Quillardet and Hofnung (1993), Yasunaga et al. (2004), Oda et al. (2009)
"Green Screen" for *GADD45a* gene induction in TK6 human cells	Hastwell et al. (2009), Jagger et al. (2009), Hughes et al. (2012)
Unscheduled DNA synthesis (UDS) in isolated rat hepatocytes or rodents in vivo	Madle et al. (1994), McQueen and Williams (1987), Butterworth et al. (1987)
Induction of mitotic recombination	Zimmermann (1992), Hoffmann (1994), Vogel and Nivard (2000), Turner et al. (2003), Reliene et al. (2007)
III. Prokaryote gene-mutation assays	
A. Bacterial reverse mutation assays:	
Salmonella/mammalian microsome assay (Ames test)	Ames et al. (1975), Kier et al. (1986), Maron and Ames (1983), Gatehouse et al. (1994), Mortelmans and Zeiger (2000), Seifried et al. (2006), Claxton et al. (2010)
E. coli WP2 tryptophan reversion assay	Gatehouse et al. (1994), Mortelmans and Riccio (2000)
Salmonella-specific base-pair substitution assay (Ames II assay)	Gee et al. (1994), Kamber et al. (2009)
E. coli lacZ-specific reversion assay	Cupples and Miller (1989), Cupples et al. (1990), Josephy (2000), Hoffmann et al. (2003)
B. Bacterial forward mutation assays:	
E. coli lacI assay	Calos and Miller (1981), Halliday and Glickman (1991)
Resistance to toxic metabolites or analogs in *Salmonella*	Jurado et al. (1994), Vlasakova et al. (2005)
IV. Assays in nonmammalian eukaryotes:	
A. Fungal assays:	
Forward mutations, reversion, and small deletions	Zimmermann et al. (1984), Crouse (2000)
Mitotic crossing over, gene conversion, and homology-mediated deletions in yeast	Zimmermann et al. (1984), Zimmermann (1992), Howlett and Schiestl (2000), Daigaku et al. (2004), Freeman and Hoffmann (2007)
Genetic detection of mitotic and meiotic aneuploidy in yeast	Zimmermann et al. (1984), Aardema et al. (1998), Howlett and Schiestl (2000), Nunoshiba et al. (2007)
B. Plant assays:	
Gene mutations affecting chlorophyll in seedlings, the *waxy* locus in pollen, or *Tradescantia* stamen-hair color	Ma et al. (2005), Grant and Owens (2006)
Chromosome aberrations and micronuclei in mitotic and meiotic cells of corn, *Tradescantia*, and other plants	Ma et al. (2005), Grant and Owens (2006), Misík et al. (2011)

(*continued*)

Table 9-2

(Continued)

ASSAYS	SELECTED LITERATURE CITATIONS
C. *Drosophila* assays:	
Sex-linked recessive lethal test in germ cells	Lee et al. (1983), Mason et al. (1987), Vogel et al. (1999)
Heritable translocation assays	Mason et al. (1987), Vogel et al. (1999)
Mitotic recombination and LOH in eyes or wings	Vogel et al. (1999), Vogel and Nivard (2000)
V. Mammalian gene-mutation assays	
A. In vitro assays for forward mutations:	
tk mutations in mouse lymphoma or human cells	Clements (2000), Seifried et al. (2006), Wang et al. (2009), Moore et al. (2011), Schisler et al. (2013)
hprt or *xprt* mutations in Chinese hamster or human cells	Li et al. (1988), DeMarini et al. (1989), Parry et al. (2005)
CD59 mutations in CHO-human hybrid A_L cells	Zhou et al. (2006), Ross et al. (2007)
B. In vivo assays for gene mutations in somatic cells:	
Mouse spot test (somatic cell specific-locus test)	Styles and Penman (1985), Lambert et al. (2005)
hprt mutations (6-thioguanine-resistance) in rodent lymphocytes	Cariello and Skopek (1993), Casciano et al. (1999), Lambert et al., 2005
Pig-a mutations (immunological detection of mutations blocking glycosylphosphatidylinositol synthesis)	Dobrovolsky et al. (2010), Miura et al. (2011), Dobo et al. (2011), Gollapudi et al. (2015)
C. Transgenic assays:	
Mutations in the bacterial *lacI* gene in Big Blue® mice and rats	Lambert et al. (2005), Singer et al. (2006)
Mutations in the bacterial *lacZ* gene in the Muta™ Mouse	Lambert et al. (2005), Singer et al. (2006)
Mutations in the phage *cII* gene in *lacI* or *lacZ* transgenic mice	Shane et al. (2000), Swiger (2001), Lambert et al. (2005)
Point mutations and deletions in the *lacZ* plasmid mouse	Lambert et al. (2005), Singer et al. (2006)
Point mutations and deletions in *gpt delta* mice and rats	Okada et al. (1999), Lambert et al. (2005), Nohmi (2016)
Forward mutations and reversions in ΦX174 transgenic mice	Valentine et al. (2010)
Inversions and deletions arising in pKZ1 mice by intrachromosomal recombination	Sykes et al. (2006)
VI. Mammalian cytogenetic assays	
A. Chromosome aberrations:	
Metaphase analysis in cultured Chinese hamster or human cells	Ishidate et al. (1988), Kirkland et al. (1990), Galloway et al. (1994), Galloway (2000), Corvi et al. (2008), Galloway et al. (2011)
Metaphase analysis of rodent bone marrow or lymphocytes in vivo	Preston et al. (1981), Kirkland et al. (1990), Tice et al. (1994)
Chromosome painting and other FISH applications in vitro and in vivo	Tucker et al. (1993b, 2005), Paccierotti and Sgura (2008)
B. Micronuclei:	
Cytokinesis-block micronucleus assay in human lymphocytes	Fenech et al. (2003), Corvi et al. (2008), Fenech (2008), Fenech et al. (2011a, 2011b)
Micronucleus assay in mammalian cell lines	Kirsch-Volders et al. (2003), Corvi et al. (2008), Galloway et al. (2011)
In vivo micronucleus assay in rodent bone marrow or blood	Krishna and Hayashi (2000), Hayashi et al. (2007), Tweats et al. (2007), Dertinger et al. (2011)
In vivo micronucleus assay in tissues other than marrow or blood	Hayashi et al. (2007), Coffing et al. (2011), Morita et al. (2011)
C. Sister chromatid exchange:	
SCE in human cells or Chinese hamster cells	Tucker et al. (1993a), Wilson and Thompson (2007)
SCE in rodent tissues, especially bone marrow	Tucker et al. (1993a)
D. Aneuploidy in mitotic cells:	
Hyperploidy detected by chromosome counting or FISH in cell cultures or bone marrow	Galloway and Ivett (1986), Natarajan (1993), Aardema et al. (1998), Paccierotti and Sgura (2008)
Micronucleus assay with centromere/kinetochore labeling in cell cultures	Lynch and Parry (1993), Natarajan (1993), Aardema et al. (1998), Fenech (2008), Paccierotti and Sgura (2008)

(*continued*)

Table 9-2

(Continued)

ASSAYS	SELECTED LITERATURE CITATIONS
Altered parameters in flow-cytometric detection of micronuclei in CHO cells	Bryce et al. (2011)
Mouse bone marrow micronucleus assay with centromere labeling	Adler (1993), Aardema et al. (1998), Krishna and Hayashi (2000)
VII. Germ cell mutagenesis	
A. Measurement of DNA damage	
Molecular dosimetry based on mutagen adducts in reproductive cells	Russell and Shelby (1985), Olsen et al. (2010), Verhofsrad et al. (2011)
UDS in rodent germ cells	Bentley et al. (1994), Sotomayor and Sega (2000)
Alkaline elution assays for DNA strand breaks in rodent testes	Bentley et al. (1994)
Comet assay in sperm and gonadal tissue	Speit et al. (2009)
B. Gene mutations	
Mouse specific-locus test for gene mutations and deletions	Russell et al. (1981), Ehling (1991), Russell and Russell (1992), Favor (1999), Russell (2004), Singer et al. (2006)
Mouse electrophoretic specific-locus test	Lewis (1991)
Dominant mutations causing mouse skeletal defects or cataracts	Ehling (1991), Selby et al. (2004)
ESTR assay in mice	Yauk (2004), Dubrova (2005), Singer et al. (2006), Somers (2006)
Germ cell mutations in transgenic assays	Lambert et al. (2005), Singer et al. (2006)
C. Chromosomal aberrations	
Cytogenetic analysis of oocytes, spermatogonia, spermatocytes, or zygotes	Kirkland et al. (1990), Tease (1992), Russo (2000), Marchetti et al. (2001)
Direct detection in sperm by FISH	Marchetti et al. (2006, 2008)
Micronuclei in mouse spermatids	Russo (2000), Hayashi et al. (2007)
Mouse heritable translocation test	Russell and Shelby (1985), Singer et al. (2006)
D. Dominant lethal mutations	
Mouse or rat dominant lethal assay	Adler et al. (1994), Singer et al. (2006)
E. Aneuploidy	
Cytogenetic analysis for aneuploidy arising by nondisjunction	Allen et al. (1986), Adler (1993), Adler et al. (1994), Aardema et al. (1998), Russo (2000), Marchetti et al. (2001)
Sex chromosome loss test for nondisjunction or breakage	Russell and Shelby (1985)
Micronucleus assay in spermatids with centromere labeling	Aardema et al. (1998)
FISH with probes for specific chromosomes in sperm	Russo (2000), Marchetti et al. (2006, 2008)

microorganisms and cultured cells. Genetic toxicology assays therefore range from inexpensive short-term tests (Zeiger, 2010) that can be performed in a few days to complicated assays for mutations in mammalian germ cells (Favor, 1999; Russell, 2004; Singer et al., 2006). Even in multicellular organisms, there has been an emphasis on designing assays that detect mutations with great efficiency (Casciano et al., 1999; Vogel et al., 1999; Lambert et al., 2005; Dobrovolsky et al., 2010). Nevertheless, there remains a gradation in which an increase in relevance for human risk entails more elaborate and costly tests (Table 9-2). The most expensive mammalian tests are typically reserved for chemicals of special importance in basic research or risk assessment, whereas the simpler assays can be applied more broadly.

Cytogenetic assays differ in design from typical gene mutation assays because of their reliance on cytological rather than genetic methods. The goal in cytogenetic methods is the unequivocal visual recognition of cells that have experienced genetic damage. The alterations measured include chromosome aberrations (Preston et al., 1981; Ishidate et al., 1988; Corvi et al., 2008), micronuclei (Hayashi et al., 2007; Corvi et al., 2008; Fenech et al., 2011a, 2011b), sister chromatid exchanges (SCEs; Tucker et al., 1993a; Wilson and Thompson, 2007), and changes in chromosome number (Aardema et al., 1998; Paccierotti and Sgura, 2008). The latter include ploidy changes (e.g., polyploid cells) and aneuploidy, in which one or a few chromosomes have been gained (i.e., hyperploidy) or lost (i.e., hypoploidy) relative to the normal chromosome number. Aneuploidy is of great interest because of its implications for human health, but assays for aneuploidy are not yet as refined or systematically applied as those for other classes of chromosomal alterations. The biological and toxicological significance of SCEs is largely unknown and as such tests for SCE induction are no longer included as part of genotoxicity assessment.

In all mutagenicity testing, one must be aware of possible sources of error. Factors to consider in the application of mutagenicity assays are the choice of suitable organisms and growth conditions, appropriate monitoring of genotypes and phenotypes,

effective experimental design and treatment conditions, inclusion of proper positive and negative controls, and sound methods of data analysis. The articles cited in Table 9-2 discuss these aspects of genetic toxicology testing. In addition, the publication by Lorge et al. (2016) provides guidance on good cell culture practices in the conduct of genetic toxicology assays using mammalian cell cultures.

Many substances that are not themselves mutagenic or carcinogenic can be activated into mutagens and carcinogens by metabolism (Guengerich, 2001; Malling, 2004). Such chemicals are called promutagens and procarcinogens. Since microorganisms and mammalian cell cultures lack many of the metabolic capabilities of intact mammals, provision must be made for metabolic activation in order to detect promutagens in many genetic assays. Incorporating an in vitro metabolic activation system derived from a mammalian tissue homogenate is the most common means of adding metabolic activation to microbial or cell culture assays (Malling and Frantz, 1973; Ames et al., 1975; Clements, 2000; Malling, 2004; Cox et al., 2016). For example, the promutagens dimethylnitrosamine and benzo[*a*]pyrene are not themselves mutagenic in bacteria, but they are mutagenic in bacterial assays if the bacteria are treated with the promutagen in the presence of a homogenate from mammalian liver.

The most widely used metabolic activation system in microbial and cell culture assays is a postmitochondrial supernatant from a rat liver homogenate, along with appropriate buffers and cofactors (Maron and Ames, 1983; Kirkland et al., 1990). The standard liver metabolic activation system is called an S9 mixture, designating a supernatant from centrifugation at 9000 g (Malling and Frantz, 1973; Maron and Ames, 1983). The S9 subcellular fraction contains microsomes and cytosol with microsomes containing enzymes necessary for Phase I metabolism (e.g., cytochrome P450s) and cytosol containing the enzymes involved in Phase II metabolism. Most of the short-term assays in Table 9-2 require exogenous metabolic activation to detect promutagens. Exceptions are assays in intact mammals and a few simpler assays that have a high level of endogenous cytochrome P450 metabolism, such as the detection of UDS or DNA strand breakage in cultured hepatocytes (Madle et al., 1994; Gealy et al., 2007).

Rat liver S9, usually prepared following induction with Aroclor-1254 (a mixture of polychlorinated biphenyls) or phenobarbital/β-naphthoflavone, provides a broad assemblage of metabolic reactions, but they are not necessarily the same as those of hepatic metabolism in an intact animal (Richardson et al., 2016). Metabolic activation systems based on homogenates from mice, guinea pigs, hamsters, or monkeys and preparations from organs other than liver have found some use in mutagenicity testing (Mortelmans and Zeiger, 2000). In some cases, these systems detect mutagenicity more efficiently than rat liver S9. However, like a homogenate from rat liver, these systems may differ from the species or organs of their origin. Therefore, alternative metabolic activation systems tend to be more useful if chosen for mechanistic reasons rather than simply testing another species or organ. Such systems include the use of intact hepatocytes (Madle et al., 1994; Storer et al., 1996; Gealy et al., 2007) to preserve the cellular compartmentalization of reactions; an in vitro system that can carry out the reduction reactions needed to detect the activity of substances whose mutagenicity requires reductive metabolism, such as some azo compounds (Mortelmans and Zeiger, 2000; Seifried et al., 2006); and the use of mammalian cells or bacteria engineered to express foreign genes that encode enzymes of metabolic activation (Sawada and Kamataki, 1998; Crespi and Miller, 1999; Josephy, 2000, 2002; Oda et al., 2009).

Besides metabolic activation, some chemicals are subject to photochemical activation. The genotoxicity of such chemicals depends on the chemical being irradiated with ultraviolet or visible light. Many of the assays listed in Table 9-2, including gene-mutation assays in bacteria and cultured mammalian cells, cytogenetic assays, and the comet assay, have been adapted so that they can measure photogenotoxic effects (Brendler-Schwaab et al., 2004; Lynch et al., 2011).

Genes encoding enzymes of xenobiotic metabolism have been incorporated by recombinant DNA technology into microorganisms or cell cultures to expand their capacity for metabolic activation. The genes incorporated into bacteria may be derived from other bacteria or from humans (Josephy, 2002; Oda et al., 2009). For example, bacterial genes that cause overexpression of *N*-acetyltransferase enhance the sensitivity of the bacteria to the mutagenicity of aromatic amines or nitroarenes (Josephy, 2002). The expression of human cytochrome P450 enzymes in *Salmonella* tester strains from the Ames assay (Josephy 2002; Yamazaki et al., 2004), a *Salmonella* SOS-induction assay (Oda et al., 2009), or *E. coli* strains of the *lacZ* reversion assay (Josephy, 2000) permits the activation of promutagens like 2-aminoanthracene and 2-aminofluorene without a S9 mixture. Mammalian cell lines have also been genetically engineered to express human Phase-I and Phase-II enzymes, including those catalyzing reactions of metabolic activation (Sawada and Kamataki, 1998; Chevereau et al., 2017). Many cell lines stably expressing a single form of P450 have been established. Mutagenesis can be measured through such endpoints as *HPRT* mutations, cytogenetic alterations, and γH2AX, and the cells are well suited to analyzing the contribution of different enzymes to the activation of promutagens (Crespi and Miller, 1999; Chevereau et al., 2017).

Metabolic activation is so central to genetic toxicology that all mutagenicity testing programs must provide for it in the choice of assays and procedures. Despite their usefulness, in vitro metabolic activation systems, however well refined, cannot mimic mammalian metabolism perfectly. There are differences among tissues in reactions that activate or inactivate foreign compounds, and organisms of the normal flora of the intestines can contribute to metabolism in intact mammals. Chemicals that induce enzyme systems or otherwise alter the physiological state can also modify the metabolism of toxicants, and the balance between activation and detoxification reactions in vitro may differ from that found in vivo.

Structural Alerts and In Silico Assays

The first indication that a chemical is a mutagen often lies in chemical structure. Potential electrophilic sites in a molecule serve as an alert to possible mutagenicity and carcinogenicity because such sites confer reactivity with nucleophilic sites in DNA (Miller and Miller, 1981; Tennant and Ashby, 1991; Snyder et al., 2004; Votano et al., 2004; Snyder and Smith, 2005). In general, genotoxic carcinogens typically are either electrophiles or can be activated to electrophilic reactive intermediates (pro-electrophiles). These compounds form covalent adducts with most of the cellular macromolecules such as DNA, RNA, or proteins at the nucleophilic centers with electron-rich heteroatoms S, O, or N (Plošnik et al., 2016). The understanding of the mechanisms of action through which electrophiles react with biological nucleophiles is based on the classical chemical reactions (S_N1, S_N1, acylation, Schiff base formation, Michael addition, and S_NAr) between electron-rich and electron-deficient centers (Plošnik et al., 2016). Strong electrophiles (e.g., nitroso compounds, epoxides, α,β- unsaturated aldehydes, *N*-sulphonyloxy-*N*-methyl-4- aminoazobenzene,

N-sulphonyloxy-*N*-acetyl-2-aminofluorene, and *N*-hydroxy-2-aminofluorene) are small, poorly polarized molecules, and their electron deficiency shows as positive electron charge. Soft electrophiles (e.g., safrole, estragole, *N*-methylol-4-aminoazobenzene) are usually large, highly polarized molecules, and their electron deficiency spreads all over the molecule (Plošnik et al., 2016).

Computer-based systems for predicting genotoxicity based on chemical structure properties are sometimes called in silico assays. These assays include computational and structural programs (Snyder and Smith, 2005; Snyder, 2009; Knudsen et al., 2011; Mahadevan et al., 2011) and the modeling of quantitative structure–activity relationships or (Q)SARs (Votano et al., 2004; Snyder and Smith, 2005; Mahadevan et al., 2011). The electrophilic theory of genotoxic carcinogenicity has led to two main (Q)SAR approaches for modeling genotoxic chemicals: (a) to identify the electrophilic functional groups or substructures, that is, to develop SAR models based on structural alerts; and (b) to find molecular descriptors that can be quantitatively related to the activity of the chemicals, that is, to develop (Q)SARs (Serafimova et al., 2010).

Structural alerts in combination with critical interpretation are a valuable initial adjunct to mutagenicity testing within the integrated approaches for testing and assessment of chemicals (Tennant and Ashby, 1991; Ashby and Paton, 1993). Although there is much skepticism that such approaches can replace biological testing, they hold promise of improving the efficiency of integrated testing strategies and reducing current levels of animal use (Guha, 2011; Dearfield et al., 2017; Wichard, 2017). The most commonly modeled endpoint for genotoxicity has been the Ames mutagenicity assay. The accuracy of Ames mutagenicity prediction is typically around 70% to 75% (Serafimova et al., 2010). (Q)SAR predictions should be interpreted by an expert with knowledge of the endpoint, the strengths and limitations of the specific model applied, the applicability domain of the model, and the reliability of prediction for the chemical of interest (Serafimova et al., 2010). Moreover, computational approaches should not be based on stand-alone basis, but on a weight-of-evidence approach including information from all available sources such as read across and in vitro test methods (Serafimova et al., 2010).

DNA Damage and Repair Assays

Some assays measure DNA damage itself, rather than mutational consequences of DNA damage. These assays belong to the category of "indicator assays" and the effects measured in these assays may or may not lead to mutational consequence. Damage to DNA may be induced directly, through such indicators as chemical adducts or strand breaks in DNA, or indirectly, through the measurement of biological repair processes. Adducts in DNA are detected by ^{32}P-postlabeling, high-performance liquid chromatography (HPLC), fluorescence-based methods, mass spectrometry, immunological methods using antibodies against specific adducts, isotope-labeled DNA binding, and electrochemical detection (Phillips et al., 2000; Farmer and Singh, 2008; Himmelstein et al., 2009). The ^{32}P-postlabeling method is highly versatile, in that it is sensitive and can be applied to diverse mutagens, but it may fall short of other methods for quantitative accuracy (Farmer and Singh, 2008). Through a combination of methods, many classes of adducts, including those of such environmentally widespread compounds as polynuclear aromatic hydrocarbons, can be detected. The measurement of adducts after human chemical exposures has proven useful in human monitoring, molecular dosimetry, and risk assessment (Phillips et al., 2000; Farmer and Singh, 2008; Himmelstein et al., 2009). Adducts in somatic cells are relevant to carcinogenesis (Himmelstein et al., 2009), whereas those in reproductive cells permit molecular dosimetry for germ cell mutagenesis (Olsen et al., 2010; Verhofsrad et al., 2011; Yauk et al., 2015).

DNA strand breakage can be measured by alkaline elution (Storer et al., 1996; Gealy et al., 2007) and electrophoretic methods. Single-cell gel electrophoresis, also called the comet assay, is a widely used, rapid method of measuring DNA damage (Fairbairn et al., 1995; Singh, 2000; Tice et al., 2000; Collins, 2004; Olive, 2009; Speit et al., 2015; Glei et al., 2016; Collins et al., 2017). In this assay, cells are incorporated into agarose on slides, lysed so as to liberate their DNA, and subjected to electrophoresis typically under alkaline conditions (pH >13) to allow detection of DNA double-strand breaks, single-strand breaks, alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and incomplete excision repair sites (Fairbairn et al., 1995; Singh, 2000; Tice et al., 2000; Pu et al., 2015). The DNA is stained with a fluorescent dye for observation and image analysis. As broken DNA fragments in the tail migrate more quickly than larger pieces of DNA of the head, a blur of fragments (a "comet") is observed when the DNA is extensively damaged. The extent of DNA damage can be estimated from the intensity of the comet tail relative to the total intensity (head plus tail) that reflects the amount of DNA breakage (Collins, 2004; OECD TG 489, 2016). Variations in the procedure permit the preferential detection of double-strand breaks under neutral conditions (Fairbairn et al., 1995; Olive, 2009) or inclusion of digestion of lesion-specific DNA repair enzymes in the procedure allows the detection of various DNA base alterations, such as oxidative base damage. (Pu et al., 2015; Glei et al., 2016; Collins et al., 2017). Comet assay has also been modified to detect DNA methylation status (Townsend et al., 2017). Finally, the high-throughput "CometChip" technique, based on the traditional comet assay, simultaneously follows DNA damage load over time in different cell lines in response to various genotoxicants (Weingeist et al., 2013; Ge et al., 2014). This method quantifies kinetics, differential sensitivity, and repair capacity within different genetic backgrounds at the level of an individual cell. Although the comet assay has proven to be a sensitive indicator of DNA damage with broad applicability, it is prone to yield false positive/uninterpretable findings under conditions of physiological and/or mechanical stress. It has been used most commonly with human lymphocytes (Fairbairn et al., 1995; Singh, 2000; Collins, 2004) and other mammalian cells (Tice et al., 2000), but it can be adapted to diverse species, including plants, worms, mollusks, fish, and amphibians (Cotelle and Férard, 1999; Lee and Steinert, 2003; Jha, 2004). This adaptability makes it well suited to use in environmental genetic toxicology. The applicability of the comet assay and other DNA damage assays to rodent testes (Bentley et al., 1994; Speit et al., 2009) makes these methods helpful in interpreting risks to germ cells.

The occurrence of DNA repair can serve as an easily measured indicator of DNA damage. Repair assays have been developed in microorganisms, cultured mammalian cells, and intact mammals (Table 9-2). Greater toxicity of a chemical in DNA-repair-deficient strains than in their repair-proficient counterparts (e.g., *rec*$^+$ and *rec*$^-$ in *Bacillus subtilis*) can serve as an indicator of DNA damage in bacteria (Takigami et al., 2002). Bacterial repair assays find occasional application, but are used less commonly today than historically. The measurement of unscheduled DNA synthesis (UDS), which is a measure of excision repair, is a mammalian DNA repair assay indicating that DNA has been damaged (Madle et al., 1994). UDS has been used with cultured hepatocytes with endogenous cytochrome P450 enzyme activities as well as tissues of intact animals, including hepatocytes (Madle et al., 1994), and germinal tissue (Bentley et al., 1994; Sotomayor and Sega, 2000). The absence

of UDS, however, does not provide evidence that DNA has not been damaged because some classes of damage are not readily excised. Based upon their analysis, Kirkland and Speit (2008) questioned the value of in vivo rodent liver UDS assay as follow-up to the positive findings of in vitro genotoxicity assays. Their analyses were based on the relative performance of three in vivo assays (liver comet, transgenic mutation, and bone marrow micronucleus) in predicting rodent carcinogens.

Besides specific DNA repair processes, the induction of general responses to genotoxic stress has been used as an indicator of genetic damage. The induction of DNA repair networks, known as the "SOS response," allows bacteria to survive sudden increases in DNA damage as detected by phage induction, colorimetry, or green fluorescent protein (GFP) reporter induction after the activation of SOS gene promoters (Quillardet and Hofnung, 1993; Yasunaga et al., 2004; Friedman et al., 2005; Michel, 2005; Oda et al., 2009). Another fluorescence-based screen, the growth arrest and DNA damage gene GADD45a-GFP assay, also called "Green Screen," detects genotoxic stress in human lymphoblastoid TK6 cells (Hastwell et al., 2009; Hughes et al., 2012). The stress response is detected by the genetic GFP reporter construct following p53-dependent expression from GADD45a promoter of the truncated *GADD45a* gene/GFP construct. The induction of fluorescence by mutagens, clastogens, and aneugens exhibits sensitivity toward genotoxicants comparable with other in vitro mammalian cell assays as well as a very high specificity (93%) (Birrell et al., 2009). The assay can be conducted with S9 metabolic activation and lends itself to automated detection by flow cytometry (Jagger et al., 2009).

Gene Mutations in Prokaryotes

The most common means of detecting mutations in microorganisms is selecting for reversion in strains that have a specific nutritional requirement differing from wild type members of the species; such strains are called auxotrophs. For example, the widely used assay developed by Bruce Ames and his colleagues is based on measuring reversion in several histidine auxotrophs in *Salmonella enterica* serovar Typhimurium, commonly called *S. typhimurium* (Ames et al., 1975).

In the Ames assay one measures the frequency of histidine-independent bacteria that arise in a histidine-requiring strain in the presence or absence of the chemical being tested. Auxotrophic bacteria are treated with the chemical of interest by one of several procedures (e.g., the standard plate-incorporation assay) and plated on medium that is deficient in histidine (Ames et al., 1975; Maron and Ames, 1983; Mortelmans and Zeiger, 2000). The assay is conducted using genetically different strains so that reversion by base pair substitutions and frameshift mutations in several DNA sequence contexts can be detected and distinguished. Since *Salmonella* does not metabolize promutagens in the same way as mammalian tissues, the assay is generally performed in the presence and absence of a rat liver S9 metabolic activation system. The Ames assay is commonly referred to as the *Salmonella–Escherichia coli*/mammalian-microsome reverse mutation assay although the activation system used is S9 (microsomes + cytosol) (OECD TG 471, 1997). Based on the current version of the OECD Ames test guideline, at least five strains of bacteria should be used that include four strains of *S. typhimurium* that revert at GC base pairs (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) and the fifth strain that reverts at AT base pair (either one of *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101), or *S. typhimurium* TA102).

The principal strains of the Ames test and their characteristics are summarized in Table 9-3. In addition to the histidine alleles that provide the target for measuring mutagenesis, the Ames tester strains contain other genes and plasmids that enhance the assay. Part I of the table gives genotypes, and Part II explains the rationale for including specific genetic characteristics in the strains. Part III summarizes the principal DNA target in each strain and the mechanisms of reversion. Taken together, the Ames strains detect a broad array of mutations, and they complement one another. For example, *E. coli* and *S. typhimurium* oxidative damage-sensitive strains TA102 and TA104, all detect the A:T-to-G:C base pair substitutions that are not detected by *hisG46* strains (Mortelmans and Zeiger, 2000). TA102 also detects chemicals that cause DNA cross-links because it has an intact excision repair system whereas the other common tester strains do not.

The most common version of the Ames assay is the plate-incorporation test (Ames et al., 1975; Maron and Ames, 1983; Mortelmans and Zeiger, 2000). In this procedure, the bacterial tester strain, the test compound (or solvent control), and the S9 metabolic activation system (or buffer for samples without S9) are added to 2 mL of molten agar containing biotin and a trace of histidine to allow a few cell divisions, mixed, and immediately poured onto the surface of a petri dish selective for histidine-independent revertants. For general testing it is recommended that at least three plates per concentration and five concentrations be used with and without S9, along with appropriate concurrent positive and negative controls (Mortelmans and Zeiger, 2000). Variations on the standard plate-incorporation assay confer advantages for some applications. These include a preincubation assay that facilitates the detection of unstable compounds and short-lived metabolites, a desiccator assay for testing volatile chemicals and gases, a microsuspension assay for working with small quantities of test materials, assays incorporating reductive metabolism rather than the conventional S9 system, and assays under hypoxic conditions (Mortelmans and Zeiger, 2000).

Although simplicity is a great merit of microbial assays, it can also be deceptive. Even assays that are simple in design and application can be performed incorrectly. For example, in the Ames assay one may see very small colonies in the petri dishes at highly toxic concentrations (Maron and Ames, 1983; Kirkland et al., 1990; Mortelmans and Zeiger, 2000). Counting such colonies as revertants would be an error because they may actually be non-revertant survivors that grew on the low concentration of histidine in the plates. Were there millions of survivors, the amount of histidine would have been insufficient to allow any of them (except real revertants) to form colonies. This artifact is easily avoided by checking that there is a faint lawn of bacterial growth in the plates; one can also confirm that colonies are revertants by streaking them on medium without histidine to be sure that they grow in its absence. Such pitfalls exist in all mutagenicity tests. Therefore, anyone performing mutagenicity tests must have detailed familiarity with the laboratory application and literature of the assay and be observant about the responsiveness of the assay in terms of laboratory historical negative and positive control values.

Although information from the Ames assay has become a standard in genetic toxicology testing, equivalent information can be obtained from other bacterial assays. Like the Ames assay, the WP2 tryptophan reversion assay in *E. coli* (Kirkland et al., 1990; Mortelmans and Riccio, 2000) incorporates genetic features that enhance assay sensitivity, can accommodate S9 metabolic activation, and performs well in many laboratories. Mutations are detected by selecting for reversion of a *trpE* allele from Trp^- to Trp^+. Its responsiveness to mutagens most closely resembles TA102 among the Ames strains (Mortelmans and Riccio, 2000).

Bacterial reversion assays are commonly used for testing purposes, but they also provide information on molecular mechanisms of mutagenesis. The broader understanding of mutational mechanisms that comes from refined genetic assays and molecular analysis of mutations can contribute to the interpretation of mutational hazards. The primary reversion mechanisms of the Ames strains,

Table 9-3

The Ames Assay: Tester Strains and Their Characteristics

I. STANDARD TESTER STRAINS OF *SALMONELLA TYPHIMURIUM AND ESCHERICHIA COLI*

STRAIN	TARGET ALLELE	CHROMOSOMAL GENOTYPE	PLASMIDS
TA1535	*hisG46*	*hisG46 rfa ΔuvrB*	None
TA100	*hisG46*	*hisG46 rfa ΔuvrB*	pKM101 (*mucAB* Ap^r)
TA1538	*hisD3052*	*hisD3052 rfa ΔuvrB*	None
TA98	*hisD3052*	*hisD3052 rfa ΔuvrB*	pKM101 (*mucAB* Ap^r)
TA1537	*hisC3076*	*hisC3076 rfa ΔuvrB*	None
TA97	*hisD6610*	*hisD6610 hisO1242 rf ΔuvrB*	pKM101 (*mucAB* Ap^r)
TA102	*hisG428*	*hisΔ (G)8476 rfa*	pKM101 (*mucAB* Ap^r); pAQ1 (*hisG428* Tc^r)
TA104	*hisG428*	*hisG428 rfa ΔuvrB*	pKM101 (*mucAB* Ap^r)
E. coli WP2 *uvrA*	*trpE*	*trpE uvrA*	None
E. coli WP2 *uvrA* (pKM101)	*trpE*	*trpE uvrA*	pKM101 (*mucAB* Ap^r)

II. GENETIC CHARACTERISTICS OF THE AMES TESTER STRAINS

CHARACTERISTIC	RATIONALE FOR INCLUSION IN THE TESTER STRAIN
Rfa	Alters the lipopolysaccharide wall, enhancing permeability to mutagens.
ΔuvrB	Deletes the excision repair system, increasing sensitivity to many mutagens; retention of excision in TA102 permits the detection of DNA cross-linking agents.
mucAB	Enhances sensitivity to some mutagens whose activity depends on the SOS system.
Ap^r	Permits selection for the presence of pKM101 by ampicillin resistance.
hisO1242	Affects regulation of histidine genes, enhancing revertibility of *hisD6610* in TA97.
His Δ (G)8476	Eliminates the chromosomal *hisG* gene, allowing detection of the reversion of *hisG428* on pAQ1 in TA102.
Tc^r	Permits selection for the presence of pAQ1 in TA102 by tetracycline resistance.

III. MECHANISMS OF REVERSION DETECTED BY THE AMES TESTER STRAINS

STRAIN	PRIMARY TARGET*	MUTATIONS DETECTED
TA1535, TA100	GGG/CCC	Base-pair substitutions, principally those beginning at G:C base pairs (G:C→A:T; G:C→T:A; G:C→C:G). These strains also detect A:T→C:G but not A:T→G:C.
TA1538, TA98	CGCGCGCG/GCGCGCGC	Frameshift mutations, especially −2 frameshifts (−GC or −CG), +1 frameshifts, other small deletions, and some complex mutations.
TA1537	GGGGG/CCCCC	Frameshift mutations, mainly −1 (−G or −C; less frequently −T), but also some +CG frameshifts.
TA97	GGGGGG/CCCCCC	Frameshift mutations, combining the specificity of TA1537 at the primary target and with some characteristics of TA98.
TA102, TA104	TAA/ATT terminating ochre mutation	Base-pair substitutions, principally those beginning at A:T base pairs (A:T→G:C; A:T→T:A; A:T→C:G), but also G:C→T:A and G:C→A:T.
E. coli WP2 *uvrA* strains	TAA/ATT terminating ochre mutation	Base-pair substitutions, principally those beginning at A:T base pairs.

**The sequences before and after the backslash represent the two complementary strands of DNA.*

summarized in Table 9-3, were initially determined by genetic and biochemical means (Maron and Ames, 1983). An ingenious method called allele-specific colony hybridization greatly facilitated the molecular analysis of revertants in the Ames assay (Koch et al., 1994), and many spontaneous and induced revertants have been cloned or amplified by the polymerase chain reaction (PCR) and sequenced (Levine et al., 1994; DeMarini, 2000).

Part III of Table 9-3 is by necessity a simplification with respect both to targets and mechanisms of reversion of the Ames strains. Some mutations that bring about reversion to histidine independence fall outside the primary target, and the full target has been found to be as much as 76 base pairs in *hisD3052* (DeMarini et al., 1998). Other revertants can arise by suppressor mutations in other genes. It has been shown that *hisG46*, *hisG428*, *hisC3076*,

hisD6610, and *hisD3052* all revert by multiple mechanisms and that the spectrum of classes of revertants may vary depending on the mutagen, experimental conditions, and other elements of the genotype (Cebula and Koch, 1990; Prival and Cebula, 1992; DeMarini et al., 1998; Mortelmans and Zeiger, 2000).

The development of *Salmonella* strains that are highly specific with respect to mechanisms of reversion has made the identification of particular base pair substitutions more straightforward. These strains (TA7001–TA7006) each revert from *his*$^-$ to *his*$^+$ by a single kind of mutation (e.g., G:C to T:A), and collectively they permit the specific detection of all six possible base pair substitutions (Gee et al., 1994; Mortelmans and Zeiger, 2000; Kamber et al., 2009). The assay, referred to as Ames II, is usually conducted using a fluctuation test in 24-well plates, rather than the plate-incorporation or preincubation method. The ability to discern mutagens and nonmutagens is comparable to the standard Ames assay with nearly 84% agreement between the two tests (Kamber et al., 2009).

Specific reversion assays are also available in *E. coli*. One system, based on reversion of *lacZ* mutations in various *E. coli* strains, measures all six possible base pair substitutions (Cupples and Miller, 1989; Josephy, 2000) and five frameshift +G, −G, −CG, +A, and −A mutations in several sequence contexts detected by growth on lactose medium (Cupples et al., 1990; Josephy, 2000; Hoffmann et al., 2003). The versatility of the *lacZ* assay has been expanded through the introduction of useful characteristics into the strains parallel to those incorporated into the Ames strains. Among the features added to the *lacZ* assay are DNA repair deficiencies, permeability alterations, plasmid-enhanced mutagenesis, and enzymes of mutagen metabolism (Josephy, 2000).

Bacterial forward mutation assays, such as selections for resistance to arabinose or to purine or pyrimidine analogs in *Salmonella* (Jurado et al., 1994; Vlasakova et al., 2005), are also used in research and testing, although less extensively than reversion assays. A versatile forward mutation assay that has contributed greatly to an understanding of mechanisms of mutagenesis is the *lacI* system in *E. coli* (Calos and Miller, 1981; Halliday and Glickman, 1991). Mutations in the *lacI* gene, which encodes the repressor of the lactose operon, are easily identified by phenotype, cloned or amplified by PCR, and sequenced. The *lacI* gene is widely used as a target for mutagenesis both in *E. coli* and in transgenic mice, and thousands of *lacI*$^-$ mutants have been sequenced.

Genetic Alterations in Nonmammalian Eukaryotes

Gene Mutations and Chromosome Aberrations Many early studies of mutagenesis used yeasts, mycelial fungi, plants, and insects as experimental organisms. Even though well-characterized genetic systems permit the analysis of a diverse array of genetic alterations in these organisms (Table 9-2), they have been largely supplanted in genetic toxicology by bacterial and mammalian systems. Exceptions are to be found where the assays in nonmammalian eukaryotes permit the study of genetic endpoints that are not readily analyzed in mammals or where the organism has special attributes that fit a particular application.

The fruit fly, Drosophila, has long occupied a prominent place in genetic research. In fact, the first unequivocal evidence of chemical mutagenesis was obtained in Scotland in 1941 when Charlotte Auerbach and J.M. Robson demonstrated that mustard gas is mutagenic in Drosophila. Drosophila continues to be used in modern mutation research (Potter et al., 2000), but its role in genetic toxicology is now more limited. The Drosophila assay of greatest historical importance is the sex-linked recessive lethal (SLRL) test. A strength of the SLRL test is that it permits the detection of recessive lethal mutations at 600 to 800 different loci on the X chromosome by screening for the presence or absence of wild type males in the offspring of specifically designed crosses (Mason et al., 1987; Vogel et al., 1999). The genetic alterations include gene mutations and small deletions. The spontaneous frequency of SLRL is about 0.2%, and a significant increase over this frequency in the lineages derived from treated males indicates mutagenesis. Although it requires screening large numbers of fruit fly vials, the SLRL test yields information about mutagenesis in germ cells, which is lacking in all microbial and cell culture systems. However, means of exposure, measurement of doses, metabolism, and gametogenesis in insects differ from those in mammalian toxicology, thereby introducing doubt about the relevance of Drosophila assays to human genetic risk. Drosophila assays are also available for studying the induction of chromosome abnormalities in germ cells, specifically heritable translocations and sex-chromosome loss (Zimmering, 1982; Mason et al., 1987; Vogel et al., 1999).

Genetic and cytogenetic assays in plants (Ma et al., 2005; Grant and Owens, 2006; Misík et al., 2011) also occupy a more restricted niche in modern genetic toxicology than they did years ago. However, plant assays continue to find use in special applications, such as in situ monitoring for mutagens and exploration of the metabolism of promutagens by agricultural plants (Gentile et al., 1986). In situ monitoring entails looking for evidence of mutagenesis in organisms that are grown in the environment of interest. Natural plant populations can also be examined for evidence of genetic damage, but doing so requires utmost precaution when characterizing the environments and defining appropriate control populations.

Mitotic Recombination In addition to its role in DNA repair, recombination (or the rearrangement between two similar or identical DNA sequences) provides critical support for the tolerance of DNA damage (Li and Heyer, 2008). The *RAD52* epistasis group genes are involved in homologous DNA recombination, and their primary structures are conserved from yeast to humans (Sonoda et al., 2001). Recombinogenic effects in yeast have long been used as a general indicator of genetic damage (Zimmermann et al., 1984), and interest in the induction of recombination has increased as recombinational events between chromosomes have been implicated in the etiology of cancer, including loss of heterozygosity (LOH) of one normal allele of tumor-suppressor genes such as *p53* (Sengstag, 1994; Reliene et al., 2007). Widely used assays for recombinogens detect mitotic crossing over and mitotic gene conversion in the yeast *Saccharomyces cerevisiae* (Zimmermann, 1992), and hundreds of chemicals have been tested for recombinogenic effects in straightforward yeast assays (Zimmermann et al., 1984). In yeast strain D7, for example, mitotic crossing over involving the *ade2* locus is detected on the basis of pink and red colony color, mitotic gene conversion at the *trp5* locus by selection for growth without tryptophan, and gene mutations by reversion of the *ilv1-92* allele (Zimmermann, 1992; Freeman and Hoffmann, 2007). Newer yeast assays have been constructed to discern whether LOH has occurred by mitotic recombination or chromosome loss (Daigaku et al., 2004; Nunoshiba et al., 2007). Strategies have also been devised to detect recombinogenic effects in human lymphocytes (Turner et al., 2003), other mammalian cells, mice, chickens, plants, and mycelial fungi (Hoffmann, 1994; Sonoda et al., 2001; Reliene et al., 2007). At least 350 chemicals have been evaluated in Drosophila somatic cell assays in which recombinogenic effects are detected by examining wings or eyes for regions in which recessive alleles are expressed in heterozygotes (Vogel et al., 1999; Vogel and Nivard, 2000).

Gene Mutations in Mammalian Cells

Mutagenicity assays in cultured mammalian cells have some of the same advantages as microbial assays with respect to speed and cost, and they use similar approaches. The most widely used assays for gene mutations in mammalian cells detect forward mutations that confer resistance to a toxic chemical. For example, mutations in the gene encoding hypoxanthine-guanine phosphoribosyltransferase (HPRT enzyme; *HPRT* gene) confer resistance to the purine analogue 6-thioguanine (Li et al., 1988; Parry et al., 2005), and thymidine kinase mutations (TK enzyme; *tk* gene) confer resistance to the pyrimidine analogue trifluorothymidine (Clive et al., 1987; Clements, 2000; Wang et al., 2009; Schisler et al., 2013). *HPRT* and *tk* mutations may, therefore, be detected by attempting to grow cells in the presence of purine analogues and pyrimidine analogues, respectively. For historical reasons, HPRT assays have most commonly been conducted in Chinese hamster cells or human cells, whereas TK assays have used mouse lymphoma cells or human cells. The mouse lymphoma assay, long used for detecting gene mutations, is now also used to detect other endpoints, including recombination, deletion, and aneuploidy (Wang et al., 2009). Forward-mutation assays typically respond to diverse mechanisms of mutagenesis, but there are exceptions such as resistance to ouabain, which only occurs by a specific alteration (DeMarini et al., 1989). Assays that do not detect various kinds of mutations are not useful for general mutagenicity testing. Genetic or molecular evidence that an assay is responsive to diverse mechanisms of mutagenesis is essential.

Instead of using selective media, flow cytometry can be used to detect gene mutations by immunological methods in mammalian cell cultures and intact animals. An in vitro assay for CD59 mutations is performed in CHO-human hybrid A_L cells based on the complement/antibody-mediated cytotoxicity principle (Zhou et al., 2006). A_L cells contain a single human chromosome 11 that expresses CD59 and other cell surface proteins along with the Chinese hamster chromosome complement of the Chinese hamster ovary (CHO) cells. Fluorescent anti-CD59 antibody is used to quantify CD59 mutant cells by flow cytometry. Besides CD59 itself, mutations in other CD genes on chromosome 11 and in their glycosylphosphatidylinositol anchor can be detected in other assays, including the in vivo *Pig-a* gene mutation assay described below (Ross et al., 2007; Krüger et al., 2016; Rees et al., 2017).

Gene Mutations In Vivo

In vivo assays involve treating intact animals and analyzing appropriate tissues for genetic effects. The choice of suitable doses, treatment procedures, controls, and sample sizes is critical in the conduct of in vivo tests. Mutations may be detected either in somatic cells or in germ cells. The latter are of special interest with respect to risk for future generations.

The mouse spot test is a traditional genetic assay for gene mutations in somatic cells (Styles and Penman, 1985; Lambert et al., 2005). Visible spots of altered phenotype in mice heterozygous for coat-colored genes indicate mutations in the progenitor cells of the altered regions. Although straightforward in design, the spot test is less used today than other somatic cell assays or than its germ cell counterpart, the mouse specific-locus test.

Cells that are amenable to positive selection for mutants when collected from intact animals form the basis for efficient in vivo mutation-detection assays analogous to those in mammalian cell cultures. Lymphocytes with mutations in the *HPRT* gene are readily detected by selection for resistance to 6-thioguanine. The HPRT assay in mice, rats, and monkeys (Casciano et al., 1999) is of special interest because it permits comparisons to the measurement of *HPRT* mutations in humans in mutational monitoring (Cole and Skopek, 1994; Albertini and Hayes, 1997; Albertini, 2001).

The *Pig-a* mutation assay is a newer assay that has versions suitable for human monitoring and laboratory studies in several species (Gollapudi et al., 2015; Ji and LeBaron, 2017). The assay detects mutations that block the transmembrane anchor glycosylphosphatidylinositol (GPI) synthesis (Dobrovolsky et al., 2010). *Pig-a* is a sex-linked, single copy gene whose gene product, the catalytic subunit of an *N*-acetyl glucosamine transferase, functions as an anchor for cell-surface proteins such as CD59, CD24, and CD55 to the exterior surface of the cytoplasmic membrane. The *Pig-a* mutation assay is based on the identification of mutant cells that have an altered repertoire of cell surface markers. Mutations in *Pig-a* can be detected in red blood cells from rats, mice, monkeys, and humans by means of fluorescent antibodies against GPI-anchored cell-surface proteins, such as CD59. The *Pig-a* mutation assay was also applied to rat cauda epididymal spermatozoa (sperm) for germ cell mutagenicity evaluation (Ji and LeBaron, 2017). Using antibodies to more than one GPI-linked marker has been suggested as a means of making the assay specific to *Pig-a* rather than also detecting mutants for a particular cell-surface protein. Frequencies can be measured by clonal growth of *Pig-a* cells using proaerolysin (ProAER) selection or by flow cytometry (Dobrovolsky et al., 2010; Dobo et al., 2011; Miura et al., 2011; Ji and LeBaron, 2017). Since this assay is performed using red blood cell, one of the challenges has been to confirm the mutant phenotype is indeed due to alteration at the DNA level (genotype) since red blood cells in mammals do not have nuceli to sequence DNA. Attempts are currently underway to collect this information by attempting to sequence the gene transcript in reticulocytes and by studying the nucleated bone marrow progenitor cells.

Besides determining whether chemicals are mutagenic, mutation assays provide information on mechanisms of mutagenesis that contributes to an understanding of mutational hazards. Base pair substitutions and large deletions, which may be indistinguishable on the basis of phenotype, can be differentiated through the use of probes for the target gene and Southern blotting, in that base substitutions are too subtle to be detectable on the blots, whereas gross structural alterations are visible (Cole and Skopek, 1994; Albertini and Hayes, 1997). Molecular analysis has been used to determine proportions of mutations ascribable to deletions and other structural alterations in several assays, including the specific-locus test for germ cell mutations in mice (Favor, 1999) and the human HPRT assay (Cole and Skopek, 1994). Gene mutations have been characterized at the molecular level by DNA sequence analysis both in transgenic rodents (Lambert et al., 2005; Singer et al., 2006) and in endogenous mammalian genes (Cariello and Skopek, 1993). Many *HPRT* mutations from human cells in vitro and in vivo have been analyzed at the molecular level and classified with respect to base pair substitutions, frameshifts, small deletions, large deletions, and other alterations (Cole and Skopek, 1994).

Transgenic Assays Transgenic animals are products of DNA technology in which the animal contains foreign DNA sequences that have been added to the genome. The foreign DNA is represented in all the somatic cells of the animal and is transmitted in the germ line to progeny. Mutagenicity assays in transgenic animals combine in vivo metabolic activation and pharmacodynamics with simple microbial detection systems, and they permit the analysis of mutations induced in diverse mammalian tissues (Lambert et al., 2005; Sykes et al., 2006; Valentine et al., 2010; OECD TG 488, 2013).

The transgenic animals that have figured most heavily in genetic toxicology are rodents that carry *lac* genes from *E. coli*. The bacterial genes were introduced into mice or rats by injecting a vector carrying the genes into fertilized oocytes generating grown up transgenic animals containing multiple copies of chromosomally integrated plasmid or phage shuttle vectors. (Lambert et al., 2005; OECD TG 488, 2013). The Mammalian Transgenic Rodent (TGR) mutation assays are performed with strains commonly referred to by their commercial names: the Big Blue® mouse and Big Blue® rat, which use *lacI* as a target for mutagenesis, and the Muta™ Mouse, which uses the *lacZ* target gene (Lambert et al., 2005; OECD TG 488, 2013). After mutagenic treatment of the transgenic animals, the *lac* genes are recovered from the animal, packaged in phage λ, and transferred to *E. coli* for mutational analysis. Mutant plaques are identified on the basis of phenotype, and mutant frequencies can be calculated for various tissues of the treated animals. The lambda *cII* locus of the transgene may be used as a second target gene in both the *lacZ* and *lacI* transgenic assays (Swiger et al., 1999; Shane et al., 2000; Swiger, 2001; Lambert et al., 2005). Its use offers technical advantages as a small, easily sequenced target in which mutations are detected by positive selection, and it permits interesting comparisons both within and between assays.

The transgenic *lacZ* plasmid mouse uses a plasmid-based system rather than a phage vector and has the advantage that deletion mutants are more readily recovered than in the phage-based *lac* systems (Lambert et al., 2005; OECD TG 488, 2013). The *gpt delta* mouse and rat transgenic animals detect two kinds of genetic events in two targets: point mutations in *gpt* detected by resistance to 6-thioguanine and *spi* (or Sensitive to P2 Interference) deletions that permit growth on P2 lysogens (Okada et al., 1999; Lambert et al., 2005; Cao et al., 2014; OECD TG 488, 2013). The *Spi*⁻ assay uniquely allows analyses of deletions and complex DNA rearrangements induced by DNA double-strand breaks using a phage vector system (Nohmi, 2016; OECD TG 488, 2013). Other transgenic assays are under development and offer the prospect of expanding the versatility of such assays (Lambert et al., 2005). These include pKZ1 mice in which inversions and deletions arising in various tissues by intrachromosomal recombination have been used to study effects of low doses of ionizing radiation (Sykes et al., 2006) and an assay that detects both forward mutations and reversion in mice that carry the genome of phage ΦX174 (Valentine et al., 2010).

Various mutagens, including alkylating chemicals, nitrosamines, procarbazine, cyclophosphamide, and polycyclic aromatic hydrocarbons have been studied in transgenic mouse assays, and mutant frequencies have been analyzed in such diverse tissues as liver, skin, spleen, kidney, bladder, small intestine, bone marrow, and testis (Lambert et al., 2005). Mutation frequencies in transgenes in testes have been compared to results in standard germ cell mutagenesis assays (Singer et al., 2006). Female germ cells are less amenable to study in transgenic assays because of the difficulty of collecting sufficient numbers of oocytes, but it has been suggested that granulosa cells from ovarian follicles may serve as a surrogate for the exposure of female germ cells to mutagens (Singer et al., 2006). Mutant frequencies have been compared to the formation of adducts in various tissues and to the site specificity of carcinogenesis and DNA repair capacity (Lambert et al., 2005). An important issue that remains to be resolved is the extent to which transgenes resemble endogenous genes. Transgenes used in routine mutation assays are transcriptionally inactive and hence differ from transcriptionally endogenous genes in their accessibility to mutagenesis as well as capability for repair. Although their mutational responses tend to be comparable (Lambert et al., 2005), some differences have been noted (Burkhart and Malling, 1993; Lambert et al., 2005), and questions have been raised about the relevance of mutations that might be recovered from dying or dead animal tissues (Burkhart and Malling, 1994). Several of the transgenic mutation assays have been adequately characterized to the point that there is an OECD test guideline for the conduct of these assays in somatic and germ cells (OECD TG 488, 2013).

Mammalian Cytogenetic Assays

Chromosome Aberrations Cytogenetic assays rely on the use of microscopy for the direct observation of the effects of interest. This approach differs sharply from the indirectness of traditional genetic assays in which one observes a phenotype and reaches conclusions about genes. It is only through the addition of DNA sequencing that genetic assays can approach the directness of cytogenetic assays.

In conventional cytogenetics, metaphase analysis is used to detect chromosomal anomalies, especially unstable chromosome and chromatid aberrations. A key factor in the design of cytogenetic assays is obtaining appropriate cell populations for treatment and analysis (Preston et al., 1981; Ishidate et al., 1988; Kirkland et al., 1990; Galloway et al., 1994). Cells with a stable, well-defined karyotype, short generation time, low chromosome number, and large chromosomes are ideal for cytogenetic analysis. For this reason, Chinese hamster cells have been used widely in cytogenetic testing. Other cells are also suitable, and human cells, especially peripheral lymphocytes, have been used extensively. Cells should be treated during a sensitive period of the cell cycle (typically S), and aberrations should be analyzed at the first mitotic division after treatment so that the sensitivity of the assay is not reduced by unstable aberrations being lost during cell division. Examples of chromosome aberrations are shown in Fig. 9-6.

Cytogenetic assays require careful attention to growth conditions, controls, dose levels, treatment conditions, and time intervals between treatment and the sampling of cells for analysis

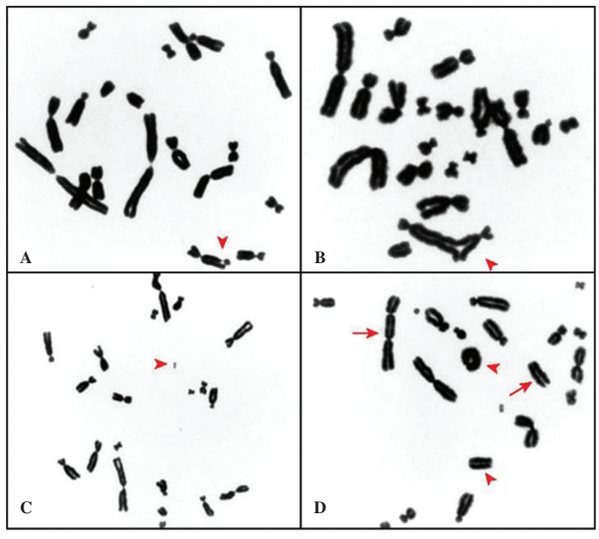

Figure 9-6. *Chromosome aberrations induced by x-rays in Chinese hamster ovary (CHO) cells.* (**A**) A chromatid deletion (▸). (**B**) A chromatid exchange called a triradial (▸). (**C**) A small interstitial deletion (▸) that resulted from chromosome breakage. (**D**) A metaphase with more than one aberration: a centric ring plus an acentric fragment (▸) and a dicentric chromosome plus an acentric fragment (→).

(Preston et al., 1981; Ishidate et al., 1988; Kirkland et al., 1990; Galloway et al., 2011). Data collection is a critical part of cytogenetic analysis. It is essential that sufficient cells be analyzed because a negative result in a small sample is inconclusive. Results should be recorded for specific classes of aberrations, not just an overall index of aberrations per cell. The need for detailed data is all the more important because of nonuniformity in the classification of aberrations and disagreement on whether small achromatic (i.e., unstained) gaps in chromosomes are true chromosomal aberrations. Gaps should be quantified but not pooled with other aberrations.

In interpreting results on the induction of chromosome aberrations in cell cultures, one must be alert to the possibility of artifacts associated with extreme assay conditions because aberrations induced under such circumstances may not be a reflection of a chemical-specific genotoxicity (Scott et al., 1991; Galloway, 2000; Galloway et al., 2011). Questionable positive results have been found at highly cytotoxic concentrations (Galloway et al., 2011), high osmolality, and pH extremes (Scott et al., 1991). The possibility that metabolic activation systems may be genotoxic also warrants scrutiny (Scott et al., 1991). Although excessively high concentrations may lead to false positive responses, the failure to test to a sufficiently high dose level may undermine the utility of a test. Therefore, testing should be extended to a concentration at which there is some cytotoxicity, such as a reduction in a replicative index or the mitotic index (the proportion of cells in division cycle). If the chemical is nontoxic, testing concentrations should extend up to an arbitrary concentration limit (Galloway et al., 2011). By a consensus of cytogeneticists and genetic toxicologists, the limit of 10 mM or 2 mg/mL, whichever is lower, has been recommended (Thybaud et al., 2017). However, the limit dose for the evaluation of human pharmaceuticals has been agreed to 1 mM (Galloway, 2017).

In vivo assays for chromosome aberrations involve treating intact animals and later collecting cells for cytogenetic analysis (Preston et al., 1981; Kirkland et al., 1990; Tice et al., 1994). The main advantage of in vivo assays is that they include mammalian metabolism, DNA repair, and pharmacodynamics. The target is typically a tissue from which large numbers of dividing cells are easily prepared for analysis. Bone marrow from rats, mice, or Chinese hamsters is most commonly used. Peripheral lymphocytes are another suitable target when stimulated to divide with a mitogen such as phytohemagglutinin. Effective testing requires dosages and routes of administration that ensure adequate exposure of the target cells, proper intervals between treatment and collecting cells, and sufficient numbers of animals and cells analyzed (Preston et al., 1981; Kirkland et al., 1990; Tice et al., 1994).

An important development in cytogenetic analysis is fluorescent in situ hybridization (FISH), in which a nucleic acid probe is hybridized to complementary sequences in chromosomal DNA (Tucker et al., 1993b, 2005; Paccierotti and Sgura, 2008). The probe is labeled with a fluorescent dye so that the chromosomal location to which it binds is visible by fluorescence microscopy, and the signal can be increased in strength by computer-enhanced processes. The use of whole-chromosome probes probed during metaphase or interphase nuclei is commonly called "chromosome painting" (Tucker et al., 1993b; Speicher and Carter, 2005; Paccierotti and Sgura, 2008). The level of sophistication has increased so much that all 24 different human chromosomes (22 autosomes, X and Y) can be individually detected (Macville et al., 1997), as can all mouse chromosomes (Liyanage et al., 1996).

Although FISH is not routinely used in genotoxicity testing, it is a valuable research tool for studying clastogens and is having a substantial impact in monitoring human populations for chromosomal damage. Fig. 9-7 shows chromosome painting and reciprocal translocations in human cancer cell. Regions of hybridization can be determined by the use of fluorescent antibodies that detect modified DNA bases incorporated during amplification or by incorporating fluorescent bases themselves during amplification. Another significant advantage of FISH methods is that the probes can be used with interphase cells/chromosomes making any tissue potentially available for analysis (Trask et al., 1993; Speicher and Carter, 2005; Vorsanova et al., 2010). Most recently, M-FISH (multicolor FISH) and M-BAND (multicolor banding) have been developed for defining chromosome abnormalities (Mackinnon and Chudoba, 2011). Alterations in tumors can also be detected on a whole-genome basis (Coleman et al., 1997; Veldman et al., 1997; Bridge and Cushman-Vokoun, 2011). The following example highlights the ability to construct breakpoint profiles of specific tumor types (Trost et al., 2006). One of the best known examples of a chromosomal breakpoint is the Philadelphia chromosome, a translocation abnormality of chromosome 22 containing a part of chromosome 9 that is often found in chronic myelogenous leukemia and sometimes in acute lymphocytic leukemia. In translocations, the involvement of specific chromosome bands that contain oncogenes or tumor-suppressor genes is also known.

Specifically, in chromosome 4 painted with a probe while the other chromosomes were counter-stained in a different color, one would see only the two homologues of chromosome 4 in the color of the probe in a normal cell. However, if there were a translocation or a dicentric chromosome and fragment involving chromosome 4, one would see three areas of fluorescence—one normal chromosome 4 and the two pieces involved in the chromosome rearrangement. Aberrations are detected only in the painted portion of the genome, but this disadvantage can be offset by painting a few chromosomes simultaneously with probes of different colors (Tucker et al., 1993b).

Micronuclei Metaphase analysis is time consuming and requires considerable skill, so simpler cytogenetic assays have been developed, of which micronucleus assays have become especially important. Micronuclei are membrane-bound structures that contain chromosomal fragments, or sometimes whole chromosomes,

Figure 9-7. *Chromosome aberrations identified by FISH.* Human breast cancer cell with aneuploidy for some chromosomes and with reciprocal translocations (identified by color switches along a chromosome).

that were not incorporated into a daughter nucleus at mitosis. Since micronuclei usually represent acentric chromosomal fragments, these are most commonly used as simple indicators of chromosomal damage. However, the ability to detect micronuclei containing whole chromosomes has led to their use for detecting aneuploidy as well. Micronucleus assays may be conducted in primary cultures of human lymphocytes (Fenech et al., 2003; Corvi et al., 2008; Fenech, 2008; Fenech et al., 2011b), mammalian cell lines (Kirsch-Volders et al., 2003; Corvi et al., 2008), or mammals in vivo (Krishna and Hayashi, 2000; Hayashi et al., 2007; Dertinger et al., 2011).

Micronucleus assays in lymphocytes have been greatly improved by the cytokinesis-block technique in which cell division is inhibited with cytochalasin B, resulting in binucleate and multinucleate cells (Fenech et al., 2003, 2011b; Kirsch-Volders et al., 2003; Fenech, 2008). In the cytokinesis-block assay in human lymphocytes, non-dividing (G_0) cells are treated with ionizing radiation or a radiomimetic chemical and then stimulated to divide with the mitogen phytohemagglutinin. Alternatively, the lymphocytes may be exposed to the mitogen first, so that the subsequent mutagenic treatment with radiation or chemicals includes the S period of the cell cycle. Cytochalasin B is added to inhibit the actin filament network formation and cytoplasmic division along with the test material when treating the cells continuously for 1.5 to 2 cell cycles in the absence of S9. For short duration exposures (e.g., 2-4 h), cytochalasin B is added at the end of treatment and the cultures are harvested also after 1.5 to 2 cell cycles. In both cases, micronuclei are counted only in binucleate cells so as to ensure that the cells have undergone a single nuclear division that is essential for micronucleus development. The assay avoids confusion owing to differences in cellular proliferation kinetics.

Although first devised using primary lymphocytes (Fenech et al., 2003; Fenech, 2008; Fenech et al., 2011b), the cytokinesis-block micronucleus assay has since been adapted for use in continuous cell cultures, including the Chinese hamster, human TK6, and mouse lymphoma cells that are widely used in other genotoxicity assays (Kirsch-Volders et al., 2003; Corvi et al., 2008). Micronucleus assays should be conducted in such a way that cellular proliferation is monitored along with the micronucleus frequency, and this is facilitated by the cytokinesis block. Reliable data have been obtained in cultured cells both with and without cytokinesis block, but scoring results only in binucleate cells after blockage of cytokinesis with cytochalasin B confers advantages with respect to the measurement of proliferation, recognizing whether an agent is cytostatic, and obtaining clear dose–response relationships (Kirsch-Volders et al., 2003).

Although micronuclei resulting from chromosome breakage comprise the principal endpoint in the cytokinesis-block micronucleus assay, the method can also provide evidence of aneuploidy, chromosome rearrangements that form nucleoplasmic bridges, inhibition of cell division, necrosis, apoptosis, and excision-repairable lesions (Fenech et al., 2003; Fenech, 2008; Fenech et al., 2011b). Micronuclei in a binucleate human lymphocyte are shown in Fig. 9-8. A recent review of the International Human Micronucleus (HUMN) Project provides a comprehensive description of standardized protocols for micronucleus assays in human lymphocytes and buccal cells together with a review of associations between micronucleus data and disease outcomes (Fenech et al., 2011a).

The in vivo micronucleus assay is often performed by counting micronuclei in immature (polychromatic) erythrocytes in the bone marrow of treated mice, but it may also be based on peripheral blood (Krishna and Hayashi, 2000; Hayashi et al., 2007). Micronuclei remain in the cell when the nucleus is extruded in the maturation of erythroblasts. In vivo micronucleus assays are increasingly used in genotoxicity testing as a substitute for bone marrow metaphase chromosome analysis. Micronucleus assays have been developed for mammalian tissues other than bone marrow and blood, including skin, duodenum, colon, liver, lung, spleen, testes, bladder, buccal mucosal cells, stomach, vagina, and fetal tissues (Coffing et al., 2011; Morita et al., 2011). Although assays in bone marrow and blood are the mainstay of genotoxicity testing, the new assays are important for mechanistic studies and research on the site specificity of genetic damage and carcinogenesis.

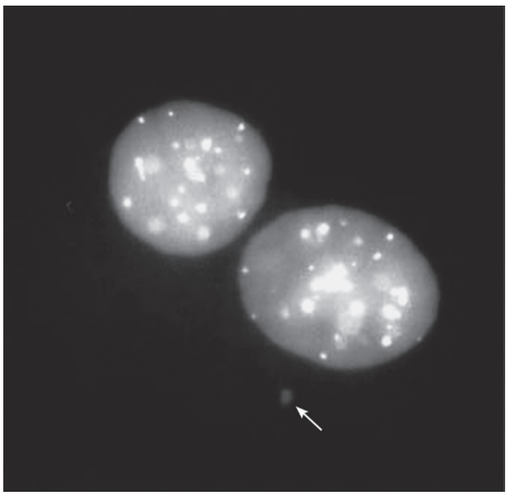

Figure 9-8. *Micronucleus in a human lymphocyte.* The cytochalasin B method was used to inhibit cytokinesis that resulted in a binucleate nucleus. The micronucleus (arrow) resulted from failure of an acentric chromosome fragment or a whole chromosome being included in a daughter nucleus following cell division. (Figure courtesy of James Allen, Jill Barnes, and Barbara Collins.)

Micronuclei are most commonly visualized through microscopy, but automated means of detecting micronuclei have been developed through the application of flow cytometry. Flow cytometric detection is effective in micronucleus assays in rodent bone marrow or blood (Dertinger et al., 2011). It can also be used to detect micronuclei in Chinese hamster CHO cells, where altered flow cytometric parameters can reveal whether the micronuclei arose primarily by chromosome breakage or by aneuploidy (Bryce et al., 2011).

Sister Chromatid Exchanges (SCE) Sister Chromatid Exchanges (SCE), in which there has been an apparently reciprocal exchange of segments between the two chromatids of a chromosome, are visible cytologically through differential staining of chromatids. Fig. 9-9 shows SCE in human cells. Many mutagens induce SCE in cultured cells and in mammals in vivo (Tucker et al., 1993a; Wilson and Thompson, 2007). Despite the convenience and responsiveness of SCE assays, data on SCE are less informative than data on chromosome aberrations. There is uncertainty about the underlying mechanisms by which SCEs are formed and how DNA damage or perturbations of DNA synthesis stimulate their formation (Preston, 1991). SCE assays are, therefore, best regarded as general indicators of mutagen exposure, analogous to DNA damage and repair assays, rather than measures of a mutagenic effect.

Aneuploidy Although assays for the induction of aneuploidy are not yet as refined as those for gene mutations and chromosome aberrations, they are being developed. The targets of aneugens

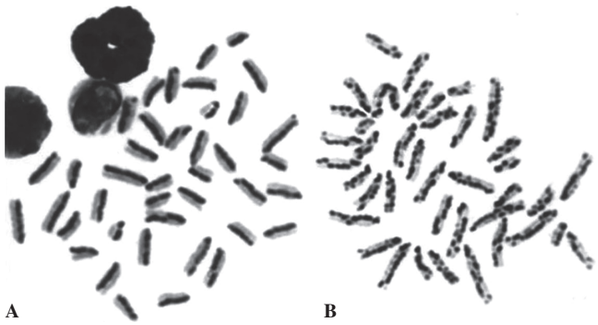

Figure 9-9. *Sister chromatid exchanges (SCEs) in human lymphocytes.* (**A**) SCE in untreated cell. (**B**) SCE in cell exposed to ethyl carbamate. The treatment results in a very large increase in the number of SCE. (Figure courtesy of James Allen and Barbara Collins.)

are often components of the mitotic or meiotic apparatus, rather than DNA. Therefore, aneugens should not be expected to overlap strongly with mutagens and clastogens. For example, a chemical that interferes with the polymerization of tubulin and thereby disrupts the formation of a mitotic spindle is likely to show specificity as an aneugen. Assays for chemicals that induce aneuploidy should thus encompass all the relevant cellular targets that are required for the proper functioning of the mitotic and meiotic process. Means of detecting aneuploidy include chromosome counting (Galloway and Ivett, 1986; Natarajan, 1993; Aardema et al., 1998; Paccierotti and Sgura, 2008), the detection of micronuclei that contain kinetochores (Lynch and Parry, 1993; Natarajan, 1993; Aardema et al., 1998; Fenech, 2008; Paccierotti and Sgura, 2008), and the observation of abnormal spindles or spindle–chromosome associations in cells in which spindles and chromosomes have been differentially stained (Parry, 1998). FISH-based assays have also been developed for the assessment of aneuploidy in interphase somatic cells (Rupa et al., 1997; Paccierotti and Sgura, 2008) and in sperm (Russo, 2000; Marchetti et al., 2006, 2008).

A complication in chromosome counting is that a metaphase may lack chromosomes because they were lost during cell preparation for analysis, rather than having been absent from the living cell. To avoid this artifact, cytogeneticists generally use extra chromosomes (i.e., hyperploidy) rather than missing chromosomes (i.e., hypoploidy) as an indicator of aneuploidy in chromosome preparations from mammalian cell cultures (Galloway and Ivett, 1986; Aardema et al., 1998) or mouse bone marrow (Adler, 1993). Techniques for counting chromosomes in intact cells may allow reliable measures of hypoploidy (Natarajan, 1993), but the detection of hyperploidy remains the norm in lieu of clear evidence that artifactual chromosome loss has been avoided. It has been suggested that counting polyploid cells, which is technically straightforward, may be an efficient way to detect aneugens (Aardema et al., 1998), but there is disagreement on the point (Parry, 1994, 1998).

Micronucleus assays can detect aneugens as well as clastogens. Micronuclei that contain whole chromosomes tend to be somewhat larger than those containing chromosome fragments, but the two categories are not readily distinguished in typically stained preparations (Natarajan, 1993). However, one can infer that a micronucleus contains a whole chromosome if it is shown to contain a kinetochore or centromeric DNA. Aneuploidy may thus be detected by means of antikinetochore antibodies with a fluorescent label or FISH with a probe for centromere-specific DNA (Lynch and Parry, 1993; Natarajan, 1993; Krishna and Hayashi, 2000; Fenech, 2008; Paccierotti and Sgura, 2008). Micronuclei containing kinetochores or centromeric DNA may be detected in cultured cells (Lynch and Parry, 1993; Aardema et al., 1998; Fenech, 2008; Paccierotti and Sgura, 2008) and in mouse bone marrow in vivo (Adler, 1993; Krishna and Hayashi, 2000). Frequencies of micronuclei ascribable to aneuploidy and to clastogenic effects may therefore be determined concurrently by tabulating micronuclei with and without kinetochores.

Germ Cell Mutagenesis

Gene Mutations Germ cell mutagenesis assays are of special interest as indicators of genetic damage that can enter the gene pool and be transmitted through generations. Mammalian germ cell assays provide the best basis for assessing risks to human germ cells and therefore hold a central place in genetic toxicology despite their relative complexity and expense. The design of the test must compensate for the fact that mutations occur at low frequency, and even the simplest animal systems face the difficulty of having a sufficiently large sample size. One can easily screen millions of bacteria or cultured cells by selection techniques, but screening large numbers of mice poses practical limitations. Therefore, a germ cell assay must offer a straightforward, unequivocal identification of mutants with minimal labor (Singer and Yauk, 2010; Yauk et al., 2015).

The mouse specific-locus test detects recessive mutations that produce easily analyzed, visible phenotypes (coat pigmentation and ear size) conferred by seven genes (Russell and Shelby, 1985; Ehling, 1991; Russell and Russell, 1992; Favor, 1999; Russell, 2004; Singer et al., 2006). Mutants may be classified as having point mutations or chromosomal alterations on the basis of genetic and molecular analysis (Favor, 1999). The assay has been important in assessing genetic risks of ionizing radiation and has been used to study various chemical mutagens. Although they have been used less extensively, there are other gene-mutation assays in mouse germ cells based on dominant mutations that cause skeletal abnormalities (Selby et al., 2004) or cataracts (Ehling, 1991) and recessive mutations that cause electrophoretic changes in proteins (Lewis, 1991).

Mammalian assays permit the measurement of mutagenesis at different germ cell stages (Favor, 1999; Russell, 2004). Late stages of spermatogenesis are often found to be sensitive to mutagenesis, but effects in spermatocytes, spermatids, and spermatozoa are transitory. Mutagenesis in stem-cell spermatogonia and resting oocytes is of special interest in genetic risk assessment because of the persistence of these stages throughout reproductive life. Chemical mutagens show specificity with respect to germ cell stages. For example, ethylnitrosourea and chlorambucil are both potent mutagens in the mouse specific-locus test, but the former induces primarily point mutations in spermatogonia, whereas the latter mostly induces deletions in spermatids (Russell and Russell, 1992). The ratio of deletions to point mutations is not only a function of the nature of the mutagen but depends on germ cell stage, as some mutagens induce higher proportions of gross alterations in late stages of spermatogenesis than in spermatogonia (Lewis, 1991; Favor, 1999; Russell, 2004).

There is currently no unequivocal evidence of induced gene mutations in human germ cells, but studies in mice leave little doubt about the susceptibility of mammalian germ cells to mutagenesis by radiation and chemicals. New molecular methods, particularly those involving the assessment of changes in tandem repeat loci (Yauk, 2004; Dubrova, 2005; Singer et al., 2006; Somers, 2006; Yauk et al., 2015), hold great promise for the development of systems that will permit the efficient detection of genetic alterations in human germ

cells. The development of methods based on expanded simple tandem repeat (ESTR) loci in mice and other species is important, in that it permits in situ monitoring for environmental mutagens and the quantification of mutagenesis after controlled exposures of laboratory animals using systems parallel to those being developed for human monitoring (Yauk, 2004; Dubrova, 2005; Wu et al., 2006; Somers, 2006). Basic research on mechanisms underlying ESTR changes is essential, as it is still unclear how ESTR changes relate to the gene mutations that have been long studied by population geneticists and are detected in classical gene-mutation assays.

Chromosomal Alterations Cytogenetic assays in germ cells are not routinely included in mutagenicity testing, but they are an important source of information for assessing risks to future generations posed by the induction of chromosome aberrations. Metaphase analysis of germ cells is feasible in rodent spermatogonia, spermatocytes, or oocytes (Kirkland et al., 1990; Tease, 1992; Russo, 2000; Marchetti et al., 2001). A micronucleus assay has also been developed in which chromosomal damage induced in meiosis is measured by the observation of rodent germ cells, principally spermatids (Russo, 2000; Hayashi et al., 2007).

Aneuploidy originating in mammalian germ cells may be detected cytologically through chromosome counting for hyperploidy (Allen et al., 1986; Adler, 1993; Aardema et al., 1998; Russo, 2000; Marchetti et al., 2001) or genetically in the mouse sex-chromosome loss test (Russell and Shelby, 1985), but these methods are not widely used in toxicological testing. A promising development is the detection of aneuploidy in the sperm of mice or rats by FISH with chromosome-specific probes (Baumgarthner et al., 1999; Russo, 2000; Marchetti et al., 2006, 2008). The presence of two fluorescent spots indicates the presence of an extra copy of the chromosome identified by the probe; probes for several chromosomes are used simultaneously so that aneuploid sperm are distinguishable from diploid sperm.

Besides cytological observation, indirect evidence for chromosome aberrations is obtained in the mouse heritable translocation assay, which measures reduced fertility in the offspring of treated males (Russell and Shelby, 1985; Singer et al., 2006). This presumptive evidence of chromosomal rearrangements can be confirmed through cytogenetic analysis. Data from the mouse heritable translocation test in postmeiotic male germ cells have been used in an attempt to quantify human germ cell risk for ethylene oxide, a mutagen used as a fumigant, sterilizing agent, and monomer in chemical syntheses (Preston et al., 1995).

Dominant Lethal Mutations The mouse or rat dominant lethal assay (Adler et al., 1994; Singer et al., 2006) has offered an extensive database on the induction of genetic damage in mammalian germ cells. In the most commonly used version of the assay, males are treated on an acute or subchronic basis with the chemical of interest and then mated with virgin females at appropriate intervals. The females are necropsied during pregnancy so that embryonic mortality may be characterized and quantified. Most dominant lethal mutations, manifested as intrauterine deaths, are thought to arise from chromosomal anomalies.

Development of Testing Strategies

Concern about adverse effects of mutation on human health, principally carcinogenesis and the induction of transmissible damage in germ cells, has provided the impetus to identify environmental mutagens. Priorities must be set for testing because it is not feasible to conduct multiple tests of all chemicals to which people are exposed. Such factors as production volumes, intended uses, the extent of human exposure, environmental distribution, and effects that may be anticipated on the basis of chemical structure or previous testing must be considered in order to ensure that compounds with the greatest potential for adverse effects receive the most comprehensive study. The most obvious use of genetic toxicology assays is screening chemicals to detect mutagens, but they are also used to obtain information on mutagenic mechanisms and dose–responses that contribute to an evaluation of hazards and risk. Besides testing pure chemicals, environmental samples are tested because many mutagens exist in complex mixtures (DeMarini 1998; Ohe et al., 2003; White, 2004). The analysis of complex mixtures often requires a combination of mutagenicity assays and refined analytical methods (White, 2004; Hewitt and Marvin, 2005). Assessment of a chemical's genotoxicity requires data from well-characterized and adequately validated assays. Assays are said to be validated when they have been shown to perform reproducibly and reliably with many compounds from diverse chemical classes in several laboratories. An evaluation of test performance and interpretability, however, sometimes extends beyond determining whether the assay effectively detects the specific endpoint that it actually measures to whether it is predictive of other endpoints of interest. For example, there is great interest in the ability of mutagenicity tests, which do not measure carcinogenicity per se, to predict whether chemicals are carcinogens.

Mutagenicity testing, combined with an evaluation of chemical structure, has been found to identify a large proportion of trans-species, multiple-site carcinogens (Tennant and Ashby, 1991; Gold et al., 1993). In contrast, some carcinogens are not detected as mutagens. Putatively non-genotoxic carcinogens often give responses that are more specific with respect to species, sites, and conditions (Ashby and Paton, 1993; Gold et al., 1993). In predicting carcinogenicity, one should consider both the sensitivity and the specificity of an assay. Sensitivity refers to the proportion of carcinogens that are positive in the assay, whereas specificity is the proportion of non-carcinogens that are negative (Tennant et al., 1987; McGregor et al., 1999). Sensitivity and specificity both contribute to the predictive reliability of an assay. The commonly held view that deficiencies in the sensitivity or specificity of individual assays may be circumvented by using assays in complementary combinations called tiers or batteries has fallen into disfavor because, rather than offsetting each other's strengths and weaknesses, genetic toxicology assays are often consistent with one another (Tennant et al., 1987; Ashby and Tennant, 1991; Kim and Margolin, 1999). The specificity of in vitro assays decreased to such an extent that nearly 95% of all noncarcinogens tested were detected as positive in at least one of the three in vitro assays (Ames, mouse lymphoma forward mutation assay, and in vitro cytogenetics; Kirkland et al., 2005).

Strategies for testing have evolved over the last few decades, such that data from a few well-chosen assays are now considered sufficient for intended applications (MacGregor et al., 2000; EFSA, 2012; Bolognesi et al., 2017; Ji et al., 2017). Rather than trying to assemble extensive batteries of complementary assays, it is prudent to emphasize mechanistic considerations in choosing assays. The bacterial Ames (OECD TG 471, 1997) test has always been considered as the preferred starting point for mutagenicity assessment and it performed reliably with thousands of compounds in laboratories throughout the world; it affords reasonable sensitivity and relatively high specificity compared to in vitro mammalian cell mutagenicity tests to detect rodent carcinogens and in vivo mutagens (Kirkland et al., 2005; Ji et al., 2017). In cases where a material cannot be adequately tested in bacteria because of its excessive toxicity (e.g., certain antimicrobial agents), mammalian cell cultures can be used to investigate mutagenic effects. Beyond gene mutations,

one should evaluate damage at the chromosomal level with a mammalian in vitro or in vivo cytogenetic assays. The preferred in vitro subset of mutagenicity and genotoxicity tests for regulatory mutagenicity assessments include bacterial reverse mutation test (OECD TG 471, 1997) and in vitro mammalian cell gene mutation tests using *Hprt* or *xprt* genes (OECD TG 476, 2016) for gene mutations; alternatively in vitro mammalian cell gene mutation tests using *tk* gene (OECD TG 490, 2015) is used to detect both gene mutations and structural chromosomal aberrations (Ji et al., 2017). For the assessment of structural chromosomal aberrations either in vitro mammalian chromosomal aberration test (OECD TG 473, 2016) or in vitro mammalian cell micronucleus test (OECD TG 487, 2016) can be used; the latter test, in addition to structural chromosomal aberrations, provides information of numerical chromosomal aberrations (Ji et al., 2017). Typically, negative outcomes in the in vitro assays would not call for in vivo follow-up, unless the intended application or regulatory jurisdiction called for it (Ji et al., 2017). Cytogenetic and transgenic mutation assays in rodents are especially useful for validating positive in vitro outcomes because they combine a well-validated genetic assay with mammalian pharmacodynamics and metabolism. The other assays in Table 9-1 offer an extensive database on chemical mutagenesis (i.e., *Drosophila* SLRL), a unique genetic endpoint (i.e., aneuploidy; mitotic recombination), applicability to diverse organisms and tissues (i.e., DNA damage assays, such as the comet assay), or special importance in the assessment of genetic risk (i.e., germ cell assays). The more extensive listing of assays in Table 9-2 provides references that can be helpful in interpreting genetic toxicology data that can be found in the scientific literature.

HUMAN POPULATION MONITORING

For cancer risk assessment considerations, the human data utilized most frequently, in the absence of epidemiologic data, are those collected from genotoxicity/mutagenicity assessments in human populations. For this purpose, the studies conducted most frequently are for chromosome aberrations, micronuclei, mutations (for several loci), and SCEs in peripheral lymphocytes. Cytogenetic alterations have also been assessed in a small number of bone marrow samples. Mutations at the *HPRT* locus have been assessed in peripheral lymphocytes. Glycophorin A variants have been studied in red blood cells. In the future, the *Pig-a* mutation assay using peripheral blood lymphocytes will likely gain popularity for human biomonitoring because of its technical simplicity.

An important component of any population monitoring study is the selection of the study groups, namely those individuals who are potentially exposed and the matched unexposed controls. The size of each study group should be sufficiently large to avoid any confounder having undue influence. It is also important to have a reliable measure to ascertain exposure(s). Certain characteristics should be matched among exposed and unexposed groups. These include age, sex, smoking status, and general dietary features. Certain characteristics are exclusionary, namely current or recent medication, radiation exposure, and certain illnesses. It is possible to develop a lengthy list of additional possible confounders of response that would make the selection of suitable study groups very difficult indeed. Study groups of 20 or more individuals can be used as a reasonable substitute for exact matching because confounders will be less influential on chromosome alteration or mutation frequency in larger groups (discussed in Au et al., 1998; Battershill et al., 2008). In some instances, it might be informative to compare exposed groups with a historical control, as well as to a concurrent control.

The magnitude of different known confounders varies considerably among studies, based in part on the size of the study populations. Some general indication of the magnitude of the effects of age and smoking status on the frequencies of chromosome aberrations and SCE is presented to illustrate the importance of accounting for confounders in the design of a population monitoring study. The comparisons presented are for large studies only. For chromosome aberrations, the frequency of aberrations has been reported in one large study to be about 50% higher in smokers (l.5 aberrations per 100 cells in smokers vs. 1.0 per 100 cells in the nonsmokers) (Galloway et al., 1986) and in another no difference between smokers and nonsmokers (Bender et al., 1988a, 1988b). The complete data set has been reviewed by Au et al. (1998). In general, the frequency of SCE is increased by about one SCE per cell in smokers compared with nonsmokers (Bender et al., 1988a, 1988b; Barale et al., 1998). The study by Barale et al. (1998) also reported a dose-response association between SCE frequency and smoking level. The differences among these studies might well be accounted for by differences among confounders in the respective groups, but it is virtually impossible to correct for these in this type of study.

Chromosome aberrations are among the most thoroughly studied effects of IR exposure (Awa, 1997). IR-induced chromosome aberrations include intrachromosomal exchanges that produce ring chromosomes or inversions, and interchromosomal exchanges, involving two chromosomes that produce dicentrics or translocations. A wide variability of the frequency of cells with chromosome aberrations between Hiroshima and Nagasaki atomic bomb survivors, different dose–response relationships in the two cities, significant effects of both time of assay, and age at the time of exposure were observed (Stram et al., 1993; Awa, 1997). Japanese atomic bomb survivor studies have demonstrated that translocation frequency is useful for the evaluation of IR dosimetry (Awa, 1997). Likewise, Chernobyl survivors exposed to very high IR doses showed complex chromosomal aberrations, with many dicentrics and reciprocal translocations (Awa, 1997).

The frequency of chromosome aberrations, particularly chromosome-type (reciprocal) exchanges, has been shown to increase with age of subject (Tucker and Moore, 1996). Galloway et al. (1986) reported an increase from 0.8 per 100 cells at about 25 years of age to about 1.5 at 60. Bender et al. (1988a, 1988b) reported an increase with age only for chromosome-type dicentric aberrations, but the increase over a broad age range was small and just statistically significant. Ramsey et al. (1995), using chromosome painting techniques, reported that individuals 50 years and older had frequencies of stable aberrations, dicentrics, and acentric fragments that were 10.6-, 3.3-, and 2.9-fold, respectively, greater than the frequency in cord bloods. Bender et al. (1988a, 1988b) did not find an increase in SCE frequency with the increasing age of the subject. The differences among the results from these large control studies emphasize the difficulty of adequately accounting for confounders (age and smoking presented here) when only a small control group is used, as is frequently the case.

Similar sources of variation have been identified for the monitoring of individuals for *HPRT* mutations. The data are reviewed in detail by Albertini and Hayes (1997). There is less information on sources of variation of glycophorin A (GPA) variants, although quite considerable inter-individual variation exists (reviewed in Cole and Skopek, 1994; Kyoizumi et al., 2005).

For cytogenetic assays (chromosome aberrations, SCEs, and micronuclei) the alterations are produced as a consequence of errors of DNA replication, as discussed in previous sections. From the nature of the alterations, assessed in traditional cytogenetic assays in which nontransmissible alterations are analyzed, it can be

established that these alterations were produced at the first in vitro S phase. Irrespective of the duration of exposure, the frequency of cytogenetic alterations will be proportional to that fraction of the DNA damage that remains at the time of in vitro DNA replication. All the DNA damage induced by potent clastogens that results in chromosome alterations is repaired within a relatively short time after exposure for G_0 human lymphocytes. Thus, for chronic exposures the lymphocyte cytogenetic assay as typically conducted is insensitive.

Reciprocal translocations using FISH methods can also be analyzed (reviewed in Awa, 1997; Tucker et al., 1997; Beskid et al., 2006; Kleinerman et al., 2006). The stable chromosome aberrations observed in peripheral lymphocytes exposed in vivo are produced in the hematopoietic stem cells or other precursor cells of the peripheral lymphocyte pool, but assessed following in vitro culture. To date, population cytogenetic monitoring studies involving the analysis of reciprocal translocations in chemically exposed individuals or radiation-exposed individuals have been rarely conducted (Lucas et al., 1992; Director et al., 1998; Smith et al., 1998; Kleinerman et al., 2006; Donner et al., 2010).

Another factor that certainly affects the utility of population monitoring data with reciprocal translocations using FISH is that the frequency of reciprocal translocations increases significantly with increasing age (Ramsey et al., 1995), but to a lesser extent for nontransmissible aberrations (Bender et al., 1988a, 1988b). Ramsey et al. (1995) provided data on the influence of other confounders on the frequency of reciprocal translocations in human groups. These confounders include smoking, consumption of diet drinks and/or diet sweeteners, exposure to asbestos or coal products, and having a previous major illness. This reemphasizes the point that the selection of study groups and accounting for confounders is essential for human population cytogenetic monitoring studies to be of utility.

Thus, very few of the published studies of cytogenetic population monitoring for individuals have analyzed the appropriate endpoint for detecting the genetic effects of long-term exposure to chemicals. It is quite surprising that positive responses have been reported for increases in unstable, chromatid aberrations because these are nontransmissible, and as noted above are induced at the first in vitro S phase. This anomaly is especially concerning when positive responses at very low levels of exposure are reported (reviewed for ethylene oxide in Preston, 1999b).

The HPRT mutation assay can assess the frequency of induced mutations in stem cells or other precursor cells because a proportion of the mutations are induced as nonlethal events. The transmissible proportion will be greater for chemicals that do not induce large deletions; this will include the majority of nonradiomimetic chemicals. Induction of mutations in lymphocyte precursor cells will lead to clonal expansion of mutations in the peripheral pool. However, assessment of the T-cell antigen receptor status of the mutant clones permits a correction for clonal expansion. The population of cells derived from any particular stem cell has a unique antigen receptor status (Albertini and Hayes, 1997). The GPA assay can similarly be used for the assessment of chronic exposures or for estimating exposures at some long time after exposure (Albertini and Hayes, 1997). The predictive value of the assay for adverse health outcome appears to be limited, but it can provide an estimate of exposure.

The potential for cytogenetic endpoints being predictive of relative cancer risk has been addressed in recent reports from the European Study Group on Cytogenetic Biomarkers and Health (Hagmar et al., 1998a, 1998b; Bonassi et al., 2004; Norppa et al., 2006). The groups selected for cytogenetic studies consisted of individuals with reported occupational exposure and unexposed controls. The association between cancer and the frequency of unstable chromosome aberrations in the study groups was not based on exposure status, but rather on the relative frequency of chromosome aberrations, namely, low (1 to 33 percentiles), medium (34 to 66 percentiles), and high (67 to 100 percentiles). In general, the higher the relative frequency of unstable aberrations, the greater the risk of cancer death for all tumors combined. The authors make it clear that there is insufficient information on exposure for it to be used as a predictor of cancer development. In fact, the data indicate that individuals with higher frequencies of chromosome aberrations for whatever reason (genetic or environmental) are *as a group* at greater risk of dying from cancer. This is very different from concluding that exposures to mutagens that result in a higher frequency of chromosome aberrations in peripheral lymphocytes leads to an increased risk of cancer, especially for specific tumor types. The relevance of exposure to mutagenic chemicals in these studies by Hagmar et al. (1998a, 1998b) is uncertain because there was no association between increased SCE frequencies, which may be indicative of higher exposure levels, and increased cancer mortality.

This latter concern was addressed by the same group (Bonassi et al., 2000) in a more recent study. The study again showed that there was a significantly increased risk for subjects with a high level of chromosome aberrations compared to those with a low level in both Nordic and Italian cohorts. Of particular relevance to risk assessment was the observation that the relationships were not affected by the inclusion of occupational exposure level or smoking. The risk for high versus low levels of chromosome aberrations was similar in individuals heavily exposed to carcinogens and in those who had never, to their knowledge, been exposed to any specific environmental carcinogen. These data highlight the need to use caution when considering the relevance of chromosome aberration data from human biomonitoring studies in cancer risk assessment.

NEW APPROACHES FOR GENETIC TOXICOLOGY

In the last 20 or so years, the field of genetic toxicology has moved into the molecular and computational era. The potential for advances in our understanding of basic cellular processes and how they can be perturbed is enormous. The ability to manipulate and characterize DNA, RNA, and proteins has been at the root of this advance in knowledge. New understanding in biological mechanism and mode of action (MOA), along with new high-content and high-throughput approaches, and increasingly sensitive analytical methods, bring new information into the field of genetic toxicology and the quantitative risk assessment processes for DNA-reactive chemicals (MacGregor et al., 2015a, 2015b; Klapacz et al., 2016). The introduction of new high-throughput screening to assess various biomarker parameters, such as DNA damage to evaluate genotoxicity, offers rapid, cost-effective, and nonlabor-intensive screening approaches (Ranganatha et al., 2016). A clear focus on molecular and biological approaches to defining and understanding consequences of DNA damage at the cellular level fits well with the 2007 NRC report, "Toxicity Testing in the 21st Century: A Vision and a Strategy" that envisions a future in which all routine testing will use cell-based in vitro assays of toxicity pathways (NRC, 2007b; Krewski et al., 2010). It is now becoming understood that complex interactions within a particular DNA repair pathway, as well as interplay between different DNA repair and DDR pathways exist that counteract the effects of ever-present background DNA damage. Together, these pathways appear sufficient to counteract mutation at low exposures/doses and/or the propagation of cells harboring significant DNA damage at least for well-studied

DNA-reactive classes (Klapacz et al., 2016). Hence, the examination of key cellular, mechanistic responses should be integrated into the designs of the mutagenic MOA studies and risk characterization processes for DNA-reactive chemicals.

Advances in Cytogenetics

Until quite recently, the analysis of chromosome alterations relied on conventional chromosome staining with DNA stains such as Giemsa or on the process of chromosome banding. Both approaches require considerable expenditure of time and a rather high level of expertise. However, chromosome banding does allow for the assessment of transmissible aberrations such as reciprocal translocations and inversions with a fairly high degree of accuracy. Knowing the induction frequency of such aberrations is very important, given that they are generally not lethal to the cell and constitute by far the major class observed in inherited genetic defects and a significant fraction of the alterations observed in tumors. In addition, because stable aberrations are transmissible from parent to daughter cell, they represent accumulated effects of chronic exposures. The more readily analyzed, but cell-lethal, nontransmissible aberrations such as dicentrics and deletions reflect only recent exposures and then only when analyzed at the first division after exposure. A more detailed discussion of these factors can be found in Preston (1999b).

There is an extensive literature on the use of FISH for tumors and in gene mapping but less on its utility for genetic toxicology studies, especially the assessment of stable chromosome aberrations at long periods after exposure or after long-term exposures. Three particular studies do, however, serve to exemplify the use of FISH in genetic toxicology.

Lucas et al. (1992) demonstrated that stable chromosomal aberrations could be detected in individuals decades after exposure to atomic bombs in Japan. How these frequencies relate to frequencies at the time of exposure is not known with any certainty, given the fact that induced frequencies were not measured because appropriate techniques were not available at that time.

Studies by Tucker et al. (1997, 2005) provided some assessment of the utility of FISH karyotyping for the analysis of radiation-induced, stable chromosome alterations at various times after exposure. The frequency of reciprocal translocations induced by γ-rays in rat peripheral lymphocytes decreased with time after exposure, reaching a plateau at 4 days that was 55% to 65% of the induced frequency and with a dose dependency (Tucker et al., 1997). Similar results were obtained for human samples (Tucker et al., 2005). These results suggest that reciprocal translocations fall into two classes, stable and unstable (cell-lethal). It is quite possible that these "unstable" translocations are lost because of the presence of other cell-lethal damage in the same cell. Additional work is required to clarify this conclusion and to extend the studies to the effects of chemicals. In addition, a recent study by Tucker and Luckinbill (2011) demonstrated how FISH translocation analysis could be used to detect extremely low doses of ionizing radiation—a few cGy per year. Such analysis could be used to estimate responses at very low doses for utility in risk assessments at similarly low doses.

FISH methods have also allowed for an accurate and sensitive assessment of chromosomal alterations present in tumors. The particular advance that makes this assessment feasible is known as comparative genomic hybridization (CGH) (Kallioniemi et al., 1992). CGH results in the ability to identify the role of chromosomal structural and numerical alterations in tumor development. The genomic instability present in all tumor types appears to have a specific genetic basis, as shown elegantly for colon cancer by Vogelstein and colleagues (Cahill et al., 1998). For CGH, tumor and control DNAs are differentially labeled with fluorescence probes and co-hybridized to normal metaphase chromosome preparations. The ratio of the fluorescence intensities of hybridized tumor and control DNA indicates regions of normal genomic content as well as those regions that are over- or underrepresented in tumors. The CGH method is being adapted for automated screening approaches using biochips (Solinas-Toldo et al., 1997; Hosoya et al., 2006). Assessing genetic alterations such as specific gene deletions in single metastatic tumor cells is feasible using a slightly different but complementary approach (Pack et al., 1997, 2005).

The types of FISH approaches described here undoubtedly indicate the direction in which cytogenetic analysis will proceed. The types of data collected will affect our understanding of how tumors develop. Data on the dose–response characteristics for a specific chromosomal alteration as a proximate marker of cancer can enhance the cancer risk assessment process by describing effects of low exposures that are below those for which tumor incidence can be reliably assessed. Cytogenetic data of the types described above can also improve extrapolation from data generated with laboratory animals to humans.

Molecular Analysis of Mutations and Gene Expression

With the advent of molecular biology techniques, the exact basis of a mutation at the level of the DNA sequence can be established. In many cases, the genetic basis of human disease can be determined even though human genes have long DNA sequences and a complex genomic arrangement. Molecular biology techniques have also enabled a distinction to be made between background mutations and those induced by specific agents. The latter observations are addressed by analyzing the mutational spectra in target genes in laboratory organisms and in humans (DeMarini, 2000; Hemminki and Thilly, 2004). For reasons of inherent sensitivity of available methods, the genes analyzed for mutations are ones for which mutated forms can be selected. The confounding factor of many normal cells, which far outnumber a few mutant cells in an exposed cellular population, can be removed by mutant selection approaches. Methods to overcome the drawback of only being able to study selectable genes have been developed, and particular ones such as ligation-mediated PCR are close to the required sensitivity level (Albertini and Hayes, 1997; Makrigiorgos, 2004; Yeh et al., 2006). For example, COLD-PCR (co-amplification at lower denaturation temperature-PCR) was developed to selectively amplify minority alleles from mixtures of wild-type and mutation-containing sequences (Li and Makrigiorgos, 2009).

A giant step forward in the ability to detect and characterize mutations at both the DNA and RNA level has been provided by the development of chip technology (Southern, 1996), array-based assay systems (Wodicka et al., 1997), and next-generation high-throughput DNA sequencing technologies at the whole genome level in a very short period of time (Greenman et al., 2007; Watson et al., 2013; Yauk et al., 2015; Du et al., 2017). With hybridization of test DNAs to oligonucleotide arrays, specific genetic alterations or their cellular consequences can be determined rapidly and automatically (Houlston and Peto, 2004; Vissers et al., 2005). Genome-wide gene expression microarray technologies now allow the measurement of changes in expression of thousands of genes at one time (Harrington et al., 2000; Elvidge, 2006; Blencowe et al., 2009). There are parallel efforts in the area of proteomics and metabolomics whereby changes in a broad range of cellular proteins

can be assessed in response to endogenous or exogenous factors, potentially leading to the development of biomarkers of effect (Aebersold et al., 2005; Robertson, 2005; Griffin, 2006; McGregor and De Souza, 2006). The biggest hurdle currently is the relative paucity of sequence data available for the world of proteins and their multiple posttranslational modifications. Certainly progress is rapid, and so methodologies akin to gene expression assessment are likely to be close at hand (Xie et al., 2011). The sensitivity of next-generation sequencing protocols is rapidly increasing and the costs are gradually coming down. Such techniques have been used to characterize the genetic alterations in a large number of tumor types, allowing for new insight and revealing unanticipated complexities in the patterns of somatic alterations in cancers (Greenman et al., 2007; Stratton et al., 2009; Watson et al., 2013). Overall, the current potential for assessing specific cellular changes following chemical exposure is enormous.

The move in the field of genetic toxicology is away from the "yes/no" approach to hazard identification and much more toward a mechanistic understanding of how a chemical or physical agent can produce adverse cellular and tissue responses. In turn, such knowledge can be used for the development of informative bioindicators representing the key events along the pathway from initial interactions with cells to adverse outcome (Jarabek et al., 2009). The move is clearly toward analysis at the whole genome level and away from single gene responses. The challenges are apparent and the solutions are being identified at a rapid pace. In a recent publication, Dearfield et al. (2017) laid the foundation for the next generation testing strategy to assess genomic damage. This strategy envisions a broader focus for assessing genomic damage instead of primarily fixating on carcinogen prediction.

CONCLUSIONS

The field of genetic toxicology has had an overall life of about 70 years and has undergone several rebirths during this period. Genetic toxicology began as a basic research field with demonstrations that ionizing radiation and chemicals could induce mutations and chromosome alterations in plant, insect, and mammalian cells. The development of a broad range of short-term assays for genotoxicity served to identify many mutagens and address the relationship between mutagens and cancer-causing agents, or carcinogens. The inevitable failure of the assays to be completely predictive resulted in the identification of nongenotoxic carcinogens. In the 1980s, genetic toxicology began to move toward providing insights into a better understanding of the mutagenic mechanisms underlying carcinogenicity and heritable effects. In recent years, genetic toxicology studies began to turn away from hazard identification alone and move toward risk-based testing (Gollapudi, 2017).

Genetic toxicology has begun to take advantage of the knowledge that cancer is a genetic disease with multiple steps, many of which require a mutation. The ability to distinguish between background and induced mutations, in some cases, can be achieved by mutation analysis at the level of DNA sequence. Next-generation sequencing technologies have allowed such whole genome mutation analysis to be accomplished in a very short time. Key cellular processes related to mutagenesis have been identified, including multiple pathways of DNA repair, cell cycle controls, and the role of checkpoints in ensuring that the cell cycle does not proceed until the DNA and specific cellular structures are checked for fidelity. These observations have enhanced our knowledge of the importance of genotype in susceptibility to cancer. Recent developments in genetic toxicology have greatly improved our understanding of basic cellular processes and alterations that can affect the integrity of the genetic material and its functions. The incorporation of key cellular, mechanistic information should be integrated into the designs of the MOA studies of various types of DNA-reactive chemicals and applied to their risk assessment processes. The ability to detect and analyze mutations in mammalian germ cells continues to improve and can contribute to a better appreciation for the long-term consequences of mutagenesis in human populations. Improvements in the qualitative assessment of mutation in somatic cells and germ cells have been paralleled by advances in the ability to assess genetic alterations quantitatively, especially in ways that enhance the cancer and genetic risk assessment process (Preston, 2005; Gollapudi, 2017). These new approaches will reduce overall uncertainty in the process, specifically for risk characterization and uncertainties surrounding exposures at environmentally relevant concentrations (Table 9-4).

Table 9-4

Overview of Global Regulatory Requirements for Genotoxicity Data

A. Agrochemicals: Booth et al. (2017)
B. Industrial chemicals: Ji et al. (2017)
C. Food contact materials: Bolognesi et al. (2017)
D. Human pharmaceuticals: Galloway (2017)
E. Medical devices: Przygoda (2017)

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REFERENCES

Aardema MJ, Albertini S, Arni P, et al. Aneuploidy: a report of an ECETOC task force. *Mutat Res*. 1998;410:3–79.

Abul-Husn NS, Owusu Obeng A, Sanderson SC, Gottesman O, Scott SA. Implementation and utilization of genetic testing in personalized medicine. *Pharmgenomics Pers Med*. 2014;7:227–240.

Aboussekhra A, Biggerstaff M, Shivji JA, et al. DNA nucleotide excision repair reconstituted with purified protein components. *Cell*. 1995;80:859–868.

Adar S, Izhar L, Hendel A, Geacintov N, Livneh Z. Repair of gaps opposite lesions by homologous recombination in mammalian cells. *Nucleic Acids Res*. 2009;37(17):5737–5748.

Adler I-D. Synopsis of the in vivo results obtained with the 10 known or suspected aneugens tested in the CEC collaborative study. *Mutat Res*. 1993;287:131–137.

Adler I-D, Shelby MD, Bootman J, et al. Summary report of the working group on mammalian germ cell tests. *Mutat Res*. 1994;312:313–318.

Aebersold R, Anderson L, Caprioli R, et al. Perspective: a program to improve protein biomarker discovery for cancer. *Proteome Res*. 2005;4:1104–1109.

Aguilar F, Harris CC, Sun T, et al. Geographic variation of p53 mutational profile in nonmalignant human liver. *Science*. 1994;264:1317–1319.

Ahnesorg P, Smith P, Jackson SP. XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell*. 2006;124:301–313.

Alapetite C, Thirion P, De la Rochefordie A, Cosset JM, Moustacchi E. Analysis by alkaline comet assay of cancer patients with severe reactions to radiotherapy: defective rejoining of radioinduced DNA strand breaks in lymphocytes of breast cancer patients. *Int J Cancer*. 1999;90:83–90.

Albertini RJ. *HPRT* mutations in humans; biomarkers for mechanistic studies. *Mutat Res*. 2001;489:1–16.

Albertini RJ. Vinyl acetate monomer (VAM) genotoxicity profile: relevance for carcinogenicity. *Crit Rev Toxicol*. 2013;43(8):671–706.

Albertini RJ, Hayes RB. Somatic cell mutations in cancer epidemiology. *IARC Sci Publ*. 1997;142:159–184.

Albertini RJ, Sweeney LM. Propylene oxide: genotoxicity profile of a rodent nasal carcinogen. *Crit Rev Toxicol*. 2007;37(6):489–520.

Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500:415–421.

Allen JW, Liang JC, Carrano AV, Preston RJ. Review of literature on chemical-induced aneuploidy in mammalian germ cells. *Mutat Res*. 1986;167:123–137.

Alyea RA, Gollapudi BB, Rasoulpour RJ. Are we ready to consider transgenerational epigenetic effects in human health risk assessment? *Environ Mol Mutagen*. 2014;55(3):292–298.

Ames BN, Gold LS. Chemical carcinogenesis: too many rodent carcinogens. *Proc Natl Acad Sci U S A*. 1990;87(19):7772–7776.

Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat Res*. 1975;31:347–364.

Araújo SJ, Tirode F, Coin F, et al. Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. *Genes Dev*. 2000;14:349–359.

Aravind L, Koonin EV. The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. *Genome Biol*. 2001;2(3):1–8.

Ashby J. Two million rodent carcinogens? The role of SAR and QSAR in their detection. *Mutat Res*. 1994;305:3–12.

Ashby J, Paton D. The influence of chemical structure on the extent and sites of carcinogenesis for 522 rodent carcinogens and 55 different human carcinogen exposures. *Mutat Res*. 1993;286:3–74.

Ashby J, Tennant RW. Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP. *Mutat Res*. 1991;257:229–306.

Ashby J, Waters MD, Preston J, et al. IPCS harmonization of methods for the prediction and quantification of human carcinogenic/mutagenic hazard, and for indicating the probable mechanism of action of carcinogens. *Mutat Res*. 1996;352:153–157.

Au WW, Gajas-Salazar N, Salama S. Factors contributing to discrepancies in population monitoring studies. *Mutat Res*. 1998;400:467–478.

Auerbach C, Robson JM. Chemical production of mutations. *Nature*. 1946;157:302.

Awa A. Analysis of chromosome aberrations in atomic bomb survivors for dose assessment: studies at the Radiation Effects Research Foundation from 1968 to 1993. *Stem Cells*. 1997;15(suppl 2):163–173.

Bansbach CE, Cortez D. Defining genome maintenance pathways using functional genomic approaches. *Crit Rev Biochem Mol Biol*. 2011;46:327–341.

Barale R, Chelotti L, Davini T, et al. Sister chromatid exchange and micronucleus frequency in human lymphocytes of 1,650 subjects in an Italian population: II. Contribution of sex, age, and lifestyle. *Environ Mol Mutagen*. 1998;31:228–242.

Barrett JC. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. *Environ Health Prospect*. 1993;100:9–20.

Bartek J, Falck J, Lukas J. CHK2 kinase—a busy messenger. *Nat Rev Mol Cell Biol*. 2001;2:877–886.

Bartek J, Lukas J. Mammalian G1- and S-phase checkpoints in response to DNA damage. *Curr Opin Cell Biol*. 2001;13:738–747.

Battershill JM, Burnett K, Bull S. Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies. *Mutagenesis*. 2008;23:423–437.

Baumgarthner A, Van Hummelen P, Lowe XR, et al. Numerical and structural chromosomal abnormalities detected in human sperm with a combination of multicolor FISH assays. *Environ Mol Mutagen*. 1999;33:49–58.

Beale G. The discovery of mustard gas mutagenesis by Auerbach and Robson in 1941. *Genetics*. 1993;134:393–399.

Becker RA, Ankley GT, Edwards SW, et al. Increasing scientific confidence in adverse outcome pathways: application of tailored Bradford-Hill considerations for evaluating weight of evidence. *Regul Toxicol Pharmacol*. 2015;72:514–537.

Becker RA, Dellarco V, Seed J, et al. Quantitative weight of evidence to assess confidence in potential modes of action. *Regul Toxicol Pharmacol*. 2017;86:205–220.

Bell DW. Our changing view of the genomic landscape of cancer. *J Pathol*. 2010;220(2):231–243.

Bender MA, Gooch PC. Persistent chromosome aberrations in irradiated human subjects. *Radiat Res*. 1962;16:44–53.

Bender MA, Preston RJ, Leonard RC, et al. Chromosomal aberration and sister-chromatid exchange frequencies in peripheral blood lymphocytes of a large human population sample. *Mutat Res*. 1988a;204:421–433.

Bender MA, Preston RJ, Leonard RC, et al. Chromosomal aberration and sister-chromatid exchange frequencies in peripheral blood lymphocytes of a large human population sample. II. Extension of age range. *Mutat Res*. 1988b;212:149–154.

Bentley KS, Sarrif AM, Cimino MC, Auletta AE. Assessing the risk of heritable gene mutation in mammals: *Drosophila* sex-linked recessive lethal test and tests measuring DNA damage and repair in mammalian germ cells. *Environ Mol Mutagen*. 1994;23:3–11.

Beranek DT. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat Res*. 1990;231:11–30.

Beskid O, Dusek Z, Solansky I, et al. The effects of exposure to different clastogens on the pattern of chromosomal aberrations detected by FISH whole chromosome painting in occupationally exposed individuals. *Mutat Res*. 2006;594:20–29.

Bavetsias V, Linardopoulos S. Aurora kinase inhibitors: current status and outlook. *Front Oncol*. 2015;5:278.

Bickel SE, Orr-Weaver TL. Holding chromatids together to ensure they go their separate ways. *Bioessays*. 1996;18:293–300.

Birrell L Cahill P, Hughes C, Tate M, Walmsley RM. GADD45a-GFP GreenScreen HC assay results for the ECVAM recommended lists of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests. *Mutat Res: Genet Toxicol Environ Mutagen*. 2009;695(1):87–95.

Bishop JM. Molecular themes in oncogenesis. *Cell*. 1991;64:235–248.

Blaisdell JO, Harrison L, Wallace SS. Base excision repair processing of radiation-induced clustered DNA lesions. *Radiat Prot Dosimetry*. 2001;97:25–31.

Blencowe BJ, Ahmad S, Lee LJ. Current-generation high-throughput sequencing: deepening insights into mammalian transcriptomes. *Genes Dev*. 2009;23:1379–1386.

Boiteux S, Guillet M. Abasic sites in DNA: repair and biological consequences in Saccharomyces cerevisiae. *DNA Repair*. 2004;3(1):1–12.

Boiteux S, Laval J. Imidazole open ring 7-methylguanine: an inhibitor of DNA synthesis. *Biochem Biophys Res Commun*. 1983;110(2):552–558.

Bolognesi C, Castoldi AF, Crebelli R, et al. Genotoxicity testing approaches for the safety assessment of substances used in food contact materials prior to their authorization in the European Union. *Environ Mol Mutagen*. 2017;58(5):361–374.

Bonassi S, Hagmar L, Strömberg U, et al. Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. *Cancer Res*. 2000;60:1619–1625.

Bonassi S, Znaor A, Norppa H, Hagmar L. Chromosomal aberrations and risk of cancer in humans: an epidemiologic perspective. *Cytogenet Genome Res*. 2004;104:376–382.

Boobis AR, Cohen S M, Dellarco V, et al. IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit Rev Toxicol*. 2006;36:781–792.

Booth ED, Rawlinson PJ, Maria Fagundes P, Leiner KA. Regulatory requirements for genotoxicity assessment of plant protection product active ingredients, impurities, and metabolites. *Environ Mol Mutagen*. 2017;58(5):325–344.

Borrego-Soto G, Ortiz-López R, Rojas-Martínez A. Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer. *Genet Mol Biol*. 2015;38(4):420–432.

Boysen G, Pachkowski BF, Nakamura J, Swenberg JA. The formation and biological significance of N7-guanine adducts. *Mutat Res*. 2009;678: 76–94.

Brendler-Schwaab S, Czich A, Epe B, et al. Photochemical genotoxicity: principles and test methods. Report of a GUM task force. *Mutat Res*. 2004;566:65–91.

Brewen JG, Preston RJ. Cytogenetic effects of environmental mutagens in mammalian cells and the extrapolation to man. *Mutat Res*. 1974;26:297–305.

Brewen JG, Preston RJ. Cytogenetic analysis of mammalian oocytes in mutagenicity studies. In: Hsu TC, ed. *Cytogenetic Assays of Environmental Mutagens*. Totowa, NJ: Allanheld, Osmun; 1982:277–287.

Bridge JA, Cushman-Vokoun AM. Molecular diagnostics of soft tissue tumors. *Arch Pathol Lab Med*. 2011;135:588–601.

Broughton BC, Berneburg M, Fawcett H, et al. Two individuals with features of both xeroderma pigmentosum and trichothiodystrophy highlight the complexity of the clinical outcomes of mutations in the XPD gene. *Hum Mol Genet*. 2001;10:2539–2547.

Brusick JD, ed. *Methods for Genetic Risk Assessment*. Boca Raton, FL: CRC Press; 1994.

Bryce SM, Avlasevich SL, Bemis JC, et al. Miniaturized flow cytpmetry-basedCHO-K1 micronucleus assay discriminates aneugenic and clastogenic modes of action. *Environ Mol Mutagen*. 2011;52:280–286.

Burkhart JG, Malling HV. Mutagenesis and transgenic systems: perspective from the mutagen, N-ethyl-N-nitrosourea. *Environ Mol Mutagen*. 1993;22:1–6.

Burkhart JG, Malling HV. Mutations among the living and the undead. *Mutat Res*. 1994;304:315–320.

Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair*. 2006;5:1042–1048.

Butterworth BE, Ashby J, Bermudez E, et al. A protocol and guide for the in vivo rat hepatocyte DNA-repair assay. *Mutat Res*. 1987;189(2):123–133.

Cadet J, Douki T, Ravanat JL. Measurement of oxidatively generated base damage in cellular DNA. *Mutat Res*. 2011;711:3–12.

Cahill D, Connor B, Carney JP. Mechanisms of eukaryotic DNA double strand break repair. *Front Biosci*. 2006;11:1958–1976.

Cahill DP, Lengauer C, Yu J, et al. Mutations of mitotic checkpoint genes in human cancers. *Nature*. 1998;392:300–303.

Calos MP, Miller JH. Genetic and sequence analysis of frameshift mutations induced by ICR-191. *J Mol Biol*. 1981;153:39–66.

Cao X, Mittelstaedt RA, Pearce MG, et al. Quantitative dose-response analysis of ethyl methanesulfonate genotoxicity in adult gpt-delta transgenic mice. *Environ Mol Mutagen*. 2014;55(5):385–399.

Cariello NF, Skopek TR. Analysis of mutations occurring at the human *hprt* locus. *J Mol Biol*. 1993;231:41–57.

Cary RB, Peterson SR, Wang J, et al. DNA looping by Ku and the DNA-dependent protein kinase. *Proc Natl Acad Sci U S A*. 1997;94:4267–4272.

Casciano DA, Aidoo A, Chen T, et al. *Hprt* mutant frequency and molecular analysis of *Hprt* mutations in rats treated with mutagenic carcinogens. *Mutat Res*. 1999;431:389–395.

Cavenee WK, Dryja TP, Phillips RA, et al. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*. 1983;305:779–784.

Cebula TA, Koch WH. Sequence analysis of *Salmonella typhimurium* revertants. In: Mendelsohn ML, Albertini RJ, eds. *Mutation and the Environment, Part D: Carcinogenesis*. New York: Wiley-Liss; 1990:367–377.

Cejka P, Stojic L, Marra G, Jiricny J. Is mismatch repair really required for ionizing radiation-induced DNA damage signaling? *Nat Genet*. 2004;36(5):432–433.

Chatterjee N, Siede W. Replicating damaged DNA in eukaryotes. *Cold Spring Harb Perspect Biol*. 2013;5:a019836.

Chatterjee N, Walker GC. Mechanisms of DNA damage, repair, and mutagenesis. *Environ Mol Mutagen*. 2017;58(5):235–263.

Chen Y, Sanchez Y. Chk1 in the DNA damage response: conserved roles from yeasts to mammals. *DNA Repair (Amst)*. 2004;3:1025–1032.

Chevereau M, Glatt H, Zalko D, Cravedi JP, Audebert M. Role of human sulfotransferase 1A1 and N-acetyltransferase 2 in the metabolic activation of 16 heterocyclic amines and related heterocyclics to genotoxicants in recombinant V79 cells. *Arch Toxicol*. 2017;91(9):3175–3184.

Cimino MC. Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. *Environ Mol Mutagen*. 2006;47:362–390.

Claxton LD, Umbuzeiro GD, Demarini DM. The *Salmonella* mutagenicity assay: the stethoscope of genetic toxicology for the 21st century. *Environ Health Perspect*. 2010;118:1515–1522.

Clements J. The mouse lymphoma assay. *Mutat Res*. 2000;455:97–110.

Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev*. 1972;52(1): 198–236.

Ceccaldi R, Liu JC, Amunugama R, et al. Homologous-recombination-deficient tumours are dependent on Polθ-mediated repair. *Nature*. 2015; 518:258–562.

Clive D, Caspary W, Kirby PE, et al. Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity. *Mutat Res*. 1987;189(2): 143–156.

Coffing S, Engel M, Dickinson D, et al. The rat gut micronucleus assay: a good choice for alternative in vivo genetic toxicology testing strategies. *Environ Mol Mutagen*. 2011;52:269–279.

Cohen SM, Arnold LL. Chemical carcinogenesis. *Toxicol Sci*. 2011; 120(S1):S76–S92.

Cohen SM, Ellwein LB. Genetic errors, cell proliferation, and carcinogenesis. *Cancer Res*. 1991;51(24):6493–6505.

Cohen SM, Purtilo DT, Ellwein LB. Ideas in pathology. Pivotal role of increased cell proliferation in human carcinogenesis. *Mod Pathol*. 1991;4(3):371–382.

Coin F, Oksenych V, Egly JM. Distinct roles for the XPB/p52 and XPD/p44 subcomplexes of TFIIH in damaged DNA opening during nucleotide excision repair. *Mol Cell*. 2007;26:245–256.

Cole J, Skopek TR. Somatic mutant frequency, mutation rates and mutational spectra in the human population in vivo. *Mutat Res*. 1994;304:33–105.

Coleman AE, Schrock E, Weaver Z, et al. Previously hidden chromosome aberrations in t(12;15)-positive BALB/c plasmacytomas uncovered by multicolor spectral karyotyping. *Cancer Res*. 1997;57:4585–4592.

Coleman KE, Huang TT. How SUMOylation fine-tunes the Fanconi anemia DNA repair pathway. *Front Genet*. 2016;7:61.

Collins A, El Yamani N, Dusinska M. Sensitive detection of DNA oxidation damage induced by nanomaterials. *Free Radic Biol Med*. 2017;107:69–76.

Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol*. 2004;26:249–261.

Colussi C, Parlanti E, Degan P, et al. The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool. *Curr Biol*. 2002;12:912–918.

Conrad DF, Keebler JE, DePristo MA, et al. Variation in genome-wide mutation rates within and between human families. *Nat Genet*. 2011;43(7):712–714.

Corvi R, Albertini S, Hartung T, et al. ECVAM retrospective validation of *in vitro* micronucleus test. *Mutagenesis*. 2008;23:271–283.

Cotelle S, Férard JF. Comet assay in genetic ecotoxicology. *Environ Mol Mutagen*. 1999;34:246–255.

Covo S, Blanco L, Livneh Z. Lesion bypass by human DNA polymerase μ reveals a template-dependent, sequence-independent nucleotidyl transferase activity. *J Biol Chem*. 2004;279(2):859–65.

Cox JA, Fellows MD, Hashizume T, White PA. The utility of metabolic activation mixtures containing human hepatic post-mitochondrial supernatant (S9) for in vitro genetic toxicity assessment. *Mutagenesis*. 2016;31(2):117–130.

Crespi CL, Miller VP. The use of heterologously expressed drug metabolizing enzymes—state of the art and prospects for the future. *Pharmacol Ther*. 1999;84:121–131.

Critchlow SE, Jackson SP. DNA end-joining from yeast to man. *Trends Biochem Sci*. 1998;23:394–398.

Croce CM. Oncogenes and cancer. *N Engl J Med*. 2008;358:502–511.

Crouse GF. Mutagenesis assays in yeast. *Methods*. 2000;22:116–119.

Cupples CG, Cabrera M, Cruz C, Miller JH. A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of specific frameshift mutations. *Genetics*. 1990;125:275–280.

Cupples CG, Miller JH. A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions. *Proc Natl Acad Sci U S A*. 1989;86:5345–5349.

d'Adda di Fagagna F. A direct role for small non-coding RNAs in DNA damage response. *Trends Cell Biol*. 2014;24(3):171–178.

Daigaku Y, Endo K, Watanabe E, et al. Loss of heterozygosity and DNA damage repair in *Saccharomyces cerevisiae*. *Mutat Res*. 2004;556:183–191.

Datta A, Jinks-Robertson S. Association of increased spontaneous mutation rates with high levels of transcription in yeast. *Science*. 1995;268:1616–1619.

Dearfield KL, Gollapudi BB, Bemis JC, et al. Next generation testing strategy for assessment of genomic damage: a conceptual framework and considerations. *Environ Mol Mutagen*. 2017;58(5):264–283.

Dearfield KL, Moore MM. Use of genetic toxicology information for risk assessment. *Environ Mol Mutagen*. 2005;46(4):236–245.

Debiak M, Nikolova T, Kaina B. Loss of ATM sensitizes against O6-methylguanine triggered apoptosis, SCEs and chromosomal aberrations. *DNA Repair*. 2004;3:359–368.

Decottignies A. Alternative end-joining mechanisms: a historical perspective. *Front Genet*. 2013;4:48.

Delaney JC, Essigmann JM. Mutagenesis, genotoxicity, and repair of 1-methyladenine, 3-alkylcytosines, 1-methylguanine, and 3-methylthymine in alkB Escherichia coli. *Proc Natl Acad Sci U S A*. 2004;101(39):14051–14056.

Delaney JC, Smeester L, Wong C, et al. AlkB reverses etheno DNA lesions caused by lipid oxidation in vitro and in vivo. *Nat Struct Mol Biol*. 2005;12:855–860.

Dellarco V, Fenner-Crisp PA. Mode of action: moving toward a more relevant and efficient assessment paradigm. *J Nutr*. 2012;142(12):2192S–2198S.

DeMarini DM. Mutation spectra of complex mixtures. *Mutat Res*. 1998;411:11–18.

DeMarini DM. Influence of DNA repair on mutation spectra in *Salmonella*. *Mutat Res*. 2000;450:5–17.

DeMarini DM, Brockman HE, de Serres FJ, et al. Specific-locus mutations induced in eukaryotes (especially mammalian cells) by radiation and chemicals: a perspective. *Mutat Res*. 1989;220:11–29.

DeMarini DM, Shelton ML, Abu-Shakra A, et al. Spectra of spontaneous frameshift mutations at the *his* D3052 allele of *Salmonella typhimurium* in four DNA repair backgrounds. *Genetics*. 1998;149:17–36.

Demple B, Harrison L. Repair of oxidative damage to DNA: enzymology and biology. *Annu Rev Biochem*. 1994;63:915–948.

Dertinger SD, Torous DK, Hayashi M, et al. Flow cytometric scoring of micronucleated erythrocytes: an efficient platform for assessing in vivo cytogenetic damage. *Mutagenesis*. 2011;26:139–145.

Director AE, Tucker JD, Ramsey MJ, Nath J. Chronic ingestion of clastogens by mice and the frequency of chromosome aberrations. *Environ Mol Mutagen*. 1998;32:139–147.

Dobrovolsky VN, Miura D, Heflich RH, et al. The in vivo *Pig-a* gene mutation assay, a potential tool for regulatory safety assessment. *Environ Mol Mutagen*. 2010;51:825–835.

Dobo KL, Fiedler RD, Gunther WC, et al. Defining EMS and ENU dose-response relationships using the Pig-a mutation assay in rats. *Mutat Res*. 2011;725:12–21.

Dogliotti E, Hainant P, Hernandez T, et al. Mutation spectra resulting from carcinogenic exposures: from model systems to cancer-related genes. *Recent Results Cancer Res*. 1998;154:97–124.

Donner EM, Wong BA, James RA, et al. Reciprocal translocations in somatic and germ cells of mice chronically exposed by inhalation to ethylene oxide: implications for risk assessment. *Mutagenesis*. 2010;25:49–55.

Dourson M, Becker RA, Haber LT, et al. Advancing human health risk assessment: integrating recent advisory committee recommendations. *Crit Rev Toxicol*. 2013;43:467–492.

Du H, Pan B, Chen T. Evaluation of chemical mutagenicity using next generation sequencing: A review. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2017;16:1-19.

Dubrova YE. Radiation-induced mutation at tandem repeat DNA loci in the mouse germline: spectra and doubling doses. *Radiat Res*. 2005;163:200–207.

Duckett DR, Drummond JT, Murchie AI, et al. Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. *Proc Natl Acad Sci U S A*. 1996;93:6443–6447.

Dusinska M, Collins AR. The comet assay in human biomonitoring: gene-environment interactions. *Mutagenesis*. 2008;23:191–205.

Earley MC, Crouse GF. The role of mismatch repair in the prevention of base pair mutations in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 1998;95(26):15487–15491.

Eastmond DA. Factors influencing mutagenic mode of action determinations of regulatory and advisory agencies. *Mutat Res*. 2012;751(1):49–63.

Eastmond DA. Recommendations for the evaluation of complex genetic toxicity data sets when assessing carcinogenic risks to humans. *Environ Mol Mutagen*. 2017;58(5):380–385.

Eastmond DA, Hartwig A, Anderson D, et al. Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme. *Mutagenesis*. 2009;24(4):341–349.

Eccles LJ, O'Neill P, Lomax ME. Delayed repair of radiation induced clustered DNA damage: friend or foe? *Mutat Res*. 2011;711:134–141.

ECHA (European Chemical Agency). *Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.7a: Endpoint specific guidance, Version 5.0*. Helsinki, Finland: European Chemicals Agency; 2016.

Echols H, Goodman MF. Fidelity mechanisms in DNA replication. *Ann Rev Biochem*. 1991;60:477–511.

EFSA. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA J*. 2012;9:2379.

Ehling UH. Genetic risk assessment. *Annu Rev Genet*. 1991;25:255–280.

Elespuru RK, Sankaranarayanan K. New approaches to assessing the effects of mutagenic agents on the integrity of the human genome. *Mutat Res*. 2007;616:83–89.

Elvidge G. Microarray expression technology: from start to finish. *Pharmacogenomics*. 2006;7:123–134.

EPA (US Environmental Protection Agency). Guidelines for carcinogen risk assessment. *Fed Reg*. 1986;51:33992–34003.

EPA (US Environmental Protection Agency). *Proposed Guidelines for Carcinogen Risk Assessment*. EPA/600P/P-92/003c. Office of Research and Development. Washington, DC: US Environmental Protection Agency; 1996.

EPA (US Environmental Protection Agency). *Guidelines for Carcinogen Risk Assessment*. Risk Assessment Forum. Washington, DC: US Environmental Protection Agency; 2005. Available at: www.epa.gov/cancerguidelines.

Erhola M, Toyokuni S, Okada K, et al. Biomarker evidence of DNA oxidation in lung cancer patients: association of urinary 8-hydroxy-2′-deoxyguanosine excretion with radiotherapy, chemotherapy, and response to treatment. *FEBS Lett*. 1997;409(2):287–291.

Evan G, Littlewood T. A matter of life and cell death. *Science*. 1998;281:1317–1322.

Evans E, Fellows J, Coffer A, Wood RD. Open complex formation around a lesion during nucleotide excision repair provides a structure for cleavage by human XPG protein. *EMBO J*. 1997;16:625–638.

Evans HJ, Prosser J. Tumor-suppressor genes: cardinal factors in inherited predisposition to human cancers. *Environ Health Perspect*. 1992;98:25–37.

Fairbairn DW, Olive PL, O'Neill KL. The comet assay: a comprehensive review. *Mutat Res*. 1995;339:37–59.

Falck J, Mailand N, Syljuåsen RG, Bartek J, Lukas J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* 2001;410(6830):842–847.

Fan J, Wilson DM III. Protein-protein interactions and posttranslational modifications in mammalian base excision repair. *Free Radic Biol Med*. 2005;38(9):1121–1138.

Fan W, Wu X. DNA polymerase lambda can elongate on DNA substrates mimicking non-homologous end joining and interact with XRCC4-ligase IV complex. *Biochem Biophys Res Commun*. 2004;323(4):1328–1333.

Farmer PB, Singh R. Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: the increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens. *Mutat Res*. 2008;659:68–76.

Favor J. Mechanisms of mutation induction in germ cells of the mouse as assessed by the specific-locus test. *Mutat Res*. 1999;428:227–236.

Favor J, Layton D, Sega G, et al. International Commission for Protection Against Environmental Mutagens and Carcinogens. Genetic risk extrapolation from animal data to human disease. A Taskgroup Report. No. 00105. *Mutat Res*. 1995;330:23–34.

Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61:759–767.

Fenech M. The micronucleus assay determination of chromosomal level DNA damage. *Methods Mol Biol*. 2008;410:185–216.

Fenech M, Chang WP, Kirsch-Volders M, et al. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronoucleus assay using isolated human lymphocyte cultures. *Mutat Res*. 2003;534:65–75.

Fenech M, Holland N, Zeiger E, et al. The HUMN and HUMNxL international collaboration projects on human micronucleus assays in lymphocytes and buccal cells—past, present and future. *Mutagenesis*. 2011a;26:239–245.

Fenech M, Kirsch-Volders M, Natarajan AT, et al. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*. 2011b;26:125–132.

Ferguson LR, Denny WA. Frameshift mutagenesis by acridines and other reversibly-binding DNA ligands. *Mutagenesis*. 1990;5:529–540.

Findlay JM, Middleton MR, Tomlinson I. A systematic review and meta-analysis of somatic and germline DNA sequence biomarkers of esophageal cancer survival, therapy response and stage. *Ann Oncol*. 2015;26(4):624–644.

Fischer AH, Young KA, DeLellis RA. Incorporating pathologists' criteria of malignancy into the evolutionary model for cancer development. *J Cell Biochem*. 2004;93:28–36.

Fix D, Canugovi C, Bhagwat AS. Transcription increases methylmethane sulfonate-induced mutations in alkB strains of *Escherichia coli*. *DNA Repair*. 2008;7(8):1289–1297.

Freeman KM, Hoffmann GR. Frequencies of mutagen-induced coincident mitotic recombinationat unlinked loci in *Saccharomyces cerevisiae*. *Mutat Res*. 2007;616:119–132.

Frick LE, Delaney JC, Wong C, Drennan CL, Essigmann JM. Alleviation of 1,N6-ethanoadenine genotoxicity by the *Escherichia coli* adaptive response protein AlkB. *Proc Natl Acad Sci U S A*. 2007;104:755–760.

Friedberg EC. *DNA Repair*. New York: Freeman; 1985.

Friedberg EC. Biological responses to DNA damage: a perspective in the new millennium. *Cold Spring Harb Symp Quant Biol*. 2000;65:593–602.

Friedberg EC, Walker GC, Siede W. *DNA Repair and Mutagenesis*. Washington, DC: ASM Press; 1995.

Friedman N, Vardi S, Ronen M, Alon U, Stavans J. Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. *PLoS Biol*. 2005;3:e238.

Fung H, Demple B. A vital role for Ape1/Ref1 protein in repairing spontaneous DNA damage in human cells. *Mol Cell*. 2005;17(3):463–470.

Futreal PA, Coin L, Marshall M, et al. A census of human cancer genes. *Nat Rev Cancer*. 2004;4(3):177–183.

Galloway SM. Cytotoxicity and chromosome aberrations in vitro: experience in industry and the case for an upper limit on toxicity in the aberration assay. *Environ Mol Mutagen*. 2000;35:191–201.

Galloway SM, Aardema MJ, Ishidate M Jr, et al. Report from working group on in vitro tests for chromosomal aberrations. *Mutat Res*. 1994;312:241–261.

Galloway SM, Berry PK, Nichols WW, et al. Chromosome aberrations in individuals occupationally exposed to ethylene oxide, and in a large control population. *Mutat Res*. 1986;170:55–74.

Galloway SM, Ivett JL. Chemically induced aneuploidy in mammalian cells in culture. *Mutat Res*. 1986;167:89–105.

Galloway S, Lorge E, Aardema MJ, et al. Workshop summary: top concentration for in vitro mammalian cell genotoxicity assays; and report from working group on toxicity measures and top concentration for in vitro cytogenetic assays (chromosome aberrations and micronucleus). *Mutat Res*. 2011;723:77–83.

Galloway SM. International regulatory requirements for genotoxicity testing for pharmaceuticals used in human medicine, and their impurities and metabolites. *Environ Mol Mutagen*. 2017;58(5):296–324.

Gatehouse D, Haworth S, Cebula T, et al. Recommendations for the performance of bacterial mutation assays. *Mutat Res*. 1994;312:217–233.

Gatenby RA, Vincent TL. An evolutionary model of carcinogenesis. *Cancer Res*. 2003;63:6212–6220.

Gealy R, Wright-Bourque JL, Kraynak AR, et al. Validation of a high-throughput in vitro alkaline elution/rat hepatocyte assay for DNA damage. *Mutat Res*. 2007;629:49–63.

Ge J, Prasongtanakij S, Wood DK, et al. CometChip: a high-throughput 96-well platform for measuring DNA damage in microarrayed human cells. *J Vis Exp*. 2014;92:e50607.

Gee P, Maron DM, Ames BN. Detection and classification of mutagens: a set of base-specific *Salmonella* tester strains. *Proc Natl Acad Sci U S A*. 1994;91:11606–11610.

Gentile JM, Gentile GJ, Plewa MJ. In vitro activation of chemicals by plants: a comparison of techniques. *Mutat Res*. 1986;164(1):53–58.

Gerkema MP, Davies WI, Foster RG, Menaker M, Hut RA. The nocturnal bottleneck and the evolution of activity patterns in mammals. *Proc Biol Sci*. 2013;280(1765):20130508.

Giglia-Mari G, Zotter A, Vermeulen W. DNA damage response. *Cold Spring Harb Perspect Biol*. 2011;3:745–778.

Gillet LC, Scharer OD. Molecular mechanisms of mammalian global genome nucleotide excision repair. *Chem Rev*. 2006;106:253–276.

Glei M, Schneider T, Schlörmann W. Comet assay: an essential tool in toxicological research. *Arch Toxicol*. 2016;90(10):2315–2336.

Gocke E, Muller L. In vivo studies in the mouse to define a threshold for the genotoxicity of EMS and ENU. *Mutat Res*. 2009;678:101–107.

Gold LS, Slone TH, Stern BR, Bernstein L. Comparison of target organs of carcinogenicity for mutagenic and non-mutagenic chemicals. *Mutat Res*. 1993;286:75–100.

Goldmacher VS, Cuzick R, Thilly WG. Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methylnitrosourea and N-methyl-N′-nitro-N-nitrosoguanidine. *J Biol Chem*. 1986;261:12462–12471.

Gollapudi B. An ongoing journey toward a risk-based testing in genetic toxicology. *Curr Opinion Toxicol*. 2017;3:71–74.

Gollapudi BB, Johnson GE, Hernandez LG, et al. Quantitative approaches for assessing dose-response relationships in genetic toxicology studies. *Environ Mol Mutagen*. 2013;54(1):8–18.

Gollapudi BB, Lynch AM, Heflich RH, et al. The in vivo Pig-a assay: a report of the International Workshop On Genotoxicity Testing (IWGT) Workgroup. *Mutat Res Genet Toxicol Environ Mutagen*. 2015;783:23–35.

Gollapudi P, Hasegawa LS, Eastmond DA. A comparative study of the aneugenic and polyploidy-inducing effects of fisetin and two model Aurora kinase inhibitors. *Mutat Res Genet Toxicol Environ Mutagen*. 2014;767:37–43.

Goodhead DT. Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. *Int J Radiat Biol*. 1994;65:7–17.

Gordon DJ, Resio B, Pellman D. Causes and consequences of aneuploidy in cancer. *Nat Rev Genet*. 2012;13:189–203.

Grant WF, Owens ET. *Zea mays* assays of chemical/radiation genotoxicity for the study of environmental mutagens. *Mutat Res*. 2006;613:17–64.

Guerard M, Baum M, Bitsch A, et al. Assessment of mechanisms driving non-linear dose-response relationships in genotoxicity testing. *Mutat Res Rev Mutat Res*. 2015;763:181–201.

Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. *Nature*. 2007;446(7132):153–158.

Griffin DK. The incidence, origin, and etiology of aneuploidy. *Int Rev Cytol*. 1996;167:263–296.

Griffin JL. Understanding mouse models of disease through metabolomics. *Curr Opin Chem Biol*. 2006;10:309–315.

Grombacher T, Mitra S, Kaina B. Induction of the alkyltransferase (MGMT) gene by DNA damaging agents and the glucocorticoid dexamethasone and comparison with the response of base excision repair. *Carcinogenesis*. 1996;17:2329–2336.

Guengerich FP. Forging the links between metabolism and carcinogenesis. *Mutat Res*. 2001;488:195–209.

Guha R. The ups and downs of structure-activity landscapes. *Methods Mol Biol*. 2011;672:101–117.

Haber JE. Partners and pathways: repairing a double-strand break. *Trends Genet*. 2000;16:259–264.

Hagmar L, Bonassi S, Strömberg U, et al. Cancer predictive value of cytogenetic markers used in occupational health surveillance programs: a report from an ongoing study by the European Study Group on Cytogenetic Biomarkers and Health. *Mutat Res*. 1998a;405:171–178.

Hagmar L, Bonassi S, Strömberg U, et al. Chromosomal aberrations in lymphocytes predict human cancer: a report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). *Cancer Res*. 1998b;58:4117–4121.

Hahn WC, Counter CM, Lundberg AS, et al. Creation of human tumor cells with defined genetic elements. *Nature*. 1999;400:464–468.

Halliday JA, Glickman BW. Mechanisms of spontaneous mutation in DNA repair-proficient *Escherichia coli*. *Mutat Res*. 1991;250:55–71.

Hamilton JP. Epigenetics: principles and practice. *Dig Dis*. 2011;29:130–135.

Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57–70.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–674.

Harrington CA, Rosenow C, Retief J. Monitoring gene expression using DNA microarrays. *Curr Opin Microbiol*. 2000;3:285–291.

Harris CC. P53: at the crossroads of molecular carcinogenesis and risk assessment. *Science*. 1993;262:1980–1981.

Hastwell PW, Webster TW, Tate M, et al. Analysis of 75 marketed pharmaceuticals using the *GADD45a-GFP "GreenScreen HC"* genotoxicity assay. *Mutagenesis*. 2009;24:455–463.

Hayashi M, MacGregor JT, Gatehouse DG, et al. In vivo erythrocyte micronucleus assay III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test. *Mutat Res*. 2007;627:10–30.

Heflich RH. Chemical mutagens. In: Li AP, Heflich RH, eds. *Genetic Toxicology: A Treatise*. Boca Raton, FL: CRC Press; 1991:143–202.

Helleday T, Lo J, van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair*. 2007;6:923–935.

Hemminki K, Thilly WG. Implications of results of molecular epidemiology on DNA adducts, their repair and mutations for mechanisms of human cancer. *IARC Sci Publ*. 2004;157:217–235.

Hewitt LM, Marvin CH. Analytical methods in environmental effects-directed investigations of effluents. *Mutat Res*. 2005;589:208–232.

Hickman MJ, Samson LD. Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents. *Proc Natl Acad Sci U S A*. 1999;96:10764–10769.

Himmelstein MW, Boogaard PJ, Cadet J, et al. Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage. *Crit Rev Toxicol*. 2009;39:679–694.

Hiom K. Coping with DNA double strand breaks. *DNA Repair*. 2010;9:1256–1263.

Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*. 2002;419(6903):135–141.

Hoffmann GR. Induction of genetic recombination: consequences and model systems. *Environ Mol Mutagen*. 1994;23(suppl 24):59–66.

Hoffmann GR, Calciano MA, Lawless BM, Mahoney KM. Frameshift mutations induced by three classes of acridines in the *lacZ* reversion assay in *Escherichia coli*: potency of responses and relationship to slipped mispairing models. *Environ Mol Mutagen*. 2003;42:111–121.

Hosoya N, Sanada M, Nannya Y, et al. Genomewide screening of DNA copy number changes in chronic myelogenous leukemia with the use of high-resolution array-based comparative genomic hybridization. *Genes Chromosomes Cancer*. 2006;45:482–494.

Houlston RS, Peto J. The search for low-penetrance cancer susceptibility alleles. *Oncogene*. 2004;23:6471–6476.

Howlett NG, Schiestl RH. Simultaneous measurement of the frequencies of intrachromosomal recombination and chromosome gain using the yeast DEL assay. *Mutat Res*. 2000;454:53–62.

Hsu TC. *Human and Mammalian Cytogenetics: A Historical Perspective*. New York: Springer-Verlag; 1979.

Hughes C, Rabinowitz A, Tate M, et al. Development of a high-throughput Gaussia luciferase reporter assay for the activation of the GADD45a gene by mutagens, promutagens, clastogens, and aneugens. *J Biomol Screen*. 2012;17(10):1302–1315.

Hunt PA. Meiosis in mammals: recombination, non-disjunction and the environment. *Biochem Soc Trans*. 2006;34:574–577.

International Program on Chemical Safety (IPCS). Overview of the IPCS collaborative study on in vivo assay systems and conclusions. In: Ashby J, de Serres FJ, Shelby MD, Margolin BH, Ishidate M Jr, Becking GC, eds. *Evaluation of Short-Term Tests for Carcinogens: Report of the International Programme on Chemical Safety's Collaborative Study on In Vivo Assays*. Vols I and II. Cambridge, UK: Cambridge University Press; 1988:1–28.

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*. 1993;363:558–561.

Ischenko AA, Saparbaev MK. Alternative nucleotide incision repair pathway for oxidative DNA damage. *Nature*. 2002;415:183–187.

Ishidate M Jr, Harnois MC, Sofuni T. A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures. *Mutat Res*. 1988;195:151–213.

Ito T, Nakamura T, Maki H, Sekiguchi M. Roles of transcription and repair in alkylation mutagenesis. *Mutat Res*. 1994;314:273–285.

Jarabek AM, Pottenger LH, Andrews LS, et al. Creating context for the use of DNA adduct data in cancer risk assessment: I. Data organization. *Crit Rev Toxicol*. 2009;39:659–678.

Jagger C, Tate M, Cahill PA, et al. Assessment of the genotoxicity of S9-generated metabolites using the GreenScreenHC *GADD45a-GFP assay*. *Mutagenesis*. 2009;24:35–50.

Jasin M, Rothstein R. Repair of strand breaks by homologous recombination. *Cold Spring Harb Perspec Biol*. 2013;5:a012740.

Jaworski A, Rosche WA, Gellibolian R, et al. Mismatch repair in Escherichia coli enhances instability of (CTG)n triplet repeats from human hereditary diseases. *Proc Natl Acad Sci U S A*. 1995;92:11019–11023.

Jha AN. Genotoxicological studies in aquatic organisms: an overview. *Mutat Res*. 2004;552:1–17.

Ji Z, Ball NS, LeBaron MJ. Global regulatory requirements for mutagenicity assessment in the registration of industrial chemicals. *Environ Mol Mutagen*. 2017;58(5):345–353.

Ji Z, LeBaron MJ. Applying the erythrocyte Pig-a assay concept to rat epididymal sperm for germ cell mutagenicity evaluation. *Environ Mol Mutagen*. 2017;58(7):485–493.

Ji Z, LeBaron MJ, Schisler MR, et al. Dose-response for multiple biomarkers of exposure and genotoxic effect following repeated treatment of rats with the alkylating agents, MMS and MNU. *Mutagenesis*. 2016;31(3):297–308.

Jiang G, Sancar A. Recruitment of DNA damage checkpoint proteins to damage in transcribed and nontranscribed sequences. *Mol Cell Biol*. 2006;26:39–49.

Jinks-Robertson S, Bhagwat AS. Transcription-associated mutagenesis. *Annu Rev Genet*. 2014;48:341–359.

Jiricny J. Eukaryotic mismatch repair: an update. *Mutat Res*. 1998;409:107–121.

Jiricny J. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol*. 2006;7:335–346.

Johnson KA. The kinetic and chemical mechanism of high-fidelity DNA polymerases. *Biochim Biophys Acta*. 2010;1804:1041–1048.

Johnson GE, Soeteman-Hernández LG, Gollapudi BB, et al. Derivation of point of departure (PoD) estimates in genetic toxicology studies and their potential applications in risk assessment. *Environ Mol Mutagen*. 2014;55(8):609–623.

Johnson RE, Haracska L, Prakash S, Prakash L. Role of DNA polymerase eta in the bypass of a (6-4) TT photoproduct. *Mol Cell Biol*. 2001;21:3558–3563.

Johnson RD, Liu N, Jasin M. Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. *Nature*. 1999;401:397–399.

Josephy PD. The *Escherichia coli lacZ* reversion mutagenicity assay. *Mutat Res*. 2000;455:71–80.

Josephy PD. Genetically-engineered bacteria expressing human enzymes and their use in the study of mutations and mutagenesis. *Toxicology*. 2002;181-182:255–260.

Jun SH, Kim TG, Ban C. DNA mismatch repair system. Classical and fresh roles. *FEBS J*. 2006;273:1609–1619.

Jurado J, Alejandre-Durán E, Pueyo C. Mutagenicity testing in *Salmonella typhimurium* strains possessing both the His reversion and Ara forward mutation systems and different levels of classical nitroreductase or o-acetyltransferase activities. *Environ Mol Mutagen*. 1994;23:286–293.

Kadyrov FA, Dzantiev L, Constantin N, Modrich P. PCNA function in the activation and strand direction of MutLα endonuclease in mismatch repair. *Cell*. 2006;126:297–308.

Kaina B, Ziouta A, Ochs K, Coquerelle T. Chromosomal instability, reproductive cell death and apoptosis induced by O6-methylguanine in Mex−, Mex+ and methylation-tolerant mismatch repair compromised cells: facts and models. *Mutat Res*. 1997;381(2):227–241.

Kallioniemi A, Kallioniemi O-P, Sudar D, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science*. 1992;258:818–821.

Kamber M, Flückiger-Isler S, Engelhardt G, et al. Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with carcinogenicity. *Mutagenesis*. 2009;24:359–366.

Kannouche PL, Wing J, Lehmann AR. Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol Cell*. 2004;14:491–500.

Kataoka H, Yamamoto Y, Sekiguchi M. A new gene (alkB) of Escherichia coli that controls sensitivity to methyl methane sulfonate. *J Bacteriol*. 1983;153:1301–1307.

Kier LE, Brusick DJ, Auletta AE, et al. The *Salmonella typhimurium*/mammalian microsomal assay: a report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res*. 1986;168:69–240.

Kim BS, Margolin BH. Prediction of rodent carcinogenicity utilizing a battery of in vitro and in vivo genotoxicity tests. *Environ Mol Mutagen*. 1999;34:297–304.

Kim ST, Sancar A.Photochemistry, photophysics, and mechanism of pyrimidine dimer repair by DNA photolyase. *Photochem Photobiol*. 1993;57(5):895–904.

Kim YJ, Wilson DM 3rd. Overview of base excision repair biochemistry. *Curr Mol Pharmacol*. 2012;5(1):3–13.

Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell*. 1996;87:159–170.

Kirkland DJ, Gatehouse DG, Scott D, et al., eds. *Basic Mutagenicity Tests: UKEMS Recommended Procedures*. New York: Cambridge University Press; 1990.

Kirkland D, Speit G. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing in vivo. *Mutat Res*. 2008;654:114–132.

Kirkland D, Aardema M, Henderson L, Müller L. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. *Mutat Res*. 2005;584(1–2):1–256.

Kirsch-Volders M, Aardema M, Elhajouji A. Concepts of threshold in mutagenesis and carcinogenesis. *Mutat Res*. 2000;464(1):3–11.

Kirsch-Volders M, Sofuni T, Aardema M, et al. Report from the in vitro micronucleus assay working group. *Mutat Res*. 2003;540:153–163.

Klapacz J, Pottenger LH, Engelward BP, et al. Contributions of DNA repair and damage response pathways to the non-linear genotoxic responses of alkylating agents. *Mutat Res Rev Mutat Res*. 2016;767:77–91.

Kleinerman RA, Romanyukha AA, Schauer DA, Tucker JD. Retrospective assessment of radiation exposure using biological dosimetry: chromosome painting, electron paramagnetic resonance and the glycophorin a mutation assay. *Radiat Res*. 2006;166:287–302.

Knudsen TB, Houck KA, Sipes NS, et al. Activity profiles of 309 ToxCast™ chemicals evaluated across 292 biochemical targets. *Toxicology*. 2011;282:1–15.

Knudson AG. Hereditary predisposition to cancer. *Ann NY Acad Sci*. 1997;833:58–67.

Koch WH, Henrikson EN, Kupchella E, Cebula TA. *Salmonella typhimurium* strain TA100 differentiates several classes of carcinogens and mutagens by base substitution specificity. *Carcinogenesis*. 1994;15:79–88.

Kolodner RD. Mismatch repair: mechanisms and relationship to cancer susceptibility. *Trends Biochem Sci*. 1995;20:397–401.

Krejci L, Chen L, Van Komen S, Sung P, Tomkinson A. Mending the break: two DNA double-strand break repair machines in eukaryotes. *Prog Nucleic Acid Res Mol Biol*. 2003;74:159–201.

Krewski D, Acosta D Jr, Andersen M, et al. Toxicity testing in the 21st century: a vision and a strategy. *J Toxicol Environ Health B Crit Rev*. 2010;13(2-4):51–138.

Krishna G, Hayashi M. In vivo rodent micronucleus assay: protocol, conduct and data interpretation. *Mutat Res*. 2000;455:155–166.

Krokan HE, Nilsen H, Skorpen F, Otterlei M, Slupphaug G. Base excision repair of DNA in mammalian cells. *FEBS Lett*. 2000;476(1-2):73–77.

Kurowski MA, Bhagwat AS, Papaj G, Bujnicki JM. Phylogenomic identification of five new human homologs of the DNA repair enzyme AlkB. *BMC Genomics*. 2003;4:48.

Krüger CT, Fischer BM, Armant O, et al. The in vitro PIG-A gene mutation assay: glycosylphosphatidylinositol (GPI)-related genotype-to-phenotype relationship in TK6 cells. *Arch Toxicol*. 2016;90(7):1729–1736.

Kyoizumi S, Kusunoki Y, Hayashi T, et al. Individual variation of somatic gene mutability in relation to cancer susceptibility: prospective study on erythrocyte glycophorin a gene mutations of atomic bomb survivors. *Cancer Res*. 2005;65:5462–5469.

Lambert IB, Singer TM, Boucher SE, Douglas GR. Detailed review of transgenic rodent mutation assays. *Mutat Res*. 2005;590:1–280.

Larson K, Sahm J, Shenkar R, Strauss B. Methylation-induced blocks to in vitro DNA replication. *Mutat Res*. 1985;150:77–84.

Laval J, Boiteux S, O'Connor TR. Physiological properties and repair of apurinic/apyrimidinic sites and imidazole ring-opened guanines in DNA. *Mutat Res*. 1990;233:73–79.

Le XC, Xing JZ, Lee J, et al. Inducible repair of thymine glycol detected by an ultrasensitive assay for DNA damage. *Science*. 1998;280:1066–1069.

LeBaron MJ, Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB. Epigenetics and chemical safety assessment. *Mutat Res*. 2010;705(2):83–95.

Lee DH, Jin SG, Cai S, Chen Y, Pfeifer GP, O′Connor TR. Repair of methylation damage in DNA and RNA by mammalian AlkB homologues. *J Biol Chem*. 2005;280:39448–39459.

Lee RF, Steinert S. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutat Res*. 2003;544:43–64.

Lee WR, Abrahamson S, Valencia R, et al. The sex-linked recessive lethal test for mutagenesis in *Drosophila melanogaster*: a report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res*. 1983;123:183–279.

Lemaître C, Soutoglou E. Double strand break (DSB) repair in heterochromatin and heterochromatin proteins in DSB repair. *DNA Repair (Amst)*. 2014;19:163–168.

Léonard A. Observations on meiotic chromosomes of the male mouse as a test of the potential mutagenicity of chemicals in mammals. In: Hollaender A, ed. *Chemical Mutagens: Principles and Methods for Their Detection*. Vol 3. New York: Plenum Press; 1973:21.

Levine JG, Schaaper RM, DeMarini DM. Complex frameshift mutations mediated by plasmid pKM101: mutational mechanisms deduced from 4-aminobiphenyl-induced mutation spectra in *Salmonella*. *Genetics*. 1994;136:731–746.

Lewis SE. The biochemical specific-locus test and a new multiple-endpoint mutation detection system: considerations for genetic risk assessment. *Environ Mol Mutagen*. 1991;18:303–306.

Lewtas J, DeMarini DM, Favor J, et al. Risk characterization strategies for genotoxic environmental agents. In: Brusick DJ ed. *Methods for GenotoxicRisk Assessment*. Boca Ratan, FL: CRC Press; 1994:125–169.

Li AP, Gupta RS, Heflich RH, et al. A review and analysis of the Chinese hamster ovary/hypoxanthine guanine phosphoribosyl transferase assay to determine the mutagenicity of chemical agents. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res*. 1988;196:17–36.

Li J, Makrigiorgos GM. COLD-PCR: a new platform for highly improved mutation detection in cancer and genetic testing. *Biochem Soc Trans*. 2009;37:427–432.

Li X, Heyer WD. Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res*. 2008;18(1):99–113.
Li Z, Sun B, Clewell RA, Adeleye Y, Andersen ME, Zhang Q. Dose-response modeling of etoposide-induced DNA damage response. *Toxicol Sci*. 2014;137(2):371–84.
Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem*. 2010;79:181–211.
Lieber MR, Ma Y, Pannicke U, Schwarz K. Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol*. 2003;4:712–720.
Lindahl T. New class of enzymes acting on damaged DNA. *Nature*. 1976;259:64–66.
Lindahl T. Instability and decay of the primary structure of DNA. *Nature*. 1993;362:709–715.
Lindahl T. Suppression of spontaneous mutagenesis in human cells by DNA base excision-repair. *Mutat Res*. 2000;462:129–135.
Liu Y, Fang Y, Shao H, Lindsey-Boltz L, Sancar A, Modrich P. Interactions of human mismatch repair proteins MutSalpha and MutLalpha with proteins of the ATR-Chk1 pathway. *J Biol Chem*. 2010;285:5974–5982.
Liyanage M, Coleman A, duManoir S, et al. Multicolour spectral karyotyping of mouse chromosomes. *Nat Genet*. 1996;14:312–315.
Lodish H, Berk A, Zipursky SL, et al. *Molecular Cell Biology*. 4th ed. New York: W. H. Freeman; 2000. Section 8.1, Mutations: Types and Causes.
Loechler EL, Green CL, Essigmann JM. In vivo mutagenesis by O6-methylguanine built into a unique site in a viral genome. *Proc Natl Acad Sci U S A*. 1984;81:6271–6275.
Lommel L, Carswell-Crumpton C, Hanawalt PC. Preferential repair of the transcribed DNA strand in the dihydrofolate reductase gene throughout the cell cycle in UV-irradiated human cells. *Mutat Res*. 1995;366:181–192.
Lorge E, Moore MM, Clements J, et al. Standardized cell sources and recommendations for good cell culture practices in genotoxicity testing. *Mutat Res*. 2016;809:1–15.
Lucas JN, Awa A, Straume T, et al. Rapid translocation analysis in humans decades after exposure to ionizing radiation. *Int J Radiat Biol*. 1992;62:53–63.
Lynch AM, Guzzie PJ, Bauer D, et al. Considerations on photochemical genotoxicity. II: report of the 2009 International Workshop on Genotoxicity Testing Working Group. *Mutat Res*. 2011;723:91–100.
Lynch AM, Parry JM. The cytochalasin-B micronucleus/kinetochore assay in vitro: studies with 10 suspected aneugens. *Mutat Res*. 1993;287:71–86.
Lynch HT, Lynch JF. Hereditary nonpolyposis colorectal cancer. *Semin Surg Oncol*. 2000;18(4):305–313.
Ma TH, Cabrera GL, Owens E. Genotoxic agents detected by plant bioassays. *Rev Environ Health*. 2005;20:1–13.
Ma Y, Pannicke U, Schwarz K, Lieber MR. Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell*. 2002;108:781–794.
MacGregor JT, Casciano D, Muller L. Strategies and testing methods for identifying mutagenic risks. *Mutat Res*. 2000;455:3–20.
MacGregor JT, Frötschl R, White PA, et al. IWGT report on quantitative approaches to genotoxicity risk assessment I. Methods and metrics for defining exposure response relationships and points of departure (PoDs). *Mutat Res Genet Toxicol Environ Mutagen*. 2015a;783:55–65.
MacGregor JT, Frötschl R, White PA, et al. IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk. *Mutat Res Genet Toxicol Environ Mutagen*. 2015b;783:66–78.
Mackinnon RN, Chudoba I. The use of M-FISH and M-BAND to define chromosome abnormalities. *Methods Mol Biol*. 2011;730:203–218.
Macville M, Veldman T, Padilla-Nash H, et al. Spectral karyotyping, a 24-colour FISH technique for the identification of chromosomal rearrangements. *Histochem Cell Biol*. 1997;108:299–305.
Madle S, Dean SW, Andrae U, et al. Recommendations for the performance of UDS tests in vitro and in vivo. *Mutat Res*. 1994;312:263–285.
Mah LJ, El-Osta A, Karagiannis TC. Gamma H2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia*. 2010;24:679–686.
Mahadevan B, Snyder RD, Waters MD, et al. Genetic toxicology in the 21st century: reflections and future directions. *Environ Mol Mutagen*. 2011;52:339–354.
Mahajan KN, McElhinny NSA, Mitchell BS, Ramsden DA. Association of DNA polymerase mu (pol mu) with Ku and ligase IV: role for pol mu in end-joining double-strand break repair. *Mol Cell Biol*. 2002;22:5194–5202.
Maillard O, Solyom S, Naegeli H. An aromatic sensor with aversion to damaged strands confers versatility to DNA repair. *PLoS Biol*. 2007;5:e79.
Makrigiorgos GM. PCR-based detection of minority point mutations. *Hum Mutat*. 2004;23:406–412.
Malling HV. Incorporation of mammalian metabolism into mutagenicity testing. *Mutat Res*. 2004;566:183–189.
Malling HV, Frantz CN. In vitro versus in vivo metabolic activation of mutagens. *Environ Health Prospect*. 1973;6:71–82.
Malu S, Malshetty V, Francis D, Cortes P. Role of non-homologous end joining in V(D)J recombination. *Immunol Res*. 2012;54(1-3):233–246.
Marchetti F, Bishop JB, Lowe X, et al. Etoposide induces heritable chromosomal aberrations and aneuploidy during male meiosis in the mouse. *Proc Natl Acad Sci U S A*. 2001;98:3952–3957.
Marchetti F, Cabreros D, Wyrobek AJ. Laboratory methods for the detection of chromosomal structural aberrations in human and mouse sperm by fluorescence in situ hybridization. *Methods Mol Biol*. 2008;410:241–271.
Marchetti F, Essers J, Kanaar R, Wyrobek AJ. Disruption of maternal DNA repair increases sperm-derived chromosomal aberrations. *Proc Natl Acad Sci U S A*. 2007;104(45):17725–17729.
Marchetti F, Massarotti A, Yauk CL, Pacchierotti F, Russo A. The adverse outcome pathway (AOP) for chemical binding to tubulin in oocytes leading to aneuploid offspring. *Environ Mol Mutagen*. 2016;57(2):87–113.
Marchetti F, Pearson FS, Bishop JB, et al. Etoposide induces chromosomal abnormalities in mouse spermatocytes and stem cell spermatogonia. *Hum Reprod*. 2006;21:888–895.
Marchetti F, Wyrobek AJ. Mechanisms and consequences of paternally-transmitted chromosomal abnormalities. *Birth Defects Res C Embryo Today*. 2005;75(2):112–129.
Margison GP, Povey AC, Kaina B, Santibanez Koref MF. Variability and regulation of O^6-alkylguanine-DNA alkyltransferase. *Carcinogenesis*. 2003;24:625–635.
Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. *Mutat Res*. 1983;113:173–215.
Marshall CJ. Tumor suppressor genes. *Cell*. 1991;64:313–326.
Martin RH, Ko E, Rademaker A. Distribution of aneuploidy in human gametes: Comparison between human sperm and oocytes. *Am J Med Genet*. 1991;39:321–331.
Martin RH, Spriggs E, Rademaker AW. Multicolor fluorescence *in situ* hybridization analysis of aneuploidy and diploidy frequencies in 225, 846 sperm from 10 normal men. *Biol Reprod*. 1996;54:394–398.
Mason JM, Aaron CS, Lee WR, et al. A guide for performing germ cell mutagenesis assays using *Drosophila melanogaster*. *Mutat Res*. 1987;189:93–102.
Mastrocola AS, Heinen CD. Nuclear reorganization of DNA mismatch repair proteins in response to DNA damage. *DNA Repair*. 2010;9:120–133.
Mathieu N, Kaczmarek N, Naegeli H. Strand- and site-specific DNA lesion demarcation by the xeroderma pigmentosum group D helicase. *Proc Natl Acad Sci U S A*. 2010;107:17545–17550.
Masutani C, Kusumoto R, Iwai S, Hanaoka F. Mechanisms of accurate translesion synthesis by human DNA polymerase eta. *EMBO J*. 2000;19:3100–3109.
Masutani C, Kusumoto R, Yamada A, et al. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature*. 1999;399:700–704.
Mazurek A, Berardini M, Fishel RJ. Activation of human MutS homologs by 8-oxo-guanine DNA damage. *Biol Chem*. 2002;277:8260–8266.
McCullough AK, Dodson ML, Lloyd RS. Initiation of base excision repair: glycosylase mechanisms and structures. *Annu Rev Biochem*. 1999;68:255–285.
McElhinny NSA, Kumar D, Clark AB, et al.Genome instability due to ribonucleotide incorporation into DNA. *Nat Chem Biol*. 2010;6(10):774–781.
McIntyre J, Podlaska A, Skoneczna A, Halas A, Sledziewska-Gojska E. Analysis of the spontaneous mutator phenotype associated with 20S proteasome deficiency in S. cerevisiae. *Mutat Res*. 2006:593:153–163.

McGregor DB, Rice JM, Venitt S, eds. *The Use of Short- and Medium-term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation.* IARC Sci Pub No. 146. Lyon, France: IARC; 1999.
McGregor E, De Souza A. Proteomics and laser microdissection. *Methods Mol Biol*. 2006;333:291–304.
McQueen CA, Williams GM. The hepatocyte primary culture/DNA repair test using hepatocytes from several species. *Cell Biol Toxicol*. 1987;3(2):209–218.
Meek ME, Bucher JR, Cohen SM, et al. A framework for human relevance analysis of information on carcinogenic modes of action. *Crit Rev Toxicol*. 2003;33:591–653.
Meek ME, Boobis A, Cote I, et al. New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis. *J Appl Toxicol*. 2014a;34(1):1–8.
Meek ME, Palermo CM, Bachman AN, North CM, Lewis RJ. Mode of action human relevance (species concordance) framework: evolution of the Bradford Hill considerations and comparative analysis of weight of evidence. *J Appl Toxicol*. 2014b;34(6):595–606.
Mellon I, Hanawalt PC. Induction of the *Escherichia coli* lactose operon selectively increases repair of its transcribed DNA strand. *Nature*. 1989;342:95–98.
Mercer WE. Checking on the cell cycle. *J Cell Biochem Suppl*. 1998;30–31:50–54.
Michel B. After 30 Years of Study, the Bacterial SOS Response Still Surprises Us. *PLoS Biol*. 2005;3(7):e255.
Miller E, Miller J. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. *Cancer*. 1981;47:2327–2345.
Miller JA, Miller EC. Ultimate chemical carcinogens as reactive mutagenic electrophiles. In: Hiatt HH, Watson JD, Winsten JA, eds. *Origins of Human Cancer*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1977:605–628.
Mimori T, Hardin JA, Steitz JA. Characterization of the DNA-binding protein antigen Ku recognized by autoantibodies from patients with rheumatic disorders. *J Biol Chem*. 1986;261:2274–2278.
Min JH, Pavletich NP. Recognition of DNA damage by the Rad4 nucleotide excision repair protein. *Nature*. 2007;449(7162):570–575.
Misík M, Ma TH, Nersesyan A, et al. Micronucleus assays with *tradescantia* pollen tetrads: an update. *Mutagenesis*. 2011;26:215–221.
Misteli T, Soutoglou E. The emerging role of nuclear architecture in DNA repair and genome maintenance. *Nat Rev Mol Cell Biol*. 2009;10(4):243–254.
Miura D, Shaddock JG, Mittelstaedt RA, et al. Analysis of mutations in the Pig-a gene of spleen T-cells from N-ethyl-N-nitrosourea-treated Fischer 344 rats. *Environ Mol Mutagen*. 2011;52:419–423.
Mladenov E, Iliakis G. Induction and repair of DNA double strand breaks: the increasing spectrum of non-homologous end joining pathways. *Mutat Res*. 2011;711:61–72.
Mocquet V, Laine JP, Riedl T, Yajin Z, Lee MY, Egly JM. Sequential recruitment of the repair factors during NER: the role of XPG in initiating the resynthesis step. *EMBO J*. 2008;27:155–167.
Modrich P. Strand-specific mismatch repair in mammalian cells. *J Biol Chem*. 1997;272:24727–24730.
Modrich P, Lahue R. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu Rev Biochem*. 1996;65:101–133.
Mohrenweiser HW. Germinal mutation and human genetic disease. In: Li AP, Heflich RH, eds. *Genetic Toxicology: A Treatise*. Boca Raton, FL: CRC Press; 1991:67–92.
Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. *Cell*. 2007;129:665–679.
Moolenaar RJ. Carcinogen risk assessment: international comparison. *Regul Toxicol Pharmacol*. 1994;20:302–336.
Moore MM, Honma M, Clements J, et al. Suitable top concentration for tests with mammalian cells: mouse lymphoma assay workgroup. *Mutat Res*. 2011;723:84–86.
Moore MM, Schoeny RS, Becker RA, White K, Pottenger LH. Development of an adverse outcome pathway for chemically induced hepatocellular carcinoma: case study of AFB1, a human carcinogen with a mutagenic mode of action. *Crit Rev Toxicol*. 2018;48(4):312–337.
Morita T, Macgregor JT, Hayashi M. Micronucleus assays in rodent tissues other than bone marrow. *Mutagenesis*. 2011;26:223–230.
Mortelmans K, Riccio ES. The bacterial tryptophan reverse mutation assay with *Escherichia coli* WP2. *Mutat Res*. 2000;455:61–69.
Mortelmans K, Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. *Mutat Res*. 2000;455:29–60.
Moser J, Kool H, Giakzidis I, et al. Sealing of chromosomal DNA nicks during nucleotide excision repair requires XRCC1 and DNA ligase III alpha in a cell-cycle-specific manner. *Mol Cell*. 2007;27:311–323.
Muller HJ. Artificial transmutation of the gene. *Science*. 1927;66:84–87.
Nagaishi M, Yamamoto T, Iinuma K, et al. Chromosome abnormalities identified in 347 spontaneous abortions collected in Japan. *J Obstet Gynaecol Res*. 2004;30:237–241.
Nakamura A, Sedelnikova OA, Redon C, et al. Techniques for gamma-H2AX detection. *Methods Enzymol*. 2006;409:236–250.
Natarajan AT. An overview of the results of testing of known or suspected aneugens using mammalian cells in vitro. *Mutat Res*. 1993;287:113–118.
National Academy of Sciences (US NAS). *Risk Assessment in the Federal Government: Managing the Process*. Washington, DC: National Academy Press; 1983.
National Research Council (NRC). *Risk Assessment in the Federal Government: Managing the Process*. Washington, DC: The National Academy Press; 1983.
National Research Council (NRC). *Science and Judgment in Risk Assessment*. Washington, DC: National Academy Press; 1994.
National Research Council (NRC). Committee on the Biological Effects of Ionizing Radiations. *Health Effects of Exposure to Low Levels of Ionizing Radiation: BEIR VII*. Washington, DC: National Academy Press; 2007a.
National Research Council (NRC). *Toxicity Testing in the 21st Century—A Vision and a Strategy*. Washington, DC: The National Academies Press; 2007b.
Nay SL, O'Connor TR. Direct repair in mammalian cells. In: Clark C, ed. *New Research Directions in DNA Repair*. London, UK: InTech; 2013:123–162.
NCRP. Ionizing Radiation Exposure of the Population of the United States NCRP Report No. 160. The National Council on Radiation Protection and Measurements, Bethesda, MD, USA. 2008. ISBN: 978-0-929600-98-7.
Neal JA, Meek A. Choosing the right path: does DNA-PK help make the decision? *Mutat Res*. 2011;711:73–86.
Nieminuszczy J, Mielecki D, Sikora A, et al. Mutagenic potency of MMS-induced 1meA/3meC lesions in *E. coli*. *Environ Mol Mutagen*. 2009;50(9):791–799.
Nohmi T. Past, present and future directions of gpt delta rodent gene mutation assays. *Food Safety*. 2016;4(1):1–13.
Noonan EM, Shah D, Yaffe MB, Lauffenburger DA, Samson LD. O6-Methylguanine DNA lesions induce an intra-S-phase arrest from which cells exit into apoptosis governed by early and late multi-pathway signaling network activation. *Integr Biol (Camb)*. 2012;4(10):1237–1255.
Norppa H, Bonassi S, Hansteen IL, et al. Chromosomal aberrations and SCEs as biomarkers of cancer risk. *Mutat Res*. 2006;600:37–45.
Nunoshiba T, Watanabe E, Takahashi T, et al. Ames test-negative carcinogen, ortho-phenyl phenol, binds tubulin and causes aneuploidyin budding yeast. *Mutat Res*. 2007;617:90–97.
Oda Y, Hirayama T, Watanabe T. Genotoxic activation of the environmental pollutant 3,6-dinitrobenzo[e]pyrene in *Salmonella typhimurium umu* strains expressing human cytochrome P450 and N-acetyltransferase. *Toxicol Lett*. 2009;188:258–262.
OECD Guideline 471. Genetic Toxicology: Bacterial Reverse Mutation Test, Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.
OECD Guideline 473. In Vitro Mammalian Chromosomal Aberration Test; adopted 29 July 2016.
OECD Guideline 476. In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes; adopted 29 July 2016.
OECD Guideline 487. In Vitro Mammalian Cell Micronucleus Test; adopted 29 July 2016.
OECD Test Guideline 488. Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays; adopted 26 July 2013.
OECD Test Guideline 489. In vivo mammalian alkaline comet assay; adopted 29 July 2016.
OECD Test Guideline 490. In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene; adopted 28 July 2015.

Ogi T, Limsirichaikul S, Overmeer RM, et al. Three DNA polymerases, recruited by different mechanisms, carry out NER repair synthesis in human cells. *Mol Cell*. 2010;37:714–727.

Ohe T, White PA, DeMarini DM. Mutagenic characteristics of river waters flowing through large metropolitan areas in North America. *Mutat Res*. 2003;534:101–112.

Okada N, Masumura K, Nohmi T, Yajima N. Efficient detection of deletions induced by a single treatment of mitomycin C in transgenic mouse *gpt* delta using the Spi(-) selection. *Environ Mol Mutagen*. 1999;34:106–111.

Olive PL. Impact of the comet assay in radiobiology. *Mutat Res*. 2009;681:13–23.

Olsen AK, Andreassen A, Singh R, et al. Environmental exposure of the mouse germ line: DNA adducts in spermatozoa and formation of de novo mutations during spermatogenesis. *PLoS One*. 2010;5:e11349.

Olsen AK, Lindeman B, Wiger R, Duale N, Brunborg G. How do male germ cells handle DNA damage? *Toxicol Appl Pharmacol*. 2005;207(2 Suppl):521–531.

Olipitz W, Wiktor-Brown D, Shuga J, et al. Integrated molecular analysis indicates undetectable change in DNA damage in mice after continuous irradiation at ~ 400-fold natural background radiation. *Environ Health Perspec*. 2012;120(8):1130–1136.

Paccierotti F, Sgura A. Fluorescence in situ hybridization for the detection of chromosome aberrations and aneuploidy induced by environmental toxicants. *Methods Mol Biol*. 2008;410:217–239.

Pack S, Vortmeyer AO, Pak E, et al. Detection of gene deletion in single metastatic tumor cells in lymph node tissue by fluorescent in-situ hybridization. *Lancet*. 1997;350:264–265.

Pack SD, Weil RJ, Vortmeyer AO, et al. Individual adult human neurons display aneuploidy: detection by fluorescence in situ hybridization and single neuron PCR. *Cell Cycle*. 2005;4:1758–1760.

Parkinson JF, Wheeler HT, McDonald KL. Contribution of DNA repair mechanisms to determining chemotherapy response in high-grade glioma. *J Clin Neurosci*. 2008;15(1):1–8.

Parry JM. Detecting and predicting the activity of rodent carcinogens. *Mutagenesis*. 1994;9:3–5.

Parry JM. Detecting chemical aneugens: a commentary to "Aneuploidy: a report of an ECETOC task force." *Mutat Res*. 1998;410:117–120.

Parry JM, Jenkins GJ, Haddad F, Bourner R, Parry EM. In vitro and in vivo extrapolations of genotoxin exposures: consideration of factors which influence dose-response thresholds. *Mutat Res*. 2000;464(1):53–63.

Parry JM, Parry EM, Johnson G, et al. The detection of genotoxic activity and the qualitative assessment of the consequences of exposures. *Exp Toxicol Pathol*. 2005;57(Suppl 1):205–212.

Paull TT, Gellert M. A mechanistic basis for Mre11-directed DNA joining at microhomologies. *Proc Natl Acad Sci U S A*. 2000;97(12):6409–6414.

Paull TT, Gellert M. The 3′ to 5′ exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol Cell*. 1998;1(7):969–979.

Philippin G, Cadet J, Gasparutto D, Mazon G, Fuchs RP. Ethylene oxide and propylene oxide derived N7-alkylguanine adducts are bypassed accurately in vivo. *DNA Repair*. 2014;22:133–136.

Phillips DH, Farmer PB, Beland FA, et al. Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ Mol Mutagen*. 2000;35:222–233.

Pieper RO. Understanding and manipulating O6-methylguanine-DNA methyltransferase expression. *Pharmacol Ther*. 1997;74:285–297.

Pottenger LH, Gollapudi BB. Genotoxicity testing: moving beyond qualitative "screen and bin" approach towards characterization of dose-response and thresholds. *Environ Mol Mutagen*. 2010;51(8-9):792–799.

Piraino SW, Furney SJ. Beyond the exome: the role of non-coding somatic mutations in cancer. *Ann Oncol*. 2016;27(2):240–248.

Pleasance ED, Cheetham RK, Stephens DJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature*. 2010;463:191–197.

Plošnik A, Vračko M, Sollner Dolenc M. Mutagenic and carcinogenic structural alerts and their mechanisms of action. *Arh Hig Rada Toksikol*. 2016;67:169–182.

Pluciennik A, Dzantiev L, Iyer RR, et al. PCNA function in the activation and strand direction of MutLα endonuclease in mismatch repair. *Proc Natl Acad Sci U S A*. 2010;107:16066–16071.

Potter CJ, Turenchalk GS, Xu T. *Drosophila* in cancer research. *Trends Genet*. 2000;16:33–39.

Prakash S, Johnson RE, Prakash L. Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu Rev Biochem*. 2005;74:317–353.

Preston RJ. Mechanisms of induction of chromosomal alterations and sister chromatid exchanges. In: Li AP, Heflich RF, eds. *Genetic Toxicology: A Treatise*. Boca Raton, FL: CRC Press; 1991:41–66.

Preston RJ. A consideration of the mechanisms of induction of mutations in mammalian cells by low doses and dose rates of ionizing radiation. *Adv Radiat Biol*. 1992;16:125–135.

Preston RJ. Aneuploidy in germ cells: disruption of chromosome mover components. *Environ Mol Mutagen*. 1996;28:176–181.

Preston RJ. Chromosomal changes. In: McGregor DB, Rice JM, Venitt S, eds. *The Use of Short- and Medium-Term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation*. IARC Sci Pub 146. Lyon, France: IARC; 1999a:395–408.

Preston RJ. Cytogenetic effects of ethylene oxide, with an emphasis on population monitoring. *Crit Rev Toxicol*. 1999b;29:263–282.

Preston RJ. Mechanistic data and cancer risk assessment: the need for quantitative molecular endpoints. *Environ Mol Mutagen*. 2005;45:214–221.

Preston RJ, Au W, Bender MA, et al. Mammalian in vivo and in vitro cytogenetic assays: a report of the U.S. EPA's Gene-Tox Program. *Mutat Res*. 1981;87:143–188.

Preston RJ, Fennell TR, Leber AP, et al. Reconsideration of the genetic risk assessment for ethylene oxide exposures. *Environ Mol Mutagen*. 1995;26:189–202.

Preston RJ, Williams GM. DNA-reactive carcinogens: mode of action and human cancer hazard. *Crit Rev Toxicol*. 2005;35:673–683.

Prival MJ, Cebula TA. Sequence analysis of mutations arising during prolonged starvation of *Salmonella typhimurium*. *Genetics*. 1992;132:303–310.

Przygoda RT. Safety assessment and global regulatory requirements for genetic toxicity evaluations of medical devices. *Environ Mol Mutagen*. 2017;58(5):375–379.

Pu X, Wang Z, Klaunig JE. Alkaline Comet Assay for Assessing DNA Damage in Individual Cells. *Curr Protoc Toxicol*. 2015;65:3–12.

Pullman A, Pullman B. Molecular electrostatic potential of the nucleic acids. *Q Rev Biophys*. 1981;14:289–380.

Putnam CD. Evolution of the methyl directed mismatch repair system in Escherichia coli. *DNA Repair (Amst)*. 2016;38:32–41.

Quillardet P, Hofnung M. The SOS chromotest: a review. *Mutat Res*. 1993;297:235–279.

Rabbitts TH. Chromosomal translocations in human cancer. *Nature*. 1994;372:143–149.

Ramsden DA, Gellert M. Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks. *EMBO J*. 1998;17(2):609–614.

Ramsey MJ, Moore DH, Briner JF, et al. The effects of age and lifestyle factors on the accumulation of cytogenetic damage as measured by chromosome painting. *Mutat Res*. 1995;338:95–106.

Ranganatha R, Chakravarthy S, Sukumaran S. High-throughput approaches for genotoxicity testing in drug development: recent advances. *Intern J High Through Screen*. 2016;2016(6):1–12.

Rattray AJ, Strathern JN. Error-prone DNA polymerases: when making a mistake is the only way to get ahead. *Annu Rev Genet*. 2003;37:31–66.

Reardon JT, Sancar A. Nucleotide excision repair. *Prog Nuclei Acid Res Mol Biol*. 2005;79:183–235.

Reed SH. Nucleotide excision repair in chromatin: damage removal at the drop of a HAT. *DNA Repair*. 2011;10:734–742.

Rees BJ, Tate M, Lynch AM, et al. Development of an in vitro PIG-A gene mutation assay in human cells. *Mutagenesis*. 2017;32(2):283–297.

Reliene R, Bishop AJR, Schiestl RH. Involvement of homologous recombination in carcinogenesis. *Adv Genet*. 2007;58:67–87.

Richardson SJ, Bai A, Kulkarni AA, Moghaddam MF. Efficiency in drug discovery: liver S9 fraction assay as a screen for metabolic stability. *Drug Metab Lett*. 2016;10(2):83–90.

Ringvoll J, Nordstrand LM, Vågbø CB, et al. Repair deficient mice reveal mABH2 as the primary oxidative demethylase for repairing 1meA and 3meC lesions in DNA. *EMBO J*. 2006;25(10):2189–2198.

Ripley LS. Mechanisms of gene mutations. In: Li AP, Heflich RH, eds. *Genetic Toxicology: A Treatise*. Boca Raton, FL: CRC Press; 1991:13–40.
Roach JC, Glusman G, Smit AF, et al. Analysis of genetic inheritance in a family quartet by whole-genome sequencing. *Science*. 2010;328(5978):636–639.
Robertson DG. Metabonomics in toxicology: a review. *Toxicol Sci*. 2005;85:809–822.
Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol*. 1999;146:905–916.
Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*. 1998;273:5858–5868.
Roszkowski K, Olinski R. Urinary 8-oxoguanine as a predictor of survival in patients undergoing radiotherapy. *Cancer Epidemiol Biomarkers Prev*. 2012;21(4):629–634.
Ross CD, French CT, Keysar SB, et al. Mutant spectra of irradiated CHO AL cells determined with multiple markers analyzed by flow cytometry. *Mutat Res*. 2007;624:61–70.
Royds JA, Iacopetta B. p53 and disease: when the guardian angel fails. *Cell Death Differ*. 2006;13:1017–1026.
Rupa DS, Schuler M, Eastmond DA. Detection of hyperdiploidy and breakage affecting the 1cen-1q12 region of cultured interphase human lymphocytes treated with various genotoxic agents. *Environ Mol Mutagen*. 1997;29:161–167.
Rupert CS. Repair of ultraviolet damage in cellular DNA. *J Cell Comparat. Physiol*. 1961;58:57–68.
Rupert CS, Goodgal SH, Herriott RM. Photoreactivation in vitro of ultraviolet-inactivated *Hemophilus influenzae* transforming factor. *J Gen Physiol*. 1958;41:451–471.
Russell LB. Effects of male germ-cell stage on the frequency, nature, and spectrum of induced specific-locus mutations in the mouse. *Genetica*. 2004;122:25–36.
Russell LB, Russell WL. Frequency and nature of specific-locus mutations induced in female mice by radiations and chemicals: a review. *Mutat Res*. 1992;296:107–127.
Russell LB, Shelby MD. Tests for heritable genetic damage and for evidence of gonadal exposure in mammals. *Mutat Res*. 1985;154:69–84.
Russell LB, Selby PB, Von Halle E, et al. The mouse specific-locus test with agents other than radiations: interpretation of data and recommendations for future work. *Mutat Res*. 1981;86:329–354.
Russell WL. X-ray-induced mutations in mice. *Cold Spring Harb Symp Quant Biol*. 1951;16:327–336.
Russo A. In vivo cytogenetics: mammalian germ cells. *Mutat Res*. 2000;455:167–189.
Sale JE. Translesion DNA synthesis and mutagenesis in eukaryotes. *Cold Spring Harb Perspect Biol*. 2013;5(3):a012708.
Sancar A. Structure and function of DNA photolyase. *Biochemistry*. 1994;33(1):2–9.
Sankaranarayanan K. Ionizing radiation and genetic risks: IX. Estimates of the frequencies of Mendelian diseases and spontaneous mutation rates in human populations: a 1998 perspective. *Mutat Res*. 1998;411:129–178.
Sankaranarayanan K. Estimation of the genetic risks of exposure to ionizing radiation in humans: current status and emerging perspectives. *J Radiat Res (Tokyo)*. 2006;47(suppl B):B57–B66.
Sankaranarayanan K, Chakraborty R, Boerwinkle EA. Ionizing radiation and genetic risks. VI. Chronic multifactorial diseases: a review of epidemiological and genetical aspects of coronary heart disease, essential hypertension and diabetes mellitus. *Mutat Res*. 1999;436:21–57.
Sawada M, Kamataki T. Genetically engineered cells stably expressing cytochrome P450 and their application to mutagen assays. *Mutat Res*. 1998;411:19–43.
Sax K. Induction by X-rays of chromosome aberrations in *Tradescantia* microspores. *Genetics*. 1938;23:494–516.
Sax K. The time factor in X-ray production of chromosome aberrations. *Proc Natl Acad Sci U S A*. 1939;25:225–233.
Sax K, Luippold H. The effects of fractional x-ray dosage on the frequency of chromosome aberrations. *Heredity*. 1952;6:127–131.
Scharer OD. Achieving broad substrate specificity in damage recognition by binding accessible nondamaged DNA. *Mol Cell*. 2007;28:184–186.
Schofield MJ, Hsieh P. DNA mismatch repair: Molecular mechanisms and biological function. *Annu Rev Microbiol*. 2003;57:579–608.
Scott D, Galloway SM, Marshall RR, et al. Genotoxicity under extreme culture conditions. *Mutat Res*. 1991;257:147–204.
Schisler MR, Moore MM, Gollapudi BB. In vitro mouse lymphoma (L5178Y Tk$^{+/-}$-3.7.2C) forward mutation assay. *Methods Mol Biol*. 2013;1044:27–50.
Seeberg E, Eide L, Bjoras M. The base excision repair pathway. *Trends Biochem Sci*. 1995;20:391–397.
Seidel SD, Sparrow BR, Kan HL, et al. Profiles of gene expression changes in L5178Y mouse lymphoma cells treated with methyl methanesulfonate and sodium chloride. *Mutagenesis*. 2004;19:195–201.
Seifried HE, Seifried RM, Clarke JJ, et al. A compilation of two decades of mutagenicity test results with the Ames *Salmonella typhimurium* and L5178Y mouse lymphoma cell mutation assays. *Chem Res Toxicol*. 2006;19:627–644.
Selby PB, Earhart VS, Garrison EM, et al. Tests of induction in mice by acute and chronic ionizing radiation and ethylnitrosourea of dominant mutations that cause the more common skeletal anomalies. *Mutat Res*. 2004;545:81–107.
Sengstag C. The role of mitotic recombination in carcinogenesis. *Crit Rev Toxicol*. 1994;24:323–353.
Serafimova R, Gatnik M, Andrew Worth A. Review of QSAR Models and Software Tools for Predicting Genotoxicity and Carcinogenicity. JRC Scientific and Technical Reports. European Commission, Joint Research Centre Institute for Health and Consumer Protection. EUR 24427 EN – 2010. 2010.
Shane BS, Smith-Dunn DL, de Boer JG, Glickman BW, Cunningham ML. Mutant frequencies and mutation spectra of dimethylnitrosamine (DMN) at the lacI and cII loci in the livers of Big Blue transgenic mice. *Mutat Res*. 2000;452(2):197–210.
Shee C, Cox BD, Gu F, et al. Engineered proteins detect spontaneous DNA breakage in human and bacterial cells. *Genes Chromosomes*. 2013;2:e01222.
Shelby MD. Human germ cell mutagens. *Environ Mol Mutagen*. 1994;23(suppl 24):30–34.
Shibata D, Peinado MA, Ionov Y, Malkhosyan S, Perucho M. Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. *Nat Genet*. 1994;6(3):273–281.
Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer*. 2003;3:155–68.
Shinde D, Lai Y, Sun F, Arnheim N. Taq DNA polymerase slippage mutation rates measured by PCR and quasi-likelihood analysis:(CA/GT)n and (A/T)n microsatellites. *Nucleic Acids Res*. 2003;31(3):974–980.
Sinha RP, Häder DP. UV-induced DNA damage and repair: a review. *Photochem Photobiol Sci*. 2002;1(4):225–236.
Sharma V, Collins LB, Clement JM, Zhang Z, Nakamura J, Swenberg JA. Molecular dosimetry of endogenous and exogenous O(6)-methyl-dG and N7-methyl-G adducts following low dose [D3]-methylnitrosourea exposures in cultured human cells. *Chem Res Toxicol*. 2014;27(4):480–482.
Shinohara A, Ogawa T. Homologous recombination and the roles of double-strand breaks. *Trends Biochem Sci*. 1995;20:387–391.
Singer TM, Lambert IM, Williams A, et al. Detection of induced male germline mutation: correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. *Mutat Res*. 2006;598:164–193.
Singer TM, Yauk CL. Germ cell mutagens: risk assessment challenges in the 21st century. *Environ Mol Mutagen*. 2010;51:919–928.
Singh NP. Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat Res*. 2000;455:111–127.
Smith MT, Zhang L, Wang Y, et al. Increased translocations and aneusomy in chromosomes 8 and 21 among workers exposed to benzene. *Cancer Res*. 1998;58:2176–2181.
Snyder RD. An update on the genotoxicity and carcinogenicity of marketed pharmaceuticals with reference to in silico predictivity. *Environ Mol Mutagen*. 2009;50:435–450.
Snyder RD, Pearl GS, Mandakas G, et al. Assessment of the sensitivity of the computational programs DEREK, TOPKAT, and MCASE in the prediction of the genotoxicity of pharmaceutical molecules. *Environ Mol Mutagen*. 2004;43:143–158.

Snyder RD, Smith MD. Computational prediction of genotoxicity: room for improvement. *Drug Discov Today*. 2005;10:1119–1124.
Sobels FH. The parallelogram: an indirect approach for the assessment of genetic risks from chemical mutagens. In: Bora KC, Douglas GR, Nestman ER, eds. *Progress in Mutation Research*. Vol 3. Amsterdam: Elsevier Biomedical Press; 1982:323–327.
Solinas-Toldo S, Lampel S, Stilgenbauer S, et al. Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances. *Genes Chromosomes Cancer*. 1997;20:399–407.
Somers CM. Expanded simple tandem repeat (ESTR) mutation induction in the male germline: lessons learned from lab mice. *Mutat Res*. 2006;598:35–49.
Sonich-Mullin C, Fielder R, Wiltse J, et al. IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul Toxicol Pharmacol*. 2001;34(2):146–152.
Sonoda E, Hochegger H, Saberi A, Taniguchi Y, Takeda S. Differential usage of nonhomologous end-joining and homologous recombination in double strand break repair. *DNA Repair (Amst)*. 2006;5:1021–1029.
Sonoda E, Takata M, Yamashita YM, Morrison C, Takeda S. Homologous DNA recombination in vertebrate cells. *Proc Natl Acad Sci U S A*. 2001;98(15):8388–8394.
Sørensen CS, Syljuåsen RG, Falck J, et al. Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. *Cancer Cell*. 2003;3(3): 247–258.
Sotomayor RE, Sega GA. Unscheduled DNA synthesis assay in mammalian spermatogenic cells: an update. *Environ Mol Mutagen*. 2000;36:255–265.
Southern EM. DNA chips: Analyzing sequence by hybridization to oligonucleotides on a large scale. *Trends Genet*. 1996;12:110–115.
Sparks JL, Chon H, Cerritelli SM, et al. RNase H2-initiated ribonucleotide excision repair. *Mol Cell*. 2012;47:980–986.
Speicher MR, Carter NP. The new cytogenetics: blurring the boundaries with molecular biology. *Nat Rev Genet*. 2005;6:782–792.
Speit G, Kojima H, Burlinson B, et al. Critical issues with the in vivo comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT). *Mutat Res Genet Toxicol Environ Mutagen*. 2015;783:6–12.
Speit G, Vasquez M, Hartmann A. The comet assay as an indicator test for germ cell genotoxicity. *Mutat Res*. 2009;681:3–12.
Srivenugopal KS, Yuan XH, Friedman HS, Ali-Osman F. Ubiquitination-dependent proteolysis of O6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochemistry*. 1996;35(4):1328–1334.
Stadler ZK, Schrader KA, Vijai J, Robson ME, Offit K. Cancer genomics and inherited risk. *J Clin Oncol*. 2014;32(7):687–698.
Stram DO, Sposto R, Preston D, Abrahamson S, Honda T, Awa AA. Stable chromosome aberrations among A-bomb survivors: an update. *Radiat Res*. 1993;136(1):29–36.
Stojic L, Mojas N, Cejka P, et al. Mismatch repair-dependent G2 checkpoint induced by low doses of SN1 type methylating agents requires the ATR kinase. *Genes Dev*. 2004;18(11):1331–1344.
Stoler DL, Chen N, Basik M, et al. The onset and extent of genomic instability in sporadic colorectal tumor progression. *Proc Natl Acad Sci U S A*. 1999 ;96:15121–15126.
Storer RD, McKelvey TW, Kraynak AR, et al. Revalidation of the in vitro alkaline elution/rat hepatocyte assay for DNA damage: improved criteria for assessment of cytotoxicity and genotoxicity and results for 81 compounds. *Mutat Res*. 1996;368:59–101.
Stracker TH, Petrini JH. The MRE11 complex: starting from the ends. *Nat Rev Mol Cell Biol*. 2011;12(2):90–103.
Stratton MR, Campbell PJ, Futreal PA. The cancer genome. *Nature*. 2009;458:719–724.
Styles JA, Penman MG. The mouse spot test: evaluation of its performance in identifying chemical mutagens and carcinogens. *Mutat Res*. 1985;154:183–204.
Sugasawa K, Akagi J, Nishi R, Iwai S, Hanaoka F. Two-step recognition of DNA damage for mammalian nucleotide excision repair: Directional binding of the XPC complex and DNA strand scanning. *Mol Cell*. 2009;36:642–653.
Sukup-Jackson MR, Kiraly O, Kay JE, et al. Rosa26-GFP direct repeat (RaDR-GFP) mice reveal tissue- and age-dependence of homologous recombination in mammals in vivo. *PLoS Genet*. 2014;10(6):e1004299.
Sung JS, Demple B. Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA. *FEBS J*. 2006;273:1620–1629.
Swenberg JA, Barrow CS, Boreiko CJ, et al. Non-linear biological responses to formaldehyde and their implications for carcinogenic risk assessment. *Carcinogenesis*. 1983;4(8):945–952.
Swenberg JA, Lu K, Moeller BC, et al. Endogenous versus exogenous DNA adducts: their role in carcinogenesis, epidemiology, and risk assessment. *Toxicol Sci*. 2011;120(Suppl 1):S130–S145.
Swiger RR. Just how does the *cII* selection system work in Muta™Mouse? *Environ Mol Mutagen*. 2001;37:290–296.
Swiger RR, Cosentino L, Shima N, Bielas JH, Cruz-Munoz W, Heddle JA. The cII locus in the MutaMouse system. *Environ Mol Mutagen*. 1999;34:201–220.
Sykes PJ, Morley AA, Hooker AM. The pKZ1 recombination mutation assay: a sensitive assay for low dose studies. *Dose-Response*. 2006;4:91–105.
Taira K, Kaneto S, Nakano K, et al. Distinct pathways for repairing mutagenic lesions induced by methylating and ethylating agents. *Mutagenesis*. 2013;28:341–350.
Takigami H, Matsui S, Matsuda T, et al. The *Bacillus subtilis* rec-assay: a powerful tool for the detection of genotoxic substances in the water environment. Prospect for assessing potential impact of pollutants from stabilized wastes. *Waste Manag*. 2002;22:209–213.
Tanaka K. Multiple functions of the S-phase checkpoint mediator. *Biosci Biotechnol Biochem*. 2010;74:2367–2373.
Tang J, Chu G. Xeroderma pigmentosum complementation group E and UV-damaged DNA-binding protein. *DNA Repair (Amst)*. 2002;1:601–616.
Tano K, Shiota S, Collier J, et al. Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O^6-alkylguanine. *Proc Natl Acad Sci U S A*. 1990;87:686–690.
Tease C. Radiation- and chemically-induced chromosome aberrations in mouse oocytes: a comparison with effects in males. *Mutat Res*. 1992; 296:135–142.
Tebbs RS, Flannery ML, Meneses JJ, et al. Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. *Dev Biol*. 1999;208(2):513–529.
Tennant RW, Ashby J. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. *Mutat Res*. 1991;257:209–227.
Tennant RW, Margolin BH, Shelby MD, et al. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. *Science*. 1987;236:933–941.
Tennant RW, Spalding J. Predictions for the outcome of rodent carcinogenicity bioassays: Identification of trans-species carcinogens and noncarcinogens. *Environ Health Perspect*. 1996;104(S5):1095–1100.
Tentner AR, Lee MJ, Ostheimer GJ, et al. Combined experimental and computational analysis of DNA damage signaling reveals context-dependent roles for Erk in apoptosis and G1/S arrest after genotoxic stress. *Mol Syst Biol*. 2012;8:568.
Thacker J. Radiation-induced mutation in mammalian cells at low doses and dose rates. *Adv Radiat Biol*. 1992;16:77–124.
Thomas AD, Fahrer J, Johnson GE, Kaina B. Theoretical considerations for thresholds in chemical carcinogenesis. *Mutat Res*. 2015;765: 56–67.
Thomas AD, Jenkins GJ, Kaina B, et al. Influence of DNA repair on nonlinear dose-responses for mutation. *Toxicol Sci*. 2013;132(1):87–95.
Thybaud V, Lorge E, Levy DD, et al. Main issues addressed in the 2014–2015 revisions to the OECD Genetic Toxicology Test Guidelines. *Environ Mol Mutagen*. 2017;58(5):284–295.
Tice RR, Agurell E, Anderson D, et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen*. 2000;35:206–221.
Tice RR, Hayashi M, MacGregor JT, et al. Report from the working group on the in vivo mammalian bone marrow chromosomal aberration test. *Mutat Res*. 1994;312:305–312.

Tomasetti C, Li L, Vogelstein B. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science*. 2017;355(6331):1330–1334.

Tomasetti C, Vogelstein B. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science*. 2015;347(6217):78–81.

Tomasetti C, Vogelstein B. On the slope of the regression between stem cell divisions and cancer risk, and the lack of correlation between stem cell divisions and environmental factors-associated cancer risk. *PLoS One*. 2017;12(5):e0175535.

Townsend TA, Parrish MC, Engelward BP, Manjanatha MG. The development and validation of EpiComet-Chip, a modified high-throughput comet assay for the assessment of DNA methylation status. *Environ Mol Mutagen*. 2017;58(7):508–521.

Trask BJ, Allen S, Massa H, et al. Studies of metaphase and interphase chromosomes using fluorescence in situ hybridizaiton. *Cold Spring Harb Symp Quant Biol*. 1993;58:767–775.

Trewick SC, Henshaw TF, Hausinger RP, Lindahl T, Sedgwick B. Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. *Nature*. 2002;419(6903):174–178.

Trost D, Hildebrandt B, Beier M, et al. Molecular cytogenetic profiling of complex karyotypes in primary myelodysplastic syndromes and acute myeloid leukemia. *Cancer Genet Cytogenet*. 2006;165:51–63.

Tucker JD, Auletta A, Cimino M, et al. Sister-chromatid exchange: second report of the Gene-Tox program. *Mutat Res*. 1993a;297:101–180.

Tucker JD, Breneman JW, Briner JF, et al. Persistence of radiation-induced translocations in rat peripheral blood determined by chromosome painting. *Environ Mol Mutagen*. 1997;30:264–272.

Tucker JD, Cofield J, Matsumoto K, et al. Persistence of chromosome aberrations following acute radiation: I, PAINT translocations, dicentrics, rings, fragments, and insertions. *Environ Mol Mutagen*. 2005;45:229–248.

Tucker JD, Luckinbill LS. Estimating the lowest detectable dose of ionizing radiation by FISH whole-chromosome painting. *Radiat Res*. 2011;175:631–637.

Tucker JD, Moore DH 2nd. The importance of age and smoking in evaluating adverse cytogenetic effects of exposure to environmental agents. *Environ Health Perspect*. 1996;104:489–492.

Tucker JD, Ramsey MJ, Lee DA, Minkler JL. Validation of chromosome painting as a biodosimeter in human peripheral lymphocytes following acute exposure to ionizing radiation in vitro. *Int J Radiat Biol*. 1993b;64:27–37.

Tung BS, McGregor WG, Wang YC, Maher VM, McCormick JJ. Comparison of the rate of excision of major UV photoproducts in the strands of the human HPRT gene of normal and xeroderma pigmentosum variant cells. *Mutat Res*. 1996;362:65–74.

Turner DR, Dreimanis M, Holt D, Firgaira FA, Morley AA. Mitotic recombination is an important mutational event following oxidative damage. *Mutat Res*. 2003;522:21–26.

Tweats DJ, Blakey D, Heflich RH, et al. Report of the IWGT working group on strategies and interpretation of regulatory *in vivo* tests I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards. *Mutat Res*. 2007;627:78–91.

UN. Globally Harmonised System of Classification and Labelling of Chemicals (GHS). ST/SG/AC.10/13/Rev.6. Sixth revised edition, United Nations New York and Geneva, 2015.

United Nations Scientific Committee on the Effects of Atomic Radiation. Hereditary effects of radiation. *UNSCEAR 2001 Report to the General Assembly, With Scientific Annex*. New York: United Nations; 2001.

Valentine CR, Delongchamp RR, Pearce MG, et al. In vivo mutation analysis using the φX174 transgenic mouse and comparisons with other transgenes and endogenous genes. *Mutat Res*. 2010;705:205–216.

Van Houten B, Albertini R. DNA damage and repair. In: Craighead JE, ed. *Pathology of Environmental and Occupational Disease*. St. Louis, MO: Mosby-Year Book; 1995:311–327.

Veldman T, Vignon C, Schrock E, et al. Hidden chromosome abnormalities in haematological malignancies detected by multicolour spectral karyotyping. *Nat Genet*. 1997;15:406–410.

Venkatachalam S, Shi YP, Jones SN, et al. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J*. 1998;17:4657–4667.

Verhofsrad N, van Oostrom CT, Zwart E, et al. Evaluation of benzo(a) pyrene-induced gene mutations in male germ cells. *Toxicol Sci*. 2011;119:218–223.

Villeneuve DL, Crump D, Garcia-Reyero N, et al. Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicol Sci*. 2014a;142(2):312–320.

Villeneuve DL, Crump D, Garcia-Reyero N, et al. Adverse outcome pathway development II: best practices. *Toxicol Sci*. 2014b;142(2):321–330.

Vinken M. The adverse outcome pathway concept: a pragmatic tool in toxicology. *Toxicology*. 2013;312:158–165.

Vissers LE, Veltman JA, van Kessel AG, Brunner HG. Identification of disease genes by whole genome CGH arrays. *Hum Mol Genet*. 2005;14:R215–R223.

Vlasakova K, Skopek TR, Glaab WE. Induced mutation spectra at the *upp* locus in *Salmonella typhimurium*: response of the target gene in the FU assay to mechanistically different mutagens. *Mutat Res*. 2005;578:225–237.

Vogel EW, Graf U, Frei HJ, Nivara MM. The results of assays in *Drosophila* as indicators of exposure to carcinogens. *IARC Sci Publ*. 1999;146:427–470.

Vogel EW, Nivard MJ. Parallel monitoring of mitotic recombination, clastogenicity and teratogenic effects in eye tissue of *Drosophila*. *Mutat Res*. 2000;455:141–153.

Vogelstein B, Kinzler KW. The multistep nature of cancer. *Trends Genet*. 1993;9(4):138–41.

Vogelstein B, Papadopoulos N, Velculescu VE, et al. Cancer genome landscapes. *Science*. 2013;339(6127):1546–1558.

Vorsanova SG, Yurov YB, Iourov IY. Human interphase chromosomes: a review of available molecular cytogenetic technologies. *Mol Cytogenet*. 2010;3:1–15.

Votano JR, Parham M, Hall LH, et al. Three new consensus QSAR models for the prediction of Ames genotoxicity. *Mutagenesis*. 2004;19:365–377.

Yoshioka K, Yoshioka Y, Hsieh P. ATR kinase activation mediated by MutSalpha and MutLalpha in response to cytotoxic O6-methylguanine adducts. *Molec Cell*. 2006;22(4):501–510.

Wallace SS. DNA damages processed by base excision repair: biological consequences. *Int J Radiat Biol*. 1994;66:579–589.

Wang J, Sawyer JR, Chen L, et al. The mouse lymphoma assay detects recombination, deletion, and aneuploidy. *Toxicol Sci*. 2009;109:96–105.

Wang Y, Qin J.MSH2 and ATR form a signaling module and regulate two branches of the damage response to DNA methylation. *Proc Natl Acad Sci U S A*. 2003;100:15387–15392.

Ward JF. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog Nucl Acid Res Mol Biol*. 1988;35:95–125.

Ward JF. The complexity of DNA damage: relevance to biological consequences. *Int J Radiat Biol*. 1994;66:427–432.

Watson IR, Takahashi K, Futreal PA, Chin L. Emerging patterns of somatic mutations in cancer. *Nat Rev Genet*. 2013;14(10):703–718.

Watson RE, Goodman JI. Epigenetics and DNA methylation come of age in toxicology. *Toxicol Sci.* 2002;67(1):11–16.

Weingeist DM, Ge J, Wood DK, et al. Single-cell microarray enables high-throughput evaluation of DNA double-strand breaks and DNA repair inhibitors. *Cell Cycle*. 2013;12(6):907–915.

West SC. Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol*. 2003;4:435–445.

Westbye MP, Feyzi E, Aas PA, et al. Human AlkB homolog 1 is a mitochondrial protein that demethylates 3-methylcytosine in DNA and RNA. *J Biol Chem*. 2008;283(36):25046–25056.

White PA, ed. The sources and potential hazards of mutagens in complex environmental matrices. *Mutat Res*. 2004;567(2–3):105–479.

White PA, Johnson GE. Genetic toxicology at the crossroads-from qualitative hazard evaluation to quantitative risk assessment. *Mutagenesis*. 2016;31(3):233–237.

Wichard JD. In silico prediction of genotoxicity. *Food Chem Toxicol*. 2017;106(Pt B):595–599.

Wiktor-Brown DM, Kwon HS, Nam YS, So PT, Engelward BP. Integrated one- and two-photon imaging platform reveals clonal expansion as a major driver of mutation load. *Proc Natl Acad Sci U S A*. 2008;105(30):10314–10319.

Wielgoss S, Barrick JE, Tenaillon O, et al. Mutation rate inferred from synonymous substitutions in a long-term evolution experiment with *Escherichia coli*. *G3*. 2011;1:183–186.

Wills JW, Johnson GE, Doak SH, et al. Empirical analysis of BMD metrics in genetic toxicology part I: in vitro analyses to provide robust potency rankings and support MOA determinations. *Mutagenesis*. 2016;31(3): 255–263.

Wilson DM, Thompson LH. Molecular mechanisms of sister-chromatid exchange. *Mutat Res*. 2007;616:11–23.

Wodicka L, Dong H, Mittmann M, et al. Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nat Biotechnol*. 1997;15:1359–1367.

Wood RD. DNA repair in eukaryotes. *Annu Rev Biochem*. 1996;65:135–167.

Wood RD, Mitchell M, Lindahl T. Human DNA repair genes, 2005. *Mutat Res*. 2005;577:275–283.

Wood RD, Mitchell M, Sgouros J, Lindahl T. Human DNA repair genes. *Science*. 2001;291(5507):1284–1289.

Wu J, Morimyo M, Hongo E, et al. Radiation-induced germline mutations detected by a direct comparison of parents and first-generation offspring DNA sequences containing SNPs. *Mutat Res*. 2006;596:1–11.

Wyatt MD, Pittman DL. Methylating agents and DNA repair responses: methylated bases and sources of strand breaks. *Chem Res Toxicol*. 2006;19(12):1580–1594.

Xie F, Liu T, Qian WJ, et al. Liquid chromatography-mass spectrometry-based quantitative proteomics. *J Biol Chem*. 2011;286:25443–25449.

Yamazaki Y, Fujita K, Nakayama K, et al. Establishment of ten strains of genetically engineered *Salmonella typhimurium* TA1538 each co-expressing a form of human cytochrome P450 with NADPH-cytochrome P450 reductase sensitive to various promutagens. *Mutat Res*. 2004;562: 151–162.

Yasunaga K, Kiyonari A, Oikawa T, et al. Evaluation of the Salmonella *umu* test with 83 NTP chemicals. *Environ Mol Mutagen*. 2004;44:329–345.

Yauk CL. Advances in the application of germline tandem repeat instability for in situ monitoring. *Mutat Res*. 2004;566:169.

Yauk CL, Aardema MJ, Benthem JV, et al. Approaches for identifying germ cell mutagens: Report of the 2013 IWGT workshop on germ cell assays(☆). *Mutat Res Genet Toxicol Environ Mutagen*. 2015;783:36–54.

Yauk CL, Lambert IB, Meek ME, Douglas GR, Marchetti F. Development of the adverse outcome pathway "alkylation of DNA in male premeiotic germ cells leading to heritable mutations" using the OECD's users' handbook supplement. *Environ Mol Mutagen*. 2015;56(9):724–750.

Yeh HC, Ho YP, Shih IeM, Wang TH. Homogeneous point mutation detection by quantum dot-mediated two-color fluorescence coincidence analysis. *Nucleic Acids Res*. 2006;34:e35.

Xanthoudakis S, Smeyne RJ, Wallace JD, Curran T. The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. *Proc Natl Acad Sci U S A*. 1996;93:8919–8923.

Zeiger E. Historical perspective on the development of the genetic toxicity test battery in the United States. *Environ Mol Mutagen*. 2010;51:781–791.

Zhang CZ, Pellman D. From mutational mechanisms in single cells to mutational patterns in cancer genomes. *Cold Spring Harb Symp Quant Biol*. 2015;80:117–137.

Zhang Q, Bhattacharya S, Conolly RB, Clewell HJ, Kaminski NE, Andersen ME. Molecular signaling network motifs provide a mechanistic basis for cellular threshold responses. *Environ Health Perspect*. 2014;122(12):1261–1270.

Zhang Q, Bhattacharya S, Pi J, Clewell RA, Carmichael PL, Andersen ME. Adaptive posttranslational control in cellular stress response pathways and its relationship to toxicity testing and safety assessment. *Toxicol Sci*. 2015;147(2):302–316.

Zhang Y, Gostissa M, Hildebrand DG, et al. The role of mechanistic factors in promoting chromosomal translocations found in lymphoid and other cancers. *Adv Immunol*. 2010;106:93–133.

Zhao H, Watkins JL, Piwnica-Worms H. Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. *Proc Natl Acad Sci U S A*. 2002;99:14795–14800.

Zheng X, Epstein A, Klein HL. Methods to study mitotic homologous recombination and genome stability. *Methods Mol Biol*. 2011;745:3–13.

Zhou H, Xu A, Gillespie JA, et al. Quantification of CD59^{-} mutants in human-hamster hybrid (AL) cells by flow cytometry. *Mutat Res*. 2006;594;113–119.

Zimmering S. A note on the utility of repair-deficient st mus302 Drosophila females in detecting chromosome loss and sex-linked recessive lethals induced in the male genome by methyl methanesulfonate (MMS) and imethylnitrosamine (DMN). *Environ Mutagen*. 1982;4(3):291–293.

Zimmermann FK. Tests for recombinogens in fungi. *Mutat Res*. 1992;284:147–158.

Zimmermann FK, von Borstel RC, von Halle ES, et al. Testing of chemicals for genetic activity with *Saccharomyces cerevisiae:* a report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res*. 1984;133:199–244.

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chapter 10

Developmental Toxicology

John M. Rogers

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Acknowledgments

References

HISTORY OF TERATOLOGY

Teratology as an empirical science has been around for millennia and encompasses the study of abnormal structural development. Conjoined twins have been depicted in marble statuary dating to 6500 BC (Warkany, 1983), and Egyptian wall paintings of human cleft palate and achondroplasia date to 5000 years ago. The origin of mythological creatures including the Cyclops and sirens may have been observations of malformed infants (Thompson, 1930; Warkany, 1977). Hippocrates and Aristotle thought that physical agents such as uterine trauma or pressure could cause birth defects. Aristotle also believed that maternal impressions and emotions could affect the developing fetus, and advised pregnant women to gaze at beautiful art to enhance their child's beauty. This idea is historically present in diverse cultures, and we now understand that maternal stress or depression during pregnancy can be deleterious (Dunkel Schetter and Tanner, 2012).

In the 16th and 17th centuries, scientific theories of causation of birth defects emerged. In 1651, William Harvey expounded the theory of developmental arrest, which posited that malformations resulted from incomplete structural development. An example was "harelip," a congenital malformation in humans that represents a normal but usually transient embryonic stage. In the early 19th century, Saint-Hilaire produced malformed chick embryos by

subjecting eggs to physical trauma or toxic exposures. Later in the 19th century, Dareste produced various malformations in chick embryos using chemical and physical agents including heat shock. Dareste found that timing was more critical than the type of insult in influencing the type of defect that resulted (Dareste, 1877, 1891). Many of the great embryologists of the 19th and 20th centuries, including Loeb, Morgan, Driesch, Spemann, and Hertwig, performed teratological experiments using various physical and chemical probes to deduce principles of normal development.

With the rediscovery of Mendel's laws in 1900, it was accepted that some birth defects were hereditary. Bateson (1894) studied animal variations as a means to elucidate evolution, suggesting that inheritance of variations was a means of speciation. He described human birth defects including polydactyly and syndactyly, supernumerary cervical and thoracic ribs, duplicated appendages, and fused kidneys. A genetic basis of birth defects was further supported by studies of inborn errors of metabolism in the early 20th century.

In the 1920s through 1940s, a variety of environmental conditions and exposures were proven to perturb development in avian, reptilian, fish, and amphibian species. Yet, at that time, mammalian development was thought to be protected from environmental conditions by the mother's body. That idea was refuted when Hale (1935) produced anophthalmia, cleft palate, and other malformations in offspring of pigs by feeding a vitamin A deficient diet. Beginning in 1940, Josef Warkany and co-workers performed a series of experiments demonstrating that maternal dietary deficiencies and other environmental factors could perturb intrauterine development in rats (Warkany and Nelson, 1940; Warkany and Schraffenberger, 1944; Warkany, 1945; Wilson et al., 1953). Since these early studies, many other chemical and physical agents have been demonstrated to cause malformations in mammals (see Warkany [1965]).

HUMAN TERATOGENS AND DEVELOPMENTAL TOXICANTS

With the development of highly sensitive tests that can detect pregnancies shortly after fertilization, recognized pregnancy loss increased to as much as 60% to 70%. Estimates of adverse outcomes include postimplantation pregnancy loss, 31%; major birth defects, 2% to 3% at birth and increasing to 6% to 7% at 1 year with continuing diagnoses; minor birth defects, 14%; low birth weight, 7%; infant mortality (prior to 1 year of age), 1.4%; and abnormal neurological function, 16% to 17% (Schardein, 2000). Brent and Beckman (1990) attributed 15% to 25% of human birth defects to genetic causes, 4% to maternal conditions, 3% to maternal infections, 1% to 2% to *deformations* (e.g., mechanical damage to normally formed structures, such as umbilical cord limb amputations), <1% to chemicals and other environmental influences, and 65% to unknown causes. These estimates are similar to those suggested by Wilson (1977). Regardless of etiology, the sum total represents a significant health burden in light of the over 4 million births annually in the United States.

Schardein (2000) estimated that over 4100 chemicals had been tested in animals for teratogenicity by that time, a third of which were shown to be non-teratogenic, 7% clearly teratogenic in more than one species, 18% "probably" teratogenic (limited testing or positive in majority of species tested) and 9% producing equivocal results. A much smaller number, about 35 to 40 chemicals, chemical classes, or conditions (Table 10-1), have been documented to be developmentally toxic in humans (Schardein and Keller, 1989; Shepard and Lemire, 2004).

How does one determine that a drug, chemical, or other agent, is a likely human teratogen? Criteria for proof of human teratogenicity, based on several sources, have been assembled by Tom Shepard (Shepard, 1994) (Table 10-2), although he stated at that time that not all human teratogens will fit these exact criteria. Shepard also described two modes of establishing an exposure as a human teratogen. The first is the rare malformation/rare exposure or case report method, sometimes referred to as the astute clinician approach (Carey et al., 2009) and the second is the epidemiological method; these have been compared and contrasted (Shepard, 1991). Examples of the case report approach include rubella, thalidomide, and diethylstilbestrol, while teratogens uncovered using the epidemiological approach include more common exposures with more common outcomes, perhaps including, for example, orofacial clefts caused by maternal smoking, or neural tube defects caused by valproic acid. These teratogens are among those summarized below. In a more contemporary example, Rasmussen et al. (2016) used Shepard's criteria to determine that the Zika virus infection during

Table 10-1

Human Developmental Toxicants

Radiation
- Atomic fallout
- Radioiodine
- Therapeutic radiation

Infections
- Cytomegalovirus
- Herpes simplex virus I and II
- Parvovirus B-19 (erythema infectiosum)
- Rubella virus
- Syphilis
- Toxoplasmosis
- Varicella virus
- Venezuelan equine encephalitis virus

Maternal trauma and metabolic imbalances
- Alcoholism
- Amniocentesis, early
- Chorionic villus sampling (before day 60)
- Cretinism
- Diabetes
- Folic acid deficiency
- Hyperthermia
- Phenylketonuria
- Rheumatic disease and congenital heart block
- Sjögren syndrome
- Virilizing tumors

Drugs and chemicals
- Aminoglycosides
- Androgenic hormones
- Angiotensin converting enzyme inhibitors: captopril, enalapril
- Angiotensin receptor antagonists: sartans
- Anticonvulsants: diphenylhydantoin, trimethadione, valproic acid, carbamazepine
- Busulfan
- Carbon monoxide
- Chlorambucil
- Cocaine
- Coumarins
- Cyclophosphamide
- Cytarabine
- Diethylstilbestrol
- Danazol
- Ergotamine
- Ethanol
- Ethylene oxide
- Fluconazole
- Folate antagonists: aminopterin, methotrexate
- Iodides
- Lead
- Lithium
- Mercury, organic
- Methimazole
- Methylene blue
- Misoprostal
- Penicillamine
- Polychlorobiphenyls
- Quinine (high dose)
- Retinoids: isotretinoin, etretinate, acitretin
- Tetracyclines
- Thalidomide
- Tobacco smoke
- Toluene
- Vitamin A (high dose)

SOURCE: Adapted from Shepard and Lemire (2004) and Schardein and Macina (2007).

Table 10-2

Shepard's Criteria for Proof of Human Teratogenicity

1. Proven exposure to agent at critical time(s) in prenatal development.
2. Consistent findings by greater or equal to two high-quality epidemiological studies; prospective studies, if possible; relative risk greater or equal to 6.
3. Careful delineation of the clinical cases. A specific defect or syndrome is helpful.
4. Rare exposure associated with rare defect. Probably three or more cases.
5. Teratogenicity in experimental animal studies important but not essential.
6. The association should make biological sense.
7. Proof in an experimental system that the agent acts in an unaltered state.

Items 1 to 3 (epidemiological approach) or 1, 3, and 4 (rare exposure/rare defect approach) are essential; items 5 to 7 are helpful but not essential.

SOURCE: Shepard (1994).

pregnancy is a human teratogen, causing microcephaly and other brain and eye malformations.

Maternal Rubella Infection

Gregg (1941) reported the first recognized case of widespread human malformations, linking an epidemic of rubella virus infection in Australia to congenital malformations including eye, heart, and ear defects, and mental retardation. Heart and eye defects were associated with maternal infection in the first 2 months of pregnancy, while hearing and speech defects and mental retardation occurred with infection in the third month (Sever, 1967). In the United States alone, it was estimated that 20,000 children were adversely affected (Cooper and Krugman, 1966). Rubella is now uncommon in developed countries due to widespread vaccination, but there have been recent outbreaks in developing countries (DeSantis et al., 2006; Chandy et al., 2011) and reported cases in developed countries as well (Vauloup-Fellous et al., 2010).

Thalidomide

Thalidomide was first marketed in Europe in 1956 and was used throughout much of the world as a sleep aid and treatment for morning sickness. There were a few reports of peripheral neuritis attributed to the drug with long-term use for up to 18 months (Fullerton and Kermer, 1961). In 1960, a striking increase in newborns with rare limb malformations was recorded in West Germany. The affected individuals had amelia (absence of the limbs) or various degrees of phocomelia (reduction of the long bones of the limbs). Congenital cardiac, ocular, otic, intestinal, and renal anomalies were also reported. In 1961, Lenz and McBride, working independently in Germany and Australia, respectively, identified thalidomide as the causative agent (McBride, 1961; Lenz, 1961, 1963). Amelia and phocomelia are rare malformations; the university clinic in Hamburg reported no cases of phocomelia between 1940 and 1959, 1 case in 1959, 30 cases in 1960, and 154 cases in 1961 (Taussig, 1962). The rarity of the malformations was a key factor in recognizing the epidemic. Thalidomide was withdrawn in November 1961, and case reports ended in mid-1962. Estimates of the total number of affected infants worldwide during this period range from 5840 (Lenz, 1988) to over 10,000 in 46 countries (Bartlett et al., 2004). Due to concerns regarding the severity of the peripheral neuropathy and subsequent questions with regard to safety in pregnancy, the U.S. Food and Drug Administration (FDA) did not approve thalidomide for use in the United States. Dr Frances Kelsey, a new FDA staff member at that time, was key to keeping the drug off the market in the United States. She later received the highest civilian honor in the United States, the medal for Distinguished Federal Civilian Service, from President John F. Kennedy, for her diligence in protecting the country. In part as a response to the thalidomide catastrophe, many countries began developing regulatory requirements for evaluating the effects of drugs during pregnancy using laboratory animals (Stirling et al., 1997). In the United States, the Segment I, II, and III testing protocols were promulgated by the FDA in 1966 (Kelsey, 1988). Current regulatory testing guidelines for developmental toxicity are discussed later in this chapter.

Thalidomide presents a variable pattern of developmental toxicity in at least 19 species in which it has been tested. Increased embryonic loss has been observed in some studies in rats, while generally no effects were reported in studies with most mouse strains. Effects more or less similar to those observed in humans have been reported for several rabbit strains and in eight of nine nonhuman primate species tested. The teratogenic dosage ranges from about 1 to 100 mg/kg, with humans being most sensitive (Schardein, 1993). Research to elucidate species and strain differences in response to thalidomide has met with limited success. Stephens (1988) summarized 24 putative mechanisms including biochemical alterations involving vitamin B, glutamic acid, acylation, nucleic acids, and oxidative phosphorylation; cellular mechanisms including cell death and cell–cell interactions; and inhibition of nerve and blood vessel outgrowth. More recent hypotheses include effects on angiogenesis (D'Amato et al., 1994; Joussen et al., 1999; Sauer et al., 2000; Stephens et al., 2000; Therapontos et al., 2009), integrins (Neubert et al., 1996), oxidative DNA damage (Parman et al., 1999), TNF-α inhibition (Argiles et al., 1998), growth factor antagonism (Stephens et al., 1998; Stephens and Fillmore, 2000), effects on redox status (Hansen et al., 1999), and inhibition of ubiquitin-mediated protein degradation (Ito et al., 2011; Ito and Handa, 2012).

Wells and colleagues (Parman et al., 1999) compared the effects of thalidomide in the pregnant mouse (a resistant species) and the pregnant rabbit (a sensitive species) and demonstrated increased DNA oxidation in rabbit, but not in mouse embryos. Pretreatment of pregnant rabbits with alpha-phenyl-*N*-*t*-butylnitrone, a free-radical scavenging agent, lowered thalidomide-induced embryonal DNA oxidation by 73%, and abolished almost all of the teratogenic effects. These results suggest that, in the sensitive rabbit, thalidomide is activated to a free-radical intermediate that is key to its teratogenicity. Hansen et al. (2002, 2004) have provided evidence that limb defects induced by thalidomide are due to altered expression of genes critical for limb outgrowth. They have demonstrated a shift in redox potential that reduces binding of a key transcription factor, NF-κB, to its response element in DNA. Binding of NF-κB is required for the normal expression of the genes *twist* and *FGF-10* in the mesenchyme and *FGF-8* in the apical ectodermal ridge of the developing limb. Inhibition of the expression of these genes by thalidomide could be predicted to cause the limb defects seen in sensitive species. Differences in redox regulation and consequent effects on redox-sensitive NF-κB may underlie species differences in teratogenicity of thalidomide.

Thalidomide may see expanded use in diseases including dermatological disorders, arthritis, multiple myeloma, diabetic retinopathy, and macular degeneration (Adler, 1994; Calabrese and Fleischer, 2000; Schwab and Jagannath, 2006; Chen et al., 2010).

Thalidomide has been approved by the FDA for inflammatory complications of Hansen's disease (leprosy) and multiple myeloma. Sadly, new cases of thalidomide embryopathy have occurred in Brazil and elsewhere consequent to its use there for leprosy and sharing of the drug by those unaware of its hazard (Vianna et al., 2013). To prevent exposure during pregnancy, the drug is only available under the Thalidomid Risk Evaluation and Mitigation Strategy (REMS), which requires certification of prescribers, patient counseling, provision of contraception, pregnancy testing, and pharmacy certification (www.thalidomidrems.com/about.html).

Ethanol/Alcoholism

The fetal alcohol syndrome (FAS) was first described by Jones and Smith in the early 1970s (Jones and Smith, 1973; Jones et al., 1973). There has since been a clear recognition and acceptance of alcohol's developmental toxicity, documented in hundreds of clinical, epidemiologic, and experimental studies of ethanol exposure during development, revealing a still broadening array of effects of alcohol on development (for reviews, see Jones [2011], Hoyme et al. [2016], Lunde et al. [2016], and McQuire et al. [2016]).

The FAS includes craniofacial dysmorphology, growth retardation, and psychomotor and intellectual deficiencies (Abel, 1982, 2006; Jones, 2011). The IQ of FAS children has been reported to average 68 (Streissguth et al., 1991a, 1991b). The molecular mechanisms underlying FAS are still poorly understood, and the possible molecular and cellular targets are many (e.g., Sulik, 2005; Smith 2010; Zeisel, 2011). Most recently, epigenetic effects of alcohol exposure during prenatal development have been reported, including effects on DNA methylation (Laufer et al., 2017) and histone modifications (Chater-Diehl et al., 2017).

Full-blown FAS has been observed primarily in children born to alcoholic mothers, and among alcoholics the incidence of FAS has been estimated at 25 per 1000 pregnancies (Abel, 1984). Estimates of the level of maternal ethanol consumption associated with FAS are a minimum of 3 to 4 oz of alcohol per day (Clarren et al., 1987; Ernhart et al., 1987). However, FAS represents the extreme end of a wide range of effects of developmental alcohol exposure now termed fetal alcohol spectrum disorders (FASD) (Koren et al., 2003; Sokol et al., 2003; Manning and Hoyme, 2007; Hellemans et al., 2010). After adjusting for smoking, gestational age, maternal height, age, parity, and sex of the child, Little (1977) found, in 800 women studied prospectively, that each ounce of ethanol consumed per day during late pregnancy corresponded to a 160-g decrease in birth weight. Streissguth et al. (1994a, 1994b), in a longitudinal prospective study of 462 children, found that the number of drinks during pregnancy was the strongest predictor of adverse effects on offspring attention, short-term memory, and performance on standardized tests. Streissguth and co-workers have also reported that prenatal alcohol exposure was associated with alcohol drinking problems at age twenty-one (Baer et al., 2003). Later life vulnerability to stress, depression, and anxiety disorders after in utero alcohol exposure has been reviewed (Hellemans et al., 2010). For reviews of the neurotoxic effects of developmental ethanol exposure, see a special issue of *Neuropsychology Review* (21(2): June 2011).

Mouse models of the FAS have been developed over the years, beginning with a model in which female mice were fed a liquid diet containing 15% to 35% ethanol-derived calories prior to and during pregnancy (Chernoff, 1977). Injection of ethanol to pregnant C57Bl/6J mice during the time that embryos are undergoing *gastrulation* (Sulik et al., 1981; Sulik and Johnston, 1983) has been used to study the etiology and pathogenesis of the craniofacial effects seen in FAS. Fetuses of mice exposed to ethanol in this manner exhibit features of FAS including microcephaly, microphthalmia, short palpebral fissures, deficiencies of the philtral region, and a long upper lip. Increased apoptosis in sensitive cell populations is a common finding in embryos of treated dams (Kotch and Sulik, 1992; Sulik, 2005; Smith, 2010).

Diethylstilbestrol

A synthetic nonsteroidal estrogen, diethylstilbestrol (DES), was used in the United States from the 1940s to the 1970s to prevent miscarriage. Seven young women between the ages of 15 and 22 were seen at Massachusetts General Hospital with clear cell adenocarcinoma of the vagina between 1966 and 1969. This type of cancer had not previously been seen in patients younger than 30. A case–control study found an association with DES exposure during the first trimester (reviewed in Poskanzer and Herbst, 1977). In 1971, the Registry of Clear Cell Adenocarcinoma of the Genital Tract of Young Females was established to track affected offspring. The appearance of genital tract tumors peaked at age 19 and declined through age 22, with risk of clear cell adenocarcinoma of the vagina and cervix of 0.14 to 1.4 per 1000 exposed pregnancies (Herbst et al., 1977). Further, noncancerous alterations in the vagina, cervix, and uterus were estimated as high as 75% (Poskanzer and Herbst, 1977). Effects on the developing female reproductive tract may be due to effects on genetic pathways governing uterine differentiation (Huang et al., 2005). Male offspring were not spared the effects of DES, as epididymal cysts, hypotrophic testes, capsular induration, low ejaculated semen volume, and poor semen quality have been reported (Bibbo et al., 1977). The latent and devastating manifestations of prenatal DES exposure have broadened our concept of the scope of potential adverse outcomes of intrauterine exposures. Yet, recent studies suggest even farther-reaching effects; Newbold et al. (2006) reported that increased susceptibility to tumors and reproductive tract abnormalities from DES exposure may be passed on to future generations of exposed pregnant mice, and Al Jishi and Sergi (2017) discuss the evidence for transgenerational effects of DES in humans.

Tobacco Smoke

Tobacco smoke and its constituents may represent the leading cause of environmentally induced developmental disease and morbidity today (Rogers, 2008) (Fig. 10-1). Despite public health programs aimed at smoking cessation, approximately one-quarter of women smokers in the United States continue to smoke during pregnancy. A large body of epidemiology provides a well-characterized picture of the adverse consequences of developmental tobacco smoke exposure, including spontaneous abortion, perinatal death, sudden infant death syndrome (SIDS), disorders of learning, behavior and attention, and lower birth weight (Fried et al., 1998; Slotkin, 1998; Tuthill et al., 1999; Haug et al., 2000; Markussen Linnet et al., 2006; Rogers, 2008). Offspring of smoking mothers have a higher risk of obesity, hypertension, and type 2 diabetes than offspring of nonsmokers (Oken et al., 2008; Suzuki et al., 2009; Bakker and Jaddoe, 2011; Cupul-Uicab et al., 2012; Riedel et al., 2014).

Mitchell and Milerad (2006) reviewed the literature on smoking and SIDS and reported a relative risk of SIDS associated with maternal smoking of 2.86 (95% confidence interval 2.77 to 2.95) before prone-sleep-position intervention (i.e., putting infants on their backs for sleep reduces the overall risk of SIDS) and 3.93 (95% confidence interval 3.78 to 4.08) after intervention programs to reduce this nonsmoking cause. These authors estimated that about one-third of SIDS deaths might have been prevented had no fetuses been exposed in utero to maternal smoking.

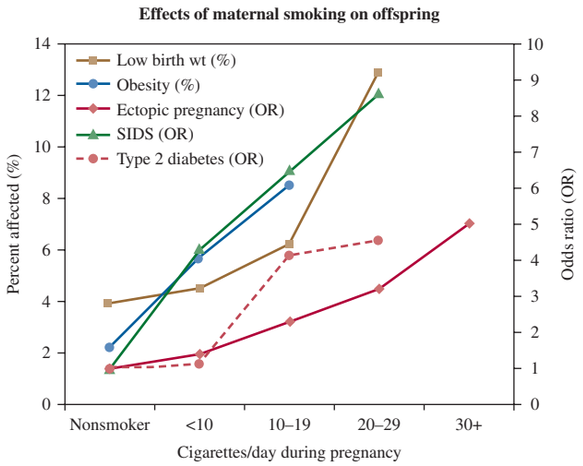

Figure 10-1. *Effects of maternal smoking on pregnancy and offspring.* These are effects for which dose–response relationships with maternal smoking have been established, but do not represent all of the effects of maternal smoking during pregnancy (see Rogers [2008]). (Plotted data are for preterm birth [spontaneous delivery at 33 to 36 weeks gestation] from Kyrklund-Blomberg and Cnattingius (1998); small for gestational age [SGA, less than 10th percentile for gestational week] from Windham et al. (2000); stillbirth from Wisborg et al. (2001); ectopic pregnancy from Handler et al. (1989); sudden infant death syndrome (SIDS) from Fleming and Blair (2007); offspring type 2 diabetes from Montgomery and Ekbom (2002); and offspring obesity [BMI > 97 percentile] from Von Kries et al., (2002).

Hackshaw et al. (2011) performed a review and meta-analysis of observational studies reporting odds ratios for nonchromosomal birth defects in offspring of women who smoked versus nonsmokers. There were 172 published articles comprising 173,687 malformed cases and 11.7 million controls. Significant associations with maternal smoking were found for these defects: cardiovascular, musculoskeletal, limb reduction, missing/extra digits, clubfoot, craniosynostosis, facial, eye, orofacial clefts, gastrointestinal, gastroschisis, anal atresia, hernia, and undescended testes. Lammer et al. (2005) found that maternal smoking was a risk factor for orofacial clefts, and that risk increased with genotypes indicating lack of enzymes involved in detoxification of smoke-derived chemicals. Gene–environment interactions may underlie the increased risk of other birth defects with maternal smoking (Shi et al., 2008). In a genome-wide association study, two genes, TBK1 on chromosome 12 and ZNF236 on chromosome 18, showed multiple single nucleotide polymorphisms associated with higher risk of nonsyndromic cleft palate in the presence of maternal smoking (Beaty et al., 2011). Perinatal exposure to tobacco smoke can also affect branching morphogenesis and maturation of the lung, leading to altered lung function (Gilliland et al., 2000; Pinkerton and Joad, 2000; Maritz and Harding, 2011). Nicotine, a primary component of tobacco smoke, is a known neuroteratogen in experimental animals and can by itself produce many of the adverse developmental effects of tobacco smoke, including impaired fertility, neurobehavioral effects, respiratory dysfunction, obesity, hypertension, and type 2 diabetes (Slotkin, 1998; Bruin et al., 2010).

The 2014 Surgeon General's report on the health consequences of smoking (U.S. DHHS, 2014) determined that there was sufficient evidence to infer a causal relationship between maternal smoking in early pregnancy and orofacial clefts, and between maternal smoking and ectopic pregnancy. In the same report, evidence was deemed suggestive but not sufficient to infer a causal relationship between maternal smoking and clubfoot, gastroschisis, atrial septal defects, spontaneous abortion, and disruptive behavioral disorders, particularly ADHD, in children.

Exposure to secondhand tobacco smoke (smoke from the lit end of cigarettes and that exhaled by smokers) also represents a significant risk to the pregnant nonsmoker and her baby, and such exposure has been associated with many of the effects caused by active maternal smoking (Windham et al., 2000; George et al., 2006; Mitchell and Milerad, 2006). Leonardi-Bee et al. (2008) found that, across many studies, exposure of nonsmoking pregnant women to secondhand smoke was associated with reduction of birthweight by 33 g or more, as well as a 22% increased risk of being of low birth weight (less than 2500 g). In another review and meta-analysis (Leonardi-Bee et al., 2011), nonsmoking pregnant women exposed to secondhand tobacco smoke had a 23% higher risk of stillbirth and a 13% higher risk of having a child with a congenital malformation.

Effects of maternal smoking on the epigenome of the developing offspring have been studied in multiple cohorts in Europe and the United States. Maternal smoking has been associated with changes in DNA methylation in cord blood and other offspring cells. DNA is methylated primarily at cytosines (to form 5-methyl cytosine) that are adjacent to a guanine in the DNA sequence (denoted as CpG). In 1062 cord blood samples from the Norwegian Mother and Child Cohort Study (MoBa), Joubert et al. (2012) discovered differential methylation for 26 CpGs (using Bonferroni correction) mapped to 10 genes. A high level of replication was achieved when a similar analysis was carried out on cord blood samples from the Newborn Epigenetics Study (NEST) cohort in Durham, North Carolina. The same 26 CpG methylation changes were found in both studies (Fig. 10-2). In a recent meta-analysis of results across 13 cohorts in the Pregnancy and Childhood Epigenetics (PACE) consortium, Joubert et al. (2016) found 568 CpG methylation changes (again, by using Bonferroni correction) between newborn blood of smoking versus nonsmoking mothers. The total number of significant CpG methylation changes was 6073 using a false discovery rate (FDR) correction of 5%. These CpGs included some genes related to normal and abnormal prenatal development as well as metabolism of xenobiotics. One of the genes most consistently affected by CpG methylation changes in these and other studies is the aryl-hydrocarbon receptor repressor (*AHRR*) gene that participates in AhR signaling involved in dioxin toxicity as well as cell growth and differentiation. This CpG in *AHRR* is currently considered a biomarker of smoking (Lee et al., 2017).

Retinoids

Excess vitamin A (retinol) has been known to be teratogenic in the rat for at least 60 years (Cohlan, 1954). Developing structures affected include the face, limbs, heart, central nervous system, and skeleton. Retinoic acid administration to the pregnant mouse (Kochhar, 1967) or hamster (Shenefelt, 1972) produces the same malformations. Understanding of the developmental effects of retinol, retinoic acid, and structurally related chemicals that bind to and activate specific nuclear receptors expanded rapidly in the 1990s (Chambon, 1994; Lohnes et al., 1994; Mendelsohn et al., 1994; Collins and Mao, 1999; Arafa et al., 2000). The teratogenic effects of vitamin A and retinoids have been reviewed (Nau et al., 1994; Collins and Mao, 1999).

A synthetic retinoid, 13-*cis*-retinoic acid (isotretinoin, Accutane), was marketed for the treatment of cystic acne beginning in 1982. Despite clear labeling of the drug at that time as FDA pregnancy category X (proven fetal risks clearly outweigh any benefit),

extensive physician and patient education, and strict requirements for prescription to women of childbearing potential, exposures of pregnant women still occurred. Infants with characteristic malformations involving the ears, heart, brain, and thymus were reported as early as 1983 (Rosa, 1983; Lammer et al., 1985). Dai et al. (1992) reported that among 115 exposed pregnancies not electively terminated, 18% ended in spontaneous abortion and 28% of live-born infants had at least one major malformation. Lammer (1992) found in another prospective study that there was nearly a doubling of the risk for premature delivery after first-trimester exposure, and about 50% of the exposed children had full-scale IQ scores below 85 at age 5. Almost 25% of women with an isotretinoin-exposed pregnancy did not recall having contraception counseling before starting their medication (Robertson et al, 2005). Rates of recommended birth control and monthly pregnancy testing were low. The Accutane brand was removed from the U.S. market in 2009, but generic isotretinoin continues to be marketed under other brand names worldwide. Adams (2010) has reviewed the neurobehavioral teratology of retinoids.

There has been concern about the use of topical retinoids during pregnancy. A recent meta-analysis ruled out a major increase in adverse pregnancy outcomes associated with use of topical retinoids, but the authors concluded that the statistical power of the analysis was inadequate to justify the use of topical retinoids during pregnancy (Kaplan et al., 2015).

Antiepileptic Drugs

Women of childbearing age that require treatment for epilepsy number over 1 million, while most current antiepileptic drugs (AEDs) have been shown to cause birth defects, cognitive impairment, and fetal death. As a class, AEDs including phenytoin, carbamazepine, and valproic acid are considered human teratogens. More recently, topiramate, used for the treatment of epilepsy as well as migraine and appetite suppression, has emerged as a teratogen strongly associated with oral clefts (Alsaad et al., 2015). Other AEDs including gabapentin, lamotrigine, oxcarbazone, levetiracetam, and zonizamide have now been used for over a decade, and studies to date suggest that these may be safer alternatives to the older AEDs or topiramate. The interested reader is directed to recent reviews (Tomson and Battino, 2005; Ornoy, 2006; Tatum, 2006; Hansen 2010; Voinescu and Pennell, 2015).

Phenytoin (diphenylhydantoin) has been used as an AED for over 70 years. Its antiepileptic effect works via inhibition of voltage-gated sodium channels. It is metabolized by the cytochrome P450 system to a reactive intermediate and then to an active metabolite. First described by Hanson and Smith (1975), the fetal hydantoin syndrome comprises craniofacial abnormalities, limb defects, growth deficiency, and mental retardation. This syndrome has been reported to occur in 5% to 10% of exposed offspring (Schardein, 2000). Putative mechanisms underlying the teratogenicity of phenytoin include folic acid deficiency and oxidative damage of tissue macromolecules (Hansen, 2010).

A report from the FDA (Rosa, 1991) linked carbamazepine use during pregnancy to neural tube defects. A later meta-analysis of 16 studies calculated a 2.89-fold increased risk of a major birth defect in offspring of pregnant women treated with carbamazepine (Matalon et al., 2002). Carbamazepine has been reported to be teratogenic in rats and mice (Schardein, 2000). An epoxide metabolite of carbamazepine was found to be teratogenic in mice in vivo (Bennett et al., 1996) but not in mouse or rat embryos cultured in vitro (Hansen et al., 1996). Thus, the teratogenicity observed in mice in vivo may have been related to maternal toxicity or to a different metabolite.

Elizabeth Robert (Robert and Guibaud, 1982) reported that of 146 cases of spina bifida aperta recorded in Lyon, France, 9 of the mothers had taken valproic acid (2-propylpentanoic acid) during the first trimester of pregnancy. The odds ratio in a case–control study was 20.6, and the estimated risk of a valproate-exposed woman having a child with spina bifida was 1.2%, similar to that for women with a previous child with a neural tube defect (CDC, 1982). The report was confirmed in other parts of the world (CDC, 1983). Several factors facilitated identification of valproate as a human teratogen, including an active birth defects registry, an

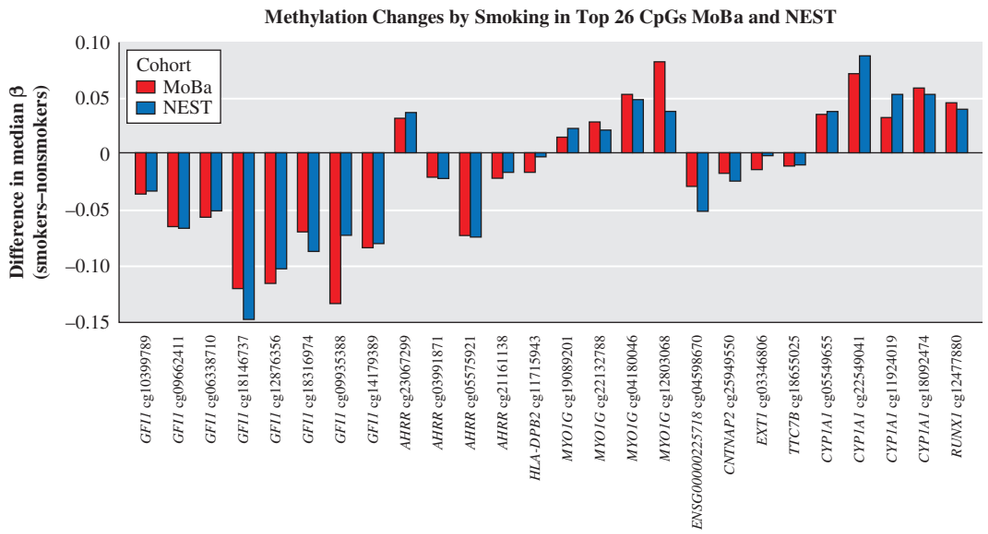

Figure 10-2. *Effects of maternal smoking on the epigenome of the newborn.* Association between maternal cotinine level and methylation of the top 26 affected CpGs (from 10 genes) in cord blood from the Norwegian mother and child (MoBa, red bars) study and the Newborn epigenetics study (NEST, blue bars). (From Joubert et al. [2012].)

interest by Robert in spina bifida, a question on anticonvulsant use in Robert's survey, and the prevalence of valproate monotherapy for epilepsy in the Lyon area (Lammer et al., 1987). Despite a great deal of research on the effects of valproate in multiple species, the mechanism of action remains elusive (Nau et al., 1991; Ehlers et al., 1992; Hauck and Nau, 1992). Use of inbred mouse strains differing in their sensitivity to valproate-induced teratogenesis has revealed several genes conferring sensitivity (Finnell et al., 1997, 2000; Bennett et al., 2000; Craig et al., 2000). Valproic acid is also a histone deacetylase inhibitor, and this may play a role in its teratogenicity (Eikel et al., 2006; Menegola et al., 2006). Similar to phenytoin, some studies have indicated a possible role of interference with folate metabolism (Hansen, 2010).

Epidemiology of Human Developmental Toxicity

Reproductive epidemiology is the study of associations between specific exposures of the father or pregnant woman and her conceptus and the outcome of pregnancy. In rare situations, such as rubella, thalidomide, isotretinoin, and Zika, where a relatively high risk exists and the outcome is a rare event, formal studies may not be needed to identify causes of abnormal birth outcomes, which are often detected by alert clinicians. The likelihood of linking a particular exposure with a series of case reports increases with the rarity of the defect, the rarity of the exposure in the population, a small source population, a short time span for study, and biological plausibility for the association (Khoury et al., 1991). In other situations, such as those with ethanol and valproic acid, associations are sought through either a case–control or a cohort approach. Both approaches require accurate ascertainment of abnormal outcomes and exposures, and a large enough effect and study population to detect an elevated risk. Therein lies one of the difficulties for epidemiologists studying abnormal reproductive outcomes. For example, it has been estimated that the monitoring of more than 1 million births would have been necessary to detect a statistically significant increase in the frequency of spina bifida following the introduction of valproic acid in the United States, where the frequency of exposure was less than 1 in 1000 pregnancies and the risk was only a doubling over the background incidence (Khoury and Holtzman, 1987). Another challenge to epidemiologists is the high percentage of human pregnancy wastage, perhaps as much as 31% in the peri-implantation period (Wilcox et al., 1988) and an additional 15% that are clinically recognized. Therefore, pregnancy failures related to a particular exposure may go undetected in the general population. Furthermore, with the availability of prenatal diagnostic procedures, some pregnancies with malformed embryos (particularly neural tube defects) are electively aborted. Thus, the incidence of abnormal outcomes at birth may not reflect the true rate of abnormalities, and the term prevalence, rather than incidence, is preferred when the denominator is the number of live births rather than total pregnancies.

Epidemiologic studies of abnormal reproductive outcomes are usually undertaken with three objectives in mind: the first is scientific research into the causes of abnormal birth outcomes and usually involves analysis of case reports or clusters; a second aim is prevention and is targeted at broader surveillance of trends by birth defect registries around the world, and the last objective is informing the public and providing assurance. In this regard, it is informative to consider the review by Schardein (1993) of the method and year by which human teratogens were detected. For 23 of 28 chemicals reviewed (including 9 cancer therapeutics, androgenic hormones, antithyroid drugs, aminoglycoside antibiotics, coumarin anticoagulants, DES, methylmercury, hydantoins, primidone, penicillamine, lithium, vitamin A, and retinoic acid), case reports presented the first evidence in humans. For two of these (DES and lithium), the case reports were soon followed by registries that provided confirmation, while for two others (methylmercury and hydantoins) follow-up epidemiology studies added support. For only four chemicals, alcohol, PCBs, carbamazepine, and cocaine, did an analytical epidemiological study provide the first human evidence. Evidence for one chemical, valproic acid, was first obtained by analysis of a birth defect registry. For the 28 chemicals in that review, human evidence of developmental toxicity preceded published animal evidence in 11 instances. Cohort studies, with their prospective exposure assessment and ability to monitor both adverse and beneficial outcomes, may be the most methodologically robust approach to identifying human developmental toxicants. The lack of cohort studies demonstrating risk for pregnancy may be in part due to the difficulty in making such associations, but may also reflect the fact that use in pregnancy is not associated with increased risk for the majority of drugs (Irl and Hasford, 2000).

Assessment of the developmental toxicity of tobacco smoke has benefitted greatly from the many epidemiological studies that have been carried out. Reasons for this success include the prevalence of smoking and secondhand smoke exposure (allowing large studies), good recall of exposure and biomarkers of exposure (e.g., the nicotine metabolite, cotinine), and comprehensive assessment of effects in offspring. The large number of studies has allowed powerful meta-analyses of the relationship between maternal smoking and birth defects (e.g., Hackshaw et al., 2011).

PRINCIPLES OF DEVELOPMENTAL TOXICOLOGY

James Wilson established principles of teratology in 1959 (Wilson, 1959) and updated them in his watershed monograph *Environment and Birth Defects* (Wilson, 1973) (Table 10-3). Despite significant advances in our knowledge of and approaches to the study of teratology, Wilson's principles have stood the test of time and remain basic to developmental toxicology. The following sections will cover these principles in some depth.

Table 10-3

Wilson's General Principles of Teratology

I. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors.
II. Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.
III. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis).
IV. The access of adverse influences to developing tissues depends on the nature of the influence (agent).
V. The four manifestations of deviant development are death, malformation, growth retardation, and functional deficit.
VI. Manifestations of deviant development increase in frequency and degree as dosage increases, from the no effect to the totally lethal level.

SOURCES: *From Wilson (1959, 1973).*

Critical Periods of Development and Susceptibility to Toxicity

Development is characterized by rapid changes in size, shape, biochemistry, physiology, and functionality. These changes are the consequence of sequential patterns of gene expression under the control of a cascade of epigenetic factors, the first of which are present in the egg prior to fertilization. These maternal factors are active in the zygote and very early embryo and then activate the embryonic genome, and sequential gene activation continues throughout development.

The embryo/fetus as a target for toxicity is continually changing as development proceeds. The concept that there are critical periods of sensitivity based on developmental stage at the time of insult is a somewhat unique consideration in developmental toxicology. While selected developmental stages are discussed here in the context of their known and potential susceptibility to toxicants, in reality development is a continuum. Comparative timing of developmental events in humans and some common laboratory species is presented in Table 10-4.

Gametogenesis is the process culminating in formation of the haploid germ cells or gametes, the egg, and sperm. Male and female gametes fuse during fertilization to form the diploid *zygote*, or one-celled embryo. The maternal and paternal genomes are not functionally equivalent in their contributions to the genome of the zygote, because the process of *imprinting* during gametogenesis confers on some genes a differential expressivity based on whether they are of maternal or paternal origin (Latham, 1999; Li and Sasaki, 2011). Imprinting involves cytosine methylation and changes in chromatin conformation, and may be susceptible to toxicants that affect these targets (Murphy and Jirtle, 2000). Both early nutrition (Waterland and Jirtle, 2003, 2004) and the estrogenic compound genistein (Dolinoy et al., 2006) have been shown to modify the methylation patterns of specific genes and cause altered phenotypes in mice. Anway et al. (2006) demonstrated a transgenerational effect of the fungicide vinclozolin in rats, in which male offspring (F1) of treated F0 females had reduced spermatogenic capacity which was transmitted to males in subsequent generations (F2 to F4) via an epigenetic mechanism. Transgenerational effects of environmental agents have been reviewed (Skinner, 2014).

Epigenetics refers to the biochemical changes that control chromatin conformation and gene expression. Epigenetic factors include DNA methylation, histone modifications, and expression of noncoding RNAs. While epigenetics provides the basis for imprinting of genes, the role of epigenetics is much broader (Rogers et al., 2010; Grace et al., 2011). Epigenetic marks may be erased and reestablished primarily during two periods of development during which broad scale demethylations of the genome are known to occur (Sasaki and Matsui, 2008). One is during migration and proliferation of the primordial germ cells (e.g., embryonic days 10.5 to 12.5 in the mouse). During this stage, imprinted genes are demethylated, followed by gender-specific remethylation during gametogenesis in offspring. The other period of widespread epigenetic reprogramming occurs after formation of the zygote and in the early embryo, with total genomic methylation being lowest at the early blastocyst stage (Hales et al., 2011). Epigenetic reprogramming represents a plausible target for environmental insults; examples of environmentally mediated epigenetic changes are emerging (Rogers et al., 2010; Perera and Herbstman, 2011; Skinner et al., 2011).

The *preimplantation* period comprises mainly an increase in cell number through a rapid series of cell divisions (*cleavage* of the zygote) with little growth in size. A stage termed the *blastocyst* follows, consisting of about a thousand cells surrounding a fluid-filled central cavity. The blastocyst may contain as few as three cells

Table 10-4

Timing of Key Developmental Events in Some Mammalian Species*

	RAT	RABBIT	MONKEY	HUMAN
Blastocyst formation	3–5	2.6–6	4–9	4–6
Implantation	5–6	6	9	6–7
Organogenesis	6–17	6–18	20–45	21–56
Primitive streak	9	6.5	18–20	16–18
Neural plate	9.5	—	19–21	18–20
First somite	10	—	—	20–21
First branchial arch	10	—	—	20
First heartbeat	10.2	—	—	22
10 Somites	10–11	9	23–24	25–26
Upper limb buds	10.5	10.5	25–26	29–30
Lower limb buds	11.2	11	26–27	31–32
Testes differentiation	14.5	20	—	43
Heart septation	15.5	—	—	46–47
Palate closure	16–17	19–20	45–47	56–58
Urethral groove closed in male	—	—	—	90
Length of gestation	21–22	31–34	166	267

**Developmental ages are days of gestation.*
SOURCE: Adapted from Shepard and Lemire (2004).

destined to give rise to the embryo proper (Markert and Petters, 1978), and these cells are within a region called the *inner cell mass.* The remainder of the blastocyst cells gives rise to membranes, trophoblast, and placenta. The fates of cells in the early embryo are not rigidly determined, and the preimplantation embryo has great restorative (regulative) growth potential (Snow and Tam, 1979). For example, Moore et al. (1968) demonstrated that individual cells from eight-celled rabbit embryos were each capable of producing normal offspring.

Toxicity during the preimplantation period is generally thought to result in either no or slight effect on growth (because of regulative growth recovery) or in death (through insurmountable damage or failure to implant). Preimplantation exposure to DDT, nicotine, or methyl-methane sulfonate results in body and/or brain weight deficits and embryo lethality, but not malformations (Fabro, 1973; Fabro et al., 1984). There are a few examples of preimplantation exposures leading to malformations. Exposure of mouse zygotes and blastocysts to the mutagens ethylene oxide (Generoso et al., 1987), ethylmethane sulfonate, ethylnitrosourea, or triethylene melamine (Generoso et al., 1988) for approximately 6 hours following fertilization resulted in malformed fetuses. Epigenetic changes were not examined, but given the developmental stages at the time of the exposure, this is a plausible target. Treatment of pregnant mice with methylnitrosourea on days 2.5, 3.5, and 4.5 of gestation resulted in neural tube defects and cleft palate (Takeuchi, 1984). Cyproterone acetate and medroxyprogesterone acetate are capable of producing malformations when administered on day 2 of gestation (Eibs et al., 1982).

Gastrulation is the process by which the trilaminar embryo is formed. *Ectoderm, mesoderm*, and *endoderm* are the resultant three germ layers. During gastrulation, cells migrate through a midline groove called the *primitive streak*, and this cell migration sets up morphogenetic fields in the embryo (Smith et al., 1994). Gastrulation is highly susceptible to teratogenesis. It is common for teratogens administered during gastrulation to produce anterior malformations, including those of the eye, brain, and face, indicative of damage to the anterior *neural plate*, one of the regions defined by the cellular migrations during gastrulation.

The formation of the neural plate in the midline ectoderm marks the onset of *organogenesis*, which as the name implies involves morphogenetic movements defining the rudiments of most structures of the body. This period extends from about the third to the eighth weeks of gestation in humans and is highly susceptible to teratogenesis. The embryo undergoes dramatic changes during this brief period, proceeding in the human from a few cell types in a trilaminar arrangement indistinguishable from most other vertebrate embryos, to a fetus clearly recognizable as human. The rapid changes during organogenesis require cell proliferation and migration, cell–cell interactions, and morphogenetic tissue remodeling. The *neural crest* cells originate at the border of the neural plate and migrate to form diverse structures throughout the embryo. Neural crest cells originating in the hindbrain migrate to form bone and connective tissues in the head (Krumlauf, 1993; Vaglia and Hall, 1999; Trainor and Krumlauf, 2001), while other neural crest cells form structures including melanocytes, enteric ganglia, adrenal medulla, and components of the cardiac outflow tract.

Within organogenesis, there are periods of peak susceptibility for each forming structure. The peak vulnerability to each malformation coincides with the timing of key developmental events in the affected structure. In general, morphogenesis proceeds from anterior to posterior in the embryo. The developmental fields for the brain and eyes are established early, and microphthalmia (small eyes) has an early critical period. Rudiments of the long bones of the limbs are established later, as is susceptibility to phocomelia (shortened limbs). There may be multiple windows of susceptibility to teratogenesis for a given structure, and the developing palate is a good illustration. The palate has multiple peaks of susceptibility during which cleft palate can be induced, one corresponding to the early establishment of the palatal folds and others corresponding to growth, elevation, and fusion of the palatal shelves. Cleft palate can be induced in mouse fetuses by maternal exposure to methanol as early as day 5 of gestation, with a peak sensitivity at day 7 and little or no sensitivity after day 9 (Rogers and Mole, 1997). In contrast, the typical peak critical period for induction of cleft palate in the mouse is between gestation days 11 and 13, during the stages of growth, elevation, and fusion of the palatal shelves. In NMRI mice, peak sensitivity to the induction of cleft palate occurred on day 11 for TCDD, day 12 for 2,4,5-trichlorophenoxyacetic acid, and day 13 for dexamethasone (Neubert et al., 1973). Detection of unexpected sensitive periods such as the one for induction of cleft palate by methanol may provide clues to the discovery of normal developmental processes. Using precisely timed administration of teratogenic dosages of retinoic acid to hamsters, Shenefelt (1972) elegantly demonstrated different gestational ages and durations of sensitive periods for induction of different malformations (Fig. 10-3).

The *fetal period,* from about day 57 to birth in humans, comprises tissue differentiation, growth, and maturation. Most organs are present and grossly recognizable, with further development during the fetal period aimed at attaining functionality prior to birth. Differentiation of the reproductive tract is one of the latest organogenetic events, with closure of the urethral groove in the male occurring at about 90 days of gestation. Toxic exposure during the fetal period is most likely to result in effects on growth and function. Functional deficits may not be apparent prenatally and may require postnatal observation and testing. Reviews of postnatal functional deficits of the central nervous system (Rodier et al., 1994; Miodovnik, 2011), immune system (Holladay and Luster, 1994; Dietert, 2008, 2014), and heart, lung, and kidneys (Lau and Kavlock, 1994) are available. Major structural alterations can occur during the fetal period, usually due to *deformations*, disruptions of previously normal structures. For example, extremities may be affected by amniotic bands, wrapping of the umbilical cord, or vascular disruptions, leading to loss of distal structures. One of the newer concepts in developmental toxicology is the idea of "developmental programming," in which the developmental environment influences the metabolic parameters of the offspring in a way that can affect life-long risk of disease (McMillen and Robinson, 2005; Gluckman et al., 2011; Barouki et al., 2012). Maternal nutrition has been the focus of much of this work, and there is a paucity of data concerning the long-term effects of developmental chemical exposure. Recently, Rogers et al. (2014) demonstrated that exposure of pregnant rats to diverse chemicals can result in increased blood pressure in offspring. Rats exposed prenatally to high dosages of ethanol have life spans shortened by about 20 weeks in females and 2.5 to 7 weeks in males (Abel et al., 1987).

Dose–Response Patterns and Thresholds for Induction of Developmental Toxicity

The relationships between growth retardation, malformations, and mortality in developmental toxicity studies are complex. These endpoints may represent a continuum of toxicity, with growth retardation, malformations, and then death occurring with increasing dosage. Malformations or death can occur without growth retardation, but this is atypical, and growth retardation and mortality often

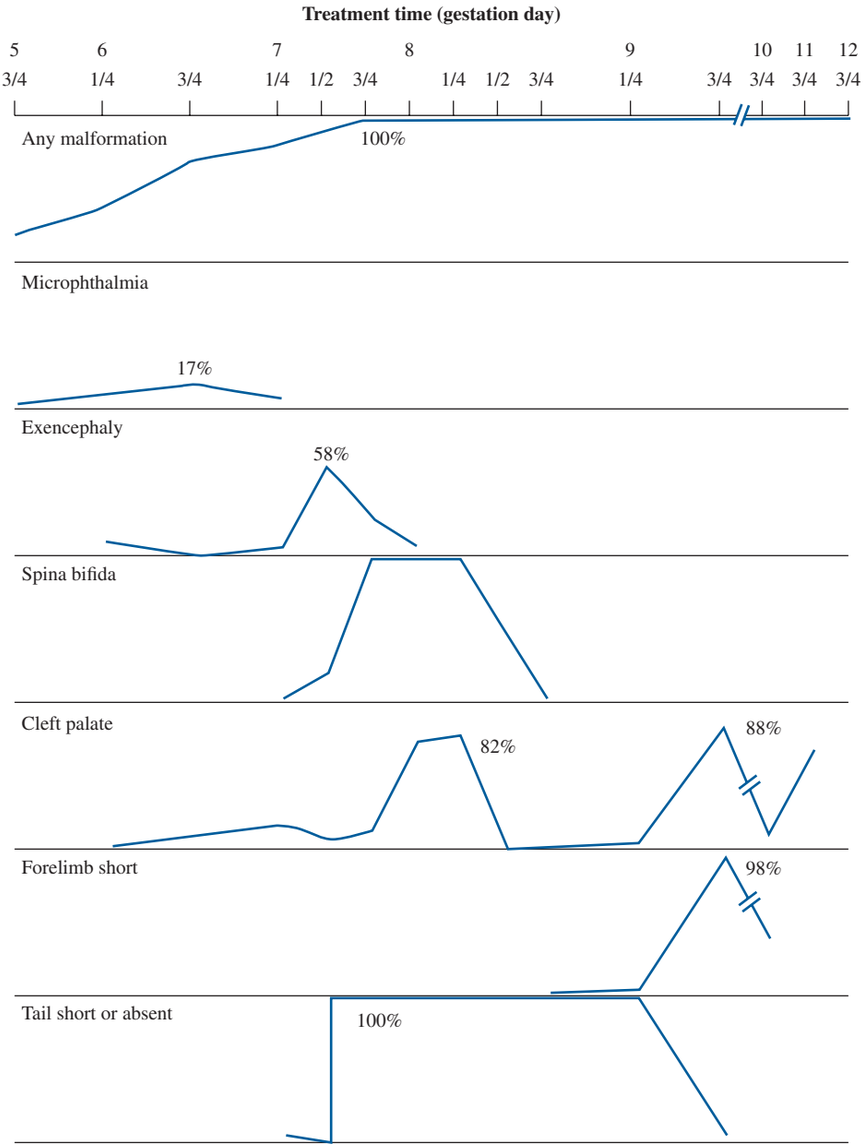

Figure 10-3. *Critical periods of sensitivity for induction of various defects by retinoic acid in the hamster.* Incidence of defects are estimates for the embryo/fetal LD_{50} maternal dosage. Note in the top panel that fewer malformations are induced on days 5 to 6, prior to organogenesis, indicating that, during this period, embryos for the most part either die or recover. Likelihood of malformation increases rapidly during gastrulation and reaches 100% during organogenesis. Peak incidence for each defect is enumerated and reflects timing of critical events in the development of each structure. (Redrawn from Shenefelt [1972].)

occur without malformations. Chemicals producing the latter pattern of response are considered embryotoxic or embryolethal but not teratogenic. Effects on functional endpoints in viable fetuses usually require postnatal assessment. Surviving birth is a rigorous test, and functional deficits may manifest as neonatal mortality.

The shape of the dose–response curve at low exposures is also important. The mammalian embryo possesses high restorative growth potential and cellular homeostatic mechanisms, which, along with maternal metabolic defenses, contribute to the conventional wisdom that mammalian developmental toxicity is a threshold phenomenon. In other words, it is assumed that there is a maternal dosage below which no increase in an adverse outcome will occur. Two experimental approaches to demonstrating a threshold were summarized by Daston (1993). One approach, exemplified by a large experiment with the herbicide 2,4,5-T (Nelson and Holson, 1978), suggests that no study is adequately powered to empirically evaluate the dose–response at very low effect levels—in this study, it was calculated that 805 litters per dose would be necessary to detect a 5% increase in resorption (early death) of embryos. The second approach is to demonstrate a threshold for the molecular mechanism underlying the observed effect. Few mechanisms of abnormal development have been thoroughly studied, but it is clear that embryonal repair capabilities and dose-dependent kinetics both support the plausibility of mechanistic thresholds. Lack of a threshold implies that exposure to any amount of a toxic chemical, even one molecule, can cause developmental toxicity. A point mutation in a critical gene could result from a single hit by a single molecule and, hypothetically, change or delete a critical gene product, leading

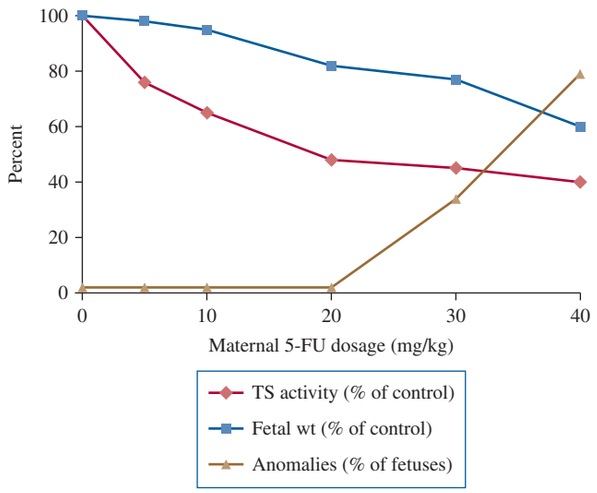

Figure 10-4. *Relationship between inhibition of embryonal thymidylate synthetase (TS) and adverse fetal outcome following maternal 5-fluorouracil (5-FU) administration on gestation day 14 in the rat.* 5-FU inhibits embryonal TS activity at dosages below 5 mg 5-FU/kg maternal body weight. Fetal weight is affected at ≥20 mg/kg and malformations increased significantly at ≥30 mg/kg. (Based on Shuey et al. [1994] and unpublished observations.)

to abnormal development. However, this would require that the molecule traverse the maternal system and the placenta and enter a critical cell in the embryo. Such an effect might be more likely at the zygote (one-cell) or the blastocyst stage (when only a few cells in the inner cell mass are embryo progenitors).

An apparent threshold for developmental toxicity based at least in part on cellular homeostatic mechanisms is suggested by studies on the teratogenicity of the cancer chemotherapeutic, 5-fluorouracil, in rats (Shuey et al., 1994). This chemical inhibits thymidylate synthetase (TS), thereby inhibiting DNA synthesis. TS inhibition is evident in the embryo at maternal dosages an order of magnitude lower than those required to produce malformations, and about fivefold below those affecting fetal growth (Fig. 10-4). The lack of developmental toxicity at dosages causing significant TS inhibition probably reflects the ability of the embryo to compensate for imbalances in cellular nucleotide pools.

For risk assessment, it is important to consider the distinction between individual and population thresholds. There is variability in sensitivity among humans, and the threshold for a population can be defined as the threshold for the most sensitive individual in the population (Gaylor et al., 1988). Although the biological target of a developmental toxicant may exhibit a threshold, background factors such as preexisting conditions or other exposures may leave individuals already at or even beyond the threshold for failure of that biological process. The concept of thresholds for reproductive toxicants has been reviewed by Piersma et al. (2011a).

MECHANISMS AND PATHOGENESIS

Mechanisms of teratogenesis include, but are not limited to, mutations, chromosomal breaks, altered mitosis, altered nucleic acid integrity or function, diminished supplies of precursors or substrates, decreased energy supplies, altered membrane characteristics, osmolar imbalance, and enzyme inhibition (Wilson, 1977). While not unique to development, such cellular insults may quickly trigger pathogenetic responses in the embryo. These pathogenetic events may include reduced cell proliferation, cell death, altered cell–cell interactions and signaling, reduced biosynthesis, inhibition of morphogenesis, or mechanical disruption of development.

A Case Study: The Teratogenicity of Cyclophosphamide

Experimentally elucidating mechanism(s) and pathogenesis of chemically induced teratogenicity can be daunting. The teratogenicity of the chemotherapeutic and immunosuppressive drug cyclophosphamide (CP) has been studied for over 50 years, and serves as a good example (Mirkes, 1985a; Ozolins, 2010). Because of its use for treating lupus, at least nine cases of human teratogenesis induced by exposure to CP during early pregnancy have been identified (Paladini et al., 2004). Experimental studies on the teratogenicity of CP illustrate approaches to understanding metabolic activation and elucidating teratogenic mechanisms and pathogenesis. Important to this work was the advent of mammalian whole embryo culture (WEC), wherein rodent or rabbit embryos are removed from the uterus at the beginning of organogenesis and grown in serum-containing media (New, 1978; Sadler and Warner, 1984; Ellis-Hutchings and Carney, 2010; Lee et al., 2012). Such embryos will grow normally in vitro for about 48 hours, completing much of organogenesis. WEC allows direct exposure, manipulation, and observation of embryos not possible in vivo; in contrast, it is devoid of maternal metabolism. Rodent WEC is also used as a screening system for developmental toxicity, which will be discussed later in the chapter.

Hepatic S9 fractions and cofactors were required for CP teratogenicity in WEC, demonstrating that it must be metabolically activated (Fantel et al., 1979; Sanyal et al. 1979). Activation was inhibited by metyrapone or carbon monoxide, indicating involvement of P450 monooxygenases. A stable derivative of the CP metabolite 4OHCP, 4-hydroperoxy-cyclophosphamide (4OOHCP), was tested in vivo (Hales, 1982) and in WEC (Mirkes, 1987). In WEC, the dysmorphology of the treated embryos was indistinguishable from that of embryos cultured with CP plus an activating system. Biotransformation of 4OHCP to phosphoramide mustard (PM) and acrolein (AC) occurs rapidly in vivo, and these metabolites, as well as 4-ketocyclophosphamide (4-ketoCP) and carboxyphosphamide (CaP), have all been studied for their teratogenicity. These experiments showed that 4OHCP was not teratogenic (Hales, 1983) and that the toxicity elicited by 4-ketoCP was unlike that of activated CP (Mirkes et al., 1981). Subsequent work on PM and AC demonstrated that the effects of PM on cultured rat embryos were the same as those of activated CP (Mirkes et al., 1981). In vivo, CP and AC administered to gestation day 13 rat embryos by intraamniotic injection caused hydrocephaly, open eyes, cleft palate, micrognathia, omphalocele, and tail and limb defects, while PM produced only tail and limb defects and hydrocephaly (Hales, 1982). These results strongly suggest that both PM and AC are teratogenic CP metabolites.

Experiments with tritiated CP revealed that 87% of bound radioactivity was in protein, 5% in DNA, and 8% in RNA (Mirkes, 1985b). CP and PM produced single-strand DNA breaks and DNA–DNA and DNA–protein cross-linking. To determine whether DNA cross-linking was required for teratogenicity, a monofunctional derivative of PM capable of producing single-strand breaks but not cross-links in DNA was tested; this derivative caused the same effects as PM (Mirkes et al., 1985). Subsequently, Little and Mirkes (1990) showed that 4-hydroperoxydechlorocyclophosphamide, a CP analog that breaks down to AC and a nonalkylating derivative of PM, did not damage DNA when embryos were exposed in WEC, but still retained its teratogenicity. They then showed that AC binds

to protein, whereas PM binds preferentially to DNA. Hales (1989) showed that PM and AC have distinctly different effects on limb buds growing in vitro. These results demonstrated different targets in the embryo for PM and AC, and that CP-induced DNA damage was caused by PM.

To illustrate pathogenesis, or how insults at the cell and molecular level translate to birth defects, consider cell cycle perturbations and cell death caused by CP. *Apoptosis* is a specific type of cell death under genetic control (Lavin and Watters, 1993); it is necessary for normal morphogenesis including sculpting the fingers and toes (Hernandez-Martinez and Covarrubias, 2011) and making appropriate functional connections between the central nervous system and distal structures. Cell proliferation is obviously a key facet of development—cells within the primitive streak of the gastrula-stage rat embryo have one of the shortest known cell cycle times of any mammalian cell, 3 to 3.5 hours (MacAuley et al., 1993). Cell proliferation rates vary spatially and temporally during development, as can be demonstrated by examining changes in the cell cycle distribution over time in different tissues during mid-to-late gestation (Fig. 10-5). Upsetting the balance between cell proliferation, cell differentiation, and apoptosis in the embryo via DNA damage may be key to the teratogenicity of CP.

CP caused an S-phase cell cycle block as well as widespread cell death in embryos of pregnant rats exposed on gestation day 10 (Fig. 10-6). Corroborating the S-phase cell cycle block, apoptosis was observed in areas of rapid cell proliferation (Chernoff et al., 1989; Francis et al., 1990). Similar embryonal cell cycle block and apoptosis were observed using activated CP in WEC (Little and Mirkes, 1992). The neuroepithelium of the embryo was sensitive to CP-induced apoptosis, while the heart was not. The neuroepithelium of the day 10 rat embryo has a cell cycle time of approximately 9.5 hours, compared to 13.4 hours in the heart. This difference is due to a longer G_0/G_1 phase in the heart cells compared to the neuroepithelium, and may contribute to the resistance of the heart to CP teratogenesis (Mirkes et al., 1989). Damage to DNA by PM occurs mainly during S-phase (Little and Mirkes, 1992), which is a relatively smaller proportion of the cell cycle in the heart than in the neuroepithelium.

Because bioactivation of CP is necessary for teratogenicity, Gomes-Carneiro et al. (2003) treated rats with an inhibitor of CYP2B1, the enzyme that activates CP, prior to CP injection. Severity and incidence of teratogenicity was reduced. Conversely, licorice extract, an upregulator of CYP2B1 expression, exacerbated the teratogenicity of CP (Park et al., 2011). Ginseng extract inhibits the CP detoxifying enzyme CYP3A, and coadministration enhanced CP teratogenicity. Thiol-containing molecules protect against alkylation, and the thiol molecules cysteine, glutathione, and Na-2-mercaptoethane sulfonate ameliorated CP teratogenicity (Ashby et al., 1976; Hales, 1981; Slott and Hales, 1986). Treatment with the antioxidant ascorbic acid attenuated CP-induced embryonic death and chromosomal damage (Kola et al., 1989), while another antioxidant, butylated hydroxyanisole, protected against CP teratogenicity in rats (Kang et al., 2003) and mice (Kim et al., 2003). Coadministration of L-carnitine or quercetin has also been shown to ameliorate the teratogenicity of CP, perhaps due to their antioxidant properties (Khaksary Mahabady et al., 2015, 2016).

The Molecular Basis of Dysmorphogenesis

Pathways of normal and abnormal development are being elucidated using molecular biology, genomics, proteomics, bioinformatics, tissue engineering, and computer modeling. Targeted gene disruption by homologous recombination (gene "knockout"), developed in the 1980s (Capecchi, 1989), has been used to study

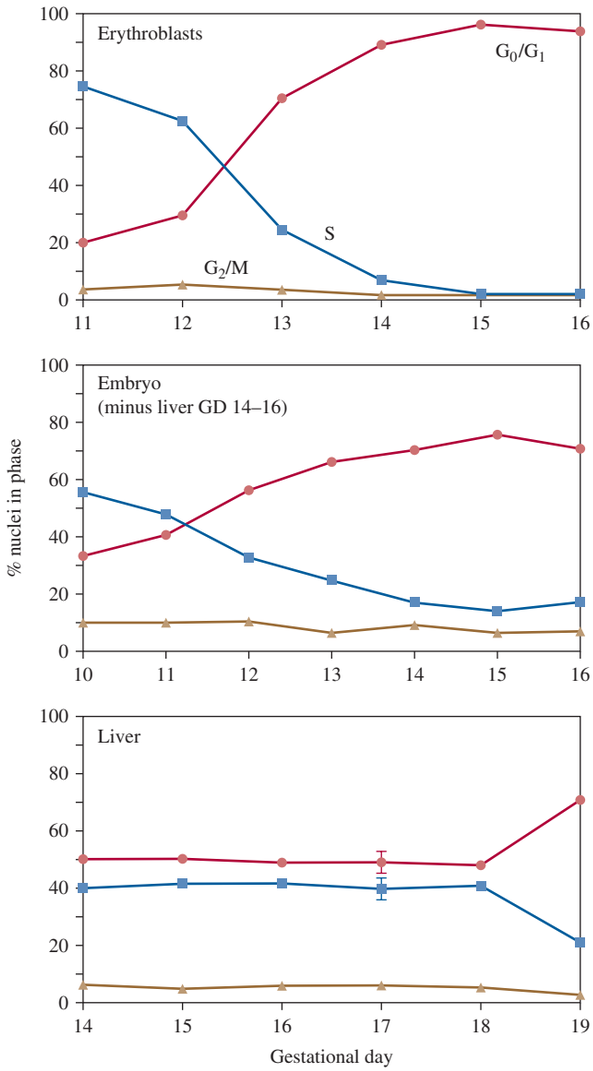

Figure 10-5. *Normal developmental changes in cell cycle distributions in erythroblasts, embryo (minus the liver after GD 13), and fetal liver. Percentages of cells in: ● G_0/G_1; ■ S; and ▲ G_2/M are shown for rat embryos between gestation days 10 and 19 (note changing x-axis range).* The proportion of cells in S-phase generally reflects proliferation rate, which decreases with developmental stage in the embryo and erythroblasts. The percentage of S-phase cells in the fetal liver remains fairly high and constant until near term, when a growth spurt occurs. (From Elstein et al. [1993].)

the loss-of-function of many gene products in developing and adult animals. An example is the retinoic acid receptor (RAR) family of nuclear ligand-inducible transcription factors that bind retinoid ligands, such as retinoic acid and 9-*cis* retinoic acid. Chambon and colleagues produced mice lacking RAR receptors either single or as combinations of knockouts. Single-receptor isoform mutants often exhibit normal development, suggesting that the receptors perform similar functions (i.e., there is functional redundancy), whereas double mutant neonates had widespread malformations of the skeleton and organs (Lohnes et al., 1994; Mendelsohn et al., 1994). The double RARγ–RARβ null mouse exhibits syndactyly, indicating that retinoic acid has a role in interdigital cell death (Dupe et al., 1999). The retinoid-X receptors (RXRα, β, and γ) bind 9-*cis* retinoic acid exclusively. RAR/RXR heterodimers form

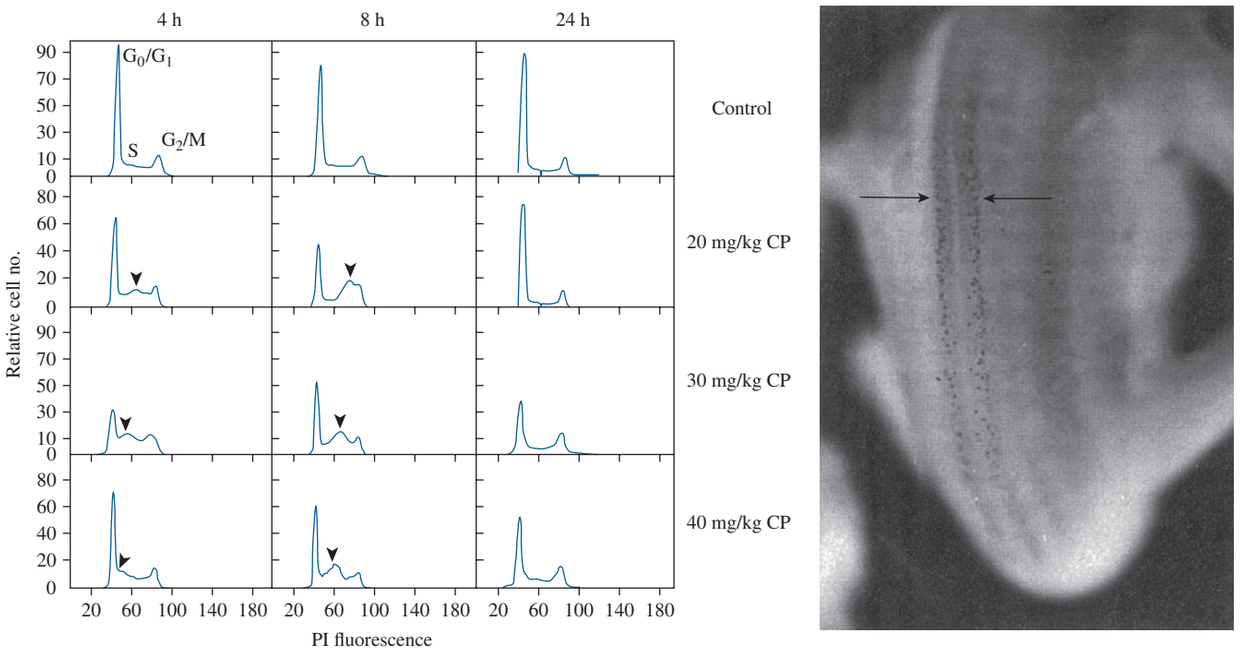

Figure 10-6. *Maternal cyclophosphamide (CP) administration on gestation day 10 in CD-1 mice produces perturbations of the embryonal cell cycle and cell death in areas of rapid proliferation. Left:* Cells are inhibited from progressing through the S (DNA synthetic) phase of the cell cycle, indicated by the abnormal population of cells (*arrowheads*) accumulating at progressively earlier stages of S-phase 4 and 8 hours after increasing maternal CP dosages. The upper panels show the normal GD 10 to 11 distributions, with the G_0/G_1, S, and G_2/M peaks identified in the upper left panel. By 24 hours postdosing, cell cycle distributions have returned to normal at 20 mg/kg, but remain abnormal at higher dosages. *Right:* Nile blue sulfate staining of a mouse embryo 24 hours after maternal CP dosing shows cell death (*stippling along either side of the midline, arrows*) in the neural tube, one of the most sensitive target sites for CP. (Adapted from Chernoff et al. [1989].)

and mediate retinoic acid signaling via transcriptional control in the embryo (Mark et al., 2006, 2009). Many spontaneous mutant and genetically engineered mouse strains are commercially available, and there is an expanding compendium of transgenic and mutants of other animals including zebrafish and frogs.

In addition to directly changing the DNA sequence using homologous recombination, it is also possible to modulate gene expression using synthetic oligonucleotides. Antisense oligonucleotides, with 15 to 25 nucleotides that are complementary to the mRNA to be disrupted (Hélène C and Toulmé JJ, 1990) can be synthesized. These probes can enter embryonal cells, and hybridization with cellular mRNA resulting in disruption of native message, such that gene function can be turned off at specific times. Using antisense oligonucleotides, Augustine et al. (1993) attenuated expression of the proto-oncogene Wnt-1 in mouse embryos developing in WEC. Exposure during neurulation produced mid- and hindbrain malformations similar to those seen in Wnt-1 null mice, as well as cardiac anomalies not observed in Wnt-1 knockouts created by homologous recombination. In mouse embryos in vitro, antisense attenuation of Wnt-3a caused anomalies of the forebrain, midbrain, and spinal cord. Simultaneously attenuating both Wnt-1 and Wnt-3a affected all brain regions and worsened the effect on the spinal cord, suggesting that these genes serve a complementary function in the development of the central nervous system. Morpholino antisense oligonucleotides contain nucleic acid analogs that resist degradation, and morpholinos have been used extensively in the study of zebrafish biology (Bedell et al., 2011). RNA interference is a more recent gene knockdown technique. Small interference (si) RNA and plasmid- and virus-encoded small RNAs can be used to downregulate the expression of specific genes posttranscriptionally (Shan, 2010). For techniques that modulate the level of mRNA, it is important to remember that selectivity and sensitivity are crucial to interpreting results of these studies. Off-target binding can occur, and appropriate controls of scrambled sequences or an unmodified vector are essential. Determining the level of mRNA change is critical because it is possible to decrease (knockdown) the level of mRNA without knocking it out. Additionally, the protein is usually the functional molecule, so it is essential to understand the relationship between changes in mRNA and protein when interpreting results. A transient decrease in mRNA may not change the level of functional protein.

Gain of gene function can also be studied by engineering genetic constructs with an appropriate promoter attached to the gene of interest. Ectopic gene expression can be ubiquitous or tissue-specific depending on the promoter selected to drive expression. Expression of the Hoxa-7 gene in mouse embryos using a chicken β-actin promoter resulted in multiple craniofacial and cervical vertebral malformations (Balling et al., 1989; Kessel et al., 1990). Transient overexpression of specific genes can be accomplished using adenoviral transduction. Hartig and Hunter (1998) injected an adenoviral vector containing either the bacterial beta-galactosidase or green fluorescent protein reporter gene under the control of the human cytomegalovirus early gene promoter into the intraamniotic space of neurulation-stage mouse embryos and achieved intense gene expression in the neuroepithelium.

Advances in gene targeting and transgenic strategies now allow modification of gene expression at specific points in development and in specific cell types. Conditional knockouts, knockdowns or knockins, inducible gene expression, and other techniques are being used to study in detail the effects of specific gene products on development (Mikkola and Orkin, 2005). Gene editing using the CRISPR-Cas9 system now allows targeting of specific sequences

in DNA for modification, and this technology has been applied to elucidating mechanisms of development. To come back to our example of retinoic acid and its receptors, Kumar et al. (2016) used CRISPR-Cas9 to produce mouse embryos with single and double nuclear receptor corepressor *Ncor1* and *Ncor2* mutants and demonstrated that *Ncor1;Ncor2* double mutants had small somites and cardiac distention similar to the phenotype of retinoic acid deficient embryos. Retinoic acid repression of *Fgf8* expression is required for different facets of morphogenesis, and this study demonstrated that NCOR1 and NCOR2 redundantly mediate the repression of *Fgf8* by retinoic acid. While much is understood about how retinoic acid turns on gene expression, little is known about mechanisms by which it represses gene expression.

Understanding the developmental consequences of altered gene expression requires understanding the temporal and spatial regulation of expression. Early studies of gene expression using in situ mRNA hybridization in tissue slices were supplanted by those using whole-mount techniques. In another approach, reporter transgenes containing a gene with a readily detectable product fused downstream of a selected regulatory region have been used to temporally map gene expression. The *Escherichia coli lacZ* (β-galactosidase) gene is commonly used for this purpose. The expression pattern of a gene can be localized by fusing the gene's upstream regulatory elements to *lacZ*, which will then be transcribed under control of those upstream elements (Zakany et al., 1990). Using these tools, it has been shown that retinoic acid (RA) can activate hox genes, and some hox genes have multiple RA response elements (RAREs). Evidence that RA-induced malformations in mouse embryos are related to changes in hox gene expression was first provided by staining of hox/*lacZ* transgenic embryos (Marshall et al., 1992). Within a few hours of RA treatment, *hoxb-1* expression extended anteriorly, suggesting that hox genes could be direct targets of RA induction. Regions of altered hox expression could be manifest as abnormal cell fate and morphogenesis (Marshall et al., 1996; Collins and Mao, 1999). Reporter genes have been used to study gene expression in zebrafish (Saydmohammed et al., 2011). In addition to mapping gene expression, cell lineage studies can be carried out by fusing *lacZ* to a constitutive regulatory sequence and introducing the construct into a somatic cell early in development. The reporter gene will then be expressed in and mark all progeny of the transfected cell. This method has been used to study postimplantation development in the mouse embryo (Sanes et al., 1986), although intracellular injection of fluorescent dyes has also proven to be reliable for cell lineage studies (Smith et al., 1994).

The use of modern biological techniques to elucidate a mechanism of chemical-induced developmental toxicity can be demonstrated by research on two perfluorinated alkyl acids (PFAAs), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). The PFAAs are a family of industrial chemicals that has been widely used as surfactants, hydrophobic and oleophobic treatments, and nonstick coatings. These chemicals are highly resistant to environmental degradation. Studies in experimental laboratory animals have demonstrated that developmental toxicity is among the most sensitive toxic effects produced by these chemicals (Lau et al., 2004). Exposure of pregnant mice to either PFOS or PFOA resulted in dose-related increases in offspring mortality during the first hours to days of life. While the toxic effects produced by PFOA and PFOS are similar, their mechanisms of action may be different. PFAAs have varied affinity for the peroxisome proliferator–activated receptor alpha (PPARα), and activation of PPARα is a putative mechanism of action for the developmental toxicity of PFAAs. To explore the role of PPARα activation in the developmental toxicity of PFOA and PFOS, Abbott and colleagues (Abbott et al., 2007, 2009; Wolf et al., 2008; Rosen et al., 2007, 2008, 2010) tested the developmental toxicity of these chemicals in PPARα null mice. Exposure of pregnant wild-type and PPARα knockout mice to PFOA revealed that neonatal mortality, the signature developmentally toxic effect of PFOA, was dependent on PPARα expression. Survival of PPARα null offspring was unaffected by maternal PFOA exposure, while survival of wild-type offspring was significantly reduced (Abbott et al., 2007). Surprisingly, when the same experiments were carried out with PFOS exposure, the PPARα null offspring were not protected against neonatal mortality (Abbott et al., 2009). Studies were undertaken to compare the ability of various PFAAs to activate mouse and human PPARα in COS-1 cells (a cell line derived from kidney cells of the African green monkey) transiently transfected with either a mouse or human PPARα-luciferase reporter gene construct, such that activation of the receptor caused light emission that could be quantified (Wolf et al., 2008). In agreement with the knockout mouse studies, PFOA activated both the mouse and human PPARα receptors at lower concentrations than did PFOS. Further evidence that PPARα activation was required for PFOA but not PFOS developmental toxicity came from studies of gene expression changes induced in fetal tissues by maternal treatment with PFOA or PFOS (Rosen et al., 2007, 2009). Although both PFOA and PFOS activated PPARα downstream genes, PFOA did so in a more robust fashion. This was also observed in liver tissue from adult mice treated with PFOA or PFOS. While PPARα knockout mice did not upregulate certain PPARα-related genes upon PFOA or PFOS treatment, the expression of other genes was changed by exposure to either of these PFAAs, indicating that the influence of either PFOA or PFOS may extend beyond activation of PPARα (Rosen et al., 2010). It remains to be determined which of the pathways downstream of PPARα are involved in the developmental toxicity of PFOA, and which non-PPARα-related pathways might be perturbed by PFOS.

MATERNAL ADAPTATIONS, METABOLISM, AND TOXICOKINETICS IN PREGNANCY

In determining the impact of chemical exposure on development, the route by which the mother is exposed, the timing of the exposure, and the extent to and from in which the chemical reaches the conceptus are important determinants of the impact on development. The maternal, placental, and embryonic compartments are distinct but interacting systems that undergo profound changes during the course of pregnancy.

Maternal Adaptations to Pregnancy

Maternal adaptations to pregnancy include changes in water and lipid content, hepatic metabolism, the gastrointestinal tract, cardiovascular system, excretory system, and the respiratory system (Hytten, 1984; Krauer, 1987; Mattison et al., 1991; Soma-Pillay et al., 2016) (Table 10-5). While these changes are necessary to maintain sufficient nutrition and eliminate waste products from the conceptus, they significantly impact the uptake, distribution, metabolism, and elimination of xenobiotics. For example, decreases in intestinal motility and increases in gastric emptying time result in longer retention of ingested chemicals in the upper gastrointestinal tract. Cardiac output in humans increases by 30% to 40% by the 27th week of pregnancy, while blood volume increases and plasma protein concentration and peripheral vascular resistance decrease. The relative increase in blood volume over red cell volume leads to borderline anemia and a generalized edema with about 70% elevation of extracellular space. Thus, the volume of distribution of a

Table 10-5

Physiological Changes in Pregnancy

SYSTEM	PARAMETER		CHANGE
Body water	Total	↑	By about 7–8 L
Body fat	Total	↑	By 3–4 kg
Gastrointestinal	Motility	↓	
	Gastric emptying time	↑	
	Acid secretion	↓	(30–50%)
	Mucous secretion	↑	
	Calcium absorption	↑	(100%)
Respiratory	Minute ventilation	↑	(40–50%)
	Alveolar ventilation	↑	
	Tidal volume	↑	(40%)
	Residual volume	↓	(20%)
	Pulmonary blood flow	↑	
Cardiovascular	Cardiac output	↑	(30–40%)
	Rate	↑	(0–20%)
	Stroke	↑	(10%)
	Peripheral blood flow	↑	
	Systemic vascular resistance	↓	(40%)
Renal	Plasma flow	↑	(40–65%)
	Glomerular filtration rate	↑	(50–85%)
Blood	Total volume	↑	(35–40%)
	Plasma volume	↑	(50%)
	Red cell mass	↑	(18%)
	Hemoglobin	↓	
	Clotting factors	↑	
	Phospholipids	↑	
	Cholesterol	↑	
	Triglycerides	↑	
	Protein	↓	
	Albumin	↓	(30%)
	Globulin	↑	
	Creatinine	↓	(55%)
	Urea	↓	(40%)
Metabolism	Metabolic rate	↑	(15%)
	Oxygen consumption	↑	(20%)
	Body temperature	↑	0.5°C
	Hepatic metabolism	↑	

SOURCES: From Hytten (1984), Krauer (1987), Mattison et al. (1991), and Soma-Pillay et al. (2016).

chemical and the amount bound by plasma proteins may change considerably during pregnancy. Renal blood flow and glomerular filtration rate also increase during pregnancy. Respiratory changes including increases in tidal volume, minute ventilation, and minute O_2 uptake can result in increased pulmonary distribution of gases and decreased time to reach alveolar steady state. Limited evidence suggests that maternal xenobiotic metabolizing enzyme activities also change during pregnancy (Juchau, 1981; Juchau and Faustman-Watts, 1983). Maternal hepatic P450 enzymes increase during pregnancy in humans; in contrast, during rat pregnancy hepatic monooxygenase activity decreases, probably due to decreased enzyme levels and competitive inhibition by circulating steroids (Neims et al., 1976). All of these maternal factors determine the amount of chemical and metabolites available to the embryo and are key to determining the extent of embryotoxicity. In one of the few studies of its type, Kimmel and Young (1983) found that a linear combination of the 45-minute and the 24-hour maternal blood concentrations was able to predict the litter response rate for pregnant rats dosed with 500 mg/kg sodium salicylate on gestation day 11. These kinetic parameters reflect the influence of peak and cumulative area under the drug concentration–time curve on the developmental toxicity of salicylate.

Role of the Placenta

The placenta is the interface between the mother and the conceptus. It plays a central role in transferring nutrients and waste products between the conceptus and mother. The placenta can mitigate embryonic xenobiotic exposure by helping to regulate blood flow, by offering a transport barrier, and by metabolizing chemicals (Slikker and Miller, 1994). Functionally, the placenta acts as a lipid membrane that permits bidirectional transfer of substances between maternal and fetal compartments. The extent of transfer depends on three major factors: the type of placentation, the physicochemical properties of the chemical, and rates of placental metabolism. Although there are marked species differences in types of placentas, orientation of blood vessels, and numbers of exchanging layers (Carney et al., 2004), these do not seem to play a dominant role in placental transfer of most chemicals.

It is important to note that virtually any substance present in the maternal plasma will cross the placenta to some extent. The passage of most drugs across the placenta seems to occur by simple passive diffusion. The rate of transfer from mother to conceptus is proportional to the diffusion constant of the drug, the concentration gradient between the maternal and embryonic plasma, the area of exchange, and the inverse of the membrane thickness (Nau, 1992). Important modifying factors to the rate and extent of transfer include lipid solubility, molecular weight, protein binding, the type of transfer (passive diffusion, facilitated, or active transport), the degree of ionization, and placental metabolism. Placental cells express mdr1a also known as P-glycoprotein (P-gp), an ATP binding cassette multidrug cellular efflux pump, and mice with a spontaneous mutation in the *Mdr1a* gene show a graded increase in susceptibility to avermectin Bla–induced cleft palate (Lankas et al., 1998). In the dosage range used in this study, homozygous mutants (*Mdr1a* –/–) were 100% susceptible to cleft palate, homozygous +/+ fetuses were totally resistant, and +/– heterozygous fetuses were of intermediate sensitivity. Weak acids appear to be rapidly transferred across the placenta during early pregnancy in rats and mice, due in part to the pH gradient between the maternal and embryonic plasma, which can trap ionized forms of the drug in the slightly more basic embryonic compartment (Nau and Scott, 1986). In humans and rabbits,

maternal serum pH is either similar to or slightly higher than the yolk sac fluid pH (Carney et al., 2004). Species differences in placental transport of immunoglobulins are an important consideration for safety assessment of biopharmaceuticals containing immunoglobulin Fc fragments, which are transported across the placenta by receptor-mediated endocytosis. The morphological path and timing of transport of immunoglobulins across the placenta vary among species. In humans and nonhuman primates, immunoglobulins are transported across the chorioallantoic placenta, and in rodents and rabbits across the inverted yolk sac. These large molecules are transported at the end of, or after, organogenesis in humans, nonhuman primates, and rabbits, while transport begins earlier in the developing rodent (DeSesso et al., 2012). Thus, the rodent could be a more conservative model for potential developmental toxicity of immunoglobulin-based biopharmaceuticals. Although smaller numbers limit the power of studies in nonhuman primates, the cynomolgus monkey is the most commonly used species for studies of the developmental toxicity of monoclonal antibody biopharmaceuticals (Pentsuk and van der Laan, 2009).

Maternal and Conceptus Xenobiotic Metabolism

The amount of xenobiotic, its form (parent or metabolite), and timing of delivery to the embryo are important components of understanding species differences in sensitivity (Nau, 1986). Despite the small size of the conceptus and the rapid rate of its development during organogenesis, there has been considerable progress in the area of embryonal toxicokinetics (Nau and Scott, 1987; Clark, 1993; Corley et al., 2003; Corley, 2010). Sensitive analytical methods can quantify metabolite formation and challenge the historical view, particularly for cytochrome P450–dependent monooxygenases, that the early embryo has low metabolic capabilities (Juchau et al., 1992). Using the WEC system, Juchau and co-workers demonstrated that the rat conceptus can bioactivate the proteratogen 2-acetylaminofluorene (2-AAF) to produce sufficient amounts of a toxic metabolite to induce dysmorphogenesis. Additionally, the proximate developmental toxicant, the 7-hydroxy metabolite, was different than the metabolite responsible for 2-AAF mutagenesis and carcinogenesis. These investigators later showed that embryos could further metabolize the 7-hydroxy metabolite to an even more toxic catechol. No previous induction was necessary for this activation step, demonstrating the presence of constitutive metabolizing enzymes in the embryo. Hines (2012) compiled findings on the ontogeny of hepatic drug metabolizing systems in humans, including significant prenatal expression for a number of enzyme classes. His survey suggested that metabolizing systems can be categorized into three classes based on the developmental trajectory of their expression (Table 10-6); Class I enzymes appear during the first trimester, remain elevated or decrease during gestation, and drop to low or no expression postnatally; Class II enzymes are expressed at a consistent level throughout gestation through adulthood; and Class III enzymes are nondetectable or low in the fetus, with expression increasing during 1 to 2 years postnatally and full expression not occurring until puberty for some Class III enzymes.

Physiologically Based Pharmacokinetic Models of Pregnancy

Physiologically based pharmacokinetic (PBPK) models provide the framework to integrate what is known about xenobiotic metabolism with physiology during pregnancy and embryo/fetal development into a quantitative description. Olanoff and Anderson (1980) described the kinetics of the antibiotic tetracycline in the pregnant rat and her litter in a model that incorporated fetal and placental growth. Gabrielson and co-workers (Gabrielson and Paalkow, 1983; Gabrielson and Larsson, 1990) were among the first investigators to develop PBPK models of pregnancy, and others have followed with an evolution of models (Fisher et al., 1989; O'Flaherty et al., 1992; Clark et al., 1993; Luecke et al., 1994, 1997; Young, 1998). The Fisher model included growth of several maternal tissues during pregnancy. The model of O'Flaherty and co-workers describes the entire period of gestation, and includes the uterus, mammary tissue, maternal fat, kidney, liver, other well-perfused maternal tissues, embryo/fetal tissues and yolk sac, and chorioallantoic placenta. This model includes changes in many compartments during pregnancy (including the embryo), as well as changes in blood flow and stage-dependent pH gradients between maternal and embryonic plasma. Transfer of xenobiotics across the placenta is diffusion-limited in this model. Luecke and co-workers developed quantitative models for human embryofetal growth for use in models of pregnancy (Luecke et al., 1994, 1997, 1999). For a review of chemical-specific PBPK models, see Corley et al. (2003) and Corley (2010).

A life-stage PBPK model was developed and integrated with adverse outcome pathways (AOPs) for developmental toxicity related to vasculogenesis, in vitro assays relevant to AOPs, and in vitro to in vivo extrapolation models as an approach to screening and prioritizing chemicals for potential risk (El-Masri et al., 2016). The PBPK model described chemical disposition during pregnancy, fetal, neonatal, infant, and adult stages to estimate a maternal

Table 10-6

Classification of Drug-Metabolizing Enzymes Based on Developmental Trajectories

CLASS I	CLASS II	CLASS III	
ADH1A	CYP2B6	ADH1B	GSTZ
CYP1A1	CYP2C19	ADH1C	FMO3
CYP3A7	CYP3A5	AOX	PON1
FMO1	EPHX1	CYP1A2	SULT2A1
GSTP	EPHX2	CYP2A6	UGT1A1
SULT1E1	GSTA	CYP2C8	UGT1A6
SULT1A3	SULT1A1	CYP2C9	UGT2B7
		CYP2D6	UGT2B15
		CYP2E1	CES1
		CYP3A4	CES2
		GSTM	

Class I enzymes appear during the first trimester, remain active during gestation, and have little or no postnatal expression; Class II enzymes are expressed at a consistent level during gestation through adulthood; Class III enzymes are low or absent in the fetus and increase during 1 to 2 years postnatally, some not being maximal until puberty. ADH, alcohol dehydrogenase; AOX, alternative oxidase; CES, carboxylesterase; CYP, cytochrome P450; EPHX, epoxide hydrolase; FMO, flavin monooxygenase; GST, glutathione S-transferase pi; GSTA, glutathione S-transferase alpha; GSTM, glutathione S-transferase Mu; GSTZ, glutathione S-transferase zeta; PON, paraoxonase; SULT, sulfotransferase; UGT, UDP glucuronosyl transferase.

SOURCE: Adapted from Hines (2012), with updates from R. Hines (personal communication).

exposure that would produce fetal blood concentrations similar to those producing toxicity in the in vitro assays. This estimate could be compared to maternal environmental exposure estimates to derive a margin of exposure.

RELATIONSHIPS BETWEEN MATERNAL AND DEVELOPMENTAL TOXICITY

All developmental toxicity ultimately results from insult to the conceptus at the cellular level, but in mammalian embryos the insult may occur through a direct effect on the embryo/fetus, indirectly through toxicity of the chemical to the mother or the placenta, or a combination of direct and indirect toxicity. Examples of maternal physiological conditions with the potential to adversely affect the conceptus include decreased uterine blood flow, anemia, under- or malnutrition, toxemia, altered organ function, autoimmune states, diabetes, electrolyte or acid–base disturbances, and elevated stress hormones (Chernoff et al., 1989; Daston, 1994). Chemical induction or exacerbation of such maternal conditions and the degree to which they contribute to abnormal development depend on maternal genetic background, age, parity, size, nutrition, disease, stress, and other health parameters and exposures (DeSesso, 1987; Chernoff et al., 1989; Rogers et al., 2005). These relationships are depicted in Fig. 10-7.

Distinguishing between direct and indirect developmental toxicity in standard developmental toxicity studies can be critical for interpreting results, as measurable maternal toxicity (e.g., decreased food or water intake, weight loss, clinical signs) is expected by design at the highest dosage level used. Such overt maternal toxicity can result from a number of underlying mechanisms, which are typically unknown. An understanding of the physiology underlying observed maternal toxicity is needed before one can begin to address the relevance of the observations for human risk assessment. Human developmental toxicants including ethanol and cocaine adversely affect the embryo/fetus predominately at maternally toxic exposures, and effects on the mother clearly contribute to their developmental toxicity. For example, the nutritional status of alcoholics is often poor and placental transfer of nutrients may be inhibited by ethanol. Chronic alcohol abuse alters maternal folate and zinc metabolism, which may play a role in the induction of fetal alcohol syndrome (Dreosti, 1993). Carney (2010) has constructed a useful decision tree for interpreting developmental toxicity observed concomitantly with maternal toxicity (Fig. 10-8).

Maternal Factors Affecting Development

Genetic Background The genetic makeup of the pregnant female has been well documented as a determinant of developmental outcome in both humans and animals. The incidence of cleft lip and/or palate (CL(P)), which in humans occurs more frequently in Caucasians than in African Americans, has been investigated in offspring of interracial couples in the United States (Khoury et al., 1983). Offspring of white mothers had a higher incidence of CL(P) than offspring of black mothers after correcting for paternal race, while offspring of white fathers did not have a higher incidence of CL(P) than offspring of black fathers after correcting for maternal race. Thus, maternal race (and presumable genotype) was the factor associated with differential risk.

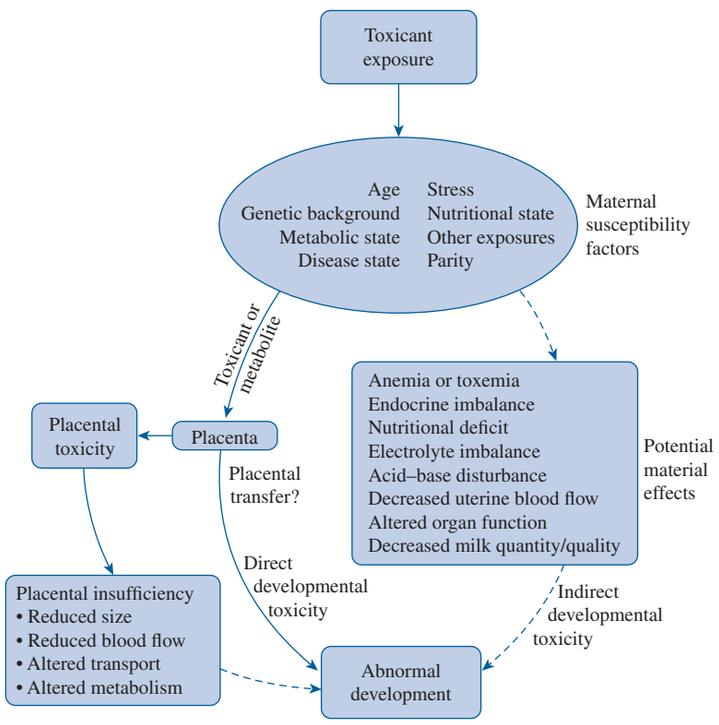

Figure 10-7. *Interrelationships between maternal susceptibility factors, metabolism, induction of maternal physiologic or functional alterations, placental transfer and toxicity, and developmental toxicity.* A developmental toxicant can cause abnormal development through any one or a combination of these pathways. Maternal susceptibility factors determine the predisposition of the mother to respond to a toxic insult, and the maternal effects listed can adversely affect the developing conceptus. Most chemicals traverse the placenta in some form, and the placenta can also be a target for toxicity. In most cases, developmental toxicity is probably mediated through a combination of these pathways.

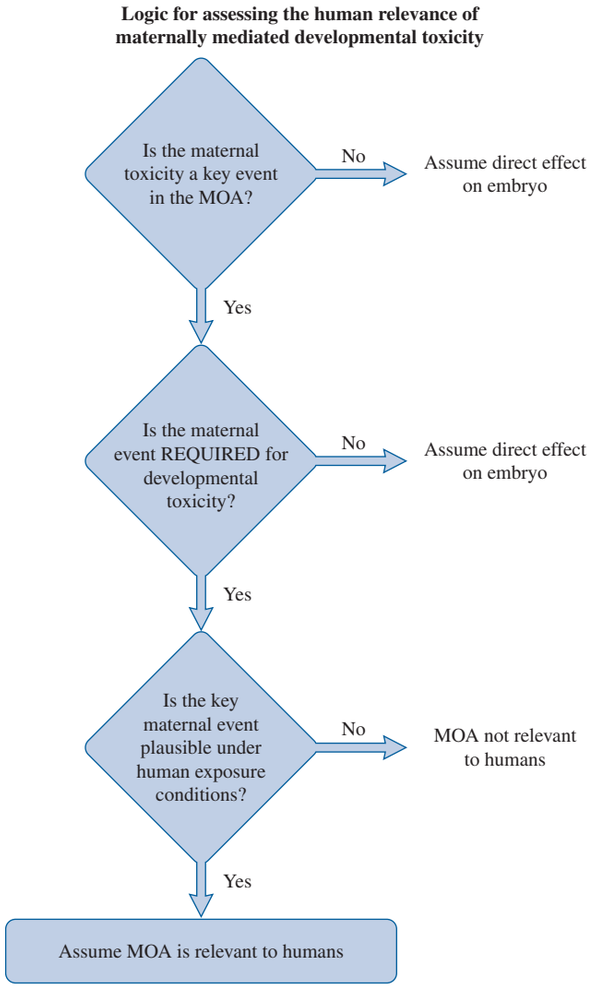

Figure 10-8. *Assessing the relevance to humans of a maternally mediated mode of action in rodents.* Flow chart for considering whether a maternally mediated mode of action (MOA) in animal studies is relevant to humans. When developmental toxicity is observed only at maternally toxic dosages, the MOA for developmental toxicity may be direct or indirect (secondary to the maternal toxicity). Maternally mediated developmental toxicity is relevant to humans if expected human exposure conditions could plausibly trigger that MOA. Knowledge of the MOA for developmental toxicity, which will likely require investigation beyond the dose–response study, is a prerequisite for using this approach. (Modified from Carney [2010].)

Two related mouse strains, A/J and CL/Fr, produced spontaneous CL(P) at 8% to 10% and 18% to 26% frequencies, respectively, and the incidence of the malformation depended on the genotype of the mother rather than that of the embryo (Juriloff and Fraser, 1980). The response of mouse embryos heterozygous for the curly-tail mutation to vitamin A also depends on the genotype of the mother (Seller et al., 1983). The teratogenicity of phenytoin has been compared in several inbred strains of mice, and again, it turned out that the susceptibility of offspring of crosses between susceptible A/J mice and resistant C57BL/6J (B6) mice was determined by the maternal, but not the embryonic genome (Hansen and Hodes, 1983).

Genes associated with susceptibility of mouse strains to valproic acid have been identified (Finnell et al., 1997; Bennett et al., 2000; Craig et al., 2000; Faiella et al., 2000; Okada et al., 2004, 2005). A study utilizing inbred B6 and DBA/2J (D2) mice to examine the role of maternal genotype in sensitivity to valproic acid (Downing et al., 2010) found that B6 mice were more susceptible to valproate-induced digital and vertebral malformations, while D2 mice were more susceptible to rib malformations. In a reciprocal cross between B6 and D2 mice, genetically identical F1 offspring responded in a manner consistent with maternal genotype: F1 offspring carried in a B6 mother had more vertebral and digital malformations while F1 carried in a D2 mother exhibited more rib malformations. It is possible that a maternal factor other than genotype was responsible for this finding.

Disease A number of maternal conditions and diseases can exert untoward effects during pregnancy. Chronic hypertension is a risk factor for the development of preeclampsia, eclampsia, and toxemia of pregnancy, and hypertension is a leading cause of pregnancy-associated maternal deaths. Uncontrolled maternal diabetes mellitus is a significant cause of prenatal morbidity. Certain maternal infections can adversely affect the conceptus (e.g., rubella virus, discussed earlier), either through indirect disease-related maternal alterations or direct transplacental infection. Cytomegalovirus infection is associated with fetal death, microcephaly, mental retardation, blindness, and deafness (MacDonald and Tobin, 1978), and maternal infection with *Toxoplasma gondii* is known to induce hydrocephaly and chorioretinitis in infants (Alford et al., 1974). The recent epidemic of microcephaly and other brain and eye defects in infants born to mothers infected during pregnancy with the Zika virus is the latest example of a maternal disease linked to birth defects (Rasmussen et al., 2016); in this case, the virus does appear to reach the brain of the fetus and to be directly toxic to neuroprogenitor cells (Faizan et al., 2016). A maternal factor common to many disease states is hyperthermia, which is a potent teratogen in experimental animals (Edwards, 1986), and there is a body of evidence associating maternal febrile illness during the first trimester of pregnancy with birth defects in humans, most notably neural tube defects, heart defects, and oral clefts (Warkany, 1986; Milunsky et al., 1992; Edwards 2006; Dreier et al., 2014; Shi et al., 2014).

Nutrition Dietary insufficiencies ranging from protein-calorie malnutrition to deficiencies of vitamins, trace elements, and/or enzyme cofactors are known to adversely affect pregnancy (Keen et al., 1993). Perhaps the most significant findings are from the landmark studies in which pregnant women at risk of having infants with neural tube defects (NTDs) were supplemented with folate (Wald, 1993; Czeizel, 2011). The largest and most convincing study was the Medical Research Council (MRC) Vitamin Study, in which supplementation with 4 mg of folic acid was associated with an over 70% reduction in recurrence of neural tube defects in previously affected mothers (MRC Vitamin Study Research Group, 1991; Bendich, 1993). The U.S. Centers for Disease Control and Prevention (CDC) recommended folate supplementation for women of childbearing age and folate supplementation of foodstuffs. In 1996, the U.S. FDA mandated enrichment of cereal grain products with folate. One study has demonstrated more than a doubling of mean serum folate concentrations across all sex and age groups since then (Dietrich et al., 2005). Yet, in the same population, less than 10% of women of childbearing age had the recommended erythrocyte folate concentration of more than 906 nmol/L shown to be associated with a reduction in NTD risk. The CDC (2004) estimated that the number of NTD-affected pregnancies in the United States declined from 4000 in 1995 to 1996 to 3000 in 1999-2000. While this survey indicates success of the folate fortification program, women capable of becoming pregnant were

urged to continue to follow the U.S. Public Health Service recommended consumption of 400 μg folate daily. Mills and Signore (2004) compared the rates of antenatal detection of NTDs in the United States (incomplete ascertainment and underprediction of NTDs) and Canada (more complete antenatal ascertainment) and determined that the studies that best identify cases of NTDs show that folic acid fortification is preventing around 50% of NTDs. Recent studies using multivitamins containing folate have achieved even better results (Czeizel et al., 2011). There is evidence that the effectiveness of folate in reducing neural tube defects may lie in its role in one carbon metabolism, particularly as a methyl donor for DNA methylation. In a mouse model of neural tube defects induced by methotrexate, a folate antimetabolite, total genomic DNA methylation was reduced in neural tube tissue of embryos with methotrexate-induced NTDs, and two genes in the Wnt pathway were especially affected (Wang et al., 2015).

Stress Diverse forms of maternal toxicity likely have in common the induction of a physiologic stress response, and understanding the effects of maternal stress on development may help interpret developmental toxicity observed in laboratory animals at maternally toxic dosages. Various forms of physical or psychological stress have been applied to pregnant animals in attempts to isolate the developmental effects of stress. Subjecting pregnant rats or mice to noise stress throughout gestation can produce developmental toxicity (Kimmel et al., 1976; Nawrot et al., 1980, 1981). Restraint stress produces increased fetal death in rats (Euker and Riegle, 1973) and cleft palate (Barlow et al., 1975), fused and supernumerary ribs, and encephaloceles in mice (Beyer and Chernoff, 1986).

Objective data on effects of stress during pregnancy in humans are difficult to obtain. Nevertheless, studies investigating maternal stress and pregnancy outcome have indicated a positive correlation between stress and adverse developmental effects including low birth weight and adverse mental health outcomes in offspring (Stott, 1973; Gorsuch and Key, 1974; Beydoun and Saftlas, 2008; Entringer et al., 2010; Dunkel Schetter and Tanner, 2012; Brunton, 2013).

Maternal and Placental Toxicity

The mother or the placenta may be the primary target for some developmental toxicants, with effects on the developing embryo/fetus occurring secondarily due to changes in the intrauterine environment or the nutrient/gas/waste exchange capabilities of the placenta.

Maternal Toxicity A retrospective analysis of relationships between maternal toxicity and specific types of prenatal effects claimed species-specific associations, yet, among rat, rabbit, and hamster studies, 22% failed to show any developmental toxicity in the presence of overt maternal toxicity (Khera, 1984, 1985). In a study designed to test the potential of maternal toxicity to affect development, Kavlock et al. (1985) administered 10 structurally unrelated compounds to pregnant mice at maternotoxic dosages. The only consistent developmental finding was an increased incidence of supernumerary ribs (ribs on the first lumbar vertebra), which occurred with 7 of the 10 compounds. Chernoff et al. (1990) dosed pregnant rats for 10 days with a series of compounds that had exhibited little or no developmental toxicity in previous studies. When these compounds were administered at maternally toxic dosages, diverse adverse developmental outcomes were noted including increased intrauterine death (two compounds), decreased fetal weight (two compounds), supernumerary ribs (two compounds), and enlarged renal pelves (two compounds). Two of the compounds produced no developmental toxicity despite substantial maternal toxicity. These diverse developmental responses led the authors to conclude that maternal toxicity characterized by weight loss or mortality was not consistently associated with any syndrome of developmental effects in the rat.

There have been a number of studies relating specific elements of maternal toxicity to developmental toxicity, including those in which the test chemical caused maternal effects that exacerbated the chemical's developmental toxicity, and instances in which developmental toxicity was thought to be secondary to maternal effects. However, clearly delineating the relative role(s) of indirect maternal and direct embryo/fetal toxicity is difficult. To follow are examples of studies in which this has been achieved to some extent.

Acetazolamide inhibits carbonic anhydrase and is teratogenic in mice (Hirsch and Scott, 1983). Although maternal weight loss was not correlated with malformation frequency, maternal hypercapnia potentiated the teratogenicity of acetazolamide. In C57Bl/6J mice, maternal hypercapnia alone resulted in right forelimb ectrodactyly, the characteristic malformation induced by acetazolamide. Correction of maternal acidosis failed to reduce developmental toxicity, suggesting that the primary teratogenic factor was elevated maternal plasma CO_2 tension (Weaver and Scott, 1984a, 1984b).

Diflunisal, an analgesic and anti-inflammatory drug, causes axial skeletal defects in rabbits. Developmentally toxic dosages resulted in severe maternal anemia (hematocrit = 20% to 24% vs. 37% in controls) and depletion of erythrocyte ATP (Clark et al., 1984). Teratogenicity, anemia, and ATP depletion were unique to the rabbit among the species studied. A single dose of diflunisal on day 5 of gestation produced maternal anemia that lasted through day 15. Concentration of the drug in the embryo was less than 5% of the peak maternal blood level, and diflunisal was cleared from maternal blood before day 9, the critical day for induction of similar axial skeletal defects by hypoxia. These results provide strong evidence that the teratogenicity of diflunisal seen in the rabbit was probably due to persistent hypoxia resulting from maternal anemia.

The anticonvulsant phenytoin can affect maternal folate metabolism in laboratory animals, which may play a role in its teratogenicity (Hansen and Billings, 1985). Heart rates were depressed by phenytoin in a dose-related manner in susceptible A/J mice but not in resistant C57Bl/6J mice (Watkinson and Millicovsky, 1983), and these authors proposed a mechanism of teratogenesis relating depressed maternal heart rate to embryonic hypoxia. Supporting studies have demonstrated that hyperoxia reduced the teratogenicity of phenytoin in mice (Millicovsky and Johnston, 1981).

Reduced uterine blood flow has been proposed as a mechanism of teratogenicity caused by hydroxyurea, which produces elevated systolic blood pressure, altered heart rate, decreased cardiac output, severely decreased uterine blood flow, and increased vascular resistance in pregnant rabbits (Millicovsky et al., 1981). Embryos exhibited craniofacial and pericardial hemorrhages immediately after treatment (Millicovsky and DeSesso, 1980a). The production of identical embryopathies by clamping the uterine vessels of pregnant rabbits for 10 minutes provided clear evidence of the role of reduced uterine blood flow in the teratogenicity of hydroxyurea (Millicovsky and DeSesso, 1980b).

Metallothionein (MT) synthesis can be induced by a variety of chemical and physical agents including metals, alcohols, urethane, endotoxin, alkylating agents, hyper- or hypothermia, and ionizing radiation (Daston, 1994). MT synthesis is also induced by endogenous mediators such as glucocorticoids and certain cytokines (Klaassen and Lehman-McKeeman, 1989). A mechanism common to the developmental toxicity of these diverse agents may

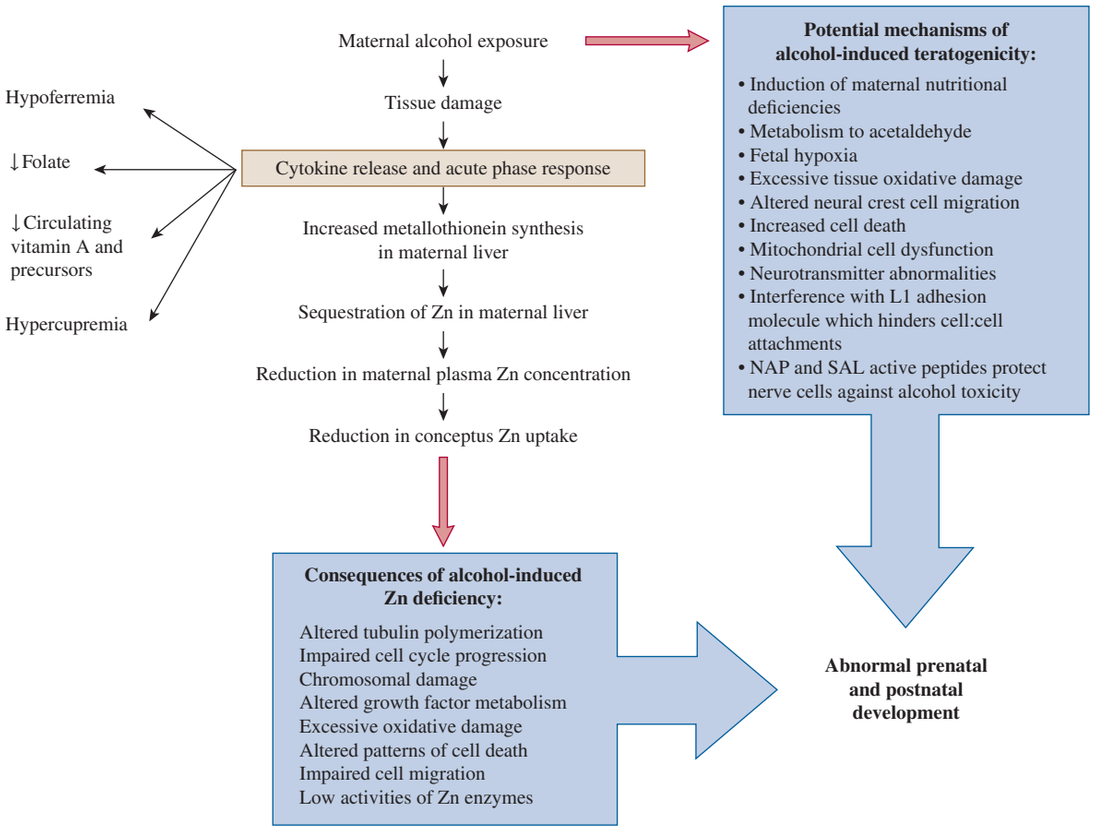

Figure 10-9. *Alcohol-induced acute phase response and sequence of events leading to conceptus zinc deficiency.* Multiple mechanisms underlie the teratogenicity of alcohol (box at upper right). Tissue damage in maternal tissues can initiate an acute phase response that includes induction of metallothionein in maternal liver, which removes available zinc from the maternal circulation, reducing availability to the conceptus. In addition to contributing to zinc deficiency in the embryo/fetus, an acute phase response can affect the metabolism of other essential nutrients, including folate, vitamin A, iron, and copper. NAP, neuroprotective octapeptide NAPVSIPQ; SAL, neurotropic nonapeptide SALLRSIPA. (Modified from Keen et al. [2010].)

be developmental Zn deficiency secondary to maternal MT induction. Induced synthesis can increase hepatic MT concentrations an order of magnitude above normal, leading to sequestration of circulating Zn in maternal liver, lower circulating Zn concentrations, and reduced Zn availability to the conceptus. Embryo/fetal Zn deficiency secondary to maternal hepatic MT induction has been demonstrated for diverse chemicals including valproic acid (Keen et al., 1989), 6-mercaptopurine (Amemiya et al., 1985, 1989), urethane (Daston et al., 1991), ethanol, and α-hederin (Taubeneck et al., 1994). Taubeneck et al. (1994) found a strong positive relationship between maternal hepatic MT induction and maternal hepatic ^{65}Zn retention, and a negative relationship between maternal MT induction and ^{65}Zn distribution to the fetuses. This is a clear example of a maternally mediated mechanism exacerbating or directly causing developmental toxicity, one that is applicable to diverse maternal insults and for which direct exposure of the conceptus to the causative agent is not necessary. Likewise, maternal ethanol exposure represents an excellent example of the interplay of indirect (maternally mediated) and direct developmentally toxic mechanisms (Fig. 10-9). Alcoholic mothers can exhibit blood alcohol levels that no doubt exert multiple direct effects on the embryo, including induction of cell death, altered neural crest cell migration, mitochondrial dysfunction, and effects on membrane integrity. At the same time, effects of chronic alcohol exposure on the mother include hepatic metallothionein induction and secondary embryofetal zinc deficiency, as described above. Additional maternal effects that may exacerbate teratogenicity include hypoferremia, reduced circulating folate, reduced circulating vitamin A, and hypercupremia. Genetic polymorphisms in both the mother and the fetus likely play roles in manifestation of fetal alcohol spectrum disorders as well, as evidenced by studies in humans and in animal models (Warren and Li, 2005). Despite literally thousands of epidemiological, clinical, and laboratory studies, the complex interplay of factors involved in the teratogenicity of ethanol is still being elucidated.

Placental Toxicity The placenta produces peptide and steroid hormones critical to the maintenance of pregnancy and the regulation of fetal and maternal metabolism, and can metabolize or store xenobiotics. Slikker and Miller (1994) listed 46 substances known to be toxic to the yolk sac or chorioallantoic placenta, including metals such as cadmium (Cd), arsenic, or mercury, cigarette smoke, ethanol, cocaine, endotoxin, and sodium salicylate. Cd is among the best studied of these, and it appears that the developmental toxicity of Cd during mid-to-late gestation involves both placental toxicity (necrosis, reduced blood flow) and inhibition of nutrient transport across the placenta. Maternal injection of Cd during late gestation resulted in fetal death within 10 hours in rats, concurrent with reduced uteroplacental blood flow, despite little cadmium entering the fetus (Parizek, 1964; Levin and Miller, 1980). The authors' conclusion that fetal death was caused by placental toxicity was supported by experiments in which fetuses were directly

injected with Cd. Despite fetal Cd burdens almost 10-fold higher than those following maternal administration, only a slight increase in fetal death was observed.

Cd is a transition metal similar in physicochemical properties to the essential metal zinc (Zn). Cd interferes with Zn transfer across the placenta (Ahokas et al., 1980; Sorell and Graziano, 1990), probably via metallothionein (MT), a metal-binding protein induced in the placenta by Cd. Because of its high affinity for Zn, MT may sequester Zn in the placenta, impeding transfer to the conceptus (induction of maternal hepatic MT by Cd or other agents can also induce fetal Zn deficiency, as discussed below). Cd inhibits Zn uptake by human placental microvesicles (Page et al., 1992), suggesting direct competition with Zn for membrane transport, and it may also competitively inhibit other Zn-dependent processes in the placenta. Coadministration of Zn ameliorates the developmental toxicity of Cd, indicating that interference of Cd with Zn metabolism is a key to its developmental toxicity (Ferm and Carpenter, 1967, 1968; Daston, 1982).

It has become evident that the placenta can undergo adaptive responses to the intrauterine milieu, and that such responses can influence the metabolic "programming" of the fetus (Myatt, 2006). Adverse conditions including hypoxia and nutritional deficiencies can lead to alterations in placental structure and function including altered expression or activity of transporters and receptors. Placental morphometry has been proposed as a biomarker of fetal programming (Thornburg et al., 2010). One role of the placenta that is key for fetal programming is as a regulator of glucocorticoid transfer between the mother and her fetus(es) (Seckl and Holmes, 2007). The enzyme 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) inactivates glucocorticoids; its activity in the placenta protects the fetus from overexposure to maternal stress hormone. In laboratory animals, lowering the activity of 11β-HSD2 by nutritional deficiency, gene ablation, or pharmacologic inhibitors leads to lower birth weight and causes increase in offspring adult blood pressure, circulating glucose concentrations, and hypothalamic-pituitary-adrenal axis activity.

DEVELOPMENTAL TOXICITY OF ENDOCRINE-DISRUPTING CHEMICALS

An "endocrine disruptor" has been broadly defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al., 1996). The Endocrine Society has stated: "an endocrine-disrupting substance is a compound, either natural or synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment" (Diamanti-Kandarakis et al., 2009). Given the critical role of hormones in directing differentiation of many tissues, the developing organism is particularly vulnerable to exposure to chemicals with hormonal (or antihormonal) activity. A wide variety of chemical classes (e.g., pesticides, herbicides, fungicides, plasticizers, surfactants, organometals, halogenated polyaromatic hydrocarbons, and phytoestrogens) have been shown to cause developmental toxicity by (1) acting as hormone receptor ligands; (2) altering hormone synthetic and metabolic enzymes; or (3) perturbing hypothalamic-pituitary release of tropic hormones. Interactions with the functions of estrogens, androgens, and thyroid hormones have been the most studied, but the underlying principles apply to other hormones as well.

Laboratory Animal Evidence

Chemicals with estrogenic activity induce pleiotropic effects, acting on diverse cell types with estrogen receptors, and can display cell- and organ-specific agonist and antagonist actions. The pattern of outcomes is generally similar across different estrogenic chemicals. Diethylstilbestrol (DES), discussed earlier, provides a well-characterized example of effects of a xenoestrogen on development, including malformations and functional alterations of the male and female reproductive tract and brain. In the CD1 mouse, effective dosages are 0.1 to 100 μg/kg on GD 9 to 16 (Newbold, 1995). At 10 to 100 μg/kg, total sterility of female offspring was noted, due in part to structural abnormalities of the oviduct, uterus, cervix, and vagina and to depletion of ovarian follicles. Male offspring show hypospadias while females exhibit excessive vaginal keratinization and vaginal tumors. Vaginal adenocarcinoma was seen at dosages as low as 2.5 μg/kg maternal body weight, and benign uterine tumors were seen at 0.1 μg/kg. In male offspring, sterility was observed at high doses, the result of retained rete testes and Müllerian duct remnants, abnormal sperm morphology and motility, cryptorchidism, abnormal reproductive tract secretions, and inflammation. DES exposure of pregnant mice also causes obesity in female offspring (Newbold et al., 2009). Other estrogenic (or antiestrogenic) developmental toxicants include estradiol (Biegel et al., 1998; Cook et al., 1998), ethinyl estradiol, antiestrogenic drugs such as tamoxifen and clomiphene citrate (Branham et al., 1988), and pesticides and industrial chemicals such as methoxychlor (Gray et al., 1989; Hall et al., 1997; Amstislavsky et al., 2003, 2004, 2006), *o,p*-DDT (Heinrichs et al., 1971), kepone (Gellert, 1978; Guzelian, 1982), dioxins (Mably et al., 1992; Gray et al., 1997a, 1997b), bisphenol A (Nagel et al., 1997; Peretz et al., 2014), and phytoestrogens such as genistein and coumestrol (Medlock et al., 1995; Rozman et al., 2006). Female offspring are generally more sensitive than males and altered pubertal development, reduced fertility, and reproductive tract anomalies are common findings.

Effects of developmental exposure to antiandrogenic chemicals are generally restricted to males, and include hypospadias, retained nipples, reduced testes and accessory sex gland weights, and decreased sperm production. Examples of chemical antiandrogens include pharmaceuticals such as the potent androgen receptor antagonist flutamide (Imperato-McGinley et al., 1992), the 5α-reductase inhibitor finasteride (Clark et al., 1990), the fungicide vinclozolin (Gray et al., 1994), and the DDT metabolite *p,p*-DDE (Kelce et al., 1995; You et al., 1998). Dibutylphthalate induces an antiandrogen phenotype in developing rats, but the effect does not appear to be due to direct interaction with the androgen receptor (Mylchreest et al., 1998, 1999). The critical developmental window for the effects of dibutylphthalate was gestation days 16 to 18 in the rat (Carruthers and Foster, 2005). Dietary exposure of pregnant rats to dibutylphthalate from gestation days 12 to 19 resulted in decreases in androgen responsive gene expression and testicular testosterone content in male fetuses and an antiandrogen phenotype in male offspring (Struve et al., 2009). A similar phenotype has been observed in offspring of dibutylphthalate-treated rabbits (Higuchi et al., 2003). Exposure of pregnant rats to mixtures of antiandrogenic chemicals have cumulative effects on development of male offspring (Rider et al., 2009, 2010; Hotchkiss et al., 2010; Hannas et al., 2011). Dose addition models appear to accurately predict the antiandrogenic effects of mixtures of phthalates (Howdeshell et al., 2015). The antiandrogenic effects of developmental exposure to phthalates (described above) has been labeled "the fetal phthalate syndrome" and Furr et al. (2014) have developed a short-term in vivo test, based on fetal testis testosterone production, that can

predict adverse effects on development of the male reproductive tract and other effects of phthalates.

Hypothyroidism during pregnancy and early postnatal development causes growth retardation, cognitive deficits, delayed eye opening, hyperactivity, and auditory defects in rodents. Polychlorinated biphenyls (PCBs) may act at several sites to lower thyroid hormone levels during development, and cause body weight and auditory deficits (Goldey et al., 1995; Goldey and Crofton, 1998). PCBs also cause learning deficits and altered locomotor activity in rodents (Eriksson et al., 1991; Schantz et al., 1995) and monkeys (Bowman, 1982; Schantz et al., 1991). The thyroid hormone synthesis inhibitor propylthiouracil (PTU) has been used to study the effects of chemically induced hypothyroidism on development of the brain (Gilbert, 2011). Addition of 1 to 3 ppm PTU to the drinking water of female rats during pregnancy and lactation produced deficits of thyroxine (T4) in the offspring, but only modest reductions in triiodothyronine (T3, the active thyroid hormone) at the high dose. Excitatory postsynaptic transmission was reduced and long-term potentiation was impaired in adult offspring despite the modest and transient effect on T3 during treatment.

Even mild thyroid deficiency during critical periods of development can have permanent adverse effects on brain development. Mild developmental thyroid insufficiency induces cortical heterotopia in rats (Goodman and Gilbert, 2007; Gilbert et al., 2014) and compromises activity-dependent neuroplasticity in the hippocampus of male rat offspring as adults (Gilbert et al., 2017).

Human Evidence

Despite the biological plausibility of effects demonstrated in numerous laboratory studies, the extent to which human health is being adversely impacted from exposures to environmental endocrine disruptors remains unclear. Reports in humans which are or may be relevant are of two types. The first type of reports are on observations of adverse effects on reproductive system development and function following exposure to chemicals with known endocrine activities that are present in medicines, contaminated food, or the workplace. These have tended to involve relatively high exposures to chemicals with known endocrine effects (e.g., DES). The second type of reports are related to epidemiologic evidence of increasing incidence of adverse reproductive and developmental outcomes having an endocrine basis. With the exception of DES, evidence is either insufficient to support a definitive link to an exposure or is variable across studies. Increasing trends have been reported for cryptorchidism (Toppari et al., 1996), hypospadias (Toppari et al., 1996; Paulozzi et al., 1997), semen quality (Carlsen et al., 1992; Skakkebaek and Keiding, 1994; Olsen et al., 1995; Auger et al., 1995; Bujan et al., 1996; de Mouzon et al., 1996; Fisch and Goluboff, 1996; Irvine et al., 1996; Vierula et al., 1996; Swann et al., 1997), and testicular cancer (Toppari et al., 1996). Recently, connections between environmental exposures and cryptorchidism and hypospadias (Toppari et al., 2010), and early onset of puberty in girls (Mouritsen et al., 2010) have been suggested. In a systematic review and meta-analysis of 185 studies across 40 years, 50 different countries, and 42,935 men, Levine et al. (2017) reported that sperm counts declined significantly between 1973 and 2011 in men from North America, Europe, Australia, and New Zealand.

Considerations of Endocrine Disruption in Screening and Testing

The findings of altered reproductive development following early-life exposures to endocrine disruptors prompted revisions of safety evaluation tests issued by the U.S. Environmental Protection Agency (EPA) (U.S. EPA, 1997). These include assessments of female estrous cyclicity, sperm parameters (total number, percent progressively motile, and sperm morphology in both the parental and F1 generations), the age at puberty in the F1 (vaginal opening in the female, preputial separation in the males); an expanded list of organs for pathology and/or histopathology to identify and characterize effects; as well as some triggered endpoints including anogenital distance in the F2 and primordial follicle counts in the parental and F1 generations. An important modification of prenatal developmental toxicity test guidelines aimed at improved detection of endocrine disruptors is the extension of the period of dosing to near the end of pregnancy in rodents in order to encompass the developmental period of urogenital tract differentiation.

REGULATORY ASSESSMENT OF RISK OF DEVELOPMENTAL TOXICITY

Experience with chemicals that have the potential to induce developmental toxicity in humans indicates that laboratory animal testing, surveillance of the human population (i.e., epidemiologic studies), and alert clinical awareness (Friedman, 2011) are all necessary to provide adequate public health protection. Laboratory animal investigations are guided both by regulatory requirements for drug or chemical marketing and by the need to understand mechanisms of toxicity.

Regulatory Guidelines for In Vivo Testing

Prior to the thalidomide tragedy, safety evaluations for reproductive effects were limited in the types of chemicals evaluated and the sophistication of the endpoints. After thalidomide, the FDA issued more extensive life-stage testing protocols (termed Segments I, II, and III) for application to a broader range of chemicals (U.S. FDA, 1966). These testing protocols, with minor variations, were adopted by regulatory agencies around the world and remained similar for nearly 30 years. Several factors including the historical experience of testing thousands of chemicals, increased knowledge of basic reproductive processes, the ever-rising cost of testing, the acknowledged redundancy and overlap of required protocols, a growing divergence in study design requirements of various countries, and the expanding international presence of the pharmaceutical and agrochemical industries succeeded in producing streamlined testing protocols that have been accepted internationally (U.S. FDA, 1994). These guidelines, the result of the International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), allow considerable flexibility in implementation depending on the particular circumstances of the chemical under evaluation. Key elements of the FDA Segment I, II, and III studies, the ICH protocols, the OECD equivalent of the FDA Segment II test, and the OECD-extended one-generation reproductive toxicity study (EOGRTS) are provided in Table 10-7. Details are available in the original publications and in several reviews (e.g., Manson, 1994; Claudio et al., 1999; Reuter et al., 2003; for the EOGRTS see OECD [2011], Moore et al. [2016], and Beekhuijzen et al. [2016a, 2016b]). Variations of these protocols include extensions of exposure to earlier or later time points in development and extensions of observations to postnatal ages with more sophisticated endpoints. For example, the EPA's Developmental Neurotoxicity Protocol for the rat includes exposure from gestation day 6 through postnatal day 10, and observation of postnatal growth, developmental landmarks of puberty (preputial separation, vaginal opening), motor activity, auditory startle, learning and memory, and neuropathology through postnatal day 60 (U.S. EPA, 1998).

Table 10-7

Summary of In Vivo Regulatory Protocol Guidelines for Evaluation of Developmental Toxicity

STUDY	EXPOSURE	ENDPOINTS COVERED	COMMENTS
Segment I: Fertility and general reproduction study	♂: 10 weeks prior to mating ♀: 2 weeks prior to mating	Gamete development, fertility, pre- and postimplantation viability, parturition, lactation	Assesses reproductive capabilities of ♂ and ♀ following exposure over one spermatogenic cycle or several estrous cycles
Segment II: Teratogenicity test	Implantation (or mating) through end of organogenesis (or term)	Viability and anatomy (external, visceral, skeletal) of fetuses prior to birth	Shorter exposure prevents metabolic adaptation and provides high exposure during gastrulation and organogenesis. Earlier dosing option for bioaccumulative agents. Later dosing to cover reproductive tract development
Segment III: Perinatal study	Last trimester of pregnancy through lactation	Postnatal survival, growth, and external morphology	To observe effects on development of major organ function during the perinatal period; may be more sensitive to adverse effects at this time
ICH 4.1.1: Fertility protocol	♂: 4 weeks prior to mating ♀: 2 weeks prior to mating	♂: Reproductive organ weights and histology, sperm counts, and motility ♀: Viability of conceptuses at mid-pregnancy or later	Improved assessment of ♂ reproductive endpoints; shorter treatment duration than Segment I
ICH 4.1.2: Prenatal and postnatal development, including maternal function	Implantation through end of lactation	Toxicity to pregnant vs. nonpregnant ♀; postnatal viability, growth, and function (behavior, maturation, reproduction)	Similar to Segment I study
ICH 4.1.3: Embryo/fetal development	Implantation through end of organogenesis	Viability and morphology (external, visceral, skeletal) of fetuses prior to birth	Similar to Segment II study. Usually conducted in two species (rodent and nonrodent)
OECD 414: Prenatal developmental	Implantation (or mating) through day prior to cesarean section	Viability and morphology (external, visceral, skeletal) of fetuses prior to birth	Similar to Segment II study. Usually conducted in two species (rodent and nonrodent)
OECD 443: Extended one-generation reproductive toxicity study	♂, ♀: 2 weeks or more prior to mating ♀: Through gestation and lactation ♂ Continue for 10 weeks	Clinical pathology and histopathology of parents and F_1; reproductive organ integrity, DNT, DIT on F_1 cohorts; triggers for F_2 production and testing	Preferred exposure route is dietary; replacement for two-generation study, using more endpoints and reducing animal use; technically challenging; flexible. See citations in text for details

DIT, developmental immunotoxicity tests; DNT, developmental neurotoxicity tests.

In 2006, the Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH, 2006) was published. REACH requires that the toxicity of chemicals marketed or manufactured in the European Union above 1 metric ton per year must be characterized. The requirements are categorized by the annual tonnage of the chemical and are more extensive as the tonnage increases (Rovida et al., 2011). Requirements for reproductive and developmental toxicity are included for chemicals with an annual tonnage of 10 metric ton or more and are expected to require the largest use of live animals under REACH, which, although it encourages use of alternative toxicity tests to reduce animal use, does not currently deem screening tests for developmental toxicity sufficient to replace required whole animal tests (Scialli, 2008; Rovida et al., 2011). Alternatives to avoid animal testing may include read-across considerations of similar chemicals and the use of existing data from published studies (Scialli and Guikema, 2012), and the use of quantitative structure–activity relationship models (Wedebye et al., 2015).

In part because of the development of pharmaceuticals for use in children, the Workshop on Testing Strategies and Design of Juvenile Animal Studies was held in 2003 (Hurtt et al., 2004). Subsequently, the U.S. FDA and the European Medicines Agency (EMA) have promulgated guidelines for determining the need for and the design of nonclinical studies in support of registering such drugs (U.S. FDA, 2006; EMA, 2008). The design of such studies is flexible and would depend in part on the intended use of the drug (e.g., age range, duration of treatment, and drug target). To assess the value of such tests, juvenile animal data were compiled from over 200 studies and reviewed in a workshop (Bailey and Marien, 2011). The consensus was that the survey demonstrated the value of juvenile studies for the development of safe pediatric drugs.

The goal of standard regulatory studies is to identify a No Observed Adverse Effect Level (NOAEL), the highest dosage level that does not produce a significant increase in adverse effects in offspring or juvenile animals. NOAELs are then used in the risk assessment process along with uncertainty factors to assess the likelihood of effects in humans given certain exposure conditions, or to set allowable exposure limits. A preferable alternative, the benchmark dose approach, is discussed later in this chapter.

Multigeneration Tests

Information pertaining to developmental toxicity can also be obtained from studies in which animals are exposed to the test substance continuously over one or more generations (e.g., OECD Test Guideline 416 or U.S. EPA 870.3800). Piersma et al. (2011b) have questioned the value of including a second generation on overall outcome of the assessment. These investigators conducted a retrospective analysis of 498 rat multigenerational studies, and found that the second-generation mating and offspring rarely provided critical information. These authors instead recommended adopting a proposed OECD-extended one-generation test (Cooper, 2009; Vogel et al., 2010) for risk assessment. The extended one-generation test is flexible and could include a second generation if certain triggers were reached in the first generation. Others have argued that the triggers may be too sensitive and will too frequently result in invoking production of the second generation (Beekhuijzen et al., 2009). The extended one-generation reproductive toxicity study (EOGRTS) was adapted by the OECD as the OECD Test Guideline 433 (OECD, 2011).

Concordance of Data Between Laboratory Animals and Humans

There have been several reviews of the similarity of responses of laboratory mammals and humans to developmental toxicants. In general, these studies support the assumption that results from laboratory tests are predictive of potential human effects. Concordance is strongest when there are positive data from more than one test species. In a quantitative sense, the few comparisons that have been made suggest that humans tend to be more sensitive to developmental toxicants than is the most sensitive test species. While concordance among species for chemicals reported as positive is high, often special steps must be taken retrospectively to produce an animal model that reflects the outcome in humans (e.g., valproic acid) (Ehlers et al., 1992).

Frankos (1985) reviewed data for 38 compounds having demonstrated or suspect developmental toxicity in humans; all except tobramycin, which caused otologic defects, were positive in at least one test species and 76% were positive in more than one test species. Predictiveness was highest in the mouse (85%) and rat (80%), with lower rates for rabbits (60%) and hamsters (40%). Frankos identified 165 chemicals with no evidence of human effects; only 29% were negative in all species tested while 51% were negative in more than one species. Schardein and Keller (1989) examined concordance by species and developmental manifestation for 51 potential human developmental toxicants that had adequate animal data (3 human developmental toxicants did not). Thalidomide received the widest testing, with data from 19 species; 53% had data from 3 species, whereas 18% had data from 4 or 5 species. Across all chemicals, the most common findings in humans, rabbits, and monkeys were spontaneous abortion and fetal/neonatal death, followed by malformations and then growth retardation. In the rat, prenatal death, growth retardation, and then malformations was the typical pattern. All species showed at least one positive response for 64% of the human developmental toxicants and, with only one exception, all of the potential human developmental toxicants showed a positive response in at least one species. Overall, the match to the human, regardless of the nature of the developmental response, was rat, 98%; mouse, 91%; hamster, 85%; monkey, 82%; and rabbit, 77%. Jelovsek et al. (1989) reviewed the predictiveness of animal data for 84 negative human developmental toxicants, 33 with unknown activity, 26 considered suspicious, and 32 considered positive. Variables considered included the response of each species, the number of positive and negative species, percent positive and negative species, and mutagenicity and carcinogenicity. The compounds were correctly classified 63% to 91% of the time based on animal data, depending upon how the suspect and unknown human toxicants were considered. The various models had a sensitivity of 62% to 75%, a positive predictive value of 75% to 100%, and a negative predictive value of 64% to 91%. A summary comparison of rodent and rabbit results for 22 human developmental toxicants has been compiled by Daston and Knudsen (2010) (Table 10-8).

Table 10-8

Developmental Toxicity Findings in Rodents and Rabbits for Known Human Developmental Toxicants

CHEMICAL	RODENT	RABBIT	HUMAN
Cyclophosphamide	+	+	+
Diazepam	+		+
Diethylstilbestrol	+	+	+
Phenytoin/trimethadione	+	+	+
Ethanol	+		+
Lithium	+/−		+
Methylmercury	+		+
13-*cis*-Retinoic acid	+	+	+
Testosterone	+	+	+
Thalidomide	−	+	+
Valproic acid	+	+	+
Warfarin	+		+
Fumonisin B_1	+	−	+
Methimazole	+/−		+
Busulfan	+		+
Enalapril/captopril	+		+
Polychlorinated biphenyls	+/−		+
Cocaine	+/−		+
Misoprostol	+	−	+
Penicillamine	+		+
Tetracycline	−		+
Toluene	+		+

The + sign indicates developmental toxicity in that species; − indicates negative findings; +/− indicates an equivocal response. Blank spaces indicate chemical not tested in that species. The type of positive finding is not necessarily the same in all species.

SOURCE: Adapted from Daston and Knudsen (2010).

Children's Health and the Food Quality Protection Act

In 1993, the National Academy of Sciences published a report entitled "Pesticides in the Diets of Infants and Children," which brought to light the fact that infants and children differ both qualitatively and quantitatively from adults in their exposure to pesticide residues in food because of different dietary composition and intake patterns and different activities (NRC, 1993). This report, along with the report from the International Life Sciences Institute, entitled "Similarities and Differences Between Children and Adults" (Guzelian et al., 1992) provided background and impetus for passage of the Food Quality Protection Act (FQPA) of 1996. The FQPA incorporates an additional 10-fold safety factor for children, cumulative effects of toxicants acting through a common mode of action, aggregate exposure (i.e., same toxicant from different sources), and endocrine disruption. The inclusion (at the discretion of the EPA) of the 10-fold factor for calculating allowable intakes for children affects most strongly the pesticide industry, whose products can appear as residues in food. Children have different diets than adults and also have activity patterns that change their exposure profile compared to adults, such as crawling on the floor or ground, putting their hands and foreign objects in their mouths, and raising dust and dirt during play. Even the level of their activity (i.e., closer to the ground) can affect their exposure to some toxicants. In addition to exposure differences, children are growing and developing, which makes them more susceptible to some types of insults. Effects of early childhood exposure, including neurobehavioral effects, obesity, diabetes, cardiovascular disease, and cancer, may not be apparent until later in life (Hanson and Gluckman, 2014).

Alternative Testing Strategies

There has been a significant ongoing effort to identify and evaluate alternative test systems to refine, reduce, and replace whole animal regulatory tests for developmental toxicity (Brannen et al., 2016). Chernoff and Kavlock (1982) proposed a short-term rodent in vivo test. Other alternative assays use cultures of cells, developing organs/structures, and whole embryos in vitro, including those of worms, flies, frogs, fish, and mammals (Chapin et al., 2008; Piersma et al., 2010). Validation of alternative tests is a major issue (Neubert, 1989; Welsch, 1990). Initially, it was proposed that alternative models would be applicable to evaluating all chemicals, and would aid in the prioritization for standard mammalian testing. Despite continued progress, this is still a work in progress. Considering the complexity of embryogenesis and the multiple cellular and molecular mechanisms and target of potential teratogens, it may be unrealistic to expect a single test, or even a small battery of tests, to accurately predict the potential developmental activity of chemicals.

The In Vivo Chernoff–Kavlock Assay The in vivo test using rodents for prescreening chemical-induced developmental toxicity (Chernoff and Kavlock, 1982) streamlines the developmental toxicity assessment. Pregnant females are exposed to a limited number of test compound dosages during the period of major organogenesis. Dosages are selected to be near those inducing maternal toxicity. Assessment focuses on evaluating the offspring over a brief neonatal period for external malformations, growth, and viability. This test has proven reliable to identify developmental toxicants over a large number of chemical agents and classes (Hardin et al., 1987), and a regulatory testing guideline is available (U.S. EPA, 1985).

Rodent and Rabbit Whole Embryo Culture The rodent WEC continues to hold promise as an alternative model system (Augustine-Rauch et al., 2016), and has been extensively used as a tool for studies of chemical mechanism of action. For WEC, rodent or rabbit conceptuses are excised from the mother at an early stage of organogenesis with the embryo and yolk sacs intact (Fig. 10-10). Typically, conceptuses are cultured for up to 48 hours (Ellis-Hutchings and Carney, 2010), after which the embryos are scored for a number of growth and developmental parameters and assigned developmental scores (e.g., rodents: Brown and Fabro, 1981; Van Maele-Fabry et al., 1990; Zhang et al., 2013; rabbits: Carney et al., 2007). As a model for assessing developmental toxicity, WEC has the advantages of directly exposing mammalian embryos to the compound of interest and being able to distribute embryos in the same litter to different treatment groups. Disadvantages include the technically difficult and time-consuming process of excising the embryos and scoring them at the end of culture, and the cost in animals for obtaining the embryos and the rat serum used as culture medium. As an alternative or in addition to morphological scoring, effects of chemicals on gene expression in the embryos have been studied to discern common patterns ("signatures") of transcriptomic effects (Luijten et al., 2010; Robinson et al., 2010, 2011a, 2011b, 2012a, 2012b, 2012c, 2012d). There are key signaling pathways and intermediates in embryological development that hold promise as potential biomarkers of developmental toxicity (Robinson and Piersma, 2013).

Mouse Embryonic Stem Cell Tests Mouse embryonic stem cell tests (mESTs) examine effects of test chemicals on viability and differentiation of mouse embryonic stem cell lines. When the stem cells are placed into hanging drop culture, they aggregate and form "embryoid bodies." Cardiomyocyte differentiation in the cultures can be quantified by examining the cultures for beating cardiomyocytes. In a different mouse embryonic stem cell test, mouse embryonic stem cells are directly plated in an adherent model in 96 well plates and test chemical applied for up to 8 days (Barrier et al., 2011; Chandler et al., 2011) (Fig. 10-10). At the end of culture, cardiomyocyte differentiation is assessed by performing in-cell Westerns for cardiac myosin heavy chain and cell number is assessed using a fluorescent dye technique. Using this technique, the effects of the Phase I chemical library in the EPA's ToxCast program have been completed and a statistical model established for molecular mechanisms associated with altered differentiation reported (Chandler et al., 2011). Mouse embryonic stem cell tests have the advantage of using established cell lines, therefore not requiring live animals. They are also fairly rapid and inexpensive. One disadvantage is that these stem cell models evaluate embryonic cells during an early window of development and do not represent the full spectrum of cellular and molecular mechanisms and targets throughout embryogenesis. Human embryonic stem cell lines and induced pluripotent stem cells are also being developed for toxicity testing, with the obvious advantage of being human cells (Krtolica et al., 2009; Vojnits and Bremer, 2010; Ilic and Ogilvie, 2017). As for whole rodent embryo culture models above, significant progress has been made in identifying perturbations of gene networks in human embryonic stem cells that are predictive of developmental toxicity (Yamane et al., 2016). If key developmental nodes of gene expression networks can be confidently identified and occur in vitro, their predictive value may be very high.

The Zebrafish Submammalian species have been used for many years in the study of normal developmental biology, and among these animal models, the African clawed frog, *Xenopus laevis* or *X. tropicalis* (Bantle, 1995; Fort et al., 2000, 2004; Fort and Paul, 2002), and the zebrafish, *Danio rerio* (Love et al., 2004; Fraysse et al., 2006; Ton et al., 2006; Yang et al., 2009; Brannen et al.,

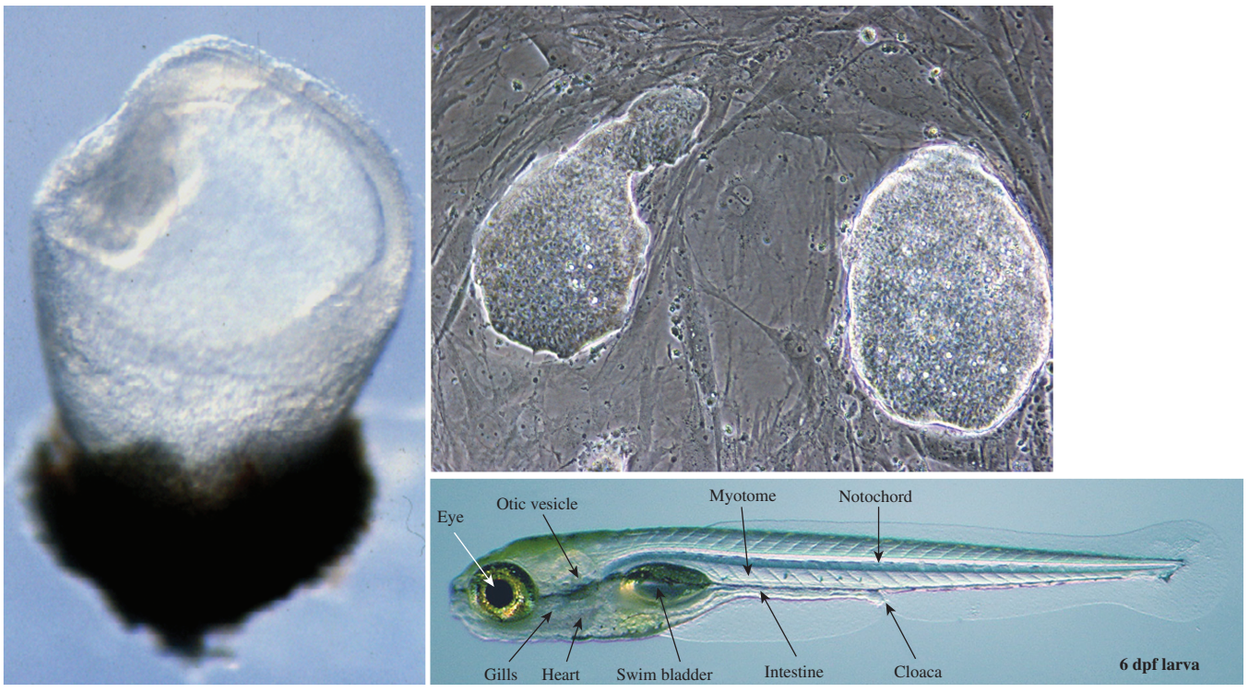

Figure 10-10. *Mouse whole embryo in culture, mouse embryonic stem cells, and zebrafish embryos are commonly used as screens for developmental toxicity. Left:* Mouse gestation day 8 embryo removed from the uterus with intact yolk sac and ectoplacental cone, ready to be cultured for up to 48 hours. (Picture courtesy of Dr E. Sidney Hunter.) *Top right:* Mouse embryonic stem cells (cell line J1) in pluripotent state maintained on a feeder layer of mitotically inactivated mouse embryonic fibroblasts. (Picture courtesy of Dr Kelly Chandler.) *Lower right:* Zebrafish larva at 6 days postfertilization (dpf). Note the transparency of the larva, facilitating observation of internal structures such as those labeled. (Zebrafish larva from Airhart et al. [2007], with permission.)

2010, 2013; McCollum et al., 2011; Sipes et al., 2011; Selderslaghs et al., 2012), have been used for developmental toxicology due to a number of advantages. Chief among the features of these species is rapid external development of the embryos, a large extant literature on their normal development, and the availability of genetic mutants and molecular biological tools for studying these embryos. In addition, they can be bred to produce large numbers of embryos in a relatively short period and are easy and inexpensive to maintain. In recent years, the zebrafish has emerged as the prominent submammalian species in use for developmental toxicity testing.

The zebrafish has gained increased usage in a number of research areas including developmental biology (e.g., Brannen et al., 2013; Truong et al., 2014; Planchart et al., 2016) and cancer research. These small fish exhibit a great deal of homology to higher vertebrates in their development, anatomy, physiology, and behavior. Their rapidly developing embryos are transparent, allowing visualization of internal anatomy (Fig. 10-10). Chemical toxicity studies in zebrafish have been comprehensively compiled and reviewed (McCollum et al., 2011). Zebrafish developmental toxicity testing can be carried out in 96-well plates, an approach used for assessment of the 309 chemical Phase I library in the EPA's ToxCast program (see below) (Padilla et al., 2012). For a review of zebrafish development and use of zebrafish as a disease model, see *Birth Defects Research Part C: Embryo Today: Reviews* 93(2), June 2011, an issue dedicated to this topic.

ToxCast The Toxicity Forecaster (ToxCast) program at the EPA Website https://www.epa.gov/chemical-research/toxicity-forecasting (Kavlock et al., 2012) includes hundreds of high-throughput assays directed at molecular and cellular targets of toxicity, a number of which are relevant to developmental toxicity. Sipes et al. (2011) evaluated statistical associations of ToxCast assay data with the mammalian developmental toxicity data in the EPA's Toxicity Reference Database, ToxRefDB. A high number of associations between the rat and rabbit in vivo data and the ToxCast data were discerned, and species-specific predictive models based on the ToxCast assays exhibited greater than 70% balanced accuracy.

Validation of Alternative Approaches to Testing for Developmental Toxicity Validation studies of the rat WEC test, the rat embryo limb bud micromass assay, and a mouse embryonic stem cell test have been carried out (Genschow et al., 2000, 2002, 2004; Brown, 2002; Piersma et al., 2004; Seiler et al., 2004; Spielmann et al., 2004). These studies involved interlaboratory evaluations of coded chemicals to assess the utility and reproducibility of assays. Analysis of the effects of chemical exposure included the development of "prediction models" which mathematically combine assay endpoints to determine which combinations and formulations are most predictive of mammalian in vivo results.

With continued development of in vitro platforms for developmental toxicity screening, the issue of proper validation of such assays is important. As discussed above, one approach is to develop lists of "positive" and "negative" reference chemicals and then test the ability of assays to properly categorize these. However, this approach does not consider how the concentrations of the chemicals in vitro compare to toxic blood or tissue concentrations in vivo. To address this gap, an approach that classifies chemical *exposures* (including concentrations) rather than chemicals per se as positive or negative has been proposed (Daston et al., 2010, 2014). This approach uses available data on toxic internal (e.g., blood or tissue) levels in vivo (human or test species) to characterize the chemical exposure as positive or negative, such that a given chemical can

be a positive at one concentration and a negative at a lower concentration. These exposure classifications can then be compared to effective concentrations in in vitro assays. A further approach combining generic physiologically based chemical kinetic models to predict in vivo target dose with in vitro toxic concentrations has been proposed for quantitative in vitro to in vivo extrapolation (Fragki et al., 2017).

Structure Activity and Read Across for Developmental Toxicity As we continue to increase our understanding of mechanisms of developmental toxicity, relationships between specific chemical structures or structural components and their effects on developing organisms are elucidated, increasing confidence in our ability to predict developmental toxicity based on chemical structure alone. This is, of course, the ultimate replacement for animal research, in that it requires only computational and bioinformatics tools. Approaches for quantitative structure–activity relationships have been developed over at least a couple of decades (Kavlock, 1990; Richard and Hunter 1996; Arena et al., 2004; Matthews et al., 2007a, 2007b; Wedebye et al., 2015). Read-across methods for predicting developmental toxicity of related chemicals can be employed using shared chemical structural components, or commonalities in their effects on the transcriptome. A good example of using shared structural components is the framework developed by Wu et al. (2013), based on known modes of action and empirical associations of chemical fragments in known developmental toxicants. This framework is a decision tree based on chemical structural features, which places chemicals into groups based on predicted receptor binding and chemical domains. The authors were able to categorize 716 developmental and reproductive toxicants into 25 categories using this expert system. A good example of classifying chemicals based on shared transcriptomal responses is the work of Rempel et al. (2015), who evaluated the effect of several histone deacetylase (HDAC) inhibitors and mercurials on the generation of neuroectoderm from human pluripotent stem cells. Their transcriptomic analysis correctly correlated gene expression with developmental toxicity among the HDAC inhibitors, and the expression of only eight genes was able to separate HDAC inhibitors from mercurials.

Contemporary and Emerging Elements of Developmental Toxicity Risk Assessment

As discussed below, there are a number of issues with using the No Adverse Effect Level (NOAEL) as the point of departure for risk assessments. Further, our present focus on apical outcomes such as malformations or lower fetal weight reveals little as to the initial target of the toxicant under study. The development of the benchmark-dose approach over the past 20 years or so has provided a clear improvement to the NOAEL, and approaches such as the development of biologically based dose–response models and adverse outcome pathways begin to piece together the early events in the etiology of abnormal development, in the hope that such knowledge will facilitate both more scientifically plausible risk assessments and relevant high-throughput assays based on key events leading to elucidated pathways.

The Benchmark Dose versus the No Observed Adverse Effect Level (NOAEL) The use of safety or uncertainty factors applied to an experimentally derived NOAEL to arrive at a presumed safe level of human exposure is predicated on the risk assessment assumption that a threshold for developmental toxicity exists (see the section "Principles of Developmental Toxicology"). A threshold should not be confused with the NOAEL, as the NOAEL is dependent entirely on the power and design of the study. Nor should the value obtained by the application of uncertainty factors to the NOAEL be confused with a threshold, as this exposure is only assumed to be without appreciable added risk.

The use of the NOAEL in the risk assessment process has been criticized for several reasons. Because it is dependent on statistical power to detect pair-wise differences between a treated and a control group, the use of larger sample sizes and more dose groups (which might better characterize the dose–response relationship) can only yield lower NOAELs, and thus better experimental designs are actually penalized by this approach. In addition, the NOAEL is limited to an experimental dose level, and an experiment might need to be repeated to develop a NOAEL for risk assessment. A final point relates to the fact that, given varying experimental designs and variability of control values, NOAELs actually represent different levels of risk in different studies.

Crump (1984) proposed using a mathematical model to estimate the lower confidence bounds on a predetermined level of risk (the "benchmark dose" [BMD]) as a means of avoiding many of the disadvantages of the NOAEL. The application of this approach to a large compilation of Segment II type data sets (Allen, et al., 1994a, 1994b; Faustman et al., 1994; Kavlock et al. 1995) demonstrated that a variety of mathematical models, including those that incorporate developmental-specific features such as litter size and intralitter correlations, can be readily applied to standard test results. On average, benchmark doses based on a 5% added risk of effect calculated on quantal endpoints (e.g., whether an implant was affected or not) were approximately equivalent to traditionally determined NOAELs. When the litter was used as the unit of response (did it contain at least one affected implant?), benchmarks calculated for a 10% added risk were most similar to the correspondingly determined NOAEL. Discrepancies between the benchmark dose and the NOAEL were most pronounced when one or more of the following conditions were present: a shallow dose–response, small sample sizes, wide spacing of experimental dosage levels, or more than the typical number of dose levels. These features tend to make determination of the NOAEL more problematic (usually higher) and the confidence limits around the maximum likelihood estimate broader (resulting in lower BMDs). Software, including the U.S. EPA's Benchmark Dose (BMDS) and Categorical Regression (CatReg), which determine whether data from separate studies can be combined into a single analysis, are available to the public at https://www.epa.gov/bmds.

Biologically Based Dose–Response Models and Adverse Outcome Pathways The introduction of statistical dose–response models for noncancer endpoints is the first step in developing quantitative, mechanistic models that will help reduce uncertainties in high-to-low dose and species-to-species extrapolation of experimental data. Biologically based dose–response models integrate pharmacokinetic information on target tissue dosimetry with molecular/biochemical responses, cellular/tissue responses, and developmental toxicity (O'Flaherty, 1997; Lau and Setzer, 2000; Lau et al., 2001; Setzer et al., 2001). Gaylor and Razzaghi (1992) proposed a model that related induction of cleft palate to fetal growth inhibition, and Gaylor and Chen (1993) proposed a model relating fetal weight and the probability of fetal abnormality. Shuey et al. (1994) presented a model using the cancer chemotherapeutic 5-fluorouracil. These investigators postulated that the developmental toxicity observed in the term fetus was due to an active metabolite (FdUMP) inhibiting the enzyme thymidylate synthetase, causing depletion of thymidine, decreased DNA synthetic rates,

reduced cell proliferation, and ultimately, reduced tissue growth and differentiation. Each step in the pathogenesis was quantified experimentally and the relationships were described by Hill equations. The individual equations were then linked in an integrated model to describe the entire relationship between administered dose and the incidence of hind limb defects (Fig. 10-11). While this is still an empirically based model, the process clearly demonstrated the utility of the approach in understanding the relative importance of various pathways of abnormal development and in providing a basis for models that incorporate species-specific response

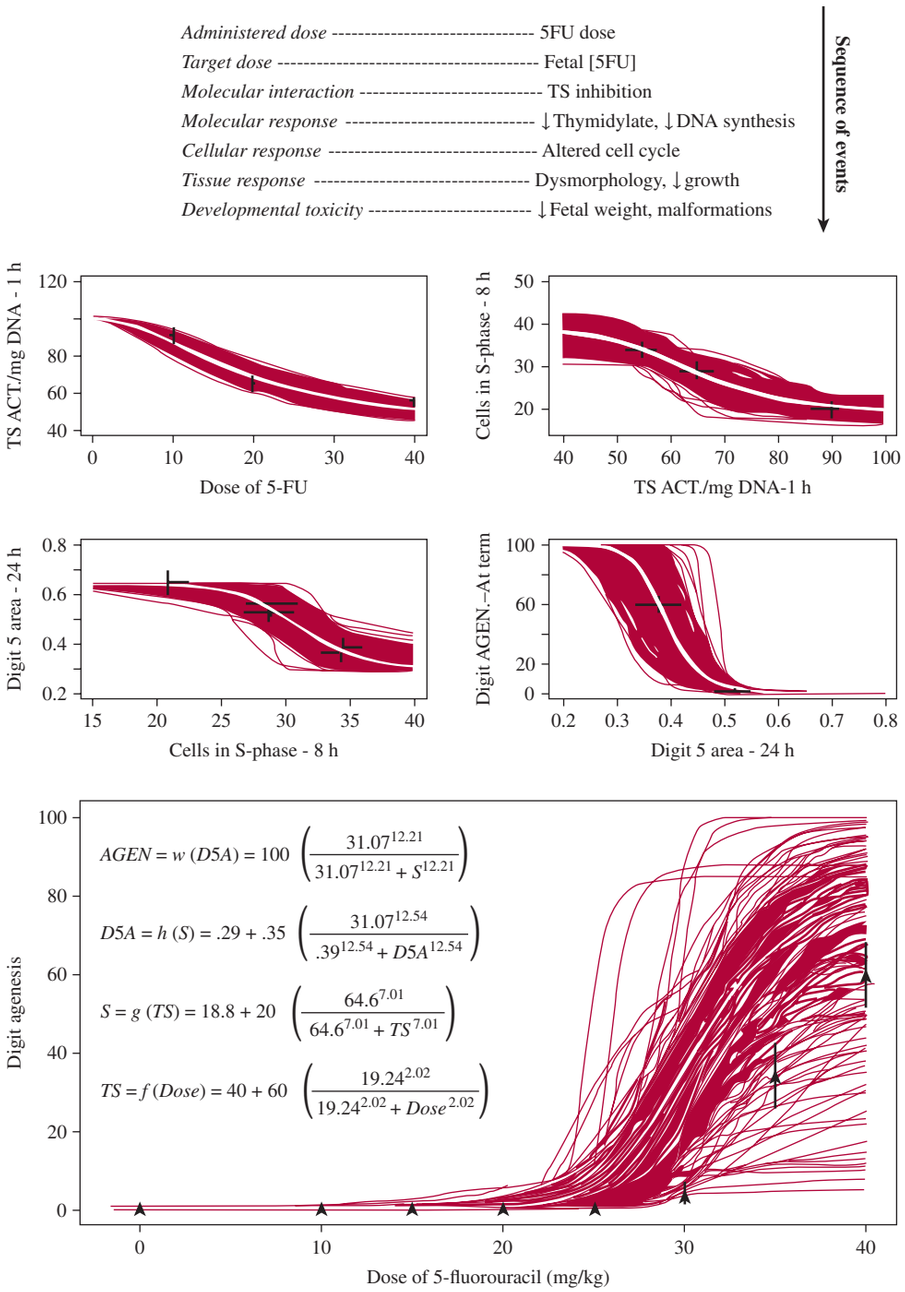

Figure 10-11. *Biologically based dose–response modeling of the developmental toxicity of 5-fluorouracil (5-FU) following maternal administration of gestation day 14 in the rat. Top:* Proposed model for the developmental toxicity of 5-FU based on thymidylate synthetase (TS) inhibition, decreased DNA synthesis, cell cycle alterations, growth deficits, and hind-limb dysmorphogenesis. *Middle:* Relationships between successive endpoints are shown in these four panels (hind-limb-bud TS activity vs. 5-FU dose, S-phase accumulation vs. TS activity, limb digit 5 area at 24 hours postdose vs. proportion of cells in S-phase, and digit dysgenesis at term vs. digit 5 area at 24 hours). Data were fitted with Hill equations. The curves were generated by Monte Carlo simulation to estimate variability around the predicted relationships. *Bottom:* Integrated model for the induction of hind-limb defects by 5-FU, generated by integration of the individual Hill equations describing the relationships between successive model endpoints as presented in the middle panels. Individual equations are listed and the curves are the results of Monte Carlo simulations of the whole sequence of event relationships. Results indicate that variability in the intermediate events can account for differences between the actual and predicted dose responses. AGEN, digit agenesis at term; D5A, digit 5 area at 24 hours; S, percent of cells in S-phase. (Adapted from Shuey et al. [1994]; images courtesy of Dr Woodrow Setzer.)

parameters. Understanding the mode of action for any developmental toxicant can greatly inform the human risk assessment process. Although the construction of BBDR models exemplifies the extent of sophistication and quantification to which such exercises can be pursued, even more qualitative information on modes of action can have a great impact on the interpretation of developmental toxicity test results. For excellent examples of the utility of mode of action information, the reader is referred to an issue of *Critical Reviews in Toxicology* (35[September–October], 2005) devoted to this topic. Developmental toxicants for which mode of action is discussed include ethylene glycol (Corley et al., 2005), nicotine (Slikker et al., 2005), phthalate esters (Foster, 2005), vinclozolin (Kavlock and Cummings, 2005), valproic acid (Wiltse, 2005), hemoglobin-based oxygen carriers (Holson et al., 2005), angiotensin-converting enzyme inhibitors (Tabacova, 2005), and propylthiouracil (Zoeller and Crofton, 2005).

The evolution of mechanistic modeling of adverse toxicological outcomes has led to the concept of adverse outcome pathways (AOPs), which are chemical-agnostic descriptions of the etiology of an adverse apical outcome such as a birth defect (Edwards et al., 2016). They are similar to BBDR models except that the chemical or agent that causes the adverse effect is not stipulated. The first step in an AOP is called the molecular initiating event, which occurs at the first interaction of the toxic moiety with the cell or organism. With adequate initiation, this is followed by a pathogenic series of key events leading to an adverse outcome. The BBDR model for 5-FU in essence contains an AOP, in which inhibition of thymidylate synthetase is the initiating event, digit agenesis is the adverse outcome, and reduced DNA synthesis and cell cycle blockage are key events. The quantitative relationships between key events are called key event relationships, represented in the 5-FU BBDR model by the Hill equations shown in Fig. 10-11.

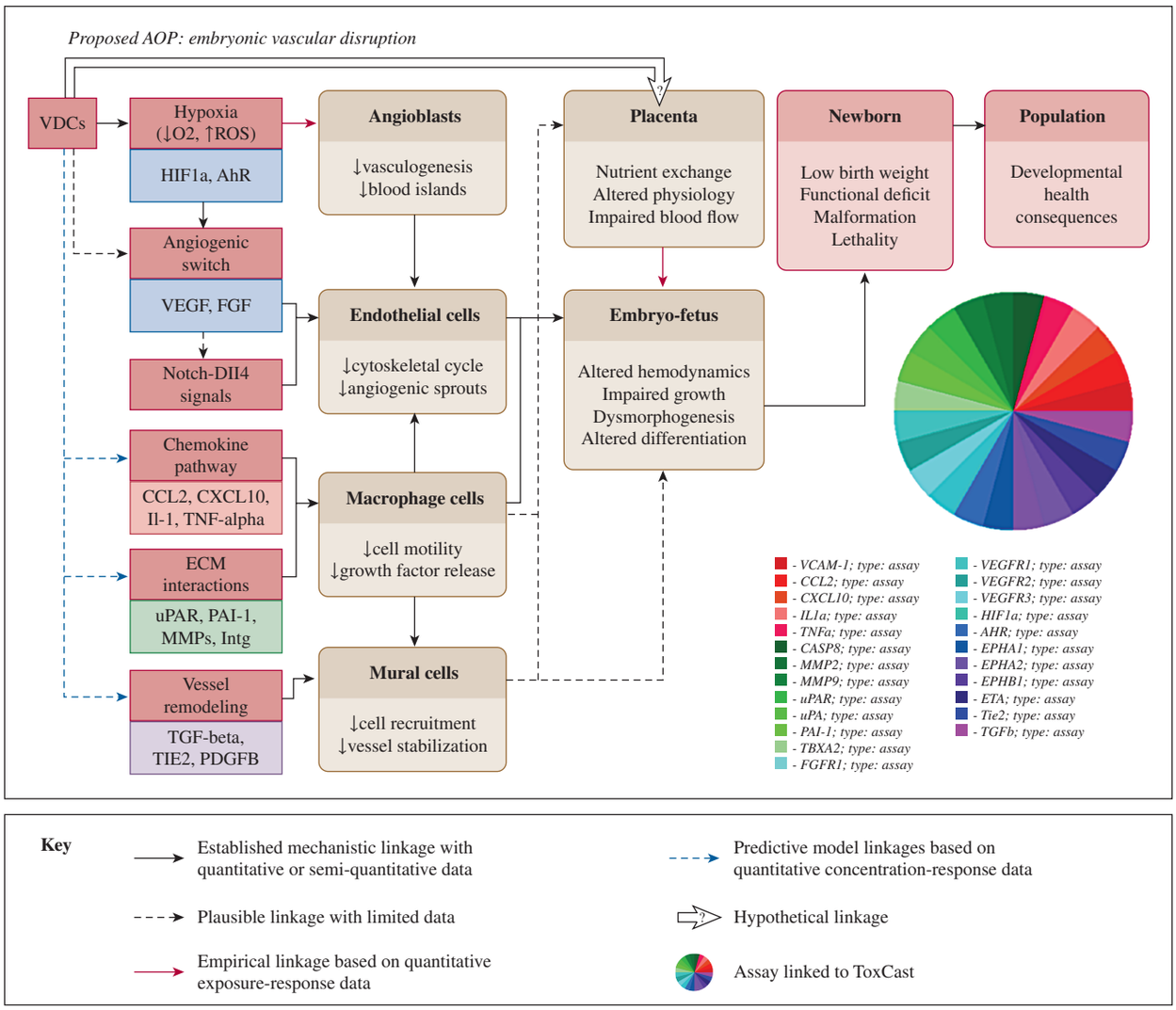

Figure 10-12. *Adverse outcome pathway (AOP) for embryonic vascular disruption.* This AOP for embryonic vascular disruption was built using high-throughput screening data from the EPA's ToxCast database. Anchor 1 (red boxes on the left side) addresses macromolecular interactions of the toxicant with elements of the pathway. Anchor 2 (red boxes on the right) refer to relevant organismal and population responses. The middle columns address intervening cellular and organ responses. The color wheel indicates sectors for 25 ToxCast assays shown that had evidence of abnormal embryonic vascular development based on genetic mouse models and mapped to previously identified critical pathways. VDC, vascular disrupting chemical. (Adapted from Knudsen and Kleinstreuer [2011].)

PATHWAYS TO THE FUTURE

In 2000, a committee assembled by the National Research Council (NRC) released its report, *Scientific Frontiers in Developmental Toxicology and Risk Assessment* (NRC, 2000), which concluded that major discoveries have been made about mechanisms of normal development that have been conserved in diverse animals. Many of these animals have been used extensively in developmental biology and genetics, including the fruit fly, roundworm, zebrafish, frog, chick, and mouse. That these key pathways have been preserved over millions of years of evolution and speciation provides a strong scientific rationale for using these animal models for developmental toxicology. These organisms are advantageous for developmental toxicity studies due to their well-known genetics and embryology, their rapid generation time, and the ability to manipulate their genetics to probe specific developmental pathways or to incorporate human genes, such as those of drug metabolizing enzymes, to answer questions of interspecies extrapolation. As discussed above, there has been a great deal of progress toward using alternative approaches for evaluating developmental toxicity. A landmark NRC report entitled *Toxicity Testing in the 21st Century: A Vision and Strategy* (NRC, 2007) laid out an approach toward revolutionizing the way that toxicity is assessed, moving from expensive and time-consuming animal studies to high-throughput in vitro assays

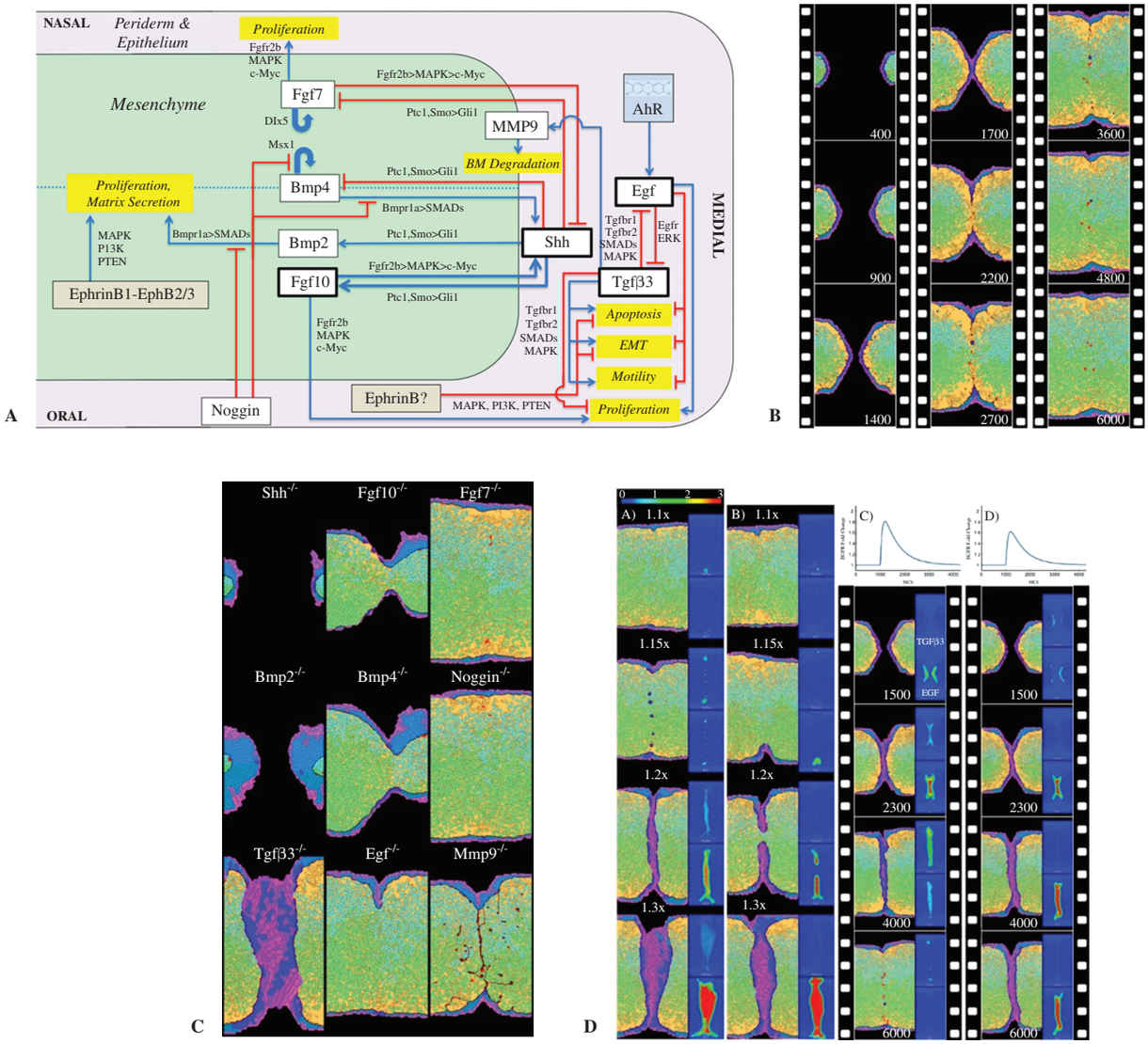

Figure 10-13. *Computational agent–based model of normal palate development and effects of virtual gene knockout or chemical toxicity.* Temporally, the model begins with the palatal shelves elevated to the horizontal position. The model includes mesenchymal, epithelial, and periderm cells, as well as basement membrane and extracellular matrix. **A**. Signaling pathways and molecules incorporated into the model and driving cellular (agent) behavior and interactions. **B.** Model simulation of normal palatogenesis, exhibiting growth (*left column top to bottom*), medial contact of the palatal shelves (*middle column top to bottom*), and dissolution of the medial edge epithelium and fusion of the shelves (*right column top to bottom*). **C.** Effects of virtual (computational) knockout of the various signaling molecules shown in panel A. Effects range from lack of growth to lack of fusion to incomplete fusion to normal fusion. **D**. Simulated fusion defects induced by TCDD exposure via AhR-mediated fold changes in EGFR (fold changes indicated in columns A and B) and by all-trans retinoic acid (columns C and D) displayed for time-dependent changes in EGFR. (Adapted from Hutson et al. [2017].)

based on cellular toxicity pathways and analyzing outputs using a systems biology approach. This report in part formed the basis for the EPA's ToxCast program discussed earlier, including assays relevant to developmental toxicity. While full implementation of such approaches is still years away, in the interim these assays are beginning to provide data for prioritizing chemicals for further study.

Advances in Genomics, Proteomics, Metabolomics, and Bioinformatics

As our knowledge of the genetic and epigenetic control of normal development has advanced immensely in the 21st century, so has the technology to examine gene expression and its control, networks of interrelated gene products, and changes in gene expression induced by alterations in the developmental environment. Advances in genomics, proteomics, metabolomics, and bioinformatics are being used to advance our understanding of health and disease, including normal and abnormal development. The ability to gather and analyze large amounts of biological data using databases and models of biological systems allows a more comprehensive approach to understanding pathways to developmental toxicity. Systems modeling and virtual tissues are among the nascent cutting-edge approaches being developed (Knudsen and Daston, 2010). An excellent example is the modeling of a proposed AOP for embryonic vascular disruption (Knudsen and Kleinstreuer, 2011). The model includes a number of potentially responsive gene networks involved in responses to hypoxia, the angiogenic switch, extracellular matrix interactions, and vessel remodeling, as well as predicted downstream consequences of disrupting these networks (Fig. 10-12). Further work on mechanisms underlying vascular dysgenesis during development can be found in Kleinstreuer et al. (2011) and Ellis-Hutchings et al. (2017).

Computational and Organotypic Models of Developing Systems

Much like the programs that allow a computer to play chess at a high level, software is being developed that can recapitulate development of tissues and organs based on the properties and relationships of cells. These so-called agent-based models (the cell is the agent) allow dynamic interactions among cells that through simulated morphogenesis can build good facsimiles of embryological structures. These models contain signaling pathways, receptors, and extracellular matrices that invoke specific cell behaviors such as adhesion, migration, mitosis, apoptosis, and differentiation. An excellent example is the recent development of a computational model of secondary palate fusion and cleft palate pathogenesis (Hutson et al., 2017) (Fig. 10-13). Signaling pathways underlying palate development are well understood and include TGF-β, BMP, FGF, EGF, SHH, noggin, and ephB. This signaling network drives the virtual palate model as diagrammed in Fig. 10-13A. The model begins at the prefusion stage at which the palatal shelves have elevated to the horizontal position, and the shelves consist of mesenchyme, basal epithelium and periderm, basement membrane, and extracellular matrix. The signaling pathways and gradients drive growth and fusion through contact of the medial edge epithelia and dissolution of the medial edge seam (Fig. 10-13B). Computational gene knockouts of signaling molecules can be executed to observe effects on phenotype (Fig. 10-13C), and predictions of the effects of chemical exposure during palatogenesis can be made based on knowledge of the underlying mechanisms of toxicity (Fig. 10-13D).

In vitro techniques for developmental toxicology and other disease studies are rapidly progressing from two-dimensional cell culture systems to complex three-dimensional "organoid" cultures (Schweiger and Jensen, 2016; Belair and Abbott, 2017). This approach promises to provide systems that have adequate complexity to recapitulate facets of morphogenesis from precursor cells not possible in current two-dimensional cell cultures. Several groups are currently developing these "organ-on-a-chip" models of organogenesis.

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REFERENCES

Abbott BD, Wolf CJ, Das KP, et al. Developmental toxicity of perfluorooctane sulfonate (PFOS) is not dependent on expression of peroxisome proliferator activated receptor-alpha (PPAR alpha) in the mouse. *Reprod Toxicol*. 2009;27:258–265.

Abbott BD, Wolf CJ, Schmid JE, et al. Perfluorooctanoic acid induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator activated receptor-alpha. *Toxicol Sci*. 2007;98:571–581.

Abel EL. Consumption of alcohol during pregnancy: a review of effects on growth and development of offspring. *Hum Biol*. 1982;54:421–453.

Abel EL. Factors affecting the outcome of maternal alcohol exposure: I. Parity. *Neurobehav Toxicol Teratol*. 1984;3:49–51.

Abel EL. Fetal alcohol syndrome: a cautionary note. *Curr Pharm Des*. 2006;12:1521–1529.

Abel EL, Church MW, Dintcheff BA. Prenatal alcohol exposure shortens life span in rats. *Teratology*. 1987; 36:217–220.

Adams J. The neurobehavioral teratology of retinoids: a 50-year history. *Birth Defects Res A Clin Mol Teratol*. 2010;88:895–905.

Adler T. The return of thalidomide. *Science News*. 1994;146:424–425.

Ahokas RA, Dilts PV Jr, LaHaye EB. Cadmium-induced fetal growth retardation: protective effect of excess dietary zinc. *Am J Obstet Gynecol*. 1980;136:216–221.

Airhart MJ, Lee DH, Wilson TD, Miller BE, Miller MN, Skalko RG. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). *Neurotoxicol Teratol*. 2007;29:652–664.

Al Jishi T, Sergi C. Current perspective of diethylstilbestrol (DES) exposure in mothers and offspring. *Reprod Toxicol*. 2017;71:71–77.

Alford CA, Stagno S, Reynolds DW. Perinatal infections caused by viruses, toxoplasma, and Treponema pallidum. In: Aldjem S, Brown AK, eds. *Clinical Perinatology*. St. Louis, MO: Mosby; 1974:31.

Allen BC, Kavlock RJ, Kimmel CA, Faustman EM. Dose response assessment for developmental toxicity: II. Comparison of generic benchmark dose models with No Observed Adverse Effect Levels. *Fundam Appl Toxicol*. 1994a;23:487–495.

Allen BC, Kavlock RJ, Kimmel CA, Faustman EM. Dose response assessment for developmental toxicity: III. Statistical models. *Fundam Appl Toxicol*. 1994b;23:496–509.

Alsaad AM, Chaudhry SA, Koren G. First trimester exposure to topiramate and the risk of oral clefts in the offspring: a systematic review and meta-analysis. *Reprod Toxicol*. 2015;53:45–50.

Amemiya K, Hurley LS, Keen CL. Effect of the anticarcinogenic drug 6-mercaptopurine on mineral metabolism in the mouse. *Toxicol Lett*. 1985;25:55–62.

Amemiya K, Hurley LS, Keen CL. Effect of 6-mercaptopurine on ^{65}Zn distribution in the pregnant rat. *Teratology*. 1989;39:387–393.

Amstislavsky SY, Amstislavskaya TG, Amstislavsky VS, et al. Reproductive abnormalities in adult male mice following preimplantation exposures to estradiol or pesticide methoxychlor. *Reprod Toxicol*. 2006;21:154–159.

Amstislavsky SY, Kizilova EA, Eroschenko VP. Preimplantation mouse embryo development as a target of the pesticide methoxychlor. *Reprod Toxicol*. 2003;17:79–86.

Amstislavsky SY, Kizilova EA, Golubitsa AN, et al. Preimplantation exposures of murine embryos to estradiol or methoxychlor change postnatal development. *Reprod Toxicol*. 2004;18:103–108.

Anway MD, Memon MA, Uzumcu M, Skinner MK. Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *J Androl*. 2006;147:5524–5541.

Arafa HM, Elmazar MM, Hamada FM, et al. Selective agonists of retinoic acid receptors: comparative toxicokinetics and embryonic exposure. *Arch Toxicol*. 2000;73:547–556.

Arena VC, Sussman NB, Mazumdar S, et al. The utility of structure–activity relationship (SAR) models for prediction and covariate selection in developmental toxicity: comparative analysis of logistic regression and decision tree models. *SAR QSAR Environ Res*. 2004;15:1–18.

Argiles JM, Carbo N, Lopez-Soriano FJ. Was tumour necrosis factor-alpha responsible for the fetal malformations associated with thalidomide in the early 1960s? *Med Hypotheses*. 1998;50:313–318.

Ashby R, Davis L, Dewhurst BB, et al. Aspects of the teratology of cyclophosphamide (NSC-26271). *Cancer Treat Rep*. 1976;60:477–482.

Auger J, Kunstmann JM, Czyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years. *N Engl J Med*. 1995;332:281–285.

Augustine K, Liu ET, Sadler TW. Antisense attenuation of Wnt-1 and Wnt-3a expression in whole embryo culture reveals roles for these genes in craniofacial, spinal cord, and cardiac morphogenesis. *Dev Genet*. 1993;500–520.

Augustine-Rauch K, Zhang CX, Panzica-Kelly JM. A developmental toxicology assay platform for screening teratogenic liability of pharmaceutical compounds. *Birth Defects Res B Dev Reprod Toxicol*. 2016;107:4–20.

Baer JS, Sampson PD, Barr HM, Connor PD, Streissguth AP. A 21-year longitudinal analysis of the effects of prenatal alcohol exposure on young adult drinking. *Arch Gen Psychiatry*. 2003;60:377–385.

Bailey GP, Marien D. The value of juvenile animal studies "What have we learned from preclinical juvenile toxicity studies?. *Birth Defects Res B Dev Reprod Toxicol*. 2011;92:273–291.

Bakker H, Jaddoe VW. Cardiovascular and metabolic influences of fetal smoke exposure. *Eur J Epidemiol*. 2011;26:763–770.

Balling R, Mutter G, Gruss P, Kessel M. Craniofacial abnormalities induced by ectopic expression of the homeobox gene Hox-1.1 in transgenic mice. *Cell*. 1989;58:337–347.

Bantle JA. FETAX: A developmental toxicity assay using frog embryos. In: Rand G, ed. *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, Risk Assessment*. 2nd ed. Washington, DC: Taylor & Francis; 1995:207–230.

Barlow SM, McElhatton PR, Sullivan FM. The relation between maternal restraint and food deprivation, plasma corticosterone, and cleft palate in the offspring of mice. *Teratology*. 1975;12:97–103.

Barouki R, Gluckman PD, Grandjean P, et al. Developmental origins of non-communicable disease: implications for research and public health. *Environ Health*. 2012;11:42.

Barrier M, Jeffay S, Nichols HP, et al. Mouse embryonic stem cell adherent cell differentiation and cytotoxicity (ACDC) assay. *Reprod Toxicol*. 2011;31:383–391.

Bartlett JB, Dredge K, Dalgleish AG. The evolution of thalidomide and its imid derivatives as anticancer agents. *Nat Rev Cancer*. 2004;4:314–322.

Bateson W. *Materials for the Study of Variation Treated with Especial Regard to Discontinuity in the Origin of Species*. London: Macmillan; 1894.

Beaty TH, Ruczinski I, Murray JC, et al. Evidence for gene–environment interaction in a genome wide study of nonsyndromic cleft palate. *Genet Epidemiol*. 2011;35:469–478.

Bedell VM, Westcot SE, Ekker SC. Lessons from morpholino-based screening in zebrafish. *Brief Funct Genomics*. 2011;10:181–188.

Beekhuijzen M, Barentsen H, Marsden E, et al. Implementing the extended one-generation reproductive toxicity study (EOGRTS): important points to consider. *Crit Rev Toxicol*. 2016a;46:332–347.

Beekhuijzen M, van Otterdijk F, Wieland W, et al. Update of OECD DART guidelines with endocrine disruptor relevant endpoints: practical considerations. *Reprod Toxicol*. 2016b;64:64–71.

Beekhuijzen M, Zmarowski A, Emmen H, et al. To mate or not to mate: a retrospective analysis of two-generation studies for evaluation of criteria to trigger additional mating in the extended one-generation design. *Reprod Toxicol*. 2009;28:203–208.

Belair DG, Abbott BD. Engineering epithelial–stromal interactions in vitro for toxicology assessment. *Toxicology*. 2017;382:93–107.

Bendich A. Folic acid and neural tube defects. Introduction to Part II. *Ann NY Acad Sci*. 1993;678:108–111.

Bennett GD, Amore BM, Finnell RH, et al. Teratogenicity of carbamazepine-10,11-epoxide and oxcarbazepine in the SWV mouse. *J Pharmacol Exp Ther*. 1996;279:1237–1242.

Bennett GD, Wlodarczyk B, Calvin JA, et al. Valproic acid–induced alterations in growth and neurotrophic factor. *Reprod Toxicol*. 2000;14:1–11.

Beydoun H, Saftlas AF. Physical and mental health outcomes of prenatal maternal stress in human and animal studies: a review of recent evidence. *Paediatr Perinat Epidemiol*. 2008;22:438–466.

Beyer PE, Chernoff N. The induction of supernumerary ribs in rodents: role of maternal stress. *Teratogenesis Carcinog Mutagen*. 1986;6:419–429.

Bibbo M, Gill W, Azizi F, et al. Follow-up study of male and female offspring of DES-exposed mothers. *Obstet Gynecol*. 1977;49:1–8.

Biegel LB, Flaws JA, Hirshfield AN et al. 90-Day feeding and one-generation reproduction study in Crl:CD BR rats with 17 beta-estradiol. *Toxicol Sci*. 1998;44:116–142.

Bowman RE. Behavioral sequelae of toxicant exposure during neurobehavioral development. In: Hunt VR, Smith MK, Worth D, eds. *Banbury Report 11: Environmental Factors in Human Growth and Development*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1982:283–294.

Branham WS, Zehr DR, Chen JJ, Sheehan DM. Uterine abnormalities in rats exposed neonatally to diethylstilbestrol, ethynyl estradiol, or clomiphene citrate. *Toxicology*. 1988;51:201–212.

Brannen KC, Chapin RE, Jacobs AC, Green ML. Alternative models of developmental and reproductive toxicity in pharmaceutical risk assessment and the 3Rs. *ILAR J*. 2016;57:144–156.

Brannen KC, Charlap JH, Lewis EM. Zebrafish teratogenicity testing. *Methods Mol Biol*. 2013;947:383–401.

Brannen KC, Panzica-Kelly JM, Danberry TL et al. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth Defects Res B Dev Reprod Toxicol*. 2010;89:66–77.

Brent RL, Beckman DA. Environmental teratogens. *Bull NY Acad Med*. 1990;66:123–163.

Brown NA. Selection of test chemicals for the ECVAM international validation study on in vitro embryotoxicity tests. European Centre for the Validation of Alternative Methods. *Altern Lab Anim*. 2002;30:177–198.

Brown NA, Fabro S. Quantitation of rat embryonic development in vitro: a morphological scoring system. *Teratology*. 1981;24:65–78.

Bruin JE, Gerstein HC, Holloway AC. Long-term consequences of fetal and neonatal nicotine exposure: a critical review. *Toxicol Sci*. 2010;116:364–374.

Brunton PJ. Effects of maternal exposure to social stress during pregnancy: consequences for mother and offspring. *Reproduction*. 2013;146:R175–189.

Bujan L, Mansat A, Pontonnier F, Mieusset R. Time series analysis of sperm concentration in fertile men in Toulouse, France between 1977 and 1992. *Br Med J*. 1996;312:471–472.

Calabrese L, Fleischer AB. Thalidomide: current and potential clinical applications. *Am J Med*. 2000;108:487–495.

Capecchi MR. Altering the genome by homologous recombination. *Science*. 1989;244:1288–1292.

Carey JC, Martinez L, Balken E, et al. Determination of human teratogenicity by the astute clinician method: review of illustrative agents and a proposal of guidelines. *Birth Defects Res A Clin Mol Teratol*. 2009;85:63–68.

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *Br Med J*. 1992;305:609–613.

Carney EW. Maternally mediated developmental toxicity. In: McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*. New York: Elsevier; 2010:163–176.

Carney EW, Scialli AR, Watson RE, et al. Mechanisms regulating toxicant disposition to the embryo during early pregnancy: an interspecies comparison. *Birth Defects Res C Embryo Today*. 2004;72:345–360.

Carney EW, Tornesi B, Keller C, et al. Refinement of a morphological scoring system for postimplantation rabbit conceptuses. *Birth Defects Res B Dev Reprod Toxicol*. 2007;80:213–222.

Carruthers CM, Foster PM. Critical window of male reproductive tract development in rats following gestational exposure to di-*n*-butyl phthalate. *Birth Defects Res B Dev Reprod Toxicol*. 2005;74:277–285.
Centers for Disease Control and Prevention (CDC). Valproic acid and spina bifida: a preliminary report–France. *MMWR*. 1982;31:565–566.
Centers for Disease Control and Prevention (CDC). Valproate: a new cause of birth defects–report from Italy and follow-up from France. *MMWR*. 1983;32:438–439.
Centers for Disease Control and Prevention (CDC). Use of vitamins containing folic acid among women of childbearing age—United States, 2004. *MMWR*. 2004;36:847–850.
Chambers CD., Scialli A. Epidemiological factors in developmental toxicology. In: McQueen CA, ed. *Comprehensive Toxicology*, 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*. New York: Elsevier; 2010:135–44.
Chambon P. The retinoid signaling pathway: molecular and genetic analyses. *Semin Cell Biol*. 1994;5:115–125.
Chandler KJ, Barrier M, Jeffay S, et al. Evaluation of 309 environmental chemicals using a mouse embryonic stem cell adherent cell differentiation and cytotoxicity assay. *PLoS One*. 2011;6:e18540.
Chandy S, Abraham AM, Jana AK, et al. Congenital rubella syndrome and rubella in Vellore, South India. *Epidemiol Infect*. 2011;139:962–966.
Chapin R, Augustine-Rauch K, Beyer B, et al. State of the art in developmental toxicity screening methods and a way forward: a meeting report addressing embryonic stem cells, whole embryo culture, and zebrafish. *Birth Defects Res B Dev Reprod Toxicol*. 2008;83:446–456.
Chater-Diehl EJ, Laufer BI, Singh SM. Changes to histone modifications following prenatal alcohol exposure: an emerging picture. *Alcohol*. 2017;60:41–52.
Chen M, Doherty SD, Hsu S. Innovative uses of thalidomide. *Dermatol Clin*. 2010;28:577–586.
Chernoff GF. The fetal alcohol syndrome in mice: an animal model. *Teratology*. 1977;15:223–230.
Chernoff N, Kavlock RJ. An in vivo teratology screen utilizing pregnant mice. *J Environ Toxicol Health*. 1982;10:541–550.
Chernoff N, Rogers JM, Alles AJ, et al. Cell cycle alterations and cell death in cyclophosphamide teratogenesis. *Teratogenesis Carcinog Mutagen*. 1989;9:199–209.
Chernoff N, Setzer RW, Miller DM, et al. Effects of chemically induced maternal toxicity on prenatal development in the rat. *Teratology*. 1990;42:651–658.
Clark DO. Pharmacokinetic studies in developmental toxicology: practical considerations and approaches. *Toxicol Meth*. 1993;3:223–251.
Clark DO, Elswick BA, Welsch F, Conolly R. Pharmacokinetics of 2-methoxyethanol and 2-methoxyacetic acid in the pregnant mouse: a physiologically based mathematical model. *Toxicol Appl Pharmacol*. 1993;121:239–252.
Clark RL, Antonello JM, Grosman SJ, et al. External genitalia abnormalities in male rats exposed in utero to finasteride, a 5α-reductase inhibitor. *Teratology*. 1990;42:91–100.
Clark RL, Robertson RT, Minsker DH, et al. Diflunisal-induced maternal anemia as a cause of teratogenicity in rabbits. *Teratology*. 1984;30:319–332.
Clarren SK, Sampson PD, Larsen J, et al. Facial effects of fetal alcohol exposure: assessment by photographs and morphometric analysis. *Am J Med Genet*. 1987;26:651–666.
Claudio L, Bearer CF, Wallinga D. Assessment of the US Environmental Protection Agency methods for identification of hazards to developing organisms: Part II. The developmental toxicity testing guideline. *Am J Ind Med*. 1999;35:554–563.
Cohlan SQ. Congenital anomalies in the rat produced by excessive intake of vitamin A during pregnancy. *Pediatrics*. 1954;13:556–567.
Collins MD, Mao GE. Teratology of retinoids. *Annu Rev Pharmacol Toxicol*. 1999;39:399–430.
Cook JC, Johnson L, O'Connor JC, et al. Effects of dietary 17β-estradiol exposure on serum hormone concentrations and testicular parameters in male Crl:CD BR rats. *Toxicol Sci*. 1998;44:155–168.
Cooper LZ, Krugman S. Diagnosis and management: congeneital rubella. *Pediatrics*. 1966;37:335–342.
Cooper RL. Current developments in reproductive toxicity testing of pesticides. *Reprod Toxicol*. 2009;28:180–187.
Corley RA. Pharmacokinetics and PBPK models. In: McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*. New York: Elsevier; 2010:27–58.
Corley RA, Mast TJ, Carney EW, et al. Evaluation of physiologically based models of pregnancy and lactation for their application in children's health risk assessments. *Crit Rev Toxicol*. 2003;33:137–211.
Corley RA, Meek ME, Carney EW. Mode of action: oxalate crystal-induced renal tubule degeneration and glycolic acid–induced dysmorphogenesis—renal and developmental effects of ethylene glycol. *Crit Rev Toxicol*. 2005;35:691–702.
Craig JC, Bennett GD, Miranda RC, et al. Ribonucleotide reductase subunit R1: a gene conferring sensitivity to valproic acid–induced neural tube defects in mice. *Teratology*. 2000;61:305–313.
Crump K. A new method for determining allowable daily intakes. *Fundam Appl Toxicol*. 1984;4:854–871.
Cupul-Uicab LA, Baird DD, Skjaerven R, et al. In utero exposure to maternal smoking and women's risk of fetal loss in the Norwegian mother and child cohort (MoBa). *Environ Health Perspect*. 2012;120:355–360.
Czeizel AE. Periconceptional folic acid–containing multivitamin supplementation for the prevention of neural tube defects and cardiovascular malformations. *Ann Nutr Metab*. 2011;59:38–40.
Czeizel AE, Dudas I, Paput L, et al. Prevention of neural-tube defects with periconceptional folic acid, methylfolate, or multivitamins? *Ann Nutr Metab*. 2011;58:263–271.
D'Amato RJ, Loughman MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci U S A*. 1994;91:4082–4085.
Dai WS, LaBraico JM, Stern RS. Epidemiology of isotretinoin exposure during pregnancy. *J Am Acad Dermatol*. 1992;26:599–606.
Dareste C. *Récherches sur la production artificielle des monstruositiés, ou essais de tératogénie expérimentale*. Paris: Reinwald; 1877.
Dareste C. *Récherches sur la production artificielle des monstruositiés, ou essais de tératogénie expérimentale*. 2nd ed. Paris: Reinwald; 1891.
Daston GP. Do thresholds exist for developmental toxicants? A review of the theoretical and experimental evidence. In Kalter H, ed. *Issues and Review in Teratology*. New York: Plenum Press; 1993:169–197.
Daston GP. Fetal zinc deficiency as a mechanism for cadmium-induced toxicity to the developing rat lung and pulmonary surfactant. *Toxicology*. 1982;24:55–63.
Daston GP. Relationships between maternal and developmental toxicity. In: Kimmel CA, Buelke-Sam J, eds. *Developmental Toxicology*. 2nd ed. New York: Raven Press; 1994:189–212.
Daston GP, Beyer BK, Carney EW, et al. Exposure-based validation list for developmental toxicity screening assays. *Birth Defects Res B Dev Reprod Toxicol*. 2014;101:423–428.
Daston GP, Chapin RE, Scialli AR, et al. A different approach to validating screening assays for developmental toxicity. *Birth Defects Res B Dev Reprod Toxicol*. 2010;89:526–530.
Daston GP, Knudsen TB. Fundamental concepts, current regulatory design and interpretation. In McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*, New York: Elsevier; 2010:3–9.
Daston GP, Overmann GJ, Taubeneck MW, Lehman-McKeeman LD, Rogers JM, Keen CL. The role of metallothionein induction and altered zinc status in maternally mediated developmental toxicity: comparison of the effects of urethane and styrene in rats. *Toxicol Appl Pharmacol*. 1991;110:450–463.
de Mouzon J, Thonneau P, Spira A, Multigner L. Semen quality has declined among men born in France since 1950. *Br Med J*. 1996;313:43.
De Santis M, Cavaliere AF, Straface G, Caruso A. Rubella infection in pregnancy. *Reprod Toxicol*. 2006;21:390–398.
DeSesso JM. Maternal factors in developmental toxicity. *Teratogenesis Carcinog Mutagen*. 1987;7:225–240.
DeSesso JM, Williams AL, Ahuja A, et al. The placenta, transfer of immunoglobulins, and safety assessment of biopharmaceuticals in pregnancy. *Crit Rev Toxicol*. 2012;42:185–210.
Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, et al. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev*. 2009;30:293–342.
Dietert RR. Developmental immunotoxicology (DIT): windows of vulnerability, immune dysfunction and safety assessment. *J Immunotoxicol*. 2008;5:401–412.

Dietert RR. Developmental immunotoxicity, perinatal programming, and noncommunicable diseases: focus on human studies. *Adv Med*. 2014;2014:867805.

Dietrich M, Brown CJ, Block G. The effect of folate fortification of cereal-grain products on blood folate status, dietary folate intake, and dietary folate sources among adult non-supplement users in the United States. *J Am Coll Nutr*. 2005;24:266–274.

Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect*. 2006;114:567–572.

Downing C, Biers J, Larson C, et al. Genetic and maternal effects on valproic acid teratogenesis in C57BL/6J and DBA/2J mice. *Toxicol Sci*. 2010;116:632–639.

Dreier JW, Andersen AM, Berg-Beckhoff G. Systematic review and meta-analyses: fever in pregnancy and health impacts in the offspring. *Pediatrics*. 2014;133:e674–688.

Dreosti IE. Nutritional factors underlying the expression of the fetal alcohol syndrome. *Ann NY Acad Sci*. 1993;678:193–204.

Dunkel Schetter C, Tanner L. Anxiety, depression and stress in pregnancy: implications for mothers, children, research, and practice. *Curr Opin Psychiatry*. 2012;25:141–148.

Dupe V, Ghyselinck NB, Thomazy V, et al. Essential roles of retinoic acid signaling in interdigital apoptosis and control of BMP-7 expression in mouse autopods. *Dev Biol*. 1999;208:30–43.

Edwards MJ. Hyperthermia as a teratogen: a review of experimental studies and their clinical significance. *Teratog Carcinog Mutag*. 1986;6:563–582.

Edwards MJ. Review: hyperthermia and fever during pregnancy. *Birth Defects Res A Clin Mol Teratol*. 2006;76:507–516.

Edwards SW, Tan YM, Villeneuve DL, et al. Adverse outcome pathways-organizing toxicological information to improve decision making. *J Pharmacol Exp Ther*. 2016;356:170–181.

Ehlers K, Sturje H, Merker HJ, et al. Valproic acid–induced spina bifida: a mouse model. *Teratology*. 1992;45:145–154.

Ehlers K, Sturje H, Merker H-J, Nau H. Spina bifida aperta induced by valproic acid and by all-*trans*-retinoic acid in the mouse: distinct differences in morphology and periods of sensitivity. *Teratology*. 1992;46:117–130.

Eibs HG, Speilman H, Hagele M. Teratogenic effects of cyproterone acetate and medroxyprogesterone treatment during the pre- and postimplantation period of mouse embryos. *Teratology*. 1982;25:27–36.

Eikel D, Lampen A, Nau H. Teratogenic effects mediated by inhibition of histone deacetylases: evidence from quantitative structure activity relationships of 20 valproic acid derivatives. *Chem Res Toxicol*. 2006;19:272–278.

Ellis-Hutchings RG, Carney EW. Whole embryo culture: a "New" technique that enabled decades of mechanistic discoveries. *Birth Defects Res B Dev Reprod Toxicol*. 2010;89:304–312.

Ellis-Hutchings RG, Settivari RS, McCoy AT, et al. Embryonic vascular disruption adverse outcomes: linking high throughput signaling signatures with functional consequences. *Reprod Toxicol*. 2017;70:82–96.

El-Masri H, Kleinstreuer N, Hines RN, et al. Integration of life-stage physiologically based pharmacokinetic models with adverse outcome pathways and environmental exposure models to screen for environmental hazards. *Toxicol Sci*. 2016;152:230–243.

Elstein KH, Zucker RM, Andrews JE, et al. Effects of developmental stage and tissue type on embryo/fetal DNA distributions and 5-fluorouracil-induced cell-cycle perturbations. *Teratology*. 1993;48:355–363.

EMA. European Medicines Agency, Committee for Human medicinal Products (CHMP). Guideline on the need for nonclinical testing in juvenile animals on human pharmaceuticals for paediatric indications. 2008. Available at: http://www.ema.europa.eu/.

Entringer S, Buss C, Wadhwa PD. Prenatal stress and developmental programming of human health and disease risk: concepts and integration of empirical findings. *Curr Opin Endocrinol Diabetes Obes*. 2010;17:507–516.

Eriksson P, Lundkvist U, Fredricksson A. Neonatal exposure to 3,4,3′,4′-tetrachlorobiphenyl: changes in spontaneous behavior and cholinergic muscarinic receptors in the adult mouse. *Toxicology*. 1991;69:27–34.

Ernhart CB, Sokol RJ, Martier S, et al. Alcohol teratogenicity in the human: a detailed assessment of specificity, critical period and threshold. *Am J Obstet Gynecol*. 1987;156:33–39.

Euker JS, Riegle GD. Effect of stress on pregnancy in the rat. *J Reprod Fertil*. 1973;34:343–346.

Fabro S. Passage of drugs and other chemicals into the uterine fluids and preimplantation blastocyst. In: Boreus L, ed. *Fetal Pharmacology*. New York: Raven Press; 1973:443–461.

Fabro S, McLachlan JA, Dames NM. Chemical exposure of embryos during the preimplantation stages of pregnancy: mortality rate and intrauterine development. *Am J Obstet Gynecol*. 1984;148:929–938.

Faiella A, Wernig M, Consalez GG, et al. A mouse model for valproate teratogenicity: parental effects, homeotic transformations, and altered HOX expression. *Hum Mol Genet*. 2000.9:227–236.

Faizan MI, Abdullah M, Ali S, et al. Zika virus–induced microcephaly and its possible molecular mechanism. *Intervirology*. 2016;59:152–158.

Fantel AG, Greenaway JC, Juchau MR, Shepard TH. Teratogenic bioactivation of cyclophosphamide in vitro. *Life Sci*. 1979;25:67–72.

Faustman EM, Allen BC, Kavlock RJ, Kimmel CA. Dose response assessment for developmental toxicity: I. Characterization of database and determination of No Observed Adverse Effect Levels. *Fundam Appl Toxicol*. 1994;23:478–486.

Ferm VH, Carpenter SJ. Teratogenic effect of cadmium and its inhibition by zinc. *Nature*. 1967;216:1123.

Ferm VH, Carpenter SJ. The relationship of cadmium and zinc in experimental mammalian teratogenesis. *Lab Invest*. 1968;18:429–432.

Finnell RH, Gelineau-van Waes J, Bennett GD, et al. Genetic basis of susceptibility to environmentally induced neural tube defects. *Ann NY Acad Sci*. 2000;919:261–277.

Finnell RH, Wlodarczyk BC, Craig JC, et al. Strain-dependent alterations in the expression of folate pathway genes following teratogenic exposure to valproic acid in a mouse model. *Am J Med Genet*. 1997;70:303–311.

Fisch H, Goluboff ET. Geographic variation in sperm counts: a potential cause of bias in studies of semen quality. *Fertil Steril*. 1996;65:1044–1046.

Fisher JW, Whitaker TA, Taylor DH, et al. Physiologically based pharmacokinetic modeling of the pregnant rat: a multiroute exposure model for trichloroethylene and its metabolite, trichloroacetic acid. *Toxicol Appl Pharmacol*. 1989;99:395–414.

Fleming P, Blair PS. Sudden infant death syndrome and parental smoking. *Early Hum Dev*. 2007;83:721–725.

Fort DJ, Paul RR. Enhancing the predictive validity of Frog Embryo Teratogenesis Assay—Xenopus (FETAX). *J Appl Toxicol*. 2002;22:185–191.

Fort DJ, Rogers RL, Thomas JH, et al. Comparative sensitivity of *Xenopus tropicalis* and *Xenopus laevis* as test species for the FETAX model. *J Appl Toxicol*. 2004;24:443–457.

Fort DJ, Stover EL, Farmer DR, Lemen JK. Assessing the predictive validity of frog embryo teratogenesis assay—*Xenopus* (FETAX). *Teratogenesis Carcinog Mutagen*. 2000;20:87–98.

Foster PM. Mode of action: impaired fetal Leydig cell function—effects on male reproductive development produced by certain phthalate esters. *Crit Rev Toxicol*. 2005;35:713–719.

Fragki S, Piersma AH, Rorije E, Zeilmaker MJ. In vitro to in vivo extrapolation of effective dosimetry in developmental toxicity testing: application of a generic PBK modelling approach. *Toxicol Appl Pharmacol*. 2017;332:109–120.

Francis BM, Rogers JM, Sulik KK, et al. Cyclophosphamide teratogenesis: evidence for compensatory responses to induced cellular toxicity. *Teratology*. 1990;42:473–482.

Frankos VH. FDA perspectives on the use of teratology data for human risk assessment. *Fundam Appl Toxicol*. 1985;5:615–622.

Fraysse B, Mons R, Garric J. Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. *Ecotoxicol Environ Saf*. 2006;63:253–267.

Fried PA, Watkinson B, Gray R. Differential effects on cognitive functioning in 9- to 12-year olds prenatally exposed to cigarettes and marihuana. *Neurotoxicol Teratol*. 1998;20:293–306.

Friedman JM. How do we know if an exposure is actually teratogenic in humans? *Am J Med Genet C Semin Med Genet*. 2011;157:170–174.

Fullerton PM, Kermer M. Neuropathy after intake of thalidomide. *Br Med J*. 1961;2:855–858.

Furr JR, Lambright CS, Wilson VS, et al. A short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse

outcome pathway, to predict disruption of sexual differentiation. *Toxicol Sci*. 2014;140:403–424.
Gabrielson JL, Larsson KS. Proposals for improving risk assessment in reproductive toxicology. *Pharmacol Toxicol*. 1990;66:10–17.
Gabrielson JL, Paalkow LK. A physiological pharmacokinetic model for morphine disposition in the pregnant rat. *J Pharmacokinet Biopharm*. 1983;11:147–163.
Gaylor DW, Chen JJ. Dose response models for developmental malformations. *Teratology*. 1993;47:291–297.
Gaylor DW, Razzaghi M. Process of building biologically based dose response models for developmental defects. *Teratology*. 1992;46: 573–581.
Gaylor DW, Sheehan DM, Young JF, Mattison DR. The threshold dose question in teratogenesis. *Teratology*. 1988;38:389–391.
Gellert RJ. Kepone, mirex, dieldrin and aldrin: estrogenic activity and the induction of persistent vaginal estrus and anovulation in rats following prenatal treatment. *Environ Res*. 1978;16:131–138.
Generoso WM, Rutledge JC, Cain KT, et al. Exposure of female mice to ethylene oxide within hours after mating leads to fetal malformations and death. *Mutat Res*. 1987;176:269–274.
Generoso WM, Rutledge JC, Cain KT, et al. Mutagen-induced fetal anomalies and death following treatment of females within hours after mating. *Mutat Res*. 1988;199:175–181.
Genschow E, Scholz G, Brown N, et al. Development of prediction models for three in vitro embryotoxicity tests in an ECVAM validation study. *In Vitro Mol Toxicol*. 2000;13:51–65.
Genschow E, Spielmann H, Scholz G, et al. The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. European Centre for the Validation of Alternative Methods. *Altern Lab Anim*. 2002;30:151–176.
Genschow E, Spielmann H, Scholz G, et al. Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests. *Altern Lab Anim*. 2004;32:209–244.
George L, Granath F, Johansson AL, Anneren G, Cnattingius S. Environmental tobacco smoke and risk of spontaneous abortion. *Epidemiology*. 2006;17:500–505.
Gilbert ME. Impact of low-level thyroid hormone disruption induced by propylthiouracil on brain development and function. *Toxicol Sci*. 2011;124:432–445.
Gilbert ME, Goodman JH, Gomez J, Johnstone AF, Ramos RL. Adult hippocampal neurogenesis is impaired by transient and moderate developmental thyroid hormone disruption. *Neurotoxicology*. 2017;59:9–21.
Gilbert ME, Ramos RL, McCloskey DP, Goodman JH. Subcortical band heterotopia in rat offspring following maternal hypothyroxinaemia: structural and functional characteristics. *J Neuroendocrinol*. 2014;26:528–541.
Gilliland FD, Berhane K, McConnell R, et al. Maternal smoking during pregnancy, environmental tobacco smoke exposure and childhood lung function. *Thorax*. 2000;55:271–276.
Gluckman PD, Hanson MA, Low FM. The role of developmental plasticity and epigenetics in human health. *Birth Defects Res C Embryo Today*. 2011;93:12–18.
Goldey ES, Crofton KM. Thyroxine replacement attentuates hypothyroxinemia, hearing loss and motor deficits following developmental exposure to Arochlor 1254 in rats. *Toxicol Sci*. 1998;45:94–105.
Goldey ES, Kehn LS, Lau C, et al. Developmental exposure to polychlorinated biphenyls (Arochlor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. *Toxicol Appl Pharmacol*. 1995;135:77–88.
Gomes-Carneiro MR, De-Oliveira AC, De-Carvalho RR, et al. Inhibition of cyclophosphamide-induced teratogenesis by beta-ionone. *Toxicol Lett*. 2003;138:205–213.
Goodman JH, Gilbert ME. Modest thyroid hormone insufficiency during development induces a cellular malformation in the corpus callosum: a model of cortical dysplasia. *Endocrinology*. 2007;148:2593–2597.
Gorsuch RL, Key MK. Abnormalities of pregnancy as a function of anxiety and life stress. *Psychosom Med*. 1974;36:352–362.
Grace CE, Kim SJ, Rogers JM. Maternal influences on epigenetic programming of the developing hypothalamic-pituitary-adrenal axis. *Birth Defects Res A Clin Mol Teratol*. 2011;91:797–805.
Gray LE, Ostby J, Ferrell J, et al. A dose–response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam Appl Toxicol*. 1989;12:92–108.
Gray LE, Ostby J, Kelce WR. A dose–response analysis of the reproductive effects of a single gestational dose of 2,3,7,8-tetrachloro-*p*-dioxin (TCDD) in male Long Evans Hooded rat offspring. *Toxicol Appl Pharmacol*. 1997a;146:11–20.
Gray LE, Ostby JS, Kelce WR. Developmental effects of an environmental anti-androgen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol*. 1994;129:46–52.
Gray LE, Wold C, Mann P, Ostby JS. In utero exposure to low doses of 2,3,7,8-tetrachloro-*p*-dioxin (TCDD) alters reproductive development of female Long Evans Hooded rat offspring. *Toxicol Appl Pharmacol*. 1997b;146:237–244.
Gregg NM. Congenital cataract following German measles in the mother. *Trans Ophthalmol Soc Aust*. 1941;3:35–40.
Guzelian PS. Comparative toxicology of chlordecone (kepone) in humans and experimental animals. *Annu Rev Pharmacol Toxicol*. 1982;22:89–113.
Guzelian PS, Henry CJ, Olin SS, eds. *Similarities and Differences Between Children & Adults: Implications for Risk Assessment*. Washington, DC: ILSI Press; 1992.
Hackshaw A, Rodeck C, Boniface S. Maternal smoking in pregnancy and birth defects: a systematic review based on 173 687 malformed cases and 11.7 million controls. *Hum Reprod Update*. 2011;17:589–604.
Hale F. Pigs born without eyeballs. *J Hered*. 1935;27:105–106.
Hales B. Comparison of the mutagenicity of cyclophosphamide and its active metabolites, 4-hydroxycyclophosphamide, phosphoramide mustard and acrolein. *Cancer Res*. 1982;42:3018–3021.
Hales B. Relative mutagenicity and teratogenicity of cyclophosphamide and two of its structural analogs. *Biochem Pharmacol*. 1983;32:3791–3795.
Hales BF. Modification of the teratogenicity and mutagenicity of cyclophosphamide with thiol compounds. *Teratology*. 1981;23:373–381.
Hales BF. Effects of phosphoramide mustard and acrolein, cytotoxic metabolites of cyclophosphamide, on mouse limb development in vitro. *Teratology*. 1989;40:11–20.
Hales BF, Grenier L, Lalancette C, et al. Epigenetic programming: from gametes to blastocyst. *Birth Defects Res A Clin Mol Teratol*. 2011;91:652–665.
Hall DL, Payne LA, Putnam JM, et al. Effect of methoxychlor on implantation and embryo development in the mouse. *Reprod Toxicol*. 1997;11:703–708.
Handler A, Davis F, Ferre C, Yeko T. The relationship of smoking and ectopic pregnancy. *Am J Public Health*. 1989;79:1239–1242.
Hannas BR, Lambright CS, Furr J, et al. Dose–response assessment of fetal testosterone production and gene expression levels in rat testes following in utero exposure to diethylhexyl phthalate, diisobutyl phthalate, diisoheptyl phthalate, and diisononyl phthalate. *Toxicol Sci*. 2011;123:206–216.
Hansen DK. Developmental toxicity of antiepileptic drugs. In: McQueen CA, ed. *Comprehensive Toxicology*, 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*. New York: Elsevier; 2010:177–187.
Hansen DK, Billings RE. Phenytoin teratogenicity and effects on embryonic and maternal folate metabolism. *Teratology*. 1985;31:363–371.
Hansen DK, Dial SL, Terry KK, et al. In vitro embryotoxicity of carbamazepine and carbamazepine-10, 11-epoxide. *Teratology*. 1996;54:45–51.
Hansen DK, Hodes ME. Comparative teratogenicity of phenytoin among several inbred strains of mice. *Teratology*. 1983;28:175–179.
Hansen JM, Carney EW, Harris C. Differential alteration by thalidomide of the glutathione content of rat vs rabbit conceptuses in vitro. *Reprod Toxicol*. 1999;13:547–554.
Hansen JM, Harris KK, Philbert MA, Harris C. Thalidomide modulates nuclear redox status and preferentially depletes glutathione in rabbit limb versus rat limb. *J Pharmacol Exp Ther*. 2002;300:768–776.
Hanson JW, Smith DW. The fetal hydantoin syndrome. *J Pediatr*. 1975;87:285–290.
Hanson MA, Gluckman PD. Early developmental conditioning of later health and disease: physiology or pathophysiology? *Physiol Rev*. 2014;94:1027–1076.
Hardin BD, Becker RJ, Kavlock RJ, et al. Overview and summary: workshop on the Chernoff/Kavlock preliminary developmental toxicity test. *Teratogenesis Carcinog Mutagen*. 1987;7:119–127.

Hartig PC, Hunter ES III. Gene delivery to the neurulating embryo during culture. *Teratology*. 1998;58:103–112.

Hauck R-S, Nau H. The enantiomers of the valproic acid analogue 2-*n*-propylpentyoic acid (4-yn-VPA): asymmetric synthesis and highly stereoselective teratogenicity in mice. *Pharm Res*. 1992;9:850–854.

Haug K, Irgens LM, Skjaerven R, et al. Maternal smoking and birthweight: effect modification of period, maternal age and paternal smoking. *Acta Obstet Gynecol Scand*. 2000;79:485–489.

Heinrichs WL, Gellert RJ, Bakke JL, Lawrence NL. DDT administered to neonatal rats induces persistent estrus syndrome. *Science*. 1971;173:642–643.

Hélène C, Toulmé JJ. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim Biophys Acta*. 1990;1049:99–125.

Hellemans KG, Sliwowska JH, Verma P, et al. Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. *Neurosci Biobehav Rev*. 2010;34:791–807.

Herbst AL, Cole P, Colton T, et al. Age-incidence and risk of diethylstilbestrol-related clear cell adenocarcinoma of the vagina and cervix. *Am J Obstet Gynecol*. 1977;128:43–50.

Hernandez-Martinez R, Covarrubias L. Interdigital cell death function and regulation: new insights on an old programmed cell death model. *Dev Growth Differ*. 2011;53:245–258.

Higuchi TT, Palmer JS, Gray LE Jr, Veeramachaneni DN. Effects of dibutyl phthalate in male rabbits following in utero, adolescent, or postpubertal exposure. *Toxicol Sci*. 2003;72:301–313.

Hines RN. Age-dependent expression of human drug-metabolizing enzymes. In: Lyubimov AV, ed. *Encyclopedia of Drug Metabolism and Interactions, 6 Volume Set*. 1st ed. Vol IV. New York: Wiley; 2012:1–33:chap 15.

Hirsch KS, Scott WJ Jr. Searching for the mechanism of acetazolamide teratogenesis. In: Kalter H, ed. *Issues and Reviews in Teratology*. Vol 1. New York: Plenum Press; 1983:309–347.

Holladay SD, Luster MI. Developmental immunotoxicology. In: Kimmel CA, Buelke-Sam J, eds. *Developmental Toxicology*. 2nd ed. New York: Raven Press, 1994:93–117.

Holson JF, Stump DG, Pearce LB, Watson RE, DeSesso JM. Mode of action: yolk sac poisoning and impeded histiotrophic nutrition—HBOC-related congenital malformations. *Crit Rev Toxicol*. 2005;35:739–745.

Hotchkiss AK, Rider CV, Furr J et al. In utero exposure to an AR antagonist plus an inhibitor of fetal testosterone synthesis induces cumulative effects on F1 male rats. *Reprod Toxicol*. 2010;30:261–270.

Howdeshell KL, Rider CV, Wilson VS, et al. Dose addition models based on biologically relevant reductions in fetal testosterone accurately predict postnatal reproductive tract alterations by a phthalate mixture in rats. *Toxicol Sci*. 2015;148:488–502.

Hoyme HE, Kalberg WO, Elliott AJ, et al. Updated clinical guidelines for diagnosing fetal alcohol spectrum disorders. *Pediatrics*. 2016;138pii:e20154256.

Huang WW, Yin Y, Bi Q, et al. Developmental diethylstilbestrol exposure alters genetic pathways of uterine cytodifferentiation. *Mol Endocrinol*. 2005;19:669–682.

Hurtt ME, Daston G, Davis-Bruno K, et al. Juvenile animal studies: testing strategies and design. *Birth Defects Res B Dev Reprod Toxicol*. 2004;71:281–288.

Hutson MS, Leung MCK, Baker NC, Spencer RM, Knudsen TB. Computational model of secondary palate fusion and disruption. *Chem Res Toxicol*. 2017;30:965–979.

Hytten FE. Physiologic changes in the mother related to drug handling. In: Krauer B, Hytten F, del Pozo E, eds. *Drugs and Pregnancy*. New York: Academic Press; 1984:7–17.

Ilic D, Ogilvie C. Concise review: human embryonic stem cells—what have we done? What are we doing? Where are we going? *Stem Cells*. 2017;35:17–25.

Imperato-McGinley J, Sanchez R, Spencer JR, et al. Comparison of the effects of the 5α-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose–response studies. *Endocrinology*. 1992;131:1149–1156.

Irl C, Hasford J. Assessing the safety of drugs in pregnancy: the role of prospective cohort studies. *Drug Saf*. 2000;22:169–177.

Irvine S, Cawood E, Richardson D, et al. Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years. *Br Med J*. 1996;312:467–471.

Ito T, Ando H, Handa H. Teratogenic effects of thalidomide: molecular mechanisms. *Cell Mol Life Sci*. 2011;68:1569–1579.

Ito T, Handa H. Deciphering the mystery of thalidomide teratogenicity. *Congenit Anom (Kyoto)*. 2012;52:1–7.

Jelovsek FR, Mattison DR Chen JJ. Prediction of risk for human developmental toxicity: how important are animal studies for hazard identification? *Obstet Gynecol*. 1989;74:624–636.

Jones KL. The effects of alcohol on fetal development. *Birth Defects Res C Embryo Today*. 2011;93:3–11.

Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. *Lancet.* 1973;2:999–1001.

Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet*. 1973;1:1267–1271.

Joubert BR, Felix JF, Yousefi P, et al. DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. *Am J Hum Genet*. 2016;98:680–696.

Joubert BR, Håberg SE, Nilsen RM, et al. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ Health Perspect*. 2012;120:1425–1431.

Joussen AM, Germann T, Kirchhof B. Effect of thalidomide and structurally related compounds on cornela angiogenesis is comparable to their teratological potency. *Graefes Arch Clin Exp Ophthalmol*. 1999;237:952–961.

Juchau MR. Enzymatic bioactivation and inactivation of chemical teratogens and transplacental carcinogens/mutagens. In: Juchau MR, ed. *The Biochemical Basis of Chemical Teratogenesis*. New York: Elsevier/North Holland; 1981:63–94.

Juchau MR, Faustman-Watts EM. Pharmacokinetic considerations in the maternal-placental unit. *Clin Obstet Gynecol*. 1983;26:379–390.

Juchau MR, Lee QP; Fantel AG. Xenobiotic biotransformation/bioactivation in organogenesis-stage conceptual tissues: implications for embryotoxicity and teratogenesis. *Drug Metab Rev*. 1992;24:195–238.

Juriloff DM, Fraser FC. Genetic maternal effects on cleft lip frequency in A/J and CL/Fr mice. *Teratology*. 1980;21:167–175.

Kang H-G, Lee C-H, Lee K-C, et al. Effects of butylated hydroxyanisole on glutathione *S*-transferase activity and cyclophosphamide-induced teratogenicity in rats. *J Toxicol Pub Health*. 2003;19:181–187.

Kaplan YC, Ozsarfati J, Etwel F, et al. Pregnancy outcomes following first-trimester exposure to topical retinoids: a systematic review and meta-analysis. *Br J Dermatol*. 2015;173:1132–1141.

Kavlock R, Chandler K, Houck K, et al. Update on EPA's ToxCast program: providing high throughput decision support for chemical risk management. *Chem Res Toxicol*. 2012;25:1287–1302.

Kavlock R, Cummings A. Mode of action: inhibition of androgen receptor function—vinclozolin-induced malformations in reproductive development. *Crit Rev Toxicol*. 2005;35:721–726.

Kavlock RJ. Structure–activity relationships in the developmental toxicity of substituted phenols: in vivo effects. *Teratology*. 1990;41:43–59.

Kavlock RJ, Allen BC, Faustman EM, Kimmel CA. Dose–response assessment for developmental toxicity: IV. Benchmark doses for fetal weight changes. *Fundam Appl Toxicol*. 1995;26:211–222.

Kavlock RJ, Chernoff N, Rogers EH. The effect of acute maternal toxicity on fetal development in the mouse. *Teratogenesis Carcinog Mutagen*. 1985;5:3–13.

Kavlock RJ, Daston GP, DeRosa D, et al. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA–sponsored workshop. *Environ Health Perspect*. 1996;104(suppl 4):715–740.

Keen CL, Bendich A, Willhite CC, eds. *Maternal Nutrition and Pregnancy Outcome*. Annals of the New York Academy of Sciences Series. Vol 678. New York: New York Academy of Sciences; 1993:372 pp.

Keen CL, Peters JM, Hurley LS. The effect of valproic acid on ^{65}Zn distribution in the pregnant rat. *J Nutr*. 1989;119:607–611.

Keen CL, Uriu-Adams JY, Skalny A, et al. The plausibility of maternal nutritional status being a contributing factor to the risk for fetal alcohol spectrum disorders: the potential influence of zinc status as an example. *Biofactors*. 2010;36:125–135.

Kelce WR, Stone CR, Laws SC, et al. Persistent DDT metabolite *p,p′*-DDE is a potent androgen receptor antagonist. *Nature*. 1995;375:581–585.
Kelsey FO. Thalidomide update: Regulatory aspects. *Teratology*. 1988;38: 221–226.
Kessel M, Balling R, Gruss P. Variations of cervical vertebrae after expression of a Hox-1.1 transgene in mice. *Cell*. 1990;61:301–308.
Khaksary Mahabady M, Gholami MR, Najafzadeh Varzi H, et al. Protective effect of quercetin on skeletal and neural tube teratogenicity induced by cyclophosphamide in rat fetuses. *Vet Res Forum*. 2016;7:133–138.
Khaksary Mahabady M, Najafzadeh Varzi H, Zareyan Jahromi S. L-carnitine protect against cyclophosphamide induced skeletal and neural tube malformations in rat fetuses. *Iran J Vet Res*. 2015;17:243–246.
Khera KS. Maternal toxicity: a possible etiological factor in embryo/fetal deaths and fetal malformations of rodent-rabbit species. *Teratology*. 1985;31:129–153.
Khera KS. Maternal toxicity—a possible factor in fetal malformations in mice. *Teratology*. 1984;29:411–416.
Khoury MJ, Erickson JD, James LM. Maternal factors in cleft lip with or without palate: evidence from interracial crosses in the United States. *Teratology*. 1983;27:351–357.
Khoury MJ, Holtzman NA. On the ability of birth defects monitoring systems to detect new teratogens. *Am J Epidemiol*. 1987;126:136–143.
Khoury MJ, James LM, Lynberg MC. Quantitative analysis of associations between birth defects and suspected human teratogens. *Am J Med Genet*. 1991;40:500–505.
Kim H-J, Lee J-E, Choi E-K, Kim Y-B. Effects of butylated hydroxyanisole on glutathione *S*-transferase activity and teratogenicity of cyclophosphamide in mice. *Korean J Lab Anim Sci*. 2003;19:120–125.
Kimmel CA, Cook RO, Staples RE. Teratogenic potential of noise in rats and mice. *Toxicol Appl Pharmacol*. 1976;36:239–245.
Kimmel CA, Young JF. Correlating pharmacokinetics and teratogenic endpoints. *Fundam Appl Toxicol*. 1983;3:250–255.
Klaassen CD, Lehman-McKeeman LD. Induction of metallothionein. *J Amer Coll Toxicol*. 1989;8:1315–1321.
Kleinstreuer NC, Judson RS, Reif DM, et al: Environmental impact on vascular development predicted by high-throughput screening. *Environ Health Perspect*. 2011;119:1596–1603.
Knudsen TB, Daston GP. Virtual tissues and developmental systems biology. In: McQueen CA ed. *Comprehensive Toxicology*. 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*. New York: Elsevier; 2010:347–358.
Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. *Birth Defects Res C Embryo Today*. 2011;93:312–323.
Kochhar DM. Teratogenic activity of retinoic acid. *Acta Pathol Microbiol Scand*. 1967;70:398–404.
Kola I, Vogel R, Spielmann H. Co-administration of ascorbic acid with cyclophosphamide (CPA) to pregnant mice inhibits the clastogenic activity of CPA in preimplantation murine blastocysts. *Mutagenesis*. 1989;4:297–301.
Koren G, Nulman I, Chudley AE, Loocke C. Fetal alcohol spectrum disorder. *CMAJ*. 2003;169:1181–1185.
Kotch LE, Sulik KK. Experimental fetal alcohol syndrome: proposed pathogenic basis for a variety of associated facial and brain anomalies. *Am J Med Genet*. 1992;44:168–176.
Krauer B. Physiological changes and drug disposition during pregnancy. In: Nau H, Scott WJ, eds. *Pharmacokinetics in Teratogenesis*. Vol 1. Boca Raton, FL: CRC Press, 1987:3–12.
Krtolica A, Ilic D, Genbacev O, et al. Human embryonic stem cells as a model for embryotoxicity screening. *Regen Med*. 2009;4:449–459.
Krumlauf R. *Hox* genes and pattern formation in the branchial region of the vertebrate head. *Trends Genet*. 1993;9:106–112.
Kumar S, Cunningham TJ, Duester G. Nuclear receptor corepressors Ncor1 and Ncor2 (Smrt) are required for retinoic acid-dependent repression of Fgf8 during somitogenesis. *Dev Biol*. 2016;418:204–215.
Kyrklund-Blomberg NB, Cnattingius S. Preterm birth and maternal smoking: risks related to gestational age and onset of delivery. *Am J Obstet Gynecol*. 1998;179:1051–1055.
Lammer EJ. Retinoids: interspecies comparisons and clinical results. In: Sundwall A, Danielsson BR, Hagberg O, et al. eds. *Developmental Toxicology—Preclinical and Clinical Data in Retrospect*. Stockholm: Tryckgruppen; 1992:105–109.
Lammer EJ, Chen DT, Hoar RM, et al. Retinoic acid induced embryopathy. *N Engl J Med*. 1985;313:837–841.
Lammer EJ, Sever LE, Oakley GP Jr. Teratogen update: valproic acid. *Teratology*. 1987;35:465–473.
Lammer EJ, Shaw GM, Iovannisci DM, Finnell RH. Maternal smoking, genetic variation of glutathione *S*-transferases, and risk for orofacial clefts. *Epidemiology*. 2005;16:698–701.
Lankas GR, Wise LD, Cartwright ME, et al. Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod Toxicol*. 1998;12:457–463.
Lary JM, Daniel KL, Erickson JD, et al. The return of thalidomide: can birth defects be prevented? *Drug Saf*. 1999;21:161–169.
Latham KE. Epigenetic modification and imprinting of the mammalian genome during development. *Curr Top Dev Biol*. 1999;43:1–49.
Lau C, Butenhoff JL, Rogers JM. The developmental toxicity of perfluoroalkyl acids and their derivatives. *Toxicol Appl Pharmacol*. 2004;198: 231–241.
Lau C, Kavlock RJ. Functional toxicity in the developing heart, lung and kidney. In: Kimmel CA, Buelke-Sam J, eds. *Developmental Toxicology*. 2nd ed. New York: Raven Press; 1994:119–188.
Lau C, Mole ML, Copeland MF, et al. Toward a biologically based dose–response model for the developmental toxicity of 5-fluorouracil in the rat: acquisition of experimental data. *Toxicol Sci*. 2001;59:37–48.
Lau C, Setzer RW. Biologically based risk assessment models for developmental toxicity. In: Tuan RS, Lo CW, eds. *Developmental Biology Protocols*. Vol II. Totowa, NJ: Humana Press; 2000:271–281.
Laufer BI, Chater-Diehl EJ, Kapalanga J, Singh SM. Long-term alterations to DNA methylation as a biomarker of prenatal alcohol exposure: from mouse models to human children with fetal alcohol spectrum disorders. *Alcohol*. 2017;60:67–75.
Lavin M, Watters D, eds. *Programmed Cell Death: The Cellular and Molecular Biology of Apoptosis*. Chur, Switzerland: Harwood Academic Publishers; 1993.
Lee DH, Hwang SH, Lim MK, et al. Performance of urine cotinine and hypomethylation of AHRR and F2RL3 as biomarkers for smoking exposure in a population-based cohort. *PLoS One*. 2017;12:e0176783.
Lee HY, Inselman AL, Kanungo J, et al. Alternative models in developmental toxicology. *Syst Biol Reprod Med*. 2012;58:10–22.
Lenz W. Kindliche Missbildungen nach Medikament-Einnahme während der Gravidität? *Dtsch Med Wochenschr*. 1961;86:2555–2556.
Lenz W. Das thalidomid-syndrom. *Fortschr Med*. 1963;81:148–153.
Lenz W. A short history of thalidomide embryopathy. *Teratology*. 1988;38:203–215.
Leonardi-Bee J, Britton J, Venn A. Secondhand smoke and adverse fetal outcomes in nonsmoking pregnant women: a meta-analysis. *Pediatrics*. 2011;127:734–741.
Leonardi-Bee J, Smyth A, Britton J, et al. Environmental tobacco smoke and fetal health: systematic review and meta-analysis. *Arch Dis Child Fetal Neonatal Ed*. 2008;93:F351–F361.
Levin AA, Miller RK. Fetal toxicity of cadmium in the rat: maternal vs. fetal injections. *Teratology*. 1980;22:1–5.
Levine H, Jorgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. *Human Reprod Update*. 2017;23:646–659.
Li Y, Sasaki H. Genomic imprinting in mammals: its life cycle, molecular mechanisms and reprogramming. *Cell Res*. 2011;21:466–473.
Little RE. Moderate alcohol use during pregnancy and decreased infant birth weight. *Am J Public Health*. 1977;67:1154–1156.
Little SA, Mirkes PE. Relationship of DNA damage and embryotoxicity induced by 4-hydroperoxydichlorocyclophosphamide in postimplantation rat embryos. *Teratology*. 1990;41:223–231.
Little SA, Mirkes PE. Effects of 4-hydroperoxycyclophosphamide (4-OOH-CP) and 4-hydroperoxydichlorocyclophosphamide (4-OOH-deClCP) on the cell cycle of postimplantation rat embryos. *Teratology*. 1992;45:163–173.
Lohnes D, Mark M, Mendelsohn C, et al. Function of the retinoic acid receptors (RARs) during development: I. Craniofacial and skeletal abnormalities in RAR double mutants. *Development*. 1994;120:2723–2748.

Love DR, Pichler FB, Dodd A, et al. Technology for high-throughput screens: the present and future using zebrafish. *Curr Opin Biotechnol*. 2004;15:564–571.

Luecke RH, Wosilait WD, Pearce BA, Young JF. A physiologically based pharmacokinetic computer model for human pregnancy. *Teratology*. 1994;49:90–103.

Luecke RH, Wosilait WD, Young JF. Mathematical analysis for teratogenic sensitivity. *Teratology*. 1997;55:373–380.

Luecke RH, Wosilait WD, Young JF. Mathematical modeling of human embryonic and fetal growth rates. *Growth Dev Aging*. 1999;63:49–59.

Luijten M, van Beelen VA, Verhoef A, et al. Transcriptomics analysis of retinoic acid embryotoxicity in rat postimplantation whole embryo culture. *Reprod Toxicol*. 2010;30:333–340.

Lunde ER, Washburn SE, Golding MC, et al. Alcohol-induced developmental origins of adult-onset diseases. *Alcohol Clin Exp Res*. 2016; 40:1403–1414.

Mably TA, Bjerke DL, Moore RW, et al. In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 3. Effects on spermatogenesis and reproductive capability. *Toxicol Appl Pharmacol*. 1992;114:118–126.

MacAuley AM, Werb Z, Mirkes PE. Characterization of the unusually rapid cell cycles during rat gastrulation. *Development*. 1993;117:873–883.

MacDonald H, Tobin JOH. Congenital cytomegalovirus infection: a collaborative study on epidemiological, clinical and laboratory findings. *Dev Med Child Neurol*. 1978;20:271–282.

Manning MA, Hoyme EH. Fetal alcohol spectrum disorders: a practical clinical approach to diagnosis. *Neurosci Biobehav Rev*. 2007;31:230–238.

Manson JM. Testing of pharmaceutical agents for reproductive toxicity. In: Kimmel CA, Buelke-Sam J, eds. *Developmental Toxicology*. 2nd ed. New York: Raven Press; 1994:379–402.

Maritz GS, Harding R. Life-long programming implications of exposure to tobacco smoking and nicotine before and soon after birth: evidence for altered lung development. *Int J Environ Res Public Health*. 2011;8:875–898.

Mark M, Ghyselinck NB, Chambon P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu Rev Pharmacol Toxicol*. 2006;46:451–480.

Mark M, Ghyselinck NB, Chambon P. Function of retinoic acid receptors during embryonic development. *Nucl Recept Signal*. 2009;7:e002.

Markert CL, Petters RM. Manufactured hexaparental mice show that adults are derived from three embryonic cells. *Science*. 1978;202:56–58.

Markussen Linnet K, Obel C, Bonde E, et al. Cigarette smoking during pregnancy and hyperactive-distractible preschoolers: a follow-up study. *Acta Paediatr*. 2006;95:694–700.

Marshall H, Morrison A, Studer M, et al. Retinoids and Hox genes. *FASEB J*. 1996;10:969–978.

Marshall H, Nonchev S, Sham MH, et al. Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature*. 1992;360:737–741.

Matalon S, Schechtman S, Goldzweig G et al. The teratogenic effect of carbamazepine: a meta-analysis of 1255 exposures. *Reprod Toxicol*. 2002;16:9–17.

Matthews EJ, Kruhlak NL, Daniel Benz R, Contrera JF. A comprehensive model for reproductive and developmental toxicity hazard identification: I. Development of a weight of evidence QSAR database. *Regul Toxicol Pharmacol*. 2007a;47:115–135.

Matthews EJ, Kruhlak NL, Daniel Benz R, et al. A comprehensive model for reproductive and developmental toxicity hazard identification: II. Construction of QSAR models to predict activities of untested chemicals. *Regul Toxicol Pharmacol*. 2007b;47:136–155.

Mattison DR, Blann E, Malek A. Physiological alterations during pregnancy: impact on toxicokinetics. *Fundam Appl Toxicol*. 1991;16:215–218.

McBride WG. Thalidomide and congenital anomalies. *Lancet*. 1961;2:1358.

McCollum CW, Ducharme NA, Bondesson M, Gustafsson J-A. Developmental toxicity screening in zebrafish. *Birth Defects Res C Embryo Today*. 2011;93:67–114.

McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev*. 2005;85:571–633.

McQuire C, Paranjothy S, Hurt L, et al. Objective measures of prenatal alcohol exposure: a systematic review. *Pediatrics*. 2016;138pii:e20160517.

Medlock KL, Branham WS, Sheehan DM. Effects of coumestrol and equol on the developing reproductive tract of the rat. *Proc Soc Exp Biol Med*. 1995;208:67–71.

Mendelsohn C, Lohnes D, Décimo D, et al. Function of the retinoic acid receptors (RARs) during development: II. Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development*. 1994;120:2749–2771.

Menegola E, Di Renzo F, Broccia ML, et al. Inhibition of histone deacetylase as a new mechanism of teratogenesis. *Birth Defects Res C Embryo Today*. 2006;78:345–353.

Mikkola HK, Orkin SH. Gene targeting and transgenic strategies for the analysis of hematopoietic development in the mouse. *Methods Mol Med*. 2005;105:3–22.

Millicovsky G, DeSesso JM. Cardiovascular alterations in rabbit embryos in situ after a teratogenic dose of hydroxyurea: an in vivo microscopic study. *Teratology*. 1980a;22:115–124.

Millicovsky G, DeSesso JM. Differential embryonic cardiovascular responses to acute maternal uterine ischemia: an in vivo microscopic study of rabbit embryos with either intact or clamped umbilical cords. *Teratology*. 1980b;22:335–343.

Millicovsky G, DeSesso JM, Kleinman LI, Clark KE. Effects of hydroxyurea on hemodynamics of pregnant rabbits: a maternally mediated mechanism of embryotoxicity. *Am J Obstet Gynecol*. 1981;140:747–752.

Millicovsky G, Johnston MC. Maternal hyperoxia greatly reduces the incidence of phenytoin-induced cleft lip and palate in A/J mice. *Science*. 1981;212:671–672.

Mills JL, Signore C. Neural tube defect rates before and after food fortification with folic acid. *Birth Defects Res A Clin Mol Teratol*. 2004;70: 844–845.

Milunsky A, Ulcickas M, Rothman KJ, et al. Maternal heat exposure and neural tube defects. *JAMA*. 1992;268:882–885.

Miodovnik A. Environmental neurotoxicants and developing brain. *Mt Sinai J Med*. 2011;78:58–77.

Mirkes PE. Cyclophosphamide teratogenesis: a review. *Teratogenicity Carcinog Mutagen*. 1985a;5:75–88.

Mirkes PE. Molecular and metabolic aspects of cyclophosphamide teratogenesis. In: Welsch F, ed. *Approaches to Elucidate Mechanisms in Teratogenesis*. Washington, DC: Hemisphere; 1987:123–147.

Mirkes PE. Simultaneous banding of rat embryo DNA, RNA and protein in cesium trifluoroacetate gradients. *Anal Biochem*. 1985b;148:376–383.

Mirkes PE, Fantel AG, Greenaway JC, Shepard TH. Teratogenicity of cyclophosphamide metabolites: phosphoramide mustard, acrolein, and 4-ketocyclophosphamide on rat embryos cultured in vitro. *Toxicol Appl Pharmacol*. 1981;58:322–330.

Mirkes PE, Greenaway JC, Hilton J, Brundrett R. Morphological and biochemical aspects of monofunctional phosphoramide mustard teratogenicity in rat embryos cultured in vitro. *Teratology*. 1985;32 :241–249.

Mirkes PE, Ricks JL, Pascoe-Mason JM. Cell cycle analysis in the cardiac and neuroepithelial tissues of day 10 rat embryos and the effects of phosphoramide mustard, the major teratogenic metabolite of cyclophosphamide. *Teratology*. 1989;39:115–120.

Mitchell EA, Milerad J. Smoking and the sudden infant death syndrome. *Rev Environ Health*. 2006;21:81–103.

Montgomery SM, Ekbom A. Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort. *BMJ*. 2002;324:26–27.

Moore NP, Beekhuijzen M, Boogaard PJ, et al. Guidance on the selection of cohorts for the extended one-generation reproduction toxicity study (OECD test guideline 443). *Regul Toxicol Pharmacol*. 2016;80:32–40.

Moore NW, Adams CE, Rowson LEA. Developmental potential of single blastomeres of the rabbit egg. *J Reprod Fertil*. 1968;17:527–531.

Mouritsen A, Aksglaede L, Sørensen K, et al. Hypothesis: exposure to endocrine-disrupting chemicals may interfere with timing of puberty. *Int J Androl*. 2010;33:346–359.

MRC Vitamin Study Research Group [prepared by Wald N with assistance from Sneddon J, Frost C, Stone R]: Prevention of neural tube defects: results of the MRC vitamin study. *Lancet*. 1991;338:132–137.

Murphy SK, Jirtle RL. Imprinted genes as potential genetic and epigenetic toxicologic targets. *Environ Health Perspect*. 2000;108(suppl 1): 5–11.

Myatt L. Placental adaptive responses and fetal programming. *J Physiol*. 2006;572:25–30.

Mylchreest E, Cattley RC, Foster PM. Male reproductive tract malformations in rats following gestational and lactational exposure to di(*n*-butyl)phthalate: an anti-androgenic mechanism? *Toxicol Sci*. 1998;43: 47–60.

Mylchreest E, Sar M, Cattley RC, Foster PM. Disruption of androgen-regulated male reproductive development by di(*n*-butyl)phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol*. 1999;156:81–95.

Nagel SC, vom Saal FS, Thayer KA, et al. Relative binding affinity–serum modified access (RBA–SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ Health Perspect*. 1997;105:70–76.

National Research Council (NRC). *Pesticides in the Diets of Infants and Children*. Washington, DC: National Academies Press; 1993.

National Research Council (NRC). *Scientific Frontiers in Developmental Toxicology and Risk Assessment*. Washington, DC: National Academies Press; 2000.

National Research Council (NRC). *Toxicity Testing in the Twenty-First Century: A Vision and a Strategy*. Washington, DC: National Academies Press; 2007.

Nau H. Species differences in pharmacokinetics and drug teratogenesis. *Environ Health Perspect*. 1986;70:113–129.

Nau H. Physicochemical and structural properties regulating placenta drug transfer. In: Polin RA, Fox WW, eds. *Fetal and Neonatal Physiology*. Vol. 1. Philadelphia, PA: Saunders; 1992:130–149.

Nau H, Chahoud I, Dencker L, et al. Teratogenicity of vitamin A and retinoids. In Blomhoff R, ed. *Vitamin A in Health and Disease*. New York: Marcel Dekker; 1994:615–664.

Nau H, Hauck R-S, Ehlers K. Valproic acid induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. *Pharmacol Toxicol*. 1991;69:310–321.

Nau H, Scott WJ. Weak acids may act as teratogens by accumulating in the basic milieu of the early mammalian embryo. *Nature*. 1986;323:276–278.

Nau H, Scott WJ. *Pharmacokinetics in Teratogenesis*. Vols I and II. Boca Raton, FL: CRC Press; 1987.

Nawrot PS, Cook RO, Hamm CW. Embryotoxicity of broadband high-frequency noise in the CD-1 mouse. *J Toxicol Environ Health*. 1981;8:151–157.

Nawrot PS, Cook RO, Staples RE. Embryotoxicity of various noise stimuli in the mouse. *Teratology*. 1980;22:279–289.

Neims AH, Warner M, Loughnan PM, Aranda JV. Developmental aspects of the hepatic cytochrome P_{450} monooxygenase system. *Annu Rev Pharmacol Toxicol*. 1976;16:427–444.

Nelson CJ, Holson JF. Statistical analysis of teratologic data: problems and recent advances. *J Environ Pathol Toxicol*. 1978;2:187–199.

Neubert D. In-vitro techniques for assessing teratogenic potential. In: Dayan AD, Paine AJ, eds. *Advances in Applied Toxicology*. London: Taylor & Francis; 1989:191–211.

Neubert D, Zens P, Rothenwallner A, Merker H-J. A survey of the embryotoxic effects of TCDD in mammalian species. *Environ Health Perspect*. 1973;5:63–79.

Neubert R, Hinz N, Thiel R, Neubert D. Down-regulation of adhesion receptors on cells of primate embryos as a probable mechanism of the teratogenic action of thalidomide. *Life Sci*. 1996;58:295–316.

New DAT. Whole embryo culture and the study of mammalian embryos during organogenesis. *Biol Rev*. 1978;5:81–94.

Newbold RR. Cellular and molecular effects of developmental exposure to diethylstilbestrol: implications for other environmental estrogens. *Environ Health Perspect*. 1995;103(suppl 7):83–87.

Newbold RR, Padilla-Banks E, Jefferson WN. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology*. 2006;147:S11–S17.

Newbold RR, Padilla-Banks E, Jefferson WN. Environmental estrogens and obesity. *Mol Cell Endocrinol*. 2009;304:84–89.

O'Flaherty EJ. Pharmacokinetics, pharmacodynamics, and prediction of developmental abnormalities. *Reprod Toxicol*. 1997;11:413–416.

O'Flaherty EJ, Scott WJ, Shreiner C, Beliles RP. A physiologically based kinetic model of rat and mouse gestation: disposition of a weak acid. *Toxicol Appl Pharmacol*. 1992;112:245–256.

OECD. Test No. 443. *Extended One-Generation Reproductive Toxicity Study*. OECD Guideline for the Testing of Chemicals. Paris: Organisation for Economic Cooperation and Development; 2011.

Okada A, Aoki Y, Kushima K, et al. Polycomb homologs are involved in teratogenicity of valproic acid in mice. *Birth Defects Res A Clin Mol Teratol*. 2004;70:870–879.

Okada A, Kushima K, Aoki Y, Bialer M, Fujiwara M. Identification of early-responsive genes correlated to valproic acid–induced neural tube defects in mice. *Birth Defects Res A Clin Mol Teratol*. 2005;73:229–238.

Oken E, Levitan EB, Gillman MW. Maternal smoking during pregnancy and child overweight: systematic review and meta-analysis. *Int J Obes (Lond)*. 2008;32:201–210.

Olanoff LS, Anderson JM. Controlled release of tetracycline–III: a physiological pharmacokinetic model of the pregnant rat. *J Pharmacokinet Biopharm*. 1980;8:599–620.

Olsen GW, Bodner KM, Ramlow JM, Ross CE, Lipshultz LI. Have sperm counts been reduced 50 percent in 50 years? A statistical model revisited. *Fertility and Sterility*. 1995;63:887–893.

Ornoy A. Neuroteratogens in man: an overview with special emphasis on the teratogenicity of antiepileptic drugs in pregnancy. *Reprod Toxicol*. 2006;22:214–226.

Ozolins TR. Cyclophosphamide and the Teratology Society: an awkward marriage. *Birth Defects Res B Dev Reprod Toxicol*. 2010;89:289–299.

Padilla S, Corum D, Padnos B, et al. Zebrafish developmental screening of the ToxCast Phase I chemical library. *Reprod Toxicol*. 2012;33:174–187.

Page K, Abramovich D, Aggett P, et al. Uptake of zinc by the human placenta microvillus border membranes and characterization of the effects of cadmium on the process. *Placenta*. 1992;13:151–162.

Paladini D, Vassallo M, D'Armiento MR, et al. Prenatal detection of multiple fetal anomalies following inadvertent exposure to cyclophosphamide in the first trimester of pregnancy. *Birth Defects Res A Clin Mol Teratol*. 2004;70:99–100.

Parizek J. Vascular changes at sites of estrogen biosynthesis produced by parenteral injection of cadmium salts: the destruction of the placenta by cadmium salts. *J Reprod Fertil*. 1964;7:263–265.

Park D, Yang YH, Choi EK, et al. Licorice extract increases cyclophosphamide teratogenicity by upregulating the expression of cytochrome P-450 2B mRNA. *Birth Defects Res B Dev Reprod Toxicol*. 2011;92:553–559.

Parman T, Wiley MJ, Wells PG. Free radical–mediated oxidative DNA damage in the mechanism of thalidomide teratogenicity. *Nat Med*. 1999;5:582–585.

Paulozzi LJ, Erickson JD, Jackson RJ. Hypospadias trends in two American surveillance systems. *Pediatrics*. 1997;100:831–834.

Pentsuk N, van der Laan JW. An interspecies comparison of placental antibody transfer: new insights into developmental toxicity testing of monoclonal antibodies. *Birth Defects Res B Dev Reprod Toxicol*. 2009;86:328–344.

Perera F, Herbstman J. Prenatal environmental exposures, epigenetics, and disease. *Reprod Toxicol*. 2011;31:363–373.

Peretz J, Vrooman L, Ricke WA, et al. Bisphenol A and reproductive health: update of experimental and human evidence, 2007–2013. *Environ Health Perspect*. 2014;122:775–786.

Piersma AH, Genschow E, Verhoef A, et al. Validation of the postimplantation rat whole-embryo culture test in the international ECVAM validation study on three in vitro embryotoxicity tests. *Altern Lab Anim*. 2004;32:275–307.

Piersma AH, Hernandez LG, van Benthem J, et al. Reproductive toxicants have a threshold of adversity. *Crit Rev Toxicol*. 2011a;41:545–554.

Piersma AH, Rorije E, Beekhuijzen ME, et al. Combined retrospective analysis of 498 rat multi-generation reproductive toxicity studies: on the impact of parameters related to F1 mating and F2 offspring. *Reprod Toxicol*. 2011b;31:392–401.

Piersma AH, van Dartel DAM, van der Ven LTM. Alternative methods in developmental toxicology. In McQueen CA, ed. *Comprehensive*

Toxicology. 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*, New York: Elsevier; 2010:293–305.

Pinkerton KE, Joad JP. The mammalian respiratory system and critical windows of exposure for children's health. *Environ Health Perspect*. 2000;108(suppl 3):457–462.

Planchart A, Mattingly CJ, Allen D, et al. Advancing toxicology research using in vivo high throughput toxicology with small fish models. *ALTEX*. 2016;33:435–452.

Poskanzer D, Herbst AL. Epidemiology of vaginal adenosis and adenocarcinoma associated with exposure to stilbestrol in utero. *Cancer*. 1977;39:1892–1895.

Rasmussen SA, Jamieson DJ, Honein MA, Petersen LR. Zika virus and birth defects—reviewing the evidence for causality. *N Engl J Med*. 2016;374:1981–1987.

REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Official Journal of the European Union L*. 2006;396, 1–849. Available at: http://home.kpn.nl/reach/downloads/reachtestingneedsfinal.pdf.

Rempel E, Hoelting L, Waldmann T, et al. A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation and optimization for histone deacetylase inhibitors. *Arch Toxicol*. 2015;89:1599–1618.

Reuter U, Heinrich-Hirsch B, Hellwig J, et al. Evaluation of OECD screening tests 421 (reproduction/developmental toxicity screening test) and 422 (combined repeated dose toxicity study with the reproduction/developmental toxicity screening test). *Regul Toxicol Pharmacol*. 2003;38:17–26.

Richard AM, Hunter ES III. Quantitative structure–activity relationships for the developmental toxicity of haloacetic acids in mammalian whole embryo culture. *Teratology*. 1996;53:352–360.

Rider CV, Furr JR, Wilson VS, et al. Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. *Int J Androl*. 2010;33:443–462.

Rider CV, Wilson VS, Howdeshell KL et al. Cumulative effects of in utero administration of mixtures of "antiandrogens" on male rat reproductive development. *Toxicol Pathol*. 2009;37:100–113.

Riedel C, Schönberger K, Yang S, et al. Parental smoking and childhood obesity: higher effect estimates for maternal smoking in pregnancy compared with paternal smoking—a meta-analysis. *Int J Epidemiol*. 2014;43:1593–1606.

Robert E, Guibaud P. Maternal valproic acid and congenital neural tube defects. *Lancet*. 1982;2:937.

Robertson J, Polifka JE, Avner M, et al. A survey of pregnant women using isotretinoin. *Birth Defects Res A Clin Mol Teratol*. 2005;73:881–887.

Robinson JF, Pennings JL, Piersma AH. A review of toxicogenomic approaches in developmental toxicology. *Methods Mol Biol*. 2012a;889:347–371.

Robinson JF, Piersma AH. Toxicogenomic approaches in developmental toxicology testing. *Methods Mol Biol*. 2013;947:451–473.

Robinson JF, Theunissen PT, van Dartel DA, et al. Comparison of MeHg-induced toxicogenomic responses across in vivo and in vitro models used in developmental toxicology. *Reprod Toxicol*. 2011a;32:180–188.

Robinson JF, Tonk EC, Verhoef A, et al. Triazole induced concentration-related gene signatures in rat whole embryo culture. *Reprod Toxicol*. 2012b;34:275–283.

Robinson JF, van Beelen VA, Verhoef A, et al. Embryotoxicant-specific transcriptomic responses in rat postimplantation whole-embryo culture. *Toxicol Sci*. 2010;118:675–685.

Robinson JF, Verhoef A, Pennings JL, et al. A comparison of gene expression responses in rat whole embryo culture and in vivo: time-dependent retinoic acid–induced teratogenic response. *Toxicol Sci*. 2012c;126:242–254.

Robinson JF, Verhoef A, Piersma AH. Transcriptomic analysis of neurulation and early organogenesis in rat embryos: an in vivo and ex vivo comparison. *Toxicol Sci*. 2012d;126:255–266.

Robinson JF, Yu X, Moreira EG et al. Arsenic- and cadmium-induced toxicogenomic response in mouse embryos undergoing neurulation. *Toxicol Appl Pharmacol*. 2011b;250:117–129.

Rodier PM, Cohen IR, Buelke-Sam J. Developmental neurotoxicology: neuroendocrine manifestations of CNS insult. In: Kimmel CA, Buelke-Sam J, eds. *Developmental Toxicology*. 2nd ed. New York: Raven Press; 1994:65–92.

Rogers JM. Tobacco and pregnancy: overview of exposures and effects. *Birth Defects Res C Embryo Today*. 2008;84:1–15.

Rogers JM, Chernoff N, Keen CL, Daston GP. Evaluation and interpretation of maternal toxicity in Segment II studies: Issues, some answers, and data needs. *Toxicol Appl Pharmacol*. 2005;207:367–374.

Rogers JM, Ellis-Hutchings RG, Grey BE, et al. Elevated blood pressure in offspring of rats exposed to diverse chemicals during pregnancy. *Toxicol Sci*. 2014;137:436–446.

Rogers JM, Ellis-Hutchings RG, Lau C. Epigenetics and the developmental origins of health and disease. In: McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*. New York: Elsevier; 2010:69–88.

Rogers JM, Mole ML. Critical periods of sensitivity to the developmental toxicity of inhaled methanol in the CD-1 mouse. *Teratology*. 1997;55:364–372.

Rosa FW. Teratogenicity of isotretinoin. *Lancet*. 1983;2:513.

Rosa FW. Spina bifida in infants of women treated with carbamazepine during pregnancy. *N Engl J Med*. 1991;324:674–677.

Rosen MB, Schmid JE, Das KP, et al. Gene expression profiling in the liver and lung of perfluorooctane sulfonate-exposed mouse fetuses: comparison to changes induced by exposure to perfluorooctanoic acid. *Reprod Toxicol*. 2009;27:278–288.

Rosen MB, Schmid JR, Corton JC, et al. Gene expression profiling in wild-type and PPAR-alpha-null mice exposed to perfluorooctane sulfonate reveals PPAR-alpha-independent effects. *PPAR Res*. 2010;2pii:794739.

Rosen MB, Thibodeaux JR, Wood CR, et al. Gene expression profiling in the lung and liver of PFOA-exposed mouse fetuses. *Toxicology*. 2007;239:15–33.

Rovida C, Longo F, Rabbit RR. How are reproductive toxicity and developmental toxicity addressed in REACH dossiers? *ALTEX*. 2011;28:273–294.

Rozman KK, Bhatia J, Calafat AM, et al. NTP-CERHR expert panel report on the reproductive and developmental toxicity of genistein. *Birth Defects Res B Dev Reprod Toxicol*. 2006;77:485–638.

Sadler TW, Warner CW. Use of whole embryo culture for evaluating toxicity and teratogenicity. *Pharmacol Rev*. 1984;36:145S–150S.

Sanes JR, Rubenstein LR, Nicolas JF. Use of a recombinant retrovirus to study postimplantation cell lineage in mouse embryos. *EMBO J*. 1986;5:3133–3142.

Sanyal MK, Kitchin KT, Dixon RL. Anomalous development of rat embryos cultured in vitro with cyclophosphamide and microsomes. *Pharmacologist*. 1979;21:A231.

Sasaki H, Matsui Y. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nat Rev Genet*. 2008;9:129–140.

Sauer H, Gunther J, Hescheler J, Wartenberg M. Thalidomide inhibits angiogenesis in embryoid bodies by the generation of hydroxyl radicals. *Am J Pathol*. 2000;56:151–158.

Saydmohammed M, Vollmer LL, Onuoha EO, et al. A high-content screening assay in transgenic zebrafish identifies two novel activators of FGF signaling. *Birth Defects Res C Embryo Today*. 2011;93:281–287.

Schantz SL, Levin ED, Bowman RE. Long-term neurobehavioral effects of perinatal PCB exposure in monkeys. *J Environ Toxicol Chem*. 1991;10:747–756.

Schantz SL, Moshtaghian J, Ness DK. Spatial learning deficits in adult rats exposed to ortho-substituted PCB congeners during gestation and lactation. *Fundam Appl Toxicol*. 1995;26:117–126.

Schardein JL. *Chemically Induced Birth Defects*. 2nd ed. New York: Marcel Dekker; 1993.

Schardein JL. *Chemically Induced Birth Defects*. 3rd ed. New York: Marcel Dekker; 2000.

Schardein JL, Keller KA. Potential human developmental toxicants and the role of animal testing in their identification and characterization. *CRC Crit Rev Toxicol*. 1989;19:251–339.
Schardein JL, Macina OT. *Human Developmental Toxicants: Aspects of Toxicology and Chemistry*. Boca Raton, FL: CRC Press; 2007:427.
Schwab C, Jagannath S. The role of thalidomide in multiple myeloma. *Clin Lymphoma Myeloma*. 2006;7:26–29.
Schweiger PJ, Jensen KB. Modeling human disease using organotypic cultures. *Curr Opin Cell Biol*. 2016;43:22–29.
Scialli AR. The challenge of reproductive and developmental toxicology under REACH. *Regul Toxicol Pharmacol*. 2008;51:244–250.
Scialli AR, Guikema AJ. REACH and reproductive and developmental toxicology: still questions. *Syst Biol Reprod Med*. 2012;58:63–69.
Seckl JR, Holmes MC. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal "programming" of adult pathophysiology. *Nat Clin Pract Endocrinol Metab*. 2007;3:479–488.
Seiler A, Visan A, Buesen R, Genschow E, Spielmann H. Improvement of an in vitro stem cell assay for developmental toxicity: the use of molecular endpoints in the embryonic stem cell test. *Reprod Toxicol*. 2004;18:231–240.
Selderslaghs IWT, Blust R, Witters HE. Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. *Reprod Toxicol*. 2012;33:142–154.
Seller MJ, Perkins KJ, Adinolfi M. Differential response of heterozygous curly-tail mouse embryos to vitamin A teratogenesis depending on maternal genotype. *Teratology*. 1983;28:123.
Setzer RW, Lau C, Mole ML, et al. Toward a biologically based dose–response model for developmental toxicity of 5-fluorouracil in the rat: a mathematical construct. *Toxicol Sci*. 2001;59:49–58.
Shan G. RNA interference as a gene knockdown technique. *Int J Biochem Cell Biol*. 2010;42:1243–1251.
Shenefelt RE. Morphogenesis of malformations in hamsters caused by retinoic acid: relation to dose and stage of treatment. *Teratology*. 1972;5:103–118.
Shepard TH. Editorial reply to "Comments on 'Moebius syndrome: animal model—human correlations and evidence for a brainstem vascular etiology'": case observation vs. epidemiology studies. *Teratology*. 1991;43:559–560.
Shepard TH. "Proof" of human teratogenicity. *Teratology*. 1994;50:97–98.
Shepard TH, Lemire RJ. *Catalog of Teratogenic Agents*. 11th ed. Baltimore, MD: Johns Hopkins University Press; 2004.
Shi M, Wehby GL, Murray JC. Review on genetic variants and maternal smoking in the etiology of oral clefts and other birth defects. *Birth Defects Res C Embryo Today*. 2008;84:16–29.
Shi QY, Zhang JB, Mi YQ, et al. Congenital heart defects and maternal fever: systematic review and meta-analysis. *J Perinatol*. 2014;34:677–682.
Shuey DL, Lau C, Logsdon TR, et al. Biologically based dose–response modeling in developmental toxicology: biochemical and cellular sequelae of 5-fluorouracil exposure in the developing rat. *Toxicol Appl Pharmacol*. 1994;126:129–144.
Sipes NS, Martin MT, Reif DM, et al. Predictive models of prenatal developmental toxicity from ToxCast high-throughput screening data. *Toxicol Sci*. 2011;124:109–127.
Sipes NS, Padilla S, Knudsen TB. Zebrafish: as an integrative model for twenty-first century toxicity testing. *Birth Defects Res C Embryo Today*. 2011;93:256–267.
Skakkebaek NE, Keiding N. Changes in semen and the testis. *Br Med J*. 1994;309:1316–1317.
Skinner MK. Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. *Mol Cell Endocrinol*. 2014;398:4–12.
Skinner MK, Manikkam M, Guerrero-Bosagna C. Epigenetic transgenerational actions of endocrine disruptors. *Reprod Toxicol*. 2011;31:337–343.
Slikker W, Miller RK. Placental metabolism and transfer: Role in developmental toxicology. In: Kimmel CA, Buelke-Sam J, eds. *Developmental Toxicology*. 2nd ed. New York: Raven Press; 1994:245–283.
Slikker W Jr, Xu ZA, Levin ED, Slotkin TA. Mode of action: disruption of brain cell replication, second messenger, and neurotransmitter systems during development leading to cognitive dysfunction—developmental neurotoxicity of nicotine. *Crit Rev Toxicol*. 2005;35:703–711.
Slotkin TA. Fetal nicotine or cocaine exposure: which one is worse? *J Pharmacol Exp Ther*. 1998;285:931–945.
Slott VL, Hales BF. Sodium 2-mercaptoethane sulfonate protection against cyclophosphamide-induced teratogenicity in rats. *Toxicol Appl Pharmacol*. 1986;82:80–86.
Smith JL, Gesteland KM, Schoenwolf GC. Prospective fate map of the mouse primitive streak at 7.5 days of gestation. *Dev Dynam*. 1994;201:279–289.
Smith SM. Alcohol and cell death. In: McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol. 12. Daston GP, Knudsen TB, eds. *Developmental Toxicology*. New York: Elsevier; 2010:223–238.
Snow MHL, Tam PPL. Is compensatory growth a complicating factor in mouse teratology? *Nature*. 1979;279:555–557.
Sokol RJ, Delaney-Black V, Nordstrom B. Fetal alcohol spectrum disorder. *JAMA*. 2003;290:2996–2999.
Soma-Pillay P, Nelson-Piercy C, Tolppanen H, Mebazaa A. Physiological changes in pregnancy. *Cardiovasc J Afr*. 2016;27:89–94.
Sorell TL, Graziano JH. Effect of oral cadmium exposure during pregnancy on maternal and fetal zinc metabolism in the rat. *Toxicol Appl Pharmacol*. 1990;102:537–545.
Spielmann H, Genschow E, Brown NA, et al. Validation of the rat limb bud micromass test in the international ECVAM validation study on three in vitro embryotoxicity tests. *Altern Lab Anim*. 2004;32:245–274.
Stephens TD. Proposed mechanisms of action in thalidomide embryopathy. *Teratology*. 1988;38:229–239.
Stephens TD, Bunde CJ, Fillmore BJ. Mechanism of action in thalidomide teratogenesis. *Biochem Pharmacol*. 2000;59:1489–1499.
Stephens TD, Bunde CJW, Torres RD, et al. Thalidomide inhibits limb development through its antagonism of IGF-I+FGF-2+heparin (abstr). *Teratology*. 1998;57:112.
Stephens TD, Fillmore BJ. Hypothesis: thalidomide embryopathy: proposed mechanism of action. *Teratology*. 2000;61:189–195.
Stirling DI, Sherman M, Strauss S. Thalidomide: a surprising recovery. *J Am Pharmacol Assoc*. 1997;NS37:307–313.
Stott DH. Follow-up study from birth of the effects of prenatal stress. *Dev Med Child Neurol*. 1973;15:770–787.
Streissguth AP, Aase JM, Clarren SK, et al. Fetal alcohol syndrome in adolescents and adults. *JAMA*. 1991a;265:1961–1967.
Streissguth AP, Barr HM, Olson HC, et al. Drinking during pregnancy decreases word attack and arithmetic scores on standardized tests: adolescent data from a population-based prospective study. *Alcohol Clin Exp Res*. 1994a;18:248–254.
Streissguth AP, Randels SP, Smith DF. A test-retest study of intelligence in patients with fetal alcohol syndrome: implications for care. *J Am Acad Child Adolesc Psychiatry*. 1991b;30:584–587.
Streissguth AP, Sampson PD, Olson HC, et al. Maternal drinking during pregnancy: attention and short-term memory in 14-year-old offspring: a longitudinal prospective study. *Alcohol Clin Exp Res*. 1994b;18:202–218.
Struve MF, Gaido KW, Hensley JB, et al. Reproductive toxicity and pharmacokinetics of di-*n*-butyl phthalate (DBP) following dietary exposure of pregnant rats. *Birth Defects Res B Dev Reprod Toxicol*. 2009;86:345–354.
Sulik KK. Genesis of alcohol-induced craniofacial dysmorphism. *Exp Biol Med*. 2005;230:366–375.
Sulik KK, Johnston MC. Sequence of developmental alterations following acute ethanol exposure in mice: craniofacial features of the fetal alcohol syndrome. *Am J Anat*. 1983;166:257–269.
Sulik KK, Johnston MC, Webb MA. Fetal alcohol syndrome: embryogenesis in a mouse model. *Science*. 1981;214:936–938.
Suzuki K, Ando D, Sato M, et al. The association between maternal smoking during pregnancy and childhood obesity persists to the age of 9–10 years. *J Epidemiol*. 2009;19:136–142.
Swann SH, Elkin EP, Fenster L. Have sperm counts declined? A reanalysis of global trend data. *Environ Health Perspect*. 1997;105:1228–1232.
Tabacova S. Mode of action: angiotensin-converting enzyme inhibition—developmental effects associated with exposure to ACE inhibitors. *Crit Rev Toxicol*. 2005;35:747–755.
Takeuchi IK. Teratogenic effects of methylnitrosourea on pregnant mice before implantation. *Experientia*. 1984;40:879–881.

Tatum WO. Use of antiepileptic drugs in pregnancy. *Expert Rev Neurother*. 2006;6:1077–1086.
Taubeneck MW, Daston GP, Rogers JM, Keen CL. Altered maternal zinc metabolism following exposure to diverse developmental toxicants. *Reprod Toxicol*. 1994;8:25–40.
Taussig HB. A study of the German outbreak of phocomelia: the thalidomide syndrome. *JAMA*. 1962;180:1106.
Therapontos C, Erskine L, Gardner ER, et al. Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. *Proc Natl Acad Sci U S A*. 2009;106:8573–8578.
Thompson CJS. *Mystery and Lore of Monsters*. London: Williams and Norgate, 1930.
Thornburg KL, O'Tierney PF, Louey S. Review: the placenta is a programming agent for cardiovascular disease. *Placenta*. 2010;31(suppl):S54–S59.
Tomson T, Battino D. Teratogenicity of antiepileptic drugs: state of the art. *Curr Opin Neurol*. 2005;18:135–140.
Ton C, Lin Y, Willett C. Zebrafish as a model for developmental neurotoxicity testing. *Birth Defects Res A Clin Mol Teratol*. 2006;76:553–567.
Toppari J, Larsen JC, Christiansen P, et al. Male reproductive health and environmental xenoestrogens. *Environ Health Perspect*. 1996;104(suppl 4):741–776.
Toppari J, Virtanen HE, Main KM, Skakkebaek NE. Cryptorchidism and hypospadias as a sign of testicular dysgenesis syndrome (TDS): environmental connection. *Birth Defects Res A Clin Mol Teratol*. 2010;88:910–919.
Trainor PA, Krumlauf R. Hox genes, neural crest cells and branchial arch patterning. *Curr Opin Cell Biol*. 2001;13:698–705.
Truong L, Mandrell D, Mandrell R, et al. A rapid throughput approach identifies cognitive deficits in adult zebrafish from developmental exposure to polybrominated flame retardants. *Neurotoxicology*. 2014;43:134–142.
Tuthill DP, Stewart JH, Coles EC, et al. Maternal cigarette smoking and pregnancy outcome. *Paediatr Perinatol Epidemiol*. 1999;13:245–253.
U.S. DHHS. *The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General*. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office of Smoking and Health; 2014. 944 pp.
U.S. EPA. Toxic substance control act testing guidelines. Final rules: preliminary developmental toxicity screen. *Fed Reg*. 1985;50:39428–39429.
U.S. EPA. *Special Report on Environmental Endocrine Disruption: An Effects Assessment and Analysis*. EPA/630/R-012. 1997; Washington, DC: U.S. Environmental Protection Agency.
U.S. EPA. *Health Effects Test Guidelines. OPPTS 870.3700. Prenatal Developmental Toxicity Study*. EPA 712-C-98-207. 1998. Washington, DC: U.S. Environmental Protection Agency.
U.S. EPA. Endocrine screening program: statement of policy. *Fed Reg*. 1998;63:71542–71568.
U.S. FDA. *Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use*. Rockville, MD: U.S. FDA; 1966.
U.S. FDA. International Conference on Harmonization: guideline on detection of toxicity to reproduction for medicinal products; availability. Notice. *Fed Reg*. 1994;59:48746–48752.
U.S. FDA. *Guidance Document. Nonclinical Safety Evaluation of Pediatric Drug Products*. Silver Spring, MD: FDA; 2006.
Vaglia JL, Hall BK. Regulation of neural crest cell populations: occurrence, distribution and underlying mechanisms. *Int J Dev Biol*. 1999;43:95–110.
Van Maele-Fabry G, Delhaise F, Picard JJ. Morphogenesis and quantification of the development of post-implantation mouse embryos. *Toxicol In Vitro*. 1990;4:149–156.
Vauloup-Fellous C, Hubschen JM, Abernathy ES, et al. Phylogenetic analysis of rubella viruses involved in congenital rubella infections in France between 1995 and 2009. *J Clin Microbiol*. 2010;48:2530–2535.
Vianna FS, Schüler-Faccini L, Leite JC, et al. Recognition of the phenotype of thalidomide embryopathy in countries endemic for leprosy: new cases and review of the main dysmorphological findings. *Clin Dysmorphol*. 2013;22:59–63.
Vierula M, Niemi M, Keiski A, et al. High and unchanged sperm counts of Finnish men. *Int J Androl*. 1996;19:11–17.
Vogel R, Seidle T, Spielmann H. A modular one-generation reproduction study as a flexible testing system for regulatory safety assessment. *Reprod Toxicol*. 2010;29:242–245.
Voinescu PE, Pennell PB. Management of epilepsy during pregnancy. *Expert Rev Neurother*. 2015;15:1171–1187.
Vojnits K, Bremer S. Challenges of using pluripotent stem cells for safety assessments of substances. *Toxicology*. 2010;270:10–17.
Von Kries R, Toschke AM, Koletzko B, Slikker W Jr. Maternal smoking during pregnancy and childhood obesity. *Am J Epidemiol*. 2002;156:954–961.
Wald N. Folic acid and the prevention of neural tube defects. In: Keen CL, Bendich A, Willhite CC, eds. Maternal Nutrition and Pregnancy Outcome. *Ann NY Acad Sci.* 1993;678:112–129.
Wang X, Guan Z, Chen Y, et al. Genomic DNA hypomethylation is associated with neural tube defects induced by methotrexate inhibition of folate metabolism. *PLoS One*. 2015;10:e0121869.
Warkany J. Manifestations of prenatal nutritional deficiency. *Vit Horm*. 1945;3:73–103.
Warkany J. Development of experimental mammalian teratology. In: Wilson JG, Warkany J, eds. *Teratology: Principles and Techniques*. Chicago, IL: University of Chicago Press; 1965:1–11.
Warkany J. History of teratology. In: *Handbook of Teratology*. Vol. 1. New York: Plenum Press; 1977:3–45.
Warkany J. Teratology: spectrum of a science. In: Kalter H, ed. *Issues and Reviews in Teratology*. Vol. 1. New York: Plenum Press; 1983:19–31.
Warkany J. Teratogen update: hyperthermia. *Teratology*. 1986;33:365–371.
Warkany J, Nelson RC. Appearance of skeletal abnormalities in the offspring of rats reared on a deficient diet. *Science*. 1940;92:383–384.
Warkany J, Schraffenberger E. Congenital malformations induced in rats by roentgen rays. *Am J Roentgenol Radium Ther*. 1944;57:455–463.
Warren KR, Li TK. Genetic polymorphisms: impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Res A Clin Mol Teratol*. 2005;73:195–203.
Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol*. 2003;23:5293–5300.
Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition*. 2004;20:63–68.
Watkinson WP, Millicovsky G. Effects of phenytoin on maternal heart rate in A/J mice: possible role in teratogenesis. *Teratology*. 1983;28:1–8.
Weaver TE, Scott WJ Jr. Acetazolamide teratogenesis: association of maternal respiratory acidosis and ectrodactyly in C57BL/6J mice. *Teratology*. 1984a;30:187–193.
Weaver TE, Scott WJ Jr. Acetazolamide teratogenesis: interactions of maternal metabolic and respiratory acidosis in the induction of ectrodactyly in C57BL/6J mice. *Teratology*. 1984b;30:195–202.
Wedebye EB, Dybdahl M, Nikolov NG, et al. QSAR screening of 70,983 REACH substances for genotoxic carcinogenicity, mutagenicity and developmental toxicity in the ChemScreen project. *Reprod Toxicol*. 2015;55:64–72.
Welsch F. Short term methods of assessing developmental toxicity hazard. In: Kalter H, ed. *Issues and Review in Teratology*. Vol. 5. New York: Plenum Press; 1990:115–153.
Wilcox AJ, Weinberg CR, O'Connor JF, et al. Incidence of early loss of pregnancy. *N Engl J Med*. 1988;319:189–194.
Wilson JG. Experimental studies on congenital malformations. *J Chronic Dis*. 1959;10:111–130.
Wilson JG. Embryotoxicity of drugs in man. In: Wilson JG, Fraser FC, eds. *Handbook of Teratology*. New York: Plenum Press; 1977:309–355.
Wilson JG. *Environment and Birth Defects*. New York: Academic Press; 1973.
Wilson JG, Roth CB, Warkany J. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency: effects of restoration of vitamin A at various times during gestation. *Am J Anat*. 1953;92:189–217.
Wiltse J. Mode of action: inhibition of histone deacetylase, altering WNT-dependent gene expression, and regulation of beta-catenin–developmental effects of valproic acid. *Crit Rev Toxicol*. 2005;35:727–738.
Windham GC, Hopkins B, Fenster L, Swann SH. Prenatal active or passive tobacco smoke exposure and the risk of preterm delivery or low birth weight. *Epidemiology*. 2000;11:427–433.
Wisborg K, Kesmodel U, Henriksen TB, et al. Exposure to tobacco smoke in utero and the risk of stillbirth and death in the first year of life. *Am J Epidemiol*. 2001;154:322–327.

Wolf CJ, Takacs ML, Schmid JE, et al. Activation of mouse and human peroxisome proliferator–activated receptor alpha by perfluoroalkyl acids of different functional groups and chain lengths. *Toxicol Sci*. 2008;106:162–171.

Wu S, Fisher J, Naciff J, et al. Framework for identifying chemicals with structural features associated with the potential to act as developmental or reproductive toxicants. *Chem Res Toxicol*. 2013;26:1840–1861.

Yamane J, Aburatani S, Imanishi S, et al. Prediction of developmental chemical toxicity based on gene networks of human embryonic stem cells. *Nucleic Acids Res*. 2016;44:5515–5528.

Yang L, Ho NY, Alshut R, et al. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. *Reprod Toxicol*. 2009;28:245–253.

You L, Casanova M, Archibeque-Engel S, et al. Impaired male sexual development in perinatal Sprague-Dawley and Long-Evans Hooded rats exposed in utero and lactationally to pp′-DDE. *Toxicol Sci*. 1998;45:162–173.

Young JF. Physiologically-based pharmacokinetic model for pregnancy as a tool for investigation of developmental mechanisms. *Comput Biol Med*. 1998;28:359–364.

Zakany J, Tuggle CK, Nguyen-Huu CM. The use of *lacZ* gene fusions in the studies of mammalian development: developmental regulation of mammalian homeobox genes in the CNS. *J Physiol Paris*. 1990;84:21–26.

Zeisel SH. What choline metabolism can tell us about the underlying mechanisms of fetal alcohol spectrum disorders. *Mol Neurobiol*. 2011;44:185–191.

Zhang C, Panzica-Kelly J, Augustine-Rauch K. The rat whole embryo culture assay using the Dysmorphology Score system. *Methods Mol Biol*. 2013;947:423–450.

Zoeller RT, Crofton KM. Mode of action: developmental thyroid hormone insufficiency—neurological abnormalities resulting from exposure to propylthiouracil. *Crit Rev Toxicol*. 2005;35:771–781.

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Unit IV

Target Organ Toxicity

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chapter 11

Toxic Responses of the Blood

Martyn T. Smith and Cliona M. McHale

Blood as a Target Organ

Hematopoiesis: The Formation of the Blood Cellular Components

Toxicology of the Erythron
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- Alterations in Red Cell Production
- Alterations in the Respiratory Function of Hemoglobin
  - *Homotropic Effects Leading to Methemoglobinemia*
  - *Heterotropic Effects*
  - *Carboxyhemoglobinemia*
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- Alterations in Erythrocyte Survival
  - *Nonimmune Hemolytic Anemia*
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Toxicology of the Leukon
- Components of Blood Leukocytes
- Evaluation of Granulocytes
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  - *Effects on Proliferation and Kinetics*
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Leukemogenesis as a Toxic Response
- Human Leukemia and Other Hematopoietic Malignancies
  - *Therapy-Related leukemia*
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- General Mechanisms of Leukemogenesis
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Toxicology of Platelets and Hemostasis
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- Toxicology of Chemicals Used to Modulate Hemostasis
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Hematotoxicity Tests
- Animal Studies
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Acknowledgments

References

BLOOD AS A TARGET ORGAN

Hematotoxicology is the study of adverse effects of chemicals, including pharmaceutical drugs, on the blood and blood-forming tissues. The vital functions that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication, make the hematopoietic system unique as a target organ. Accordingly, hematotoxicity ranks alongside toxicity to the liver and kidney as among the most important considerations in assessing risks to humans from pharmaceutical, occupational, and environmental chemical exposures.

The blood can be considered as a tissue in its own right, as it comprises around 7% of the body weight of a typical adult who has 4.7 to 5.5 L of blood. Some of the vital functions that the blood performs include the delivery of oxygen to tissues throughout the body, maintenance of vascular integrity, and provision of the many affector and effector immune functions necessary for host defense. All of these functions require a prodigious proliferative and regenerative capacity. Each of the various blood cells (erythrocytes, granulocytes, and platelets) is produced at a rate of approximately 1 to 3 million/s in a healthy adult and several times that rate in conditions where demand for these cells is high, as in hemolytic anemia or suppurative inflammation (Kaushansky, 2006). As with intestinal mucosa and gonads, this characteristic makes hematopoietic tissue a particularly sensitive target for cytoreductive or antimitotic drugs, such as those used to treat cancer, infection, and immune-mediated disorders. This tissue is also susceptible to secondary effects of toxic chemicals that affect the supply of nutrients, such as iron; the clearance of toxicants and metabolites, such as urea; or the production of vital growth factors, such as erythropoietin and granulocyte colony-stimulating factor (G-CSF).

The consequences of direct or indirect damage to blood cells and their precursors are predictable and potentially life-threatening. They include hypoxia, hemorrhage, and infection. These effects may be subclinical and slowly progressive or acute and fulminant, with dramatic clinical presentations. In cancer treatment and other clinical settings, hematotoxicity is usually assessed in the context of risk versus benefit. It may be used to define dosage in treatment modalities in which these effects are limiting, such as those employing certain anticancer, antiviral, and antithrombotic drugs.

While limited hematotoxicity may be an unavoidable side effect of treatments for serious illnesses, it is unacceptable in treatments for less serious illnesses, such as mild hypertension or arthritis, or following exposure to contaminated foods or environmental contaminants. Risk-versus-benefit decisions involving hematotoxicity may be controversial, especially when the incidence of these effects is very low. Irrespective of whether the effect is linked to the pharmacologic action of the drug, as with cytoreductive or thrombolytic agents, or unrelated to its intended action, the right balance between risk and benefit is not always clear.

Hematotoxicity may be regarded as *primary,* where one or more blood components are directly affected, or *secondary,* where the toxic effect is a consequence of other tissue injury or systemic disturbances. Primary toxicity is regarded as among the more common serious effects of xenobiotics, particularly drugs (Vandendries and Drews, 2006). Secondary toxicity is exceedingly common, due to the propensity of blood cells to reflect a wide range of local and systemic effects of toxicants on other tissues. These secondary effects on hematopoietic tissue are often more reactive or compensatory than toxic, and provide the toxicologist and clinician with an important and accessible tool for monitoring and characterizing toxic responses.

HEMATOPOIESIS: THE FORMATION OF THE BLOOD CELLULAR COMPONENTS

The production of blood cells, or hematopoiesis, is a highly regulated sequence of events by which blood cell precursors proliferate and differentiate to meet the relentless needs of oxygen transport, host defense and repair, hemostasis, and other vital functions described previously. The bone marrow is the principal site of hematopoiesis in humans and most laboratory and domestic animals. However, the lung has also recently been shown to be a major site of hematopoiesis and harbors blood stem cells that can repopulate the bone marrow (Lefrancais et al., 2017). The spleen has little function in blood cell production in the healthy human but plays a critical role in the clearance of defective or senescent cells, as well as in host defense. In the human fetus, hematopoiesis can be found in the liver, spleen, bone marrow, thymus, and lymph nodes. The bone marrow is the dominant hematopoietic organ in the latter half of gestation and the major blood-cell-producing organ at birth (Moore, 1975). All marrow is active, or "red marrow," at birth (Hudson, 2006). During early childhood, hematopoiesis recedes in long bones and, in adults, is confined to the axial skeleton and proximal humerus and femur (Custer and Ahlfeldt, 1932). The marrow in the distal long bones becomes "yellow" or fatty. When demand for blood cell production is great, as with certain disease states, fatty marrow can be reactivated as sites of hematopoiesis (Fig. 11-1). This can be useful in toxicology studies as a marker of sustained hematopoietic stress, as exemplified in studies on the hematopathology of cephalosporin toxicity in the dog (Bloom et al., 1987). Under extreme conditions, embryonic patterns of hematopoiesis may reappear as *extramedullary hematopoiesis* (Young and Weiss, 1997).

While the central function of bone marrow is hematopoiesis and lymphopoiesis, bone marrow is also one of the sites of the mononuclear phagocyte system (MPS), contributing monocytes that differentiate into a variety of MPS cells located in liver (Kupffer cells), spleen (littoral cells), lymph nodes, and other tissues. Conventional histologic and cytologic sampling of bone marrow reveals a very limited picture of an exceedingly complex tissue containing erythroid, granulocytic, megakaryocytic, MPS, and lymphoid precursors in varied stages of maturation; stromal cells; and vasculature all encased by bone (Fig. 11-1). Routine examinations of such specimens in the laboratory cannot possibly reveal the sophisticated interactions that mediate lineage commitment, proliferation, differentiation, acquisition of functional characteristics, and trafficking that result in the delivery of mature cells to the circulation, as required in sickness and in health. Hematopoietic stem cells (HSCs) reside in bone marrow niches, specialized microenvironments that include a range of types of support cells including mesenchymal stromal cells, vascular endothelial cells, and non-myelinating Schwann cells that interact with HSC. Together with osteoblasts, macrophages, and megakaryocytes, these support cells regulate HSC fate, including quiescence or proliferation and differentiation in response to dynamic physiological and pathological states (Morrison and Scadden, 2014; Asada et al., 2017). HSC regulation is mediated by complex interactions among transmembrane receptors, secreted factors, and extracellular matrix molecules (Domingues et al., 2017a). CXCL12 (also known as stromal cell-derived factor-1, SDF-1), produced by mesenchymal stromal cells, vascular cells, and osteoblasts, is a key regulator of HSC maintenance proliferation and migration, via its receptor CXCR4, as reviewed (Nagasawa, 2015). Other important growth factors include niche-derived transforming growth factor-1 (TGF-β1), stem cell factor (SCF), and thrombopoietin (TPO), the latter produced by megakaryocytes. Receptors and cell adhesion molecules (including cadherins, selectins, and integrins) are expressed by HSC and interact with extracellular matrix components (such as fibronectin, laminin, and collagen) and with other niche cells. Signaling via the aryl hydrocarbon receptor (AhR) expressed on HSC is an important regulator of hematopoiesis and prolonged and/or inappropriate stimulation of AhR activity was proposed to disrupt the regulation of HSCs (Gasiewicz et al., 2014). Other signaling pathways, notably Wnt/β-catenin, also regulate stem cell growth, function, and death (Ahmadzadeh et al., 2016). Earlier studies assessed HSC activity by testing for blood cell repopulation in an animal following irradiation. Newer methodologies such as monoclonal antibodies and fluorescence activated cell sorting (FACS) enable the purification of hematopoietic stem and progenitor cells (HSPCs) on the basis of cell surface markers.

All of the cellular components of the blood are derived from pluripotent HSCs (Fig. 11-2). This leads to a hierarchical model of hematopoiesis in which HSCs differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLP) that ultimately generate the entire range of mature blood cells. CLPs give rise to B cells, T cells, and natural killer (NK) cells. CMPs differentiate into platelets (also known as thrombocytes), erythrocytes, and mast cells and into myeloblast cells that give rise to monocytes (which ultimately become macrophages), neutrophils, basophils, and eosinophils.

As reviewed by Seita and Weissman (Seita and Weissman, 2010), hematopoiesis is more complex than illustrated in Fig. 11-2. HSCs have self-renewal capability and they give rise to multipotent progenitors (MPPs) that lack self-renewal capability. HSCs, progenitors, and other cells in the hierarchy can be identified by antibodies directed against distinct combinations of cell surface antigens in a process known as immunophenotyping. Coupling of the antibodies to fluorescent markers enables fluorescent-activated cell sorting (FACS). The markers distinguishing cells in the mouse and human hierarchies were previously summarized, for example, human HSCs were characterized as $Lin^{-}CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ and MPPs as $Lin^{-}CD34^{+}CD38^{-}CD90^{-}CD45RA^{-}$ (Seita and Weissman, 2010), but refinements continue to be made, as reviewed (McCracken et al., 2016).

A variety of suffixes are commonly used in describing altered hematopoiesis. These include -emia, with reference to the blood (e.g., uremia, septicemia, anemia); -penia, decreased number of blood cells (e.g., leukopenia, pancytopenia); -cytosis, increased number of blood cells (e.g., granulocytosis, thrombocytosis, erythrocytosis).

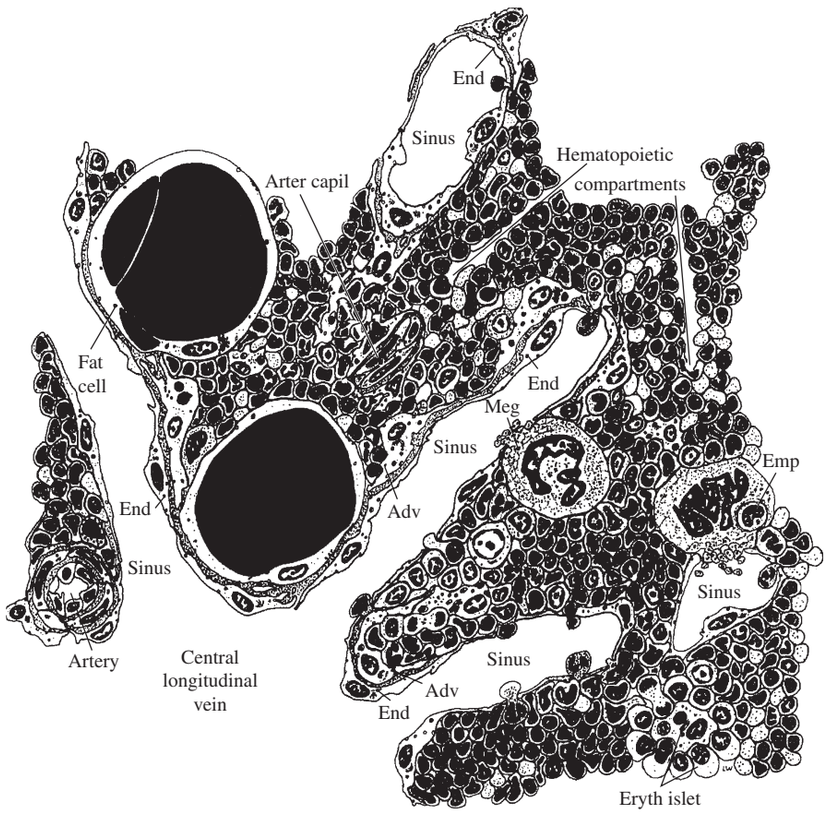

Figure 11-1. *Bone marrow schema.* Several venous sinuses (Sinus), cut longitudinally, drain into the central longitudinal vein, cut in cross-section. A branch of the nutrient artery (Artery) and an arterial capillary (Arter Capil) are present. The circulation in the bone marrow, as in the other tissues of the body save the spleen, is "closed," that is, there is endothelial continuity from artery into vein. Veins in bone marrow have in common with veins elsewhere the primary function of returning blood to the heart. Marrow veins, in addition, possess the distinctive function of receiving blood cells produced and stored in the marrow and carrying them to thymus or spleen, or into the general circulation, for further maturation, widespread distribution, and function. The hematopoietic compartments of the bone marrow consist of hematopoietic cells in varying stages of differentiation supported by a fibroblastic stroma. They lie between the most proximal veins, termed venous sinuses or vascular sinuses. When hematopoiesis is rather quiet and few nascent blood cells cross the wall of vascular sinuses, moving from hematopoietic compartments into the sinus lumen, the wall of the sinus tends to be trilaminar, consisting of endothelium (end), wispy basement membrane (in stipple), and adventitial reticular cells (adv) that form an incomplete outermost layer and branch out into the hematopoietic compartment, forming a scaffolding enclosing and supporting the hematopoietic cells. Thus, adventitial reticular cells are both vascular, as the outermost wall of the vascular sinus, and stromal, branching into the perivascular hematopoietic space, holding the vascular sinus in place and supporting hematopoietic cells. Where hematopoietic cell traffic across the wall of the venous sinus is heightened, the adventitial cell cover is retracted and a larger expanse of endothelium, covered only by wisps of basement membrane, is exposed to the hematopoietic cells, facilitating their transmural cell passage. Where transmural cell passage is greatly reduced, adventitial cells accumulate fat and become rounded and bulky, now termed adipocytes, impeding hematopoietic cell passage, and occupying space in the hematopoietic compartment that, when they transform again to adventitial cells flattened upon veins, yield to hematopoiesis. These fibroblastic stromal cells in the marrow of central bones can modulate readily to and from adventitial cell and adipocyte and retain their granulocyte inductive capacities in either form. In the distal limb and tail bones, where there is little hematopoiesis, they assume the adipocyte form in such large numbers that the marrow is grossly yellow. These adipocytes lose fat only in marked hematopoietic stress, as in spherocytic and other severe anemias where this marrow becomes hematopoietic and grossly red. In such stress, moreover, barrier cells may augment or replace adventitial reticular cells and even endothelial cells. Thus, adventitial cells/adipocytes, by their disposition and bulk, mechanically regulate hematopoiesis and blood cell delivery. In addition, they do so in a subtle manner, through paracrine secretion of several small-protein regulatory factors termed cytokines, which include interleukins. (Reprinted from Young and Weiss [1997], with permission from the authors and Elsevier Science.)

TOXICOLOGY OF THE ERYTHRON

The erythron (pronounced: er´ĭthron) consists of the circulating red blood cells (erythrocytes; RBCs) in the blood, their precursors, and all the body elements concerned in their production.

The Erythrocyte

Erythrocytes make up 40% to 45% of the circulating blood volume and serve as the principal vehicle of transportation of oxygen from the lungs to the peripheral tissues. In addition, erythrocytes are involved in the transport of carbon dioxide from tissues to the lung, maintenance of a constant pH in blood, and regulation of blood flow to tissues (Hsia, 1998; Kim-Shapiro et al., 2005). Erythrocytes also regulate vascular homeostasis through their ability to transport, produce, and secrete the vasoactive compounds nitric oxide (NO) (Porro et al., 2014). Erythrocytes express high levels of complement receptor 1 (CR1), through which they regulate the complement pathway and clear C3-opsonized immune complexes. The number of CR1 molecules is decreased with normal aging of erythrocytes and in systemic lupus erythematosus (SLE), HIV infection, and some hemolytic anemias (Khera and Das, 2009). Erythrocytes can also act as a carrier and/or reservoir for drugs and toxicants (Schrijvers et al., 1999) and are being explored as delivery systems for drugs, biological, and nanoparticles (Villa et al., 2016). The effect of xenobiotics on erythrocytes has been extensively evaluated, because of both the ready access to these cells and the

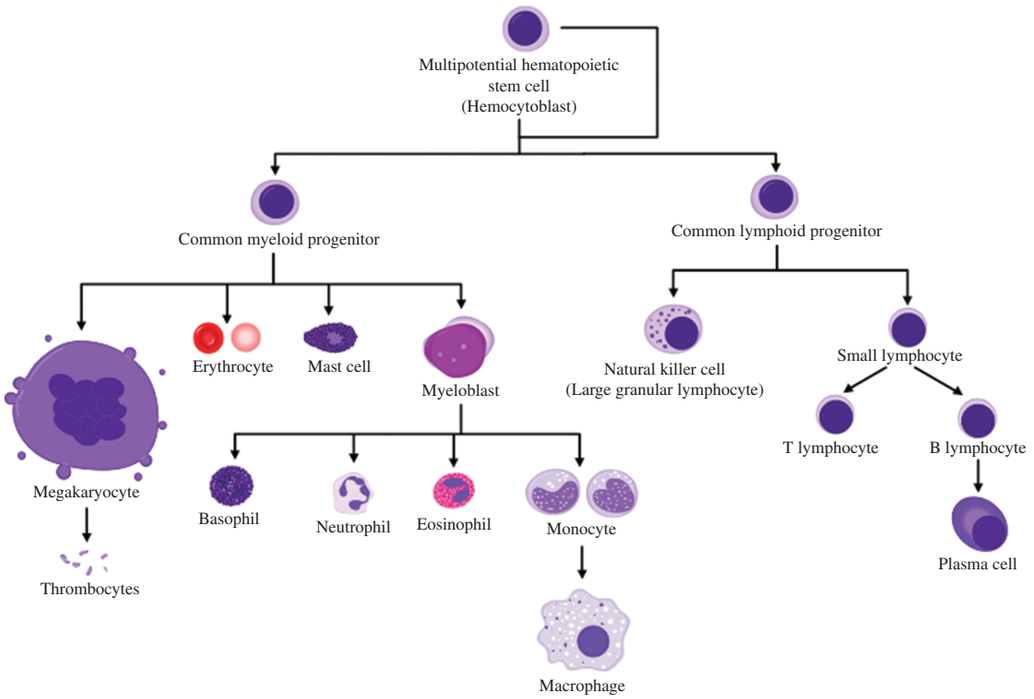

Figure 11-2. *Schematic of hematopoiesis.* All mature blood cells are derived from pluripotent HSCs in a hierarchical process. HSCs differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLP) that ultimately generate the entire range of mature blood cells. CLPs give rise to B cells, T cells, and natural killer (NK) cells. CMPs differentiate into platelets (also known as thrombocytes), erythrocytes, and mast cells and into myeloblast cells that give rise to monocytes (which ultimately become macrophages), neutrophils, basophils, and eosinophils. Reproduced from Wikimedia Commons.

frequency with which xenobiotics cause changes in these critically important cells.

Xenobiotics may affect the production, function, and/or survival of erythrocytes. These effects are most frequently manifest as a change in the circulating red cell mass, usually resulting in a decrease (anemia). Occasionally, chemicals that increase oxygen affinity lead to an increase in red cell mass (erythrocytosis), which can be caused by polycythemia (an overproduction of RBCs in absence of an appropriate stimulus, e.g., altitude), but this is distinctly less common. However, a known inappropriate chemical stimulus for erythropoiesis is cobalt ion (Davis and Fields, 1958), which induces transcription of erythropoietin gene via activation of hypoxia-inducible factor (Maxwell and Salnikow, 2004). Erythrocytosis caused by polycythemia was commonly seen, along with other symptoms, in the epidemics of beer drinker's cardiomyopathy in the 1960s after cobalt was added into some brands of beer to stabilize foam (Morin et al., 1969). Shifts in plasma volume can alter the relative concentration of erythrocytes/hemoglobin and can be easily confused with true anemia or erythrocytosis.

There are two general mechanisms that lead to true anemia—either decreased production or increased erythrocyte destruction. Both mechanisms may be operative in some disorders, or a combination may arise due to the imposition of a second disorder on a compensated underlying problem. For example, patients with compensated congenital hemolytic anemias are very susceptible to additional insults that may precipitate an acute drop in a previously stable red cell mass, such as parvovirus infection-associated suppression of erythropoiesis.

Evaluation of a peripheral blood sample can provide evidence for the underlying mechanism of anemia. The usual parameters of a complete blood count (CBC)—including the RBC count, hemoglobin concentration (Hbg), and hematocrit (also referred to as packed cell volume [PCV])—can establish the presence of anemia. Two additional parameters helpful in classifying an anemia are the mean corpuscular volume (MCV) and the reticulocyte count. Increased destruction is usually accompanied by an increase in reticulocytes (young erythrocytes containing residual RNA), which are easily enumerated. Reticulocyte counting may also be useful in conditions associated with decreased production, particularly when assessing response to therapy. Other readily performed parameters helpful in the evaluation of the human erythron include erythrocyte morphology (e.g., megaloblastic changes, erythrocyte fragmentation, sickled RBCs); serum concentration of haptoglobin, lactate dehydrogenase, free hemoglobin, vitamin B_{12}, folate, iron, and ferritin; direct and indirect red cell antiglobulin tests; and bone marrow morphology (Prchal, 2006; Ryan, 2006). Newer techniques to evaluate erythrocyte function include time-lapse interferometry to quantify membrane fluctuations at the nanometer scale and thus cell stiffness (Bhaduri et al., 2014) and metabolomics (Srivastava et al., 2017).

Alterations in Red Cell Production

Erythrocyte production is a continuous process that is dependent on frequent cell division and a high rate of hemoglobin synthesis. Human adult hemoglobin (hemoglobin A), the major constituent of the erythrocyte cytoplasm, is a tetramer composed of two α-globin and two β-globin chains, each with a heme residue located in a stereospecific pocket of the globin chain. Synthesis of hemoglobin is dependent on coordinated production of globin chains and heme moieties. Abnormalities that lead to decreased hemoglobin synthesis are relatively common (e.g., iron deficiency) and are often associated with a decrease in the MCV and hypochromasia (increased central pallor of RBCs on stained blood films due to the low hemoglobin concentration).

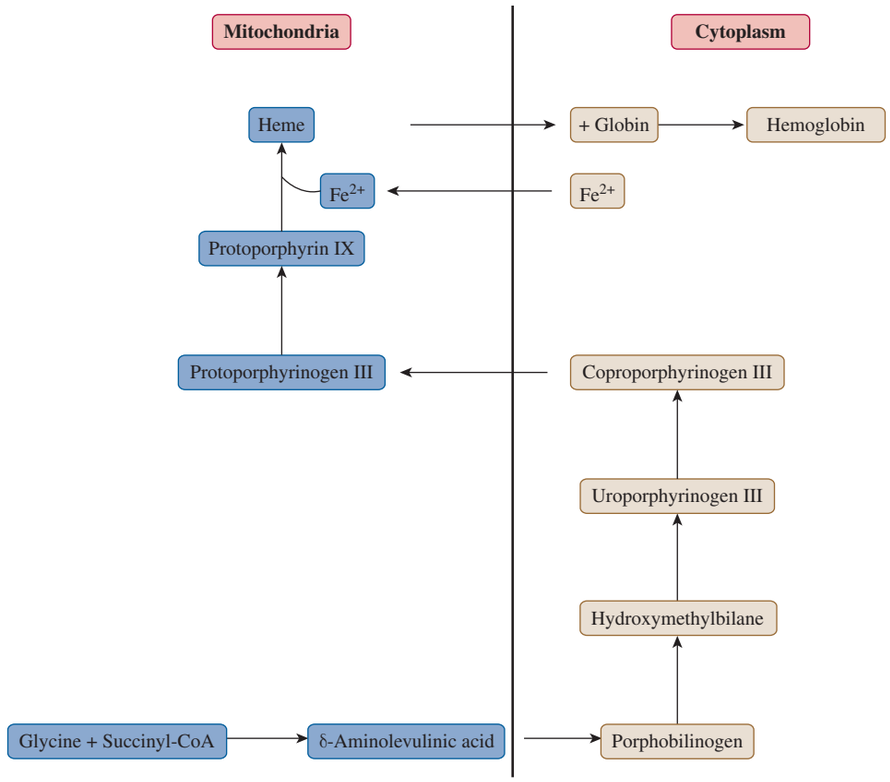

Figure 11-3. *The synthesis of heme involves a series of reactions that occur in the cytoplasm and mitochondria of erythroblasts.* The initial step in the pathway is the mitochondria synthesis of δ-aminolevulinic acid, a step that is commonly affected by xenobiotics, including lead. Ferrochelatase catalyzes the incorporation of ferrous iron into the tetrapyrrole protoporphyrin IX. Inhibition of the synthetic pathway leading to protoporphyrin IX, as occurs in the sideroblastic anemias, can cause an imbalance between iron concentration and ferrochelatase activity, resulting in iron deposition within mitochondria. Mitochondrial accumulation of iron is the hallmark lesion of the sideroblastic anemias.

An imbalance between α- and β-chain production is the basis of congenital thalassemia syndromes and results in decreased hemoglobin production and microcytosis (Weatherall, 2006). Xenobiotics can affect globin chain synthesis and alter the composition of hemoglobin within erythrocytes. The most well-known example is hydroxyurea, which increases the synthesis of γ-globin chains. The γ -globin chains are a normal constituent of hemoglobin during fetal development, replacing the β chains in the hemoglobin tetramer (hemoglobin F, $\alpha_2\ \gamma_2$). Hemoglobin F has a higher affinity for oxygen than hemoglobin A and can protect against crystallization (sickling) of deoxyhemoglobin S in sickle cell disease (Steinberg, 2006).

Synthesis of heme requires incorporation of iron into a porphyrin ring (Fig. 11-3) (Ponka, 1997; Napier et al., 2005). Iron deficiency is usually the result of dietary deficiency or increased blood loss. Drugs that contribute to blood loss, such as nonsteroidal anti-inflammatory drugs, with their increased risk of gastrointestinal ulceration and bleeding, may potentiate the risk of developing *iron deficiency anemia.* Defects in the synthesis of porphyrin ring of heme can lead to *sideroblastic anemia,* with its characteristic accumulation of iron in bone marrow erythroblasts. The accumulated iron precipitates within mitochondria in a complex with mitochondrial ferritin, causing the characteristic staining pattern of ringed sideroblasts evident on iron stains such as Prussian blue (Cazzola et al., 2003). A number of xenobiotics (Table 11-1) can interfere with one or more of the steps in erythroblast heme synthesis and result in sideroblastic anemia (Fiske et al., 1994; Alcindor and Bridges, 2002; Beutler, 2006a, 2006b, 2006c). The most well-known of these is lead, which inhibits two enzymes in the heme synthesis pathway, aminolevulinic acid dehydratase (ALAD) and ferrochelatase (Fig. 11-3).

Table 11-1

Xenobiotics Associated with Sideroblastic Anemia

Ethanol	Chloramphenicol
Isoniazid	Copper chelation/deficiency
Pyrazinamide	Zinc intoxication
Cycloserine	Lead intoxication

Hematopoiesis requires active DNA synthesis and frequent mitoses. Folate and vitamin B_{12} are necessary to maintain synthesis of thymidine for incorporation into DNA (Fig. 11-4). Deficiency of folate and/or vitamin B_{12} results in *megaloblastic anemia,* with its characteristic morphologic and biochemical changes (Table 11-2), which commonly affect erythroid, myeloid, and megakaryocytic lineages. A number of xenobiotics may contribute to a deficiency of vitamin B_{12} and/or folate (Table 11-3), leading to megaloblastic anemia. Drugs that cause megaloblastic anemia have a number of different mechanisms of action including modulation of purine metabolism, interference with pyrimidine synthesis, decreased absorption of folic acid, folate analogue activity, decreased absorption of vitamin B_{12}, and increased excretion of vitamin B_{12} (Hesdorffer and Longo, 2016).

Many of the antiproliferative drugs used in the treatment of malignancy predictably inhibit hematopoiesis, including

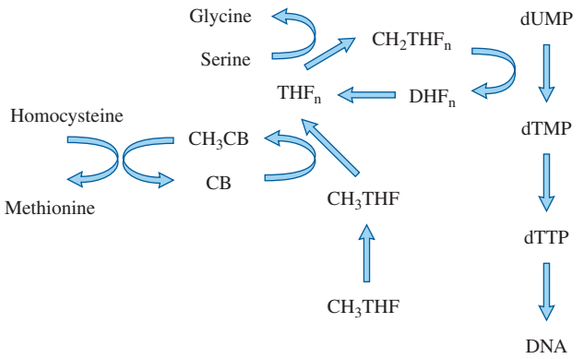

Figure 11-4. *Both tetrahydrofolate (THF) and cobalamin (CB, or vitamin B12) are necessary for the synthesis of thymidine (dTMP) for incorporation into DNA.* Folate enters the cell as a monoglutamate (CH_3THF) but is transformed to a polyglutamate within the cell, a step that helps prevent leakage of folate back across the cell membrane. However, CH_3THF cannot be conjugated with glutamate. CB is necessary for demethylation of the folate, allowing formation of conjugated (polyglutamate) folate (THF_n). In the absence of CB, folate levels within the cell drop, causing a functional deficiency of folate and impairing synthesis of thymidine.

Table 11-2

Laboratory Features of Megaloblastic Anemia

MORPHOLOGY	BIOCHEMISTRY
Peripheral blood	Peripheral blood
Pancytopenia	Decreased B_{12} and/or folate
Macrocytosis (↑ MCV)	Increased LD
Oval macrocytes	Antiparietal cell antibodies
Hypersegmented neutrophils	Antibody to intrinsic factor
Variation in RBC shape	Increased serum iron
Bone marrow	Hypokalemia
Erythroid hyperplasia	
Megaloblastic anemia	
Giant band neutrophils	
Giant metamyelocytes	

erythropoiesis. The resulting bone marrow toxicity may be dose-limiting, as previously discussed. Drugs, such as amifostine, have been developed that may help protect against the marrow toxicity of these drugs (Phillips, 2002). Amifostine protects cells by quenching free radicals, promoting DNA repair by donating hydrogen, and inducing cellular hypoxia (Kouvaris et al., 2007).

Drug-induced *aplastic anemia* may represent either a predictable or idiosyncratic reaction to a xenobiotic. This life-threatening disorder is characterized by peripheral blood pancytopenia, reticulocytopenia, and bone marrow hypoplasia (Young, 1999, 2000; Vandendries and Drews, 2006). Agents such as benzene and radiation have a *predictable* effect on hematopoietic progenitors, and the resulting aplastic anemia corresponds to the magnitude of the exposure to these agents (Smith, 2010). In contrast, idiosyncratic aplastic anemia does not appear to be related to the dose of the agent initiating the process. A long list of chemicals has been associated with the development of aplastic anemia (Table 11-4), many of which have been reported in only a few patients and their association is tentative. The principal mechanisms of aplastic anemia, as with the more common agranulocytosis, are still debated.

Table 11-3

Xenobiotics Associated with Megaloblastic Anemia

B_{12} DEFICIENCY	FOLATE DEFICIENCY
Colchicine	Ampicillin
Cycloserine	Antimetabolites
Ethanol	Chloramphenicol
Isoniazid	Cholestyramine
Metformin	Carbamazepine
Neomycin	Erythromycin
Omeprazole	Ethanol
Paraaminosalicylic acid	Phenobarbital
Sodium nitroprusside	Phenytoin
Zidovudine	Primidone
	Quinine
	Tetracyclines
	Triamterene
PURINE METABOLISM	**PYRIMIDINE SYNTHESIS**
Azathioprine	Gemcitabine
Thioguanine	Hydroxyurea
Mercaptopurine	Methotrexate
Fludarabine	Mercaptopurine
Pentostatin	Fluorouracil
Methotrexate	Nitrous Oxide

Immune mechanisms have long been thought to contribute to the development of both idiosyncratic disorders, as discussed in greater detail in the next section. However, it has been, up until recently, difficult to obtain definitive evidence for humoral and/or cellular mechanisms of marrow suppression (Young, 2000; Vandendries and Drews, 2006). Both toxicities may be immune-mediated, often involving proteins altered by reactive metabolite–mediated damage (Zhang et al., 2011). Immune-mediated destruction of hematopoietic stem/progenitor cells in aplastic anemia is mediated by a dysregulated T-cell repertoire and abnormal production of cytokines, with individual susceptibility influenced by polymorphisms in cytokine genes and human leucocyte antigen (HLA) alleles (Zeng and Katsanis, 2015).

Pure red cell aplasia is a syndrome in which the decrease in marrow production is limited to the erythroid lineage (Fisch et al., 2000; Djaldetti et al., 2003). It is an uncommon disorder that may be due to genetic defects, infection (parvovirus B19), immune-mediated injury, myelodysplasia, drugs, or other toxins. As pure red cell aplasia occurs sporadically and infrequently, the linkage between drug exposure and pathogenesis of the aplasia remains speculative for some agents. The drugs most clearly implicated and for which there are multiple case reports, include isoniazid (antimycobacterial, anti-tuberculosis), phenytoin (antiepileptic), and azathioprine (immunosuppressant). The mechanism of drug-induced pure red cell aplasia is unknown, but some evidence suggests that it may also be immune-mediated. Patients with drug-induced red cell aplasia should not be reexposed to the purported offending chemical. As noted above, pure red cell aplasia may also occur as a consequence of an immune response to therapeutic erythropoietin.

Alterations in the Respiratory Function of Hemoglobin

Human blood is red in color, ranging from bright red when oxygenated in the arteries to a dark, almost blackish-red when deoxygenated in the veins. It owes its color to hemoglobin, to which oxygen

Table 11-4

Drugs and Other Chemicals Associated with the Development of Aplastic Anemia

Chloramphenicol	Organic arsenicals	Quinacrine
Methylphenylethylhydantoin	Trimethadione	Phenylbutazone
Gold	Streptomycin	Benzene
Penicillin	Allopurinol	Tetracycline
Methicillin	Sulfonamides	Chlortetracycline
Sulfisoxazole	Sulfamethoxypyridazine	Amphotericin B
Mefloquine	Ethosuximide	Felbamate
Carbimazole	Methylmercaptoimidazole	Potassium perchlorate
Propylthiouracil	Tolbutamide	Pyrimethamine
Chlorpropamide	Carbutamide	Tripelennamine
Indomethacin	Carbamazepine	Diclofenac
Meprobamate	Chlorpromazine	Chlordiazepoxide
Mepazine	Chlorophenothane	Parathion
Thiocyanate	Methazolamide	Dinitrophenol
Bismuth	Mercury	Chlordane
Carbon tetrachloride	Cimetidine	Metolazone
Azidothymidine	Ticlopidine	Isoniazid
Trifluoperazine	D-Penicillamine	

binds. Deoxygenated blood is darker due to the difference in color between deoxyhemoglobin and oxyhemoglobin. Hemoglobin is necessary for effective transport of oxygen and carbon dioxide between the lungs and tissues. The respiratory function of hemoglobin has been studied in detail, revealing an intricately balanced system for the transport of oxygen from the lungs to the tissues (Hsia, 1998). Electrostatic charges hold the globin chains of deoxyhemoglobin in a "tense" (T) conformation characterized by a relatively low affinity for oxygen. Binding of oxygen alters this conformation to a "relaxed" (R) conformation that is associated with a 500-fold increase in oxygen affinity. Thus, the individual globin units show cooperativity in the binding of oxygen, resulting in the familiar sigmoid shape to the oxygen dissociation curve (Fig. 11-5). Similarly, the release of the first oxygen molecule facilitates the release of the second, third, and fourth oxygen molecules. The ability of hemoglobin to safely and efficiently transport oxygen is dependent on both intrinsic (homotropic) and extrinsic (heterotropic) factors that affect the performance of this system.

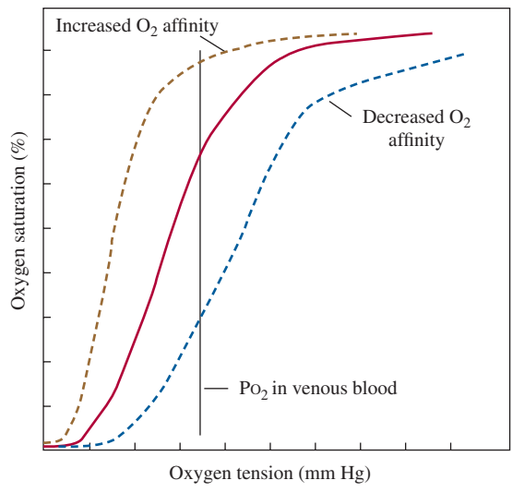

Figure 11-5. *The normal oxygen dissociation curve (solid line) has a sigmoid shape due to the cooperative interaction between the four globin chains in the hemoglobin molecule.* Fully deoxygenated hemoglobin has a relatively low affinity for oxygen. Interaction of oxygen with one heme-iron moiety induces a conformational change in that globin chain. Through surface interactions, that conformational change affects the other globin chains, causing a conformational change in all of the globin chains that increases their affinity for oxygen. Homotropic and heterotropic parameters also affect the affinity of hemoglobin for oxygen. An increase in oxygen affinity results in a shift to the left in the oxygen dissociation curve. Such a shift may decrease oxygen delivery to the tissues. A decrease in oxygen affinity results in a shift to the right in the oxygen dissociation curve, facilitating oxygen delivery to the tissues. In healthy resting adults the extraction of oxygen from capillary blood is about 25%.

Homotropic Effects Leading to Methemoglobinemia One of the most important homotropic properties of oxyhemoglobin is the slow but consistent oxidation of heme iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state to form methemoglobin (Percy et al., 2005). The change in oxidation state renders methemoglobin unable to bind and transport oxygen. In addition, the presence of methemoglobin in a hemoglobin tetramer has allosteric effects that increase the affinity of oxyhemoglobin for oxygen, resulting in a leftward shift of the oxygen dissociation curve (Fig. 11-6). The combination of decreased oxygen content and increased affinity may significantly impair delivery of oxygen to tissues when the concentration of methemoglobin rises beyond critical levels (Hsia, 1998; Percy et al., 2005).

Not surprisingly, the normal erythrocyte has metabolic mechanisms for reducing heme iron back to the ferrous state; these mechanisms are normally capable of maintaining the concentration of methemoglobin at less than 1% of total hemoglobin (Percy et al., 2005). In humans, there are two mechanisms by which erythrocytes counteract the formation of methemoglobin. The most predominant is via nicotine adenine dinucleotide (NADH)-methemoglobin reductase (also known as cytochrome b5 reductase), which converts 95% to 99% of methemoglobin to hemoglobin. The second mechanism is via nicotine adenine dinucleotide phosphate (NADPH)-methemoglobin reductase, which requires a cofactor or electron receptor such flavin to reduce methemoglobin. This pathway usually accounts for less than 5% of the reduction of methemoglobin,

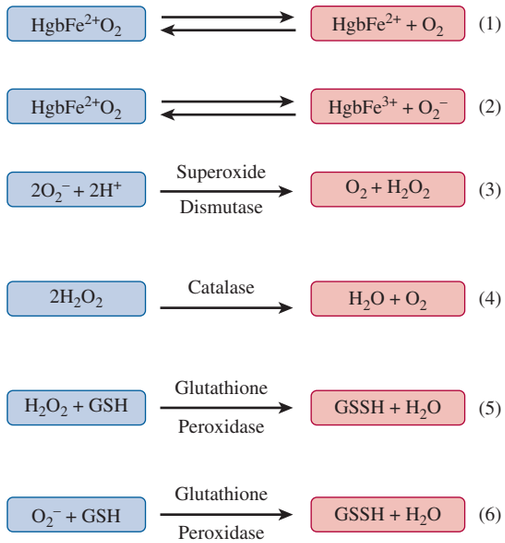

Figure 11-6. *Oxygen normally exchanges with the ferrous iron of deoxyhemoglobin (Eq. (1)).* Oxygen can "capture" one of the iron electrons, resulting in the generation of methemoglobin ($HgbFe^{3+}$) and superoxide (O_2^-) (Eq. (2)). Superoxide must be detoxified or it can lead to oxidative injury within the cell. The pathways involved include superoxide dismutase (Eq. (3)), catalase (Eq. (4)), and glutathione peroxidase (Eqs. (5) and (6)). A supply of reduced glutathione (GSH) is necessary to prevent excessive oxidative injury.

but its activity can be greatly enhanced by methylene blue, which is reduced to leukomethylene blue by NADPH-methemoglobin reductase. Leukomethylene blue then reduces methemoglobin to deoxyhemoglobin.

A failure of these control mechanisms leads to increased levels of methemoglobin, or *methemoglobinemia,* in which the arterial blood becomes a characteristic chocolate-brown color. The most common cause of methemoglobinemia is exposure to an oxidizing xenobiotic that overwhelms the NADH-methemoglobin reductase system. A large number of chemicals and therapeutic agents may cause methemoglobinemia (Table 11-5) (Coleman and Coleman, 1996; Bradberry et al., 2001; Bradberry, 2003). Medications—including local anesthetics, antineoplastic agents, antibiotics, and antimalarials—are the most common cause of methemoglobinemia in clinical practice. Nitrates are present in industrial/household products, in drinking water as a result of fertilizer or animal waste run-off, and in foods such as vegetables and cured meats. Nitrates are toxic through their conversion to nitrate before and/or after ingestion (Greer et al., 2005). Nitrate toxicity can lead to reflex tachycardia and orthostatic hypotension. Infants under 3 months of age are especially susceptible to nitrite-induced methemoglobinemia for several reasons (Greer et al., 2005; Savino et al., 2006). Infants have lower activity of NADH-cytochrome b5 reductase. Gastric pH is lower in infants than in adults enabling increased proliferation of intestinal flora and enhanced conversion of nitrate to nitrite. Nitrites more readily oxidize fetal hemoglobin than adult hemoglobin to methemoglobin. Finally, infants consume large quantities of water relative to their body weight and may have proportionately greater exposures as formula, juices, etc.

The chemicals in Table 11-5 may be divided into direct oxidizers, which are capable of inducing methemoglobin formation when added to erythrocytes in vitro or in vivo, and indirect oxidizers, which do not induce methemoglobin formation when exposed to

Table 11-5

Xenobiotics Associated with Methemoglobinemia

THERAPEUTIC AGENTS	ENVIRONMENTAL AGENTS
Benzocaine	Nitrites
Lidocaine	Nitrates
Prilocaine	Nitrobenzenes
Dapsone	Aniline d+ and aniline derivatives
Amyl nitrate	Butyl nitrite
Isobutyl nitrite	Potassium chlorate
Nitroglycerine	Gasoline additives
Primaquine	Aminobenzenes
Sulfonamide	Nitrotoluenes
Phenacetin	Trinitrotoluene
Nitric oxide	Nitroethane
Phenazopyridine	*ortho*-Toluidine
Metoclopramide	*para*-Toluidine
Flutamide	β-Naphthol disulfonate
Silver nitrate	
Quinones	
Methylene blue	

erythrocytes in vitro but do so after metabolic modification in vivo. Nitrites appear to be able to interact directly with ferrous hemoglobin to facilitate oxidation of heme iron, but the precise mechanism that leads to methemoglobin formation is unknown for many of the other substances listed in Table 11-5.

The development of methemoglobinemia may be slow and insidious or abrupt in onset, as with the use of some topical anesthetics (Shamriz et al., 2014; Tran and Koo, 2014; Barash et al., 2015). Most patients tolerate low levels (<10%) of methemoglobin without clinical symptoms. Cyanosis is often evident when the methemoglobin concentration exceeds 5% to 10%. Levels above 20% are generally clinically significant and some patients may begin to manifest symptoms related to tissue hypoxemia at methemoglobin levels between 10% and 20%. The severity of clinical manifestations increases as the concentration rises above 20% to 30%, with methemoglobin levels above 70% being life-threatening. Intravenous administration of 1 to 2 mg/kg methylene blue is effective in rapidly reversing methemoglobinemia through activation of the NADPH-methemoglobin reductase pathway (Clifton and Leikin, 2003). The effect of methylene blue is dependent on an adequate supply of NADPH. Consequently, methylene blue is not effective in patients with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency because of the decreased capacity to form NADPH (Coleman and Coleman, 1996; Bradberry, 2003).

Methemoglobin can also combine reversibly with a variety of chemical substances, including cyanide, sulfides, peroxides, fluorides, and azides. The interaction of cyanide and methemoglobin is leveraged in two ways. Formation of cyanmethemoglobin by reaction of hemoglobin with potassium ferricyanide is a standard method for measurement of hemoglobin concentration. In the treatment of cyanide poisoning, nitrate is administered to form methemoglobin, which then binds free cyanide, sparing other critical cellular respiratory enzymes such as cytochrome oxidase (Cummings, 2004). Other treatments for cyanide poisoning include sulfur donors (e.g., sodium thiosulfate) and direct binding agents (e.g., hydroxocobalamin), as reviewed (Petrikovics et al., 2015). Regional variations exist in the approach to treatment. In the United States, sodium nitrite and sodium thiosulfate and hydroxocobalamin are favored, while in Europe, hydroxocobalamin is the predominant

treatment. The current approaches have limitations. They are all administered intravenously, limiting their use in a mass exposure event. Sodium nitrate can cause hypotension and methemoglobinemia while sodium thiosulfate has a slow onset of action. Thus, there is a need for newer approaches.

Heterotropic Effects There are three major heterotropic effectors of hemoglobin function: pH, erythrocyte 2,3-bisphosphoglycerate (2,3-BPG, formerly designated 2,3-diphosphoglycerate) concentration, and temperature (Hsia, 1998). A decrease in pH (e.g., lactic acid, carbon dioxide) lowers the affinity of hemoglobin for oxygen, that is, it causes a right shift in the oxygen dissociation curve, facilitating the delivery of oxygen to tissues (Fig. 11-5). As bicarbonate and carbon dioxide equilibrate in the lung, the hydrogen ion concentration decreases, increasing the affinity of hemoglobin for oxygen and facilitating oxygen uptake. Thus, the buffering capacity of hemoglobin also serves to improve oxygen uptake and delivery.

The binding site for 2,3-BPG is located in a pocket formed by the 2 β chains of a hemoglobin tetramer. Binding of 2,3-BPG to deoxyhemoglobin results in stabilization of the "T" conformation, with reduced oxygen affinity (a shift to the right of the oxygen dissociation curve). The conformational change induced by binding of oxygen alters the binding site for 2,3-BPG and results in release of 2,3-BPG from hemoglobin. This facilitates uptake of more oxygen for delivery to tissues. The concentration of 2,3-BPG increases whenever there is tissue hypoxemia but may decrease in the presence of acidosis or hypophosphatemia. Thus, hypophosphatemia may result in a left shift of the oxygen dissociation curve.

Clofibrate and bezafibrate (lipid lowering drugs used to treat hyperlipidemia) are capable of lowering the oxygen affinity of hemoglobin, analogous to 2,3-BPG (Poyart et al., 1994). However, the association constant of bezafibrate for hemoglobin is too low for there to be a meaningful effect in vivo.

The oxygen affinity of hemoglobin decreases as the body temperature increases (Hsia, 1998). This facilitates delivery of oxygen to tissues during periods of extreme exercise and febrile illnesses associated with increased temperature. Correspondingly, oxygen affinity increases during hypothermia, which may lead to decreased oxygen delivery under these conditions. This must be taken into consideration during surgical procedures during which there is induction of deep hypothermia.

Carboxyhemoglobinemia The respiratory function of hemoglobin may also be impaired by blockade of the ligand binding site following interaction with other substances, most notably carbon monoxide (CO) (Hsia, 1998). CO has a relatively low rate of association with deoxyhemoglobin but has high affinity once bound. The affinity is about 200 times that of oxygen, and thus persistent exposure to a low level of CO (e.g., 0.1%) may lead to 50% saturation of hemoglobin. Binding of carbon monoxide also results in stabilization of the hemoglobin molecule in the high-affinity "R" conformation. Consequently, the oxygen dissociation curve is shifted to the left, further compromising oxygen delivery to the tissues. Low amounts of CO are produced at low levels by the body through the metabolism of heme and equilibrate across the pulmonary capillary/alveolar bed. At these low levels, CO is necessary for cell signaling and function and holds therapeutic potential (Fix et al., 2015).

The major sources of significant exogenous exposure to CO are smoking and burning of fossil fuels (including automobiles), particularly in enclosed spaces. Smoking tobacco or marijuana elevates levels of carboxyhemoglobin in the blood turning it bright cherry red. Smoking during pregnancy may also result in significant levels of carboxyhemoglobin being formed in fetal blood and diminished oxygenation of fetal tissues. At levels below 10% carboxyhemoglobin (resulting from 35 ppm exposure), headache and dizziness may occur after 6 to 8 hours of constant exposure (Struttmann et al., 1998). Other symptoms of toxicity, such as weakness, dizziness, shortness of breath, headache, nausea, and blurred vision begin to appear within a few hours when carboxyhemoglobin levels reach 20%. Patients may have a bluish-tinted skin. Levels of 50% to 80% carboxyhemoglobin may be lethal. Diagnosis of acute CO poisoning can be challenging if there is a delay of a few hours between exposure and blood sampling due to the short half-life of carboxyhemoglobin and carboxyhemoglobin levels may differ among individuals exposed to the same levels of carbon monoxide. This means that there can be a relatively poor correlation between carboxyhemoglobin levels and the symptoms of CO poisoning. The key to therapy is removal from the source of CO and provision of an adequate supply of oxygen; hyperbaric hyperoxia may be used in serious cases (Ryter and Otterbein, 2004; Kao and Nanagas, 2005). It is now known that CO has additional effects other than carboxyhemoglobin formation, including roles in nitric oxide release and reactive oxygen species formation (Roderique et al., 2015). CO also interacts with other heme-containing proteins including myoglobin and cytochromes. Relapses in persons recovering from acute CO poisoning are most likely due to CO storage in myoglobin (Dolan, 1985). Adjunct and combination therapeutic approaches are under consideration to counteract some of these other mechanisms of toxicity.

Sulfhemoglobin and Hydrogen Sulfide Poisoning Sulfhemoglobin is a greenish pigment formed when hydrogen sulfide gas is bubbled through blood, but it plays no role in acute hydrogen sulfide poisoning. It has, however, been observed in patients exposed to high doses of the azo-dye phenazopyridine (Gopalachar et al., 2005) and the sulfonamide drug sumatriptan (Flexman et al., 2007). In the latter case report, the blood of the patient overdosed with sumatriptan was green in color. Methemoglobin also forms a complex with hydrosulfide ion, known as sulfmethemoglobin, which is analogous to cyanmethemoglobin formed after exposure to cyanide. Hydrogen sulfide poisoning is almost identical to cyanide poisoning, but sulfide also produces local tissue reactions such as conjunctivitis and pulmonary edema (Guidotti, 2015). As with cyanide-poisoning, nitrite-induced methemoglobinemia is used to treat hydrogen sulfide poisoning and provides considerable protection and antidotal effects in animals and humans. Acute poisoning with hydrogen sulfide is primarily a hazard in the oil and gas industry, agriculture, sewage and animal waste handling, construction (asphalt operations and disturbing marshy terrain), and other settings where organic material decomposes under reducing conditions (producing sewer gas), as well as in geothermal operations (Guidotti, 2015).

Alterations in Erythrocyte Survival

The normal survival of erythrocytes in the circulation is about 120 days (Dessypris, 1999). During this period the erythrocytes are exposed to a variety of oxidative injuries and must negotiate the tortuous passages of the microcirculation and the spleen. This requires a deformable cell membrane and energy to maintain the sodium–potassium gradients and repair mechanisms (Van Wijk and van Solinge, 2005). Very little protein synthesis occurs during this time, as erythrocytes are anucleate when they enter the circulation and residual mRNA is rapidly lost over the first 1 to 2 days in the circulation. Consequently, senescence occurs over time until the aged erythrocytes are removed by the spleen, where the iron is recovered for reutilization in heme synthesis. Any insult

that increases oxidative injury, decreases metabolism, or alters the membrane may cause a decrease in erythrocyte concentration and a corresponding anemia.

Nonimmune Hemolytic Anemia

Microangiopathic Anemias Intravascular fragmentation of erythrocytes gives rise to *microangiopathic hemolytic anemia* (Baker, 2006). The hallmark of this process is the presence of schistocytes (fragmented RBCs) in the peripheral blood. These abnormal cellular fragments are usually promptly cleared from the circulation by the spleen. Thus, their presence in peripheral blood samples indicates either an increased rate of formation or abnormal clearance function of the spleen. The formation of fibrin strands in the microcirculation is a common mechanism for RBC fragmentation. This may occur in the setting of disseminated intravascular coagulation, sepsis, the hemolytic-uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP). The erythrocytes are essentially sliced into fragments by the fibrin strands that extend across the vascular lumen and impede the flow of erythrocytes through the vasculature. Excessive fragmentation can also be seen in the presence of abnormal vasculature, as occurs with damaged cardiac valves, arteriovenous malformations, vasculitis, and widely metastatic carcinoma (Baker, 2006). The high shear associated with malignant hypertension may also lead to RBC fragmentation. Around 170 cases of cancer-related microangiopathic hemolytic anemia cases were reported in the literature as reviewed (Lechner and Obermeier, 2012), the majority associated with metastatic solid cancers and a minority with lymphoma. Cocaine use has been associated with microangiopathic hemolytic anemia, possibly via cocaine-induced vasoconstriction, vascular damage, platelet activation, and procoagulation (Odronic et al., 2014).

Oxidative Hemolysis Molecular oxygen is a reactive and potentially toxic chemical species; consequently, the normal respiratory function of erythrocytes generates oxidative stress on a continuous basis. The major mechanisms that protect against oxidative injury in erythrocytes include NADH-methemoglobin reductase, superoxide dismutase, catalase, and the glutathione pathway (Coleman and Coleman, 1996; Njalsson and Norgren, 2005). As indicated previously, a small amount of methemoglobin is continuously formed during the process of loading and unloading of oxygen from hemoglobin. Formation of methemoglobin is associated with formation of superoxide free radicals, which must be detoxified to prevent oxidative injury to hemoglobin and other critical erythrocyte components. Under physiologic conditions, superoxide dismutase converts superoxide into hydrogen peroxide, which is then metabolized by catalase and glutathione peroxidase (Fig. 11-6).

A number of xenobiotics, particularly compounds containing aromatic amines, are capable of inducing oxidative injury in erythrocytes (Table 11-6) (Bradberry, 2003; Percy et al., 2005). These chemicals appear to potentiate the normal redox reactions and are capable of overwhelming the usual protective mechanisms. The interaction between these xenobiotics and hemoglobin leads to the formation of free radicals that denature critical proteins, including hemoglobin, thiol-dependent enzymes, and components of the erythrocyte membrane. In the presence of hydrogen peroxide and xenobiotics such as hydroxylamine, hydroxamic acid, and phenolic compounds, a reactive ferryl (Fe^{4+}) hemoglobin intermediate may be formed according to the following reaction:

$$H_2O_2 + Hgb - Fe^{3+} \rightarrow H_2O + Hgb(\bullet +) - Fe^{4+} - O^{-}.$$

In this intermediate, referred to as compound 1, tyrosine may donate the extra electron, turning it into a reactive free radical. Compound 1 may undergo further reaction with organic compounds

Table 11-6

Xenobiotics Associated with Oxidative Injury

Arsine	Copper
Acetanilide	Phenylhydrazine
Naphthalene	Nitrobenzene
Nitrofurantoin	Phenacetin
Sulfamethoxypyridazine	Phenol
Aminosalicylic acid	Hydroxylamine
Sodium sulfoxone	Methylene blue
Dapsone	Toluidine blue
Phenazopyridine	Furazolidone
Primaquine	Nalidixic acid
Chlorates	Sulfanilamide
Sulfasalazine	

(AH_2 in the following equations) to yield additional free radicals according to the following reactions:

$$Hgb(\bullet +) - Fe^{4+} - O + AH_2 \rightarrow Hgb - Fe^{4+} - O^{-} + AH\bullet + H^{+};$$
$$Hgb - Fe^{4+} - O^{-} + AH_2 + H^{+} \rightarrow Hgb - Fe^{3+} + AH\bullet + H_2O.$$

Hemoglobin contains free exposed cysteines (β93) that are critical for the structural integrity of the molecule. Oxidation of these groups can denature hemoglobin and decrease its solubility. The oxidized, denatured hemoglobin species comprise what has been designated sulfhemoglobin. The denatured hemoglobin can form aggregates that bind to the cell membrane to form inclusions called *Heinz bodies,* a hallmark of oxidative injury to erythrocytes (Jandl, 1987). Heinz bodies are usually visualized by use of phase contrast microscopy or supravital stains such as crystal violet or more recently by flow cytometry (Palasuwan et al., 2017). These membrane-associated inclusions impair the deformability of the erythrocyte membrane and thus impede movement of erythrocytes through the microcirculation and the spleen. Heinz bodies are effectively removed from the erythrocyte by the spleen, so they are not often observed in peripheral blood samples from patients despite ongoing oxidative injury. However, the culling of Heinz bodies can alter the morphology of the affected cells, giving rise to what are called "bite" cells and "blister" cells, which may provide an important clue as to the ongoing process (Yoo and Lessin, 1992). These cells look as though a portion of the cytoplasm had been cut away. Heinz body formation can be induced by in vitro exposure to oxidizing agents and patients with oxidative hemolysis often show increased in vitro formation of Heinz bodies.

Oxidative denaturation of the globin chain decreases its affinity for the heme group, which may dissociate from the globin chain during oxidative injury (Kumar and Bandyopadhyay, 2005). Free heme itself is toxic to cells and can induce tissue injury through formation of reactive oxygen species. The ferric iron in the heme ring may react with chloride to form a complex called hemin. Hemin is hydrophobic and intercalates into the erythrocyte membrane from which it is removed by interaction with albumin. However, if the rate of hemin formation exceeds the rate of removal by albumin, hemin accumulates in the membrane, where it can cause rapid lysis of the erythrocyte.

The generation of free radicals may also lead to peroxidation of membrane lipids (Jandl, 1987; Kumar and Bandyopadhyay, 2005). This may affect the deformability of the erythrocyte and the permeability of the membrane to potassium. The alteration of the Na^+/K^+ gradient is independent of injury to the Na^+/K^+ pump and is potentially lethal to the affected erythrocyte. Oxidative injury also

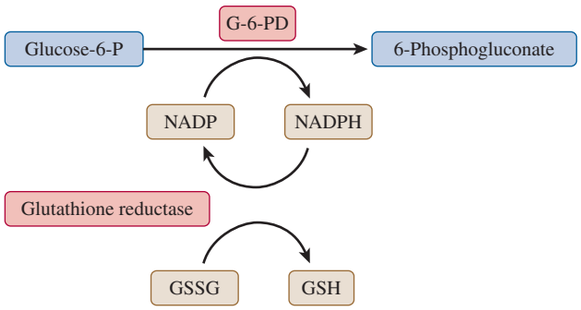

Figure 11-7. *The hexose monophosphate shunt in the erythrocyte is critical for generation of NADPH, which helps maintain an intracellular supply of reduced glutathione (GSH).* With a deficiency of glucose-6-phosphate dehydrogenase (G-6-PD), the rate-limiting step in this pathway, the cellular levels of GSH are reduced. Such cells show increased susceptibility to oxidative injury. Acute exposure of such cells to an oxidizing agent can result in rapid hemolysis.

impairs the metabolic machinery of the erythrocyte, resulting in a decrease in the concentration of ATP (Tavazzi et al., 2000). Damage to the membrane can also permit leakage of denatured hemoglobin from the cell. Such free denatured hemoglobin can be toxic on its own. Free hemoglobin may irreversibly bind nitric oxide, resulting in vasoconstriction. Released hemoglobin may form nephrotoxic hemoglobin dimers, leading to kidney damage.

Oxidative injury thus results in a number of changes that decrease the viability of erythrocytes. Protection against many of the free radical–induced modifications is mediated by reduced glutathione, along with antioxidant defense enzymes including superoxide dismutase, catalase, and glutathione peroxidase, and glutathione reductase (Njalsson and Norgren, 2005; Perrone et al., 2012) (see Chap. 3). Formation of reduced glutathione is dependent on NADPH and the hexose monophosphate shunt (Fig. 11-7). Significant oxidative injury usually occurs when the concentration of the xenobiotic is high enough (due to either high exposure or decreased metabolism of the xenobiotic) to overcome the normal protective mechanisms, or, more commonly, when there is an underlying defect in the protective mechanisms.

The most common enzyme defect associated with oxidative hemolysis is G-6-PD deficiency, a relatively common X-linked disorder characterized by alterations in the primary structure of G-6-PD that diminish its functional activity (Beutler, 1996). It is often clinically asymptomatic until the erythrocytes are exposed to oxidative stress. The stress may come from the host response to infection or exposure to xenobiotics. The level of G-6-PD normally decreases as the erythrocytes age. Over 140 mutations have been identified (Mason et al., 2007; Cappellini and Fiorelli, 2008) and high-frequency variants are found in African, Mediterranean, and Asiatic populations (Porter et al., 1964). G-6-PD deficiency may have developed as a protection against malaria, which is endemic in these regions (Luzzatto et al., 1969). In the African type of G-6-PD deficiency, the enzyme is less stable than normal; thus, the loss of activity is accelerated compared with normals. In the Mediterranean type of G-6-PD deficiency, the rate of loss of enzyme activity is even higher. Consequently, the older erythrocytes with the lowest levels of G-6-PD are most susceptible to hemolysis, with the degree of hemolysis affected by the residual amount of enzyme activity as well as the magnitude of the oxidative injury.

Erythrocyte-reduced glutathione is rapidly depleted on exposure to an oxidizing chemical in patients with G-6-PD deficiency. This leads to the series of oxidative injuries described above with the development of intravascular and extravascular hemolysis. Oxidative hemolysis is usually reversible if the process is promptly recognized and the offending chemical is removed. Occasionally the hemolysis may be sufficiently severe to result in death or serious morbidity (e.g., renal failure). A connection between fava bean ingestion and hemolytic anemia was recognized around 510 BC, by the philosopher and mathematician Pythagoras who forbade his followers from eating the *Vicia faba*. The link was later confirmed and called favism and recognized as being associated with G-6-PD deficiency (Mason et al., 2007; Ho et al., 2014). Following fava bean ingestion, the generation of divicine and isouramil, from vicine and convicine, leads to peroxide production and GSH oxidation and depletion (Winterbourn, 1989; Winterbourn et al., 1989). The resulting anemia is acute and severe. In a similar manner to fava beans, the malaria drug primaquine causes hemolysis in G-6-PD-deficient individuals (Alving et al., 1956; Beutler, 1996; Howes et al., 2013; Watson et al., 2017). Copper toxicity also causes hemolysis and methemoglobinemia in G-6-PD deficient individuals (Moore and Calabrese, 1980; Yang et al., 2004). Copper generates free radicals and inhibits enzymes of erythrocyte metabolism including NADPH-methemoglobin reductase (Clopton and Saltman, 1997).

Deficiency of glutathione synthetase can also lead to hemolytic anemia due to the reduced intracellular concentration of glutathione (Njalsson and Norgren, 2005; Ristoff and Larsson, 2007; Soylu Ustkoyuncu et al., 2017).

Nonoxidative Chemical-Induced Hemolysis Some xenobiotics act by both oxidative and nonoxidative mechanisms while some are associated with hemolysis without significant oxidative injury (Beutler, 2006a, 2006b, 2006c). Arsenic hydride is a gas that is formed during several industrial processes. Inhalation of the gas can result in severe hemolysis, with anemia, jaundice, and hemoglobinuria. Arsine has oxidative effects and also possibly acts on the Na^+/K^+ pump mechanism, leading to swelling and hemolysis (Pakulska and Czerczak, 2006). Stibine, a colorless gas and principal covalent hydride of antimony that is used to dope silicon with antimony in the semiconductor industry, causes severe hemolysis but the mechanism is unknown. Lead poisoning is associated with defects in heme synthesis and a shortening of erythrocyte survival. The cause of the hemolysis is uncertain, but lead can cause membrane damage and interfere with the Na^+/K^+ pump (Suwalsky et al., 2003; Rehman, 2013). These effects may cause premature removal of erythrocytes from the circulation. Excess copper has oxidative effects as mentioned above and also exerts inhibitory effects on the hexose monophosphate shunt and the Embden–Meyerhof pathway. Ingestion of excess chromium may result in hemolytic anemia and thrombocytopenia, although the mechanism is not known (Cerulli et al., 1998; Ray and Sarkar, 2012). However, hexavalent chromium also leads to eryptosis—apoptosis-like cell death—through increased Ca^{2+} entry and depleted cytosolic ATP in erythrocytes, rather than hemolysis (Lupescu et al., 2012). Significant hemolysis may also occur with biologic toxins found in insect and snake venoms, through unknown mechanisms (Beutler, 2006a, 2006b, 2006c).

Immune Hemolytic Anemia Immunologic destruction of erythrocytes is mediated by the interaction of IgG or IgM antibodies with antigens expressed on the surface of the erythrocyte. In the case of autoimmune hemolytic anemia the antigens are intrinsic components of the patient's own erythrocytes. A large number of drugs have been associated with enhanced binding of immunoglobulin to the erythrocyte surface and shortened RBC survival (Arndt and Garratty, 2005).

A number of mechanisms have been implicated in xenobiotic-mediated antibody binding to erythrocytes (Arndt and Garratty,

2005). Some drugs, of which penicillin is a prototype, appear to bind to the surface of the cell, with the "foreign" drug acting as a *hapten* and eliciting an immune response. The antibodies that arise in this type of response only bind to drug-coated erythrocytes. Other drugs, of which quinidine is a prototype, bind to components of the erythrocyte surface and induce a conformational change in one or more components of the membrane. This type of interaction can give rise to a confusing array of antibody specificities. Some of the antibodies recognize only the *drug–membrane component* complex; others are specific for the membrane component, but only when drug is present; while still others may recognize the membrane component in the presence or absence of the drug. An additional mechanism, for which α-methyldopa is a prototype, results in production of a *drug-induced autoantibody* that cannot be distinguished from the antibodies arising in idiopathic autoimmune hemolytic anemia. The mechanism for induction of this group of antibodies is not understood, but may be related to development of an autoimmune response. A variant of this type of response is the augmentation of autoimmune hemolytic anemia that may occur during therapy of some lymphoproliferative disorders. Autoimmune phenomena, including autoimmune hemolytic anemia, are known to occur in lymphoproliferative disorders such as chronic lymphocytic leukemia (CLL). Treatment of these disorders with some drugs has been associated with worsening of the hemolytic anemia (Gonzalez et al., 1998). It has been hypothesized that therapy further disrupts regulation of the autoimmune phenomenon, allowing increased antibody production.

Some xenobiotics are associated with *nonspecific deposition of proteins* on erythrocytes. This was first associated with cephalosporins (beta-lactam antibacterials) but has also been seen with other drugs, including cisplatin (antineoplastic) and the beta-lactam antibacterials sulbactam and clavulanate (Arndt and Garratty, 2005). Immunoglobulin and complement proteins may be among the proteins deposited on the erythrocyte surface. These proteins may cause a positive direct antiglobulin test, suggesting a drug-induced antibody response. However, there is no evidence of a drug-dependent antibody in the patient's serum, and drug-treated erythrocytes may bind antibody from normal nondrug exposed serum. This form of antibody deposition is generally not associated with hemolysis, although the possibility of hemolysis related to this type of reaction has been raised.

Drug-induced intravascular hemolysis is often a dramatic clinical event and may be associated with fever, chills, back pain, hypotension, a rapid fall in hemoglobin concentration, a decrease in serum haptoglobin, a marked increase in serum lactate dehydrogenase, and hemoglobinuria (Arndt and Garratty, 2005). The clinical picture of extravascular hemolysis depends on the rate of hemolysis but is usually less dramatic. Often there is evidence of reticulocytosis, polychromasia, spherocytosis, a moderate increase in serum lactate dehydrogenase, and an increase in serum bilirubin. Serologic studies usually show evidence of IgG and/or complement on the surface of erythrocytes, although it may be difficult to document that antibody binding is drug-dependent. The mainstay of therapy in patients with drug-induced hemolytic anemia is removal of the offending agent and avoidance of reexposure.

TOXICOLOGY OF THE LEUKON

Components of Blood Leukocytes

The leukon consists of leukocytes, or white blood cells. They include granulocytes, which may be subdivided into neutrophils, eosinophils, and basophils; monocytes; and lymphocytes. Granulocytes and monocytes are nucleated ameboid cells that are phagocytic. They play a central role in the inflammatory response and host defense. Unlike the RBCs, which reside exclusively within blood, granulocytes and monocytes generally pass through the blood on their way to the extravascular tissues, where they reside in large numbers. Circulating mature neutrophils undergo senescence, and the consequent increased membrane expression of CXCR4 leads them to migrate to different tissues in response to the chemokine CXCL12 (Martin et al., 2003; Eash et al., 2009). In mice, around a third of senescent neutrophils home to the bone marrow via CXCL12/CXCR4 chemotaxis, and undergo apoptosis and phagocytosis by macrophages (Furze and Rankin, 2008a, 2008b). The engulfing macrophages produce G-CSF thus stimulating neutrophil production (Furze and Rankin, 2008a, 2008b; Summers et al., 2010). The glucocorticoid-regulated protein, annexin A1, was recently shown to modulate neutrophil maturation and senescent neutrophil homing to the bone marrow (Dalli et al., 2012; Machado et al., 2014; Machado et al., 2016).

Granulocytes are defined by the characteristics of their cytoplasmic granules as they appear on a blood smear stained with a polychromatic (Romanovsky) stain. Neutrophils, the largest component of blood leukocytes, are highly specialized in the mediation of inflammation and the ingestion and destruction of pathogenic microorganisms. The turnover of neutrophils is enormous and increases dramatically in times of inflammation and infection, elevating the number of these cells released from the bone marrow. Eosinophils and basophils modulate inflammation through the release of various mediators and play an important role in other homeostatic functions. All these are influenced by cellular and humoral immune responses.

In the world of clinical and experimental toxicology, the neutrophil is the focus of concern when evaluating granulocytes as possible targets for drug and nontherapeutic chemical effects. Eosinophils and basophils are far more difficult to study, with changes in these populations most frequently associated with reactions to other target organs or systemic toxicity. Examples include the eosinophilia observed with the toxic oil syndrome that resulted from exposure to rapeseed oil denatured with aniline utilized in northwestern Spain (Kilbourne et al., 1991), and the eosinophilia–myalgia syndrome associated with λ-tryptophan preparations contaminated with 1,1-ethylidene-bis[tryptophan] (Varga et al., 1992). Peripheral eosinophilia is often but not reliably observed with hypersensitivity reactions to drugs (Roujeau, 2005), while tissue eosinophilia can be diagnostic, in the context of a suggestive clinical course, in conditions such as drug-induced cutaneous vasculitis (Bahrami et al., 2006) and eosinophilic pneumonia (Flieder and Travis, 2004). This variability in systemic response can be genetically predisposed, as demonstrated in studies using transgenic mice on genetic restrictions in people afflicted by the aforementioned toxic oil syndrome (Gallardo et al., 2005). The time course of the reaction can also influence whether eosinophilia can be demonstrated in hypersensitivity disease (Roujeau, 2005).

Evaluation of Granulocytes

The most informative test to assess the neutrophil compartment is the blood neutrophil count. Accurate interpretation requires an understanding of neutrophil kinetics and the response of this tissue to physiologic and pathologic changes. In the blood, neutrophils are distributed between *circulating* and *marginated* pools, which are of equal size in humans and in constant equilibrium (Athens et al., 1961). A blood neutrophil count assesses only the circulating pool, which remains between 1800/μL and 7500/μL in a healthy adult human (Dale, 2006).

This constancy is remarkable, considering that as many as 10^{11} neutrophils are released from the marrow daily, that this circulating pool represents only 1% of the total body neutrophils (Semerad et al., 2002), and that the circulating half-life of these cells is only approximately 6 hours (Cartwright et al., 1964). How this extraordinary regulation is achieved is not fully understood. The upregulation and downregulation of chemokine receptors is involved in the release of neutrophils from the bone marrow and their return following senescence (Martin et al., 2003). IL-23, IL-17, and G-CSF modulate niche size, with consequent effects on hematopoietic stem/progenitor cells and their fates. An expanded view of the regulation of neutrophil homeostasis has been developed recently and involves a role for the innate immune system in sensing and regulating granulopoiesis and a dependence on danger receptors, macrophages, and circadian rhythms (Schuettpelz et al., 2014; Wirths et al., 2014).

Neutrophil kinetics and response to disease vary substantially among animal species (Feldman et al., 2006). Thus, a thorough understanding of these features in any animal model used in investigative toxicology is required before informed interpretations can be made. In humans, clinically significant neutropenia occurs when the blood neutrophil count is less than 1000/μL, but serious recurrent infections do not usually occur until counts fall below 500/μL (Dale, 2006). Morphologic assessment of peripheral blood granulocytes can be helpful in characterizing neutropenia. In humans and most healthy animal species, mature (segmented) and a few immature (band) neutrophils can be identified on blood films stained with Wright or Giemsa stain. During inflammation, a "shift to the left" may occur, which refers to an increased number of immature (nonsegmented) granulocytes in the peripheral blood, which may include bands, metamyelocytes, and occasionally myelocytes. During such times, neutrophils may also show "toxic" granulation, Döhle bodies, and cytoplasmic vacuoles. These morphologic changes may be prominent in sepsis or as a result of drug or chemical intoxication. In order to fully characterize such changes or understand the pathogenesis of the abnormality, bone marrow must be examined using marrow aspirates and biopsies. These provide information on rates of production, bone marrow reserves, and abnormalities in cell distribution, and occasionally specific clues as to etiology. In vitro stem cell assays may be used to assess the granulocyte progenitor cell compartment, which may include colony-forming-units granulocyte/monocyte (CFU-GM) performed in a semisolid medium, such as agar or methylcellulose, which contains appropriate growth factors, as discussed later in this chapter. Normal human marrow specimens contain approximately 50 to 1000 CFU-GM per 10^6 nucleated cells cultured (Liesveld and Lichtman, 1997). Marrow stem cell reserves can be assessed in vitro after administration of G-CSF (Demirer and Bensinger, 1995), which stimulates increased production and release of neutrophil precursors. Glucocorticoids (Peters et al., 1972) and epinephrine (Babior and Golde, 1995) may also be used for this purpose but are rarely used in a clinical setting.

CXCL12, the CXC chemokine ligand of CXCR4, mediates the retention of myeloid stem cells within their bone marrow niche (Laurence, 2006; Sharma et al., 2011), as well as mature neutrophils within the bone marrow pool (Semerad et al., 2002). Plerixafor is an inhibitor of CXCR4 that, when administered with G-CSF, can cause a transient release of neutrophils and CD34+ (stem) cells into the circulation (Liles et al., 2005). The latter can be collected and re-engrafted to form new functioning bone marrow (Broxmeyer et al., 2005). Plerixafor is approved for mobilization in combination with G-CSF in patients with non-Hodgkin lymphoma and multiple myeloma (Pusic and DiPersio, 2010) and works even in very poor mobilizers (Mohty et al., 2017). Several newer classes of CXCR4 antagonists are in development (Domingues et al., 2017b). The homing and retention of such hematopoietic stem/progenitor cells is influenced by complex and highly coordinated interactions among adhesion molecules, cytokines, growth factors, and regulatory cofactors that are only partially understood (Sharma et al., 2011). The ability to manipulate this system, as in the above experiments and through the many recombinant proteins under development, will continue to provide important research, diagnostic, and therapeutic tools for the hematologist, oncologist, and toxicologist.

The degree of proliferation in the granulocyte compartment can also be assessed using older techniques that employ ^{3}H-thymidine suicide assays or DNA-binding dyes with fluorescence-activated cell sorting analyses (Keng, 1986). In recent years, neutrophils have been found to more plastic and heterogeneous than previously thought (Kruger et al., 2015; Scapini et al., 2016; Marini et al., 2017). CD10 was identified as a novel surface marker with the potential to distinguish T-cell suppressive and T-cell stimulatory neutrophils in peripheral blood (Kruger et al., 2015).

Toxic Effects on Granulocytes

The toxicologist is concerned with the effect of xenobiotics on granulocytes as relates to proliferation (granulopoiesis) and kinetics, the extent to which a drug or chemical contaminant can impair the vital functions these cells perform, and how neutrophils mediate or exacerbate inflammatory disease or other target organ toxicity. The last two areas are discussed in Chap. 12, as they relate to their role as important effector cells of the immune system. However, it is difficult to separate effects on granulopoiesis and neutrophil kinetics from that of function. Both are complex and highly regulated, as discussed above, through an array of growth factors, chemokines, cytokines, and interactions with monocytes, dendritic cells, and lymphocytes in a bidirectional, multicompartmental manner (Nathan, 2006). Such complexity is not surprising, given the daunting task these cells perform, which is elegantly described in a review by Nathan (2006): "The [neutrophil] must remain non-sticky as it hurtles through the arterial and arteriolar circulation; then it must squeeze through capillaries smaller in diameter than itself, without allowing collision, friction, or distortion to activate it. A fraction of the population must adhere tightly enough to the normal endothelium of post-capillary venules to resist being washed away in the circulation, but loosely enough to roll while scouting for evidence of tissue damage and microbial infection. If such evidence is received, the cell must crawl to a boundary between endothelial cells, penetrate the junctions and the underlying basement membrane without damaging these structures, move up the chemotactic gradient, and decide whether its original information remains valid. If the answer is negative, the cell must execute itself by apoptosis. If the answer is positive, the cell must attempt to engulf and destroy microbes. If it cannot locate microbes quickly, it must attempt to destroy them at a distance by releasing every weapon at its disposal." Many of the mediators and interactions that enable this feat have now become targets for the therapeutic dysregulation of these processes, which has led to the development of candidate drugs that may prove to be uniquely efficacious and/or toxic, as discussed below.

Effects on Proliferation and Kinetics

As with other hematopoietic tissue, the high rate of proliferation of neutrophils makes their progenitor and precursor granulocyte pool particularly susceptible to inhibitors of mitosis. Such effects by cytotoxic drugs are generally nonspecific, as they similarly affect cells of the dermis, gastrointestinal tract, and other rapidly dividing tissues. Agents that affect both neutrophils and monocytes pose a greater risk for toxic sequelae, such as infection (Dale, 2006). Such effects tend to be

dose-related, with mononuclear phagocyte recovery preceding neutrophil recovery (Arneborn and Palmblad, 1982).

Myelotoxicity in clinical medicine and preclinical safety studies today is most commonly seen with cytoreductive cancer chemotherapy drugs. However, this is changing, as more cancer cell–targeted, normal-tissue-sparing anticancer agents are being developed. In fact, most published reviews today on drug-induced neutropenia and agranulocytosis do not even address these effects associated with such cytotoxic drugs. Accordingly, the term commonly refers to *idiosyncratic reactions,* most often secondary to accelerated immune-mediated destruction of neutrophils and their progenitors, as discussed below. The toxicity associated with cytotoxic drugs, however, remains important in that it is often dose-limiting with serious manifestations that include febrile neutropenia associated with life-threatening infections (Kuderer et al., 2002). These drugs vary in terms of their mechanism, the kinetics of the cytopenias they induce, and how individual patients or animals respond. Most act to inhibit DNA synthesis or directly attack its integrity through the formation of DNA adducts or enzyme-mediated breaks (Chabner et al., 2006). While cytoreductive drugs such as alkylating agents, cisplatin, and nitrosoureas can be toxic to both resting and actively dividing cells, nonproliferating cells such as metamyelocytes, bands, and mature neutrophils are relatively resistant (Friberg and Karlsson, 2003). Generally, stem cells cycle slowly and are therefore minimally affected by a single administration of a cytotoxic drug such as 5-fluorouricil; however, such exposure can stimulate cycling activity, making these cells more vulnerable to doses administered 3 to 5 days later (Harrison and Lerner, 1991). Sustained exposure to drugs affecting slowly cycling stem cells is thought to cause more prolonged myelosuppression, similar to that observed with idiosyncratic toxic neutropenia (Tannock, 1986). Recent studies suggest that these features are shared by leukemic stem cells, which is the basis for the more sophisticated cancer interventions and regimens under development today (Eramo et al., 2008; Ricci-Vitiani et al., 2010; Vermeulen et al., 2010).

Finally, there is considerable variation among individuals as regards susceptibility to bone marrow toxicity, related to drug metabolism as with 5-fluorouracil (Sundman-Engberg et al., 1998) and 6-mercaptopurine (Chabner et al., 2006), gender (Huang et al., 2007; Gamazon et al., 2011), and other factors. Based on these and other data, including plasma drug concentrations, "semi-mechanistic" pharmacokinetic/pharmacodynamic models of myelosuppression have been developed to tailor doses and treatment regimens to individual patients (Friberg and Karlsson, 2003). Pharmacokinetic monitoring is now routinely performed with some anticancer treatment regimens, particularly high-dose methotrexate (Chabner et al., 2006).

Two innovations have had a dramatic impact on cancer chemotherapy and the dose-limiting myelotoxicity associated with these drugs: (1) the aforementioned development of drugs with cancer cell–specific molecular targets that are relatively bone marrow sparing, such as those that target aberrant growth factor receptor signaling, apoptosis, angiogenesis, and other metabolic, immune, inflammatory, and mutation-promoting pathways that selectively advantage tumor cells (Hanahan and Weinberg, 2011); and (2) the use of hematopoietic growth factors, the cotreatment with which mitigates or successfully rescues patients from the effects of myelosuppression (Andres et al., 2010).

Lindane, an insecticide used to treat seeds and soil, has been associated with leukopenia (Parent-Massin et al., 1994). It is cytotoxic for human CFU-GMs at concentrations observed in blood and adipose tissue from exposed human subjects. An example of chemicals affecting mature cells is methyl methacrylate monomer, which has been used in orthopedic surgical procedures and is cytotoxic to both neutrophils and monocytes at clinically relevant concentrations (Dahl et al., 1994).

Chemicals that affect granulocyte kinetics can cause neutropenia or neutrophilia that has variable toxicologic significance. The effects of epinephrine and glucocorticoids on granulocyte kinetics were discussed previously. Dexamethasone has long been known to cause neutrophilia through enhanced release of mature neutrophils from the bone marrow and demargination (release of neutrophils from the endovascular lining of the blood vessel into circulation), with the latter being the largest contributor to the expanded circulating pool (Nakagawa et al., 1998). It is now clear that the reduced margination of neutrophils is mediated by multiple effects, including altered chemotaxis, expression of adhesion molecules, and the release of mediators from other cells (Caramori and Adcock, 2005; Barnes, 2006). It is widely assumed that inhibition of margination and homing (migration to the organ of origin, i.e., bone marrow) are among the important mechanisms of the anti-inflammatory and immunosuppressive effects of these widely used drugs.

Effects on Function While there are a variety of disorders associated with defects in the parameters of neutrophil function discussed above, demonstrable in vivo effects associated with drugs and nontherapeutic chemicals are surprisingly few (Borregaard and Boxer, 2006). Examples include ethanol and glucocorticoids, which impair phagocytosis and microbe ingestion in vitro and in vivo (Brayton et al., 1970). Iohexol and ioxaglate, components of radiographic contrast media, have also been reported to inhibit phagocytosis (Lillevang et al., 1994). Superoxide production, required for microbial killing and chemotaxis, has been reported to be reduced in patients using parenteral heroin as well as in former opiate abusers on long-term methadone maintenance (Mazzone et al., 1994).

In addition to glucocorticoids, several drugs and nontherapeutic chemicals have been shown to inhibit neutrophil chemotaxis. Examples include macrolide antibiotics, which suppress the expression of the adhesion molecule ICAM (Tamaoki, 2004); zinc salts, which are found in antiacne preparations (Dreno et al., 1992); the insecticide chlordane (Miyagi et al., 1998); and mercuric chloride/methylmercuric chloride (Contrino et al., 1988). More common is the activation of neutrophils with the potential for proinflammatory consequences, specifically through increased phagocytosis, O_2^- production, or both. Examples include the environmental contaminants sodium sulfite, mercuric chloride, chlordane, and toxaphene (Girard, 2003).

Idiosyncratic Toxic Neutropenia Of greater concern are chemicals that unexpectedly damage neutrophils and granulocyte precursors—particularly to the extent of inducing *agranulocytosis,* which is characterized by a profound depletion in blood neutrophils to less than 500/μL (Pisciotta, 1973). The term was first used by Schultz in 1922 to describe patients with severe sore throat associated with a marked reduction of granulocytes, followed by sepsis and death (Schultz, 1922). Drug association was first demonstrated through rechallenge experiments performed on two patients with aminopyrine-induced agranulocytosis, who developed leucopenia within 2 hours of reexposure (Madison and Squier, 1934). A later study demonstrated that blood transferred from an agranulocytosis patient to normal controls resulted in a rapid drop in neutrophil count, suggesting a role for a preformed blood factor(s) such as antibodies (Moeschlin and Wagner, 1952). Such toxicity occurs in specifically conditioned individuals, and is therefore termed "idiosyncratic." While rare, the seriousness of this disorder, as with aplastic anemia, makes it among the most important hematotoxicities, as

Table 11-7

Stages of Granulocytopoiesis: Site of Xenobiotic-Induced Cellular Damage

STAGE OF DEVELOPMENT	DISEASE	OFFENDING DRUGS
Uncommitted (totipotential) stem cell CFU-S	Aplastic anemia	Chloramphenicol Gold salts Phenylbutazone Phenytoin Mephenytoin Carbamazepine
Committed stem cell CFU-G CFU-E BFU-E	Aplastic anemia Agranulocytosis Pure red cell aplasia	Carbamazepine Chlorpromazine Carbamazepine Clozapine Phenytoin
Morphologically recognizable precursors	Hypoplastic marrow	Most cancer chemotherapy agents
Dividing pool Promyelocyte Myelocyte	Hypoplastic marrow	Chloramphenicol Alcohol
Nondividing pool Metamyelocytes, bands PMNs	Agranulocytosis	Clozapine Phenothiazines, etc.
Peripheral blood lysis PMNs	Agranulocytosis	Clozapine, etc. Aminopyrine
Tissue pool		

SOURCE: Modified from Pisciotta (1997), with permission from Elsevier Science.

regards regulatory scrutiny and risk:benefit considerations, which justifies the detailed discussion below. Acute agranulocytosis is attributed to drugs in >70% of cases (Garb, 2007).

Mechanisms of idiosyncratic damage often do not relate to pharmacologic properties of the parent drug, which makes managing this risk a particular challenge to hematologists and toxicologists, as discussed later in this chapter. Preclinical toxicology is rarely predictive of these effects, which are generally detected and characterized following exposure of a large population to the drug (Dieckhaus et al., 2002; Szarfman et al., 2002). Idiosyncratic drug-induced neutropenia may be dose-related and involve a nonselective disruption of protein synthesis or cell replication resulting in agranulocytosis, as discussed below. Alternatively, and more commonly, it may not be dose-dependent (as qualified below), in which case it is usually thought to be allergic or immunologic in origin. The latter has been observed with many drugs, and is more frequently observed in women, older patients, and patients with a history of allergies (Dale, 2006).

Idiosyncratic xenobiotic-induced agranulocytosis may involve a sudden depletion of circulating neutrophils concomitant with exposure, which may persist as long as the chemical or its metabolites persist in the circulation. Hematopoietic function is usually restored when the chemical is detoxified or excreted. Suppression of granulopoiesis, however, is more prevalent than peripheral lysis of neutrophils and is asymptomatic unless sepsis supervenes (Pisciotta, 1973). The onset of leukopenia in the former is more gradual, but may be precipitous if lysis of circulating neutrophils also occurs. The pattern of the disease varies with the stage of granulopoiesis affected, which has been well defined for several drugs that cause bone marrow toxicity (Table 11-7). Toxicants affecting uncommitted stem cells induce total marrow failure, as seen in aplastic anemia, which generally carries a worse prognosis than chemicals affecting more differentiated precursors, such as CFU-G. It is thought that, in the latter case, surviving uncommitted stem cells eventually produce recovery, provided that the risk of infection is successfully managed during the leukopenic episodes (Pisciotta, 1973).

The incidence of drug-induced idiosyncratic agranulocytosis ranges from 2 to 15 cases per million patients exposed to drugs per year (Andres et al., 2002). While all drugs may be causative, the most commonly incriminated drugs include antithyroid agents and antibiotics, particularly sulfonamides (Andres et al., 2002; Berliner et al., 2004). An extensive case–control study on drug-induced agranulocytosis in Barcelona, Spain, followed 177 community cases (representing 78.73 million person-years), which were compared with 586 sex-, age-, and hospital-matched control subjects previously treated with the same putative drugs (Ibanez et al., 2005). The annual incidence was 3.46:1 million, which increased with age. The fatality and mortality rates were 7.0% and 0.24:1 million, respectively. The drugs most frequently implicated (in decreasing order of odds ratio) were ticlopidine hydrochloride (antithrombotic), calcium dobesilate (vasoprotective, antivaricose), antithyroid drugs, metamizole (analgesic), and spironolactone (diuretic). Other drugs associated with significant risk were pyrithyldione (psycholeptic), cinepazide (peripheral vasodilator), aprindine hydrochloride (antiarrhythmic), carbamazepine (antiepileptic), sulfonamides (intestinal antiinfective), phenytoin and phenytoin sodium (antiepileptic), β-lactam antibiotics, erythromycin stearate and erythromycin ethylsuccinate (anti-infectives), and diclofenac sodium (nonsteroidal anti-inflammatory). These drugs accounted for 68.6% of all cases.

Some drugs commonly implicated in the past, such as phenylbutazone (nonsteroidal anti-inflammatory, antirheumatic), chloramphenicol (antibiotic), and ticlopidine (antithrombotic), are used less commonly today due to this and other toxicity. Curiously, the incidence of this idiosyncratic reaction has not changed in the western hemisphere over the past 30 years, despite this evolution of putative drugs, suggesting that host factors are critical in the pathogenesis of the toxicity (Tesfa et al., 2009).

The severity of the neutropenia often causes severe sepsis or localized infections, such as sore throat, pneumonia, or various cutaneous infections. Prior to the use of hematopoietic growth factors, the mortality was 10% to 20% (Julia et al., 1991); with appropriate management it is now less than 5% (Andres et al., 2010).

Clozapine-induced agranulocytosis (CIAG) is unique, as a genetic predisposition has been established (Yunis et al., 1995; Turbay et al., 1997). Prior to an aggressive risk management program that included careful screening of prospective patients and early detection through hematologic monitoring, the incidence of agranulocytosis with this highly efficacious atypical antipsychotic was as high as 1% to 2%. This measure reduced the incidence to 0.38% and the mortality by 90% (Honigfeld et al., 1998). As reviewed, evidence from genetic association studies shows that the human leukocyte antigen (HLA) region plays a role in susceptibility to CIAG but multiple genes are likely to be involved (de With et al., 2017).

Mechanisms of Toxic Neutropenia Because cases of drug-induced neutropenia are relatively rare, sporadic, or transient, studies on the pathogenesis of this hematotoxicity have been limited. Toxic neutropenia has historically been classified according to mechanism as *immune-mediated* or *nonimmune-mediated.* There has long been a debate in the literature as to whether the principal mechanism of idiosyncratic drug-induced neutropenia (including agranulocytosis) is immune-mediated, or subsequent to the generation of toxic metabolites—both involving "preconditioning" through genetically determined immune responses to, or metabolism of, the putative drug, respectively. Both are consistent with the aforementioned early observations of Madison and Squier (1934) where transfusion of blood from affected patients to normal subjects induced neutropenia, presumably involving a humoral factor—presumably an antibody. There is now consensus that the mechanism of most drug-induced idiosyncratic diseases, including hepatotoxicity, Stevens–Johnson syndrome, agranulocytosis, and aplastic anemia, usually is immune-mediated, often involving altered proteins through reactive metabolite–mediated damage (Zhang et al., 2011). The Th17 T lymphocyte is emerging as an important mediator of this through a cellular immune mechanism demonstrated in aplastic anemia (de Latour et al., 2010; Platanias, 2010). Two hypotheses as to the mechanism (or alternative pathogeneses) for these idiosyncratic reactions that have emerged based on observations over the past 10 years include the *hapten hypothesis* and the *danger hypothesis* (Zhang et al., 2011). The former involves a reactive metabolite binding to a protein making it "foreign," which in turn induces an immune response that leads to the toxicity. The latter has a reactive metabolite damaging a cell, which elicits an immune response against the drug or an autoimmune response. The "perfect storm" in the rare individual in which these reactions occur is thought to be caused by preconditioned (or individual-specific) circumstances that drive both the metabolism of the drug and the immune reactions to the altered proteins. Consistent with these hypotheses is the fact that aplastic anemia and the more common agranulocytosis can be induced by many of the same drugs, most of which can be oxidized to reactive metabolites by the myeloperoxidase system of neutrophils, macrophages, and/or their precursors (Uetrecht, 1990).

The incidence of xenobiotic-induced immune neutropenia is considerably less than that of immune hemolytic anemias (Vandendries and Drews, 2006). In immune-mediated neutropenia, antigen–antibody reactions lead to destruction of peripheral neutrophils, granulocyte precursors, or both. As with RBCs, an immunogenic xenobiotic can act as a hapten, where the chemical must be physically present to cause cell damage, or may induce immunogenic cells to produce antineutrophil antibodies that do not require the drug to be present (Salama et al., 1989). Also like immune hemolytic anemia, drug-induced *autoimmune* neutropenia has been observed (Capsoni et al., 2005). Examples of drugs that have been implicated include fludarabine (antineoplastic, antimetabolite) (Stern et al., 1999), propylthiouracil (antithyroid) (Sato et al., 1985), and rituximab (antineoplastic, immunomodulator) (Voog et al., 2003). Xenobiotic-induced immune-mediated damage may also be cell-mediated (Pisciotta, 1973).

Detection of xenobiotic-induced neutrophil antibodies is also considerably more difficult than that of RBCs or platelets. This is because the neutrophil is relatively fragile and short-lived, and becomes easily activated (Palmblad et al., 2001). There is no good test for direct antigranulocyte antibodies comparable to the Coombs (antiglobulin) test. Several assays have been used, which can be grouped into four categories: those measuring endpoints of leukoagglutination, cytotoxic inhibition of neutrophil function, immunoglobulin binding, and those using cell-mediated mechanisms. Among the specific challenges these assays pose are the tendency of neutrophils to stick to each other in vitro, attract immunoglobulin nonspecifically to their surface, and reflect membrane damage through indirect and semiquantitative changes (Pisciotta, 1973). Most neutrophil-associated antibody assays applied clinically today employ flow cytometry, with the indirect granulocyte immunofluorescence test being the most promising (Sella et al., 2010). The reader is referred elsewhere for a more detailed discussion of other assays for immune-mediated neutrophil damage (Hagen et al., 1993; Palmblad et al., 2001; Nifli et al., 2006; Sella et al., 2010).

Some nonimmune-mediated toxic neutropenias have long been known to have a genetic predisposition (Pisciotta, 1973). Direct damage may cause inhibition of granulopoiesis or neutrophil function. It may entail failure to detoxify or excrete a xenobiotic or its metabolites, which subsequently build up to toxic proportions (Gerson et al., 1983; Uetrecht, 1990; Gerson and Meltzer, 1992). Some studies suggest that a buildup of toxic oxidants generated by leukocytes can result in neutrophil damage, as with the reactive intermediates derived from the interaction between clozapine and neutrophils. The resulting superoxide and hypochlorous acid production by the myeloperoxidase system are thought to contribute to clozapine-induced neutropenia (Uetrecht, 1990). Accumulation of nitrenium ion, a metabolite of clozapine (antipsychotic drug) that causes a depletion of ATP and reduced glutathione, rendering the neutrophil highly susceptible to oxidant-induced apoptosis, is now thought to be the principal mechanism of this disorder (Williams et al., 2000).

Examples of agents associated with immune and nonimmune neutropenia/agranulocytosis are listed in Table 11-8.

LEUKEMOGENESIS AS A TOXIC RESPONSE

Human Leukemia and Other Hematopoietic Malignancies

Leukemia is the sixth leading cause of cancer deaths among males and females in the United States (ACS, 2013). Leukemias arise when hematopoietic stem or progenitor cells (HSC/HPC) in the

Table 11-8

Examples of Toxicants that Cause Immune and Nonimmune Idiopathic Neutropenia

DRUGS ASSOCIATED WITH WBC ANTIBODIES	DRUGS NOT ASSOCIATED WITH WBC ANTIBODIES
Aminopyrine	Isoniazid
Propylthiouracil	Rifampicin
Ampicillin	Ethambutol
Metiamide	Allopurinol
Dicloxacillin	Phenothiazines/
Phenytoin	Chlorpromazine
Aprindine	Flurazepam
Azulfidine	Hydrochlorothiazide
Chlorpropamide	
Chlorpromazine/phenothiazines	
Procainamide	
Nafcillin	
Tolbutamide	
Lidocaine	
Methimazole	
Levamisole	
Gold	
Quinidine	
Clozapine	

SOURCE: Modified from Pisciotta (1997), with permission from Elsevier Science.

bone marrow undergo uncontrolled proliferation or clonal expansion and cannot differentiate normally into mature blood cells (Sawyers et al., 1991). Leukemias are broadly characterized as myeloid or lymphoid depending on the lineage of origin. Based on the stage of differentiation and rate of clonal expansion, they are also characterized as acute (rapid onset, immature blast cells) or chronic (more gradual onset over months or years, more mature cells). Using this basic classification, four major types of leukemia are commonly referred to —acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) also known as acute nonlymphocytic leukemia (ANLL), chronic lymphoblastic leukemia (CLL), and chronic myeloid leukemia (CML). Within these categories numerous subtypes exist and are classified according to morphologic, cytogenetic, immunophenotypic, and more recently, molecular characteristics, according to the French-American-British (FAB) or World Health Organization (WHO) classification systems (Bennett et al., 1985; Levine and Bloomfield, 1992; WHO, 2001, 2008; Haferlach et al., 2005).

The WHO classification system provides a comprehensive updated diagnostic framework for classifying CLL, CML, ALL, AML, myeloproliferative neoplasms (MPN), and myelodysplastic syndromes (MDS), along with various subtypes of these disorders (Vardiman et al., 2002; Vardiman et al., 2009; Campo et al., 2011; Arber et al., 2016; Bennett, 2016; Swerdlow et al., 2016). The program identifies new entities and variants based on emerging findings from clinical and scientific studies. The WHO AML classification utilizes, among other characteristics, molecular features, particularly recurrent cytogenetic abnormalities and specific gene mutations. In the WHO classification, the term "myeloid" includes all cells belonging to the granulocytic (neutrophil, eosinophil, basophil), monocytic/macrophage, erythroid, megakaryocytic, and mast cell lineages. In this scheme, a myeloid neoplasm with 20% or more blasts in the blood or marrow is considered to be AML. AML can arise from a pre-existing MDS, which represent myeloid clonal hemopathies with relatively heterogeneous spectrums of presentation. The major clinical problems in these disorders are morbidities caused by cytopenias and the potential for MDS to evolve into AML. MDS are a diverse group of bone marrow disorders in which the bone marrow does not produce enough healthy blood cells. MDS is primarily a disease of the elderly (most patients are older than age 70) (Rollison et al., 2008). CML is now considered a form of MPN (Arber et al., 2016).

Therapy-Related leukemia More than half of all leukemias occur in people with no obvious prior exposures of relevance and are termed de novo leukemias. *Secondary leukemias* are those that develop in individuals who have previously been exposed to radiation, industrial chemicals, or chemotherapeutic drugs or who previously had an MDS/MPN. *Therapy-related MDS* and *AML* (t-MDS and t-AML) arise following cytotoxic therapy for an unrelated malignancy or autoimmune disease. They account for approximately 10% of all cases of AML and MDS (WHO, 2008) and are distinct from their de novo counterparts with regard to onset, prognosis, and response to therapy. In a recent national study in Denmark, the frequencies of secondary AML and t-AML were 19.8% and 6.6%, respectively (Granfeldt Ostgard et al., 2015). In the United States t-AML is thought to accounts for 10% of all diagnosed AML (Churpek and Larson, 2013).

Childhood Leukemia Adult leukemias are predominantly myeloid and originate in pluripotent stem or progenitor cells. In contrast, 30% of pediatric acute leukemias are AML and 70% are ALL. Childhood ALL (cALL) arises in naïve pre-B (B-ALL) or pre-T progenitor cells (T-ALL) that are present in large numbers in early life and undergoing immune education. B-ALL can be stratified by differentiation stage; for example, proB-ALL or preB-ALL) (Wiemels, 2012). Childhood AML can affect both immature (FAB subtype M0) and mature lineage-committed types, such as erythroblastic or megakaryoblastic leukemia (subtypes M6 and M7, respectively). ALL and AML can be further stratified according to molecular cytogenetics (Pandita et al., 2015; Yohe, 2015), which is related to prognosis. Studies have shown that the underlying aberrations occur in two distinct stages, with oncogenic fusion proteins generated in utero, and subsequent cooperating hits of a genetic, epigenetic, or immune nature occurring after birth (Wiemels, 2008). Childhood leukemia is the second highest cause of cancer death in children 0 to 14 years old and first in those 15 to 19 years old (ACS, 2017).

General Mechanisms of Leukemogenesis

As illustrated in Fig. 11-8, leukemogenesis is a multistep process involving the acquisition of various combinations of chromosomal, genetic, and epigenetic aberrations that transform a normal stem cell into a leukemic stem cell (LSC) (Look, 2005; De Kouchkovsky and Abdul-Hay, 2016; Gore and Weinstein, 2016; Khaled et al., 2016; Bullinger et al., 2017). The resulting altered gene expression patterns disrupt critical hematopoietic processes such as signal transduction, alternative splicing, and epigenetics, and give rise to cellular phenotypes such as increased proliferation or survival, block of differentiation, and immortalization (Kitamura et al., 2016). Combinations of aberrations inform the WHO classification system (Vardiman et al., 2009; Campo et al., 2011; Bennett, 2016).

Chromosomal Aberrations Several kinds of chromosomal aberration can occur and they alter the function of genes in the affected region. A translocation is an abnormality in which chromosome regions are swapped between chromosomes. Translocations are designated as t(a;b), where part of chromosome a is translocated to

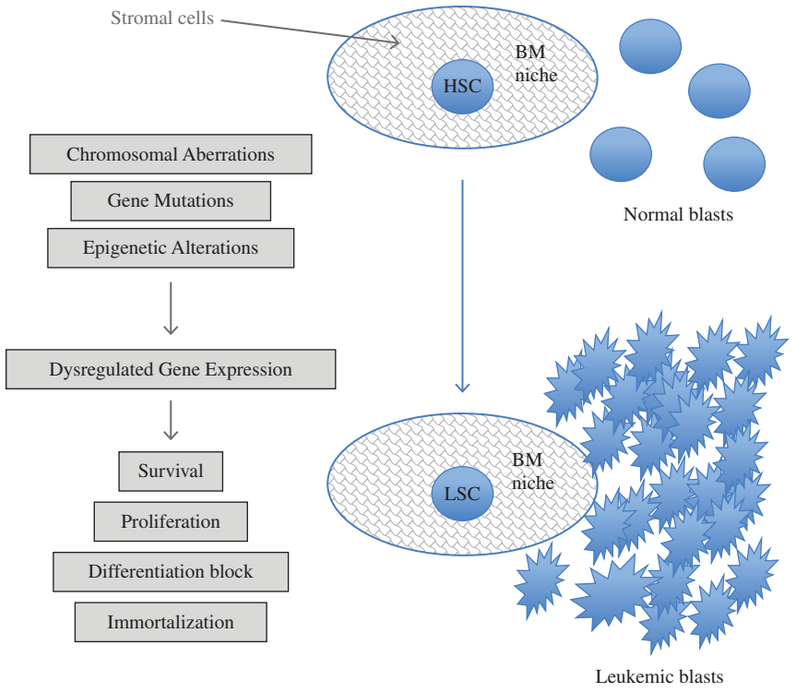

Figure 11-8. *Pathogenesis of leukemia.* Leukemogenesis is a multistep process involving the acquisition of various combinations of chromosomal, genetic, and epigenetic aberrations that transform a normal HSC into an LSC. The resulting altered gene expression patterns disrupt critical hematopoietic processes such as signal transduction, alternative splicing, and epigenetics, and give rise to cellular phenotypes such as increased proliferation or survival, block of differentiation, and immortalization. Thus, the bone marrow becomes replete with leukemic blasts and LSCs that replenish them.

chromosome b and vice versa. Chromosomal deletion, designated as del(#p or #q), involves the loss of a chromosomal region and an inversion, designated as inv(#) involves the end-to-end reversal of a region within a chromosome.

Many leukemia patients exhibit chromosomal alterations in their tumor cells (Pedersen-Bjergaard et al., 2006; Pedersen-Bjergaard et al., 2008; Mrozek et al., 2009; Vardiman et al., 2009; Qian et al., 2010; Campo et al., 2011; De Kouchkovsky and Abdul-Hay, 2016; Saleem and Yusoff, 2016; Taylor et al., 2017). Chromosomal aberrations in AML (Yang et al., 2017) and ALL (Jabbour et al., 2015) have been reviewed. Common aberrations in AML include loss or gain of one or more chromosomes (e.g., monosomy 7 and trisomy 8, respectively), recurrent chromosomal translocations such as t(8;21) and t(15;17), deletions, and inversions such as inv(16). In ALL, t(9;22) frequently occurs in adults whereas t(12;21) is more common in children.

Altered function of the fusion genes resulting from a translocation may have consequent effects on growth and maturation. For example, t(8;21)—which occurs in 12% of de novo AML and up to 40% of AML FAB subtype M2—fuses the RUNX1 gene on chromosome 21 with the RUNX1T1 gene on chromosome 8. The fusion product blocks maturation of the myeloid progenitor cells by interfering with transcription of RUNX1 target genes. t(12;21), present in around 25% of childhood precursor B cell ALL (Inaba et al., 2013), results in the ETV6-RUNX1 fusion gene which generates an aberrant transcription factor that represses gene expression (Teppo et al., 2016).

Gene Mutations Mutations in a recurring set of genes in HSC/HPC (Mehdipour et al., 2015; Marjanovic et al., 2016; Shin et al., 2016) that alter cell growth, apoptosis, differentiation, epigenetic processes, and tumor suppression characterize AML (Cancer Genome Atlas Research, 2013; Dohner et al., 2015). The most common recurrent AML mutations are in FLT3, NPMI, DNMT3A, IDH1, IDH2, TET2, and RUNX1 (Cancer Genome Atlas Research, 2013; Papaemmanuil et al., 2016). Mutations in ASXL1, DNMT3A, TET2, and IDH1/2 lead to epigenetic deregulation and are considered to be early initiating events (Chan and Majeti, 2013; Hirsch et al., 2016). Studies using recent next generation sequencing technologies have shown that over 70% of AML cases have mutations in genes encoding DNA methylation or histone modifiers (Mehdipour et al., 2015). Alternative splicing is an important contributor to the diversity and function of the hematopoietic cellular repertoire (Pritsker et al., 2005). Mutations in genes such as SRSF2, SF3B1, and U2AF1 lead to aberrant splicing, a major driver of leukemogenesis (Hahn et al., 2015).

Epigenetic Modifications Epigenetic modifications likely play an important role in leukemogenesis (Pedersen-Bjergaard et al., 2006; Nervi et al., 2008; Liu et al., 2011) and altered epigenetic signatures such as DNA methylation and miRNA expression have also been seen in many types of leukemia (Zheng et al., 2004; Pedersen-Bjergaard et al., 2006; Nervi et al., 2008; Khalaj et al., 2014; Yeh et al., 2016). Long noncoding RNAs, important regulators of gene expression, may also contribute to the initiation and development of AML (Wei and Wang, 2015).

Leukemic Stem Cells and the Bone Marrow Niche Leukemia-related aberrations arise in HSC/HPC and in other cellular components of the bone marrow niches where HSC/HPC reside. The acquisition of increased self-renewal capability in HSC/HPC leads to the generation of leukemic stem cells (LSCs) resembling multilineage progenitors that initiate and maintain AML (Passegue et al., 2003; Reinisch et al., 2015). LSCs can evade programmed

cell death and removal. For example, altered cell-surface expression of CD47 on AML LSCs inhibits phagocytosis by macrophages (Majeti et al., 2009); CD47 is also abnormally expressed in MDS patients at a high-risk of progressing to AML (Jiang et al., 2013). Disruption of aryl hydrocarbon receptor (AhR) signaling, which regulates stem cell cycling, regulation, and quiescence, may play a role in the etiology and/or progression of certain hematopoietic diseases (Casado et al., 2011). Toxicity-induced aberrations in stromal cells may lead to disruption of signals from the bone marrow niche (Ehninger and Trumpp, 2011). The bone marrow niche microenvironment enables the survival and proliferation of AML blasts (Shafat et al., 2017).

Leukemogenic Agents and Mechanisms of Leukemogenesis

Around 25% of the 100 agents identified as human carcinogens in humans by IARC are known to induce lymphohematopoietic cancers (IARC, 2010). Of the 25%, many are antineoplastic drugs, but the list includes industrial chemicals, various forms of radiation, immunosuppressive drugs, and infectious agents. The established nongenetic causes of AML and MDS are ionizing radiation, cytotoxic chemotherapeutic drugs, tobacco smoking, and occupational exposure to benzene or high levels of formaldehyde. Some studies suggest that smoking a pack-a-day of cigarettes increases the risk of AML by about 50% or 1.5-fold (Moorman et al., 2002; Kasim et al., 2005). Smoking is, therefore, a relatively weak, though established cause of AML as it doesn't cause a doubling of the risk in most studies even at high levels of exposure. Pesticide exposure and obesity have also been associated with AML in a number of studies but not consistently. They remain possible causes of AML.

Table 11-9 provides details of known (IARC Group 1) leukemogenic agents and their likely modes of action, adapted from EPA data (U.S. EPA, 2012).

Cytotoxic Chemotherapeutic Agents Today it is thought that approximately 10% of AMLs in the United States are therapy-related (Churpek and Larson, 2013). The main associated therapeutic agents include alkylating agents, topoisomerase II inhibitors, antimetabolite, and antitubulin drugs. The risk of t-MDS/t-AML varies considerably with the therapeutic regimen and ranges from 0.8% to 24% (U.S. EPA, 2012; Bhatia, 2013). Alkylating agents, such as nitrogen mustards, and antimetabolites induce bone marrow damage primarily characterized by pancytopenia, similar to the effects of ionizing radiation. The main dose-limiting toxicity associated with the majority of anticancer drugs is therapy-related myelosuppression (Parchment et al., 1998).

Most *alkylating agents* used in cancer chemotherapy (e.g., melphalan, cyclophosphamide, nitrogen mustard, busulfan, chlorambucil, carboplatin, cisplatin, dacarbazine, procarbazine, carmustine, mitomycin C, thiotepa, lomustine) can cause MDS and/or AML (Bhatia, 2013; Zahid et al., 2016). *Alkylating agents* typically induce AMLs that are preceded by a myelodysplastic phase and are often characterized by loss of all or part of chromosome 5 or 7, complex karyotypes, and mutations in p53 (Le Beau et al., 1986; Karp and Sarkodee-Adoo, 2000; Churpek and Larson, 2013; Zahid et al., 2016). Monofunctional alkylating agents, such as nitrosoureas, dacarbazine and temozolomide, transfer an alkyl group to DNA bases to form lesions that result in the formation of mutations, secondary DNA double-stranded breaks, and eventual cytotoxicity (Drablos et al., 2004; Allan and Travis, 2005; Kaina et al., 2007). Bifunctional alkylators, such as cyclophosphamide, melphalan and chlorambucil, induce the formation of cross-links within and between DNA lesions in addition to alkylated base lesions (Sill et al., 2011). The resulting double-stranded DNA breaks can lead to chromosomal translocations, insertions, inversions, and loss-of-heterozygosity (Richardson and Jasin, 2000; Helleday et al., 2008).

Topoisomerase II poisons kill cells by increasing levels of covalent enzyme-cleaved DNA complexes that are normal reaction intermediates. Drugs such as etoposide, doxorubicin, and mitoxantrone are used to treat a variety of cancers and autoimmune conditions. Unfortunately, their use is also associated with the development of specific leukemias. Regimens that include etoposide or doxorubicin are linked to the occurrence of AMLs that feature rearrangements at chromosomal band 11q23 (Pendleton et al., 2014). Similar rearrangements are seen in infant leukemias and are associated with gestational diets that are high in naturally occurring topoisomerase II-active compounds (Spector et al., 2005). Finally, regimens that include mitoxantrone and epirubicin are linked to acute promyelocytic leukemias that feature t(15;17) rearrangements (Joannides et al., 2011). Compared with alkylating agents, topoisomerase II poison-associated AMLs have shorter latencies and often lack a preceding myelodysplastic phase.

Patients treated with the *antimetabolite drugs* azathioprine, 6-thioguanine, and 6-mercaptopurine have an increased risk of developing AML (Bo et al., 1999; Karran, 2006; Yenson et al., 2008). Risks are higher in patients with low thiopurine *S*-methyltransferase activity and may also involve aberrant mismatch repair and microsatellite instability (Bo et al., 1999; Karran, 2006). Many patients treated with antimetabolite drugs have loss of chromosome 7 or partial loss of chromosome 5 or 7 ($5q^-$ or $7q^-$) (Yenson et al., 2008).

As t-AML is the dominant hematopoietic disorder associated with drug or chemical exposure (Casciato and Scott, 1979; Anderson et al., 1981; Irons, 1997), followed by t-MDS, these diseases may represent a continuum of toxic response (Irons, 1997; Look, 2005). In 2008, Pedersen-Bjergaard reviewed the genetics of de novo MDS/AML and t-MDS/AML and found strong similarities in cytogenetic abnormalities and gene mutations (Pedersen-Bjergaard et al., 2008). However, more t-AML and t-MDS cases (up to 50%) have a *complex karyotype* (more than three aberrations) (Mauritzson et al., 2000; Rossi et al., 2000). Subtypes with frequent, high-risk cytogenetic aberrations have a poorer prognosis (Godley and Larson, 2008). Pederson-Bjergaard identified eight separate pathways that appear to lead either directly to t-AML or t-MDS/AML (Pedersen-Bjergaard et al., 2006). The direct induction of oncogenic aberrations, genetic instability, and accumulation of complex aberrations, represent one mechanism of t-AML/MDS. Recent experimental and clinical data show that the selection by chemotherapeutic drugs of a pre-existing treatment-resistant HSC clone with TP53 mutation may be another important mechanism (Wong et al., 2015). Specific TP53 mutations were found at low frequency in hematopoietic cells 3 to 6 years before the onset of therapy-related disease. TP53 is the most frequently mutated gene in t-AML/t-MDS, being present in one-third of patients (Christiansen et al., 2001; Shih et al., 2013).

Radiation Exposure to ionizing radiation has been associated with several types of leukemia (IARC, 2000, 2001; UNSCEAR, 2000a, 2000b; Ron, 2003; NRC, 2006). Children who survived the atomic bombs in Hiroshima and Nagasaki were at an elevated risk of radiation-induced leukemia (Ron et al., 1994; Preston et al., 2004). Leukemogenesis associated with radiation involves myelotoxicity, immune suppression, chromosomal aberrations, and genetic mutations. Increased risk of leukemia has also been reported following the use of radiation for diagnostic tests, for the treatment of benign diseases, and following radiotherapy for cancer (Ron, 1998, 2003).

Table 11-9

Characteristics of Selected Known Human Leukemia-Inducing Agents (IARC Group 1)

AGENT	CLASS	LEUKEMIA TYPE	MYELO-TOXICITY	CHROMOSOME ABERRATIONS	ACTIVATION	PRIMARY MECHANISM
(1-(2-)Chlorethyl)-3-(4-methylcyclohexyl) nitrosourea (Methyl-CCNU, Semustine)	Therapeutic agent	ANLL	+	n/d	Degrades to direct- acting alkylating and carbamoylating agents	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations
1,3-Butadiene	Industrial chemical	Lympho-hematopoietic cancers	−	−	Bioactivated to mono- and bifunctional alkylating agents	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations
1,4-Butanediol dimethanesulfonate (Busulfan, Myleran)	Therapeutic agent	ANLL	+	SCA	Direct-acting bifunctional alkylating agent	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations
Benzene	Industrial and environmental chemical	ANLL, ALL, CLL	+	SCA, aneuploidy	Metabolized into reactive protein and DNA-binding species	Likely multiple metabolites and modes of action involved
Chlorambucil	Therapeutic agent	ANLL	+	SCA	Direct-acting bifunctional alkylating agent	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations
Cyclophosphamide	Therapeutic agent	ANLL	+	SCA	Bioactivated to bifunctional alkylating agent and acrolein	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations
Etoposide	Therapeutic agent	ANLL	+	MN	Topoisomerase II- poison	Mutation resulting from chromosomal breakage and translocations
Formaldehyde	Industrial and environmental chemical	Myeloid	+/−	+/−	Direct-acting, forms DNA-protein cross-links	Unknown, possibly mutation resulting from DNA binding and/or chromosomal alterations
Melphalan	Therapeutic agent	ANLL	+	SCA	Direct-acting bifunctional alkylating agent	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations

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MOPP therapy	Therapeutic agent combination	ANLL	+	SCA	A direct-acting bifunctional and an indirect monofunctional alkylating agent, a microtubule inhibitor, and a glucocorticoid	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations
Paint (occupation, maternal)	Occupational exposure	Childhood ALL	n/d	n/d	Possibly DNA reactive chemicals	Unknown, assumed to be mutation resulting from DNA binding and/or chromosomal alterations and/or immunosuppression
Radiation (alpha, beta)	Therapeutic, energy, and military uses	ANLL, CML, ALL	+	SCA	Direct and indirect DNA damage	Mutation resulting from DNA damage and/or chromosomal alterations
Radiation (x-ray, gamma)	Therapeutic, energy, and military uses	ANLL, CLL, ALL	+	SCA	Direct and indirect DNA damage	Mutation resulting from DNA damage and/or chromosomal alterations
Rubber manufacturing occupation	Occupational exposure	Leukemia	n/d	SCA	Possibly DNA reactive chemicals	Unknown, assumed to be mutation resulting from DNA binding and/or chromosomal alterations and/or immunosuppression
Thio-TEPA (tris(1-aziridinyl)-phosphine)	Therapeutic agent	Leukemia	+	SCA	Direct-acting trifunctional alkylating agent. Also, metabolized to monofunctional alkylating agent aziridine	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations
Tobacco smoking	Lifestyle use	ANLL	n/d	SCA	Direct and indirect DNA damage	Mutation resulting from DNA damage and/or chromosomal alterations
Tobacco smoking (parental)	Parental use	Childhood ALL	n/d	n/d	Direct and indirect DNA damage	Mutation resulting from DNA damage and/or chromosomal alterations
Treosulfan	Therapeutic agent	ANLL	+	n/d	Converts to a mono- and bifunctional alkylating agent	DNA reactive; mutation resulting from DNA binding and/or chromosomal alterations

Abbreviations: n/d, not determined, insufficient data; SCA, structural chromosome aberrations.
SOURCE: Adapted from data in EPA (U.S. EPA, 2012).

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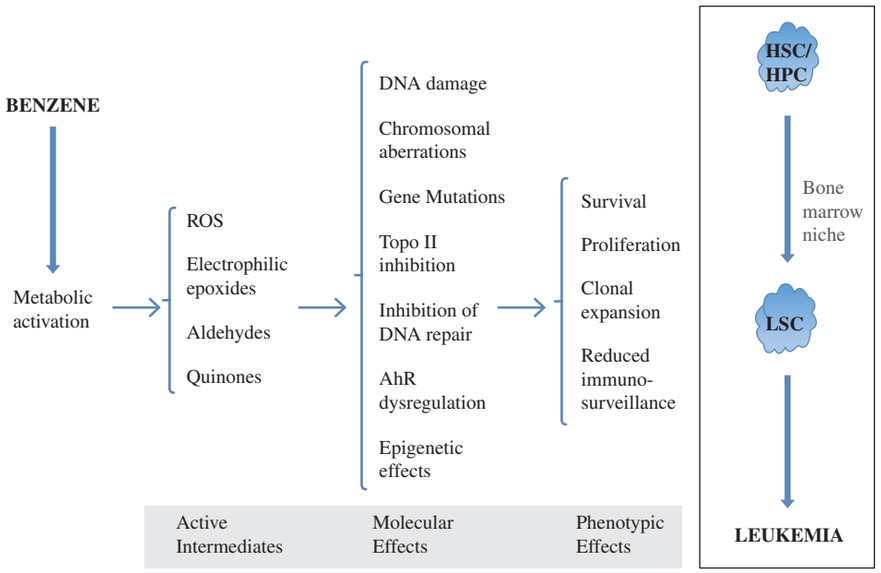

Figure 11-9. *Probable mechanism of leukemia induction by benzene.* Schematic representation of the active intermediates of benzene toxicity and their molecular and phenotypic effects in HSC/HPCs and bone marrow niche cells that lead to the generation of LSCs and leukemia development.

Occupational and Environmental Exposures Benzene and formaldehyde are among the few occupational/environmental exposures that have been classified as known human leukemogens (Table 11-9).

Benzene Benzene is an industrial chemical, a component of gasoline and cigarette smoke, a contaminant in mixtures of organic solvents and glues, and a widespread environmental contaminant. Despite its regulation, it has recently been estimated that exposure to benzene may cause as much as 8% to 16% of the AML and MDS cases in the general U.S. population (Poynter et al., 2017). The hematotoxic properties of benzene were first demonstrated more than a century ago in 1897 (Santesson, 1897), and by the 1920s it was well known that benzene was a dangerous toxicant in the workplace (Selling, 1916; Hamilton, 1931). The first case report of benzene-induced leukemia was published in 1928 (Delore and Borgomano, 1928), multiple additional case reports and series appeared in the 1930s to 1960s. In 1961, Cronkite described benzene as one of two known leukemogens (Cronkite, 1961) the other being ionizing radiation and by the late 1970s specifically designed epidemiological studies had clearly established benzene as a cause of human leukemia (Infante et al., 1977; Rinsky et al., 1981). A number of specific studies have established the relationship between benzene exposure and MDS (Strom et al., 2005; Lv et al., 2011; Schnatter et al., 2012; Copley et al., 2017).

Multiple metabolites and mechanisms are likely to be involved in benzene-induced leukemogenesis (ATSDR, 2007; IARC, 2012; McHale et al., 2012), as illustrated in Fig. 11-9. Benzene is metabolized in the liver to form reactive metabolites such as benzene oxide, benzene dihydrodiol epoxide, and the phenols: catechol, benzenetriol, and hydroquinone, which are subsequently metabolized in the bone marrow to produce benzoquinone metabolites (Snyder and Hedli, 1996). Reactive species include benzene epoxide, benzene dihydrodiol epoxide, *t,t*-muconaldehyde, 1,2- and 1,4-benzoquinone. Genotoxicity and myelotoxicity are induced by benzene metabolites and by reactive oxygen species (ROS) formation via redox cycling of reactive intermediates (Sammett et al., 1979; Kolachana et al., 1993; Valentine et al., 1996; Ahmad Khan, 2007).

Benzene targets pluripotent bone marrow HSC and reduces the numbers of circulating blood cells in experimental animals (Snyder, 1988). Sustained exposure to high levels in people can lead to pancytopenia and, in severe cases, aplastic anemia (Goldstein, 1977; Aksoy, 1988; Snyder, 2012). In occupationally exposed workers including those exposed to levels below the U.S. occupational exposure limit, hematotoxicity—manifest as reduced numbers of total white blood cells, granulocytes, lymphocytes, B cells, and platelets, was reported (Lan et al., 2004). The number of circulating hematopoietic myeloid progenitor cells was also suppressed.

Benzene is mutagenic to spleen and bone marrow in mice (Ward et al., 1992; Mullin et al., 1995; Provost et al., 1996; Albertini et al., 2010). However, increased gene-duplicating mutations rather than gene-inactivating mutations were detected in the glycophorin A gene in exposed workers (Rothman et al., 1995). Benzene induces structural chromosomal aberrations, and to a lesser degree numerical aberrations, in experimental animals (Eastmond et al., 2001; ATSDR, 2007; IARC, 2012). Chromosome breakage and aneuploidy have been seen in multiple studies of the lymphocytes of exposed workers (Zhang et al., 2002). Benzene exposure has been associated with higher levels of chromosomal changes commonly observed in AML, including 5q/5 or 7q/7, þ8 and t(8;21) in the peripheral blood lymphocytes of highly exposed workers (Smith et al., 1998; Zhang et al., 1999; Zhang et al., 2002). Monosomy 7 and 8 were produced in a dose-dependent manner in blood progenitor cells (Zhang et al., 2012). Benzene-induced AMLs share cytogenetic aberrations with t-AML induced by alkylating agents (Irons and Stillman, 1996; Zhang et al., 1998; Zhang et al., 2012) and topoisomerase II inhibitors (Smith et al., 1998). Benzene and its metabolites can inhibit topoisomerase II in vitro and in vivo (Chen and Eastmond, 1995; Eastmond et al., 2001; Mondrala and Eastmond, 2010).

As well as targeting critical genes and pathways through the induction of genetic and chromosomal abnormalities and genomic instability in HSC/HPC, additional mechanisms of benzene-induced

hematotoxicity and leukemogenesis have been proposed, including altered proliferation and differentiation of HSCs, oxidative stress, altered epigenetics and transcriptomics, bone marrow niche dysregulation, altered AhR signaling, and reduced immunosurveillance (McHale et al., 2012; Greim et al., 2014).

Formaldehyde Formaldehyde is an important industrial chemical in the global economy but also a ubiquitous pollutant in the environment. It is used as a tissue preservative and in the manufacture of many consumer products, including pressed wood furniture and flooring. Globally, millions of people are occupationally and environmentally exposed to formaldehyde (Zhang et al., 2009). Formaldehyde is classified as a leukemogen by IARC and United States National Toxicology Program, particularly for its association with myeloid neoplasms, based on epidemiological and mechanistic evidence (IARC, 2012).

Occupational exposure to formaldehyde in healthy workers led to a decrease in complete blood cell counts and reduced numbers of lymphocytes, granulocytes, and platelets (Zhang et al., 2010c), aneuploidy of chromosome 5 and 7, and structural aberrations in chromosome 5 in myeloid progenitor cells (Zhang et al., 2010c; Lan et al., 2015). Toxicity of formaldehyde to blood-forming systems, including mature blood cells, bone marrow cells, and hematopoietic stem/progenitor cells (HSCs/HPCs), has been further examined in experimental animal models. Formaldehyde exposure in vivo reduced the number of nucleated bone marrow cells and the counts of granulocyte-macrophage colony-forming units (CFU-GM) colonies cultured from mouse bone marrow (Wei et al., 2017), but increased the number of bone marrow megakaryocytes (Zhang et al., 2016).

Formaldehyde is genotoxic and induces DNA damage in the form of DNA adducts (Lu et al., 2011), protein-adducts (Regazzoni et al., 2017), and DNA-protein cross-links (DPCs). It also induces chromosome changes, including chromosomal aberrations (CA), sister chromatid exchanges (SCEs), and micronuclei (MN) (Zhang et al., 2010a). These changes have been reported in treated human cells in vitro, in exposed animals, and in the peripheral blood lymphocytes of exposed people, though some inconsistent results exist in the literature (Zhang et al., 2010a).

The mechanisms of formaldehyde-induced leukemogenesis are not well understood. Due to its highly reactive nature, formaldehyde is unlikely to mimic classic leukomogens (such as benzene) and exert hematotoxic/genotoxic damage directly in the bone marrow. Potential alternative mechanisms have been proposed in which inhaled formaldehyde could act by inducing mutations or pre-mutagenic lesions in (1) HSCs and HPCs circulating in peripheral blood proximal to the lungs; or (2) primitive pluripotent stem cells present within the nasal turbinates and/or olfactory mucosa (Zhang et al., 2009). In both cases, the damaged stem cells could travel to the bone marrow and initiate leukemogenesis, either through normal trafficking or trafficking enhanced by formaldehyde-induced cytotoxicity. The recent finding that the lung is a reservoir for HSC/HPC, capable of moving bidirectionally between the lung and bone marrow (Lefrancais et al., 2017), presents the possibility of a third alternative mechanism in which formaldehyde damages HSC/HPC in the lung. The mechanisms of formaldehyde-induced leukemogenesis require experimental validation.

Causes of Childhood Leukemia Multiple risk factors influence childhood leukemia, particularly in utero and in early life. In utero exposure to low-dose radiation from medical x-rays is a well-known risk factor (Linet et al., 2009). Exposures to pesticides through home use or paternal occupational exposure, paternal preconception smoking, solvents, and traffic emissions also have consistently been associated with disease risk (Metayer et al., 2013; Boothe et al., 2014; Filippini et al., 2015; Carlos-Wallace et al., 2016; Whitehead et al., 2016). In addition, there is evidence of associations between childhood leukemia and several different potential metrics of benzene exposure (Carlos-Wallace et al., 2016). Infant AML with chromosome 11q23 rearrangements is associated with maternal diets high in naturally occurring topoisomerase II-active agents, including soy. Apart from the latter, the mechanisms underlying these associations are unclear. The most common translocation in ALL, ETV6-RUNX1, appears to be associated with parental smoking (Metayer et al., 2013) and home paint use (Scelo et al., 2009). Prenatal and early-life tobacco smoke exposure was recently shown to increase the frequency of ALL-associated gene deletions in children who develop ALL (de Smith et al., 2017).

Factors That Modulate Leukemia Risk

Modifiers of leukemia risk by leukemia-inducing agents and their importance to risk assessment have been discussed previously (U.S. EPA, 2012). These include metabolism, pharmacokinetics, DNA-adduct type and repair, as well as individual and age-related susceptibility factors. For example, genetic variation can modulate the response to hematotoxic and leukemogenic agents. In multiple candidate gene association studies, susceptibility to benzene-induced hematotoxicity has been shown to be mediated by polymorphisms in genes involved in benzene metabolism, oxidative stress, DNA repair and genomic maintenance, innate immunity, cytokines, cellular adhesion molecules (Lan et al., 2004, 2005, 2009; Shen et al., 2011; Carbonari et al., 2016). One of these polymorphisms in the enzyme NAD(P)H:quinone oxidoreductase (NQO1) lowers or eliminates its activity and increases susceptibility to benzene-induced hematotoxicity (Rothman et al., 1997), as well as overall risk of de novo AML (Li et al., 2014) and therapy-induced myeloid neoplasms (Larson et al., 1999). Thus, knowing an AML case's NQO1 genetic variants does not help a differential diagnosis distinguish whether an AML is related to benzene exposure or arose de novo idiopathically from no known cause.

TOXICOLOGY OF PLATELETS AND HEMOSTASIS

Hemostasis is a multicomponent system responsible for preventing the loss of blood from sites of vascular injury and maintaining circulating blood in a fluid state. Loss of blood is prevented by formation of stable hemostatic plugs mediated by the procoagulant arm of hemostasis. This procoagulant response is normally limited to sites of vascular injury by the multicomponent regulatory arm of hemostasis. The dynamically modulated balance between procoagulant and regulatory pathways permits a rapid, localized response to injury. The major constituents of the hemostatic system include circulating platelets, a variety of plasma proteins, and vascular endothelial cells. The role of other cells in hemostasis, especially leukocytes, has been documented (Lane et al., 2005; Monroe and Hoffman, 2005). Alterations in these components or systemic activation of this system can lead to the clinical manifestations of deranged hemostasis, including excessive bleeding and thrombosis. The hemostatic system is a frequent target of therapeutic intervention as well as inadvertent expression of the toxic effect of a variety of xenobiotics. This section briefly reviews the inadvertent effects of xenobiotics on hemostasis and the toxic effects of chemicals used to manipulate the hemostatic system.

Table 11-10

Mechanism of Immune-Mediated Thrombocytopenia

PROBABLE MECHANISM	PROTOTYPIC AGENT	ANTIGEN/EPITOPE	PLATELET EFFECT	CLINICAL EFFECT
Hapten-directed antibody	Penicillin	Drug	Opsonization ↑ Clearance	Bleeding
Acquired antibody to drug-induced epitope	Quinidine	Drug–GP Ib/IX/V Drug–GP IIb/IIIa	Opsonization ↑ Clearance	Bleeding
Natural antibody to drug-induced epitope	Abciximab	GP IIb/IIIa	Opsonization ↑ Clearance	Bleeding
Induction of autoimmune antibodies	Gold	Platelet membrane	Opsonization ↑ Clearance	Bleeding
Immune complex	Heparin	PF 4–heparin complex	Platelet activation and aggregation ↑ Clearance	Thrombosis
Thrombotic thrombocytopenic purpura (TTP)	Ticlopidine	vWF-cleaving protease	Platelet activation Platelet aggregation ↑ Clearance	Microvascular thrombosis Microangiopathic hemolytic anemia
Hemolytic uremic syndrome (HUS)	Mitomycin	Unknown	Platelet activation and aggregation ↑ Clearance	Microvascular thrombosis Microangiopathic hemolytic anemia Renal failure

Toxic Effects on Platelets

The Thrombocyte Platelets are essential for formation of a stable hemostatic plug in response to vascular injury. They initially adhere to the damaged wall through binding of von Willebrand factor (vWF) with the platelet glycoprotein Ib/IX/V (GP Ib/IX/V) receptor complex (Jurk and Kehrel, 2005). Ligand binding to GP Ib/IX/V or interaction of other platelet agonists (e.g., thrombin, collagen, ADP, thromboxane A_2) with their specific receptors initiates biochemical response pathways that lead to shape change, platelet contraction, platelet secretion of granule contents, activation of the GP IIb/IIIa receptor, and externalization of phosphatidylserine (Jurk and Kehrel, 2005). Activation of the GP IIb/IIIa receptor permits fibrinogen and other multivalent adhesive molecules to form cross-links between nearby platelets, resulting in platelet aggregation. Xenobiotics may interfere with the platelet response by causing thrombocytopenia or interfering with platelet function; some agents are capable of affecting both platelet number and function.

Thrombocytopenia Like anemia, thrombocytopenia may be due to decreased production or increased destruction. Thrombocytopenia is a common side effect of intensive chemotherapy, due to the predictable effect of antiproliferative agents on hematopoietic precursors, including those of the megakaryocytic lineage. It is a clinically significant component of idiosyncratic xenobiotic-induced aplastic anemia, as discussed previously. Indeed, the initial manifestation of aplastic anemia may be mucocutaneous bleeding secondary to thrombocytopenia.

Exposure to xenobiotics may cause increased immune-mediated platelet destruction through several mechanisms (Table 11-10). Initially, immune thrombocytopenia was thought to be an antibody-mediated disease (Harrington et al., 1951) with either the xenobiotic acting as a hapten after binding to the platelet surface or by the xenobiotic causing a neoepitope to be formed on a platelet membrane glycoprotein. These actions were thought to elicit an antibody response, with the responding antibody binding resulting in removal of the platelet from the circulation by the mononuclear phagocytic system. However, a variety of lymphocyte abnormalities are also found in patients with immune thrombocytopenia, including skewing of T helper cells to an autoimmune phenotype, poor activity of regulatory T cells or regulatory B cells, and also direct killing of platelets by cytotoxic T cells (Cooper, 2017), suggesting an important role for T and B cells in xenobiotic-induced immune thrombocytopenia. Antibodies and T cells may also attack megakaryocytes, causing reduced platelet production (Cooper, 2017).

More than 100 drugs have been associated with causing immune thrombocytopenia, but recent reviews of available data show that carbamazepine, eptifibatide, ibuprofen, quinidine, quinine, oxaliplatin, rifampicin, sulfamethoxazole, trimethoprim, and vancomycin are probably the most frequently implicated (Curtis, 2014). The example of oxaliplatin is informative, as this can induce thrombocytopenia by both immune and nonimmune mechanisms. Thrombocytopenia is a significant problem for cancer patients being treated with this third-generation platinum drug as it occurs, for example, in more than 70% of patients with colorectal cancer receiving oxaliplatin. In most patients this effect is moderate in nature, but in 3% to 4% of patients it produces severe grade 3 to 4 thrombocytopenia causing life-threatening bleeding (Woo et al., 2015). The main cause of thrombocytopenia related to oxaliplatin use is myelosuppression, which represents asymptomatic thrombocytopenia. In some patients, hepatic sinusoid damage by oxaliplatin leads to portal hypertension and resultant splenic sequestration of platelets. Immune-mediated thrombocytopenia rarely occurs during oxaliplatin infusion but results in the severe life-threatening grade 3 to 4 thrombocytopenia that causes bleeding, hypersensitivity reactions, or hemolysis. These symptoms and thrombocytopenia occur suddenly after administration of oxaliplatin and can be treated by stopping oxaliplatin infusion. However, the rareness

of the immune-mediated effect sometimes makes it difficult to recognize (Woo et al., 2015).

Heparin-induced thrombocytopenia (HIT) represents another mechanism of immune-mediated platelet destruction (Evans and Gomes, 2017). This disorder is due to the development of antibodies that react with a multimolecular complex formed by the interaction between heparin and a protein, usually platelet factor 4 (PF 4) (Warkentin and Greinacher, 2004; Davoren and Aster, 2006). When the relative concentration of heparin to PF 4 is appropriate, formation of this complex is associated with exposure of a neoepitope on PF 4 (or another target protein) and development of an IgG response to the neoepitope. The IgG then binds to the PF 4–heparin complex to form an immune complex that binds to the platelet Fc receptor, FcγRIIa. Clustering of platelet FcγRIIa by the immune complex activates biochemical signaling pathways mediated by the cytoplasmic domain of FcγRIIa. This results in platelet activation and aggregation. During the process of platelet activation, platelet microparticles that promote thrombin generation are released. Consequently, HIT is associated with both thrombocytopenia and an increased risk of arterial and venous thrombosis. Other drug–antibody complexes (e.g., streptokinase–IgG) may trigger platelet activation and thrombocytopenia through a similar mechanism (Deckmyn et al., 1998; McRedmond et al., 2000).

Thrombotic thrombocytopenic purpura (TTP) is a rare and life-threatening thrombotic microangiopathy characterized by microangiopathic hemolytic anemia, severe thrombocytopenia, and organ ischemia linked to disseminated microvascular platelet rich-thrombi (Joly et al., 2017). TTP is specifically related to a severe deficiency in ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13), the specific von Willebrand (vWF) factor-cleaving protease. ADAMTS13 deficiency is most frequently acquired via ADAMTS13 autoantibodies, but rarely, it is inherited via mutations of the *ADAMTS13* gene. The pathogenesis of TTP appears to be related to the ability of unusually large vWF multimers to activate platelets, even in the absence of significant vascular damage (Lian, 2005). Although these large multimers are normally secreted into blood by endothelial cells, they are rapidly processed into smaller multimers by ADAMTS13, which is present in plasma. Acquired TTP is associated with the development of an antibody that inhibits this protease, permitting the very large vWF multimers to persist in the circulation (Lian, 2005; Veyradier and Meyer, 2005). Consequently, these multimers bind to platelet GP Ib/IX/V and induce platelet activation and aggregation. The organ failure and hemolysis in TTP is due to the formation of platelet-rich microthrombi throughout the circulation. A recent review described the association of more than 70 drugs with TTP (Al-Nouri et al., 2015). In this systematic review, 22 drugs had evidence of a definite association with TTP, but only 3 of these accounted for the majority of cases reported; namely quinine, cyclosporine, and tacrolimus (Al-Nouri et al., 2015). In some cases, drug-induced TTP is related to development of antibodies to ADAMTS13, whereas in other cases it may be due to direct endothelial cell toxicity from the drug.

Hemolytic uremic syndrome (HUS) is a disorder characterized by clinical features similar to those of TTP, with microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. HUS is usually categorized as typical, caused by Shiga toxin-producing *Escherichia coli* (STEC) infection, as atypical HUS (aHUS), usually caused by uncontrolled complement activation, or as secondary HUS with a coexisting disease (Jokiranta, 2017). Typical HUS (i.e., STEC-HUS) follows a gastrointestinal infection with STEC, whereas aHUS is associated primarily with mutations or autoantibodies leading to dysregulated complement activation. Among the 30% to 50% of patients with HUS who have no detectable complement defect, some have either impaired diacylglycerol kinase ε (DGKε) activity, cobalamin C deficiency, or plasminogen deficiency. Some have secondary HUS with a coexisting disease or trigger such as autoimmunity, transplantation, cancer, infection, certain drugs, or pregnancy. The common features in STEC-HUS, aHUS, and secondary HUS are simultaneous damage to endothelial cells, intravascular hemolysis, and activation of platelets leading to a procoagulative state, formation of microthrombi, and tissue damage (Jokiranta, 2017). Desmopressin, a vasopressin analog, is an example of nonimmune-mediated increased platelet destruction. It induces a two- to fivefold increase in the plasma concentration of vWF and factor VIII. It is commonly used in the treatment of patients with von Willebrand disease and other mild bleeding syndromes (Federici, 2008). Desmopressin has been associated with the development or accentuation of thrombocytopenia in some patients with type 2B von Willebrand disease. The thrombocytopenia in such cases is related to the release of an abnormal vWF from endothelial cells. The abnormal vWF has enhanced affinity for GP Ib/IX/V and the interaction of the vWF with its receptor leads to platelet clearance from the circulation (Mannucci, 1998).

Toxic Effects on Platelet Function Platelet function is dependent on the coordinated interaction of a number of biochemical response pathways. A variety of drugs and foods have been found to inhibit platelet function, either in vivo or in vitro (Abrams, 2006). Major drug groups that affect platelet function include nonsteroidal anti-inflammatory drugs, β-lactam-containing antibiotics, cardiovascular drugs, particularly β-blockers, psychotropic drugs, anesthetics, antihistamines, and some chemotherapeutic drugs. The effect of these drugs can vary between individuals, perhaps due to subclinical variations in underlying platelet function.

Xenobiotics may interfere with platelet function through a variety of mechanisms. Some drugs inhibit the phospholipase A_2/cyclooxygenase pathway and synthesis of thromboxane A_2 (e.g., nonsteroidal anti-inflammatory agents). Other drugs appear to interfere with the interaction between platelet agonists and their receptors (e.g., antibiotics, ticlopidine, clopidogrel). As the platelet response is dependent on a rapid increase in cytoplasmic calcium, any chemical that interferes with translocation of calcium may inhibit platelet function (e.g., calcium channel blockers). Occasionally, drug-induced antibodies will bind to a critical platelet receptor and inhibit its function. The functional defect induced by such antibodies may potentiate the bleeding risk associated with the xenobiotic-induced thrombocytopenia. In some cases, the mechanism of inhibition is not known.

Laboratory tests of platelet function, such as bleeding time, light transmission platelet aggregation, lumiaggregometry, impedance aggregometry on whole blood, and platelet activation investigated by flow cytometry, are traditionally utilized for diagnosing hemostatic disorders and managing patients with platelet and hemostatic defects (Paniccia et al., 2015). The effect of xenobiotics on platelet function can be studied in vivo with these methods, but several effects can also be used following in vitro exposure of platelets to the chemical of interest and/or its metabolites. The most common method of assessing platelet function is turbidimetric platelet aggregation using platelet-rich plasma, but several devices that are much simpler to use have recently become available (i.e., PFA-100, VerifyNow System, Multiplate Electrode Aggregometry [MEA], Paniccia et al., 2015).

Toxic Effects on Fibrin Clot Formation

Coagulation Fibrin clot formation is the result of sequential activation of a series of serine proteases that culminates in the formation of thrombin (Lane et al., 2005; Monroe and Hoffman, 2005).

Table 11-11

Conditions Associated with Abnormal Synthesis of Vitamin K–Dependent Coagulation Factors

Warfarin and analogs	Intravenous α-tocopherol
Rodenticides (e.g., brodifacoum)	Dietary deficiency Cholestyramine resin
Broad-spectrum antibiotics	Malabsorption syndromes
N-Methyl-thiotetrazole cephalosporins	

Thrombin is a multifunctional enzyme that converts fibrinogen to fibrin; activates factors V, VIII, XI, XIII, protein C, and platelets; and interacts with a variety of cells (e.g., leukocytes and endothelial cells), activating cellular signaling pathways (Lane et al., 2005).

The most common toxic effect of xenobiotics on fibrin clot formation are related to a decreased level of one or more of the critical proteins necessary for this process. The decrease in clotting factor activity may be due to decreased synthesis of the protein(s) or increased clearance from the circulation. Decreased synthesis is most often a reflection of hepatocellular damage or interference with vitamin K metabolism, as discussed below, whereas increased clearance is usually associated with the development of an antibody to a specific coagulation factor.

Decreased Synthesis of Coagulation Proteins The majority of proteins involved in the coagulation cascade are synthesized in the liver. Therefore, any chemical that impairs liver function may cause a decrease in production of coagulation factors. The common tests of the coagulation cascade, the prothrombin time (PT) and activated partial thromboplastin time (aPTT), may be used to screen for liver dysfunction and a decrease in clotting factors. These assays are often performed as part of the safety evaluation of a new chemical entity. The half-life of clotting factors in the circulation varies significantly, with factor VII having the shortest half-life (3 to 4 hours). Therefore, with acute toxicity (e.g., acetaminophen overdose), the effect on blood coagulation may be first seen as a decrease in the level of factor VII. Such a decrease would lead to prolongation of the PT with a normal aPTT. With a more chronic process, the PT, the aPTT, or both may be affected.

Factors II, VII, IX, and X are dependent on vitamin K for their complete synthesis (Stafford, 2005). Anything that interferes with vitamin K metabolism may lead to a deficiency of these factors and a bleeding tendency. This may occur with xenobiotics that interfere with absorption of vitamin K from the intestine or with chemicals that interfere with the reduction of vitamin K epoxide (Table 11-11). The combination of antibiotic therapy and limited oral intake is a common cause of acquired deficiency of vitamin K–dependent proteins among hospitalized patients (Chakraverty et al., 1996). The "super rodenticides" are another cause of acquired vitamin K deficiency (Chua and Friedenberg, 1998; Berry et al., 2000). These chemicals have a very prolonged half-life in vivo; thus, the coagulation defect may persist for weeks or months following exposure. Rodenticide exposure may occur accidentally, as part of a Munchausen syndrome, in association with a suicide attempt, or as part of a homicide attempt. At times it may be important to distinguish between a true vitamin K deficiency and interference with the reduction of vitamin K epoxide. This is most readily accomplished by measuring the level of vitamin K and vitamin K epoxide in serum or plasma. In the case of vitamin K deficiency, both vitamin K and vitamin K epoxide are decreased, whereas in the case of inhibition of vitamin K reduction, vitamin K epoxide is significantly increased. Specific rodenticides may be measured using HPLC techniques, but it is important to specify which active chemical (e.g., brodifacoum) should be measured, as the assays may not show cross-reactivity between chemicals.

Table 11-12

Relationship Between Xenobiotics and the Development of Specific Coagulation Factor Inhibitors

COAGULATION FACTOR	XENOBIOTIC
Thrombin	Topical bovine thrombin Fibrin glue
Factor V	Streptomycin Penicillin Gentamicin Cephalosporins Topical bovine thrombin
Factor VIII	Penicillin Ampicillin Chloramphenicol Phenytoin Methyldopa Nitrofurazone Phenylbutazone
Factor XIII	Isoniazid Procainamide Penicillin Phenytoin Practolol
von Willebrand factor	Ciprofloxacin Hydroxyethyl starch Valproic acid Griseofulvin Tetracycline Pesticides

Increased Clearance of Coagulation Factors Idiosyncratic reactions to xenobiotics include the formation of antibodies that react with coagulation proteins. These antibodies bind to the coagulation factor, forming an immune complex that is rapidly cleared from the circulation and resulting in deficiency of the factor. The antibody is often reversible over time if the initiating agent is withdrawn. However, during the acute phase, these patients may have life-threatening bleeding. The factors that are most often affected include factors VIII, V, and XIII, vWF, prothrombin, and thrombin (Table 11-12) (Lollar, 2004, 2005). Many of these antibodies inhibit the functional activity of the coagulation factor in addition to increasing the rate of clearance. Other antibodies have been demonstrated to have catalytic activity, resulting in proteolysis of the target coagulation factor (Ananyeva et al., 2004).

Lupus anticoagulants are antibodies that interfere with in vitro phospholipid-dependent coagulation reactions (Bertolaccini et al., 2004). Although it was once hypothesized that these antibodies were directed against phospholipid, it is now evident that lupus anticoagulants are directed against phospholipid-binding proteins, including prothrombin and β_2-glycoprotein 1. These antibodies usually do not cause a deficiency of any specific coagulation factor. However,

in vivo, these antibodies can potentiate procoagulant mechanisms and interfere with the protein C system. Consequently, these antibodies have been associated with an increased risk of thrombosis (Bertolaccini et al., 2004). The development of lupus anticoagulants has been seen in association with a variety of medications, including procainamide, chlorpromazine, and hydralazine.

Toxicology of Chemicals Used to Modulate Hemostasis

Patients with bleeding or thrombotic problems are commonly encountered in clinical practice. A variety of agents are available to treat such patients, ranging from recombinant hemostatic proteins to chemical entities that modulate the activity of the coagulation system. The major toxicologic reactions to plasma-derived products are infectious diseases (e.g., hepatitis C) and allergic reactions, which can be severe. The use of some products, such as activated concentrates of vitamin K–dependent proteins (e.g., Autoplex and FEIBA), has been associated with the development of disseminated intravascular coagulation and/or thrombosis in some patients (Mannucci, 1998).

Oral Anticoagulants Oral anticoagulants (warfarin) interfere with vitamin K metabolism by preventing the reduction of vitamin K epoxide, resulting in a functional deficiency of reduced vitamin K (Ansell et al., 2004). These drugs are widely used for prophylaxis and therapy of venous and arterial thrombosis. The therapeutic window for oral anticoagulants is relatively narrow, and there is considerable interindividual variation in the response to a given dose. A number of factors, including concurrent medications and genetics, affect the individual response to oral anticoagulants (Ansell et al., 2004; D'Andrea et al., 2005; Rojas et al., 2005). For these reasons, therapy with these drugs must be routinely monitored to maximize both safety and efficacy. This is routinely performed with the PT, with results expressed in terms of the international normalized ratio (INR).

A number of xenobiotics, including foods, have been found to affect the response to oral anticoagulants (Ansell et al., 2004). Perhaps the most common mechanism for interference with oral anticoagulants is mediated by inhibition of CYP2C9 (Rojas et al., 2005). Other mechanisms of interference include induction of CYP2C9, which tends to diminish the effect of warfarin by shortening its half-life; interference with absorption of warfarin from the gastrointestinal tract; displacement of warfarin from albumin in plasma, which temporarily increases the bioavailability of warfarin until equilibrium is reestablished; diminished vitamin K availability, due to either dietary deficiency or interference with the absorption of this lipid-soluble vitamin; and inhibition of the reduction of vitamin K epoxide, which potentiates the effect of oral anticoagulants.

Just as other drugs interfere with the action of oral anticoagulants, administration of oral anticoagulants may affect the activity of other medications, particularly those that are metabolized by CYP2C9. Dicumarol administration prolongs the half-life of chlorpropamide and phenytoin, resulting in hypoglycemia in the case of chlorpropamide and an increased plasma drug concentration in the case of phenytoin. Bis-hydroxycoumarin, but not warfarin, potentiates the activity of tolbutamide, resulting in enhanced hypoglycemia (Harder and Thurmann, 1996).

Oral anticoagulants have been associated with the development of warfarin-induced skin necrosis (Ansell et al., 2004). This disorder is due to the development of extensive microvascular thrombosis in the affected skin. This uncommon toxic effect is thought to be related to a rapid drop in protein C following administration of the drug, resulting in impaired protein C function. The risk of developing warfarin-induced skin necrosis increases with the dose of warfarin used to initiate therapy, particularly when the initial dose exceeds 10 mg per day; this is one of the reasons that loading doses of warfarin are no longer recommended.

Vitamin K is necessary for the synthesis of proteins other than the coagulation-related factors, including osteocalcin, a major component of bone. Perhaps because of this, long-term administration of warfarin has been associated with bone demineralization (Ansell et al., 2004; Stafford, 2005). This effect can be important in patients with borderline bone density. Administration of warfarin during pregnancy, particularly the first 12 weeks of pregnancy, is associated with congenital anomalies in 25% to 30% of exposed infants (Bates et al., 2004). Many of the anomalies are related to abnormal bone formation. It is thought that warfarin may interfere with synthesis of proteins critical for normal structural development.

The last decade has seen development and increasing clinical use of the direct oral anticoagulants (DOACs), orally bioavailable direct inhibitors of either coagulation factor Xa (e.g., rivaroxaban, apixaban), or thrombin (i.e., dabigatran). As with any anticoagulant therapy, bleeding risk exists, although these newer agents are anticipated to begin to replace warfarin (Crowther, 2017). The DOACs do not require routine monitoring like warfarin, and reversal strategies are FDA-approved or emerging, depending on the drug (Pollack et al., 2015; Connolly et al., 2016). Because these new agents work by directly inhibiting specific coagulation proteases, and not by altering vitamin K levels, adverse effects related to changes in vitamin K are limited, although these newer drugs are not without potential for drug–drug interaction (Di Minno et al., 2017).

Heparin Heparin is a widely used anticoagulant for both prophylaxis and therapy of acute venous thromboembolism (Hirsh and Raschke, 2004). In many hospitals, the majority of patients are exposed to this potent anticoagulant at some point during their hospitalization. The major complication associated with heparin therapy is bleeding, a direct manifestation of its anticoagulant activity. The risk of bleeding is related to the intensity of therapy, the patient's body mass and underlying condition, and the presence of other hemostatic defects (e.g., thrombocytopenia).

As discussed in the section "Toxic Effects on Platelets," heparin administration is also associated with the development of heparin-induced thrombocytopenia (HIT). For unknown reasons, this complication occurs more frequently with heparin derived from bovine sources than with that derived from porcine sources. The incidence of HIT is also significantly higher in patients receiving unfractionated heparin than it is in patients receiving low-molecular-weight heparin (Warkentin and Greinacher, 2004; Davoren and Aster, 2006).

Long-term administration of heparin is associated with an increased risk of clinically significant osteoporosis (Hirsh and Raschke, 2004; Dinwoodey and Ansell, 2006). The mechanism underlying the development of osteoporosis in these patients is not known. Patients may suffer from spontaneous vertebral fractures and demineralization of long bones of the arms and legs. The risk of osteoporosis may be less with low-molecular-weight heparin as compared with that with unfractionated heparin.

Heparin administration may also cause a transient rise in serum transaminases, suggesting significant liver dysfunction (Guevara et al., 1993). However, the rise is rapidly reversible on discontinuation of heparin and may reverse even before heparin is discontinued. The elevation of serum transaminases has not been associated with chronic liver dysfunction. The mechanism of heparin-induced increase in transaminases is not known.

In 2008, serious injuries and deaths were associated with the use of heparin originating in China. It was determined that certain

sources of heparin were contaminated with oversulfated chondroitin sulfate (OSCS). The presence of OSCS is believed to be responsible for the observed severe allergic reactions, gastrointestinal disturbances, and hypotension (Blossom et al., 2008).

Fibrinolytic Drugs Fibrinolytic drugs are used in the treatment of acute thromboembolic disease with the goal of dissolving the pathogenic thrombus (Collen and Lijnen, 2005). Each of these drugs works by converting plasminogen, an inactive zymogen, to plasmin, an active proteolytic enzyme. Plasmin is normally tightly regulated and is not freely present in the circulation. However, administration of fibrinolytic drugs regularly results in the generation of free plasmin leading to systemic fibrin(ogen)olysis. The toxicology of the fibrinolytic drugs can be divided into toxic effects of systemic plasmin activation and toxic effects of the activators themselves.

Systemic fibrinolysis is associated with the development of a complex coagulopathy characterized by a decrease in fibrinogen, factors V and VIII, and α_2-antiplasmin; an increase in circulating fibrin split products; degradation of platelet GP Ib/IX/V and IIb/IIIa; degradation of endothelial cell glycoproteins; degradation of fibronectin and thrombospondin; and prolongation of the PT, aPTT, and thrombin time (Hajjar, 2006). All of these effects potentiate the risk of bleeding. Anatomic locations that are frequently involved in bleeding complications include the cerebral circulation and sites of recent vascular access. As systemic plasmin can lyse physiologic as well as pathologic thrombi, reactivation of bleeding from sites of vascular access is not uncommon. Platelet inhibitors and heparin are commonly used in conjunction with fibrinolytic therapy to prevent recurrent thrombosis. As one might expect, the concurrent use of anticoagulants with systemic fibrinolysis may contribute to the risk of bleeding (Menon et al., 2004).

Another complication associated with fibrinolysis is recurrent thrombosis at the site of pathologic thrombosis. While rethrombosis may be related to underlying damage to the vascular wall, there is some evidence that fibrinolytic therapy may contribute to this process. For example, plasmin, in appropriate concentrations, can actually induce platelet activation (McRedmond et al., 2000). This process may be mediated by plasmin or streptokinase/plasminogen cleavage of the platelet thrombin receptor (protease activated receptor-1). Cleavage of the receptor is associated with activation of the platelet biochemical signaling pathways. There is sufficient "cross-talk" between the fibrinolytic system and the contact system of coagulation that one could also anticipate increased thrombin generation occurring as a result of fibrinolytic therapy (Schmaier et al., 1999).

Streptokinase is a protein derived from group C β-hemolytic streptococci and is antigenic in humans. Antibody formation to streptokinase occurs commonly in association with streptococcal infections as well as exposure to streptokinase. Acute allergic reactions may occur in 1% to 5% of patients exposed to streptokinase, and these allergic reactions may consist of minor symptoms such as hives and fever as well as major, life-threatening anaphylactic reactions. In addition, delayed hypersensitivity reactions associated with severe morbidity may occur (Siebert et al., 1992; Curzen et al., 1998). Allergic reactions also occur with other fibrinolytic drugs containing streptokinase (e.g., anisoylated plasminogen–streptokinase complex) or streptokinase-derived peptides. The immune complex formed by IgG and streptokinase is capable of binding to and clustering platelet FcγRIIa, initiating platelet activation and aggregation (McRedmond et al., 2000).

Urokinase and recombinant tissue plasminogen activator (t-PA) are generally not associated with allergic reactions. However, work is progressing on a number of genetically engineered forms of t-PA. Whether or not such mutant forms of t-PA are immunogenic has not been firmly established (Collen and Lijnen, 2005).

Inhibitors of Fibrinolysis Inhibitors of fibrinolysis are commonly used to control bleeding in patients with congenital abnormalities of hemostasis, such as von Willebrand disease. Tranexamic acid and ε-aminocaproic acid are small molecules that block the binding of plasminogen and plasmin to fibrin and other substrate proteins through interaction with lysine binding sites on plasmin(ogen). Although relatively well tolerated, there is some evidence that administration of these chemicals may increase the risk of thrombosis due to the inhibition of the fibrinolytic system (Mannucci, 1998). In a single case, intravenous infusion of ε-aminocaproic acid in a patient with chronic renal failure was associated with acute hyperkalemia (Perazella and Biswas, 1999).

Aprotinin is a naturally occurring polypeptide inhibitor of serine proteases. It is usually derived from bovine material and consequently is immunogenic when administered to humans. Aprotinin is given by intravenous infusion, as it is inactive when given orally. Allergic reactions in response to aprotinin have been reported, ranging from minor cutaneous manifestations to anaphylactic reactions (Peters and Noble, 1999). A more recent observational study compared clinical outcomes after cardiac surgery performed with aprotinin, tranexamic acid, or ε-aminocaproic acid with outcomes after cardiac surgery without an inhibitor of fibrinolysis (Mangano et al., 2006). In this study, use of antifibrinolytics was associated with decreased blood loss. However, use of aprotinin was associated with a significant increase in end-organ damage, including renal, cardiac, and cerebral events. The design and results of this study have been questioned, with calls for prospective randomized comparator studies (Sedrakyan et al., 2006). The results also point out the intricate balance within the hemostatic system and the potential for problems when modulating the activity of one portion of this system.

HEMATOTOXICITY TESTS

Assessing the risk that exposure to new drugs, chemical products, and other agents poses to humans—in terms of significant toxic effects on hematopoiesis and the functional integrity of blood cells and hemostatic mechanisms—can be logistically and intellectually challenging. This is due in part to the complexity of hematopoiesis and the range of important tasks that these components perform, as previously discussed.

Animal Studies

Conventional hematotoxicity testing in animals assesses peripheral blood cell parameters (complete blood counts) and histopathology of bone marrow and other hematopoietic tissues. Specific tests used vary with the phase or objective of the evaluation (acute, subacute, chronic), the intended use of the chemical, and what is understood or suspected regarding the toxicologic profile of the xenobiotic. Ideally, the studies in aggregate should provide information on the effects of single- and multiple-dose exposure on erythrocyte parameters (RBC, Hbg, PCV, MCV, MCHC), leukocyte parameters (WBC and absolute differential counts), thrombocyte counts, screening coagulation tests (PT, aPTT), peripheral blood cell morphology, and bone marrow cytologic and histologic features (Bloom, 1993; Weingand et al., 1996; Lund, 2000). Best practices for evaluation of bone marrow toxicity in nonclinical studies were developed through a joint effort of the American Society for Veterinary Clinical Pathology and the Society of Toxicologic Pathology (Bone Marrow Working Group) (Reagan et al., 2011). Cytological or flow cytometric evaluation of bone marrow is useful

Table 11-13

Examples of Problem-Driven Tests Used to Characterize Hematologic Observations in Preclinical Toxicology

Flow cytometry (see text)
Heinz body preparation
Cell-associated antibody assays (erythrocyte, platelet, neutrophil)
Erythrocyte osmotic fragility test
Erythrokinetic/ferrokinetic analyses
Cytochemical/histochemical staining
Electron microscopy
In vitro hematopoietic clonogenic assays
Platelet aggregation
Plasma fibrinogen concentration
Clotting factor assays
Thrombin time
Bleeding time

to characterize effects on different hematopoietic lineages or stem or progenitor cells. Examples of additional tests that can be used to better characterize findings from initial screening efforts or to more fully explore a class-specific effect or other hematotoxicologic potential of concern (Bloom, 1993) are listed in Table 11-13.

Important considerations for animal toxicity studies include the selection of the appropriate animal model. Rats and mice are commonly used species and both have been well characterized hematologically (Valli and McGrath, 1997; Moore, 2000a, 2000b). However, their small size precludes serial sampling of blood and bone marrow. Larger species, such as the dog and monkey, enable serial sampling and are hematologically more similar to humans, as regards hematopoiesis and blood cell kinetics, which in the monkey extends to immunohematologic features (Shifrine and Wilson, 1980; Ladiges et al., 1990). However, interanimal hematologic variability is an issue in monkeys, particularly in wild-caught primates.

Interspecies differences limit the relevance of animal studies to human hematotoxicity. Conventional peripheral blood cell parameters in animals have limited predictive value for human hematotoxicity. Animal studies may be even less predictive for human recombinant proteins, monoclonal antibodies, and targets that are specific to human tissue and disorders, or for idiosyncratic iatrogenic disease in humans, such as aplastic anemia and agranulocytosis. Mice are not relevant for testing toxicity of infectious agents with exclusively human or primate hosts.

Interspecies differences in sensitivity of bone marrow cells to anticancer drugs were previously reported (Erickson-Miller et al., 1997; Pessina et al., 2003; Masubuchi, 2006). In order to improve the relevance of the mouse model for human hematotoxicity, a humanized bone marrow xenograft mouse model was developed (Cai et al., 2011). It was validated with a temozolomide-based regimen that is known to have dose limiting toxicity in the clinic. Flow cytometric analysis indicated that human bone marrow cells were more sensitive to treatment than mouse bone marrow cells and that all lineages were affected. This model enables the screening of the response of bone marrow cells in their microenvironment.

Most of IARC's Group 1 leukemia- and lymphoma-inducing agents were reported previously to be carcinogenic in rodent bioassays (Eastmond, 1997), suggesting a good correlation between animal and humans. However, many of the leukemias or lymphomas induced in mice were T-cell leukemias or lymphomas, rather than AML, the predominant human form. The difference in tumor types may be related to species differences in hematopoiesis and/or immune surveillance (Eastmond, 1997).

The strains of animals widely used in animal studies are genetically similar and do not approximate human genetic variability for gene by environment studies. Genetically diverse mouse models such as Collaborative Cross strains (Threadgill et al., 2011) and Diversity Outbred mice (Churchill et al., 2012) have been developed to address this. They enable the examination of effects of environmental exposures against variable genetic backgrounds. For example, two sulfotransferase genes were identified as candidate susceptibility genes related to benzene-induced genotoxicity in bone marrow cells using Diversity Outbred mice (French et al., 2015). However, there are no human homologs of these sulfotransferase genes in humans, indicating that findings in rodents must be interpreted cautiously.

Human Population Studies

Treatment with drugs and therapeutics can lead to *idiosyncratic reactions* that cause toxicity in a small number of susceptible individuals (Patton and Duffull, 1994; Dieckhaus et al., 2002) and may include aplastic anemia, thrombocytopenia, hemolysis, and leukopenia, which may or may not be immune-mediated (Salama et al., 1989). Examples of iatrogenic blood dyscrasias detected through postmarketing surveillance include the hemolysis and thrombocytopenia associated with the antibiotic temafloxacin, the aplastic anemia linked to the antiepileptic felbamate, the hemolysis caused by the antidepressant nomifensine, and the agranulocytosis associated with the antiarrhythmic aprindine.

The WHO has established criteria for grading hematotoxicity (WHO, 1979), which are summarized in Table 11-14. These have been particularly useful in establishing and communicating treatment strategies and guidelines for chemicals known to suppress hematopoiesis (cytoreductive oncolytic, immunosuppressive, and antiviral agents, etc.) and for which this limiting toxicity is used to establish maximum tolerated doses for individual patients.

Idiosyncratic reactions may be detected in Phase III clinical trials or through postmarketing surveillance, such as the MedWatch program introduced by the FDA in 1993 (Szarfman et al., 1997). Global adverse event data, including serious hematotoxicity, are provided to the WHO; this information is compiled by a computer-based recording system employing WHO terminology and system and organ classifications for adverse reactions (Edwards et al., 1990). Data mining techniques enable the quantitative analysis of large public databases (Szarfman et al., 2002; Hopstadius et al., 2008) or pharmaceutical company databases (Egberts et al., 2002; Lehman et al., 2007) for detection of drug-adverse event relationships. These approaches can detect concealed associations (Lehman et al., 2007) and are recognized as important tools in pharmacovigilance (Almenoff et al., 2005; Hauben et al., 2005).

In Vitro Bone Marrow Assays

In vitro methods for assessing potential hematotoxicity are faster and less expensive than in vivo studies, while providing mechanistic data. Suppressive effects on HSC can be assessed using short-term clonogenic assays including burst-forming-unit erythroid (BFU-E), colony-forming-unit erythroid (CFU-E), colony-forming-unit granulocyte/monocyte (CFU-GM), colony-forming-unit megakaryocyte (CFU-MK), and colony-forming-unit granulocyte, erythroid, megakaryocyte, monocyte (CFU-GEMM), which have been developed for several laboratory animal species (Pessina et al.,

Table 11-14

WHO Grading Criteria for Subacute and Acute Hematotoxicity

HEMATOLOGIC PARAMETERS (ADULTS)	GRADE 0	GRADE 1	GRADE 2	GRADE 3	GRADE 4
Hemoglobin (g/dL) (nmol/L)	11.0 (6.8)	9.5–10.5 (6.5–6.7)	8.0–9.4 (4.95–5.8)	6.5–7.9 (4.0–4.9)	6.5 (4.0)
Leukocytes (1000/μL)	4.0	3.0–3.9	2.0–2.9	2.0–1.9	1.0
Granulocytes (1000/μL)	2.0	1.5–1.9	1.0–1.4	0.5–0.9	0.5
Platelets (1000/μL)	100	75–99	50–74	25–49	<25
Hemorrhage, blood loss	None	Petechiae	Mild	Gross	Debilitating

SOURCE: Data from WHO (1979).

2005). These assays enable the examination of tightly controlled exposure concentrations and durations effects on the myeloid, erythroid, and megakaryocytic lineages.

In vitro clonogenic assays have a number of advantages. Most important is the ability to test human hematopoietic cells directly in a preclinical setting, thus obviating extrapolation considerations. In vitro assays can be used to test combinations of chemicals as well as their metabolites and effects of serum and other cell components, such as lymphocytes (Pisciotta, 1997). Adaptations to incorporate metabolic activation have been developed such as incorporation of liver microsomes and cofactors as a metabolic system into the CFU-GM assay protocol (Dal Negro et al., 2006). A coculture model utilizing HepG2 liver spheroids as a source of metabolic enzymes and mesenchymal cells was used to assess mesenchymal stem cell (MSC) damage and was validated using chemotherapeutic agents, both prodrugs and active drugs (May et al., 2012). When used in a preclinical setting in combination with in vivo testing, the predictive value of these assays is enhanced, for example, the in vitro component has been used for therapeutic index-based screening to identify less myelosuppressive analogs of anticancer and antiviral drugs, structure–toxicity relationships, and new-drug lead candidates (Deldar and Stevens, 1993; Parchment et al., 1993; Pessina et al., 2003).

In vitro clonogenic assays in risk assessment have been useful for making practical interspecies comparisons regarding sensitivity to a particular agent or group of drugs or chemicals. Comparisons to the sensitivity of human cells can be made that have implications for the relative predictive value of various animal models for hematotoxicity in humans. Examples include the resistance of murine CFU-GM to the anticancer drug topotecan relative to that of the canine and human cells (Deldar and Stevens, 1993). This is consistent with the early observations of Marsh—that the dog is a particularly predictive model for the myelosuppression associated with anticancer drugs in humans (Marsh, 1985). Thus, while some chemicals show comparable suppressive activity across species lines (doxorubicin, pyrazoloacridine, hepsulfan, cyclopentenyl cytosine), others, such as camptothecins, carboxyamidotriazole, and fostriecin, show differences of as much as three log concentrations (Horikoshi and Murphy, 1982; Du et al., 1991; Reagan et al., 1993). Using CFU-GM-, BFU-E-, and CFU-E-clonogenic assays, aflatoxins B1 and M1 were reported to induce dose-dependent toxic effects on human and mouse stem cells, with higher sensitivity in human cells, and had greater impacts on myeloid and the immature erythroid lineages in both species (Roda et al., 2010).

One of the limitations of the assays is the low-throughput capacity. While the most commonly applied clonogenic techniques still employ viscous or semisolid matrices and culture supplements that promote differentiation of the hematopoietic cells (Erickson-Miller et al., 1997; McMullin et al., 1998), some newer techniques use liquid culture. In the automated non-clonogenic fluorometric microculture cytotoxicity assay (FMCA), human CD34 cells are incubated in 384-well microplates with drugs in liquid culture, supplemented with GM-stimulating cytokines, after which cell survival is assessed (Haglund et al., 2010). Extensive validation with known myelotoxic drugs found similar 50% inhibitory concentrations (IC(50)) after 7 days of culture as standard proposed FMCA-GM assays. Other assays measure intracellular ATP as a measure of proliferation. For example, HALO® (Hemotoxicity Assays via Luminescence Output) is an ATP-based bioluminescence assay that is conducted in 96-well culture plates and is completed in half the time of the manual clonogenic assay (Rich and Hall, 2005). Fourteen different lymphohematopoietic populations from three different tissues (peripheral blood, bone marrow, and cord blood) and from five different species (human, nonhuman primate, dog, rat, and mouse) can be assayed. HALO® has been extensively used for screening and studying various compounds (Rich and Hall, 2005; Parent-Massin et al., 2010). Platforms that enable the liquid culture of HSC/HPC can be used in conjunction with multiple other assays to understand mechanism of action, including mitochondrial dysfunction, genotoxicity (micronucleus assay), proliferation, differentiation, oxidative stress, cytokine release, chemotaxis/migration, oxidative stress, apoptosis, etc. Other commercially available products considerably improve the throughput of clonogenic assays (Yadav et al., 2016).

Because myelotoxicity, and particularly suppression of granulopoiesis, is a major limitation in administering anticancer drugs, and is often used to determine the optimal dose, the clonogenic assay for CFU-GM has received the most attention as a preclinical tool for predicting this response. It is the only assay that has been validated by an international study supported by the European Centre for Validation of Alternative Methods (ECVAM) (Pessina et al., 2003). Analytical validation was completed for the human and mouse assays, and a predictive model was developed to calculate the maximum tolerated dose (MTD) in humans using data from the mouse assay that is adjusted for the established interspecies variation (Pessina et al., 2001). The model was applied in an international blind trial to 20 drugs that included 14 antineoplastic drugs, the antiviral drugs zidovudine and acyclovir, and the pesticide lindane (Pessina et al., 2003). It predicted the MTD for all 20 chemicals, although extrapolation on the regression curve out of the range of the actual drug doses tested was required to derive the IC90 for 10 of these drugs. Since then, the Scientific Advisory Committee of the ECVAM endorsed the CFU-GM assay for predicting acute neutropenia in humans as a substitute to using a second species, such as the dog, for this purpose (24th meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 2006). Subsequent studies have confirmed the predictive value of this assay in both anticancer drugs (Masubuchi, 2006)

and nononcology candidate drugs (Horn et al., 2008). The latter study illustrates the species specificity encountered and the importance of integrating the in vivo and in vitro approaches required to fully characterize the hematotoxicity observed in preclinical safety assessment today.

While this discussion has focused on in vitro hematopoietic clonogenic assays in the context of risk assessment, these assays have also proven to be extraordinarily useful tools for investigating mechanisms of toxic cytopenia in humans (Deldar, 1994). Parchment and Murphy (1997) review the application of these to four categories of hematologic toxicity observed clinically: (1) the reversible cytopenia following acute exposure to a cytotoxic or cytostatic chemical; (2) the permanent loss in the production of a mature blood cell type(s); (3) cytosis, or the dramatic increase in blood cell counts following single or repeated toxicant exposure; and (4) the progressive loss of one or more blood cell lineages during chronic exposure to a toxicant. In all these circumstances, in vitro and ex vivo hematopoietic clonogenic assays have proven useful in understanding the mechanism(s) of these toxic effects and formulating strategies for risk management and treatment. Clonogenic assays have been used to show the effects of iron overload on hematopoietic progenitor cells in myelodysplastic syndrome (MDS) patients; proliferation of BFU-E but not CFU-GM was suppressed (Hartmann et al., 2013). Clonogenic assays have shown that bone marrow-derived HSC/HPC that are present in circulating peripheral blood are suppressed in human populations exposed to benzene and formaldehyde (Lan et al., 2004; Zhang et al., 2010c). Further, chromosomal abnormalities were detected in the resulting colonies (Zhang et al., 2010c, 2012). As circulating HSC/HPC originate in bone marrow, these assays allow the indirect assessment of bone marrow-derived cells in healthy, human subjects without using invasive sampling methods.

Emerging Technologies

Toxicogenomics Toxicogenomics involves the assessment by omic technologies of the genome, transcriptome, epigenome, proteome, and metabolome, of animals, people, or in vitro models that have been exposed to toxic agents. For example, analysis of the transcriptome by microarray, and more recently by next-generation sequencing (NGS), in the peripheral blood cells of benzene-exposed factory workers has identified potential biomarkers of exposure and early effect and mechanisms of action, even at levels below the occupational exposure limit (McHale et al., 2016). NGS revealed that benzene altered the expression of miRNAs related to hematopoiesis and hematological malignancies in HSC/HPC of exposed mice (Wei et al., 2015). Genomics studies using highly sensitive, error-corrected NGS methodologies detected low-frequency, leukemia-related mutations in the peripheral blood of individuals after treatment with cytotoxic chemotherapeutic drugs for a primary malignancy, up to 12 years before the diagnosis of therapy-related AML or therapy-related MDS (Wong et al., 2015; Young et al., 2015). Activity-based protein profiling or chemoproteomics can map targets of toxicants and delineate toxicological mechanisms (Ford et al., 2017).

Application of toxicogenomics in exposed human populations can inform risk assessment by elucidating mechanisms of action underlying exposure-related disease, identifying susceptible individuals, and revealing responses at low doses (McHale et al., 2010; Zhang et al., 2010b; McHale et al., 2012). Robust experimental design incorporating precise, individual exposure measurements, adequate power, relevant phenotypic endpoints, and range of exposure levels is necessary (McHale et al., 2016). Integrative omic analysis of various data types can provide more detailed pathway and network information, identify more robust biomarkers, and elucidate mechanisms of toxicity and disease, thus aiding risk characterization (Gohlke et al., 2009; Enfield et al., 2012; Shivarov and Bullinger, 2014; Nardini et al., 2015; van Breda et al., 2015). As more studies are performed and incorporated into databases such as the Comparative Toxicogenomics Database (CTD) and Chemical Effects in Biological Systems (CEBS), data can be mined for classification of newly tested chemicals (hazard identification), and, for investigating the dose–response, inter-relationship among genes, environment, and disease in a systems biology approach (risk characterization). For example, CTD pathway data was used to discriminate chemical leukemogens from non-leukemogens (Thomas et al., 2012).

3D Bone Marrow Niche Models Research groups have attempted to develop 3D models of the bone marrow niche using biomimetic materials, such as collagen microspheres, hydrogels, decellularized extracellular matrix extracts, and synthetic polyacrylates, as scaffolds (Kim et al., 2015; Dhami et al., 2016; Xu et al., 2016; Houshmand et al., 2017). Although progress has been made, key challenges remain in replicating the complex interactions between HSCs and marrow microenvironments and precisely controlling the biochemical and physical niches that are critical in supporting hematopoiesis.

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REFERENCES

Abrams CS. Acquired qualitative platelet disorders. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006:1833–1855.

ACS. *Cancer Facts & Figures 2013*. Atlanta, GA: American Cancer Society; 2013.

ACS. *Cancer Facts & Figures 2017*. Atlanta, GA: American Cancer Society; 2017.

Ahmad Khan H. Benzene's toxicity: a consolidated short review of human and animal studies. *Hum Exp Toxicol*. 2007;26:677–685.

Ahmadzadeh A, Norozi F, Shahrabi S, Shahjahani M, Saki N. Wnt/Beta-Catenin Signaling in Bone Marrow Niche. *Cell Tissue Res*. 2016;363:321–335.

Aksoy M. Benzene Haematotoxicity. In: Aksoy M, ed. *Benzene Carcinogenicity*. Boca Raton, FL: CRC Press; 1988:59–112.

Al-Nouri ZL, Reese JA, Terrell DR, Vesely SK, George JN. Drug-induced thrombotic microangiopathy: a systematic review of published reports. *Blood*. 2015;125:616–618.

Albertini RJ, Judice SA, Recio L, Walker VE. Hprt mutant frequency and P53 gene status in mice chronically exposed by inhalation to benzene. *Chem Biol Interact*. 2010;184:77–85.

Alcindor T, Bridges KR. Sideroblastic anaemias. *Br J Haematol*. 2002;116:733–743.

Allan JM, Travis LB. Mechanisms of therapy-related carcinogenesis. *Nat Rev Cancer*. 2005;5:943–955.

Almenoff J, Tonning JM, Gould AL, et al. Perspectives on the use of data mining in pharmaco-vigilance. *Drug Saf*. 2005;28:981–1007.

Alving AS, Carson PE, Flanagan CL, Ickes CE. Enzymatic deficiency in primaquine-sensitive erythrocytes. *Science*. 1956;124:484–485.

Ananyeva NM, Lacroix-Desmazes S, Hauser CA, et al. Inhibitors in hemophilia A: mechanisms of inhibition, management and perspectives. *Blood Coagul Fibrinolysis*. 2004;15:109–124.

Anderson RL, Bagby GC Jr, Richert-Boe K, Magenis RE, Koler RD. Therapy-related preleukemic syndrome. *Cancer*. 1981;47:1867–1871.

Andres E, Kurtz JE, Martin-Hunyadi C, et al. Nonchemotherapy drug-induced agranulocytosis in elderly patients: the effects of granulocyte colony-stimulating factor. *Am J Med*. 2002;112:460–464.

Andres E, Maloisel F, Zimmer J. The role of haematopoietic growth factors granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor in the management of drug-induced agranulocytosis. *Br J Haematol*. 2010;150(1):3–8.

Ansell J, Hirsh J, Poller L, et al. The pharmacology and management of the vitamin K antagonists: the seventh ACCP conference on antithrombotic and thrombolytic therapy. *Chest*. 2004;126:204S–233S.

Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127:2391–2405.

Arndt PA, Garratty G. The changing spectrum of drug-induced immune hemolytic anemia. *Semin Hematol*. 2005;42:137–144.

Arneborn P, Palmblad J. Drug-induced neutropenia—a survey for Stockholm 1973–1978. *Acta Med Scand*. 1982;212:289–292.

Asada N, Takeishi S, Frenette PS. Complexity of bone marrow hematopoietic stem cell niche. *Int J Hematol*. 2017;106:45–54.

Athens JW, Haab OP, Raab SO, et al. Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. *J Clin Invest*. 1961;40:989–995.

ATSDR. *Toxicological Profile for Benzene*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2007.

Babior BM, Golde DW. Production, distribution and fate of neutrophils. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, eds. *Williams Hematology*. 5th ed. New York: McGraw-Hill; 1995:773–779.

Bahrami S, Malone JC, Webb KG, et al. Tissue eosinophilia as an indicator of drug-induced cutaneous small-vessel vasculitis. *Arch Dermatol*. 2006;142:155–161.

Baker KR. Hemolytic anemia resulting from physical injury to red cells. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. New York: McGraw-Hill; 2006:709–716.

Barash M, Reich KA, Rademaker D. Lidocaine-induced methemoglobinemia: a clinical reminder. *J Am Osteopath Assoc*. 2015;115:94–98.

Barnes PJ. Corticosteroids: the drugs to beat. *Eur J Pharmacol*. 2006;533:2–14.

Bates SM, Greer IA, Hirsh J, et al. Use of antithrombotic agents during pregnancy: the seventh ACCP conference on antithrombotic and thrombolytic therapy. *Chest*. 2004;126:627S–644S.

Bennett JM. Changes in the updated 2016: WHO Classification of the myelodysplastic syndromes and related myeloid neoplasms. *Clin Lymphoma Myeloma Leuk*. 2016;16:607–609.

Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French–American–British Cooperative Group. *Ann Intern Med*. 1985;103:620–625.

Berliner N, Horwitz M, Loughran TP Jr. Congenital and acquired neutropenia. In: Broudy VC, Berliner N, Larson RA, Leung LL, eds. *American Society of Hematology Education Program Book*. Washington, DC: American Society of Hematology; 2004:63–79.

Berry RG, Morrison JA, Watts JW, et al. Surreptitious superwarfarin ingestion with brodifacoum. *South Med J*. 2000;93:74–75.

Bertolaccini ML, Hughes GR, Khamashta MA. Revisiting antiphospholipid antibodies: from targeting phospholipids to phospholipid binding proteins. *Clin Lab*. 2004;50:653–665.

Beutler E. G6pd: population genetics and clinical manifestations. *Blood Rev*. 1996;10:45–52.

Beutler E. Hemolytic anemias resulting from infections with microorganisms. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006a:723–727.

Beutler E. Hemolytic anemias resulting from chemical and physical agents. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006b:717–721.

Beutler E. Hereditary and acquired sideroblastic anemias. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006c:823–828.

Bhaduri B, Kandel M, Brugnara C, Tangella K, Popescu G. Optical assay of erythrocyte function in banked blood. *Sci Rep*. 2014;4:6211.

Bhatia S. Therapy-related myelodysplasia and acute myeloid leukemia. *Semin Oncol*. 2013;40:666–675.

Bloom JC. Principles of hematotoxicology: laboratory assessment and interpretation of data. *Toxicol Pathol*. 1993;21:130–134.

Bloom JC, Lewis HB, Sellers TS, et al. The hematopathology of cefonicid- and cefazedone-induced blood dyscrasias in the dog. *Toxicol Appl Pharmacol*. 1987;90:143–155.

Blossom D, Kallen A, Patel PR, et al. Outbreak of adverse reactions associated with contaminated heparin. *N Engl J Med*. 2008;359:2674–2684.

Bo J, Schroder H, Kristinsson J, et al. Possible carcinogenic effect of 6-mercaptopurine on bone marrow stem cells: relation to thiopurine metabolism. *Cancer*. 1999;86:1080–1086.

Boothe VL, Boehmer TK, Wendel AM, Yip FY. Residential traffic exposure and childhood leukemia: a systematic review and meta-analysis. *Am J Prev Med*. 2014;46:413–422.

Borregaard N, Boxer LA. Disorders of neutrophil function. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006: 921–957.

Bradberry SM. Occupational methaemoglobinaemia. Mechanisms of production, features, diagnosis and management including the use of methylene blue. *Toxicol Rev*. 2003;22:13–27.

Bradberry SM, Aw TC, Williams NR, et al. Occupational methaemoglobinaemia. *Occup Environ Med*. 2001;58:611–615.

Brayton RG, Stokes PE, Schwartz MS, et al. Effect of alcohol and various diseases on leukocyte mobilization, phagocytosis and intracellular bacterial killing. *N Engl J Med*. 1970;282:123–128.

Browning E. *Toxicity and Metabolism of Industrial Solvents*. London: Elsevier; 1965.

Broxmeyer HE, Orschell CM, Clapp DW, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med*. 2005;201:1307–1318.

Bullinger L, Dohner K, Dohner H. Genomics of Acute Myeloid Leukemia Diagnosis and Pathways. *J Clin Oncol*. 2017;35:934–946.

Cai S, Wang H, Bailey B, et al. Humanized Bone Marrow Mouse Model as a Preclinical Tool to Assess Therapy-Mediated Hematotoxicity. *Clin Cancer Res*. 2011;17:2195–2206.

Campo E, Swerdlow SH, Harris NL, et al. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood*. 2011;117:5019–5032.

Cancer Genome Atlas Research N. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368:2059–2074.

Cappellini MD, Fiorelli G. Glucose-6-Phosphate dehydrogenase deficiency. *Lancet*. 2008;371:64–74.

Capsoni F, Sarzi-Puttini P, Zanella A. Primary and secondary autoimmune neutropenia. *Arthritis Res Ther*. 2005;7:208–214.

Caramori G, Adcock I. Anti-inflammatory mechanisms of glucocorticoids targeting granulocytes. *Curr Drug Targets Inflamm Allergy*. 2005;4:455–463.

Carbonari D, Chiarella P, Mansi A, et al. Biomarkers of susceptibility following benzene exposure: influence of genetic polymorphisms on benzene metabolism and health effects. *Biomark Med*. 2016;10:145–163.

Carlos-Wallace FM, Zhang L, Smith MT, Rader G, Steinmaus C. Parental, in utero, and early-life exposure to benzene and the risk of childhood leukemia: a meta-analysis. *Am J Epidemiol*. 2016;183:1–14.

Cartwright GE, Athens JW, Wintrobe MM. The kinetics of granulopoiesis in normal man. *Blood*. 1964;24:780–803.

Casado FL, Singh KP, Gasiewicz TA. Aryl hydrocarbon receptor activation in hematopoietic stem/progenitor cells alters cell function and pathway-specific gene modulation reflecting changes in cellular trafficking and migration. *Mol Pharmacol*. 2011;80:673–682.

Casciato DA, Scott JL. Acute leukemia following prolonged cytotoxic agent therapy. *Medicine*. 1979;58:32–47.

Cazzola M, Invernizzi R, Bergamaschi G, et al. Mitochondrial ferritin expression in erythroid cells from patients with sideroblastic anemia. *Blood*. 2003;101:1996–2000.

Cerulli J, Grabe DW, Gauthier I, et al. Chromium picolinate toxicity. *Ann Pharmacother*. 1998;32:428–431.

Chabner BA, Wilson W, Supko J. Pharmacology and toxicity of antineoplastic drugs. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006:247–274.

Chakraverty R, Davidson S, Peggs K, et al. The incidence and cause of coagulopathies in an intensive care population. *Br J Haematol*. 1996;93:460–463.

Chan SM, Majeti R. Role of Dnmt3a, Tet2, and Idh1/2 Mutations in Pre-Leukemic Stem Cells in Acute Myeloid Leukemia. *Int J Hematol*. 2013;98:648–657.

Chen H, Eastmond DA. Topoisomerase inhibition by phenolic metabolites: a potential mechanism for benzene's clastogenic effects. *Carcinogenesis*. 1995;16:2301–2307.

Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *J Clin Oncol*. 2001;19:1405–1413.

Chua JD, Friedenberg WR. Superwarfarin poisoning. *Arch Intern Med*. 1998;158:1929–1932.

Churchill GA, Gatti DM, Munger SC, Svenson KL. The diversity outbred mouse population. *Mamm Genome*. 2012;23:713–718.

Churpek JE, Larson RA. The evolving challenge of therapy-related myeloid neoplasms. *Best Pract Res Clin Haematol*. 2013;26:309–317.

Clifton J, Leikin JB. Methylene blue. *Am J Ther*. 2003;10:289–291.

Clopton DA, Saltman P. Copper-specific damage in human erythrocytes exposed to oxidative stress. *Biol Trace Elem Res*. 1997;56:231–240.

Coleman MD, Coleman NA. Drug-induced methaemoglobinaemia. Treatment issues. *Drug Saf*. 1996;14:394–405.

Collen D, Lijnen HR. Thrombolytic agents. *Thromb Haemost*. 2005;93:627–630.

Connolly SJ, Milling TJ Jr, Eikelboom JW, et al. Andexanet alfa for acute major bleeding associated with factor Xa inhibitors. *N Engl J Med*. 2016;375:1131–1141.

Contrino J, Marucha P, Ribaudo R, et al. Effects of mercury on human polymorphonuclear leukocyte function in vitro. *Am J Pathol*. 1988;132:110–118.

Cooper N. State of the art—how I manage immune thrombocytopenia. *Br J Haematol*. 2017;177:39–54.

Copley GB, Schnatter AR, Armstrong TW, et al. Hospital-based case-control study of MDS subtypes and benzene exposure in Shanghai. *J Occup Environ Med*. 2017;59:349–355.

Cronkite EP. Evidence for radiation and chemicals as leukemogenic agents. *Archives of Environmental Health*. 1961;3:297–303.

Crowther M. The direct oral anticoagulants: can we finally stop using rat poison? *Blood Advances*. 2017;1:980.

Cummings TF. The treatment of cyanide poisoning. *Occup Med*. 2004;54:82–85.

Curtis BR. Drug-induced immune thrombocytopenia: incidence, clinical features, laboratory testing, and pathogenic mechanisms. *Immunohematology*. 2014;30:55–65.

Curzen N, Haque R, Timmis A. Applications of thrombolytic therapy. *Intensive Care Med*. 1998;24:756–768.

Custer RP, Ahlfeldt FE. Studies on the structure and function of bone marrow. *J Lab Clin Med*. 1932;17:960–962.

D'Andrea G, D'Ambrosio RL, Di Perna P, et al. A polymorphism in the VKORC1 gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. *Blood*. 2005;105:645–649.

Dahl OE, Garvik LJ, Lyberg T. Toxic effects of methylmethacrylate monomer on leukocytes and endothelial cells in vitro. *Acta Orthop Scand*. 1994;65:147–153.

Dal Negro G, Vandin L, Bonato M, Sciuscio D. Toward refinement of the colony-forming unit-granulocyte/macrophage clonogenic assay: inclusion of a metabolic system. *Toxicol In Vitro*. 2006;20:743–749.

Dale DC. Neutropenia and neutrophilia. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006:907–955.

Dalli J, Jones CP, Cavalcanti DM, et al. Annexin A1 regulates neutrophil clearance by macrophages in the mouse bone marrow. *FASEB J*. 2012;26:387–396.

Davis JE, Fields JP. Experimental production of polycythemia in humans by administration of cobalt chloride. *Proc Soc Exp Biol Med*. 1958;99:493–495.

Davoren A, Aster RH. Heparin-induced thrombocytopenia and thrombosis. *Am J Hematol*. 2006;81:36–44.

De Kouchkovsky I, Abdul-Hay M. Acute myeloid leukemia: a comprehensive review and 2016 update. *Blood Cancer J*. 2016;6:e441.

de Latour RP, Visconte V, Takaku T, et al. Th17 immune responses contribute to the pathophysiology of aplastic anemia. *Blood*. 2010;116:4175–4180.

de Smith AJ, Kaur M, Gonseth S, et al. Correlates of prenatal and early-life tobacco smoke exposure and frequency of common gene deletions in childhood acute lymphoblastic leukemia. *Cancer Res*. 2017;77:1674–83.

de With SAJ, Pulit SL, Staal WG, Kahn RS, Ophoff RA. More than 25 years of genetic studies of clozapine-induced agranulocytosis. *Pharmacogenomics J*. 2017;17:304–311.

Deckmyn H, Vanhoorelbeke K, Peerlinck K. Inhibitory and activating human antiplatelet antibodies. *Baillieres Clin Haematol*. 1998;11:343–359.

Deldar A. Drug-induced blood disorders: review of pathogenetic mechanisms and utilization of bone marrow cell culture technology as an investigative approach. *Curr Topics Vet Res*. 1994;1:83–101.

Deldar A, Stevens CE. Development and application of in vitro models of hematopoiesis to drug development. *Toxicol Pathol*. 1993;21:231–240.

Delore P, Borgomano C. Leucemie aigue au cours de l'intoxication benzenique: sur l'origine toxique de certaines leucemies aigues et leur relations avec les anemies graves. *J Med Lyon*. 1928;9:227–233.

Demirer T, Bensinger WI. Optimization of peripheral blood stem cell collection. *Curr Opin Hematol*. 1995;2:219–226.

Dessypris EN. Erythropoiesis. In: Lee CR, Foerster J, Lukens J, Paraskevas P, Greer JP, Rodgers GM, eds. *Wintrobe's Clinical Hematology*. 10th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 1999:169–192.

Dhami SP, Kappala SS, Thompson A, Szegezdi E. Three-dimensional ex vivo co-culture models of the leukaemic bone marrow niche for functional drug testing. *Drug Discov Today*. 2016;21:1464–1471.

Di Minno A, Frigerio B, Spadarella G, et al. Old and new oral anticoagulants: food, herbal medicines and drug interactions. *Blood Rev*. 2017;31:193–203.

Dieckhaus CM, Thompson CD, Roller SG, Macdonald TL. Mechanisms of idiosyncratic drug reactions: the case of felbamate. *Chem Biol Interact*. 2002;142:99–117.

Dinwoodey DL, Ansell JE. Heparins, low-molecular-weight heparins, and pentasaccharides. *Clin Geriatr Med*. 2006;22:1–15, vii.

Djaldetti M, Blay A, Bergman M, et al. Pure red cell aplasia—a rare disease with multiple causes. *Biomed Pharmacother*. 2003;57:326–332.

Dohner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. *N Engl J Med*. 2015;373:1136–1152.

Dolan MC. Carbon monoxide poisoning. *CMAJ*. 1985;133:392–399.

Domingues MJ, Cao H, Heazlewood SY, Cao B, Nilsson SK. Niche extracellular matrix components and their influence on HSC. *J Cell Biochem*. 2017a.

Domingues MJ, Nilsson SK, Cao B. New agents in HSC mobilization. *Int J Hematol*. 2017b;105:141–152.

Drablos F, Feyzi E, Aas PA, et al. Alkylation damage in DNA and RNA—repair mechanisms and medical significance. *DNA Repair (Amst)*. 2004;3:1389–1407.

Dreno B, Trossaert M, Boiteau HL, et al. Zinc salts effects on granulocyte zinc concentration and chemotaxis in acne patients. *Acta Derm Venereol*. 1992;72:250–252.

Du DL, Volpe DA, Grieshaber CK, Murphy MJ Jr. Comparative toxicity of fostriecin, hepsulfam and pyrazine diazohydroxide to human and murine hematopoietic progenitor cells in vitro. *Invest New Drugs*. 1991;9:149–157.

Eash KJ, Means JM, White DW, Link DC. Cxcr4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood*. 2009;113:4711–4719.

Eastmond DA. *Chemical and radiation leukemogenesis in humans and rodents and the value of rodent models for assessing risks of lymphohematopoietic Cancers*. Washington, DC: U.S. Environmental Protection Agency; 1997.

Eastmond DA, Schuler M, Frantz C, et al. Characterization and mechanisms of chromosomal alterations induced by benzene in mice and humans. *Res Rep Health Eff Inst*. 2001:1–68; discussion 69–80.

Edwards IR, Lindquist M, Wiholm BE, Napke E. Quality criteria for early signals of possible adverse drug reactions. *Lancet*. 1990;336:156–158.

Egberts AC, Meyboom RH, van Puijenbroek EP. Use of measures of disproportionality in pharmacovigilance: three Dutch examples. *Drug Saf*. 2002;25:453–458.

Ehninger A, Trumpp A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med*. 2011;208:421–428.

Enfield KS, Pikor LA, Martinez VD, Lam WL. Mechanistic roles of noncoding RNAs in lung cancer biology and their clinical implications. *Genet Res Int*. 2012;2012:737416.

Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ*. 2008;15:504–514.

Erickson-Miller CL, May RD, Tomaszewski J, et al. Differential toxicity of camptothecin, topotecan and 9-aminocamptothecin to human, canine, and murine myeloid progenitors (CFU-GM) in vitro. *Cancer Chemother Pharmacol*. 1997;39:467–472.

Evans NS, Gomes M. Heparin-induced thrombocytopenia (Hit). *Vasc Med*. 2017:1358863X17714340.

Federici AB. The use of desmopressin in von willebrand disease: the experience of the first 30 Years (1977–2007). *Haemophilia*. 2008;14(suppl 1):5–14.

Feldman BF, Zinkle JG, Jain NC, eds. *Schalm's Veterinary Hematology*. 5th ed. Ames, IA: Blackwell Publishing; 2006.

Filippini T, Heck JE, Malagoli C, Del Giovane C, Vinceti M. A review and meta-analysis of outdoor air pollution and risk of childhood leukemia. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2015;33:36–66.

Fisch P, Handgretinger R, Schaefer HE. Pure red cell aplasia. *Br J Haematol*. 2000;111:1010–1022.

Fiske DN, McCoy HE III, Kitchens CS. Zinc-induced sideroblastic anemia: report of a case, review of the literature, and description of the hematologic syndrome. *Am J Hematol*. 1994;46:147–150.

Fix SM, Borden MA, Dayton PA. Therapeutic gas delivery via microbubbles and liposomes. *J Control Release*. 2015;209:139–149.

Flexman AM, Del Vicario G, Schwarz SK. Dark green blood in the operating theatre. *Lancet*. 2007;369:1972.

Flieder DB, Travis WD. Pathologic characteristics of drug-induced lung disease. *Clin Chest Med*. 2004;25:37–45.

Ford B, Bateman LA, Gutierrez-Palominos L, et al. Mapping proteome-wide targets of glyphosate in mice. *Cell Chem Biol*. 2017;24:133–140.

French JE, Gatti DM, Morgan DL, et al. Diversity outbred mice identify population-based exposure thresholds and genetic factors that influence benzene-induced genotoxicity. *Environ Health Perspect*. 2015;123:237–245.

Friberg LE, Karlsson MO. Mechanistic models for myelosuppression. *Invest New Drugs*. 2003;21:183–194.

Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. *Immunology*. 2008a;125:281–288.

Furze RC, Rankin SM. The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse. *FASEB J*. 2008b;22:3111–3119.

Gallardo S, Cardaba B, Posada M, et al. Toxic oil syndrome: genetic restriction and immunomodulatory effects due to adulterated oils in a model of HLA transgenic mice. *Toxicol Lett*. 2005;159:173–181.

Gamazon ER, Im HK, O'Donnell PF, et al. Comprehensive evaluation of the contribution of X chromosome genes to platinum sensitivity. *Mol Cancer Ther*. 2011;10(3):472–480.

Garb E. Non-chemotherapy drug-induced agranulocytosis. *Expert Opin Drug Saf*. 2007;6(3):323–335.

Gasiewicz TA, Singh KP, Bennett JA. The Ah receptor in stem cell cycling, regulation, and quiescence. *Ann N Y Acad Sci*. 2014;1310:44–50.

Gerson SL, Meltzer H. Mechanisms of clozapine-induced agranulocytosis. *Drug Saf*. 1992;7(suppl 1):17–25.

Gerson WT, Fine DG, Spielberg SP, et al. Anticonvulsant-induced aplastic anemia: increased susceptibility to toxic drug metabolites in vitro. *Blood*. 1983;61:889–893.

Girard D. Activation of human polymorphonuclear neutrophils by environmental contaminants. *Rev Environ Health*. 2003;18:75–89.

Godley LA, Larson RA. Therapy-related myeloid leukemia. *Semin Oncol*. 2008;35:418–429.

Gohlke JM, Thomas R, Zhang Y, et al. Genetic and environmental pathways to complex diseases. *BMC Syst Biol*. 2009;3:46.

Goldstein BD. Benzene toxicity: a critical evaluation: hematotoxicity in humans. *J Toxicol Environ Health Suppl*. 1977;2:69–105.

Gonzalez H, Leblond V, Azar N, et al. Severe autoimmune hemolytic anemia in eight patients treated with fludarabine. *Hematol Cell Ther*. 1998;40:113–118.

Gopalachar AS, Bowie VL, Bharadwaj P. Phenazopyridine-induced sulfhemoglobinemia. *Ann Pharmacother*. 2005;39:1128–1130.

Gore AV, Weinstein BM. DNA methylation in hematopoietic development and disease. *Exp Hematol*. 2016;44:783–790.

Granfeldt Ostgard LS, Medeiros BC, Sengelov H, et al. Epidemiology and clinical significance of secondary and therapy-related acute myeloid leukemia: a national population-based cohort Study. *J Clin Oncol*. 2015;33:3641–3649.

Greer FR, Shannon M, American Academy of Pediatrics Committee on N, American Academy of Pediatrics Committee on Environmental H. Infant methemoglobinemia: the role of dietary nitrate in food and water. *Pediatrics*. 2005;116:784–786.

Greim H, Kaden DA, Larson RA, et al. The bone marrow niche, stem cells, and leukemia: impact of drugs, chemicals, and the environment. *Ann N Y Acad Sci*. 2014;1310:7–31.

Guevara A, Labarca J, Gonzalez-Martin G. Heparin-induced transaminase elevations: a prospective study. *Int J Clin Pharmacol Ther Toxicol*. 1993;31:137–141.

Guidotti TL. Hydrogen sulfide intoxication. *Handb Clin Neurol*. 2015;131:111–133.

Haferlach T, Kern W, Schnittger S, Schoch C. Modern diagnostics in acute leukemias. *Crit Rev Oncol Hematol*. 2005;56:223–234.

Hagen EC, Ballieux BE, van Es LA, et al. Antineutrophil cytoplasmic autoantibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenetic consequences. *Blood*. 1993;81:1996–2002.

Haglund C, Aleskog A, Hakansson LD, et al. The Fmca-Gm assays, high throughput non-clonogenic alternatives to CFU-GM in preclinical hematotoxicity testing. *Toxicol Lett*. 2010;194:102–107.

Hahn CN, Venugopal P, Scott HS, Hiwase DK. Splice factor mutations and alternative splicing as drivers of hematopoietic malignancy. *Immunol Rev*. 2015;263:257–278.

Hajjar A. Fibrinolysis and thrombolysis. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006:1833–1855.

Hamilton A. Benzene (benzol) poisoning. General review. *Arch Pathol lab Med*. 1931;11:434.

Hanahan D, Weinberg R. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–674.

Harder S, Thurmann P. Clinically important drug interactions with anticoagulants. An update. *Clin Pharmacokinet*. 1996;30:416–444.

Harrington WJ, Minnich V, Hollingsworth JW, et al. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med*. 1951;38:1–10.

Harrison DE, Lerner CP. Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-fluorouracil. *Blood*. 1991;78:1237–1240.

Hartmann J, Braulke F, Sinzig U, et al. Iron overload impairs proliferation of erythroid progenitors cells (BFU-E) from patients with myelodysplastic syndromes. *Leuk Res*. 2013;37:327–332.

Hauben M, Patadia V, Gerrits C, Walsh L, Reich L. Data mining in pharmacovigilance: the need for a balanced perspective. *Drug Saf*. 2005;28:835–842.

Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer*. 2008;8:193–204.

Hesdorffer CS, Longo DL. Drug-induced megaloblastic anemia. *N Engl J Med*. 2016;374:696–697.

Hirsch PY, Tang R, et al. Genetic hierarchy and temporal variegation in the clonal history of acute myeloid leukaemia. *Nat Commun*. 2016;7:12475.

Hirsh J, Raschke R. Heparin and low-molecular-weight heparin: the seventh ACCP conference on antithrombotic and thrombolytic therapy. *Chest*. 2004;126:188S–203S.

Ho HY, Cheng ML, Chiu DT. Glucose-6-phosphate dehydrogenase—beyond the realm of red cell biology. *Free Radic Res*. 2014;48:1028–1048.

Honigfeld G, Arellano F, Sethi J, et al. Reducing clozapine-related morbidity and mortality: 5 years of experience with the Clozaril National Registry. *J Clin Psychiatry*. 1998;59(suppl 3):3–7.

Hopstadius J, Noren GN, Bate A, Edwards IR. Impact of stratification on adverse drug reaction surveillance. *Drug Saf*. 2008;31:1035–1048.

Horikoshi A, Murphy MJ Jr. Comparative effects of chemotherapeutic drugs on human and murine hematopoietic progenitors in vitro. *Chemotherapy*. 1982;28:480–501.

Horn TL, Harder JB, Johnson WD, et al. Integration of in vivo and in vitro approaches to characterize the toxicity of antalarmin, a corticotropin-releasing hormone receptor antagonist. *Toxicology*. 2008;248:8–17.

Houshmand M, Soleimani M, Atashi A, et al. Mimicking the acute myeloid leukemia niche for molecular study and drug screening. *Tissue Eng Part C Methods*. 2017;23:72–85.

Howes RE, Battle KE, Satyagraha AW, Baird JK, Hay SI. G6pd deficiency: global distribution, genetic variants and primaquine therapy. *Adv Parasitol*. 2013;81:133–201.

Hsia CC. Respiratory function of hemoglobin. *N Engl J Med*. 1998;338:239–247.

Huang RS, Kistner EO, Bleibel WK, et al. Effect of population and gender on chemotherapeutic agent-induced cytotoxicity. *Mol Cancer Ther*. 2007;6(1):31–36.

Hudson G. Bone-marrow volume in the human foetus and newborn. *Br J Haematol*. 2006;11:446–452.

IARC. *A Review of Human Carcinogens, Part F: Chemical Agents and Related Occupations, Iarc Monographs on the Evaluation of Carcinogenic Risks to Humans*. Lyons: International Agency for Research on Cancer; 2012.

IARC. *Ionizing Radiation, Part 1: X- and Gamma (G)-Radiation, and Neutrons*. Lyon: International Agency for Research on Cancer; 2000.

IARC. *Ionizing Radiation, Part 2: Some Internally Deposited Radionuclides*. Lyon: International Agency for Research on Cancer; 2001.

IARC. *Overall Evaluations of Carcinogenicity to Humans*. Lyon, France: International Agency for Research on Cancer; 2010.

Ibanez L, Vidal X, Ballarin E, et al. Population-based drug-induced agranulocytosis. *Arch Int Med*. 2005;165(8):869–874.

Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. *Lancet*. 2013;381:1943–1955.

Infante PF, Rinsky RA, Wagoner JK, Young RJ. Leukaemia in benzene workers. *Lancet*. 1977;2:76–78.

Irons RD. Leukemogenesis as a toxic response. In: Sipes IG, McQueen AC, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997:175–199.

Irons RD, Stillman WS. The process of leukemogenesis. *Environ Health Perspect*. 1996;104(suppl 6):1239–1246.

Jabbour E, O'Brien S, Konopleva M, Kantarjian H. New insights into the pathophysiology and therapy of adult acute lymphoblastic leukemia. *Cancer*. 2015;121:2517–2528.

Jandl JH. *Blood*. Boston, MA: Little Brown & Co; 1987.

Jiang H, Fu R, Wang H, et al. Cd47 is expressed abnormally on hematopoietic cells in myelodysplastic Syndrome. *Leuk Res*. 2013;37:907–910.

Joannides M, Mays AN, Mistry AR, et al. Molecular pathogenesis of secondary acute promyelocytic leukemia. *Mediterr J Hematol Infect Dis*. 2011;3:e2011045.

Jokiranta TS. HUS and atypical HUS. *Blood*. 2017;129:2847–2856.

Joly BS, Coppo P, Veyradier A. Thrombotic thrombocytopenic purpura. *Blood.* 2017;129:2836–2846.

Julia A, Olona M, Bueno J, et al. Drug-induced agranulocytosis: prognostic factors in a series of 168 episodes. *Br J Haematol*. 1991;79:366–371.

Jurk K, Kehrel BE. Platelets: physiology and biochemistry. *Semin Thromb Hemost*. 2005;31:381–392.

Kaina B, Christmann M, Naumann S, Roos WP. Mgmt: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair (Amst)*. 2007;6:1079–1099.

Kao LW, Nanagas KA. Carbon monoxide poisoning. *Med Clin North Am*. 2005;89:1161–1194.

Karp JE, Sarkodee-Adoo CB. Therapy-related acute leukemia. *Clin Lab Med*. 2000;20:71–81, ix.

Karran P. Thiopurines, DNA damage, DNA repair and therapy-related cancer. *Br Med Bull*. 2006;79–80:153–170.

Kasim K, Levallois P, Abdous B, Auger P, Johnson KC. Lifestyle factors and the risk of adult leukemia in Canada. *Cancer Causes Control*. 2005;16:489–500.

Kaushansky K. Lineage-specific hematopoietic growth factors. *N Engl J Med*. 2006;354:2034–2045.

Keng PC. Use of flow cytometry in the measurement of cell mitotic cycle. *Int J Cell Cloning*. 1986;4:295–311.

Khalaj M, Tavakkoli M, Stranahan AW, Park CY. Pathogenic Microrna's in Myeloid Malignancies. *Front Genet*. 2014;5:361.

Khaled S, Al Malki M, Marcucci G. Acute myeloid leukemia: biologic, prognostic, and therapeutic insights. *Oncology (Williston Park)*. 2016;30:318–329.

Khera R, Das N. Complement receptor 1: disease associations and therapeutic implications. *Mol Immunol*. 2009;46:761–772.

Kilbourne EM, Posada de la Paz M, Abaitua B, et al. Toxic oil syndrome: a current clinical and epidemiologic summary, including comparisons with the eosinophilia–myalgia syndrome. *J Am Coll Cardiol*. 1991;18:711–717.

Kim J, Lee H, Selimovic S, Gauvin R, Bae H. Organ-on-a-Chip: development and clinical prospects toward toxicity assessment with an emphasis on bone marrow. *Drug Saf*. 2015;38:409–418.

Kim-Shapiro DB, Gladwin MT, Patel RP, et al. The reaction between nitrite and hemoglobin: the role of nitrite in hemoglobin-mediated hypoxic vasodilation. *J Inorg Biochem*. 2005;99:237–246.

Kitamura T, Watanabe-Okochi N, Enomoto Y, et al. Novel working hypothesis for pathogenesis of hematological malignancies: combination of mutations-induced cellular phenotypes determines the disease (cMIP-DD). *J Biochem*. 2016;159:17–25.

Kolachana P, Subrahmanyam VV, Meyer KB, et al. Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells in vitro and in the bone marrow in vivo. *Cancer Res*. 1993;53: 1023–1026.

Kouvaris JR, Kouloulias VE, Vlahos LJ. Amifostine: the first selective-target and broad-spectrum radioprotector. *Oncologist*. 2007;12:738–747.

Kruger P, Saffarzadeh M, Weber AN, et al. Neutrophils: between host defence, immune modulation, and tissue Injury. *PLoS Pathog*. 2015;11:e1004651.

Kuderer NM, Cosler L, Crawford J. Cost and mortality associated with febrile neutropenia in adult cancer patients. *Proc Am Soc Clin Oncol*. 2002;21:250a.

Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett*. 2005;157:175–188.

Ladiges WC, Storb R, Thomas ED. Canine models of bone marrow transplantation. *Lab Anim Sci*. 1990;40:11–15.

Lan Q, Smith MT, Tang X, et al. Chromosome-wide aneuploidy study of cultured circulating myeloid progenitor cells from workers occupationally exposed to formaldehyde. *Carcinogenesis*. 2015;36:160–167.

Lan Q, Zhang L, Li G, et al. Hematotoxicity in workers exposed to low levels of benzene. *Science*. 2004;306:1774–1776.

Lan Q, Zhang L, Shen M, et al. Large-scale evaluation of candidate genes identifies associations between DNA repair and genomic maintenance and development of benzene hematotoxicity. *Carcinogenesis*. 2009;30:50–58.

Lan Q, Zhang L, Shen M, et al. Polymorphisms in cytokine and cellular adhesion molecule genes and susceptibility to hematotoxicity among workers exposed to Benzene. *Cancer Res*. 2005;65:9574–9581.

Lane DA, Philippou H, Huntington JA. Directing thrombin. *Blood*. 2005;106:2605–2612.

Larson RA, Wang Y, Banerjee M, et al. Prevalence of the inactivating 609c—>T polymorphism in the Nad(P)H:quinone Oxidoreductase (NQO1) gene in patients with primary and therapy-related myeloid leukemia. *Blood*. 1999;94:803–807.

Laurence AD. Location, movement and survival: the role of chemokines in haematopoiesis and malignancy. *Br J Haematol*. 2006;132:255–267.

Le Beau MM, Albain KS, Larson RA, et al. Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes and acute nonlymphocytic leukemia: further evidence for characteristic abnormalities of chromosomes No. 5 and 7. *J Clin Oncol*. 1986;4:325–345.

Lechner K, Obermeier HL. Cancer-related microangiopathic hemolytic anemia: clinical and laboratory features in 168 reported cases. *Medicine (Baltimore)*. 2012;91:195–205.

Lefrancais E, Ortiz-Munoz G, Caudrillier A, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*. 2017;544:105–109.

Lehman HP, Chen J, Gould AL, et al. An evaluation of computer-aided disproportionality analysis for post-marketing signal detection. *Clin Pharmacol Ther*. 2007;82:173–180.

Levine EG, Bloomfield CD. Leukemias and myelodysplastic syndromes secondary to drug, radiation, and environmental exposure. *Semin Oncol*. 1992;19:47–84.

Li C, Liu Y, Wei S, Zhou Y. A meta-analysis of the association between nqo1 c609t variation and acute myeloid leukemia risk. *Pediatr Blood Cancer*. 2014;61:771–777.

Lian EC. Pathogenesis of thrombotic thrombocytopenic purpura: ADAMTS13 deficiency and beyond. *Semin Thromb Hemost*. 2005;31:625–632.

Liesveld JL, Lichtman MA. Evaluation of granulocytes and mononuclear phagocytes. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997:123–144.

Liles WC, Rodger E, Broxmeyer HE, et al. Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. *Transfusion*. 2005;45:295–300.

Lillevang ST, Albertsen M, Rasmussen F, et al. Effect of radiographic contrast media on granulocyte phagocytosis of Escherichia coli in a whole blood flow cytometric assay. *Invest Radiol*. 1994;29:68–71.

Linet MS, Kim KP, Rajaraman P. Children's exposure to diagnostic medical radiation and cancer risk: epidemiologic and dosimetric considerations. *Pediatr Radiol*. 2009;39(suppl 1):S4–S26.

Liu C, Li B, Cheng Y, et al. Mir-21 plays an important role in radiation induced carcinogenesis in balb/c mice by directly targeting the tumor suppressor gene big-H3. *Int J Biol Sci*. 2011;7:347–363.

Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost*. 2004;2:1082–1095.

Lollar P. Pathogenic antibodies to coagulation factors. Part II. Fibrinogen, prothrombin, thrombin, factor V, factor XI, factor XII, factor XIII, the protein C system and von Willebrand factor. *J Thromb Haemost*. 2005;3:1385–1391.

Look AT. Molecular pathogenesis of MDS. In: Berliner N, Lee SJ, Linenberger M, Vogelsang GB, eds. *American Society of Hematology Education Program Book*. Washington, DC: American Society of Hematology; 2005:156–160.

Lu K, Moeller B, Doyle-Eisele M, McDonald J, Swenberg JA. Molecular dosimetry of N2-Hydroxymethyl-Dg DNA adducts in rats exposed to formaldehyde. *Chem Res Toxicol*. 2011;24:159–161.

Lund JE. Toxicologic effects on blood and bone marrow. In: Feldman BF, Zinkl JG, Jain NC, eds. *Schalm's Veteriary Hematology*. 5th ed. Ames, IA: Blackwell Publishing; 2000:44–50.

Lupescu A, Jilani K, Zelenak C, et al. Hexavalent chromium-induced erythrocyte membrane phospholipid asymmetry. *Biometals*. 2012;25:309–318.

Luzzatto L, Usanga FA, Reddy S. Glucose-6-phosphate dehydrogenase deficient red cells: resistance to infection by malarial parasites. *Science*. 1969;164:839–842.

Lv L, Lin G, Gao X, et al. Case-control study of risk factors of myelodysplastic syndromes according to WHO Classification in a Chinese population. *Am J Hematol*. 2011;86:163–169.

Machado ID, Santin JR, Drewes CC, et al. Alterations in the profile of blood neutrophil membrane receptors caused by in vivo adrenocorticotrophic hormone actions. *Am J Physiol Endocrinol Metab*. 2014;307:E754–E763.

Machado ID, Spatti M, Hastreiter A, et al. Annexin A1 is a physiological modulator of neutrophil maturation and recirculation acting on the Cxcr4/Cxcl12 pathway. *J Cell Physiol*. 2016;231:2418–2427.

Madison FW, Squier T. The etiology of primary granulocytopenia (agranulocytic angina). *J Am Med Assoc*. 1934;102:755–763.

Majeti R, Chao MP, Alizadeh AA, et al. Cd47 Is an Adverse Prognostic Factor and Therapeutic Antibody Target on Human Acute Myeloid Leukemia Stem Cells. *Cell*. 2009;138:286–299.

Mangano DT, Tudor IC, Dietzel C. The risk associated with aprotinin in cardiac surgery. *N Engl J Med*. 2006;354:353–365.

Mannucci PM. Hemostatic drugs. *N Engl J Med*. 1998;339:245–253.

Marini O, Costa S, Bevilacqua D, et al. Mature Cd10+ and immature Cd10- neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood*. 2017;129:1343–1356.

Marjanovic I, Kostic J, Stanic B, et al. Parallel targeted next generation sequencing of childhood and adult acute myeloid leukemia patients reveals uniform genomic profile of the disease. *Tumour Biol*. 2016;37:13391–13401.

Marsh JC. Correlation of hematologic toxicity of antineoplastic agents with their effects on bone marrow stem cells: interspecies studies using an in vivo assay. *Exp Hematol*. 1985;13(suppl 16):16–22.

Martin C, Burdon PC, Bridger G, et al. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity*. 2003;19:583–593.

Mason PJ, Bautista JM, Gilsanz F. G6pd deficiency: the genotype-phenotype association. *Blood Rev*. 2007;21:267–283.

Masubuchi N. Risk assessment of human myelotoxicity of anticancer drugs: a predictive model and the in vitro colony forming unit granulocyte/macrophage (CFU-GM) assay. *Pharmazie*. 2006;61:135–139.

Mauritzson N, Johansson B, Albin M, et al. Survival time in a population-based consecutive series of adult acute myeloid leukemia—the prognostic impact of karyotype during the time period 1976–1993. *Leukemia*. 2000;14:1039–1043.

Maxwell P, Salnikow K. Hif-1: an oxygen and metal responsive transcription factor. *Cancer Biol Ther*. 2004;3:29–35.

May JE, Morse HR, Xu J, Donaldson C. Development of a novel, physiologically relevant cytotoxicity model: application to the study of chemotherapeutic damage to mesenchymal stromal cells. *Toxicol Appl Pharmacol*. 2012;263:374–389.

Mazzone A, Mazzucchelli I, Fossati G, et al. Granulocyte defects and opioid receptors in chronic exposure to heroin or methadone in humans. *Int J Immunopharmacol*. 1994;16:959–967.

McCracken MN, George BM, Kao KS, et al. Normal and neoplastic stem cells. *Cold Spring Harb Symp Quant Biol*. 2016;81:1–9.

McHale CM, Smith MT, Zhang L. Application of transcriptomics in exposed human populations: benzene as an example. In: Waters MD, Thomas RS, eds. *Predictive Carcinogenicity: Current Issues and Future Applications*. Cambridge, U.K.: Royal Society of Chemistry; 2016:352–370.

McHale CM, Zhang L, Hubbard AE, Smith MT. Toxicogenomic profiling of chemically exposed humans in risk assessment. *Mutat Res*. 2010;705:172–183.

McHale CM, Zhang L, Smith MT. Current understanding of the mechanism of benzene-induced leukemia in humans: implications for risk assessment. *Carcinogenesis*. 2012;33:240–252.

McMullin MF, Buckley O, Magill MK, Irvine AE. Long-term bone marrow culture profiles in patients with myelodysplastic syndromes are not explicable by defective apoptosis. *Leuk Res*. 1998;22:735–740.

McRedmond JP, Harriott P, Walker B, et al. Streptokinase-induced platelet activation involves antistreptokinase antibodies and cleavage of protease-activated receptor-1. *Blood*. 2000;95:1301–1308.

Mehdipour P, Santoro F, Minucci S. Epigenetic alterations in acute myeloid leukemias. *FEBS J*. 2015;282:1786–1800.

Menon V, Harrington RA, Hochman JS, et al. Thrombolysis and adjunctive therapy in acute myocardial infarction: the seventh ACCP conference on antithrombotic and thrombolytic therapy. *Chest*. 2004;126:549S–575S.

Metayer C, Zhang L, Wiemels JL, et al. Tobacco smoke exposure and the risk of childhood acute lymphoblastic and myeloid leukemias by cytogenetic subtype. *Cancer Epidemiol Biomarkers Prev*. 2013;22:1600–1611.

Miyagi T, Lam KM, Chuang LF, et al. Suppression of chemokine-induced chemotaxis of monkey neutrophils and monocytes by chlorinated hydrocarbon insecticides. *In Vivo*. 1998;12:441–446.

Moeschlin S, Wagner K. Agranulocytosis due to the occurrence of leukocyte-agglutinin; pyramidon and cold agglutinins. *Acta Haematol*. 1952;8:29–41.

Mohty M, Drillat P, Grouin JM, et al. Addition of plerixafor to G-CSF is useful to achieve efficient collection even in very poor mobilizers: hope for patients with diminished hematopoietic function. *Bone Marrow Transplant*. 2017;52:1049–1050.

Mondrala S, Eastmond DA. Topoisomerase Ii inhibition by the bioactivated benzene metabolite hydroquinone involves multiple mechanisms. *Chem Biol Interact*. 2010;184:259–268.

Monroe DM, Hoffman M. What does it take to make the perfect clot? *Arterioscler Thromb Vasc Biol*. 2005;25:2463–2469.

Moore DM. Hematology of the Rat. In: Feldman BF, Zinkl JG, Jain NC, eds. *Schalm's Veterniary Hematology*. 5th ed. Ames, IA: Blackwell Publishing; 2000a:1210–1218.

Moore DM. Hematology of the mouse. In: Feldman BF, Zinkl JG, Jain NC, eds. *Schalm's Veterniary Hematology*. 5th ed. Ames, IA: Blackwell Publishing; 2000b: 1219–1224.

Moore GS, Calabrese EJ. G6pd-deficiency: a potential high-risk group to copper and chlorite ingestion. *J Environ Pathol Toxicol*. 1980;4:271–279.

Moore MAS. Embryologic and phylogenetic development of the haematopoietic system. *Adv Biosci*. 1975;16:87–103.

Moorman AV, Roman E, Cartwright RA, Morgan GJ. Smoking and the risk of acute myeloid leukaemia in cytogenetic subgroups. *Br J Cancer*. 2002;86:60–62.

Morin Y, Tetu A, Mercier G. Quebec beer-drinkers' cardiomyopathy: clinical and hemodynamic aspects. *Ann N Y Acad Sci*. 1969;156:566–576.

Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014;505:327–334.

Mrozek K, Radmacher MD, Bloomfield CD, Marcucci G. Molecular signatures in acute myeloid leukemia. *Curr Opin Hematol*. 2009;16:64–69.

Mullin AH, Rando R, Esmundo F, Mullin DA. Inhalation of benzene leads to an increase in the mutant frequencies of a laci transgene in lung and spleen tissues of mice. *Mutat Res*. 1995;327:121–129.

Nagasawa T. Cxcl12/Sdf-1 and Cxcr4. *Front Immunol*. 2015;6:301.

Nakagawa M, Terashima T, D'yachkova Y, et al. Glucocorticoid-induced granulocytosis: contribution of marrow release and demargination of intravascular granulocytes. *Circulation*. 1998;98:2307–2313.

Napier I, Ponka P, Richardson DR. Iron trafficking in the mitochondrion: novel pathways revealed by disease. *Blood*. 2005;105:1867–1874.

Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol*. 2006;6:173–182.

Nardini C, Dent J, Tieri P. Editorial: multi-omic data integration. *Front Cell Dev Biol*. 2015;3:46.

Nervi C, Fazi F, Grignani F.Oncoproteins, heterochromatin silencing and micrornas: a new link for leukemogenesis. *Epigenetics*. 2008;3:1–4.

Nifli AP, Notas G, Mamoulaki M, et al. Comparison of a multiplex, bead-based fluorescent assay and immunofluorescence methods for the detection of ANA and ANCA autoantibodies in human serum. *J Immunol Methods*. 2006;311:189–197.

Njalsson R, Norgren S. Physiological and pathological aspects of GSH metabolism. *Acta Paediatr*. 2005;94:132–137.

NRC. *Health Risks from Exposure to Low Levels of Ionizing Radiation*, Beir Committee on the Biological Effects of Ionizing Radiation, National Research Council. Washington, DC: 2006.

Odronic S, Quraishy N, Manroa P, et al. Cocaine-induced microangiopathic hemolytic anemia mimicking idiopathic thrombotic thrombocytopenic purpura: a case report and review of the literature. *J Clin Apher*. 2014;29:284–289.

Pakulska D, Czerczak S. Hazardous effects of arsine: a short review. *Int J Occup Med Environ Health*. 2006;19:36–44.

Palasuwan D, Palasuwan A, Charoensappakit A, Noulsri E. A novel flow cytometry-based method of analyzing heinz bodies. *Int J Lab Hematol*. 2017;39:68–75.

Palmblad J, Papadaki HA, Eliopoulos G. Acute and chronic neutropenias. What is new? *J Intern Med*. 2001;250:476–491.

Pandita A, Harish R, Digra SK, et al. Molecular cytogenetics in childhood acute lymphoblastic leukemia: a hospital-based observational study. *Clin Med Insights Oncol*. 2015;9:39–42.

Paniccia R, Priora R, Liotta AA, Abbate R. Platelet function tests: a comparative review. *Vasc Health Risk Manag*. 2015;11:133–148.

Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic classification and prognosis in acute myeloid leukemia. *N Engl J Med*. 2016;374:2209–2221.

Parchment RE, Gordon M, Grieshaber CK, et al. Predicting hematological toxicity (myelosuppression) of cytotoxic drug therapy from in vitro tests. *Ann Oncol*. 1998;9:357–364.

Parchment RE, Huang M, Erickson-Miller CL. Roles for in vitro myelotoxicity tests in preclinical drug development and clinical trial planning. *Toxicol Pathol*. 1993;21:241–250.

Parchment RE, Murphy MJ. Human hematopoietic stem cells: laboratory assessment and response to toxic injury. In: Sipes IG, McQueen AC, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997: 303–320.

Parent-Massin D, Hymery N, Sibiril Y. Stem cells in myelotoxicity. *Toxicology*. 2010;267:112–117.

Parent-Massin D, Thouvenot D, Rio B, et al. Lindane haematotoxicity confirmed by in vitro tests on human and rat progenitors. *Hum Exp Toxicol*. 1994;13:103–106.

Passegue E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci U S A*. 2003;100(suppl 1):11842–11849.

Patton WN, Duffull SB. Idiosyncratic drug-induced haematological abnormalities. incidence, pathogenesis, management and avoidance. *Drug Saf*. 1994;11:445–462.

Pedersen-Bjergaard J, Andersen MK, Andersen MT, Christiansen DH. Genetics of therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia*. 2008;22:240–248.

Pedersen-Bjergaard J, Christiansen DH, Desta F, Andersen MK. Alternative genetic pathways and cooperating genetic abnormalities in the pathogenesis of therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia*. 2006;20:1943–1949.

Pendleton M, Lindsey RH Jr, Felix CA, et al.. Topoisomerase Ii and leukemia. *Ann N Y Acad Sci*. 2014;1310:98–110.

Perazella MA, Biswas P. Acute hyperkalemia associated with intravenous epsilon-aminocaproic acid therapy. *Am J Kidney Dis*. 1999;33:782–785.

Percy MJ, McFerran NV, Lappin TR. Disorders of oxidised haemoglobin. *Blood Rev*. 2005;19:61–68.

Perrone S, Tataranno ML, Stazzoni G, Del Vecchio A, Buonocore G. Oxidative injury in neonatal erythrocytes. *J Matern Fetal Neonatal Med*. 2012;25:104–108.

Pessina A, Albella B, Bueren J, et al. Prevalidation of a model for predicting acute neutropenia by colony forming unit granulocyte/macrophage (CFU-GM) assay. *Toxicol In Vitro*. 2001;15:729–740.

Pessina A, Albella B, Bayo M, et al. Application of the CFU-GM assay to predict acute drug-induced neutropenia: an international blind trial to validate a prediction model for the maximum tolerated dose (MTD) of myelosuppressive xenobiotics. *Toxicol Sci*. 2003;75:355–367.

Pessina A, Malerba I, Gribaldo L. Hematotoxicity testing by cell clonogenic assay in drug development and preclinical trials. *Curr Pharm Des*. 2005;11:1055–1065.

Peters DC, Noble S. Aprotinin: an update of its pharmacology and therapeutic use in open heart surgery and coronary artery bypass surgery. *Drugs*. 1999;57:233–260.

Peters WP, Holland JF, Senn H, et al. Corticosteroid administration and localized leukocyte mobilization in man. *N Engl J Med*. 1972;286:342–345.

Petrikovics I, Budai M, Kovacs K, Thompson DE. Past, present and future of cyanide antagonism research: from the early remedies to the current therapies. *World J Methodol*. 2015;5:88–100.

Phillips GL. The potential of amifostine in high-dose chemotherapy and autologous hematopoietic stem cell transplantation. *Semin Oncol*. 2002;29:53–56.

Pisciotta AV. Immune and toxic mechanisms in drug-induced agranulocytosis. *Semin Hematol*. 1973;10:279–310.

Pisciotta AV. Response of granulocytes to toxic Injury. In: Sipes IG, McQueen AC, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997:145–158.

Platanias LC. Abnormalities in Th17 cells in aplastic anemia. *Blood*. 2010;116:4039–4040.

Pollack CV Jr, Reilly PA, Eikelboom J, et al. Idarucizumab for dabigatran reversal. *N Engl J Med*. 2015;373:511–520.

Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood*. 1997;89:1–25.

Porro B, Eligini S, Squellerio I, Tremoli E, Cavalca V. The red blood cell: a new key player in cardiovascular homoeostasis? Focus on the nitric oxide pathway. *Biochem Soc Trans*. 2014;42:996–1000.

Porter IH, Boyer SH, Watson-Williams EJ, et al. Variation of glucose-6-phosphate dehydrogenase in different populations. *Lancet*. 1964;1:895–899.

Poyart C, Marden MC, Kister J. Bezafibrate derivatives as potent effectors of hemoglobin. *Methods Enzymol*. 1994;232:496–513.

Poynter JN, Richardson M, Roesler M, et al. Chemical exposures and risk of acute myeloid leukemia and myelodysplastic syndromes in a population-based study. *Int J Cancer*. 2017;140:23–33.

Poynter JN, Richardson M, Roesler M, et al. Chemical exposures and risk of acute myeloid leukemia and myelodysplastic syndromes in a population-based study. *Int J Cancer*. 2017;140:23–33.

Prchal JT. Clinical manifestations and classification of erythrocyte disorders. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006:411–418.

Preston DL, Pierce DA, Shimizu Y, et al. Effect of recent changes in atomic bomb survivor dosimetry on cancer mortality risk estimates. *Radiat Res*. 2004;162:377–389.

Pritsker M, Doniger TT, Kramer LC, Westcot SE, Lemischka IR. Diversification of stem cell molecular repertoire by alternative splicing. *Proc Natl Acad Sci U S A*. 2005;102:14290–14295.

Provost GS, Mirsalis JC, Rogers BJ, Short JM. Mutagenic response to benzene and tris(2,3-dibromopropyl)-phosphate in the lambda laci transgenic mouse mutation assay: a standardized approach to in vivo mutation analysis. *Environ Mol Mutagen*. 1996;28:342–347.

Pusic I, DiPersio JF. Update on clinical experience with Amd3100, an Sdf-1/Cxcl12-Cxcr4 inhibitor, in mobilization of hematopoietic stem and progenitor cells. *Curr Opin Hematol*. 2010;17:319–326.

Qian Z, Joslin JM, Tennant TR, et al. Cytogenetic and genetic pathways in therapy-related acute myeloid leukemia. *Chem Biol Interact*. 2010;184:50–57.

Ray RR, Sarkar NK.Light and scanning electron microscopic studies on chromium-induced anemia in a murine model. *Bull Environ Contam Toxicol*. 2012;88:10–14.

Reagan WJ, Handy V, McKamey A, et al. Effects of doxorubicin on the canine erythroid and myeloid progenitor cells and bone marrow microenvironment. *Comp Haematol Int*. 1993;3:96–101.

Reagan WJ, Irizarry-Rovira A, Poitout-Belissent F, et al. Best practices for evaluation of bone marrow in nonclinical toxicity studies. *Toxicol Pathol*. 2011;39:435–448.

Regazzoni LG, Grigoryan H, Ji Z, et al. Using lysine adducts of human serum albumin to investigate the disposition of exogenous formaldehyde in human blood. *Toxicol Lett*. 2017;268:26–35.

Rehman SU. Effect of lead on lipid peroxidation, phospholipids composition, and methylation in erythrocyte of human. *Biol Trace Elem Res*. 2013;154:433–439.

Reinisch A, Chan SM, Thomas D, Majeti R. Biology and clinical relevance of acute myeloid leukemia stem cells. *Semin Hematol*. 2015;52:150–164.

Ricci-Vitiani L, Pallini R, Biffoni M, et al. Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. *Nature*. 2010;468:824–828.

Rich IN, Hall KM. Validation and development of a predictive paradigm for hemotoxicology using a multifunctional bioluminescence colony-forming proliferation assay. *Toxicol Sci*. 2005;87:427–441.

Richardson C, Jasin M. Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature*. 2000;405:697–700.

Rinsky RA, Young RJ, Smith AB. Leukemia in benzene workers. *American Journal of Industrial Medicine*. 1981;2:217–245.

Ristoff E, Larsson A. Inborn errors in the metabolism of glutathione. *Orphanet J Rare Dis*. 2007;2:16.

Roda E, Coccini T, Acerbi D, Castoldi AF, Manzo L. Comparative in vitro and ex-vivo myelotoxicity of aflatoxins b1 and m1 on haematopoietic progenitors (BFU-E, CFU-E, and CFU-GM): species-related susceptibility. *Toxicol In Vitro*. 2010;24:217–223.

Roderique JD, Josef CS, Feldman MJ, Spiess BD. A modern literature review of carbon monoxide poisoning theories, therapies, and potential targets for therapy advancement. *Toxicology*. 2015;334:45–58.

Rojas JC, Aguilar B, Rodriguez-Maldonado E, et al. Pharmacogenetics of oral anticoagulants. *Blood Coagul Fibrinolysis*. 2005;16:389–398.

Rollison DE, Howlader N, Smith MT, et al. Epidemiology of myelodysplastic syndromes and chronic myeloproliferative disorders in the united states, 2001–2004, using data from the naaccr and seer programs. *Blood*. 2008;112:45–52.

Ron E. Ionizing radiation and cancer risk: evidence from epidemiology. *Radiat Res*. 1998;150:S30–S41.

Ron E. Cancer Risks from medical radiation. *Health Phys*. 2003;85:47–59.

Ron E, Preston DL, Mabuchi K, Thompson DE, Soda M. Cancer incidence in atomic bomb survivors. part iv: comparison of cancer incidence and mortality. *Radiat Res*. 1994;137:S98–S112.

Rossi G, Pelizzari AM, Bellotti D, et al. Cytogenetic analogy between myelodysplastic syndrome and acute myeloid leukemia of elderly patients. *Leukemia*. 2000;14:636–641.

Rossi G, Pelizzari AM, Bellotti D, Tonelli M, Barlati S. Cytogenetic analogy between myelodysplastic syndrome and acute myeloid leukemia of elderly patients. *Leukemia*. 2000;14:636–641.

Rothman N, Haas R, Hayes RB, et al. Benzene induces gene-duplicating but not gene-inactivating mutations at the glycophorin a locus in exposed humans. *Proc Natl Acad Sci U S A*. 1995;92:4069–4073.

Rothman N, Smith MT, Hayes RB, et al. Benzene poisoning, a risk factor for hematological malignancy, is associated with the NQO1 609c—>T mutation and rapid fractional excretion of chlorzoxazone. *Cancer Res*. 1997;57:2839–2842.

Roujeau JC. Clinical heterogeneity of drug hypersensitivity. *Toxicology*. 2005;209:123–129.

Ryan DH. Examination of the blood. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006:11–19.

Ryter SW, Otterbein LE. Carbon monoxide in biology and medicine. *Bioessays*. 2004;26:270–280.

Salama A, Schutz B, Kiefel V, Breithaupt H, Mueller-Eckhardt C. Immune-mediated agranulocytosis related to drugs and their metabolites: mode of sensitization and heterogeneity of antibodies. *Br J Haematol*. 1989;72:127–132.

Salama A, Schutz B, Kiefel V, et al. Immune-mediated agranulocytosis related to drugs and their metabolites: mode of sensitization and heterogeneity of antibodies. *Br J Haematol*. 1989;72:127–132.

Saleem M, Yusoff NM. Fusion genes in malignant neoplastic disorders of haematopoietic system. *Hematology*. 2016;21:501–512.

Sammett D, Lee EW, Kocsis JJ, Snyder R. Partial hepatectomy reduces both metabolism and toxicity of benzene. *J Toxicol Environ Health*. 1979;5:785–792.

Santesson GG. Uber chronische vergiftungen mit steinkohlen benzin. Vier todes falle. *Archives of Hygiene*. 1897;31:336–376.

Sato K, Miyakawa M, Han DC, et al. Graves' disease with neutropenia and marked splenomegaly: autoimmune neutropenia due to propylthiouracil. *J Endocrinol Invest*. 1985;8:551–555.

Savino F, Maccario S, Guidi C, et al. Methemoglobinemia caused by the ingestion of courgette soup given in order to resolve constipation in two formula-fed infants. *Ann Nutr Metab*. 2006;50:368–371.

Sawyers CL, Denny CT, Witte ON. Leukemia and the disruption of normal hematopoiesis. *Cell*. 1991;64:337–350.

Scapini P, Marini O, Tecchio C, Cassatella MA. Human neutrophils in the saga of cellular heterogeneity: insights and open questions. *Immunol Rev*. 2016;273:48–60.

Scelo G, Metayer C, Zhang L, et al. Household exposure to paint and petroleum solvents, chromosomal translocations, and the risk of childhood leukemia. *Environ Health Perspect*. 2009;117:133–139.

Schmaier AH, Rojkjaer R, Shariat-Madar Z. Activation of the plasma kallikrein/kinin system on cells: a revised hypothesis. *Thromb Haemost*. 1999;82:226–233.

Schnatter AR, Glass DC, Tang G, Irons RD, Rushton L. Myelodysplastic syndrome and benzene exposure among petroleum workers: an international pooled analysis. *J Natl Cancer Inst*. 2012;104:1724–1737.

Schrijvers D, Highley M, De Bruyn E, Van Oosterom AT, Vermorken JB. Role of red blood cells in pharmacokinetics of chemotherapeutic agents. *Anticancer Drugs*. 1999;10:147–153.

Schuettpelz LG, Borgerding JN, Christopher MJ, et al. G-CSF regulates hematopoietic stem cell activity, in part, through activation of toll-like receptor signaling. *Leukemia*. 2014;28:1851–1860.

Schultz W. About specific sore throat. *Dtsch Med Wochenschr*. 1922;48:1495–1496.

Sedrakyan A, Atkins D, Treasure T. The risk of aprotinin: a conflict of evidence. *Lancet*. 2006;367:1376–1377.
Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med*. 2010;2:640–653.
Sella R, Flomenbilt L, Goldstein I, et al. Detection of anti-neutrophil antibodies in autoimmune neutropenia of infancy: a multicenter study. *Isr Med Assoc J*. 2010;12:91–96.
Selling L. Benzol as a leucotoxin. studies on the degeneration and regeneration of the blood and haematopoietic organs. *Johns Hopkins Hosp Rep*. 1916;17:83–148.
Semerad CL, Liu F, Gregory AD, et al. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity*. 2002;17:413–423.
Shafat MS, Gnaneswaran B, Bowles KM, Rushworth SA. The bone marrow microenvironment—home of the leukemic blasts. *Blood Rev*. 2017;31:277–286
Shamriz O, Cohen-Glickman I, Reif S, Shteyer E. methemoglobinemia induced by lidocaine-prilocaine cream. *Isr Med Assoc J*. 2014;16:250–254.
Sharma M, Afrin F, Satija N, et al. Stromal-derived factor-1/CXCR4 signaling: indispensable role in homing and engraftment of hematopoietic stem cells in bone marrow. *Stem Cells Dev*. 2011;20(6):933–946.
Shen M, Zhang L, Lee KM, et al. Polymorphisms in genes involved in innate immunity and susceptibility to benzene-induced hematotoxicity. *Exp Mol Med*. 2011;43:374–378.
Shifrine M, Wilson FD. *The Canine as a Biomedical Research Model: Immunological, Hematological and Oncological Aspects*. Springfield: US Department of Commerce Information Center; 1980.
Shih AH, Chung SS, Dolezal EK, et al. Mutational analysis of therapy-related myelodysplastic syndromes and acute myelogenous leukemia. *Haematologica*. 2013;98:908–912.
Shin SY, Lee ST, Kim HJ, et al. Mutation profiling of 19 candidate genes in acute myeloid leukemia suggests significance of Dnmt3a mutations. *Oncotarget*. 2016;7:54825–54837.
Shivarov V, Bullinger L. Expression profiling of leukemia patients: key lessons and future directions. *Exp Hematol*. 2014;42:651–660.
Siebert WJ, Ayres RW, Bulling MT, et al. Streptokinase morbidity—more common than previously recognised. *Aust N Z J Med*. 1992;22:129–133.
Sill H, Olipitz W, Zebisch A, Schulz E, Wolfler A. Therapy-related myeloid neoplasms: pathobiology and clinical characteristics. *Br J Pharmacol*. 2011;162:792–805.
Smith MT. Advances in understanding benzene health effects and susceptibility. *Annu Rev Public Health*. 2010;31:133–148, 132 p following 148.
Smith MT, Zhang L, Wang Y, et al. Increased translocations and aneusomy in chromosomes 8 and 21 among workers exposed to benzene. *Cancer Res*. 1998;58:2176–2181.
Snyder C. Experimental benzene toxicity. In: Aksoy M, ed. *Benzene Carcinogenesis*. Boca Raton, FL: CRC Press; 1988:30–32.
Snyder R. Leukemia and benzene. *Int J Environ Res Public Health*. 2012;9:2875–2893.
Snyder R, Hedli CC. An overview of benzene metabolism. *Environ Health Perspect*. 1996;104(suppl 6):1165–1171.
Soylu Ustkoyuncu P, Mutlu FT, Kiraz A, Tag Balkis Z, Yel S. A rare cause of neonatal hemolytic anemia: glutathione synthetase deficiency. *J Pediatr Hematol Oncol*. 2017;40:e45–e49.
Spector LG, Xie Y, Robison LL, et al. Maternal diet and infant leukemia: the DNA topoisomerase ii inhibitor hypothesis: a report from the children's oncology group. *Cancer Epidemiol Biomarkers Prev*. 2005;14:651–655.
Srivastava A, Evans KJ, Sexton AE, Schofield L, Creek DJ. Metabolomics-based elucidation of active metabolic pathways in erythrocytes and HSC-derived reticulocytes. *J Proteome Res*. 2017;16:1492–1505.
Stafford DW. The vitamin K cycle. *J Thromb Haemost*. 2005;3:1873–1878.
Steinberg MH. Pathophysiologically based drug treatment of sickle cell disease. *Trends Pharmacol Sci*. 2006;27:204–210.
Stern SC, Shah S, Costello C. Probable autoimmune neutropenia induced by fludarabine treatment for chronic lymphocytic leukaemia. *Br J Haematol*. 1999;106:836–837.
Strom SS, Gu Y, Gruschkus SK, Pierce SA, Estey EH. Risk factors of myelodysplastic syndromes: a case-control study. *Leukemia*. 2005;19:1912–1918.
Struttmann T, Scheerer A, Prince TS, Goldstein LA. Unintentional carbon monoxide poisoning from an unlikely source. *J Am Board Fam Pract*. 1998;11:481–484.
Summers C, Rankin SM, Condliffe AM, et al. Neutrophil kinetics in health and disease. *Trends Immunol*. 2010;31:318–324.
Sundman-Engberg B, Tidefelt U, Paul C. Toxicity of cytostatic drugs to normal bone marrow cells in vitro. *Cancer Chemother Pharmacol*. 1998;42:17–23.
Suwalsky M, Villena F, Norris B, et al. Effects of lead on the human erythrocyte membrane and molecular models. *J Inorg Biochem*. 2003;97:308–313.
Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127:2375–2390.
Szarfman A, Machado SG, O'Neill RT. Use of Screening algorithms and computer systems to efficiently signal higher-than-expected combinations of drugs and events in the US FDA's spontaneous reports database. *Drug Saf*. 2002;25:381–392.
Szarfman A, Talarick L, Levine JG. Analysis and risk assessment of hematological data from clinical trials. In: Sipes IG, McQueen AC, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997: 363–379.
Tamaoki J. The effects of macrolides on inflammatory cells. *Chest*. 2004;125:41S–50S.
Tannock IF. Experimental chemotherapy and concepts related to the cell cycle. *Int J Radiat Biol Relat Stud Phys Chem Med*. 1986;49:335–355.
Tavazzi B, Di PD, Amorini AM, et al. Energy metabolism and lipid peroxidation of human erythrocytes as a function of increased oxidative stress. *Eur J Biochem*. 2000;267:684–689.
Taylor J, Xiao W, Abdel-Wahab O.Diagnosis and classification of hematologic malignancies on the basis of genetics. *Blood*. 2017;130:410–423.
Teppo S, Laukkanen S, Liuksiala T, et al. Genome-wide repression of erna and target gene loci by the etv6-runx1 fusion in acute leukemia. *Genome Res*. 2016;26:1468–1477.
Tesfa D, Keisu M, Palmblad J. Idiosyncratic drug-induced agranulocytosis: possible mechanisms and management. *Am J Hematol*. 2009;84:428–434.
Thomas R, Phuong J, McHale CM, Zhang L. Using bioinformatic approaches to identify pathways targeted by human leukemogens. *Int J Environ Res Public Health*. 2012;9:2479–2503.
Threadgill DW, Miller DR, Churchill GA, de Villena FP. The collaborative cross: a recombinant inbred mouse population for the systems genetic era. *ILAR J*. 2011;52:24–31.
Tran AN, Koo JY. Risk of systemic toxicity with topical lidocaine/prilocaine: a review. *J Drugs Dermatol*. 2014;13:1118–1122.
Turbay D, Lieberman J, Alper CA, et al. Tumor necrosis factor constellation polymorphism and clozapine-induced agranulocytosis in two different ethnic groups. *Blood*. 1997;89:4167–4174.
U.S. EPA. *Lymphohematopoietic Cancers Induced by Chemicals and Other Agents: Overview and Implications for Risk Assessment (Final Report)*. Washington, DC: U.S. Environmental Protection Agency; 2012.
Uetrecht J. Drug metabolism by leukocytes and its role in drug-induced lupus and other idiosyncratic drug reactions. *Crit Rev Toxicol*. 1990;20:213–235.
UNSCEAR. *Sources and Effects of Ionizing Radiation, Vol. 1, Report of the United Nations Scientific Committee on the Effects of Atomic Radiation to the General Assembly and Annexes*. 2000a.
UNSCEAR. *Sources and Effects of Ionizing Radiation, Vol. 2, Report of the United Nations Scientific Committee on the Effects of Atomic Radiation to the General Assembly and Annexes*. 2000b.
Valentine JL, Lee SS, Seaton MJ, et al. Reduction of benzene metabolism and toxicity in mice that lack Cyp2e1 expression. *Toxicol Appl Pharmacol*. 1996;141:205–213.
Valli VE, McGrath JP. Comparative leukocyte biology and toxicology. In: Sipes IG, McQueen AC, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997:201–215.
van Breda SG, Wilms LC, Gaj S, et al. The exposome concept in a human nutrigenomics study: evaluating the impact of exposure to a complex mixture of phytochemicals using transcriptomics signatures. *Mutagenesis*. 2015;30:723–731.
Van Wijk R, van Solinge WW. The energy-less blood cell is lost: erythrocyte enzyme abnormalities of glycolysis. *Blood*. 2005;106:4034–4042.

Vandendries ER, Drews RE. Drug-associated disease: hematologic dysfunction. *Crit Care Clin*. 2006;22:347–355.

Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood*. 2002;100:2292–2302.

Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (Who) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–951.

Varga J, Uitto J, Jimenez SA. The cause and pathogenesis of the eosinophilia—myalgia syndrome. *Ann Intern Med*. 1992;116:140–147.

Vermeulen L, De Sousa F, Melo E, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol*. 2010;12:468–476.

Veyradier A, Meyer D. Thrombotic thrombocytopenic purpura and its diagnosis. *J Thromb Haemost*. 2005;3:2420–2427.

Villa CH, Anselmo AC, Mitragotri S, Muzykantov V. Red blood cells: supercarriers for drugs, biologicals, and nanoparticles and inspiration for advanced delivery systems. *Adv Drug Deliv Rev*. 2016;106:88–103.

Voog E, Morschhauser F, Solal-Celigny P. Neutropenia in patients treated with rituximab. *N Engl J Med*. 2003;348:2691–2694.

Ward JB Jr, Ammenheuser MM, Ramanujam VM, et al. The Mutagenic effects of low level sub-acute inhalation exposure to benzene in Cd-1 mice. *Mutat Res*. 1992;268:49–57.

Warkentin TE, Greinacher A. Heparin-induced thrombocytopenia: recognition, treatment, and prevention: the seventh ACCP conference on antithrombotic and thrombolytic therapy. *Chest*. 2004;126:311S–337S.

Watson J, Taylor WR, Menard D, Kheng S, White NJ. Modelling primaquine-induced haemolysis in G6pd deficiency. *Elife*. 2017;6.

Weatherall D. Disorders of globin synthesis in the thalassemias. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*. 7th ed. New York: McGraw-Hill; 2006:663–666.

Wei C, Wen H, Yuan L, et al. Formaldehyde induces toxicity in mouse bone marrow and hematopoietic stem/progenitor cells and enhances benzene-induced adverse effects. *Arch Toxicol*. 2017;91:921–933.

Wei H, Zhang J, Tan K, et al. Benzene-induced aberrant mirna expression profile in hematopoietic progenitor cells in C57bl/6 mice. *Int J Mol Sci*. 2015;16:27058–27071.

Wei S, Wang K. Long noncoding RNAs: pivotal regulators in acute myeloid leukemia. *Exp Hematol Oncol*. 2015;5:30.

Weingand K, Brown G, Hall R, et al. Harmonization of animal clinical pathology testing in toxicity and safety studies. *Fundam Appl Toxicol*. 1996;29:198–201.

Whitehead TP, Metayer C, Wiemels JL, Singer AW, Miller MD. Childhood leukemia and primary prevention. *Curr Probl Pediatr Adolesc Health Care*. 2016;46:317–352.

WHO. *Pathology and Genetics: Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2001.

WHO. *Who Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon, France: IARC Press; 2008.

WHO. *World Health Organization Handbook for Reporting Results of Cancer Treatments*. Offset Publication No. 48. Geneva: WHO; 1979.

Wiemels J. Chromosomal translocations in childhood leukemia: natural history, mechanisms, and epidemiology. *J Natl Cancer Inst Monogr*. 2008:87–90.

Wiemels J. Perspectives on the causes of childhood leukemia. *Chem Biol Interact*. 2012;196:59–67.

Williams DP, Pirmohamed M, Naisbitt DJ, et al. Induction of metabolism-dependent and -independent neutrophil apoptosis by clozapine. *Mol Pharmacol*. 2000;58:207–216.

Winterbourn CC. Inhibition of autoxidation of divicine and isouramil by the combination of superoxide dismutase and reduced glutathione. *Arch Biochem Biophys*. 1989;271:447–455.

Winterbourn CC, Cowden WB, Sutton HC. Auto-oxidation of dialuric acid, divicine and isouramil. Superoxide dependent and independent mechanisms. *Biochem Pharmacol*. 1989;38:611–618.

Wirths S, Bugl S, Kopp HG. Neutrophil homeostasis and its regulation by danger signaling. *Blood*. 2014;123:3563–3566.

Wong TN, Ramsingh G, Young AL, et al. Role of Tp53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature*. 2015;518:552–555.

Woo HS, Lee KH, Yoon PH, et al. Oxaliplatin-induced immune-mediated thrombocytopenia: a case report. *Cancer Res Treat*. 2015;47:949–953.

Xu Y, Shan W, Li X, et al. A synthetic three-dimensional niche system facilitates generation of functional hematopoietic cells from human-induced pluripotent stem cells. *J Hematol Oncol*. 2016;9:102.

Yadav NK, Shukla P, Omer A, et al.. Alternative methods in toxicology: CFU assays application, limitation and future prospective. *Drug Chem Toxicol*. 2016;39:1–12.

Yang CC, Wu ML, Deng JF. Prolonged hemolysis and methemoglobinemia following organic copper fungicide ingestion. *Vet Hum Toxicol*. 2004;46:321–323.

Yang JJ, Park TS, Wan TS. Recurrent cytogenetic abnormalities in acute myeloid leukemia. *Methods Mol Biol*. 2017;1541:223–245.

Yeh CH, Moles R, Nicot C. Clinical significance of micrornas in chronic and acute human leukemia. *Mol Cancer*. 2016;15:37.

Yenson PR, Forrest D, Schmiegelow K, et al.. Azathioprine-associated acute myeloid leukemia in a patient with Crohn's disease and thiopurine s-methyltransferase deficiency. *Am J Hematol*. 2008;83:80–83.

Yohe S. Molecular genetic markers in acute myeloid leukemia. *J Clin Med*. 2015;4:460–478.

Yoo D, Lessin LS. Drug-associated "bite cell" hemolytic anemia. *Am J Med*. 1992;92:243–248.

Young AL, Wong TN, Hughes AE, et al. Quantifying ultra-rare pre-leukemic clones via targeted error-corrected sequencing. *Leukemia*. 2015;29:1608–1611.

Young KM, Weiss L. Hematopoiesis: structure–function relationships in bone marrow and spleen. In: Sipes IG, McQueen AC, Gandolfi AJ, eds. *Comprehensive Toxicology*. Oxford: Pergamon Press; 1997:11–34.

Young NS. Acquired aplastic anemia. *JAMA*. 1999;282:271–278.

Young NS. Hematopoietic cell destruction by immune mechanisms in acquired aplastic anemia. *Semin Hematol*. 2000;37:14.

Yunis JJ, Corzo D, Salazar M, et al. HLA associations in clozapine-induced agranulocytosis. *Blood*. 1995;86:1177–1183.

Zahid MF, Parnes A, Savani BN, et al.. Therapy-related myeloid neoplasms—what have we learned so far? *World J Stem Cells*. 2016;8:231–242.

Zeng Y, Katsanis E. The complex pathophysiology of acquired aplastic anaemia. *Clin Exp Immunol*. 2015;180:361–370.

Zhang L, Eastmond DA, Smith MT. The nature of chromosomal aberrations detected in humans exposed to benzene. *Crit Rev Toxicol*. 2002;32:1–42.

Zhang L, Freeman LE, Nakamura J, et al. Formaldehyde and leukemia: epidemiology, potential mechanisms, and implications for risk assessment. *Environ Mol Mutagen*. 2010a;51:181–191.

Zhang L, Lan Q, Ji Z, et al. Leukemia-related chromosomal loss detected in hematopoietic progenitor cells of benzene-exposed workers. *Leukemia*. 2012;26:2494–2498.

Zhang L, McHale CM, Rothman N, et al. Systems biology of human benzene exposure. *Chem Biol Interact*. 2010b;184:86–93.

Zhang L, Rothman N, Wang Y, et al. Increased aneusomy and long arm deletion of chromosomes 5 and 7 in the lymphocytes of chinese workers exposed to benzene. *Carcinogenesis*. 1998;19:1955–1961.

Zhang L, Rothman N, Wang Y, et al. Benzene increases aneuploidy in the lymphocytes of exposed workers: a comparison of data obtained by fluorescence in situ hybridization in interphase and metaphase cells. *Environ Mol Mutagen*. 1999;34:260–268.

Zhang L, Steinmaus C, Eastmond DA, Xin XK, Smith MT. Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms. *Mutat Res*. 2009;681:150–168.

Zhang L, Tang X, Rothman N, et al. Occupational exposure to formaldehyde, hematotoxicity, and leukemia-specific chromosome changes in cultured myeloid progenitor cells. *Cancer Epidemiol, Biomarkers Prev*. 2010c;19:80–88.

Zhang X, Liu F, Chen X, et al. Involvement of the immune system in idiosyncratic drug reactions. *Drug Metab Pharmacokinet*. 2011;26(1):47–59.

Zhang Y, McHale CM, Liu X, et al. Data on megakaryocytes in the bone marrow of mice exposed to formaldehyde. *Data Brief*. 2016;6:948–952.

Zheng S, Ma X, Zhang L, et al. Hypermethylation of the 5′ cpg island of the *FHIT* gene is associated with hyperdiploid and translocation-negative subtypes of pediatric leukemia. *Cancer Res*. 2004;64:2000–2006.

chapter 12

Toxic Responses of the Immune System

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Immunotoxicity of Biologics

New Frontiers and Challenges in Immunotoxicology

Acknowledgments

References

INTRODUCTION

Immunotoxicology can be most simply defined as the study of adverse effects on the immune system resulting from occupational, inadvertent, or therapeutic exposure to drugs, environmental chemicals, and, in some instances, biological materials. Studies in animals and humans have indicated that the immune system is comprised of potential target organs, and that damage to this system can be associated with morbidity and even mortality. Indeed, in some instances, the immune system can be compromised (decreased lymphoid cellularity, alterations in lymphocyte subpopulations, decreased host resistance, altered specific immune function responses) in the absence of observed toxicity in other organ systems. These studies coupled with tremendous advances made in immunology and molecular biology have led to a steady and exponential growth in our understanding of immunotoxicology during the past 40 years. Recognition by regulatory agencies that the immune system is an important, as well as sensitive, target organ for chemical- and drug-induced toxicity is another indication of the growth of this subdiscipline of toxicology. With the availability of sensitive, reproducible, and predictive tests, it is now apparent that the inclusion of immunotoxicity testing represents a significant adjunct to routine safety evaluations for therapeutics, biologics, and chemicals now in development.

Understanding the impact of toxic responses on the immune system requires an appreciation of its role, which may be stated succinctly as the preservation of integrity. It is a series of delicately balanced, complex, multicellular, and physiologic mechanisms that allow an individual to distinguish foreign material (i.e., "nonself") from "self," and to neutralize, eliminate, and/or coexist with the foreign matter. One definition of nonself is essentially anything other than that encoded in one's own germline genome (Nathan, 2006). Examples of self are all the tissues, organs, and cells of the body. Examples of nonself include a variety of opportunistic pathogens, such as bacteria and viruses, and transformed cells or tissues (i.e., tumors). Nonself might also include drugs, xenografts, or allergens, such as pollen. The immune system is characterized by a virtually infinite repertoire of specificities, highly specialized effectors, complex regulatory mechanisms, and an ability to travel throughout the body. The great complexity of the mammalian immune system is an indication of the importance, as well as the difficulty, of its role. If the immune system fails to recognize as nonself an infectious entity or neoantigens expressed by a newly arisen tumor, then the host is in danger of rapidly succumbing to the unopposed invasion. This aspect of immune competence is the reason why the immune system is often synonymous with "host defense." Alternatively, if some integral bodily tissue is not identified as self, then the immune system is capable of turning its considerable defensive capabilities against that tissue, and elicitation of autoimmune disease may ensue. This aspect of immune competence emphasizes the tremendous destructive potential that is associated with the host defense mechanisms of the immune system. The cost to the host of these mistakes, made in either direction, may be quite high. The fact that mistakes can occur in either direction is an indication that immunotoxicology should be considered as a continuum (Fig. 12-1). At the center of the concept of the continuum is the recognition that immune responses in the normal human population can vary by more than two standard deviations (Luebke et al., 2004). Because the cost of mistakes in immune competence can be so high, and because of the tremendous diversity involved in the identification of self versus nonself, a complex array of organs, cells, soluble factors, and their interactions has evolved to regulate this system and minimize the frequency of errors in either direction. Due to the potentially profound effects resulting from disruption of the delicately balanced immune system, there is a need to understand the cellular, biochemical, and molecular mechanisms of xenobiotic-induced immune modulation.

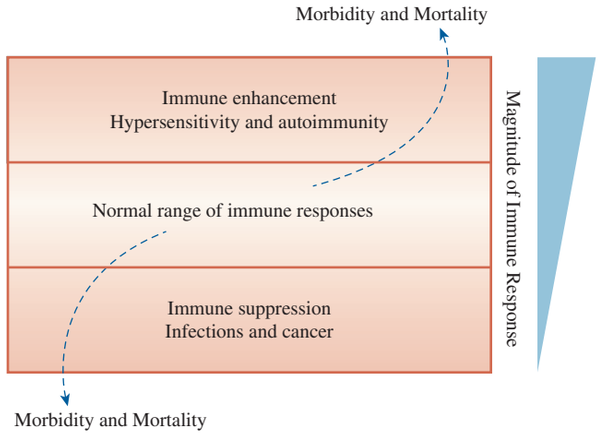

Figure 12-1. *The continuum of immunotoxicology.* Immune toxicity results from xenobiotic-induced suppression or enhancement of immune function.

This chapter provides (1) an overview of basic concepts in immunology (structure, components, and functions), which are important to the understanding of the impact xenobiotics may have on the exposed individual; (2) a summary of selected current methods utilized to assess immune function; and (3) a brief review of current information on the immune modulation (immune suppression, immune enhancement, hypersensitivity, and autoimmunity) induced by a variety of xenobiotics. This chapter is not meant to be an immunology textbook, nor an exhaustive review of the mechanisms of immunotoxicity of a myriad of xenobiotics. For detailed information on immunology, the reader is referred to three texts: the first edited by Paul, *Fundamental Immunology* (7th edition, 2013), the second written by Delves, Martin, Burton, and Roitt, *Roitt's Essential Immunology* (13th edition, 2017), and the third written by Murphy and Weaver, *Janeway's Immunobiology* (9th edition, 2016). For a more comprehensive review of immunotoxicology, the reader is referred to three texts: the first edited by Vohr and colleagues, *Encyclopedic Reference of Immunotoxicology* (2nd edition, 2016), the second edited by House and Luebke, *Immunotoxicology and Immunopharmacology, Target Organ Toxicity Series* (3rd edition, 2007), and the third edited by Corsini and Van Loveren, *Molecular Immunotoxicology* (1st edition, 2014). The reader might also find the list of abbreviations in Table 12-1 helpful.

THE IMMUNE SYSTEM

Unlike most organ systems, the immune system has the unique quality of not being confined to a single site within the body. It comprises numerous lymphoid organs (Table 12-2) and many different cellular populations and soluble factors with a variety of functions focused on providing immunity against nonself or exogenous substances (i.e., antigens). Immunity can be classified into two functional divisions: innate immunity and acquired (adaptive) immunity (Table 12-3). Innate immunity has historically been characterized as a first-line defense response with little immunological memory. Therefore, in healthy adults, the magnitude of the innate immune response to a foreign organism is similar for a secondary

Table 12-1

Abbreviations

TERM	ABBREVIATION	TERM	ABBREVIATION
Antibody-dependent cell-mediated cytotoxicity	ADCC	Lipopolysaccharide	LPS
Antibody forming cell	AFC	Local lymph node assay	LLNA
Antigen presenting cell	APC	Major histocompatibility complex	MHC
Antibody secreting cell	ASC	Membrane attack complex	MAC
Aryl hydrocarbon receptor	AhR	Mixed lymphocyte response	MLR
B-cell receptor	BCR	Neutrophil extracellular trap	NET
B-regulatory cell	Breg	National Toxicology Program	NTP
Carboxyfluorescein succinimidyl ester	CFSE	Natural killer cell	NK
CD40 ligand	CD40L	Natural T regulatory cell	nTreg
Cell-mediated immunity	CMI	NOD-like receptors	NLR
Central nervous system	CNS	Non-human primates	NHP
Cluster of differentiation	CD	Nuclear factor of activated T cells	NFAT
Colony stimulating factor	CSF	Nuclear factor κB	NF-κB
Common lymphoid progenitor	CLP	Pathogen-associated molecular patterns	PAMPs
Common myeloid progenitor	CMP	Pattern recognition receptors	PRR
Concanavalin-A	Con-A	Peripheral blood mononuclear cell	PBMC
Conventional dendritic cell	cDC	Phytohemagglutinin	PHA
Cyclooxygenase	COX	Plaque forming cell	PFC
Cytotoxic T lymphocyte	CTL	Plasmacytoid dendritic cell	pDC
Danger-associated molecular patterns	DAMPs	Polymerase chain reaction	PCR
Delayed-type hypersensitivity	DTH	Pre/postnatal development	PPND
Dendritic cell	DC	Reactive oxygen species	ROS
Developmental and reproductive toxicology	DART	RIG-like receptors	RLR
Developmental immunotoxicology	DIT	Reverse transcriptase	RT
Embryo fetal development	EFD	Sheep red blood cells	sRBCs
Experimental autoimmune encephalomyelitis	EAE	Single nucleotide polymorphism	SNP
Fc receptor	FcR	Systemic lupus erythematosus	SLE
Hematopoietic stem cell	HSC	T-cell-dependent antibody response	TDAR
Human lymphocyte activation	HuLa	T-cell-independent antibody response	TIAR
Human peripheral blood	HPB	T-cell receptor	TCR
Immunoglobulin	Ig	T-helper cell	Th
Interferon	IFN	Toll-like receptor	TLR
Inducible T regulatory cell	iTreg	T-regulatory cell	Treg
Innate lymphoid cell	ILC	Transforming growth factor	TGF
Interleukin	IL	Tumor necrosis factor	TNF
Keyhole limpet hemocyanin	KLH		

Table 12-2

Organization of the Immune System: Lymphoid Tissue

CLASSIFICATION	LYMPHOID ORGANS
Primary	Bone marrow Thymus
Secondary	Spleen Lymph nodes Peyer's patches Skin-associated lymphoid tissue (SALT) Mucosal-associated lymphoid tissue (MALT) Gut-associated lymphoid tissue (GALT) Bronchial-associated lymphoid tissue (BALT) Nasal-associated lymphoid tissue (NALT)
Tertiary	Cells lining the genitourinary tract Lymphoid neogenesis in nonlymphoid organs

or a tertiary challenge as it is for the primary exposure. By contrast, acquired (adaptive) immunity is characterized by antigen specificity and immunological memory. Thus, in healthy adults, the speed and magnitude of the acquired immune response to a foreign organism is greater for a secondary challenge than it is for the primary challenge.

All immune cells originate from the bone marrow, which is considered a primary lymphoid organ. The bone marrow contains a microenvironment capable of supporting immune cell production. As such the bone marrow is the site of origin of the pluripotent and self-renewing hematopoietic stem cell (HSC) from which all other hematopoietic cells are derived. While immunologists differ on how to define and identify the hematopoietic intermediates, Fig. 12-2 shows one model of hematopoiesis (adapted from Baltimore et al. [2008] and Chaplin [2010]). During gestation, the HSC is found in the embryonic yolk sac and fetal liver; eventually, it migrates to the bone marrow. Within the bone marrow, the HSC developmentally commits to either the lymphoid or myeloid lineages by giving rise to the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP), which differentiate into various types of hematopoietic cells that mediate innate and adaptive immunity.

Table 12-3

Innate versus Adaptive Immunity

CHARACTERISTIC	INNATE IMMUNITY	ACQUIRED IMMUNITY
Cells involved	Neutrophils Macrophages NK/NKT cells DC TCRγδ	T cells B cells Macrophages DC
Primary soluble mediators	Complement Perforin/Granzyme Acute-phase proteins IFN-α/β Other cytokines	Antibody Cytokines Perforin/Granzyme
Specificity of response	Limited	Very high specificity
Receptors	TLR complement receptors NLR FcR TCRγδ	TCRαβ TCRγδ BCR
Response enhanced by repeated antigen challenge	No	Yes

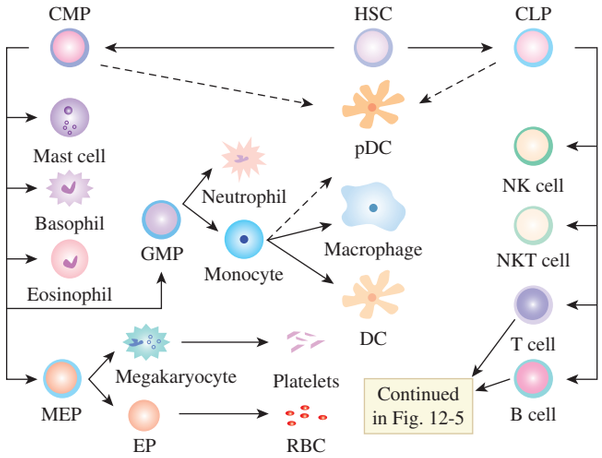

Figure 12-2. *Development of the cellular components of the immune system.* All immune cells initially develop in the bone marrow. The HSC differentiates into two main progenitors from which all other cells develop. The progenitor for pDCs is still unclear (dashed lines). CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; EP, erythrocyte precursor; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; MEP, megakaryocyte-erythrocyte progenitor; pDC, plasmacytoid dendritic cell; RBC, red blood cells.

Because effective immunity is the result of a coordinated multifunctional system, the various cells that comprise innate and acquired immunity must interact and communicate. Cells interact with antigens and each other via receptors, and often produce soluble factors termed cytokines and chemokines as means of cell–cell communication. The resulting actions by cells receiving messages in the form of cytokines and chemokines are diverse and include cellular activation, initiation or termination of intracellular signaling events, proliferation, differentiation, migration, trafficking, or effector functions. It is important to note that while some of these molecules might be constitutively expressed, most are inducible in response to antigens, cellular stressors, or other cytokines. Thus, many cytokines and chemokines are not stored in the cell, but are tightly regulated, often at the transcriptional level, so that they are quickly generated on demand. Moreover, many cytokines share common receptor subunits, which allows greater flexibility for a cell's responsiveness to these extracellular signals. A partial list of cytokines and a brief description of the cell types that release and are acted upon by these various mediators is provided in Table 12-4.

Innate Immunity

As stated above, innate immunity acts as a first line of defense against anything nonself. With respect to infectious agents, the innate immune system eliminates most potential pathogens before significant infection occurs. The innate immune system includes physical and biochemical barriers both inside and outside the body, as well as immune cells designed for host defense responses and soluble factors.

Externally, the skin provides an effective barrier, as most organisms cannot penetrate intact skin. Most infectious agents enter the body through the respiratory system, gut, or genitourinary tract. Innate defenses present to combat infection from pathogens entering through the respiratory system include mucus secreted along the nasopharynx, the presence of lysozyme in most secretions, and cilia lining the trachea and main bronchi. In addition, reflexes such as coughing, sneezing, and elevation in body temperature are also a part of innate immunity. Pathogens that enter the body via the digestive tract are met with severe changes in pH (acidic) within the stomach and a host of commensal bacteria (i.e., microbiota) living in the intestines that play a protective role.

Cellular Components: Neutrophils, Macrophages, Dendritic Cells, Natural Killer Cells, NKT Cells, γδ T Cells, and B-1 B Cells Several cell types are involved in innate immunity, and in fact, some also provide critical signals to initiate adaptive immune responses (Tables 12-3 and 12-5). Neutrophils (also known as polymorphonuclear leukocytes or PMNs) are phagocytic cells that develop from the myeloid lineage of HSCs. Neutrophils enter the bloodstream where they circulate for about 10 hours and then enter the tissues where they perform effector functions for about 1 to 2 days. Neutrophils are capable of passing between the endothelial cells of the blood vessels (i.e., extravasation) and thereby represent a primary line of defense against pathogens. They are excellent phagocytic cells and can eliminate most microorganisms through the release of various reactive oxygen species (ROS), such as superoxide, singlet oxygen, ozone, hydrogen peroxide, and hydroxyl radicals. Their phagocytic activity (i.e., ability to internalize foreign materials) is greatly enhanced by the presence of complement and antibody deposited on the surface of the foreign target (see the section "Antigen Recognition"). Neutrophils are also important in the induction of an inflammatory response (see the section "Inflammation").

Table 12-4

Cytokines: Sources and Functions in Immune Regulation

CYTOKINE	SOURCE	PHYSIOLOGIC ACTIONS
IL-1	Macrophages Epithelial cells	Activation and proliferation of T cells Pro-inflammatory Induces fever and acute-phase proteins Induces synthesis of pro-inflammatory cytokines
IL-2	T cells	Primary T-cell growth factor Growth factor for B cells and NK cells
IL-4	Th2 cells Mast cells	Proliferation of activated Th2 and B cells B-cell differentiation and IgE isotype switching Antagonizes IFN-γ Inhibits Th1 responses
IL-5	Th2 cells Mast cells	Proliferation and differentiation of eosinophils
IL-6	Macrophages Th2 cells B cells Endothelial cells	Enhances B-cell differentiation and Ig secretion Induction of acute-phase proteins by liver Pro-inflammatory Proliferation of T cells and increased IL-2 receptor expression
IL-10	Tregs Bregs Macrophages	Inhibits T-cell and macrophage responses
IL-12	DCs Macrophages	Activates NK cells Induces Th1 responses
IL-13	Th2 cells	Stimulates B-cell growth Inhibits Th1 responses
IL-17	Th17 cells NK cells γδ T cells Neutrophils	Pro-inflammatory Inhibits Tregs
IL-22	Th17 cells ILCs	Activates neutrophils Mucosal protection
IFN-α/β (Type I IFN)	Leukocytes DCs Fibroblasts	Induction of MHCI expression Antiviral activity Stimulation of NK cells
IFN-γ	T cells NK cells	Induction of MHCI and MHCII Activates macrophages
TGF-β	Macrophages Megakaryocytes T cells	Enhances monocyte/macrophage chemotaxis Enhances wound healing: angiogenesis, fibroblast Proliferation, deposition of extracellular matrix Inhibits T and B-cell proliferation Inhibits antibody secretion Primary inducer of isotype switch to IgA
GM-CSF	T cells Macrophages Endothelial cells Fibroblasts	Stimulates growth and differentiation of monocytes and granulocytes

SOURCE: Information on selected cytokines taken from Murphy and Weaver (2016).

Also important in the innate response are macrophages. Macrophages are terminally differentiated monocytes, which develop from the myeloid lineage of HSCs (Fig. 12-2). Upon exiting the bone marrow, monocytes circulate within the bloodstream for about 1 day. At that time, they begin to distribute to the various tissues where they can then differentiate into distinct macrophage populations. Tissue-specific macrophages include Kupffer cells (liver), alveolar macrophages (lung), microglial cells (CNS), and peritoneal and splenic macrophages. These tissue-specific macrophages have distinct properties and vary in the extent of surface receptors, oxidative metabolism, and expression of major histocompatibility complex class II (MHCII), due to the factors

Table 12-5

Characteristics of Selected Immune Cells

PROPERTIES	MACROPHAGE	DC	T CELLS	B CELLS	NK CELLS
Phagocytosis	Yes	Yes	No	Yes	No
Adherence	Yes	Yes	No	No	No
Surface receptors:					
Antigen receptors	No	No	Yes	Yes	No
Complement	Yes	Yes	No	Yes	Yes
Fc region of Ig	Yes	Yes	Some	Yes	Yes
Surface markers	CD16 CD11b CD64	CD11c CD11b	CD4 CD8 CD3	Ig CD19 B220	CD16 CD11b
Proliferation in response to:					
Allogeneic cells (MLR)	No	No	Yes	No	No
LPS	No	No	No	Yes	No
PHA	No	No	Yes	No	No
Con A	No	No	Yes	No	No
Anti-Ig + IL-4	No	No	No	Yes	No
Anti-CD3 + IL-2	No	No	Yes	No	No
CD40L + cytokines	Yes	Yes	No	Yes	No
Effector functions:					
Antibody production	No	No	No	Yes	No
Cytokine production	Yes	Yes	Yes	Yes	Yes
Bactericidal activity	Yes	Yes	No	No	No
Tumor cell cytotoxicity	Yes	Yes	Yes	No	Yes
Immunologic memory	No	No	Yes	Yes	No
Suppressor activity	Yes	No	Yes	Yes	No

present within the microenvironment in which the cells reside. Macrophages can be classified as classically activated macrophages (M1), which are pro-inflammatory, or alternatively activated macrophages (M2), which are efficient at apoptotic cell removal and tissue remodeling and repair (reviewed by Martinez and Gordon [2014]). Macrophages also act as antigen-presenting cells (APCs; see the section "Antigen Recognition").

Another critical innate cell is the dendritic cell (DC; also see the section "Antigen Processing"). DCs are critical to innate immunity because they survey their environment for pathogens, then mature to become highly efficient APCs. Thus, they provide a bridge between initial detection of an infectious agent and elicitation of adaptive immune responses. DCs can be classified into several subsets and it is thought that the various subsets develop from either CLPs or CMPs, although the exact details are still unclear (reviewed by Watowich and Liu [2010]). Although not reviewed here, a challenge with lineage definition is the extensive heterogeneity of cell surface proteins expressed by DC populations, including varying expression of CD11b, CD11c, MHCII, CD4, CD8, and CD205 (reviewed by Watowich and Liu [2010]). All DCs possess efficient APC activity and cytokine production (Watowich and Liu, 2010), but may also possess specialized functions. Plasmacytoid dendritic cells (pDCs), for instance, secrete large amounts of Type I interferons (IFNs), such as IFN-α, which is necessary for T-cell differentiation and antiviral activity (Sachdeva et al., 2008).

Like other immune cells, NK cells are derived from a CLP (Fig. 12-2). NK cells belong to a larger family of cells defined as innate lymphoid cells (ILCs), which play roles in innate immunity and inflammation, particularly during the initial phases of inflammation prior to T-cell differentiation (Spits and Di Santo, 2011; Vanaudenaerde et al., 2011). NK cells have two major functions, cytokine production and destroying virus-infected or neoplastic target cells. NK cells are a predominant source of IFN-γ, which helps mature DCs and activate macrophages (Vivier et al., 2011). Thus, NK cells also help to bridge innate-adaptive immunity. NK cells mediate both antibody-independent and antibody-dependent cellular cytotoxicity (ADCC) using a variety of mechanisms, including perforin and granzyme, Fas ligand, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), and TNF-α. ADCC occurs via an FcR (FcγRIIIA), which is present on most NK cell subsets. Invariant NKT (iNKT) cells are one such subset of NK cells that express both NK- and T-cell markers and are restricted to recognition of microbial glycolipids presented by CD1d, a MHCI-like molecule (Diana and Lehuen, 2009).

Another cell type that has been found to bridge innate-adaptive immunity is the $\gamma\delta$ T cell. These cells migrate predominantly to "exposed" tissues, including skin, lung, gut, and reproductive organs, and are also expressed highly in the liver (Bonneville et al., 2010). In part through the expression of Toll-like receptors (TLRs), which are discussed in more detail below, $\gamma\delta$ T cells can acquire effector functions similar to NK cells (cytokine production and cytotoxicity). There is also a subpopulation of B cells (i.e., $CD5^+$ or B-1 cells) that bridge between innate immunity and adaptive immunity. Unlike the conventional B-2 cells, B-1 cells predominate in embryonic life and are later found mostly in the peritoneal and pleural cavities. B-1 cells are self-renewing and spontaneously produce polyspecific IgM antibodies (i.e., antibodies directed primarily against capsular polysaccharide antigens) independent of T-cell help (Baumgarth, 2011).

Historically, innate immunity was defined as nonspecific. It is now clear that innate cells express receptors that respond to soluble components (e.g., Fc or complement receptors) or to certain antigenic motifs. Pattern recognition receptors are a family of receptors that recognize pathogen-derived molecules (pathogen-associated molecular patterns [PAMPs]) or cell-derived molecules produced in response to cellular stress (danger-associated molecular patterns [DAMPs]). Receptors that recognize PAMPs or DAMPs include TLRs, NOD-like receptors (NLRs), and RIG-like receptors (RLRs) (Davis et al., 2011). Several TLRs are expressed extracellularly, and several are expressed intracellularly in endosomes, in order to protect against extracellular and intracellular pathogens, respectively. Moreover, this diverse localization of TLRs allows cells to respond to a variety of pathogenic components, such as bacterial cell wall lipids, single- and double-stranded nucleic acids, or fungal and parasitic products (Chang, 2010). Functional consequences of TLRs include expression of adhesion molecules, chemokines, or cytokines to stimulate T- or B-cell differentiation, enhance phagocytosis, or induce maturation of DCs (Chang, 2010). NLRs and RLRs are expressed in many innate cells. Several family members of NLRs participate in a complex called the inflammasome, which activates inflammatory caspases to enhance production of mature interleukin (IL)-1β and IL-18 (Martinon et al., 2009).

Soluble Components: Acute-Phase Proteins, Granzyme, Perforin, and Complement Acute-phase proteins, such as serum amyloid A, serum amyloid P, and C-reactive protein, participate in an acute-phase response to infection by binding bacteria and facilitating complement activation. Granzyme and perforin work in conjunction, with perforin disrupting the target cell membrane, allowing granzyme to enter and mediate cell lysis by several mechanisms.

The complement system is a family of about 30 serum proteins, which functions to destroy the membranes of infectious agents, facilitate phagocytosis of foreign materials, and promote an inflammatory response (reviewed by Stoermer and Morrison [2011]; also see the section "Inflammation"). Complement activation occurs with each component sequentially acting on other components in a manner similar to the blood-clotting cascade (Fig. 12-3). Proximal components of the cascade are often modified serine proteases, which activate the system but have limited substrate specificity. Several components are capable of binding to microbial membranes and serve as ligands for complement receptors on phagocytic cells to facilitate phagocytosis, although a primary pathway of complement activation occurs after antibody binds a pathogen. The final components, which are related structurally, are also membrane-binding proteins that form the membrane attack complex (MAC), which enters into the membrane and disrupts membrane integrity.

Specifically, three pathways have been identified in activation of the complement cascade. The classical complement pathway is initiated when antibody binds antigen on the microorganism. The classical pathway then proceeds by activating a C1 subunit serine protease, subsequently recruiting C4, C2, and C3. Various cleavages ultimately result in C3b surface binding to the surface of the microorganism and release of C3a, a pro-inflammatory mediator (see the section "Inflammation"). Microorganism-bound C3b can then be recognized by complement receptors on phagocytic cells, which engulf and destroy the microorganism. It is also at this point that the alternative pathway can be activated and amplifies the complement-mediated killing of the microorganism. Finally, C3b mediates recruitment of C5, which is cleaved, generating C5b on the surface and releasing C5a, another pro-inflammatory mediator (see the section "Inflammation"). Microorganism-bound C5b recruits C6 and

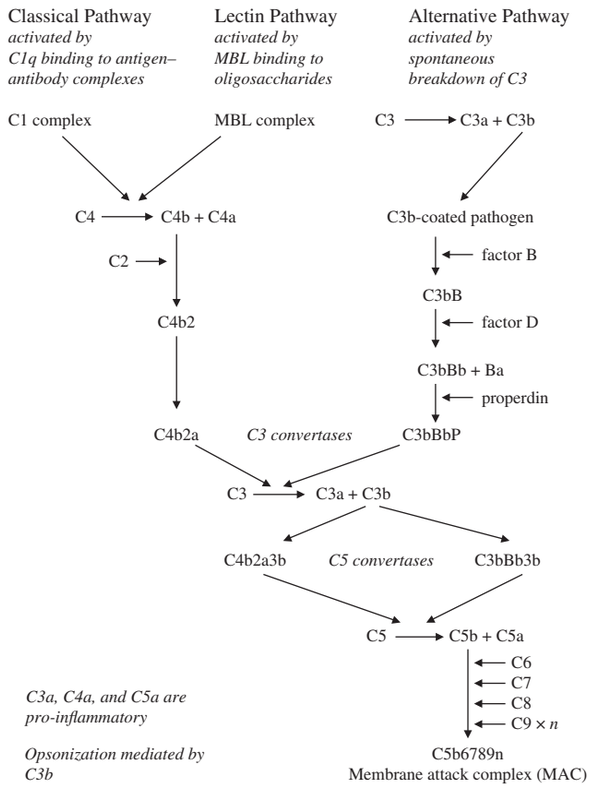

Figure 12-3. *The complement cascade.* The complement cascade can be activated in three different ways. Cytolysis occurs via generation of the MAC. Various complement proteins generated along the pathway are either pro-inflammatory (C3a, C5a) or result in opsonization (C3b).

C7, which together form a complex and recruit C8. C9 is ultimately recruited, polymerizes, and forms a pore in the membrane of the microorganism, causing its death. In addition to the classical and alternative pathways, complement-mediated lysis is also activated through the lectin pathway, in which binding of mannose-binding lectin (MBL) to the surface of the microorganism activates the pathway and converges with the classical pathway at C4 (Fig. 12-3).

A functional outcome of complement activation, in addition to cytolysis and generation of pro-inflammatory mediators, is opsonization, the process by which microorganisms are coated by opsonins (predominantly IgM and IgG antibodies and C3b), rendering them vulnerable to phagocytosis by cells (usually macrophages and neutrophils) (Dunkelberger and Song, 2010). Therefore, in combination with C3b opsonization and IgG antibodies recognizing extracellular antigens, phagocytic cells efficiently engulf the microbe through complement receptors or Fc receptors specific for IgG.

Antigen Recognition

A critical step in the initiation of acquired immunity is the ability of immune cells to recognize nonself. Nonself molecules need to be processed and presented by APCs to initiate the cell-mediated and humoral response by T and B cells, respectively.

Antigen As stated earlier, a nonself substance that can be recognized by the immune system is called an antigen (also referred to as an immunogen or allergen). Antigens are usually (but not absolutely) biological molecules that can be cleaved and rearranged for presentation to other immune cells. Generally, antigens are about

Table 12-6

Properties of Immunoglobulin Classes and Subclasses

CLASS	MEAN SERUM CONCENTRATION (MG/ML)	HUMAN HALF-LIFE DAYS	BIOLOGICAL PROPERTIES
IgG			Complement fixation (selected subclasses) Crosses placenta
IgG_1	9	21	
IgG_2	3	20	
IgG_3	1	7	
IgG_4	1	21	
IgA	3	6	Secretory antibody
IgM	1.5	10	Complement fixation Efficient agglutination
IgD	0.03	3	Possible role in antigen-triggered lymphocyte differentiation
IgE	0.0001	2	Allergic responses (mast cell degranulation)

10 kDa or larger in size. Smaller antigens are termed "haptens" and must be conjugated with carrier molecules (larger antigens) in order to elicit a specific response. However, once an initial response is made, the hapten can induce subsequent responses in the absence of the carrier.

Antibodies Antibodies are produced by B cells and are defined functionally by the antigen with which they react, and by their subtype, termed "isotype" (IgM, IgD, IgG, IgE, and IgA; Table 12-6). Thus, an IgM antibody directed against sheep red blood cells (sRBCs) is called anti-sRBC IgM. Because the immune system generates antibodies to thousands of antigens with which the host may or may not ever come into contact, antibodies of unknown specificity are referred to as immunoglobulin (e.g., serum immunoglobulin or serum IgM) until defined by their specific antigen (e.g., anti-sRBC IgM).

The ability of the immune system to generate antibody to thousands of antigens is the result of somatic recombination, in which the germline DNA that encodes for antibodies is rearranged in B cells. All Igs are made up of heavy and light chains and of constant and variable regions. For the light chain genes, two separate gene segments (V and J) are combined to form the variable region, which is then joined to one constant region. For the heavy chain genes, three separate gene segments (V, D, and J) are combined to form the variable region, which is then joined to the constant region. There are several light chain V and J gene segments, and several heavy chain V, D, and J gene segments, which can rearrange in various combinations to contribute to the immense genetic diversity of the Ig genes (Fig. 12-4). Finally, the five types of Ig are dependent on which heavy chain constant region is transcribed and translated (heavy chain genes μ, γ, ε, δ, or α encode for the IgM, IgG, IgE,

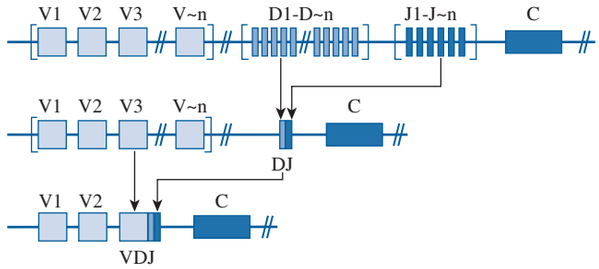

Figure 12-4. *Ig recombination.* An example of VDJ recombination that might occur for Ig heavy chain in B cells or TCR β chain in T cells. Ig light chain in B cells or TCR-α chain in T cells is similar, but does not include D gene segments.

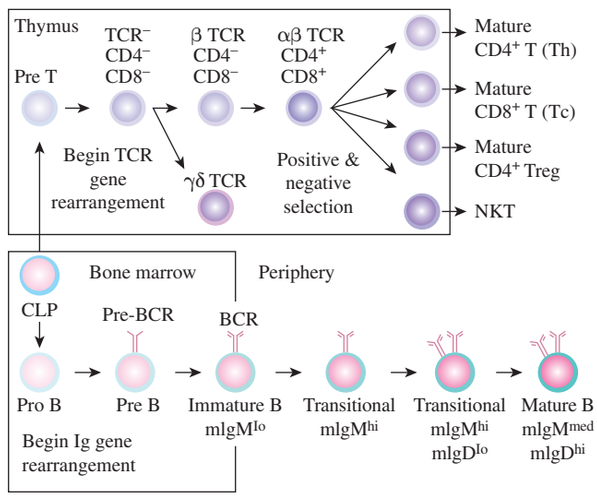

Figure 12-5. *Development and differentiation of T and B cells.* T cells develop in the thymus and B cells develop in the bone marrow. For T cells, an initial step is commitment to either αβ or γδ TCR. After positive and negative selection, T cells acquire either CD4 or CD8 to become Th or CTL, respectively. T cells can also become natural Tregs (nTregs) or NKT cells. The B-cell lineage depicted here is of the conventional B-2 lineage. B-1 cells are not depicted since these cells arise from a distinct precursor not found in the bone marrow, which might be a precursor that can generate B-1 or myeloid cells. mIg refers to membrane-bound immunoglobulin.

IgD, or IgA heavy chain proteins, respectively). Immunoglobulin gene rearrangement occurs in the bone marrow during B-cell development (Fig. 12-5). B-cell maturation also involves the establishment of central tolerance (i.e., nonreactive to self-antigens) to prevent the maturation of lymphocytes that recognize self-antigens. For B cells, central tolerance occurs in the bone marrow and simply involves the removal of self-reactive cells.

The variable regions, which are contained within the Fab regions of the antibody molecule, determine antibody specificity and interact with antigen (Fig. 12-6). The Fc region mediates various effector functions, such as complement activation (IgM and some IgG subclasses) and phagocyte binding (via Fc receptors). Antibodies possess several functions: (1) opsonization, which is coating of a pathogen with antibody to enhance Fc receptor–mediated endocytosis by phagocytic cells; (2) initiation of the classic pathway of complement-mediated lysis; (3) neutralization of viral infection by binding to viral particles and preventing further infection; and (4) enhancement of the specificity of effectors of cell-mediated immunity (CMI) by binding to specific antigens on target

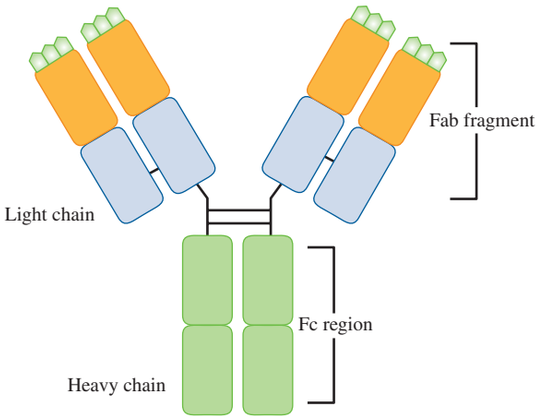

Figure 12-6. *Ig structure.* Igs are comprised of two heavy chains and two light chains, which are connected by disulfide bonds. Gold areas are variable regions and green areas are antigen recognition regions.

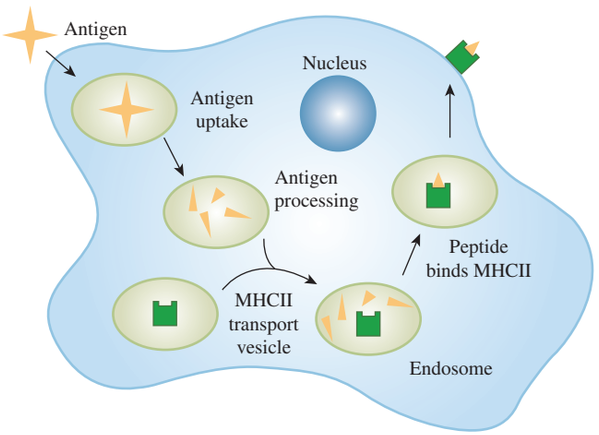

Figure 12-7. *Antigen processing by the MHCII pathway.* Antigen is engulfed by an APC (DC, macrophage, or B cell), degraded and loaded onto MHCII. The MHCII–peptide complex is then expressed on the surface of the APC for presentation to $CD4^+$ Th cells.

cells, which are then recognized and eliminated by effector cells such as natural killer (NK) cells or cytotoxic T lymphocytes (CTLs).

Antigen Processing In order to elicit an acquired immune response to a particular antigen, that antigen must be taken up and processed by accessory cells for presentation to lymphocytes. As introduced earlier, accessory cells that perform this function are termed APCs and include macrophages, DCs, and B cells. Of these, the most proficient APC is the DC. There are several subtypes of DC, including plasmacytoid (pDCs), conventional (cDCs; can be resident or migratory depending on whether they migrate to the site of infection), and specialized DCs in the skin called Langerhans cells. In addition, there are follicular DCs, which predominantly mediate T-cell-independent stimulation of B cells in germinal centers (Vinuesa et al., 2010).

In most tissues in the absence of antigenic stimulation, DCs are in an immature state, during which they efficiently capture antigens. DCs can mature in response to antigen itself, TLR agonists such as lipopolysaccharide (LPS), or cytokines (e.g., granulocyte-macrophage colony-stimulating factor [GM-CSF], or TNF-α) (Banchereau and Steinman, 1998). Upon maturation, DCs migrate to lymph nodes and present antigen to T cells. Thus, the DC–T-cell interaction is critical for the development of an acquired immune response.

Immature DCs internalize the antigen either by phagocytosis, pinocytosis, or by receptor-mediated endocytosis (via antigen, Fc, or complement receptors), after which the DCs mature to express high levels of both classes of MHC (MHCI and MHCII) to stimulate innate and adaptive immune responses, and costimulatory molecules to facilitate interaction with T cells (Banchereau and Steinman, 1998). Interestingly, Langerhans cells express high levels of MHCII even in a resting state (i.e., nonactivated) (Kaplan, 2010). Following internalization, antigen is processed (intracellular denaturation and catabolism) through several cytoplasmic compartments, and a piece of the antigen (peptide fragments about 20 amino acids in length) becomes physically associated with MHCII (Fig. 12-7). This MHCII–peptide complex is then transported to the surface of the cell and can interact in a specific manner with T lymphocytes. For most APCs, an immunogenic determinant is expressed on the surface of the APC within an hour after internalization, although this is slightly longer for B cells (3 to 4 hours). In addition to processing and presentation, pieces of processed antigen may be expelled into the extracellular space. These pieces of processed antigen can then bind in the peptide groove of empty MHCII on the surface of other APCs for the presentation of that peptide fragment to lymphocytes.

In addition to antigen processing and presentation via MHCII, some antigens may be processed and presented via MHCI. Although the pathways share some similarities in that short peptide fragments of antigens are generated, loaded onto MHC, and presented on the surface of cells to T lymphocytes, major differences between the MHCI and MHCII pathways are (1) antigens processed and presented via MHCI are not limited to professional APC; (2) all nucleated cells express MHCI; (3) the mechanisms by which the antigen is processed and loaded onto MHCI are slightly different than MHCII; (4) the MHCI antigenic peptides are usually smaller, often 8 to 10 amino acids in length; (5) the MHCI antigens to be processed are usually aberrantly expressed proteins, such as viral-associated proteins or mutated proteins; and (6) MHCI facilitates antigen presentation to $CD8^+$ T cells, whereas MHCII facilitates antigen presentation to $CD4^+$ T cells. MHCI antigen processing and presentation is the major pathway by which virally infected cells are detected and killed by the acquired immune system.

Regardless of the MHC utilized to present antigens to lymphocytes, T cells are able to recognize antigen in the context of MHC with their T-cell receptor (TCR). Similar to Ig, the ability of T cells to specifically recognize thousands of antigens is due to somatic recombination. All TCRs are comprised of two different subunits, each encoded from a distinct gene (most abundant T-cell population expresses the αβ subunits, but γδ also exist). All TCR subunits are made up of constant and variable regions. For α subunits, two separate gene segments (V and J) are combined to form the variable region, which is then joined to a constant region. For β subunits, three separate gene segments (V, D, and J) are combined to form the variable region, which is then joined to a constant region. Similar to the Ig genes, there are several α subunit V and J gene segments, and several β subunit V, D, and J gene segments (Fig. 12-4). TCR gene rearrangement occurs during T-cell and iNKT cell development in the thymus. iNKT cells express an invariant TCR that recognizes microbial glycolipids in the context of the MHCI-like molecule CD1d.

Although CLP cells originate in the bone marrow and give rise to B cells, NK cells, T cells, and iNKT cells, the latter two cell types do not develop in the bone marrow. Instead T-cell progenitors migrate from the bone marrow to the thymus, (i.e., a primary

lymphoid tissue specific for T-cell development; Figs. 12-2 and 12-5). The maturation process for T cells involves the establishment of central tolerance, which is a state of non-responsiveness to self. It is complex for T cells and involves both positive and negative selection resulting in mature T cells that recognize self-peptide loaded MHC (or CD1d for iNKT cells) on the host's cells (i.e., positive selection) but not too strongly. T cells that recognize self too strongly are deleted from the T-cell repertoire (termed negative selection).

With regard to T and B cells, key events that occur following antigen encounter are (1) specific antigen recognition either in the context of MHCI or MHCII for T cells or through the Ig receptor for B cells; (2) cellular activation and initiation of intracellular signaling cascades that contribute to production and release of cytokines and other cellular mediators; (3) clonal expansion (proliferation) of antigen-specific cells; and (4) differentiation of antigen-stimulated lymphocytes into effector and memory cells. These processes are detailed below.

Acquired (Adaptive) Immunity

If the primary defenses against infection (innate immunity) are breached, the acquired arm of the immune system is activated and produces a specific immune response to each infectious agent, which usually eliminates the infection. This branch of immunity is also capable of remembering the pathogen and can protect the host from future infection by the same agent. Therefore, the two key features that define acquired immunity are specificity and memory. Acquired immunity may be further subdivided into humoral and cell-mediated immunity (CMI). Humoral immunity is directly dependent on the production of antigen-specific antibody by B cells and involves the coordinated interaction of APCs, T cells, and B cells. CMI is that part of the acquired immune system in which effector cells, such as phagocytic cells, T helper cells, regulatory T cells, APCs, cytotoxic T cells, or memory T cells, play the critical role(s) without antibody involvement.

Mature, naive, or virgin lymphocytes (those T and B cells that have never undergone antigenic stimulation) are first brought into contact with exogenously derived antigens within the highly organized microenvironment of the spleen and lymph nodes, which are secondary lymphoid organs. These organs can be thought of as biological sieves. The spleen serves as a filter for the blood, removing both foreign antigens and any circulating dead cells and cellular debris. The lymph nodes are part of a network of lymphatic veins that filter antigens from the fluid surrounding the tissues of the body. In addition to the spleen and lymph nodes, other secondary lymphoid tissues associated with the skin, gut, bronchioles, or nasal cavity are referred to as associated lymphoid tissues (abbreviated SALT, GALT, BALT, and NALT, respectively; Table 12-2). MALT is a general term for all mucosal-associated lymphoid tissues. Included in the GALT are isolated lymphoid follicles and specialized structures in the small intestine called Peyer's patches, both of which collect antigens from the gastrointestinal tract. The associated lymphoid tissues tend to have more exposure to antigen and greater plasticity (i.e., increase or decrease in size and/or number) than lymph nodes. The plasticity of lymphoid organs is underscored by the detection of lymphoid neogenesis in nonlymphoid organs during chronic inflammation. The new lymphoid tissue is classified as tertiary lymphoid tissue, and its development is reversible if the inflammatory response is resolved. A role for innate lymphocytes (ILCs) in development of tertiary lymphoid tissue has recently been identified (Pearson et al., 2012).

Cellular Components: Antigen-Presenting Cells, T Cells, and B Cells APCs, which were discussed previously in the section "Antigen Processing," include professional APCs such as B cells, macrophages, and DCs. Although all cells may act as APCs with internal antigen processing through the MHCI pathway, what distinguishes a professional APC from the others is the ability to internalize external antigens and process them through the MHCII pathway for presentation to T helper cells.

B cells are not only capable of serving as professional APCs, but they are also the effector cells of humoral immunity, producing a number of Ig isotypes with varying specificities and affinities. Like other immune cells, the B cell develops in the bone marrow from the HSC and becomes committed to the B-cell lineage when the cell begins to rearrange its Ig genes, as described in the section "Antigen Recognition" (Figs. 12-4 and 12-5). Following successful rearrangement of the heavy chain, these cells express Ig heavy chains in their cytoplasm and are termed pre-B cells. Successful rearrangement of the light chain results in expression of membrane-bound IgM in association with Igα and Igβ to form the B-cell receptor (BCR). Expression of membrane IgM and IgD BCRs indicates a mature B cell. B-1 B cells predominate in embryonic life and are later found mostly in the intestinal mucosa (Hardy, 2006). These cells are probably associated with innate immunity and are long-lived, self-renewing cells that produce high levels of polyspecific IgM (Baumgarth, 2011). Mature B cells of the conventional B-2 subset are found in the lymph nodes, spleen, and peripheral blood. Upon antigen binding to the BCR, the mature B cell becomes activated and, after proliferation, undergoes differentiation into either an antibody-forming cell (AFC) or a memory B cell. AFCs, also known as plaque-forming cells (PFCs), antibody-secreting cells (ASCs), or plasma cells, actively secrete antibody specific for the antigen that initially bound and activated the BCR. A broad description of several B-cell characteristics can be found in Table 12-5.

In a manner analogous to B cells, at a specified time following their commitment to the T-cell lineage, T-cell precursors migrate from the bone marrow to the thymus where they begin to rearrange their TCRs, as described in the section "Antigen Recognition" (Fig. 12-5). The TCR consists of two chains, either $\alpha\beta$ or $\gamma\delta$. The majority of T cells express the $\alpha\beta$ TCR, which is critical for the recognition of antigens in the context of MHC. A much smaller population of T cells express a $\gamma\delta$ TCR. The $\gamma\delta$ T cells differ from $\alpha\beta$ T cells in that they do not require antigen processing or presentation by MHC but instead directly bind intact host and pathogen proteins. Like the B-1 lineage, the $\gamma\delta$ T cells are expressed first in embryonic development and may be associated with innate immunity (see the section "Innate Immunity"). T cells committed to the $\alpha\beta$ lineage will express the surface markers CD4 and CD8. CD4 and CD8 are co-receptors expressed by $\alpha\beta$ T cells and are involved in the interaction of the T cell with MHC. T cells expressing $\alpha\beta$ TCR and both CD4 and CD8 are termed immature double-positive cells ($CD4^+$/$CD8^+$). These immature cells then undergo positive and negative selection to (1) select TCRs with affinity for self-MHC (positive selection) and eliminate cells that do not produce a functional TCR or produce TCRs with no affinity for self-MHC or (2) eliminate cells that strongly bind MHC plus self-peptide (negative selection). Double-positive cells that survive positive and negative selections will give rise to T cells that lose expression of either CD4 or CD8 and enter the periphery as mature single-positive cells ($CD4^+$ or $CD8^+$) with a high level of $\alpha\beta$ TCR expression. This rigorous selection process produces T cells that can recognize MHC plus foreign peptides and eliminates autoreactive T cells. Additionally, expression of CD4 or CD8 will determine to which MHC class the $\alpha\beta$ TCR will bind. CD4 will facilitate binding to MHCII expressed on APCs; T cells expressing CD4 (T helper cells [Th]) help activate other cells of the adaptive immune response. CD8 will facilitate binding to MHCI, which is expressed on all nucleated cells;

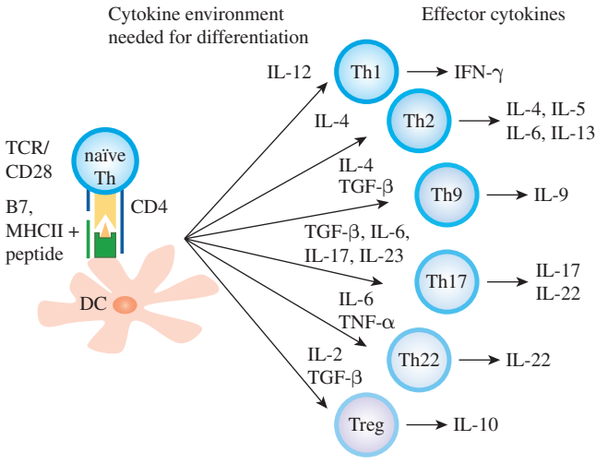

Figure 12-8. *The cytokine environment drives different Th populations.* Various Th cells (and T regulatory cells, Tregs) are differentially induced in response to the cytokine milieu. Th1 cells drive CTL responses; Th2 cells drive humoral immune responses; Th9, Th17, and Th22 all produce pro-inflammatory cytokines; Tregs control other T-cell responses.

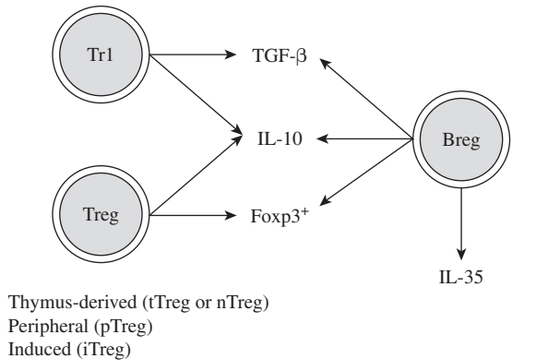

Figure 12-9. *Tregs and Bregs.* There are at least three different subpopulations of Tregs and Bregs, all of which suppress immune function via IL-10 or TGF-β. Tregs can be natural (thymus-derived) or induced in the periphery.

generally, T cells expressing CD8 mediate cell killing (cytotoxic T lymphocytes [CTL]). A broad description of several T-cell characteristics (specific to αβ T cells) can be found in Table 12-5. In contrast to αβ T cells, the γδ T cells do not express CD4 or CD8 and therefore do not interact with MHC and do not undergo positive or negative selection.

Mature T cells are found in the lymph nodes, spleen, and peripheral blood. Upon binding of the TCR to antigen in the context of MHC, the T cell becomes activated and, after proliferation, undergoes differentiation into either an effector cell or a memory T cell. Effector Th cells can subsequently differentiate into several phenotypes depending on the cytokine milieu (Fig. 12-8), two of which, Th1 or Th2, dictate whether CMI or humoral immunity will predominate, respectively. Th1 cells predominantly express IL-2, IFN-γ, and lymphotoxin, which promote CMI and humoral defense against intracellular invaders. Th2 cells predominantly express IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13, which promote humoral defense against extracellular invaders. Although the two populations are not mutually exclusive, they do negatively regulate each other, such that a strong Th1 response suppresses a Th2 response and vice versa.

Th17, Th9, and Th22 cells have been shown to be critical for inflammatory responses (see the section "Inflammation"), and several studies including autoimmune models and clinical studies support a role of Th17 cells in inflammation and autoimmune diseases. Modulation of Th17 cellular function by xenobiotics may alter these disease states. Conversely, a better understanding of the relationship between Th17 and specific disease states has led to new therapeutics in the treatment of autoimmune and inflammatory diseases (Hemdan et al., 2010; Kimura and Kishimoto, 2011; Bartlett and Million, 2015).

The ability of APCs, B cells, and T cells to communicate with each other is dependent on a variety of receptor–ligand interactions between cell types. These interactions also help dictate the type of immune response (i.e., humoral vs. CMI) and the magnitude of the immune response. For example, TCR binding by antigen presented in the context of MHC is the major interaction that must occur between APCs and T cells to initiate an acquired immune response. Subsequent to this initial interaction, costimulatory molecules must be engaged on both the T cells and APCs to sustain and direct the immune response. One of the best characterized interactions occurs between CD28 on the T cells and CD80 or CD86 (B7.1 and B7.2) on the APCs, which provides a more robust immune response as measured by clonal expansion and cytokine (e.g., IL-2) production. Later in the response (2 to 3 days), T cells express CTLA-4, which exhibits higher affinity for CD80/CD86 than does CD28, and the CTLA-4–CD80/CD86 interaction serves as an immune checkpoint to suppress the T-cell response. Other important interactions between APCs and T cells include (1) CD40 and CD40 ligand (CD40L), which sustains clonal expansion and differentiation of the T cells and provides activation signals to the APC; (2) inducible T-cell costimulator (ICOS [CD278], a member of the CD28 superfamily) and ligand for ICOS, which sustains clonal expansion and differentiation of the T cells and induces IL-10 production; and (3) 4-1BB (CD137/Ly63, a member of the TNF receptor superfamily) and 4-1BB ligand, which sustains clonal expansion of the T cells and helps to activate the APC. CD40 ligand, ICOS, and 4-1BB are expressed on T cells. Therapeutic drugs that bind some of these costimulatory surface molecules have been developed and utilized clinically to either suppress immune function for transplantation and autoimmune therapy or stimulate immune function for cancer treatment, which will be discussed in the section "Therapeutic Drugs." For simplicity, in figures throughout the chapter, only the CD28–B7 interaction is depicted.

The duration and extent of an acquired immune response is also controlled by specialized regulatory cells found in both the T-cell and B-cell lineages, and there are considerable functional similarities between the different regulatory subsets found within both lineages (reviewed by Noh and Lee [2011]) (Figs. 12-8 and 12-9). For the T-cell lineage, there is a small population of $CD4^+$ cells that develop into T-regulatory cells (Tregs), which help to control various immune responses, including those directed against self. Tregs are generally identified as $CD4^+CD25^+Foxp3^+$, which are defined as natural Tregs (nTregs) if they develop in the thymus (Lio and Hsieh, 2011), but can also be induced (iTregs) in the periphery in response to IL-2 and TGF-β (Horwitz et al., 2008). Tregs can suppress $CD4^+$ and $CD8^+$ IL-2 production and proliferation, and in vivo have been shown to suppress autoimmune disease (Sakaguchi et al., 2009). Immune suppression by Tregs is likely a multistep process, which might involve direct cell killing via granzyme or perforin, induction of intracellular cAMP, or affecting cell surface expression of critical proteins, such as CD80/CD86. For instance, Foxp3 induces high levels of CTLA-4, which would interact with target cell CD80/CD86 and prevent target cell

CD80/CD86 from interacting with CD28 on other stimulatory cells (reviewed by Sakaguchi et al. [2009]). It has also been suggested that the high expression level of CD25 on Tregs might act as a reservoir for IL-2 in an immune synapse microenvironment, thereby preventing IL-2 from stimulating surrounding cells (Sakaguchi et al., 2009). In addition to Tregs, other regulatory T-cell subsets have been identified such as the IL-10-producing, FoxP3$^-$ Tr1 cells and the TGF-β-producing Th3 cells; all three regulatory subsets appear to be involved in regulating immune responses (Noh and Lee, 2011). Regardless of the processes that direct Treg (or perhaps Tr1 and Th3) suppression of immune function, their induction has been identified as one mechanism by which drugs and xenobiotics might result in immune suppression (Marshall and Kerkvliet, 2010; Ohkura et al., 2011). Like the regulatory T cells, several subsets of regulatory B cells (Bregs) are emerging and several reviews have been devoted to outlining the experimental evidence for specific regulatory B-cell subpopulations and their generally suppressive role in hypersensitivity and autoimmune diseases. Specifically, Bregs have been identified in controlling immune diseases such as allergic rhinitis (Xu et al., 2016b), collagen-induced arthritis (Park et al., 2016), chronic graft versus host disease (Sarvaria et al., 2016), and autoimmune disease (Huarte et al., 2016; Korniotis et al., 2016; Lehmann-Horn et al., 2016; Menon et al., 2016). The mechanism by which Bregs act involves induction of apoptosis-triggering molecules such as Fas ligand or production of immunosuppressive cytokines, IL-10 and IL-35. The regulatory T- and B-cell subsets also appear to reciprocally activate or suppress each other and may cooperatively control immune responses. The interplay between regulatory T- and B-cell subsets and their impact on immune responses, including autoimmune and allergic diseases, are important areas of investigation that will likely lead to a better understanding of immune pathologies including those induced by drugs and other xenobiotics.

Humoral and Cell-Mediated Immunity As described earlier, there are two branches of acquired immunity, humoral immunity and CMI. Humoral immunity is that part of the acquired immune system in which antibody is involved. In general, B cells produce antibodies specific to an antigen, which may act to opsonize or neutralize the invader, or the antibodies act to recruit other factors, such as the complement cascade. CMI is that part of the acquired immune system in which various effector cells perform a wide variety of functions to eliminate invaders. Often, these two branches are coordinated, such as activation of Th cells that produce specific cytokines that enhance B-cell proliferation and differentiation to secrete more antibody. A general diagram of the cellular interactions involved in a humoral immune response is given in Fig. 12-10. The production of antigen-specific IgM requires 3 to 5 days after the primary (initial) exposure to antigen (Fig. 12-11). Upon secondary antigenic challenge, the B cells undergo isotype switching, producing primarily IgG antibody, which is of higher affinity for the activating antigen due to a process called somatic hypermutation and affinity maturation. Somatic hypermutation involves introducing point mutations within the DNA of the variable region to induce conformational changes that may result in a higher affinity interaction between antigen and antibody. In addition, there is a higher serum antibody titer associated with a secondary antibody response. CMI functions include delayed-type hypersensitivity (DTH) and cell-mediated cytotoxicity. Cell-mediated cytotoxicity responses may occur in numerous ways: (1) MHC-dependent recognition of specific antigens (such as viral particles or tumor proteins) by CTLs or iNKT cells (CTL depicted in Fig. 12-12); (2) indirect antigen-specific recognition by the binding of Fc receptors

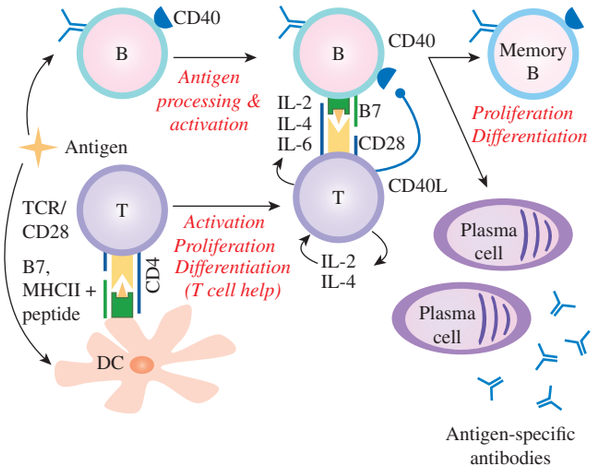

Figure 12-10. *Cellular interactions in the humoral immune response.* Antigen is engulfed by an APC (usually DC) and the antigenic peptide is presented to CD4$^+$ T cells in the context of MHC II. CD4$^+$ T cells then become activated, proliferate, and differentiate into Th cells, which release cytokines to help B cells that had also encountered the same antigen. B cells become activated, proliferate, and differentiate into memory B cells or antigen-producing plasma cells.

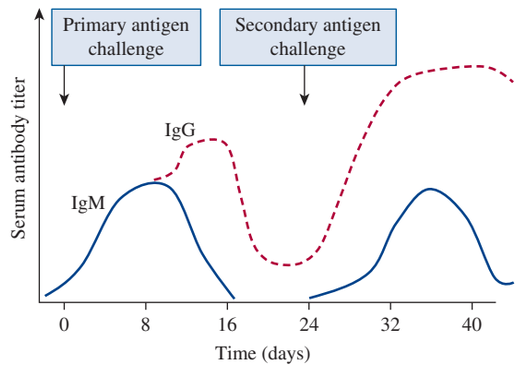

Figure 12-11. *Kinetics of humoral immune responses.* In response to primary antigen encounter, IgM is produced within several days. In response to secondary antigen encounter, IgG is produced more rapidly, and to a higher magnitude, than IgM.

on NK cells to antibodies coating target cells; and (3) receptor-mediated recognition of complement-coated foreign targets by macrophages.

In cell-mediated cytotoxicity, the CTL, iNKT, or NK effector cell binds in a specific manner to the target cell. The majority of CTLs express CD8 and recognize either foreign MHCI on the surface of allogeneic cells or antigen in association with self-MHCI (e.g., viral particles). Fig. 12-12 shows the cell-mediated CTL response. NK cell recognition of target cells is also antigen-specific because the mechanism of recognition involves binding of Fc receptors on NK cells to the Fc portion of antigen-specific antibody that coats the target cell; a process referred to as ADCC. Once the CTL or NK cells interact with the target cell, the effector cell undergoes cytoplasmic reorientation so that cytolytic granules are oriented along the side of the effector, which is bound to the target. The effector cell then releases the contents of these granules onto the target cell. The target cell may be damaged by the perforins or enzymatic contents of the cytolytic granules. In addition, CTLs induce

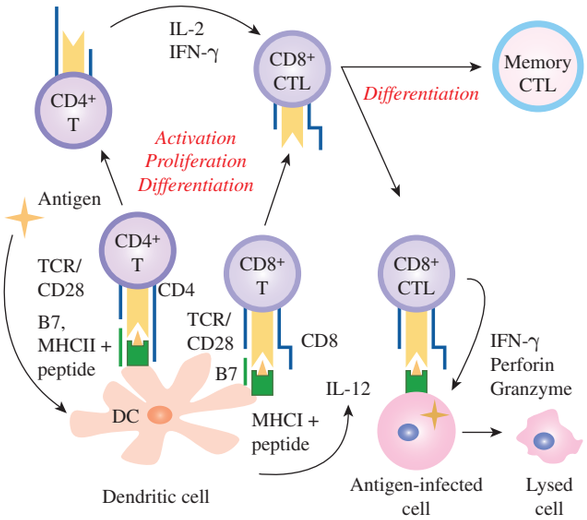

Figure 12-12. *Cellular interactions in the CTL response.* Antigen is engulfed by an APC (usually DC) and the antigenic peptide is presented to $CD4^+$ and $CD8^+$ T cells in the context of MHC II and I, respectively. $CD4^+$ T cells then become activated, proliferate, and differentiate into Th cells, which release cytokines to help $CD8^+$ T cells that had also encountered the same antigen. Especially in the presence of IL-12 produced by the DC, $CD8^+$ T cells become activated, proliferate, and differentiate into CTL that can kill other antigen-infected cells.

the target to undergo apoptosis through activation of the Fas and cytotoxic cytokine (i.e., TNF-α and lymphotoxin) pathways. Once it has degranulated, the effector cell can release the dying target and move on to kill other target cells. Notably, many of the soluble factors critical to the innate immune system are also primary effectors in the acquired immune system, including ROS, granzyme, perforin, cytokines, and chemokines, again demonstrating the interplay among the various arms of the immune system.

Inflammation

Inflammation, simply defined, refers to a complex reaction to injury, irritation, or foreign invaders characterized by pain, swelling, redness, and heat. Inflammation involves various stages, including release of chemotactic factors following the insult, increased blood flow, increased capillary permeability allowing for cellular infiltration, followed by either an acute resolution of tissue damage or persistence of the response that might contribute to fibrosis or subsequent organ failure (Serhan and Savill, 2005). It is important to emphasize that while inflammation is a natural reaction to repair tissue damage or attack foreign invaders, the process often results in destruction of adjacent cells and/or tissues. Thus, there is overwhelming evidence that inflammation plays a critical role in many diseases, including asthma, multiple sclerosis, cardiovascular disease, Alzheimer disease, bowel disorders, and cancer. In addition, inflammation exacerbates idiosyncratic reactions to drugs and other chemicals (reviewed by Ganey et al. [2004]).

Cellular Components: Macrophages, Neutrophils, ILCs, and T Cells Many of the cellular components described earlier are critical to initiation and maintenance of an inflammatory response. Major cellular contributors to an inflammatory response are macrophages, neutrophils, ILCs, and T cells. Neutrophils are often the first, and most numerous, responders to sites of insult. In response to either host- or pathogen-derived signals, neutrophils secrete chemotactic factors to recruit other pro-inflammatory cells, such as macrophages, to the area. IL-17 produced early by various sources, including NKT cells and ILCs, is a critical pro-inflammatory cytokine that rapidly induces neutrophil and macrophage influx to the area of insult (Vanaudenaerde et al., 2011). Macrophages can be activated by a variety of mechanisms at the site of insult, such as activation via TLR, pro-inflammatory cytokines, or recognition of opsonized particles by Fc receptors or complement receptors. Macrophages and neutrophils also induce apoptosis of cells in the insult area through the release of nitric oxide and other ROS, resulting in disruption of extracellular structures that compromise tissue structure and function (reviewed by Duffield [2003]). Both neutrophils and macrophages are phagocytic cells and can contribute to clearing of apoptotic cells. Later in the inflammatory response, T cells are critical for generating an adaptive immune response. T cells are attracted to the insult area by adhesion molecules and integrins, and are activated in response to antigen presented in the context of MHC, often by a DC. As described above, depending on the signals that the T cell receives from the cytokine milieu, distinct subpopulations of T cells are induced (Fig. 12-8). Chronic inflammatory states are maintained with activation and differentiation of Th2 and Th17 cells, which drive chronic eosinophilic and neutrophilic inflammation, respectively (Jutel and Akdis, 2011). Th9 and Th22 cells also likely contribute to chronic inflammation, but their roles are not yet completely understood.

Of all Th subsets, Th17 cells have certainly been shown to play a critical role in inflammation (Kimura and Kishimoto, 2011). In contrast to cells involved in the innate response (NK or NKT cells, γδ T cells, or other ILCs) that produce IL-17, Th17 cells are part of the adaptive immune response, and produce IL-17, IL-22, and TNF-α (Vanaudenaerde et al., 2011). They are induced in the presence of IL-6 and TGF-β, conditions that are unfavorable for Tregs, thereby exacerbating the pro-inflammatory response through suppression of Treg differentiation and promotion of chronic neutrophilic infiltration (Awasthi and Kuchroo, 2009). Th17 cells are defined by the expression of retinoid-related orphan receptors RORγ and RORα. Interestingly, Th17 cells also express high levels of aryl hydrocarbon receptor (AhR) (Veldhoen et al., 2008), providing an additional cellular target for immune modulation by AhR ligands (see the section "Halogenated Aromatic Hydrocarbons").

There are several other soluble factors that contribute to inflammation that warrant discussion. In addition to ROS that are released from both macrophages and neutrophils to compromise membrane integrity, a plethora of cytokines and chemokines contribute to the inflammatory process. As mentioned in the section "Innate Immunity," some pro-inflammatory cytokines (IL-1β and IL-18) are produced in a mature form as a result of the activation of the inflammasome (Martinon et al., 2009). In fact, it has been demonstrated that inflammation as a result of disease (i.e., influenza) or toxicity (i.e., silicosis) depends on inflammasome activation (Cassel et al., 2008; Ichinohe et al., 2009). An additional discussion regarding the role of the inflammasome in immune responses to inhaled particles is provided in "Inhaled Substances." The actions of various pro-inflammatory cytokines include inducing fever or activating T cells and macrophages (IL-1); stimulating B- or T-cell proliferation (IL-6); inducing neutrophilia (IL-17); and increasing vascular permeability or inducing apoptosis (TNF-α). Many pro-inflammatory cytokines also induce acute-phase proteins, such as C-reactive protein. C-reactive protein binds to several ligands, including phosphatidylcholine on the membranes of cells, Fc receptors, and C1q (part of the complement C1 complex), which activates the classical complement cascade (reviewed by Marnell et al. [2005]). Complement participates in inflammation through inappropriate and sustained

activation of the cascade, ultimately leading to destruction of cells through the MAC (Fig. 12-3). Furthermore, complement fragments, such as C3a and C5a, act as chemotactic factors to recruit other phagocytic cells to sites of insult (macrophages, neutrophils, eosinophils, or mast cells) or induce degranulation (basophils, eosinophils, or mast cells) (Dunkelberger and Song, 2010). Finally, prostaglandins and other eicosanoids possess various pro-inflammatory actions, including increased blood flow and pain (prostaglandin E2), increase in vascular permeability (prostacyclin I2), and stimulation of endothelial inflammatory responses (thromboxane A2) (reviewed by Ricciotti and FitzGerald [2011]). Prostaglandins contribute to hyperalgesia and it is for this reason that cyclooxygenase-2, which converts arachidonic acid to other bioactive prostaglandins, is an attractive therapeutic target to reduce inflammation and pain (see the section "Therapeutic Drugs: Anti-inflammatory Drugs").

As mentioned above, it is undisputed that chronic inflammation plays a critical role in many disease states, including asthma, multiple sclerosis, cardiovascular disease, Alzheimer disease, bowel disorders, and cancer. Chronic inflammation could result in high circulating levels of pro-inflammatory cytokines, complement fragments, prostaglandins, and thromboxanes. An inflammatory cascade can be established because many of these mediators induce expression of others (i.e., TNF-α induces expression of IL-1 and IL-6 or complement fragment C3a causes chemotaxis of phagocytic cells). Moreover, the sustained presence of pro-inflammatory cytokines could exacerbate ROS production from neutrophils and macrophages. As seen in the next section, the underlying pro-inflammatoryresponse also plays a critical role in immune-mediated disease.

Immune-Mediated Disease

Recall that the purpose of the immune system is to preserve the integrity of the individual from disease states, whether infectious, parasitic, or cancerous, through both cellular and humoral mechanisms. In so doing, the ability to distinguish self from nonself plays a predominant role. However, situations arise in which the individual's immune system responds in a manner producing tissue damage, resulting in a self-induced disease. These disease states fall into two categories: (1) hypersensitivity, or allergy, and (2) autoimmunity. Hypersensitivity reactions result from the immune system responding in an exaggerated or inappropriate manner after repeated exposure to the offending antigen. These reactions have been subdivided by Coombs and Gell (1975) into four types, which represent four different mechanisms leading to tissue damage. In the case of autoimmunity, mechanisms involve a break down in self-recognition resulting in the production of autoreactive antibodies and/or TCRs recognizing self-antigens as nonself, ultimately culminating in tissue damage and disease.

Hypersensitivity

Classification of Hypersensitivity Reactions One characteristic common to all four types of hypersensitivity reactions is the necessity of prior exposure leading to sensitization in order to elicit a reaction upon subsequent challenge. In the case of Types I, II, and III, prior exposure to antigen leads to the production of allergen-specific antibodies (IgE, IgM, or IgG) and, in the case of Type IV, the generation of allergen-specific memory T cells. Fig. 12-13 illustrates the mechanisms of hypersensitivity reactions as classified by Coombs and Gell (1975). Although not completely understood, regulation of Ig production is dependent in part on the characteristics of the antigen, the genetics of the individual, and environmental factors. The mechanisms of antibody production in hypersensitivity reactions are identical to those described earlier

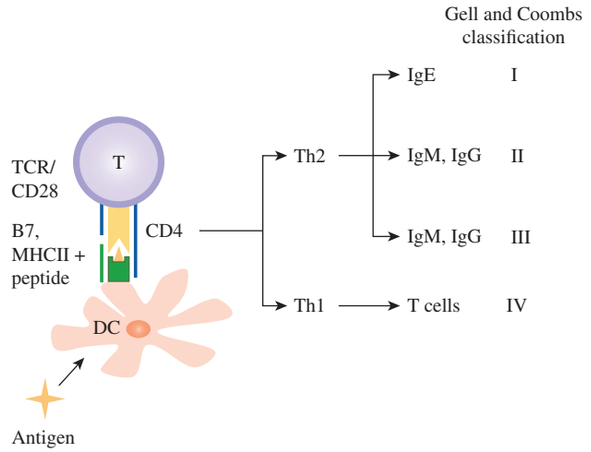

Figure 12-13. *Overview of classification of hypersensitivity reactions.* Hypersensitivity reactions are mediated via T cells and antibody production.

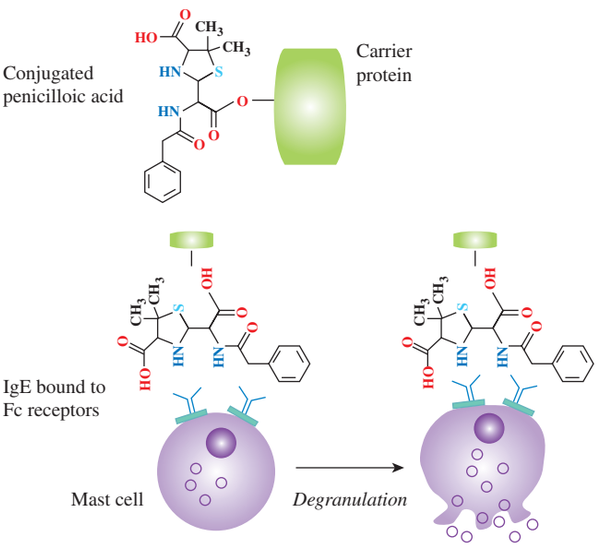

Figure 12-14. *Type I hypersensitivity reaction.* Metabolized penicillin is a hapten that conjugates with a protein. The conjugated hapten crosslinks IgE antibodies on mast cells. IgE crosslinking causes mast cell degranulation, releasing histamine and other pro-inflammatory mediators.

in the chapter (Fig. 12-10). A brief description of the four types of hypersensitivity reactions is presented below.

Type I (Immediate or IgE-Mediated Hypersensitivity) Using penicillin as an example, Fig. 12-14 depicts the major events involved in a Type I hypersensitivity reaction (what most people think of as "allergy" and is clinically referred to as "atopy"). Sensitization occurs as the result of dermal exposure to antigens or by exposure to antigens through the respiratory or gastrointestinal tract. Most people would mount an IgM, IgG, or IgA immune response to these antigens and clear them without causing any allergic symptoms. It is unclear why these antigens become allergens in certain individuals who respond by mounting an IgE immune response instead, but the mechanism appears to involve genetic and/or environmental determinants and likely some type of triggering event (e.g., acute pathogen exposure and emotional stress). IgE production is highest in lymphatic tissues that drain sites of exposure (i.e., tonsils, bronchial lymph nodes, and intestinal lymphatic tissues, including Peyer's patches) and is low in

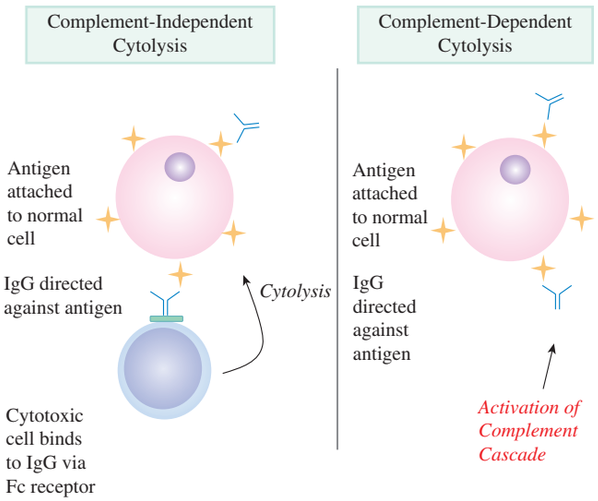

Figure 12-15. *Type II hypersensitivity reactions.* In complement-independent cytolysis, antigen becomes attached to a normal cell, which can be recognized by IgG. A cell capable of cytolysis (CTL, NK cell) binds to IgG via its Fc receptor and kills the antigen-coated cell. In complement-dependent cytolysis, antigen becomes attached to a normal cell, which can be recognized by IgG. Complement gets activated by the classical pathway (antigen–antibody complexes).

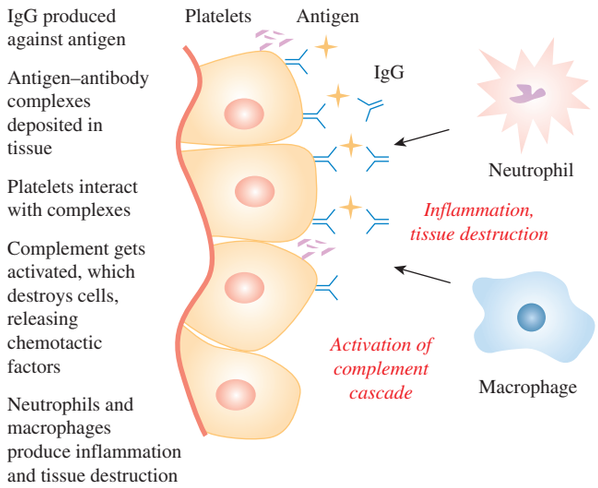

Figure 12-16. *Type III hypersensitivity reactions.* IgG is produced against an antigen and antigen–antibody complexes form, which can become deposited in tissue. Complement gets activated by the classical pathway (antigen–antibody complexes) and platelets also interact with complexes. Following complement-mediated cytolysis, released chemotactic factors attract neutrophils and macrophages, causing additional inflammation and tissue damage.

the spleen. Compared to other Igs, IgE has a low serum concentration and a short serum half-life (Table 12-6). Once produced, soluble IgE not only binds to local tissue mast cells, but also enters the circulation, where it binds to circulating mast cells, basophils, and tissue mast cells at distant sites. Once an individual is sensitized, reexposure to the antigen results in binding to IgE on local mast cells and degranulation with the release of preformed mediators and cytokines that recruit and activate circulating eosinophils, basophils, macrophages, and neutrophils leading to the synthesis and release of more cytokines and of leukotrienes and thromboxanes. These mediators promote vasodilation, bronchial constriction, and inflammation. Clinical manifestations can vary from urticarial skin reactions (wheals and flares) to signs of hay fever, including rhinitis and conjunctivitis, to more serious diseases, such as asthma and potentially life-threatening anaphylaxis. These responses may begin within minutes of reexposure to the offending antigen; therefore, Type I hypersensitivity is often referred to as immediate hypersensitivity.

Type II (Antibody-Dependent Cytotoxic Hypersensitivity) Type II hypersensitivity is IgG or IgM-mediated. A foreign antigen attached to the surface of a cell or tissue may mediate the antibody response. Conversely, an antibody response could be mediated by an autoantibody due to a breakdown in tolerance and the resulting response would be part of an autoimmune disease (e.g., autoimmune hemolytic anemias and Goodpasture syndrome). Fig. 12-15 shows the mechanisms of action for complement-independent and complement-dependent cytotoxic reactions. Tissue damage may result from the direct action of cytotoxic cells, such as macrophages, neutrophils, or eosinophils, linked through the Fc receptor to antibody-coated target cells (complement-independent) or by antibody-induced activation of the classic complement pathway. Complement activation may result in C3b binding to the target cell surface with C3b acting as a recognition site for effector cells. Alternatively, the MAC may be bound to the target cell surface, resulting in direct cell lysis.

Type III (Immune Complex–Mediated Hypersensitivity) Type III hypersensitivity reactions also involve IgG or IgM. The distinguishing feature of Type III is that, unlike Type II, in which Ig production is against specific cellular or tissue-associated antigen, Ig production is against soluble antigen in the serum (Fig. 12-16). This allows for the formation of circulating immune complexes composed of a lattice of antigen and Ig, which may result in widely distributed tissue damage in areas where immune complexes are deposited. The most common location is the vascular endothelium in the lung, joints, and kidneys. The skin and circulatory systems may also be involved. Pathology results from the inflammatory response initiated by the activation of complement. Macrophages, neutrophils, and platelets attracted to the deposition site contribute to the tissue damage. As with Type II hypersensitivity, responses similar to Type III hypersensitivity can be induced in autoimmune diseases due to autoantibodies directed against soluble antigens such as double-stranded DNA or small nuclear proteins as seen with systemic lupus erythematosus (SLE). In addition, antibody responses directed to protein therapeutics/biologics can lead to the formation of immune complexes with the drug and, if the appropriate size, can result in Type III hypersensitivity responses. These responses can manifest either as infusion reactions following dosing due to complement activation and cytokine release, loss of platelets, or tissue damage (Krishna and Nadler, 2016).

Type IV (Cell-Mediated Hypersensitivity) Type IV, or DTH responses, can be divided into three classes: contact hypersensitivity, tuberculin-type hypersensitivity, and hypersensitivity pneumonitis. The following discussion is focused on contact hypersensitivity using the classical example of contact hypersensitivity seen following poison ivy exposure. Contact hypersensitivity is initiated by topical exposure, and the associated pathology is primarily epidermal. It is characterized clinically by an eczematous reaction at the site of allergen contact and, like Type I through III responses, consists of two phases: sensitization and elicitation. However, in this case sensitization is the result of the development of activated and memory T cells as opposed to antibody production (Figs. 12-17 and 12-18). Sensitization occurs when a hapten

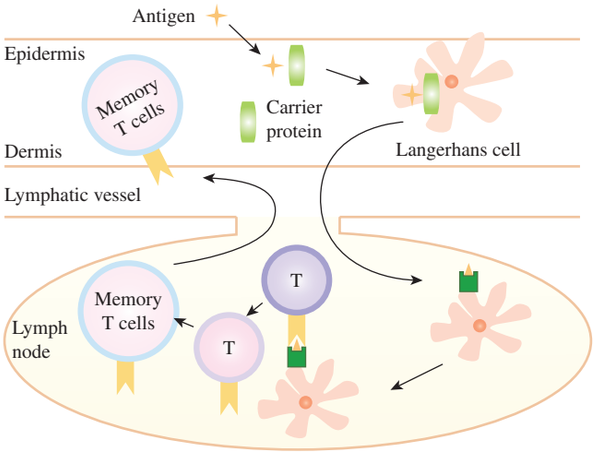

Figure 12-17. *Type IV hypersensitivity sensitization.* A hapten permeates the epidermis and forms a complex with a carrier protein. The conjugated hapten gets engulfed by Langerhans DCs, which migrate to the lymph node and present antigen to $CD4^+$ T cells in the context of MHCII. $CD4^+$ T cells activate, proliferate, and differentiate into Th cells and memory T cells.

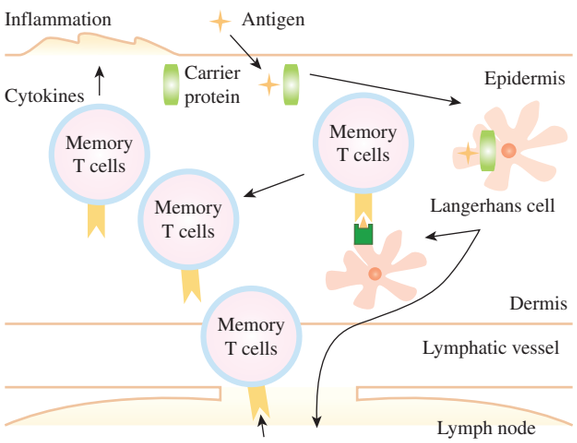

Figure 12-18. *Type IV hypersensitivity elicitation.* Upon secondary exposure to the hapten, Langerhans DCs either migrate to the lymph node or directly present antigen to memory T cells. Memory T cells become activated and produce cytokines to stimulate pro-inflammatory cytokine release from other cell types, such as keratinocytes.

penetrates the epidermis and forms a complex with a protein carrier. The hapten–carrier complex is processed by Langerhans DCs that migrate out of the epidermis to the local lymph nodes. There, the APC presents the processed antigen to $CD4^+$ Th cells, leading to clonal expansion and the generation of memory T cells including memory $CD8^+$ CTL, which appear to play a major role in the elicitation phase.

Upon secondary contact, Langerhans DCs present the processed hapten–carrier complex to memory $CD8^+$ CTL in either the skin or the lymph nodes. These activated T cells then secrete cytokines that bring about further proliferation of T cells and generation of CTLs, as well as increased expression of adhesion molecules on the surface of keratinocytes and endothelial cells in the dermis. Both the expression of adhesion molecules and the secretion of pro-inflammatory cytokines by T cells and keratinocytes facilitate the recruitment of inflammatory cells into the skin, resulting in erythema and the formation of papules and vesicles. In addition and under certain circumstances, intracellular proteins can be modified by lipid-soluble chemicals that might readily cross the cell membrane. These cells then present modified peptides on their cell surface in conjunction with MHCI molecules. $CD8^+$ cytotoxic T cells recognize these foreign peptides and cause tissue damage by either direct cytotoxic action (CTL) or the secretion of cytokines that further promote the inflammatory response.

Table 12-7

Sites of Autoimmune Reactions

SITE/TARGET	DISEASE EXAMPLE
Nuclei—antibodies against DNA or histones	Systemic lupus erythematosus
Red blood cells	Hemolytic anemia
Striated muscle—cholinergic receptors	Myasthenia gravis

Autoimmunity Autoimmune disease occurs when the reactions of the immune system are directed against the body's own tissues, and, as with hypersensitivity, is characterized by a genetic susceptibility. Autoimmune diseases may be tissue-specific, in which the damage is associated with a specific type of tissue or a specific organ, or tissue-nonspecific, in which the signs and symptoms are associated with several organs and tissues. The targets from the perspective of the primary sites of tissue damage in autoimmune disease are many and varied (Table 12-7). Both humoral immunity and CMI can be involved as effector mechanisms in causing the damage in autoimmune conditions. The terms "hypersensitivity" and "autoimmunity" are often confused and are certainly interrelated. Based on their definitions, a hypersensitivity response can be a mechanism by which an autoimmune disease is produced.

In the section on hypersensitivity presented above, two mechanisms by which host tissues are damaged by the host's own immune system were discussed (Types II and III), resulting in autoimmune-like disease. It is important to emphasize that in the case of hypersensitivity reactions, unaltered self-antigens are not the target of the immune mechanisms, but damage occurs to cells bearing a hapten on their membranes, or nonspecifically to innocent bystander cells in close proximity to antigen–antibody complexes that activate the complement cascade. For example, damage produced in autoimmune Goodpasture disease is similar to that induced by Type III hypersensitivity reactions in the lung due to trimellitic anhydride (TMA) exposure (see the section "Acid Anhydrides"). Although the resulting pathology may be very similar for autoimmune reactions and hypersensitivity, the precipitating mechanisms for true autoimmune disease are distinct from hypersensitivity. Specifically, in cases of autoimmunity, self-antigens are the target of the immune response, and in the case of chemical-induced autoimmunity, the disease state is induced by a modification of host tissues or immune cells by the chemical and not the chemical acting as an antigen/hapten as in hypersensitivity reactions.

Mechanisms of Autoimmunity As discussed earlier, the rearrangement and recombination of the genes that comprise Ig and TCR result in tremendous diversity in the potential antigen recognition of B cells and T cells, respectively. Ideally, during development those lymphocytes recognizing self-antigens will largely be deleted by negative selection as central tolerance is established. Autoreactive clones that escape central tolerance and migrate to

the periphery are normally controlled by peripheral tolerance mediated by various mechanisms that ultimately induce anergy or clonal deletion. For autoimmune disease to occur, an autoreactive clone must escape central tolerance, pass into the periphery, and bind with specificity to its self-antigen; then mechanisms of peripheral tolerance must fail, and the autoreactive clone must induce a detrimental immunological response. Clearly, induction of autoimmunity is multifaceted and likewise has been associated with several mechanisms primarily related to insufficient peripheral tolerance. Conversely, defects in central tolerance are rarely encountered. However, an example of autoimmunity due to defective central tolerance is the disease autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, which results from a defective transcriptional regulator called autoimmune regulator (AIRE). For the negative selection process in the thymus, AIRE induces the expression of a wide variety of self-antigens normally found in the periphery. Loss of AIRE results in release of autoreactive T cells to the periphery. Several mechanisms have been associated with the breakdown of peripheral tolerance and prime events for the onset of autoimmune disease. These mechanisms include inflammation; molecular mimicry by pathogen antigens; inherent defects in T or B cells including regulatory subsets, APCs, cytokines, or complement; and epitope spreading. For example, increasing evidence supports a potential breakdown of peripheral tolerance during an inflammatory response, which may be mediated by the inappropriate maturation of immature DCs that process both pathogenic antigens and self-antigens released during the inflammatory response. Self-antigens could then be presented to naive autoreactive T cells via MHC. With molecular mimicry, a pathogen presents epitopes that resemble those on self-antigens, and because the pathogen will induce a strong inflammatory response, peripheral tolerance may be overridden. Additionally, interference of normal immune regulation by Tregs or Bregs could create an environment conducive to autoimmune disease. Finally, epitope spreading may occur during an immune response due to tissue damage and the release of new epitopes that were hidden either because they were sequestered from the immune system or their expression levels were insufficient to induce negative selection or peripheral tolerance mechanisms.

Effector mechanisms involved in autoimmune disease can be the same as those described earlier for Types II and III hypersensitivity or, in the case of pathology associated with solid tissues, including organs, they may involve $CD8^+$ CTL. Tissue damage associated with CTL may be the result of direct cell membrane damage and lysis, or the result of cytokines produced and released by the T cell. TNF-β (lymphotoxin) has the ability to kill susceptible cells, and IFN-γ may increase the expression of MHCI on cell surfaces, making them more susceptible to $CD8^+$ cells. Cytokines may also be chemotactic for macrophages, which can cause tissue damage directly or indirectly through the release of pro-inflammatory cytokines. As is the case with hypersensitivity reactions, autoimmune disease is often the result of more than one mechanism working simultaneously.

Developmental Immunology

A sequential series of carefully timed and coordinated developmental events, beginning early in embryonic/fetal life and continuing through the early postnatal period, is required to establish a functional immune system in all mammals, including humans (Fig. 12-19). The immune system develops initially from a population of pluripotent HSCs that are generated early in gestation from uncommitted mesenchymal stem cells in the intraembryonic splanchnopleure surrounding the heart. This early population of HSCs gives rise to all circulating blood cell lineages, including cells of the immune system, via migration through an orderly series of tissues, and a dynamic process that involves continual differentiation of lineage-restricted stem cells. Lymphoid-hematopoietic progenitor cells are established via the migration of these cells from intraembryonic mesenchyme to fetal liver and fetal spleen, and ultimately, the relocation of these cells in late gestation to bone marrow and thymus. The latter two organs are the primary sites of lymphopoiesis and appear to be unique in providing the microenvironment factors necessary for the development of functionally competent immune cells. These lineage-restricted stem cells expand to form a pool of highly proliferative progenitor cells that are capable of continual renewal of short-lived functional immunocompetent cells (Fig. 12-2), and that ultimately provide the necessary cellular capacity for effective immune responsiveness, and the necessary breadth of the immune repertoire (Good, 1995).

It is important to recognize that immune system development does not cease at birth, and that immunocompetent cells continue to be produced from proliferating progenitor cells in the bone marrow and thymus (Fig. 12-2). Mature immunocompetent cells leave these primary immune organs and migrate via the blood to the secondary immune organs: spleen, lymph nodes, and mucosal lymphoid tissues. Because birth occurs at various stages of fetal maturity in different species, the significance of parturition as a landmark in the development of the immune system can vary from species to species (Holsapple et al., 2003). As such, direct comparison of immune functional development between humans and animals is complicated by differences in the maturity of the immune system before and after parturition. This difference has been linked to the length of gestation (Holladay and Smialowicz, 2000), in that animals with short gestation periods (e.g., mice, rats, rabbits, and hamsters) have relatively immature immune systems at birth compared to humans. The onset of functional immune competence depends on the specific parameter being measured, and varies across species with striking differences noted between rodents and humans (Holsapple et al., 2003). The immune system of children continues to develop postnatally until 5 to 12 years of age (Miyawaki et al., 1981). Exposure to specific antigens during the perinatal period results in a rapidly expanding accumulation of lymphocyte specificities in the pool of memory cells in secondary lymphoid tissues. As thymic function wanes and thymocytes are no longer produced in that tissue, it is this pool of memory B and T cells that maintains immune competence for the life of the individual. Senescence of immunity is associated with reductions in both innate and acquired immune responses to antigens during the last quartile of life. This failure of the immune response is due, in part, to a continual reduction in the production of newly formed cells, and to the decreased survival of long-lived memory cells in lymphoid tissues. The concept of immune senescence is discussed further below.

One feature of the developing immune system that clearly distinguishes it from the mature immune system, especially during gestation, is the role played by organogenesis. Defects in the development of the immune system due to heritable changes in the lymphoid elements have provided clinical and experimental examples of the devastating consequences of impaired immune development (Rosen et al., 1995). Therefore, the effects of chemicals on the genesis of critical immune organs in the developing fetus may be more important than effects on these tissues after having been populated by hematopoietic and lymphoid cells. However, a chemical that induces this kind of developmental structural abnormality in the fetus would legitimately be classified as a teratogen, prompting the question as to how many known teratogens would have an impact on organs essential to the normal functioning of the immune system.

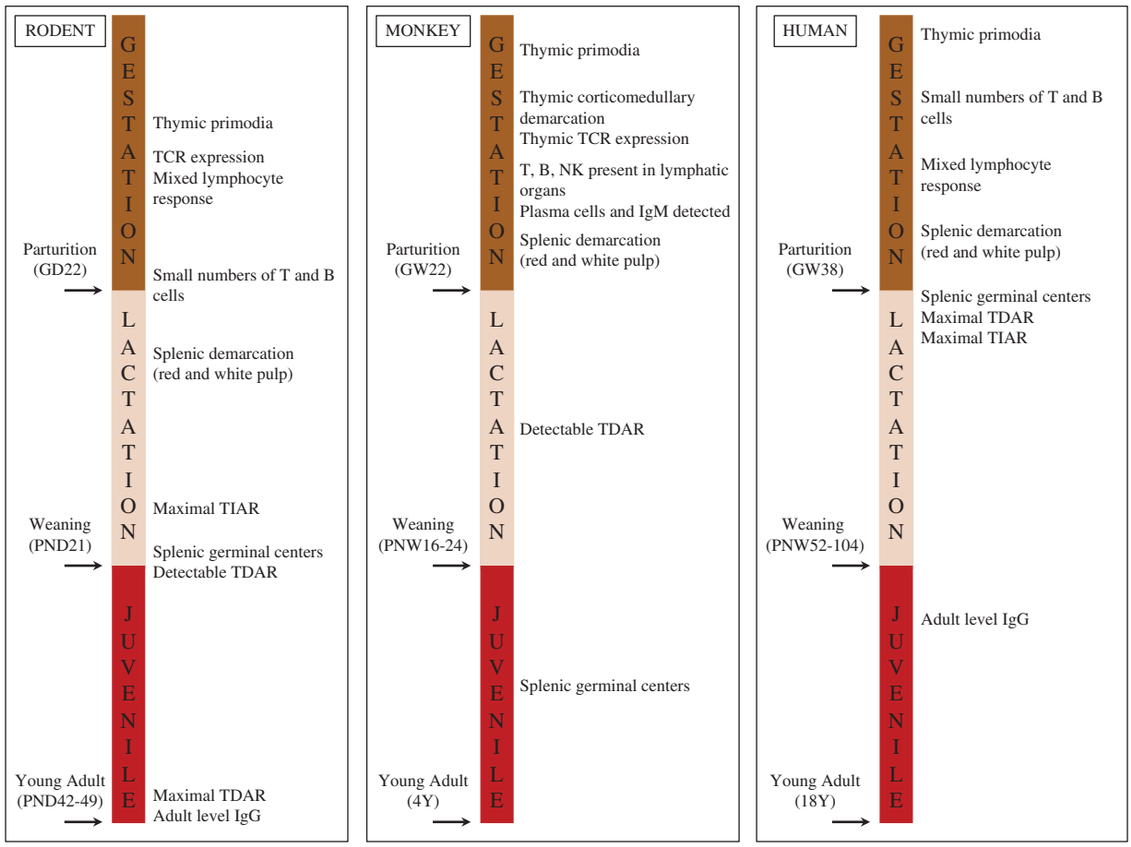

Figure 12-19. *Developmental immunology.* Comparison of major developmental milestones in human, monkey, and mouse. Species-specific timelines for gestation, lactation, and juvenile stages are also presented. GD, gestational day; GW, gestational week; PND, postnatal day; PNW, postnatal week.

At the other end of the spectrum from the development of the immune system during gestation and the perinatal period is the status of the immune system in the aging population. Unlike the developing immune system, there have been very few studies that have addressed the impact of specific xenobiotics on the aging immune system. Nonetheless, it is known that the immune system of older people is usually perceived as declining in fidelity and efficiency with age resulting in an increased susceptibility to infectious diseases and pathological conditions relating to inflammation (e.g., cardiovascular disease and Alzheimer's Disease) or autoreactivity (e.g., rheumatoid arthritis, RA) (Caruso et al., 2009). Immune senescence has been defined as the impairment in immunity as a result of age-associated changes in function in a variety of cell types. It is a phenomenon of decreased function, involving changes in both innate and adaptive immunity, and a disbalance between the two arms (Pawelec et al., 2010). Virtually, all components of the immune system are affected (Ongradi and Kovesdi, 2010). The decline in the B-cell compartment is responsible for the decreased effectiveness of vaccines in older people, and the decline in immune surveillance (e.g., the T-cell compartment and NK-cell function) is associated with an increased incidence in malignancy. Additionally, aging has been associated with a shift from the Th1 to the Th2 cytokine profile in response to immune stimulation, and the overproduction of Th2 cytokines could augment B-cell production of autoreactive antibodies. Studies in humans and mice have shown that TLR expression and function decline with age resulting in the decreased production of pro-inflammatory cytokines and chemokines. From a mechanistic standpoint, a number of factors have been proposed as contributing to the onset and severity of immune senescence including the accumulation of damage to lymphocytes by oxidative stress, persistent microbial infections with immunosuppressive viruses that establish latency, such as cytomegalovirus and Epstein–Barr virus, and lifestyle factors such as smoking, alcohol use, and poor nutrition. An improved understanding of immune dysfunction in human aging will increase the probability of discovering means to restore appropriate function and alleviate the burden of infectious disease later in life (Ongradi and Kovesdi, 2010).

Neuroendocrine Immunology

There is overwhelming evidence that cytokines, neuropeptides, neurotransmitters, and hormones, as well as their receptors, mediate interactions between the CNS, endocrine, and immune systems. Because receptors for neuropeptides, neurotransmitters, and hormones are present on lymphoid cells, some chemicals exert their immunomodulatory effects indirectly on the immune system by acting to modulate the activity of the nervous or endocrine systems. In addition, immune cells secrete peptide hormones and neurotransmitters, which can have autocrine (immune system) and paracrine (endocrine and nervous systems) effects. Similar to the complexity of the cytokine network, the effects of various hormones and neurotransmitters are too vast to discuss here. However, it is evident, even from this brief overview of the immune system and its potential interaction with other biological systems, that immune responses are complex processes involving multiple cell types and mediators (reviewed by Kaplan et al. [2015]).

ASSESSMENT OF IMMUNOLOGIC INTEGRITY

For many years, it has been widely established that xenobiotics can exert significant effects on the immune system as evidenced by changes in circulating and lymphoid organ-associated leukocyte number, proliferative capacity, and in effector functions including defense against a broad array of bacterial, parasitic, and viral pathogens. During the establishment of the subdiscipline of immunotoxicology, a significant emphasis was placed on the development of a standardized battery of tests to evaluate immune competence. Among the unique features of the immune system is the ability of immune cells to be removed from the body and to function in vitro. This unique quality makes it possible to comprehensively evaluate the actions of xenobiotics on the immune system employing in vivo, ex vivo, and in vitro approaches to dissect the cellular, biochemical, and molecular mechanisms of action of xenobiotics. While standard toxicological endpoints such as organ weights, cellularity, and enumeration of cell subpopulations are important components in assessing when an agent is capable of altering the immune system, by far the most sensitive indicators of immunotoxicity are the tests that induce the various cell types within the immune system to respond functionally to exogenous stimuli (White, 1992; Descotes, 2004). Employing such a battery of functional assays whereby different cell types can be evaluated not only for their effector functions, but also in certain cases for their ability to participate as accessory cells in an immune response can provide important mechanistic information concerning which cell type(s) within the immune system are, in fact, targeted by a xenobiotic. This section focuses on selected in vivo, ex vivo, and in vitro tests currently used for evaluating immunotoxicity, as well as models and approaches that can be utilized for elucidation of the mechanism of action.

The current state-of-the-science of immunotoxicity testing can be found in *Immunotoxicology Strategies for Pharmaceutical Safety Assessment* by Herzyk and Bussier (1st edition, 2008) or in *Immunotoxicity Testing: Methods and Protocols (Methods in Molecular Biology)* by Dietert (2010).

General Assessment

Central to any series of studies evaluating immune competence is the inclusion of standard toxicological studies, because any immunologic finding should be interpreted in conjunction with effects observed on other target organs. Standard toxicological studies that are usually evaluated include body and selected organ weights, general observations of overall animal health, selected serum chemistries, hematologic parameters, and status of the bone marrow (ability to generate specific colony-forming units). In addition, histopathology of lymphoid organs, such as the spleen, thymus, and lymph nodes, may provide insight into potential immunotoxicants. General guidelines for the histopathological examination of each of these lymphoid organs have been reviewed (Elmore, 2010).

Because of the unique nature of the immune system, there are several experimental approaches that may be taken to assess immunotoxicity and to evaluate the mechanisms of action of xenobiotics. These are depicted in Fig. 12-20 and vary with respect to in vivo or in vitro exposure, immunologic challenge (i.e., the stimulus employed to induce an immune response), or immunologic evaluation (immune assay). As an example, Fig. 12-20(b) is an ex vivo assay in which xenobiotic exposure and antigen challenge occur in vivo and the immune response is evaluated in vitro. In contrast, as depicted in Fig. 12-20(c), splenocytes can be isolated after in vivo

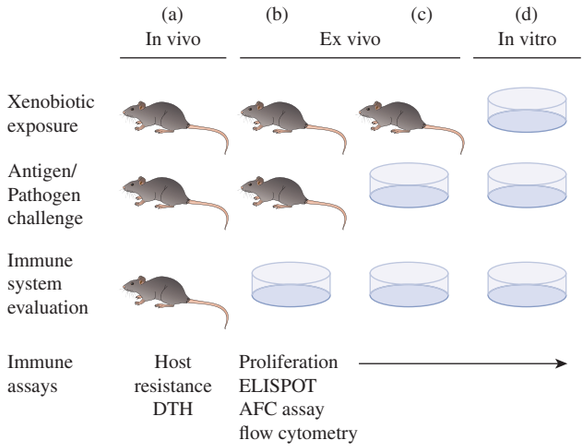

Figure 12-20. *Approaches for assessing the immunotoxicity of xenobiotics.* The ability to stimulate immune cells in vitro allows for much flexibility in the assessment of immunotoxicity of xenobiotics.

treatment with a xenobiotic, sensitized with an antigen in vitro and evaluated in vitro; or as depicted in Fig. 12-20(d), splenocytes can be removed from a naive (untreated) animal, exposed to a xenobiotic and antigen in vitro, and evaluated in vitro. If splenocytes are sensitized in vitro with sheep red blood cells (sRBCs) to generate antibody forming cells (AFCs), this is known as the Mishell–Dutton assay (Mishell and Dutton, 1967).

Functional Assessment

Innate Immunity As described earlier, innate immunity encompasses all those immunologic responses that are not induced through antigen receptors (i.e., TCR or BCR) and for which there is limited immunological memory. These responses include phagocytosis of pathogens and release of ROS as a killing mechanism by macrophages, neutrophils, and monocytes; recognition of tumor cells by NK cells; and the lytic activity of the components of the complement cascade.

To evaluate phagocytic activity in vitro or ex vivo, plate-based or flow cytometric methods can be used to measure the amount of fluorophore-labeled foreign matter engulfed by phagocytic cells. For example, phagocytic cells, isolated from tissues including blood, peritoneal cavity fluid, and bronchial alveolar lavage fluid that adhere to tissue culture plates or whole blood containing monocytes and granulocytes, are incubated with fluorophore-labeled particles such as opsonized *Escherichia coli* or polystyrene beads in the absence or presence of additional stimuli (e.g., mitogens such as phorbol myristate acetate (PMA) or chemotactic peptide *N*-formyl-methionine-leucine-phenylalanine [fMLP]). To quantify the overall degree of fluorescence or the number of particles engulfed, cell samples are then analyzed by a microplate fluorimeter, fluorescent microscopy, or flow cytometry.

During phagocytosis, ROS are generated in response to soluble antigens to kill the pathogen. There are several in vitro or ex vivo methods available to measure this functional response, termed respiratory burst, that are based on quantifying the production of intracellular or extracellular ROS such as free oxygen radicals, nitric oxides, or hydrogen peroxide. These methods, which can be plate- or flow cytometry–based, quantify the fluorescence elicited by oxidation of a fluorogenic substrate. As with the phagocytosis assays, phagocytic adherent cells isolated from tissues including blood, peritoneal cavity fluid, and bronchial alveolar lavage fluid or

whole blood, which contains monocytes and neutrophils, are incubated with a substrate (e.g., dihydrorhodamine 123 or 6-carboxy-2,7-dichlorodihydrofluorescien diacetate) that is internalized. The cells are then incubated with a respiratory burst stimulator such as phorbol esters, opsonized *E. coli*, or zymosan and the amount of fluorescence is recorded using a microplate fluorimeter, or by flow cytometry that also allows for the identification of the cell type(s) affected (Freebern et al., 2013).

Phagocytosis can also be assessed in vivo. An older method involved intravenous injection of radiolabeled sRBCs (^{51}Cr-sRBCs), which are removed from the circulation by tissue macrophages and sequestered for degradation in organs such as the liver, spleen, lymph nodes, lung, and thymus. Clearance of the ^{51}Cr-sRBCs is monitored by sampling the peripheral blood. After steady state has been attained, organs are assayed using a gamma counter to assess uptake of the ^{51}Cr-sRBCs. Similar to in vitro methods, newer methods to assess in vivo phagocytosis involve injection of fluorophore-labeled particles, including nanoparticles or bacteria (Tartaro et al., 2015).

When phagocytic cells encounter antigens, especially those associated with bacteria or a virus, they undergo activation, which is induced, in part, through interactions with TLRs. Extracellular antigens are processed and presented in association with MHCII, while intracellular antigens are processed and presented in association with MHCI, as described in the section "Antigen Processing." Concomitant with antigen encounter by the phagocyte, there is an increase in the expression of MHCI and/or MHCII as well as the costimulatory molecules CD80/CD86. The simultaneous increase in MHC molecules in combination with CD80 and CD86 is critically important in order for the phagocytic cell to drive T-cell effectors ($CD4^+$ and $CD8^+$ T cells). To further assess phagocytic cell function and the influence of an immunotoxicant, the upregulation of MHCI and/or MHCII and CD80 and CD86 can be readily quantified by flow cytometry (see the section "Flow Cytometric Analysis") after the activation of phagocytic cells, primarily those of the monocytic lineage. These measurements are especially informative when performed in combination with cell surface markers that are capable of identifying monocytic lineage subpopulations such as tissue macrophages, conventional DC, and plasmacytoid DC because they provide information on whether the immunotoxicant interferes with the activation and/or differentiation of the phagocytic cell and its antigen presenting capability. The types of macrophages and DCs, their hematopoietic origin, and a general discussion of the different assays used to assess their functional status have been reviewed (Barnett and Brundage, 2010; Satpathy et al., 2012).

Evaluation of the ability of NK cells to lyse tumor cells is achieved using species-specific tumor cell lines (e.g., murine YAC-1 or human K562 cell line) as a tumor target for an in vitro cytotoxicity assay. Tumor cells are radiolabeled with ^{51}Cr and incubated in specific effector-to-target ratios with splenocytes or peripheral blood mononuclear cells (PBMCs) from xenobiotic-exposed and nonexposed animals. During an incubation step, NK cells (effectors) lyse the ^{51}Cr-tumor cells, releasing ^{51}Cr into the supernatant. At the end of the incubation, culture plates are centrifuged and the supernatant is removed and counted by a gamma counter. After correcting for spontaneous release (which should be less than 10%), specific release of ^{51}Cr is calculated for each effector-to-target ratio and compared to the specific release from control animals. To avoid the use of radionucleotides, a number of flow cytometry–based assays have been developed utilizing fluorescent stains to identify live and dead target cells. One such assay involves the co-culture of NK cells with targets cells that have been stained with the fluorescent green dye 3,3′- dioctadecyloxacarbocyanine perchlorate (DiO) (Kane et al., 1996). Utilizing DiO allows for readily discriminating between target and nontarget cells while propidium iodide is used to distinguish between live and dead cells. The various methods for NK assays used in immunotoxicity testing have been reviewed (Li, 2010).

More recently, NK cell activity is also assessed by quantifying the expression of lysosome-associated membrane protein-1 (LAMP-1, CD107a). As degranulation of NK cells occurs, secretory lysosomes are released and CD107a is transported to the surface of the NK cells. Once on the surface, CD107a can be detected by antibody binding and allow for the identification of activated NK cells, thus serving as a biomarker for NK cell functional activity. Specifically, human or nonhuman primate (NHP) NK cells (PBMC or whole blood) are co-cultured with either K562 cells or PMA and ionomycin and the percentage of CD3, CD56/CD159 (NK cell surface markers), and CD107a positive cells are quantified by flow cytometry.

Acquired Immunity: Humoral The AFC assay is a sensitive indicator of immunologic integrity for several reasons. It is a test of the ability of the host to mount an antibody response to a specific antigen. When a T-cell-dependent antigen (an antigen that requires T cells to help B cells make antibody) like sRBC is used, this response, referred to as the T-cell-dependent antibody response (TDAR), requires the coordinated interaction of several different immune cells: APC (macrophages and DCs), T cells, and B cells. Therefore, functional impairment of any one of these cell types (e.g., antigen processing and presentation, cytokine production, proliferation, or differentiation) can alter the ability of B cells to produce antigen-specific antibodies. Other antigens, termed T-cell-independent antigens, such as dinitrophenyl-Ficoll or trinitrophenyl-LPS, can also be used that bypass the requirement for accessory T-cell help in eliciting antibody production by B cells, and is thus termed a T-cell-independent antibody response (TIAR). Although the AFC assay in response to sRBC was developed in rodents almost half a century ago, its sensitivity in identifying immunotoxicants has resulted in worldwide application for the purpose of hazard identification, which extends to present day (see the section "Historical Perspective on Regulatory Guidance in Immunotoxicology"). Indeed, an overview of the TDAR to sRBCs was described as the "gold standard" for evaluating the potential adverse effects of xenobiotics on the immune system (White et al., 2010).

A standard AFC assay involves immunizing control and xenobiotic-exposed mice either by intravenous or intraperitoneal injection with sRBCs. The antigen is taken up in the spleen and an anti-sRBC antibody response is induced. In the mouse, the peak day of antibody production is 4 days after immunization. Historically, the antibody response has been quantified by enumerating the number of plasma cells (also known as AFCs or ASCs) present in the spleen that are secreting anti-sRBC antibodies. This can be accomplished by removing the spleen and making a spleen cell suspension, which is then mixed with sRBC, complement, and melted agar. This mixture is poured onto a petri dish and covered with a cover slip. After the agar solidifies, the petri dishes are incubated for 3 hours at 37°C. B cells that have been appropriately activated by the antigen and have differentiated into plasma cells during the 4 days post antigen immunization are now actively secreting large quantities of anti-sRBC IgM antibody. When the complement and the secreted IgM coat sRBCs that surround the plasma cells, areas of hemolysis (plaques) appear against a lawn of sRBC that can be enumerated. At the center of each plaque is a single plasma cell

(AFC). Data are usually presented as IgM AFCs per million splenocytes. IgG AFCs can also be enumerated by slight modifications of this same assay. This isotype switching (from IgM to IgG) is important in secondary responses in which memory B cells respond more quickly to an antigen.

A second method to enumerate AFCs is by ELISPOT. This method is similar to the plaque assay described above with the exception that the splenocytes isolated from sRBC-sensitized mice are incubated on ELISPOT microtiter plates coated with either antimouse IgM or sRBC membranes to capture the secreted IgM. After incubation, the spleen cells are removed using a washing buffer followed by incubation with an enzyme-conjugated antimouse IgM antibody. A second washing step is used to remove unbound enzyme-conjugated antimouse IgM followed by addition of the chromogen substrate. The site at which each IgM secreting cell was present in the well of the ELISPOT microtiter plate is now visualized by the formation of a "spot" through the enzymatic conversion of the chromogenic substrate. Typically enumeration is performed using an ELISPOT reader that can rapidly quantify the number of spots (number of plasma cells) as well as the size and intensity (quantity of IgM being secreted) of the spots. It is noteworthy that in addition to enumeration of AFCs, the ELISPOT method can be applied to quantification of any secreted protein for which a suitable capture antibody exists. In evaluations of immunocompetent cells, the ELISPOT is especially useful for enumerating cytokine and chemokine secreting cells in which the desired endpoint is effector cell enumeration.

A third way the anti-sRBC humoral immune response can be evaluated is ex vivo using serum from peripheral blood of immunized mice and an enzyme-linked immunosorbent assay (ELISA) or other immunoassay methods. Although the optimal response is delayed by 1 to 2 days (compared to the AFC assay), this approach takes into account antigen-specific antibody secreted by B cells in the spleen as well as B cells residing in the bone marrow and lymph nodes. Peripheral blood is collected from mice (or other experimental animals) 6 days postimmunization with sRBCs. Serum from each sample is serially diluted and incubated in microtiter plates that have been coated with sRBC membranes or antimouse IgM, similar to that described above for the ELISPOT assay. After incubation of the test sera and a wash step, an enzyme-conjugated monoclonal antibody (the secondary antibody) against IgM (or IgG) is added. This antibody recognizes the IgM (or IgG) and binds specifically to that antibody. After incubation and a wash step, the chromogenic substrate is added. Conversion of the substrate by the enzyme conjugated to the secondary antibody results in a color change, which can be quantified by quantifying absorbance with a plate reader. Because this is a kinetic assay (color develops over time and is dependent upon the concentration of anti-sRBC antibody in the test sera), it is important to establish control concentration–response curves so that data can be evaluated in the linear range of the curve. In addition to evaluating serum, the supernatant from in vitro AFC assays can also be evaluated by ELISA. An advantage of the ELISA over the AFC assay and ELISPOT is the ability to attain a greater degree of flexibility, because serum or supernatant samples can be stored frozen for analysis at a later date. In contrast, a disadvantage of the ELISA approach is that it provides no information on the actual number of plasma cells that were induced by sRBC sensitization. Other T-cell-dependent antigens, including keyhole limpet hemocyanin (KLH) and vaccines (e.g., tetanus toxoid or hepatitis B surface antigen), are routinely used in multiple species including humans to drive an antigen-specific antibody response and to assess the potential for immunotoxicity (Plitnick and Herzyk, 2010). In larger species than mice, blood samples can be collected over multiple time points following immunization allowing for an assessment of the kinetics of the antibody responses, including different antibody isotypes.

B cell to plasma cell differentiation can also be monitored by flow cytometry. This can be accomplished utilizing intracellular staining for IgM (or other antibody isotypes such as IgG) to identify IgM^{high} cells in combination with the transmembrane proteoglycan, CD138, also termed syndecan-1, which is a hallmark of a fully differentiated plasma cell (Costes et al., 1999). It is noteworthy that even in resting B cells (i.e., not activated with antigen), background levels of intracellular IgM are readily detectable because the monomeric form of IgM serves as the BCR. For this reason, the designation IgM^{low} typically refers to a resting B cell versus IgM^{high}, which is indicative of high-level IgM expression as exhibited by plasma cells.

One final assay measures the ability of B cells to undergo blastogenesis and proliferation, which are critical steps in the generation of an antibody response. Lymphocyte proliferation is most often quantified either using a radioisotope-based assay or by flow cytometry. Splenocytes are stimulated in microtiter plates with a monoclonal antibody to surface Ig in the presence of IL-4, or with a B-cell mitogen such as LPS. Proliferation is evaluated 2 to 3 days after stimulation. In the radioisotope-based assay, proliferation is quantified by measuring incorporation of ^{3}H-thymidine into the DNA of the cultured cells. Data are usually expressed as mean counts per minute (cpm) for each treatment group. In the flow cytometry–based assay, cells are stained with one of several membrane permeable dyes such as carboxyfluorescein succinimidyl ester (CFSE). CFSE is a colorless dye that passively diffuses into the cell. Once inside the cell, CFSE undergoes cleavage by intracellular esterases to yield carboxyfluorescein succinimidyl, which is highly fluorescent and covalently reacts with intracellular amines. As the cell divides, the intensity of staining in each of the two newly formed daughter cells decreases by approximately half, as well as in every cell division thereafter. The number of cell divisions each cell has undergone is inversely proportional to fluorescence intensity. Alternatively cells can be treated with (5-ethynyl-2′-deoxyuridine [EDU]), which is a thymidine analog that incorporates into DNA during DNA synthesis. It can be subsequently detected using a click chemistry–based reaction that contains a fluorescent dye, which can also be detected by flow cytometry. For EDU, proliferation is directly proportional to fluorescence intensity. T-cell proliferative responses can be quantified using the same methodologies as described above with the exception that T-cell-specific stimuli are used to induce T-cell proliferation. Often in broad-based immunotoxicological evaluations, B-cell proliferative responses are measured in conjunction with T-cell proliferation.

Acquired Immunity: Cell-Mediated (CMI) While there are numerous assays used to assess CMI, three primary tests are used routinely in mice in the National Toxicology Program (NTP) test battery (see the section "The National Toxicology Program (NTP) Tier Approach"). The test battery includes the CTL assay, DTH response, and the T-cell proliferative responses to TCR stimulation (anti-CD3 plus IL-2), mitogens (phytohemagglutinin [PHA] and concanavalin-A [ConA]), and allogeneic cell antigens (mixed lymphocyte responses [MLR]). While these assays developed for mice use splenocytes, as described below, many of these assays have been adapted for larger species, including humans, using PBMCs.

The CTL assay measures the in vitro ability of splenic T cells to recognize allogeneic target cells (MHC mismatched) by

evaluating the ability of the CTLs to proliferate and then lyse the target cells. Splenocytes are incubated with P815 mastocytoma cells, which serve as target cells. The target cells are pretreated with mitomycin C or are irradiated so that they cannot proliferate. During this sensitization phase, the CTLs recognize the targets and undergo proliferation. Five days after sensitization, the CTLs are harvested and incubated in microtiter plates with radiolabeled (^{51}Cr) P815 mastocytoma cells. During this elicitation phase, the CTLs that have acquired memory recognize the foreign MHCI on the P815 cells and lyse the targets. At the end of the incubation, the culture plates are centrifuged, the supernatant is removed, and radioactivity released into the supernatant is quantified by a gamma counter. After correcting for spontaneous release, the percent cytotoxicity is calculated for each effector-to-target ratio and compared to that from control animals. Several flow cytometry–based methods that avoid the use of radionucleotides have also been developed to assay CTL activity the use of radionucleotides. One approach is similar to the approach described for the NK cell assay above, and involves the labeling of target cells with a fluorescent dye (e.g., DiO or CFSE) in order to discern target cells from effectors. After co-culture of target cells and CTL effectors, target cell viability is assessed by propidium iodide staining or using one of several other commercially available stains to assess viability. Another alternative nonradioactive surrogate assay widely used in place of measuring direct target cell killing is quantification of IFN-γ-expressing CD8$^+$ T cells by flow cytometry using intracellular staining. An inherent limitation to this last approach is that direct target cell killing is not assessed and it is assumed that all IFN-γ^+ CD8$^+$ cells identified are CTL effectors (see the section "Flow Cytometric Analysis"). Use of the CTL assay to evaluate the effects of xenobiotics on CMI function has been discussed by Burleson et al. (2010).

The DTH response evaluates the ability of memory T cells to recognize foreign antigen, proliferate, and migrate to the site of the infection, where they secrete cytokines and chemokines that promote the influx of other inflammatory cells. The assay quantifies the influx of mononuclear cells into the sensitization site. During xenobiotic exposure, mice are sensitized subcutaneously, between the shoulders, with two doses of KLH. On the last day of exposure, mononuclear cells are labeled in vivo with an IV injection of ^{125}I-5-iododeoxyuridine. One day later, mice are challenged intradermally in one ear with KLH. Twenty-four hours after challenge, animals are euthanized, the ears are biopsied, and radiolabeled cells are counted in a gamma counter. Data are expressed as a stimulation index, which represents the cpm of ^{125}I activity in the challenged ear divided by the cpm in the unchallenged ear. Alternative nonradioactive methods include systemic sensitization with sRBC, KLH or bovine serum albumin (BSA), followed by challenge (with the same antigen) in one footpad. Saline injection in the contralateral footpad serves as the control. Swelling into the challenged footpad as compared to the saline-injected footpad indicates activation of the DTH response (Dietert et al., 2009). It is important to note that alterations of DTH functional response may be influenced by disruption of either Th1-driven, antigen-dependent T-cell activation or mobilization of sensitized T cells to a local site (Dietert et al., 2010). DTH assays have also been developed for the NHP (Price, 2008; Bouchez et al., 2012).

T cells play a central role in CMI and their ability to undergo clonal expansion through proliferation is critical to this role. Several mechanisms exist to evaluate proliferative capacity. The MLR measures the ability of T cells to recognize foreign MHCI and MHCII on splenocytes from an MHC-incompatible mouse (allogeneic cells) and undergo proliferation. For example, splenocytes from B6C3F1 mice (responders) are incubated with splenocytes from mitomycin C–treated or irradiated DBA/2 mice (stimulators). Proliferation is evaluated 4 to 5 days after stimulation by quantifying uptake of ^{3}H-thymidine into the DNA of the cultured responder cells. Cells are collected from each well using a cell harvester and counted in a scintillation counter. Data may be expressed as either the mean cpm for each treatment group or as a stimulation index in which the index is calculated by dividing the cpm of wells containing responders and stimulators by the cpm of wells containing responders alone. Alternatively, cell proliferation can be quantified by flow cytometry using membrane permeable dyes such as CFSE or EDU (see the section "Flow Cytometric Analysis").

General T-cell proliferation can be evaluated in a manner similar to that described above for B cells (Table 12-5). Splenocytes or PBMC are stimulated in microtiter plates with monoclonal antibodies directed to the CD3 complex of the TCR (anti-CD3) and the CD28 T-cell co-receptor (anti-CD28), or with the T-cell mitogens Con A or PHA. Proliferation is evaluated 2 to 3 days after stimulation by quantifying uptake of ^{3}H-thymidine into the DNA of the cultured T cells or other nonradioactive methods. Data are usually expressed as mean cpm for each treatment group. As discussed above, proliferation can also be quantified by flow cytometry. These studies are usually done in conjunction with B-cell proliferative responses described above. In addition, T-cell activation can be measured by the upregulation of activation markers such as CD25 or CD69 as assessed by flow cytometry.

Flow Cytometric Analysis One of the most rapidly advancing areas and powerful tools in immunotoxicology has been the application of flow cytometry to investigations of immune modulation by xenobiotics. In the most general sense, flow cytometry is a method that employs light scatter, fluorescence, and absorbance measurements to analyze large numbers of cells (typically 5000 to 100,000 per sample) on an individual basis. Most commonly, fluorochrome-conjugated monoclonal antibodies raised against a specific protein of interest are employed for detection. The strength of the approach is that a wide variety of measurements can be made on large numbers of cells, individually, rapidly, and with a high level of precision. In addition, methods are also available that allow for the analysis of specific proteins in cell-free preparations such as cell lysates, biological fluids, and culture supernatants. A broad selection of polyclonal and monoclonal antibodies to cell surface markers, intracellular proteins, and secreted proteins are available (see Table 12-8).

The most common application of flow cytometry in immunotoxicology is to enumerate specific leukocyte populations and subpopulations. For example, antibodies are available to the T-cell surface proteins CD4, CD8, and CD3 (among others). Because flow cytometers can detect light emission of multiple wavelengths simultaneously, multiple-colored fluorochromes can be used concurrently facilitating an analysis of more than one protein simultaneously in a given sample (multicolor/parameter analysis). This approach can be used to provide insight into which specific T-cell subsets are targeted after exposure to a xenobiotic and to identify putative effects on T-cell maturation. Similarly, antibodies are readily available to identify other leukocyte subpopulations including antibodies to surface Ig, CD19, and B220 (the CD45 phosphatase on B cells) for enumerating B cells; Mac1 and F4/80 for macrophages; CD16/CD56 for NK cells in humans; and CD161 (NK1.1) for NK cells in mice. Surface marker analysis of heterogeneous cell preparations can reveal significant alterations in lymphoid subpopulations, and in many instances this is indicative of alterations in immunologic integrity. Indeed, an indicator of disease progression in AIDS can be monitored by changes in CD4$^+$ T-cell numbers. In their benchmark

paper, Luster et al. (1992) reported that in conjunction with two or three functional tests, the enumeration of lymphocyte subsets can greatly enhance the detection of immunotoxic chemicals. However, it is important to emphasize that although surface marker analysis can identify changes in leukocyte populations, functional analysis of the immune system is more definitive for the detection of immunotoxicity because the ability of immunocompetent cells to mount an effector response is assessed directly. Table 12-8 provides a list of common epitopes used to identify leukocyte populations in the mouse, rat, and human. In addition, flow cytometry is routinely used to purify and isolate leukocyte subpopulations from heterogeneous cellular preparations.

With the use of tetramers, flow cytometry can also identify antigen-specific T cells, especially those that recognize MHCI-restricted epitopes. Tetramers consist of four biotinylated MHC molecules connected by streptavidin. The MHC molecules include an antigenic peptide, usually of known sequence, that is known to activate an antigen-specific T cell. The tetramer is also conjugated to a fluorochrome that can be detected with a flow cytometer. Thus, tetramers will interact with its cognate T cell to allow for identification and quantification of antigen-specific T cells. One limitation with this technology is that the specific MHC (i.e., haplotype) must be known, as does the peptide sequence. An example is a tetramer to which the peptide SIINFEKL is bound, which specifically recognizes ovalbumin-specific $CD8^+$ T cells in mice with a $H\text{-}2K^b$ haplotype.

With technical advancements of flow cytometers (i.e., an increased number of wavelengths that can be simultaneously detected, enhanced sensitivity of detection, and more rapid analysis) coupled with a steady growth of applications, reagents, and methods, flow cytometry has become an integral tool in elucidating the cellular and molecular mechanisms of action produced by immunotoxicants. Current applications go well beyond strictly evaluating cell surface markers and include measurements of cellular viability, cell cycle, cell proliferation, intracellular free calcium, induction of apoptosis, DNA strand breaks (TUNEL assay), intracellular proteins (cytosolic and nuclear), membrane potential, intracellular pH, oxidative stress, and membrane lipophilicity (reviewed by Shapiro [2003], Hawley and Hawley [2004], and Bacher and Scheffold [2015]). Of particular utility in assessments of immune cell signaling and effector function has been the development of protocols and antibodies for intracellular staining of permeabilized cells. In the case of effector function, flow cytometry can be used to quantify the expression of multiple cytokines and/or chemokines simultaneously in individual cells by intracellular staining. Because activated leukocytes often secrete multiple regulatory factors simultaneously, termed "multifunctional," using intracellular staining in conjunction with extracellular staining for phenotypic markers to identify specific cell populations can provide important insights into the magnitude and profile of effects exerted by an immunotoxicant on specific cell types and populations. Measurements of regulatory factors in combination with phenotypic markers can be extended to also include markers of cell activation or differentiation to identify at which stage during effector cell generation an immunotoxicant is acting. For an example of intracellular cytokine staining see Karmaus et al. (2011). A second application of intracellular staining in flow cytometry has been for investigating cell signaling. The complex networks and pathways that control cellular activities can be studied on an individual cell basis to provide static or kinetic information in specific cell types. For example, by using intracellular staining, the abundance of specific signaling proteins, cytosolic and nuclear, can be quantified. In addition to signaling protein abundance, posttranslational modifications of proteins represent an important mechanism for transducing regulatory signals. One of the most extensively studied is protein phosphorylation and dephosphorylation, which often results in the activation or inactivation of protein function. The development of antibodies capable of distinguishing the point at which proteins possess phospho-groups at specific regulatory sites has made it possible to study modulation of distinct regulatory pathways by immunotoxicants. Because flow cytometers have the ability to detect 6 to 8 colors simultaneously, with more advanced instruments possessing 18 color capacity, it is possible to measure multiple signaling events and/or network interactions in single cells. This capability coupled with the fact that the flow cytometer can evaluate thousands of cells per second allows for accurate and reproducible detection of even rare events occurring in small subsets of leukocytes. Moreover, flow cytometry has numerous advantages over other approaches such as Western blotting and immunoprecipitation because of the rapidity of analysis and in not being a bulk lysis method in which all cells in a given sample are pooled for analysis to render results that are representative of the overall mean within the entire cell population rather than in individual cells. For examples of applying the measurement of intracellular phosphoproteins and transcription factors by flow cytometry to studying mechanisms of immune modulation see North et al. (2010) and Lu et al. (2011).

Another major advancement in flow cytometry–based analyses has been the development of fluorescent microspheres that are individually identified by the instrument. By coating the surface of microspheres with various concentrations of two fluorescent dyes, sets of microspheres can be generated with each set possessing a unique spectral signature. Subsequently, various materials (i.e., proteins, antibodies, or nucleic acids) can be covalently conjugated to the surface of these microspheres in order to create unique detection systems. This technology is being widely applied for analyzing a broad variety of soluble cellular components including proteins in cell free preparations by flow cytometry. For example, up to 15 different cytokines can be quantified simultaneously in

Table 12-8

Phenotypic Markers to Identify Immune Cells

IMMUNE CELL TYPE	HUMAN	MOUSE	RAT
B Cell	CD19	CD19	Surface IgM Surface IgG
T Cell	CD3	CD3	CD3
T-helper Cell	CD4	CD4	CD4
Cytotoxic T lymphocyte	CD8	CD8	CD8
γδT Cell	γδTCR	γδTCR	γδTCR
NK Cell	CD16 CD56	NK1.1	CD161
Monocyte	CD14	CD11b	CD11b/c
Macrophage	CD11b	F4/80	CD11b
Dendritic cell	CD1c CD83 MHCII	CD11c MHCII	CD11b/c CD103 MHCII
Plasmacytoid dendritic cell	CD303		

cell supernatants or biological fluids (e.g., brochoalveolar lavage fluid, BALF). A major advantage of this method over more traditional approaches, such as ELISA-based assays, is that only small amounts of biological samples and reagents are required because multiple proteins are assayed simultaneously. The same technology is also being applied for analyzing cell lysates for changes in protein phosphorylation in investigations of cell signaling.

A recent flow cytometry advancement is the ability to quantify gene-specific mRNA levels in individual cells using a technique termed PrimeFlow®. The methodology is based on the principle of using antisense probes containing 20 to 40 oligonucleotides that hybridize to target mRNA. Using branched-DNA technology, the signal is amplified by binding of preamplifiers then amplifiers and finally the fluorochrome-conjugated probes, thus allowing for sufficient amplification to facilitate accurate quantification of gene-specific mRNA transcripts. The current technology will accommodate detection of two different mRNA species simultaneously. Quantification of modestly expressed mRNA species is presently a limitation of this technique. PrimeFlow® has a wide array of applications in immunotoxicology including identifying cell types that are the source of regulatory proteins (e.g., cytokine and chemokines), as these are typically made on demand and regulated at the transcriptional level.

Flow cytometry has become a powerful tool for characterizing the cellular and molecular mechanisms associated with immunotoxicants. For information concerning specific flow cytometry protocols and applications beyond those provided by commercial vendors, the reader is directed to several references including *Practical Flow Cytometry in Haematology: 100 Worked Examples* by Leach et al. (2015), the 3rd edition of *Methods in Molecular Biology: Flow Cytometry Protocols* by Hawley and Hawley (2016), and the Purdue University Cytometry Laboratories Web site www.cyto.purdue.edu/, which hosts a blog to which one can submit questions to the broader community of flow cytometry users for technical assistance.

Measurements of Cytokines and Cytokine Profiling As discussed in the earlier part of this chapter, development, maturation, differentiation, and effector responses of the immune system are highly dependent on a multitude of small, secreted proteins termed cytokines. In most cases, these immunological processes are controlled by the production of multiple cytokines, some of which are released simultaneously, whereas others are released in a very defined temporal sequence. Many of these cytokines are produced by T cells and are the mechanism by which a wide variety of functions by T cells are mediated. Due to the importance of cytokines in regulating the immune system, xenobiotics that alter the production and release of these mediators can significantly affect immune competence. Therefore, quantification of multiple cytokines, often referred to as cytokine profiling, has become routine in immunotoxicology and can provide significant insights into the mechanisms by which a xenobiotic produces its immunotoxicity. For example, cytokine profiling has been explored as an approach for identifying chemical allergens, either contact sensitizers, which typically induce a Th1 profile of cytokines (IL-2, IFN-γ, and TNF-β), or respiratory sensitizers, which typically produce a strong Th2 cytokine profile (IL-4, IL-5, IL-6, and IL-10) (Mosmann and Coffman, 1989; Mosmann et al., 1991). Cytokines can be quantified in cell culture supernatants or biological fluids (i.e., serum or BALF) by ELISA or by multiplex assays. Quantification of test samples is accomplished by comparison to a standard curve employing recombinant cytokine standards. The aforementioned ELISPOT assay can also be used to quantify the number of cells producing the cytokine of interest. Likewise flow cytometry can be used to identify cytokine-producing cells by intracellular staining or to quantify cytokines in media or biological fluids, with the main advantage being that the source of the cytokine(s) can be identified using the former approach, and that many cytokines can be assayed simultaneously from one sample using the latter approach (see the section "Flow Cytometric Analysis"). Because cytokines are regulated transcriptionally and then actively synthesized by cells at the time they are secreted, rather than existing as stored proteins, measurements of cytokine mRNA levels has become another common approach of assessing which cytokines are being expressed at a given time, and the putative effects xenobiotics exert on their regulation and expression. Real-time polymerase chain reaction (PCR) is most commonly used for quantifying mRNA levels of specific cytokines as well as for other genes of interest in cells or tissues but other approaches, such as RNAseq, are used for more global assessments. Major advantages to quantifying cytokine expression at the mRNA rather than protein level include significantly greater sensitivity, the ability to quantify expression in solid tissues, and the capability to rapidly design appropriate reagents (PCR primers) specific for any cytokine. A major disadvantage is that changes at the mRNA level for a given cytokine may not necessarily correlate with changes in protein. Another disadvantage compared to flow cytometric determinations by intracellular staining is not knowing the cell type(s) from which the cytokines are being produced. One additional limitation to cytokine measurements by ELISA or via quantification of cytokine mRNA levels is that neither of these approaches provides information concerning the biological activity of the proteins being measured. Additionally, as described by Corsini and House (2010), multicytokine analysis still needs to be standardized in terms of optimum sources for analysis, protocols, and quality control issues, such as the use of reference standards and the expression of results.

Cytokine Release Assays Therapeutic monoclonal antibodies and pleiotropic cytokines have the potential to lead to the activation of a number of immune cells (primarily T cells and monocytes/macrophages) by the initiation of a cascade that causes the release of cytokines, including TNF-α, IFN-γ, and IL-6. Cytokine release syndrome (also known as cytokine storm) can be characterized by flu-like symptoms, fever, chills, headache, back pain, hypotension, and organ failure. Cytokine release is, at least in part, responsible for several drug-induced immune-mediated adverse reactions described in the literature including first-dose reactions, infusion reactions, tumor-lysis syndrome, and systemic inflammatory response syndrome and, under physiological conditions, can contribute to the pathology following infection with certain pathogens (Gribble et al., 2007). In 2005, an anti-CD28 super costimulatory agonist (TGN1412 monoclonal antibody), which bypasses the need for antigen signaling and leads to polyclonal T-cell expansion, was administered to six healthy male volunteers in a first-in-human trial and within 90 minutes of the first dose there was a profound impact on the immune system that resulted in an acute cytokine release, systemic inflammatory response, and ultimately multiorgan failure (Suntharalingam et al., 2006). This response was not predicted by in vitro human cell–based assays or from toxicology studies conducted in NHPs, highlighting the limitations of these approaches to always predict adverse effects. The mechanisms responsible for cytokine release by monoclonal antibodies can include the cross-linking and clustering of antigen targets on the immune cells by the Fab arms, by interaction of the Fc region of the monoclonal antibodies with Fc gamma receptors on NK cells, monocytes, and/or neutrophils, or by ADCC. To evaluate for the potential to induce

Table 12-9
Models of Host Resistance

MODEL	PATHOGEN
Bacterial	*Listeria monocytogenes* *Streptococcus pneumoniae*
Parasite	*Trichinella spiralis*
Fungal	*Candida albicans*
Viral	Influenza A2
Tumor	B16F10 melanoma

SOURCES: From Burleson and Burleson (2010) and Luebke (2010).

cytokine release in humans, monoclonal antibodies are often evaluated using an in vitro cytokine release assay with human cells. An overview of current practices for conducting cytokine release assays was published (Finco et al., 2014). Methods include whole blood or PBMCs and presentation of monoclonal antibodies either in solution, immobilized by coating onto assay plates, or via capture with anti-Fc antibodies coated to assay plates. Briefly, the biotherapeutic is incubated with cells from multiple human donors for up to 72 hours and the supernatant evaluated for a panel of cytokines with the response being compared to the appropriate positive and negative controls. The specific method utilized should be scientifically justified based on the pharmacology of the drug.

Host Resistance Assays Host resistance assays represent a way of assessing how xenobiotic exposure affects the ability of the host to combat infection by a variety of pathogens. Although host resistance studies provide significant insight into the mechanisms by which an immunotoxicant is acting, these assays are not used as a first or only choice for evaluating immune competence. The results from host resistance assays are typically more variable than other immune function assays already discussed and therefore require markedly greater numbers of animals in order to obtain statistical power. The increased number of animals required coupled with challenging animals with a live pathogen also raises ethical considerations as well as cost. In addition, as with other immune function tests, no single host resistance model can predict overall immune competence of the host, primarily because each model uses different mechanisms for elimination of various pathogens. A representative list of host resistance models is shown in Table 12-9. Typically, three challenge levels of pathogen (approximating the LD_{20}, LD_{50}, and LD_{80}) for each concentration of xenobiotic are used in order to be able to detect both increases and decreases in resistance. Endpoint analyses are lethality (for bacterial and viral pathogens), changes in tumor burden, and increased or decreased parasitemia. In host resistance studies, it is also important to consider the following: (1) strain, route of administration, and challenge levels of the pathogen; (2) strain, age, and sex of the host; (3) physiologic state of the host and the pathogen; and (4) time of challenge with the pathogen (prior to, during, or after xenobiotic exposure). All of these can have significant effects on the results from any individual study.

A major advantage in applying host resistance models to investigations of immunotoxicity is the ability to study the effects of an immunotoxicant within the context of the intact immune system encompassing all its diversity as it acts to protect the host against a bona fide pathogen. It is important to emphasize that the immune system possesses significant redundancy (i.e., multiple mechanisms to provide defense against invading microorganisms). Therefore, even if a particular cell type or effector response has been compromised by exposure to an immunotoxicant, other components of the immune system might provide partial or complete protection to the host from pathogen challenge. In addition, certain pathogens are restricted to specific tissues or anatomical sites, for example, influenza virus, which is typically restricted to airways. Using host resistance models permits the study of immunotoxicants and their effects within the environment and in the context of the tissue targeted by the pathogen (Buchweitz et al., 2007). A variety of host resistance models have been reviewed including bacterial challenge models (Burleson and Burleson, 2010), viral host resistance models (Freebern, 2010), parasite challenge models (Luebke, 2010), and tumor challenge models (Ng et al., 2010).

Assessment of Developmental Immunotoxicology (DIT) Interest in DIT is predicated on the recognition that the developing immune system represents a novel target for xenobiotic-induced toxicity that presents some special considerations when it comes to assessment. The concept that any of a number of dynamic changes associated with the developing immune system might provide periods of unique susceptibility to chemical perturbation has been reviewed (Dietert et al., 2000; Holladay and Smialowicz, 2000). This unique susceptibility may be manifested as a (1) qualitative difference, in the sense that a chemical could affect the developing immune system without affecting the adult immune system; (2) quantitative difference, in the sense that a chemical could affect the developing immune system at lower doses than the adult immune system; or (3) temporal difference, in the sense that a chemical could produce either a more persistent effect in younger animals than adults, or trigger a delayed effect (i.e., the consequences of early exposure are not manifested until early adulthood). As a result, while effective assessment of DIT should certainly draw upon the prior experience gained from adult-exposure immunotoxicity assessments, it is important to examine the database of known immune changes that reflect the potential for unique susceptibility and are specific for the developing immune system (Dietert and Piepenbrink, 2006b). It is also noteworthy that development of leukocyte progenitors from bone marrow stem cells ultimately giving rise to mature immunocompetent cells is a life-long cell renewal process not unique to early life stages.

One of the most comprehensive reports to date attempted to compare the immunotoxicity following developmental or adult exposure to the following compounds: diethylstilbestrol, diazepam, lead, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), and tributyltin oxide (Luebke et al., 2006a). The selection of these five compounds was reported to be based on the availability of some human data. The authors concluded that for all five chemicals, the developing immune system was found to be at greater risk than the adult, either because lower doses produced immunotoxicity, adverse effects were persistent, or both. However, it is noteworthy that no developmental immunotoxicants have been identified that have not also been immunomodulatory in the adult animal. Importantly, this observation may merely reflect the state-of-the-science; that is, all published DIT studies to-date have been conducted on known adult immunotoxicants, and systematic studies comparing the effects across multiple developmental windows are still uncommon.

A better understanding of the developing immune system, particularly, an understanding of critical developmental hallmarks, has prompted some to speculate about the existence of five critical windows of vulnerability as outlined in Fig. 12-21 (Dietert et al., 2000). The first window encompasses a period of hematopoietic stem cell

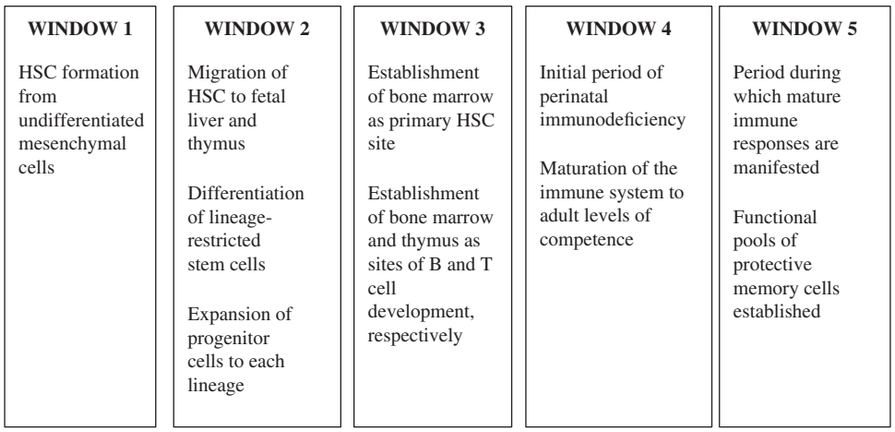

Figure 12-21. *DIT windows of sensitivity.* Five developmental windows of sensitivity to immunotoxicity have been identified.

formation from undifferentiated mesenchymal cells. Exposure of the embryo to toxic chemicals during this period could result in failures of stem cell formation, abnormalities in production of all hematopoietic lineages, and altered immunocompetence. The second window is characterized by migration of hematopoietic cells to the fetal liver and thymus, differentiation of lineage-restricted stem cells, and expansion of progenitor cells for each leukocyte lineage. This developmental window is likely to be particularly sensitive to agents that interrupt cell migration, adhesion, and proliferation. The critical developmental events during the third window are the establishment of bone marrow as the primary hematopoietic site and the establishment of the bone marrow and the thymus as the primary lymphopoietic sites for B cells and T cells, respectively. The fourth window addresses the critical periods of immune system functional development, including the initial period of perinatal immunodeficiency, and the maturation of the immune system to adult levels of competence. The final window addresses the subsequent period during which mature immune responses are manifest, and functional pools of protective memory cells are established.

Most recently, considerable attention has been focused on the perinatal period (i.e., prior to and just after birth) because this window of development is known to be replete with dynamic immune changes, many of which do not occur in adults (Dietert and Piepenbrink, 2006b). Indeed, one reality associated with DIT windows is that the developing immune system exists in an unbalanced state through the latter portion of gestation with certain functional CMI capacities deliberately impaired. In fact, Taylor et al. (2006) demonstrated that placentally induced immune skewing via the release of Fas-ligand-containing exosomes is one hallmark of a successful pregnancy brought to full term. Upon birth, restoring effective immune balance through the enhancement of Th1 capacity in the newborn is critical for protecting childhood health (Holt et al., 2005; Yun and Lee, 2005). Prenatal maturation and functional skewing of the fetal immune system followed by the rapid reversal of the imbalance at birth have features that are not effectively modeled using adult exposure-assessment (Dietert and Piepenbrink, 2006b), and DIT is best viewed as a continuum of alterations. Suppression of the developing immune system, manifested as increased susceptibility to infections and cancer, is not the only concern. Immunotoxic changes that increase the risk for allergic or autoimmune responses in later life should also be considered (Edwards and Cooper, 2006; Selgrade et al., 2006; Yeatts et al., 2006). Adding to the complexity is the demonstration that some developmental immunotoxicants seem capable of inducing targeted immune suppression, while at the same time elevating the risk of allergy and/or autoimmunity (Haggqvist et al., 2005). Because either significant immune suppression or disrupted immune regulation is a concern and needs to be detected, the most effective methodology for assessing DIT must also be capable of assessing significant changes in immune balance.

In spite of the increased interest in assessing the potential for developmental immunotoxicity, it must be emphasized that neither validated nor widely accepted methods currently exist for evaluating the effects of a chemical on the developing immune system. Several workshops have summarized consensus thinking concerning DIT evaluation (Dietert et al., 2000; Holsapple, 2002b; Luster et al., 2003; Van Loveren and Piersma, 2004; Holsapple et al., 2005; Ladics et al., 2005) and a number of reviews have been written (Holsapple et al., 2003, 2004, 2007; Luebke et al., 2006a; Dietert and Piepenbrink, 2006b; Dietert and Holsapple, 2007; Burns-Naas et al., 2008; Dietert and Burns-Naas, 2008; Weinstock et al., 2010; Winans et al., 2011). Additionally, other reviews have dealt with issues concerning immunotoxicity evaluation across various life stages (Germolec et al., 2004; Holsapple et al., 2005; Ladics et al., 2005; Ravel and Descotes, 2005). Below is a brief discussion of critical issues requiring consideration in establishing a testing framework.

In constructing a DIT testing framework, one of the first points to consider is the selection of an animal model. Consistently, the rat has been identified as the preferred species for evaluations of DIT, largely due to the fact that the rat has been utilized extensively in guideline developmental and reproductive toxicology testing. However, the NHP is often employed for many biopharmaceuticals where the NHP is the only relevant pharmacologic species. A review of the anatomical and functional differences in the immune systems of the mouse, rat, dog, primate, and human, and their use as models for DIT testing concluded that the developing immune systems of mice and humans have been best characterized to date, and immune ontogeny in the mouse and rat is likely similar (Holsapple et al., 2003). Another important consideration when selecting a species is that the development of the immune system in the rodent is delayed relative to the human, and how this differential maturation will impact data extrapolation for predicting human risk. For example, some developmental landmarks observed in utero in humans occur after parturition in the rat.

A second consideration when constructing a framework for assessing DIT concerns sex-specific effects. Results from perinatal exposure to xenobiotics suggest that significant sex-based differences in immunotoxic sensitivity are common and are at least

as prevalent, if not more frequent, compared with the incidence observed following adult exposure-assessment (Luebke et al., 2006a; Dietert and Piepenbrink, 2006b). As such, for the evaluation of DIT, testing of both sexes may be critical.

A third major consideration in a DIT testing framework is consideration of exposure. There is general agreement that the best exposure protocol is one where exposure occurs across all nonadult developmental windows followed either by immediate assessment or assessment after a few weeks (Luster et al., 2003; Holsapple et al., 2005; Ladics et al., 2005). Exposure to pregnant dams has been a hallmark feature of most DIT protocols, and the maternal influences on exposure to the fetus/newborn pups would be dependent on transfer of the xenobiotic either across the placenta or via lactation. The gestational (e.g., transplacental) and lactational periods in the rat would result in exposure from conception to early postweaning in the pup, approximately 3 weeks of age. Direct exposure of pups via the diet would generally commence at about 3 weeks after birth. An unresolved issue is whether direct exposure of the pups, which is generally accepted as a routine procedure at around postnatal day 7, should occur during the lactational period as well (Ladics et al., 2005); however, any decisions concerning this issue will require consideration of how humans would be exposed and the specific properties of the chemical being studied. For example, if exposure is only oral and there is no reason to believe that the lactational transfer differs significantly for a class of compounds between rat and human, then direct dosing of pups could be initiated postweaning. In addition, information on pharmacokinetic and dosimetry could be useful in determining whether any direct dosing of pups during lactation is necessary. However, it is important to emphasize that this type of information is not routinely available for most xenobiotics.

A fourth consideration in creating a DIT testing framework concerns which specific endpoints to measure. Immune organs, such as the thymus, spleen, lymph nodes, and/or bone marrow, are not typically assessed in routine developmental and reproductive toxicology studies. However, it has been the consensus of a number of workshops that histopathological evaluation of these immune organs could be easily integrated into existing protocols and is occurring for pharmaceuticals when there is a cause for concern (Luster et al., 2003; Holsapple et al., 2005). There is still uncertainty whether routine histopathology is sufficiently sensitive to detect all potential immunotoxic effects, especially when the unique characteristics of the developing immune system, as discussed above, are considered. Indeed, there are examples where morphometric histopathologic findings do not predict functional impairments due to toxicity produced on the developing immune system (Hussain et al., 2005). Ultimately, while the use of specific, rather than general, histopathology has been recommended, it has been also suggested that functional tests be employed in the assessment of DIT. Unfortunately, few if any functional assays have been validated for detection of DIT, even for routine assays such as the TDAR, which has largely been confined to adult exposure protocols. Results published on several chemicals and drugs in recent years suggest that functional tests are a frontline priority for perinatal immunotoxicity detection and that a combination of at least two functional tests, such as a multi-isotype TDAR, and a CMI response assay, such as the DTH assay and/or CTL or NK cytotoxicity assay, should be paired with histopathological analysis and phenotypic analysis of lymphocyte subsets using flow cytometry (Dietert and Piepenbrink, 2006b).

One final point regarding DIT needs to be considered. As developmental immunotoxicity protocols are inserted into existing toxicology testing regimes, such as developmental and reproductive toxicology protocols, immunization protocols are often incorporated. This approach has raised concerns among those evaluating other physiological systems (e.g., reproductive and neurological) in terms of potential immunization-induced changes. However, investigations addressing this potential by determining the impact of the incorporation of immunotoxicological functional assays on standard toxicological studies in rats have been largely negative. Ladics et al. (1995) showed that immunization did not significantly alter the weights or morphology of routine protocol tissues with the exception of the spleen (i.e., which manifested the anticipated increases in the numbers and size of germinal centers). In a subsequent study by this same group, immunization did not alter any hematological or clinical chemistry parameters, or lymphocyte subset numbers (i.e., measured on peripheral blood with a flow cytometer), and did not mask the anticipated hepatotoxic effects of subchronic exposure to carbon tetrachloride (Ladics et al., 1998). An additional component of this discussion is the reality that the species being modeled, the human, has immunizations as a routine lifelong component of preventative medicine. As such, to avoid all immunizations in animal evaluation protocols does not closely simulate the childhood experience. Additionally, until the immune system is asked to respond specifically to a foreign antigen, the capacity to detect immunotoxicity may be severely limited. Therefore, immunization and a range of functional T-cell-dependent immune response evaluations seem necessary as components of an effective DIT assessment.

Assessment of Hypersensitivity Responses As emphasized throughout this chapter, immunotoxicology has evolved to the point where an ever-increasing number of studies are being conducted to characterize the immunotoxicity of a variety of xenobiotics using standard immunotoxicological parameters. Depending on the specific drug/chemical, the exposure conditions, the species being tested, and the immune parameter being measured, the outcomes can be manifested as decreases, increases, or no effect. While there is no question that the most frequent observation has been immune suppression, there is also no doubt that some examples of immune stimulation have been reported, a profile consistent with the concept of immunotoxicology existing as a continuum (Fig. 12-1). However, this observation can trigger some important questions. Should a treatment-related increase in an immune parameter such as the TDAR be considered an adverse response? Should an increase in the antibody response to a neoantigen be considered a harbinger of autoimmune potential? There simply is no consensus regarding the correct way to interpret an increase in immune parameters such as the TDAR, which were primarily developed to characterize the immunosuppressive potential of xenobiotics. The adverse consequences of exaggerated immune function do reflect an inability to recognize self, and are generally depicted as hypersensitivity and autoimmunity.

Drugs and chemicals that are capable of eliciting an immune response are generally low-molecular-weight substances possessing some inherent reactivity. There is a genetic susceptibility to hypersensitivity responses in that not all individuals react to the same drugs and chemicals. For the most part, the xenobiotic cannot be considered an antigen, simply because by itself it is not capable of stimulating an immune response. Instead, the xenobiotic becomes a hapten, which triggers the immune response in some tissue in the host. This property is called the sensitizing potential of the hapten and is associated with its inherent reactivity. Hapten-specific immune responses are therefore triggered only in the presence of the hapten–carrier complex and can be mediated either by humoral immunity or CMI. The damage associated with any type of hypersensitivity response can be directed against the tissue that

is bound by the hapten. One of the most important and challenging problems in the field of immunotoxicology is determining the potential for chemicals to induce immune modulation and, in the current context, to demonstrate the sensitizing potential of xenobiotics. Thus, it becomes essential to have validated predictive animal models, and to understand the underlying mechanisms of action. The following is a discussion of the currently used methods of predicting the most frequently occurring hypersensitivity reactions to chemicals, Type I and Type IV, which are most often manifested as respiratory hypersensitivity and contact sensitization, respectively.

Assessment of Respiratory Hypersensitivity in Experimental Animals Methods for assessing the potential to induce respiratory hypersensitivity have been reviewed (Holsapple et al., 2006). The objectives of this review were to describe the appropriate methods for identifying and characterizing respiratory hazards and risks and to identify the key data gaps and related research needs. The review addressed these objectives from the perspectives of proteins, chemicals, and drugs, and emphasized the important roles played by IgE and Th2 cell–mediated responses, while recognizing that damage to the respiratory tract can be triggered either by nonspecific irritation or by other specific immune-mediated mechanisms (i.e., not IgE- or Th2-mediated).

Animal models for respiratory hypersensitivity has been reviewed (Pauluhn, 2005). Current assays utilize two phases: induction/sensitization and challenge/elicitation. The induction phase usually includes multiple exposures to the test compound (sensitization) via the respiratory tract (i.e., inhalation and intranasal or intratracheal instillation) or by dermal contact. There are advantages and disadvantages to each. Inhalation more closely represents environmental exposure by allowing for chemical contact with the upper as well as the lower respiratory tract. However, the equipment required is expensive and difficult to maintain. Exposure via the intranasal route is easily accomplished and allows for distribution of antigen to the upper and lower respiratory tract; however, studies have shown that a large proportion of the material can be recovered from the stomach (Robinson et al., 1996). In contrast, intratracheal instillation results in exposure to the lower respiratory tract only. The observation that a predominant respiratory sensitizer would still trigger an IgE response when applied topically has suggested an important interplay between Type I and Type IV hypersensitivity reactions. The basis for this observation can be accounted for by a cytokine network model, which involves important cross-talk between humoral immunity and CMI. Basically, a chemical with the capability of being a respiratory sensitizer will trigger an IgE response regardless of its route of exposure because it "selects" or supports the development of a Th2-dependent response, with the associated cytokine profile: IL-4, IL-5, IL-10, and IL-13. In contrast, a chemical that lacks the capability of being a respiratory sensitizer but can still trigger contact dermatitis will select or support a Th1-dependent response, with the associated cytokine profile including IL-2 and IFN-γ.

The challenge (elicitation) phase involves exposure to the chemical (hapten), the homologous protein conjugate of the hapten, or the antigen. Endpoints to characterize a positive response range from the induction of Igs (i.e., total IgE for chemicals or specific IgE for protein antigens), cytokines, or lymphokines in serum, to pathophysiological responses, including the influx of inflammatory cells and the onset of bronchoconstriction along with the associated changes in respiratory functional parameters. The latter may be accomplished by visual inspection of the animals' respiratory pattern or more quantitatively by plethysmography. With plethysmography, changes in the respiratory rate, tidal volume, and plethysmographic pressure can be measured. However, plethysmography in guinea pigs, rats, and mice can be technically challenging and labor intensive. In addition, there is some controversy over the interpretation of measurements made from whole body plethysmography, and whether these measurements truly reflect changes in airway mechanics (Lundblad et al., 2002; Mitzner and Tankersley, 2003).

None of the currently applied animal models duplicate all features of human asthma, and most of the current animal models were developed for studying specific hypersensitivity responses to high-molecular-weight protein allergens (Pauluhn, 2005). Fewer animal models have been developed for use in the area of chemically induced respiratory allergy, which is a key data gap (Holsapple et al., 2006). One of the limitations associated with low-molecular-weight models is that often these compounds must conjugate with host proteins to become antigenic (i.e., form hapten–carrier complexes). Often, a challenge with the conjugated chemical is necessary to induce a pulmonary response. Adding this variable can make the analysis of test results more difficult. False-negative results may occur due to variability in test article conjugation, and chemical conjugates are also necessary to measure specific immunologic responses.

The majority of the current animal models are based on antibody-mediated events occurring as a reflection of the induction phase (Pauluhn, 2005). In certain cases, immunologic sensitization may be confirmed by the detection of antigen-specific antibody; however, subsequent challenge does not produce clinical signs of respiratory distress. It is also possible to detect pulmonary sensitization in animal models where there is no detectable antigen-specific antibody production. In these cases, CMI or other mechanisms may be involved, or there may be difficulty in antibody detection.

Guinea pig models have been most frequently used for detection of pulmonary reactions to chemicals because this species is known to respond vigorously to appropriate stimuli by developing an asthmatic-like bronchial spasm. In the guinea pig, as in the human, the lung is the major shock organ for anaphylactic response. Like humans, the guinea pig also demonstrates immediate- and late-onset allergic reactions as well as bronchial hyperreactivity and eosinophil influx and inflammation. The major difference in the mechanism of pulmonary responses between humans and guinea pigs is that the antibody involved in Type I reactions in humans is IgE but predominantly IgG1 in guinea pigs. The key features of this animal model involve protocols using single or repeated inhalation or cutaneous (i.e., primarily topical or intradermal) exposures followed by a 21-day rest period (Pauluhn, 2005). After the rest period, inhalation challenge with the hapten or antigen is performed, thereby focusing on a measurement of the elicitation phase of the response (Karol et al., 1994). Respiratory patterns are often measured in whole-body plethysmographs, as discussed above. A common pathologic accompaniment of increased airway hyperactivity is prolonged eosinophil-rich inflammatory leukocyte infiltration into the lungs of guinea pigs after inhalation challenge of the protein or hapten-conjugate. One of the disadvantages of using guinea pigs is the lack of reagents needed to identify cells and mediators in respiratory allergy, which has hampered mechanistic studies.

Murine models, in which the reagent availability is not an issue, have become more frequently utilized in the evaluation of respiratory hypersensitivity, and two approaches have been described. The first approach capitalizes on the fact that, like humans, IgE is the major anaphylactogenic antibody in mice, and focuses on the induction of total serum IgE (Dearman et al., 1998). The second approach capitalizes on the aforementioned cytokine network and has been referred to as cytokine fingerprinting (Dearman

et al., 2002, 2003; Plitnick et al., 2002, 2003). Both approaches have relied on dermal application of potential allergens/sensitizers, and on the theoretical foundation that chemical allergens induce divergent immune responses characteristic of the selective activation of discrete T-cell subpopulations (Pauluhn, 2005). Contact allergens, such as 2,4-dinitrochlorobenzene, do not cause sensitization of the respiratory tract, but instead trigger an immune response in mice that is consistent with the preferential activation of Th1 cells (i.e., little to no increase in total serum IgE and the production of IL-2 and IFN-γ in the draining lymph nodes). In contrast, topical sensitization to chemical respiratory allergens, such as trimellitic anhydride (TMA), trigger an immune response in mice that is consistent with the preferential activation of Th2 cells (i.e., moderate to marked increase in total serum IgE and the production of IL-4, IL-5, IL-10, and IL-13 in the draining lymph nodes). It is important to emphasize that while both the mouse total serum IgE test and cytokine profiling hold much promise, neither approach can be considered validated at this time.

In 2014, the Health and Environmental Sciences Institute's Immunotoxicology Technical Committee held a workshop on the assessment of respiratory sensitization to discuss the current state-of-the-science for identification and characterization of respiratory sensitizer hazard; to identify information facilitating development of validated standard methods and frameworks; and to consider the regulatory and practical risk management needs (North et al., 2016). While scientific progress has occurred in the development of nontraditional assessment models (in silico and in vitro), an understanding of some of the more fundamental pathophysiologic characteristics such as the role of IgE in human chemical sensitization has not evolved from a decade ago. Additionally, how to best assess and characterize chemical respiratory sensitization remains elusive. The workshop acknowledged the need for a tiered approach, but in the absence of validated methods for assessment, the weight-of-evidence assessment based on available animal, human, and mechanistic data remains the best available option, such as that described in the World Health Organization (WHO) *Guidance for Immunotoxicity Risk Assessment for Chemicals* (WHO, 2012).

Assessment of IgE-Mediated Hypersensitivity Responses in Humans Described below are methods of human Type I hypersensitivity testing. These test results, in conjunction with a relevant history and physical exam, can be diagnostic of IgE-mediated pulmonary disease. Two skin tests are available for immediate hypersensitivity testing. In both, the measured endpoint is a "wheal and flare" reaction (i.e., the result of edema and erythema subsequent to the release of preformed inflammatory mediators). The prick–puncture test introduces very small amounts of antigen under the skin and, owing to the reduced chance of systemic reaction, is recommended as a screening test. For test compounds not eliciting a reaction, the intradermal test using dilute concentrations of antigen may be used, but there is a higher risk of systemic reactions. The reader is referred to an additional text for a more detailed description of testing methods (Demoly et al., 1998).

In vitro serologic tests, ELISAs, and radioallergosorbent tests may also be used to detect the presence of antigen-specific antibody in the patient's serum. These tests do not pose a risk of adverse reactions and may be used in situations where standardized reagents for skin testing are not available. Serologic testing is often used in population-based epidemiologic studies. Additionally, bronchial provocation tests can be performed by having the patient inhale an antigen into the bronchial tree and evaluating his or her pulmonary response. In some cases this may be the only way to demonstrate that a test article is capable of producing an asthmatic response. Care must be taken in these test situations in that it is possible to produce severe asthmatic reactions or anaphylaxis in sensitized individuals.

Assessment of Contact Hypersensitivity in Experimental Animals Classically, the potential for a chemical to produce contact hypersensitivity has been assessed by the use of guinea pig models. These tests vary in their method of application of the test article, in the dosing schedule, and in the utilization of adjuvants (Klecak, 1987). The two most commonly utilized guinea pig models, the Büehler test (Buehler, 1965) and the guinea pig maximization test (Magnusson and Kligman, 1969), are described briefly below.

In the Buehler test, the test article is applied to the shaven flank and covered with an occlusive bandage for 6 hours. This procedure is repeated on Days 7 and 14. On day 28, a challenge dose of the test article is applied to a shaven area on the opposite flank and covered with an occlusive dressing for 24 hours. At 24 and 48 hours after the patch is removed, test animals are compared with vehicle-treated controls for signs of edema and erythema. The guinea pig maximization test differs in that the test article is administered by intradermal injection, an adjuvant is employed, and irritating concentrations are used. Animals are given pairs of intradermal injections at a shaven area on the shoulders. One pair of injections contains adjuvant alone, one pair contains test article alone, and one pair contains the test article mixed with adjuvant. Seven days following injection, after the area is reshaven, the test article is applied topically and an occluded patch is applied for 48 hours. In cases where the test article at the given concentration is nonirritating, the area is pretreated with 10% sodium lauryl sulfate 24 hours before the patch is applied to produce a mild inflammatory response. Two weeks following topical application, the animals are challenged on the shaven flank with a nonirritating concentration of the test article, which remains under an occluded patch for 24 hours. Then, after 24 and 48 hours, the test site is examined for signs of erythema and edema, two well-recognized indicators of cutaneous inflammation and contact dermatitis. However, the endpoints for evaluation in the guinea pig assays are visual and subjective, and it is difficult to assess irritating or colored compounds using these models.

Efforts have been made to develop and establish more quantitative and immunologically based assay methods in other species, focusing mainly on the mouse, again primarily because of the availability of reagents and techniques to conduct mechanistic studies. Gad et al. (1986) developed the mouse ear-swelling test, which uses a quantitative measurement of ear thickness as an endpoint. Animals are sensitized by topical application of the test article for 4 consecutive days to abdominal skin that has been prepared by intradermal injection of adjuvant and tape stripping. On day 10, the animals are challenged by topical application of the test article to one ear and vehicle to the contralateral ear. Measurements are made of ear thickness 24 and 48 hours later. A positive response is considered anything above a 20% increase in thickness of the treated ear over the control ear. Notably, Thorne et al. (1991) showed that dietary supplementation with vitamin A enhanced the mouse ear-swelling assay in the absence of adjuvants, injections, or occlusive patches.

The assays described above evaluate the elicitation phase of the response in previously sensitized animals. In contrast, the mouse local lymph node assay (LLNA) identifies contact allergens as a function of the events occurring during the induction (sensitization) phase of a hypersensitivity response. The origins, development, evaluation, and eventual validation of the LLNA have been described in a comprehensive review (Kimber et al., 2002). In this assay, the induction phase of contact sensitization is measured by the incorporation of ^{3}H-thymidine into proliferating lymphocytes in lymph nodes draining the site where the test article has been applied. Animals are dosed by topical application of the test article to the

ears for 3 consecutive days. The animals are rested for 2 days and then injected intravenously with 20 μCi of ^{3}H-thymidine. Five hours later, animals are sacrificed, the draining lymph nodes are dissected out, and single-cell suspensions are prepared and radioassayed. With consideration of dose–response and statistical significance, a threefold increase in ^{3}H-thymidine cpm in chemically exposed animals over vehicle control animals (i.e., the stimulation index) is considered to be a positive response. As described later in this chapter, because of pressures over the use of animals, a number of new in vitro methods to assess sensitization potential have emerged.

The LLNA offers several advantages over the guinea pig assays in that (1) it has the potential to reduce the number of animals required and reduces animal distress; (2) it provides quantitative data that allow for statistical analysis; and (3) it provides dose–response data. The latter qualities of the LLNA have facilitated its integration into comparisons of potency across individual chemicals and/or within a chemical class. These additional advantages of the LLNA have facilitated its integration into risk assessments for contact dermatitis (Felter et al., 2003). Additionally, because the LLNA evaluates the induction phase of the immune response, it is more applicable to mechanistic studies. As an example, some compounds capable of producing contact sensitization also induce IgE production and subsequent respiratory hypersensitivity. Using three known allergenic diisocyanates (diphenylmethane-4,4′,-diisocyanate; dicyclohexylmethane-4,4′-diisocyanate; and isophorone diisocyanate), Dearman et al. (1992) showed that all three known contact sensitizers induced lymphocyte proliferation in the draining lymph node but that only diphenylmethane-4,4′,-diisocyanate, a known respiratory sensitizer, induced elevated levels of serum IgE and IgG2b. These types of results have prompted a number of investigators to propose that the LLNA could be considered as the first step in a safety evaluation process for allergenic potential (i.e., as a method to identify sensitization potential of a chemical) with other methods being deployed to distinguish between skin and respiratory sensitizing activity (Holsapple et al., 2006).

Assessment of Contact Hypersensitivity in Humans Human testing for contact hypersensitivity reactions is by skin patch testing. Patch testing allows for the diagnostic production of acute lesions of contact hypersensitivity by the application of a suspected allergen to the skin. Patches containing specified concentrations of the allergen in the appropriate vehicle are applied under an occlusive patch for 48 hours in most test protocols. Once the patch is removed and enough time elapses for the signs of mechanical irritation to resolve (approximately 30 minutes), the area is read for signs of erythema, papules, vesicles, and edema. Generally, the test is read again at 72 hours and in some cases signs may not appear for up to 1 week or more. Detailed information on patch testing has been reviewed (Mydlarski et al., 1998).

Human repeat insult patch tests are available as predictive tests in humans. Like predictive testing in animal models, there are many variations in attempts to increase the sensitivity of these procedures. These include preparation of the induction site by either tape-stripping, the application of an irritating concentration of sodium lauryl sulfate, or use of high concentrations of the test article for induction of sensitization. In general, the application of multiple occlusive patches, up to 10 for 48 hours each at the same site, is followed by a rest period and then challenge takes place under an occlusive patch at a different site. Positive reactions are scored in the same manner as for diagnostic patch tests.

Assessment of Autoimmune Responses As described in the preceding section, exaggerated immune responses can be mediated by two entirely different types of interactions between the immune system and xenobiotics. One type of interaction has been described in the preceding section in which the xenobiotic is a hapten and the immune system plays an active role in eliciting a hypersensitivity response. The immune system can also be a passive target for the enhancing effects of drugs and chemicals, such as occurs when a xenobiotic mimics or causes the aberrant production of immunomodulatory cytokines, or when a xenobiotic disrupts the regulatory mechanisms which serve to protect self (i.e., disrupt a suppressor mechanism). Additional mechanisms have been presented in the section "Immune-Mediated Disease: Autoimmunity."

Another way that xenobiotics can enhance immune function is by acting as an adjuvant, which is defined as any substance that nonspecifically enhances the immune response to an antigen. The classic adjuvant is complete Freund's adjuvant, which is a water-in-oil emulsion containing killed *Mycobacterium tuberculosis*. The effectiveness of adjuvants in enhancing immune responses can be demonstrated by the fact that animals are often injected with complete Freund's adjuvant to increase the production of antigen-specific antibodies. However, because adjuvants produce severe side effects, there is a desire to develop an adjuvant that is safe in humans and can be used in conjunction with vaccines or immunotherapy. The specific mechanisms for the actions of adjuvants, including complete Freund's adjuvant, are not known, but ongoing studies are addressing the role of various components, including TLRs and the inflammasome (Eisenbarth et al., 2008; Seubert et al., 2011; Neumann et al., 2014).

In the context of testing strategies, the situation with autoimmunity is much more complex than with hypersensitivity responses. Animal models exist for a number of autoimmune diseases, and autoimmunity has been clearly demonstrated in humans. Therefore, the existence of autoimmune disease, and the expected consequences cannot be denied. However, the ability of drugs and chemicals to exacerbate or trigger autoimmune disease in either animal models or humans is poorly understood. In fact, of all the possible consequences of immunotoxicity, autoimmunity is unquestionably the least understood. Primarily because of the strong genetic component in the susceptibility to autoimmunity, deciphering the exact role of xenobiotics in the induction of these conditions has proven to be very difficult. The following is a brief review of some of the currently used methods of predicting the potential of a xenobiotic to trigger or exacerbate autoimmunity leading to an inappropriate immune response to self-tissue antigens that can be associated with the generation of autoantibodies and/or autoreactive T cells.

While a wide variety of animal species have been utilized to study autoimmune disease, rodents have been most common, and rodent models fall into three categories: genetically predisposed animal models; animal models in which the autoimmune disease is produced by immunization with specific antigens; and animal models in which the disease is chemically induced (Germolec, 2005). Examples of genetically predisposed animal models include the nonobese diabetic (NOD) mouse, the F1 cross between the New Zealand black (NZB) and New Zealand white (NZW) mouse, and the MRL/lpr mouse. The NOD model has been used to study type 1 diabetes, specifically the T-cell autoimmune response, the role of B-cell antigen presentation and the role of cytokines in disease progression. The NZBxNZW F1 and the MRL/lpr mouse models have been used to study human SLE. The NZBxNZW F1 model has been used to map the specific susceptibility loci and to assess the importance of B-cell hyperactivity and T-cell involvement in autoantibody production in the development of SLE (reviewed by Germolec [2005]). The important role of apoptosis in negative selection has been studied in the MRL/lpr mouse model, in which a genetic defect results in a mutation in the *Fas* gene. Although

both models exhibit characteristics of human SLE, including high levels of serum immunoglobulins (i.e., antinuclear and anti-DNA antibodies), as well as the immune-mediated nephritis, the MRL/lpr mouse model also exhibits rheumatoid factor autoantibodies and inflammatory joint disease characteristic of an arthritic response.

Arthritis can also be induced in susceptible rat strains by immunization with complete Freund's adjuvant containing killed *M. tuberculosis* in oil. Immunization of susceptible mouse strains (i.e., those containing H-2^q or H-2^r alleles of the MHC) with type II collagen or cartilage glycoproteins, in the presence of adjuvant, can induce pathology similar to human rheumatoid arthritis. In collagen-induced models of arthritis, the immune response is directed against specific connective tissue antigens, while the complete Freund's adjuvant-induced response is directed against a mycobacterium heat-shock protein, with the observed pathology resulting from cross-reactive destruction of a proteoglycan found in joints (Germolec, 2005). Experimental autoimmune encephalomyelitis (EAE) can be induced in a number of species by immunization with myelin-derived peptides in complete Freund's adjuvant. This model has been used in rodents to characterize the role of T helper cell–mediated autoimmune disease characterized by perivascular lymphocyte infiltration into the CNS and the destruction of the myelin nerve sheath resulting in paralysis, similar to human multiple sclerosis.

One of the most commonly used models of chemically induced autoimmunity is the Brown Norway rat model, in which animals are injected with mercuric chloride. While the selection of doses is such that exposure produces no overt signs of toxicity, Brown Norway rats develop an immunologically mediated disease characterized by T-cell-mediated polyclonal B-cell activation and autoantibodies to laminin, collagen IV, and other components of the glomerular basement membrane similar to human autoimmune glomerulonephritis. Numerous mouse strains have also been used to evaluate the development of autoantibodies following exposure to mercury, gold, and cadmium (Selgrade et al., 1999). Other examples of xenobiotics that have been demonstrated to be associated with autoimmune disease are provided in the section "Xenobiotic-Induced Hypersensitivity and Autoimmunity."

Molecular Biology Approaches to Immunotoxicology

As in all of the biological sciences, the continuing evolution of molecular biology–based methods and technologies have vastly expanded the tools available to immunotoxicologists. In general, molecular biology approaches have been thus far employed primarily in the investigation and elucidation of mechanisms of immunotoxicity rather than for identifying immunotoxicants. As these approaches become more refined and sophisticated with time, their application will surely expand. A primary application of molecular biology in immunotoxicology has been to identify genes whose expression has been altered by a xenobiotic, often termed gene expression profiling, and/or to quantify the magnitude to which gene expression has been changed due to some treatment. As already discussed, methods for assessing changes in gene expression have been particularly useful for studies of the immune system due to the fact that many of the immunological mediators produced by leukocytes (e.g., cytokines, chemokines, and immunoglobulins) are regulated transcriptionally (i.e., synthesized and secreted on demand) rather than being maintained in cells as stored products. Toward this end, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) has been the principal method currently used to assess changes in mRNA levels for specific genes in tissues and cells, although PrimeFlow® (see the section "Flow Cytometric Analysis") and RNA-Seq methods are now being commonly used as well.

A routinely employed methodology for characterizing effects on gene transcription has been the use of reporter assays. Reporter assays are being widely used to discern whether xenobiotic-induced changes in mRNA levels for specific genes are due to alterations at the level of transcription versus mRNA stability. Likewise, reporters can be used to characterize the effects of xenobiotics on specific transcription factors acting through defined regulatory elements. The approach involves construction of a DNA plasmid possessing the 5′ untranslated regulatory region of the gene of interest that has been ligated to the translated region of a reporter gene. Commonly used reporter genes are typically enzymes, because their expression can be easily assayed. Moreover, studies in mammalian systems commonly employ reporter genes of insect or bacterial origin, thus eliminating the need to differentiate between endogenous and ectopic expression. The most widely used reporter genes are firefly luciferase and bacterial β-galactosidase. Most often, reporter assays are performed by transient transfection into cell lines. This approach has been extensively used to study the effects of leukocyte activation stimuli and xenobiotics on the regulation of promoter and enhancer regions of cytokine and Ig genes. In spite of the important mechanistic information that can be gained from these types of studies, significant challenges often arise in utilizing reporter assays to study leukocytes. Transfection of primary leukocytes, especially lymphocytes, yielding both high transfection efficiency and good cell viability is extremely difficult. An additional complicating factor concerns the fact that lymphocytes can only be viably maintained in culture for short periods (approximately 24 hours) in the absence of activation, which limits the duration the cells can be given to recover after transfection. Likewise, it is not uncommon for T- and B-cell-derived lines to be resistant to transfection in spite of the many commercial transfection reagents presently available and new refinements made to electroporation instruments. In most cases transfection conditions must be optimized for transfection efficiency and cell viability for each cell line or preparation using a control plasmid (i.e., a plasmid possessing strong constitutive expression).

At least two methods can be used to achieve gene-specific silencing, which allows one to address the role of various genes in elucidation of mechanism of immunotoxicity. First, RNA interference employs small interfering double-stranded RNAs (siRNA). RNA duplexes of 21 nucleotides possessing two nucleotide 3′ overhanging ends are transfected into the cell and incorporate into a nuclease complex known as the RNA-induced silencing complex (RISC). Through an ATP-dependent mechanism, the duplexes are unwound and separated into their sense and antisense strands. RISC is directed to the target mRNA through homologous binding of the unwound antisense siRNA strand to the target mRNA, which then undergoes cleavage by an endonuclease termed Slicer. The ensuing destruction of the target gene mRNA results in the posttranslational silencing of gene expression. This approach provides a rapid mechanism by which the involvement of a specific gene product can be linked to biochemical and functional events induced by a xenobiotic in a given cell type, including leukocytes (Sandy et al., 2005). Major challenges in the application of this methodology can exist including the need to identify an effective and specific siRNA and that delivery of the siRNA via transfection can be difficult in leukocytes as discussed above. Second, CRISPR (clustered regularly-interspaced short palindromic repeats)-Cas9 is a relatively new technology used for gene editing. It allows for targeted genomic cleavage; that is, introduction of double-stranded DNA breaks at

specific sites. Cells are transfected with a plasmid that expresses a 20-base pair RNA complementary to the gene of interest. This 20-base pair RNA is part of a guide RNA that serves to place the Cas9 endonuclease in proximity of the gene to be edited. Cas9 then cleaves both DNA strands, which can be repaired either by nonhomologous end joining (NHEJ) or homology-directed repair (HDR). If the DNA strand breaks are repaired by NHEJ, random mutations are inserted effectively knocking out the targeted gene. If additional DNA with matching flanking regions is introduced, then the DNA strand breaks can be repaired by inserting the DNA, which can result in a gene knockout, a gene correction, or even gene insertion. While this technology is quicker, cheaper, and more accurate than the siRNA technique described above, it too is limited by transfection efficiency for effective delivery of the guide RNA and Cas9 protein.

An area of research that has not yet had broad application to the field of immunotoxicology is proteomics. Proteomics can be defined as research that aims to identify, quantify, and classify the function of proteins produced by given genomes. In addition to the systematic identification and characterization of proteins, a potentially important application of proteomics is in the characterization of protein–protein interactions, especially as they relate to the elucidation of signal transduction mechanisms. Strategies have been proposed for the functional analysis of signal transduction, termed phosphoproteomics (Morandell et al., 2006). Unfortunately, a major drawback in the application of proteomics includes the lack of an experimental platform currently available to systematically measure the diverse properties of proteins in a high-throughput approach. Presently, most proteomic methods are based on mass spectrometry and require expensive instrumentation, information-technology infrastructure, and highly specialized personnel. In addition, the complexity of the proteosome is enormous as illustrated by Aebersold when he compared the human genome, which is comprised of approximately 30,000 genes, to human serum, which alone has been estimated to contain approximately 500,000 different protein species (Aebersold, 2003). Nevertheless, the application of proteomics to study proteins is occurring routinely in cell-based systems.

As stated above, the use of molecular biology–based methods in immunotoxicology to date has been primarily for understanding mechanisms of action of known immunotoxicants. Much research is currently focused on quantifying changes in mRNA levels (e.g., cytokines) and elucidating mechanisms responsible for those xenobiotic-induced changes. Similarly, changes in mRNA expression profiles may be useful as possible biomarkers of exposure. Proteomics and genomics, combined with bioinformatics, are making it possible to evaluate chemically induced alterations in entire pathways and signaling networks. The utility of molecular biology tools such as proteomics and genomics in elucidating mechanisms of action is obvious. More challenging will be the application of these tools for identifying new or suspected immunotoxicants (Luebke et al., 2006b).

Mechanistic Approaches to Immunotoxicology

Once an agent has been identified as being an immunotoxicant, it may be necessary to further characterize the mechanism by which it exerts its effects on the immune system. Toward this end, a unique aspect of the immune system that greatly facilitates investigations aimed at delineating the underlying mechanisms of action is the ability to study the immune system in the intact animal as well as by removing immune cells from the intact animal and studying their function in vitro. Using a combination of these two approaches, a general strategy has been successfully employed by a wide number of laboratories for characterizing mechanisms of immunotoxic action by xenobiotics and involves the following steps: (1) identifying the cell type(s) targeted by the agent, (2) determining whether the effects are mediated by the parent compound or by a metabolite of the parent, (3) determining whether the effects are mediated directly or indirectly by the xenobiotic, and (4) elucidating the molecular events responsible for altered leukocyte function. The significance of each and the experimental strategies typically employed are discussed below.

Identification of the Cell Type or Type(s) Targeted

With few exceptions, the first objective toward elucidation of the mechanism(s) responsible for immunotoxicity is the identification of the cell type(s) affected by the xenobiotic. Because the immune system is comprised of a wide variety of cell types with broad, and often, overlapping effector functions, identification of the specific cell type(s) affected allows for the selection of appropriate approaches and techniques to employ to further elucidate the mechanism of action.

The approaches available for the identification of the cell types targeted by a specific agent are numerous but typically originate with the employment of one or more of the functional assays within the immunotoxicology tier testing battery (see the section "The National Toxicology Program (NTP) Tier Approach"). Each of the functional assays in the immunotoxicology tier testing battery provides information on accessory and/or effector cell function. For example, the AFC response to the T-cell-dependent antigen, sRBC, requires macrophages and $CD4^+$ T cells (Th cells) to function as accessory cells, and B cells to serve as the effector cells. Therefore, if any one of these three cell types is adversely affected by an immunotoxicant, changes in the magnitude of the AFC response would be observed as compared to control (i.e., enhancement or suppression). Similarly, the CTL response requires APC and $CD4^+$ T cells (Th cells) as accessory cells and $CD8^+$ T cells (CTL) as the effector cells. Functional assays can be further refined to provide additional information concerning the targeted cell types by employment of various defined antigens requiring different cellular cooperativity to elicit an effector response.

The AFC response has been especially useful in identifying cellular targets. Historically, a comparison between the T-cell-dependent versus T-cell-independent antigens was employed to identify cell types affected by a xenobiotic. However, the current strategy utilized for identifying targets is to perform cell-specific isolations, either by a flow cytometry–based cell sorter or magnetic bead isolation kits. The primary advantage of cell sorting by flow cytometry is that it yields an exceptionally high purity of cell populations and subpopulations. The primary disadvantages include (1) practical limitations concerning the total number of cells that can be collected within a reasonable period of time due to the rate at which the instrument analyzes and collects the cells, and (2) often flow cytometry–based sorting uses positive selection (i.e., the cells are bound by a cell-specific antibody) for identifying the desired cell population being collected. The second and more commonly used approach utilizes antibodies directed at surface antigens unique to specific leukocyte populations and subpopulations that have been covalently conjugated to magnetic beads. Using the conjugated magnetic beads and a magnet, large numbers of highly pure cell populations can be isolated rapidly, by positive or negative selection, without requirements for expensive instrumentation. Both cell-sorting techniques provide purified populations of cells, which can be isolated from vehicle and treated animals for direct assessment or reconstituted in various combinations for evaluation in functional assays.

Determination of Whether Immunotoxicity Is Mediated by the Parent or by a Metabolite of the Parent Compound A critical aspect in the elucidation of the mechanism of toxicity for any compound regardless of the target or tissue is whether the adverse effects are mediated by the parent form of the compound or its metabolite. An understanding of the role of metabolism is especially critical when studying immunotoxicants as it will dictate the experimental approaches that can be utilized. In general, leukocytes possess very modest levels of drug metabolizing enzymes, especially those within the cytochrome P-450 family. Therefore, those xenobiotics that are metabolically bioactivated to an immunosuppressive form will in most cases not exhibit their immunotoxic profile of activity when added directly to cultured leukocytes. In fact, an important indication that metabolic activation may be a requisite mechanistic event is the observation that a xenobiotic is immunotoxic following in vivo administration while exhibiting no immunotoxic activity when added directly to cultured leukocytes. An example of such a xenobiotic is the therapeutic drug, cyclophosphamide. When administered in vivo, cyclophosphamide is a potent immunosuppressant that suppresses adaptive immune responses while having no effect when added directly to cultured leukocytes.

In order to assess whether metabolic bioactivation is required for immunotoxicity, several different approaches can be employed. One approach is to determine whether pre- or co-treatment with either an inducer or inhibitor of the enzymes suspected of being involved in the metabolism of the xenobiotic, modify the immunotoxicity produced in vivo. Similarly, in vitro approaches have also been used to assess the role of metabolism for an immunotoxicant. Specifically, these approaches utilize various in vitro metabolic activation systems such as S9 liver homogenates or isolated liver microsomes, which can activate the xenobiotic when incorporated with leukocyte cultures. Alternatively, freshly isolated primary hepatocytes can be co-cultured with leukocytes in the presence of the xenobiotic. Although primary hepatocytes most closely simulate the metabolic activity observed in vivo, this approach is also the most technically challenging because the approach is critically dependent on the isolation of viable and metabolically active hepatocytes. Cyclophosphamide is typically employed as a positive control in all three of the aforementioned in vitro activation systems to confirm metabolic activity. The metabolic activation systems discussed above can also be employed for conducting mechanistic studies in vitro that cannot be performed in the intact animal to further characterize immunotoxicants requiring metabolic bioactivation.

Determination of Whether the Effects Are Mediated Directly or Indirectly by the Xenobiotic or a Metabolite of the Xenobiotic In most instances, immunotoxicants mediate their effects by interacting directly with the immune system. These direct actions may include structural alterations in lymphoid organs or on the cellular composition of lymphoid organs, on the expression of regulatory molecules on the immune cell surface, and/or by altering intracellular biochemical or molecular events (Table 12-10). However, some xenobiotics mediate changes in immune competence through an indirect action on the immune system. Under these circumstances, changes in immune competence

Table 12-10

Possible Mechanisms of Chemical-Induced Immune Modulation

TYPE OF EFFECT	MECHANISM	EXAMPLES
Direct	Functional changes	Altered antibody-mediated responses
		Altered cell-mediated responses
		Altered release of preformed mediators
		Altered host resistance
		Inability of one or more cell types to perform a required activity, for example
		Production of antibody
		Release of cytokines
		Processing and presentation of antigen
		Proliferation and differentiation
		Receptor-mediated signal transduction
	Structural changes	Alterations in surface receptors or ligands
		Alteration in expression of receptors or ligands
		Alteration in peptide binding to MHC
		Histopathologic changes in lymphoid organs
	Compositional changes	Alterations in $CD3^+$, $CD4^+$, $CD8^+$, $B220^+$, and/or Ig^+ in spleen
		Alterations in $CD4^+$, $CD8^+$, $CD4^+/CD8^+$, and/or $CD4^-/CD8^-$ in thymus
		Changes in hematologic cellular parameters
		Alterations in circulating Ig
		Alterations in CFU profile in bone marrow
	Metabolic activation	Conversion to a toxic metabolite
Indirect	Effects secondary to other target organ toxicity	Induction of acute-phase proteins as a result of liver injury
		Increased corticosteroid release from the adrenal gland
	Hormonal changes	Alteration in neuroendocrine regulation
		Alteration in autonomic output from the CNS
		Altered release of steroids from sex organs

are mediated through the release of an immunomodulatory factor resulting from the actions of the immunotoxicant on cells or tissues other than the immune system. One tissue most often implicated in indirectly modulating the immune system is the liver, as it is the source of a broad and extremely diverse group of proteins, including acute-phase proteins. Acute-phase proteins are a family of serum proteins produced by the liver in response to inflammation or infection (see the section "Inflammation"). Acute-phase proteins are believed to have evolved as part of an immediate survival response to systemic infection. Acute-phase proteins are also associated with downregulation of the immune system and are therefore believed to play a role in maintaining immune homeostasis. A second example of indirect actions on the immune system is stress as well as agents that alter the regulation of the hypothalamic-pituitary-adrenal axis, thus leading to changes in hormonal homeostasis. Deregulation of hormonal homeostasis, especially increased circulating levels of glucocorticoids, can markedly decrease immune competence (see the section "Neuroendocrine Immunology").

The elucidation of indirect mechanisms of action by an immunotoxicant can be challenging by virtue that the effect is indirect and mediated by one or more circulating immunomodulatory serum factors. The involvement of an immunomodulatory serum factor can be partially deduced by two distinguishing features. First, the profile of immunotoxicity observed after in vivo administration of the causative agent is different from that produced by its direct addition to leukocytes in culture. Second, the profile of immunotoxicity observed after in vivo administration of the agent can be mimicked by direct addition of serum from the treated animal to naive leukocytes in culture. Third, the involvement of a metabolite of the parent form of the chemical has been ruled out. Confirmation of the involvement of a specific serum factor is most often accomplished in vitro by abrogating the immunotoxic activity produced by serum from treated animals using neutralizing antibodies directed against the suspected serum factor.

Elucidation of the Molecular Mechanism Numerous methodologies are available to evaluate cellular and molecular mechanisms of action and those continue to increase with the availability of new technologies, new animal models including transgenic and knockouts, and an ever-expanding list of reagents and molecular probes. Due to the broad nature of this topic area, this section is devoted to a general discussion of considerations and strategies aimed at the elucidation of molecular mechanisms. The discussion will be directed at B cells and T cells for illustrative purposes but is certainly applicable to other cell types comprising the immune system.

When considering the molecular mechanism by which a xenobiotic alters the function of a mature lymphocyte, a practical strategy is to first identify at which stage of leukocyte function the agent is acting (i.e., antigen recognition/signaling through the antigen receptor, cellular activation, proliferation, or differentiation). As discussed in the earlier sections of this chapter, lymphocytes have evolved a number of specialized mechanisms by which B and T cells recognize antigen. Common to both cell types is that immediately after recognition of an antigen, there are a number of biochemical events triggered, including changes in protein phosphorylation and activation of a variety of kinases, fluxes of ions, and changes in the level of cyclic nucleotides. Because lymphocytes undergo a rapid and robust rise in intracellular calcium almost immediately after antigen-receptor binding, changes in calcium flux prior to and immediately following stimulation by an antigen provide important insights on whether an agent alters the most proximal stage of leukocyte function. Similarly changes in the phosphorylation status of the intracellular domain of membrane-associated proteins (e.g., CD3 complex, CD4 and CD8 on T cells, and CD79, CD45, and CD22 on B cells) and the kinases that mediate the phosphorylation (e.g., lyk and fyn) can be investigated to study the most proximal events associated with triggering of antigen receptors. Polyclonal activators are often employed to simultaneously activate large numbers of lymphocytes facilitating the evaluation of changes in these early biochemical events. For B cells, antibodies directed against the BCR in combination with CD40L can be used. For T cells, antibodies against the TCR/CD3 complex and the coreceptor CD28 can be used.

The stage termed lymphocyte activation is functionally defined and refers to that period which begins when the T cell has initiated a response to an activation stimulus and ends when there is robust transcription of cytokines, such as the T-cell autocrine/paracrine growth factor, IL-2, and upregulation of proteins on the cell surface including CD40L, CD69, and CD25 (alpha chain of the IL-2 receptor). In B cells, it is the period that begins with the cell responding to an activation signal culminating in the upregulation of activation markers, such as CD80 (B7.1), CD86 (B7.2), CD69, and increased MHCII. All of the aforementioned changes associated with B-cell and T-cell activation can be readily monitored and quantified in individual cells by flow cytometry. Investigations directed at elucidating the molecular events deregulated by xenobiotics during lymphocyte activation represent one of the most intensively studied areas presently in immunotoxicology as many different immunotoxicants affect this stage of lymphocyte function.

Clonal expansion is a critical phase of the immune response as it ensures that a sufficient number of antigen-specific B- and T-effector cells are generated to respond effectively to combat a pathogen. In light of the importance of clonal expansion in mounting an effective immune response, the effect xenobiotics exert on lymphocyte proliferation has been widely used to determine whether a xenobiotic has immunotoxic properties. As with measurements directed at other stages of the immune response, it is often convenient to employ polyclonal B-cell and T-cell activators for investigating effects on proliferative responses. Due to a significant increase in the understanding of the cell cycle, coupled with the availability of reagents for measuring specific proteins involved in the regulation of cell cycle, it is now possible to functionally characterize and dissect the mechanism and specific intracellular targets affected by xenobiotics that disrupt lymphocyte proliferation. Measurements of changes in cell-cycle-associated regulatory proteins are most commonly achieved by employing flow cytometry.

The final stage of the immune response involves differentiation into a mature effector or memory cell. For B and T cells, terminal differentiation into effector cells can be best assessed by measurements of effector function in conjunction with the upregulation and downregulation of proteins on the cell surface. Antibody production and upregulation of CD138 (also known as syndecan-1) are most often used as indicators of B-cell differentiation into effector cells. It is noteworthy that high-level antibody secretion by human B cells precedes CD138 upregulation but in both the mouse and human, CD138 expression is considered the hallmark of plasma cell formation. By contrast, either cytokine production (Th cells and CTL) or lysis of target cells expressing viral or tumor proteins (CTL) is used to indicate T-cell differentiation. A number of commonly used immune function methods for assessing B- and T-cell effector functions have been described earlier in this section.

Once the specific stage of lymphocyte function being altered by a specific xenobiotic has been established, experiments can be designed to identify the specific intracellular proteins affected by the xenobiotic and the molecular mechanism by which it is modulated. Cell line models can be invaluable tools for studies involving cell signaling and gene regulation.

Cell Line Models in Immunotoxicology Cell line–based models are being widely used for identifying xenobiotics possessing the potential of producing immunotoxicity as well as for conducting in-depth investigations aimed at elucidating molecular mechanisms for immunotoxicity. Significant advantages and disadvantages exist when applying cell line–based models, some of which are unique to investigations of the immune system. A brief discussion of the strengths and limitations of cell line–based models in the context of studies of immunotoxicants is provided below.

Although there are many advantages to cell line–based models, the most important characteristic is that all of the cells are derived from the same clone, thus providing a relatively homogenous cellular preparation. The homogeneity of the model is especially useful for studies directed at characterizing signal transduction pathways as well as gene expression profiling due to the greater likelihood of obtaining reproducible results. There are a number of advantages of cell line–based models that are especially useful in immunotoxicology. Primary leukocytes whether isolated from blood or lymphoid organs are highly heterogeneous in their cellular composition. Purification of these primary cell preparations into specific cell types is expensive, can be labor intensive, and with most isolation methods typically yields 50% to 75% efficiency (i.e., 25% to 50% of the desired cells are lost during the purification process). Purification efficiency can become a critical issue when utilizing small rodents such as mice in which the number of animals per assay might be significantly increased due to the loss of cells being recovered in the cell isolation procedure. When employing cell line models, typically there is no limitation on the number of cells available for a given study. Another important consideration in the case of primary lymphocytes is that they are only viable in culture for relatively brief periods of time (approximately 24 hours) in the absence of an activation signal. Therefore, extended preincubation periods in culture with an immunotoxicant or other response modifiers that do not activate primary lymphocytes cannot be performed. Primary leukocytes, especially lymphocytes, are also difficult to transfect and often yield poor transfection efficiency and/or viability. The combination of poor transfection efficiency and limited culture duration in the absence of activation make reporter assays, transient ectopic gene expression and utilization of siRNA to silence gene expression, challenging in primary lymphocytes. Conversely, cell line–based models, especially of the lymphoid lineage, in most cases can be readily transfected with suitable efficiency and viability to conduct reporter assays, ectopic gene expression, and gene silencing with siRNA. It is noteworthy that primary cells of the myeloid lineage are more suited for long-term culture and transfection-based experimental approaches as they have less stringent requirements for activation to be maintained in culture for extended periods. In such instances, it may be more advantageous to employ primary cells. Lastly, cell line–based models are also now being widely adapted for high-throughput screening due to the reproducibility of results obtained with these models and the ease in which cell lines can be maintained and manipulated.

In spite of the advantages discussed above, there are numerous disadvantages and limitations inherent in utilizing cell line models for characterizing immunotoxicants. The most important consideration when utilizing cell line models is that by definition a cell line is an abnormal population of cells that has undergone a change rendering it capable of dividing indefinitely in culture. Because cell lines are continuously dividing, in most cases they are not good models for studying immunotoxicants that act by altering cell proliferation and/or regulators of the cell cycle. The aberrant nature of cell lines may also extend to a loss of function through one or more of its cognate receptors. Lastly, it is critical that cell lines are carefully monitored and characterized for changes in function and morphology after repeated passage in culture. Additional important considerations when selecting a cell line for mechanistic studies is the capacity of the cell line to perform a given effector function and the stimuli to which the model will respond. Toward this end a number of cell lines have been extensively characterized and widely utilized that are capable of induced effector functions, including cytokine production, antibody secretion, and release of a wide variety of mediators. Table 12-11 provides examples of some commonly used cell lines in immunotoxicology. As discussed above, with some cell lines, induction of an effector function may only be achievable by using pharmacological activators (e.g., phorbol ester plus calcium ionophore) that bypass extracellular receptors, or by agents that are polyclonal activators (e.g., LPS) that do not activate directly through antigen receptors. Again these are characteristics of the models that need to be considered in the context of how the models will be used and for what specific purpose. In most cases, the utilization of cell line–based models can be extremely useful but results should always be confirmed, when possible, in primary leukocytes because cell lines are aberrant models.

Animal Models: Transgenic, Knockout, and Humanized/SCID The developments in molecular biology have not only permitted the evaluation of specific genes or arrays of genes but also allowed for the manipulation of the embryonic genome, creating

Table 12-11

Cellular Models for Immunotoxicology

CELL LINE	CELL TYPE	SPECIES	EFFECTOR RESPONSE	STIMULUS
EL4	T cell	Mouse	IL-2	Phorbol ester
Jurkat E6	T cell	Human	IL-2	Phorbol ester + Ca ionophore or αCD3 + αCD28
CHI2.LX	B cell	Mouse	IgM	LPS
BCL-1	B cell	Mouse	IgM	LPS
SKW	B cell	Human	IgM	LPS or αIgM + CD40L
Daudi	B cell	Human	None (signal transduction)	αIgM
RAW 264.7	Macrophage	Mouse	Nitric oxide, IL-1	LPS
J774.1	Macrophage	Mouse	Nitric oxide, IFN-γ	LPS
U937	Macrophage	Human	TNF-α, IL-1α, IL-6, IL-8	LPS

transgenic and knockout mice. As a consequence of transgenic technology, complex immune responses can be dissected into their components. In this way, the mechanisms by which immunotoxicants act can be better understood. Mice engineered to express nonself genes (e.g., human MHCI) or overexpress self-genes (e.g., constitutively active transcription factors) are termed "transgenic," and can be used to address the role of a certain protein in immunotoxicology. In contrast, mice lacking certain proteins (e.g., receptors, transcription factors, or cytokines) are termed "knockouts," and can be used for similar mechanistic studies. Numerous transgenic and knockout mice have been engineered and are available to investigators worldwide. Of particular interest and potential utility in the area of immunotoxicology is what has been termed "humanized" mice. Humanized mice refer to immune-deficient mice that have been reconstituted either with human hematopoietic stem cells to support the development of a fully human immune system or with mature cells to evaluate immune regulation, hematopoiesis, hypersensitivity, and autoimmunity. Severe combined immunodeficient (SCID) mice, which are mouse T- and B-cell deficient due to a VDJ (variable region gene segments) recombination defect, are common models into which human immune cells/system are established. In addition, SCID mice are backcrossed with a variety of mouse models in order to eliminate various arms of the immune system (e.g., $\beta 2M^{null}$/SCID eliminate MHCI interactions) prior to reconstitution with human cells (reviewed by Thomsen et al. [2005]). Although their use in mechanistic studies is obvious, SCID/hu mice have also been utilized for evaluation of efficacy of antiviral drugs for HIV/AIDS (reviewed by Taggart et al. [2004]). There are still considerations, however, for broader application of these humanized mouse models for immunotoxicological assessment of compounds. For instance, one must consider how these animals compare with standard animal models with respect to time course of action, dose response, pharmacokinetics, and other factors of chemical toxicity. With the hope that these humanized mouse models can be used to identify new or suspected immunotoxicants, and mechanisms of action, one must also consider whether they are as sensitive, predictive, and/or cost-effective as traditional animal models.

Approaches to the Assessment of Human Immunotoxicity

As emphasized throughout this chapter, animal models have been extensively utilized to characterize immunotoxicity, and it is widely recognized that chemicals that produce immunotoxicity in animals have the potential to produce immune effects in the human population. In spite of similarities between the rodent and human immune systems, which has facilitated our ability to make decisions on the immunotoxic potential of test materials based on the results of these experimental models, there is also an increasing recognition of distinct, although often subtle, differences in the composition and functional responses of the immune system between animal species. These differences have primarily come to light in studies of specific leukocyte populations, the phenotypic markers they express, and by the regulatory factors they secrete. Whether these differences in immune system composition and response to stimuli result in significant differences across species in sensitivity to immunotoxicants is unclear. It is important to emphasize that to date there are no known instances of significant human immunotoxicity that has resulted due to false-negative results from rodent-based immunotoxicity testing. However, there is limited human immunotoxicology data on test materials other than those directly intended for human immunotherapy.

One of the obvious and most important goals of an experimental immunotoxicity testing strategy is to enable the best extrapolations between the results generated in the laboratory animal models and the potential risk of immunotoxicity in humans. The overall strategy to date has been to utilize in vivo and in vitro data from animal studies and, when available, in vitro data using human leukocytes in order to predict the in vivo effects of an immunotoxicant in humans. If in vitro human data are not available, then safety decisions are made solely on animal data.

Even if human in vitro data are available when assessing the immunotoxicity of a test article, typically it is limited with the majority of data coming from assessments made in animal models, and often from rodents. Therefore, it is recognized that extrapolations of alterations in immune function observed in laboratory animals to human health is associated with various uncertainties. For example, experimental animals are often inbred, which certainly lessens interanimal variability thereby simplifying the statistical evaluation of observations. Additionally, laboratory studies in rodents are highly controlled for environment, diet, and health status. By contrast, humans are a highly outbred species with a high degree of interindividual variability in immune response, and any human study must take into account extreme variability in all of the controlled parameters. In fact, it has been estimated that the overall immunocompetence of the individual is affected by age, sex, genetic factors, use of certain medications, drug/alcohol use, smoking history, stress, and nutritional status, and that these factors can account for variability in the "normal" human population (Luebke et al., 2004).

There is a clear association between compromised immune function and an increased incidence of infectious and neoplastic disease in humans, as is observed in individuals with primary immune deficiencies (reviewed by Rezaei et al. [2011]). However as noted above, a major limitation of immunotoxicity risk assessment has been the lack of human data (Descotes, 2006). In the context of xenobiotics, the majority of such data comes from immunotherapeutic drugs, which were intentionally designed to influence the immune system. The aforementioned association has been established primarily with a standard battery of parameters used extensively in clinical immunology studies, including routine complete blood counts and differentials, phenotypic characterization of surface markers, and more detailed analysis of leukocyte populations using flow cytometry, lymphoproliferative assays (i.e., using a number of stimuli including mitogens such as ConA and PHA, recall antigens such as tetanus toxoid, anti-CD3 monoclonal antibody, and allogeneic lymphocytes in an MLR), and DTH responses using a test panel of recall antigens. While these endpoints are sufficient to detect immunodeficiencies associated with either congenital disorders or immunosuppressive drug therapy, they may not possess the necessary sensitivity to detect the more subtle consequences of xenobiotic-induced immunotoxicity. Interestingly, Luebke et al. (2004) noted that in contrast to individuals with severe forms of primary or secondary immunodeficiency in whom infections to opportunistic pathogens (i.e., members of the herpes viruses family, certain protozoans, *Candida albicans*, a yeast, and *Pneumocystis carinii*, a fungus) are seen, humans with low to moderate suppression of immune function are more susceptible to infection with pathogens associated with infections common in the general population (i.e., encapsulated bacteria like *Streptococcus*, and influenza viruses). As a result, several proposals have been put forth to reevaluate the way immune competence is assessed in humans. Most of these testing strategies have incorporated plans to measure the primary response to a new antigen, and several of these testing strategies have recommended using newly developed vaccines as the new antigen (Van Loveren et al., 2001). Vaccine response rates have been successfully used to characterize the impact of chronic stress on human

immunocompetence (Kiecolt-Glaser et al., 1996; Glaser et al., 2000). Importantly, in spite of the limitations in human data, there is no question that environmental chemicals are associated with immunotoxicity in humans (Veraldi et al., 2006; Selgrade, 2007).

It is anticipated that the development, validation, and utilization of more predictive methods to assess immunotoxicology in humans will be increasingly important in the future. This is due in part to the recognition that investigations conducted exclusively using in vitro and in vivo laboratory animal models may not always be predictive of toxicity in humans, and in part due to advancements in reagents and technology, making the study of human primary leukocytes more feasible. An additional factor has been the significant expansion in the development of new therapeutic agents specifically targeting the human immune system as discussed elsewhere in this chapter. Collectively there has been increasing interest in establishing predictive in vitro methods using human lymphocytes.

As will be discussed in the section "The National Toxicology Program (NTP) Tier Approach," a cornerstone of immunotoxicologic assessments using rodents has been a tier testing battery of immune function assays capable of evaluating the effects of xenobiotics on a host of different immunological parameters. Such an approach has been necessary due to the diversity of cell types and functions that are involved in maintaining immune competence (Luster et al., 1988). In fact there is strong historical evidence, from examination of the NTP's immunotoxicology database, that no one immune function assay or endpoint can reliably identify all immunotoxicants. Toward this end a multi-assay approach, analogous to that currently used for evaluations in the mouse, could be employed for immunotoxicity evaluations using human primary leukocytes. Such a human tiered testing approach could be used as an adjunct to animal-based immunotoxicity testing.

Many if not most in vitro immune function assays initially developed using mouse leukocytes have been adapted for human primary leukocytes. Perhaps the most challenging has been assays to assess the primary antibody response. Toward this end several strategies have been applied. For example, a polyclonal IgM AFC response model was developed using the combination of CD40 ligand (CD40L) plus recombinant cytokines because it effectively drives both primary human and mouse B cells in a physiologically relevant manner to mimic TDAR in vivo (Lu et al., 2009). IgM-secreting cells are enumerated by ELISPOT. Alternatively, supernatant IgM is quantified by ELISA. A second assay, the human lymphocyte activation (HuLa) assay, measures an influenza antigen-specific response using frozen-stored human PBMC (Collinge et al., 2010). Although proliferation is the primary endpoint in the HuLa, flow cytometry can be used to characterize the proliferating lymphocyte subsets (e.g., $CD4^+$ and $CD8^+$ T cells and B cells), and ELISPOT can be used to enumerate influenza-specific ASCs. A third in vitro model, termed Modular Immune In vitro Construct (MIMIC), uses the circulating immune cells of individual donors to recapitulate each individual human immune response by maintaining the autonomy of the donor and represents a physiologically accurate model of the human immune system (Higbee et al., 2009). Importantly, all three of these models have been used to characterize the dose–response characteristics and potency of a number of immunosuppressive drugs and nondrug chemicals.

For illustrative purposes it is useful to briefly compare and contrast the in vitro T-cell-dependent IgM AFC response, which uses mouse spleen cells and is often considered one of the gold standard assays for characterizing immunotoxicants, to the human CD40L-induced IgM AFC assay, which utilizes purified human peripheral blood (HPB) B cells. Unlike the widely used T-cell-dependent IgM AFC response assay, which uses a heterogeneous leukocyte preparation from the mouse spleen and sRBC as the sensitizing antigen, the CD40L drives IgM production using purified HPB B cells or primary mouse B cells, thus allowing for direct comparisons of sensitivity of immunotoxicants between mouse and human B cells (Lu et al., 2009). Importantly, there are a number of significant and fundamental differences between the sRBC IgM AFC response and the CD40L-induced IgM AFC response. First, the CD40L response has no accessory cell involvement (i.e., T cells or APCs), because CD40L stimulation is provided either through the direct addition of rCD40L or by co-culturing B cells with a genetically engineered CD40L-expressing fibroblast cell line (CD40L-L cells) plus recombinant T-cell cytokines. Therefore, the CD40L assay only provides information on B-cell function. In contrast, the sRBC IgM AFC response assay requires functional T cells and APC for IgM production. From the perspective of hazard identification, the sRBC assay has greater capacity to detect immunotoxicants, because in addition to B cells, the interactions of two other cell types and their consequent accessory cell functions are required in comparison to the CD40L assay. However, mechanistically, modulation of the CD40L-induced IgM response provides definitive identification of B-cell immunotoxicants, so mechanisms of B-cell immunotoxicology can be investigated in the absence of potential confounders associated with the presence of other cell types. In fact, using CD40L plus cytokines, the effects of immunotoxicants on B cells can be studied temporally throughout the entire continuum, from activation to differentiation, using flow cytometry. Second, the sRBC IgM AFC response is antigen-specific and the CD40L-induced IgM response is not. In fact, presently there is no established antibody assay in which a primary antigen-specific TDAR can be elicited in human primary leukocytes. One of the major challenges in establishing such an assay is that there are very few circulating naive B cells present in peripheral blood and even fewer that possess BCRs specific for any given antigen. Because all B cells constitutively express CD40, the CD40L-induced IgM response circumvents the requirement for antigen specificity and induces a polyclonal B-cell response. Antigen-specific secondary antibody responses are more feasible, especially in response to recall antigens, such as tetanus toxoid. Because a large portion of the human population has undergone repeated and regular vaccination throughout their life (e.g., tetanus), HPB B cells possessing BCRs specific for vaccine-associated antigens in these individuals have been clonally expanded and can be readily activated and quantified in vitro. The disadvantage to this approach is that memory B cells, which are the cells that drive the secondary immune responses to recall antigens, have been found to be less sensitive to modulation by immunotoxicants. In light of this, the CD40L-induced IgM response has been primarily conducted with naive HPB B cells after removal of memory B cells.

Three extensively studied B-cell immunotoxicants in mice, arsenic, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), and TCDD, were evaluated using the CD40L-induced IgM AFC response (Lu et al., 2009, 2010). All three immunotoxicants were found to suppress the CD40L-induced IgM AFC response in mouse splenic B cells, with the magnitude of suppression being comparable to that previously reported using the sRBC IgM AFC response (Holsapple et al., 1986; Kawabata and White, 1989; Burns et al., 1991). BPDE and TCDD were also found to markedly suppress the CD40L-induced IgM AFC response in HPB B cells, and with a similar magnitude of suppression being observed between mouse and human B cells. In contrast, arsenic did not suppress the CD40L-induced IgM AFC response in HPB B cells. These limited results using only three B-cell immunotoxicants suggest that the mouse may not always serve as a reliable surrogate for predicting immune toxicity in humans.

In addition to the primary antibody response, a number of immune function assays using primary human leukocytes have historically been widely employed such as the MLR, which provides information on $CD4^+$ and $CD8^+$ T-cell function, in the clinical setting to determine histocompatibility between donors and recipients for tissue transplantation. Similarly, B-cell and T-cell lymphoproliferative responses, which assess lymphocyte clonal expansion, have been used to assess human lymphocyte function. Collectively, these assays represent a foundation for a human leukocyte immunotoxicology testing battery. The possibility of developing better translational biomarkers could facilitate the progression from preclinical studies to clinical studies in drug development.

Regulatory Approaches to the Assessment of Immunotoxicity

The maturity and acceptance of any subdiscipline of toxicology can frequently be directly correlated to the level of interest being demonstrated by the regulatory community. This section provides a brief review of the history of regulatory approaches to immunotoxicity. It is important to note that the regulatory approaches to immunotoxicity have evolved with the science, and the timing of the introduction of specific guidelines has generally reflected the state-of-the-science. Because of the importance of the evolution of the science, this section begins with an overview of one of the most comprehensive databases for immunotoxicology.

The National Toxicology Program (NTP) Tier Approach Although the concept of required immunotoxicity testing is a relatively recent development, the recognition that the immune system is a potential target organ prompted a consideration of the need to assess the potential of immunotoxicity for some time. All testing strategies to date have recognized the complexity of the immune system as a target organ, and that no single immune parameter can be used with sufficient confidence to test for the hazard of immunotoxicity. Therefore, historically immunotoxicity has been assessed using a battery of assays, which are typically structured in a multi-tiered approach. Importantly, studies conducted by the NTP have indicated that immunotoxicity can be assessed with a finite number of assays. Luster et al. (1988) originally described the selection of a battery of tests used by the NTP to screen for potential immunotoxicants. The result was a tiered approach to assess immunotoxicity, which is summarized in Table 12-12.

Tier I tests provide assessment of general toxicity (e.g., immunopathology, hematology, and body and organ weights) as well as some assays to assess immune functional capability (e.g., proliferative responses, TDAR, CTL assay, and NK assay). Tier I was designed to detect potential immunotoxic compounds at concentrations that do not produce overt toxicity. Tier II tests were designed to further define an immunotoxic effect, and included functional tests for secondary antibody responses, enumeration of lymphocyte populations, and host resistance models.

In the original NTP effort, over 50 chemicals were studied, and included a variety of chemical classes, such as catalysts, solvents, dyes, lubricants, pesticides, disinfectants, drugs, food additives, and natural products (Luster et al., 1988). It is important to emphasize that the chemicals selected for study by the NTP were nominated for testing because of a suspicion that they might target the immune system. These studies were conducted in young adult rodents, principally the mouse, and involved comparative studies across multiple labs. The experimental design emphasized that regardless of the specific immune parameters included in a testing strategy, the interpretation for immunotoxicity could only be made in the context of a well-designed study from the perspective of the dose–response relationship. The approach by Luster and co-workers also emphasized the importance of avoiding high doses (i.e., triggered less than a 10% reduction in body weight) because these types of exposures will increase the likelihood that indirect mechanisms of immunotoxicity may be involved.

Table 12-12

Tier Approach for Immunotoxicology Testing

TESTING LEVEL	PROCEDURES
Tier I	Hematology Body weight Organ weights (spleen, thymus, kidney, liver) Spleen cellularity Bone marrow cellularity and CFU Immunopathology AFC assay Proliferative responses NK assay
Tier II	Surface marker analysis Secondary (IgG) AFC assay CTL assay DTH response Host resistance studies

Table 12-13

Suggested Testing Configurations: Three Tests with 100% Concordance

TDAR	DHR	Surface markers
TDAR	NK	DHR
TDAR	NK	Thymus:body weight
TDAR	DHR	Thymus:body weight
Surface markers	NK	DHR
Surface markers	DHR	T-cell mitogens
Surface markers	DHR	Thymus:body weight
Surface markers	DHR	LPS response

SOURCE: Luster et al. (1988), modified, with permission.

Several testing configurations were ultimately defined that would minimize the number of immune tests needed, yet could still provide a high degree of sensitivity for detecting potential immunotoxicants. These configurations are depicted in Table 12-13. Specifically, the results indicated that none of the assays measured were 100% predictive alone. The top three assays in terms of detecting immunotoxicants were an assessment of lymphocyte subpopulations by flow cytometric analysis (e.g., 83% concordance), the TDAR (e.g., 78% concordance), and an assessment of NK cell activity (e.g., 69% concordance) (Luster et al., 1992). In regards to the comparison between the two most sensitive assays, it is important to note that a change in flow cytometric analysis of any of the lymphocyte subsets measured (e.g., total B cells, total T cells, $CD4^+$ T cells, or $CD8^+$ T cells) would be sufficient to establish the

concordance of flow cytometry, while the concordance of the TDAR was determined solely by the number of AFCs. Subsequent analysis indicated that an application of the full extent of the tiered approach was not necessary. The NTP studies indicated that several combinations of only two tests could give more than 90% concordance, and that a number of combinations of just three tests gave 100% concordance. In this regard, it is important to note that flow cytometric analysis of lymphocyte subsets, and the TDAR were consistently involved in both the two-test concordance of more than 90%, and in the three-test concordance of 100% (Luster et al., 1993).

Subsequent results also indicated a good correlation between the results from functional tests and host resistance models (Luster et al., 1993), which indicated that the latter were not necessary to adequately identify immunosuppressive potential. The impacts of the NTP database are clear, and one only needs to consider the structure of the immunotoxicity guidelines that have been established to see the impact of the NTP results. Specifically, after several years of international debate, the importance of including functional immunotoxicity assessments in regulatory studies has been emphasized, as opposed to relying solely on histopathology as an indicator of further testing needs. Moreover, as described further below, the specific immune functional tests in most, if not all, immunotoxicity regulatory guidelines reflect the concordance results described above, and include the TDAR, immune cell phenotyping by flow cytometry, and the NK cell assay.

While the original NTP studies in the 1980s were intended to develop and validate methods to evaluate modulation of immune function, the emphasis was clearly focused on immunosuppression. The NTP contract to assess the "Potential for Environmental and Therapeutic Agents to Induce Immunotoxicity" is still assessing chemicals for potential immunomodulation at the rate of four chemicals per year for screening (e.g., range-finding) studies and two chemicals per year for definitive (e.g., full protocol) studies. Importantly, the current approaches by the NTP reflect the state-of-the-science of immunotoxicology, in that two developmental immunotoxicity studies, four contact hypersensitivity studies, and one study assessing the potential of a xenobiotic-associated change in an autoimmune disease model are assessed each year.

In 2009, the NTP provided another critical component to our understanding of xenobiotic-induced immunotoxicity when they released their *Explanation of Levels of Evidence for Immune System Toxicity* (NTP, 2009) as an approach to judging the quality of results in a study purporting to establish xenobiotic-induced immunotoxicity. In the document, which was prepared by a panel of experts representing the academic, government, and industry sectors of the scientific community, the NTP emphasized the importance of recognizing that the "levels of evidence" statements that are described reflected only immunologic hazard, and that the actual determination of risk to humans required exposure data that were not covered in the document. Five categories of evidence of immune system toxicity were used to summarize the strength of the evidence observed in each experiment being considered: two categories for positive results (clear evidence and some evidence); one category for uncertain findings (equivocal evidence); one category for no observable effects (no evidence); and one category for experiments that cannot be evaluated because of major design or performance flaws (inadequate study). Each category included one or more bullet points for additional clarification. The category for clear evidence is worthy of specific mention.

Clear Evidence of Toxicity to the Immune System:

- Is demonstrated by data that indicate a dose-related effect (considering the magnitude of the effect and the dose–response) on more than one functional parameter and/or a disease resistance assay that is not a secondary effect of overt systemic toxicity, or
- Is demonstrated by data that indicate dose-related effects on one functional assay and additional endpoints that indicate biological plausibility (NTP, 2009).

The significance of this category is that clear evidence of immunotoxicity can be established without the need to conduct a host resistance assay, and that mechanistic data to establish biological plausibility is weighed very heavily. The NTP report also indicates that negative results, in which the study animals do not exhibit evidence of immunotoxicity, do not necessarily imply that a test article is not an immune system toxicant, but only that the test article is not an immune system toxicant under the specified experimental conditions. Finally, the report indicates that positive results demonstrating that a test article causes immunotoxicity in laboratory animals under the conditions of the study are assumed to be relevant in humans, unless data are available which demonstrate otherwise.

Historical Perspective on Regulatory Guidance in Immunotoxicology The history of regulatory guidance in immunotoxicology was reviewed by House (2005). As noted above, the TDAR has been the consensus choice for a functional endpoint to identify immunotoxicity hazard in most, if not all, regulatory guidelines. A reasonable question to address is what accounts for the sensitivity, predictivity, and (perceived) utility of the TDAR. The simplest answer is that TDAR requires the orchestrated coordination and cooperativity of multiple cell types (e.g., APCs, Th cells, and B cells) and the manifestation of most, if not all, of the biological functions important to immunocompetence. As noted throughout this chapter, the discipline of immunotoxicology continues to evolve along with advances in immunology and molecular biology. However, in the context of providing an effective screen for immunotoxic hazards, nothing can compare to the time-tested and mechanistically understood specific antibody response to a well-characterized T-dependent antigen.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) of the U.S. Environmental Protection Agency (EPA) published guidelines entitled *Biochemicals Test Guidelines: OPPTS 880.3550 Immunotoxicity* in 1996. These guidelines described the preferred study design for an exceptionally thorough evaluation of the potential immunotoxicity in biochemical pest control agents. These guidelines described a panel of tests that included standard toxicology tests as well as immune functional tests assessing both humoral immunity and CMI. OPPTS 880.3550 clearly presented a very comprehensive approach to immunotoxicity; but a second document, *Biochemicals Test Guidelines: OPPTS 880.3800 Immune Response*, was needed to provide the rationale for when these studies should be conducted. The 880 series of immunotoxicity guidelines would arguably detect any type of immunotoxic potential by pesticides. However, the comprehensive nature of these guidelines rendered them prohibitively expensive and time-consuming. The EPA released the *Health Effects Test Guidelines: OPPTS 870.7800 Immunotoxicity* in 1998 (EPA, 1998). These guidelines described the approach to immunotoxicology testing for nonbiochemical agents regulated by the EPA. The testing approach in OPPTS 870.7800 reflected the continued evolution of the science of immunotoxicology and called for a more limited, case-by-case approach than previously described by the earlier more comprehensive guidelines. The cornerstone of OPPTS 870-7800 was a functional test, the primary TDAR. If the chemical was shown to produce significant suppression of the humoral response, then surface marker assessment by flow cytometry may be performed. If the chemical was not shown to produce suppression of the humoral response, then an assessment of innate immunity (e.g., specifically, the NK

cell activity assay) may be performed. The specific criteria for conducting these "optional" tests have never been identified. The tests do not represent a comprehensive assessment of immune function but are intended to complement assessment made in routine toxicity testing (e.g., hematological assessments, lymphoid organ weights, and histopathology).

Internationally, the Organization for Economic Cooperation and Development (OECD) has not as yet adopted specific guidelines for immunotoxicity assessment. As reviewed by House (2005), the OECD Guideline 407, entitled *Repeated Dose 28-day Oral Toxicity Study in Rodents* while not specific for immunotoxicology, includes a variety of toxicological endpoints that can provide early evidence of immune system alterations. To date, the OECD Guideline 407 has not been modified. Moreover, while the OECD published a template for immunotoxicity studies on its Web site in 2011, there are still no corresponding OECD test guidelines for immunotoxicity.

The earliest immunotoxicity guidelines from the Food and Drug Administration (FDA) were centered on food additives as the *Draft Redbook II* in 1993. This document, although never finalized, contained an extensive description of immunotoxicology testing. In general, the Redbook guidelines reflected the tiered approach to immunotoxicology (Table 12-12), as described in greater detail below. Specifically, the Redbook emphasized a step-wise approach that began with expanded studies utilizing data obtained in standard toxicology testing as initial indicators of immunotoxicity. Progressively more complicated immunological tests were prescribed using an approach that was very much case-by-case, with each new level of testing predicated on positive results in the preceding level. In 2002, the FDA Center for Drug Evaluation and Research (CDER) released its document entitled *Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs* (FDA, 2002). This document is arguably the most comprehensive description of approaches to immunotoxicology. Not only does the FDA CDER *Guidance for Industry* describe the full spectrum of adverse events associated with the immunotoxicology continuum, including immune suppression, immunogenicity, hypersensitivity, autoimmunity, and adverse immune stimulation, this document provides approaches at the level of specific methodology for evaluating each event. As with the earlier document from the FDA, the new *Guidance for Industry* advocated the use of information derived from standard repeat-dose toxicity studies to provide the earliest indicators of immunotoxicity. The review by House (2005) also addressed the current status of regulatory guidance for biologicals, vaccines, devices, and radiological agents within the FDA.

Internationally, perhaps one of the most significant advances in our approach to the assessment of immunotoxicity with human pharmaceuticals has been a guidance entitled *S8 Immunotoxicity Studies for Human Pharmaceuticals*, which was prepared under the auspices of the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceutical for Human Use (EMA, 2006). The objectives of this guideline are twofold: (1) to provide recommendations on nonclinical testing approaches to identify compounds that have the potential for immunotoxicity, and (2) to provide guidance on a weight-of-evidence decision-making approach for immunotoxicity testing. The guidance applies to unintended immune suppression and immune enhancement, excluding allergenicity or drug-specific autoimmunity. The ICH S8 guideline is based on a cause for concern approach, using a review of factors such as the following: results from Standard Toxicity Studies (STS), pharmacological properties of the drug, the intended patient population, structural similarities to known immune modulators, the disposition of the drug, and clinical information.

As emphasized in the section "Introduction," immunotoxicity exists in a continuum (Fig. 12-1). By and large, more effort has been invested in the validation of studies to address the immunosuppressive part of the continuum, and there is no doubt that much attention is paid to immune suppression in the majority of guidance documents (House, 2005). Nonetheless, it is important to determine xenobiotic-induced changes in immune function in both directions. As noted by House (2005), hypersensitivity is the most common type of immune modulation in humans resulting from exposure to xenobiotics. One of the most significant advances in our approach to assessing the potential hypersensitivity of xenobiotics has been the LLNA. The LLNA was the first assay to be evaluated by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM). Because of the ICCVAM evaluation, the LLNA is now an accepted assay and discussed in considerable detail in the OECD 429 Guideline, *Skin Sensitization: Local Lymph Node Assay*, and the EPA document, *OPPTS 870.2600 Skin Sensitization*.

While the mouse LLNA is the preferred and accepted method for assessing skin sensitization of most substances, a number of in vitro models have emerged because of the desire to further reduce the number of animals used in these types of studies. Not too surprisingly, these efforts have been driven mostly in Europe. Specifically, the European Union Reference Laboratory for alternatives to animal testing and European Committee for the Validation of Alternative Methods (EURL–ECVAM) have endorsed as valid the following three nonanimal test methods: the Direct Peptide Reactivity Assay (ECVAM, 2013), the KeratinoSENS™ (ECVAM, 2014), and the human Cell Line Activation Test (ECVAM, 2015). The Direct Peptide Reactivity Assay (DRPA) addresses haptenation, a key mechanism in the development of skin sensitization/allergy/contact dermatitis (ECVAM, 2013). The EURL–ECVAM determined that the KeratinoSENS™ appears to be a reliable test method that provides information on the ability of a chemical to activate the electrophilic and oxidative-stress response signaling pathway mediated by the transcription factor Nrf2 (nuclear factor E2–related factor 2), which has been shown to be a relevant pathway in skin sensitization (ECVAM, 2014). Finally, because the activation of dendritic cells (typically measured by the expression of cell surface markers and secretion of chemokines and cytokines) is recognized as a key event in skin sensitization, the human Cell Line Activation Test (h-CLAT) was developed. Specifically, the h-CLAT measures the upregulation of the CD86 and CD54 markers of DC activation in THP-1 cells, a human monocytic leukemia cell line (ECVAM, 2015).

Recent Advances in Regulatory Guidance on Immunotoxicology There have been two areas over the last few years that have impacted the status of immunotoxicology testing, which are either legislative demands or because of the continued evolution of the science.

As noted in the sections "Molecular Biology Approaches to Immunotoxicology" and "Mechanistic Approaches to Immunotoxicology," the use of in vitro and cell-based tissue culture methods have always played an important role in the science of immunotoxicology. Of course, it has always been recognized that the use of these alternative approaches are consistent with the 3Rs of reducing, replacing, and refining the use of animal studies. However, recent legislative actions such as the implementation of Registration, Evaluation and Authorization of Chemicals (REACH) and of the Cosmetics Directive in Europe have focused renewed and required attention on in vitro approaches to all types of toxicity assessment, including immunotoxicity, especially in Europe

(Carfi et al., 2007; Galbiati et al., 2010; Lankveld et al., 2010). Some recent specific examples of the utility of in vitro approaches to assess immunotoxicity include an improved in vitro cytokine release assay using human PBMC (Kooijman et al., 2010), and some renewed interest in the Mishell–Dutton culture system that has been described earlier in this chapter in the section "Methods to Assess Immune Competence: General Assessment" (Mishell and Dutton, 1967; Fischer et al., 2011). Not too surprisingly, Fischer et al. (2011) conclude that the investigation of in vitro antibody responses is a sensitive and reliable approach for the detection of a compound-specific effect on the immune system. They also conclude that the implementation of this approach in routine toxicology would enable the refinement of existing in vivo studies by reducing the number of animals. However, somewhat surprising is their conclusion that the in vitro Mishell–Dutton culture system for measuring specific antibody responses of primary cells may replace the ex vivo TDAR in the future. A significant concern pertaining to the utilization of this strictly in vitro approach is that leukocytes possess remarkably limited drug metabolizing capability and as such chemicals that are activated by metabolism to an immunotoxic form would not be detected utilizing this strategy.

As described in the section "Developmental Immunology," the interest in DIT testing has increased over the last years, with considerable attention being devoted to this area of study by the scientific and regulatory communities on a global scale. While it is important to emphasize that there are currently no regulatory guidelines specifically focused on assessment of DIT for either drugs or nondrug chemicals, there are some general guidelines included in both developmental and reproductive toxicity (DART) and general immunotoxicology guidance documents. Evaluations of DIT began to be required as part of the U.S. EPA OPPTS test guideline for the two-generation reproductive toxicity study (EPA, 1998), which requires collection of spleen and thymus weights in one pup per sex per litter in F_1 and F_2 weanlings. Another example of this trend in the context of assessing the effects of chemicals on the developing immune system is the paper by Cooper et al. (2006), in which the authors described an expanded one-generation DART protocol that included the assessment of immunotoxicology, among other endpoints, following exposure during critical windows of development. More recently, Vogel et al. (2010) described a modular one-generation reproduction study that included an immunotoxicity module as a flexible testing system for regulatory safety assessment, and highlighted the application of such an approach to the EU's REACH program. Based on these kinds of proposed studies, the U.S. EPA and the Dutch authorities convened a working group to revise the OECD 415 protocol to embrace some of the principles articulated by Cooper et al. (2006) and by Vogel et al. (2010). Subsequently, the OECD released Test Guidance 443, which is an extended one-generation reproductive toxicity study protocol (OECD, 2010) that included three cohorts, with the third cohort devoted to an assessment of the potential impact of chemical exposure on developmental immunotoxicity. Cohort 3 includes a minimum of 10 males and 10 females, with a primary TDAR conducted at postnatal day 56. The significance of the TDAR is interpreted in the context of additional immunologic indicators, including bone marrow cellularity, weight and histopathology of lymphoid tissues, and lymphocyte subset distribution. This guidance represents the most robust assessment of the potential hazard of a chemical utilizing in utero, lactational, and juvenile exposure.

In its 2002 *Guidance for Industry*, the FDA noted that all drugs that are immunotoxic in adult animals and are to be given to pregnant women should be evaluated for DIT (FDA, 2002). Endpoints recommended to be assessed were hematology and lymphoid organ weights on F_1 pups in standard DART studies. More recently, a number of regulatory guidelines have also emerged to address the safety of drugs intended for pediatric indications, including the FDA *Guidance for Industry Nonclinical Safety Evaluation of Pediatric Drug Products* (FDA, 2006; Tassinari et al., 2011), and the European Medicines Agency *Guideline on the Need for Nonclinical Testing in Juvenile Animals on Human Pharmaceuticals for Pediatric Indications* (EMA, 2008; Carleer and Karres, 2011). In Japan, the Ministry of Health, Labor, and Welfare (MHLW) started to prepare their *Guideline for Nonclinical Safety Studies in Juvenile Animals* in 2010 (Shimomura, 2011). In general, juvenile toxicity studies are conducted to identify potential latent signs of toxicity, to characterize the potential for chronic developmental toxicity that cannot be thoroughly assessed through standard DART protocols, and to ensure that children are not unnecessarily exposed to potentially adverse risk factors. The primary objective of juvenile animal toxicity studies of pharmaceuticals is to obtain safety data, including information on the potential for adverse effects on postnatal growth and development (Cappon et al., 2009). Indeed, the FDA guidance from their CDER addresses "… the role and timing of animal studies in the safety evaluation of therapeutics intended for the treatment of pediatric patients," and emphasizes the following premise: "It is thought that organ systems at highest risk for drug toxicity are those that undergo significant postnatal development" (FDA, 2006). Importantly, the immune system was identified in the FDA *Guidance for Industry* as an important endpoint.

While the OECD 443 protocol described above has been designed to assess the effects of chemical exposure in rats, more recently, evaluations of DIT for pharmaceuticals in NHPs have been conducted as part of embryo-fetal development (EFD), pre/postnatal development (PPND), or juvenile studies. With regard to critical immune system windows, the enhanced PPND (ePPND) study design in NHPs appears optimal for DIT testing, and has been shown to have similar benefit to the testing of monoclonal antibodies (Stewart, 2009) and biopharmaceuticals (Martin et al., 2009). Additional perspective on the state-of-the-science of juvenile immunotoxicology studies for pharmaceuticals is provided in a paper by Holsapple and O'Lone (2012).

In March 2012, the World Health Organization (WHO)/International Programme on Chemical Safety (IPCS) published the first harmonized guidance for immunotoxicity risk assessment for chemicals (WHO, 2012). The guidance aims to improve immunotoxicity risk assessment of chemicals to reduce or prevent human exposure to immunotoxic concentrations of chemicals and thereby protect public health. The WHO/IPCS *Guidance for Immunotoxicity Risk Assessment for Chemicals* provides background information on the immune system and its perturbation by xenobiotics and step-by-step weight-of-evidence approaches to assess the risk for the different types of immunotoxicity from chemical exposures. A table of entry points for the risk assessment process is included to help the risk assessor to identify the type(s) of immunotoxicity suggested by human or animal data and decide which of the risk assessment schemes (immune suppression, immune stimulation, autoimmunity, or allergenicity) should be followed. In some cases, chemicals may exert different or partly overlapping types of immunotoxicity, and each should be addressed. Additionally, a review and risk assessment guidance for each type of immunotoxicity are presented. The guidance also includes six case-studies of selected immunotoxic chemicals to illustrate how the risk assessment guidance can be used for assessing the risk for the various areas of immunotoxicity. This guidance is intended for adoption and use by regulatory authorities and other risk assessment bodies, industry, research institutions, and others involved in chemical risk assessment (WHO, 2012).

IMMUNE MODULATION BY XENOBIOTICS

The very nature of the immune system with its diversity of cell types, presence of various cell types in every tissue of the body, dependence upon proliferation and differentiation for effector functions, and necessity to maintain immune function homeostasis renders it susceptible to modulation by a wide variety of xenobiotics. Although many of the xenobiotics discussed below exhibit immunosuppressive actions, it is important to realize that many of these chemicals are actually immunomodulatory; that is, they might produce both immune suppression and immune enhancement (in the absence of true hypersensitivity or autoimmunity). Of course, one cannot ignore the chemicals that do produce true hypersensitivity and/or autoimmunity and some examples of these are discussed later in the section "Xenobiotic-Induced Hypersensitivity and Autoimmunity." Regardless of the end effect (immune suppression, immune enhancement, hypersensitivity, or autoimmunity) of a particular xenobiotic on the immune system, several common themes exist regarding the mechanism by which these chemicals act. First, the mechanisms by which a xenobiotic affects immune function are likely to be multifaceted, involving several proteins, signaling cascades, or receptors. In fact, for several of the xenobiotics discussed below, there is evidence to suggest that xenobiotic-specific effects on the immune system are both receptor-dependent and receptor-independent. Second, whether a xenobiotic produces a particular immune effect might depend on the concentration or dose of the xenobiotic, the mode and/or magnitude of cellular stimulation, and the kinetic relationship between exposure to the xenobiotic and exposure to the immune stimulant (i.e., antigen, mitogen, or pharmacological agent). Third, xenobiotic exposures rarely occur one chemical at a time; thus, the effects and/or mechanisms observed might be attributable to several chemicals or classes of chemicals. Finally, determination of immune system effects and/or mechanisms by xenobiotics in humans might be further confounded by the physiological or immunological state of the individual. Despite these variables, the application of general, functional, and mechanistic tools discussed above have driven the determination of some of the effects and mechanisms for several xenobiotics.

Halogenated Aromatic Hydrocarbons

Few classes of xenobiotics have been as extensively studied for immunotoxicity as the halogenated aromatic hydrocarbons (HAHs; reviewed by Kerkvliet and Burleson [1994], Holsapple [1996], Kerkvliet [2002], and Bock [2013]). The HAHs include the polychlorinated biphenyls (PCBs), the polybrominated biphenyls (PBBs), the polychlorinated dibenzofurans (PCDFs), and the polychlorinated dibenzodioxins (PCDDs). Substantial evidence has accumulated that demonstrates the immune system to be a sensitive target for toxicity by these chemicals. Derived from a variety of animal models, primarily rodents, this evidence includes thymic atrophy, pancytopenia, cachexia, immune suppression, and tumor promotion. There is also epidemiological evidence suggesting immunotoxicity by the HAHs can also occur in humans (Weisglas-Kuperus et al., 1995, 2000, 2004; Geusau et al., 2001; Baccarelli et al., 2002; Kim et al., 2003; Pesatori et al., 2003; Miyashita et al., 2011; Kramer et al., 2012; Saurat et al., 2012; Kuwatsuka et al., 2014; Liu et al., 2015a); however, significant immune suppression has not been associated conclusively with specific alterations of human immune function. The majority of the biochemical and toxic effects, including immunotoxicity, produced by the HAHs are mediated via HAH binding to the cytosolic aryl hydrocarbon receptor (AhR) (White and Birnbaum, 2009) (for a detailed description of the AhR signaling pathway refer to Chap. 6, "Biotransformation of Xenobiotics"). The role of the AhR in mediating HAH toxicity has been supported by structure–activity relationship studies using HAH congeners with low to high affinity for the AhR, animal models expressing an AhR with high or low affinity for HAH ligand, chemical AhR antagonists, and/or AhR knockout animal and cellular models.

Polychlorinated Dibenzodioxins By far the majority of the investigations into the immunotoxic potential and mechanisms of action of the HAHs have focused on TCDD, primarily because this chemical is the most potent of the HAHs, binding the AhR with the highest affinity. The effects of TCDD on immune function have been demonstrated to be among the earliest and most sensitive indicators of TCDD-induced toxicity (reviewed by Holsapple et al. [1991a, 1991b], Kerkvliet and Burleson [1994], Holsapple [1996], Kerkvliet [2002], and Bock [2013]). TCDD is an environmental contaminant formed primarily as a by-product of the manufacturing process that uses chlorinated phenols or during the combustion of chlorinated materials. It is usually associated with the production of herbicides such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and Agent Orange (a 1:1 combination of 2,4-dichlorophenoxyacetic acid [2,4-D] and 2,4,5-T). Other sources include pulp and paper manufacturing (chlorine bleaching), automobile exhaust (leaded gasoline), combustion of municipal and industrial waste, and the production of PCBs.

Like other HAHs, exposure to TCDD results in severe lymphoid atrophy. TCDD appears to target immature hematopoietic stem cells and impairs B-cell maturation as well as thymic seeding of progenitor cells (Gasiewicz et al., 2014; Li et al., 2017). Indeed a hallmark response to TCDD exposure is thymic involution, which is AhR-dependent and besides decreased thymic seeding involves cell cycle arrest and increased apoptosis of immature T cells (Greenlee et al., 1985; Laiosa et al., 2003; Camacho et al., 2005). Because thymus-derived cells play an integral role in tumor surveillance and host resistance, the earliest studies on TCDD-induced immunotoxicity focused on changes in CMI. CTL development and activity is significantly decreased after exposure to TCDD and is dependent on AhR expression in both $CD4^+$ and $CD8^+$ T cells (Kerkvliet et al., 2002). Additional studies have supported a role for TCDD-induced Treg generation in the suppression of the CTL response (Funatake et al., 2005, 2008). Similarly, AhR activation by TCDD or by the tryptophan-derived endogenous AhR ligand, 2-(1′H-indole-3′carbonyl)-thiazole-4-carboxylic acid methyl ester, induced the generation of Tregs in EAE, which produced a reduction in severity of disease (Quintana et al., 2008). These findings are in contrast to the observation that 6-formylindolo[3,2-*b*]carbazole (FICZ), also a tryptophan-derived high-affinity AhR ligand, induced IL-17 production by Th17 cells and increased the severity of disease in the same EAE autoimmune model (Quintana et al., 2008; Veldhoen et al., 2008). Importantly, Th17 cells and Tregs negatively cross-regulate each other and this observation raises what appears to be a dichotomy, in that AhR activation in the same model of autoimmunity can produce very different outcomes and may be in part explained by the ligand that activates the AhR (Quintana et al., 2008; Veldhoen et al., 2008, 2009; Singh et al., 2011; Hayes et al., 2014; Liu et al., 2014). Moreover, transcriptome analysis suggests that the AhR mRNA is highly expressed in Th17 cells as compared to the much lower expression in Th1 and Th2 cells (Quintana et al., 2008; Veldhoen et al., 2008; Trifari et al., 2009; Durant et al., 2010; Liu et al., 2013). In regulatory T cells, nTregs and iTregs express much lower AhR mRNA levels than Th17 but higher than Th1 or Th2 cells; in contrast, Tr1 cells have comparable AhR mRNA as

Th17 cells (Quintana et al., 2008; Veldhoen et al., 2008; Apetoh et al., 2010; Durant et al., 2010; Liu et al., 2013; Gagliani et al., 2015). Additionally, Th22 cells have been identified as targets of AhR ligands, particularly due to the prominent role of the AhR in regulating IL-22 expression. Th22 and Th17 cells have a protective or pathogenic role in epithelial barrier organs, and it is becoming clear that the diet and microbiota generate AhR ligands that influence mucosal health, perhaps by altering Th22 and Th17 function. However, Type 3 innate lymphoid cells (ILC3) are also likely players in AhR-mediated modulation of mucosal immunity. ILC3 cells are the largest producers of IL-22 in the intestine and AhR knockout results in decreased numbers of ILC3 cells and altered gut physiology, as well as increased susceptibility to infection (Jeuken et al., 2003; Brembilla et al., 2011; Kiss et al., 2011; Lee et al., 2011; Basu et al., 2012; Qiu et al., 2012, 2013; Spits et al., 2013; Yeste et al., 2014; Azizi et al., 2015; Nikoopour et al., 2015). Additionally, AhR ligands induce protection against colon damage in colitis animal models, which may be due to enhanced Treg, and Th22 and ILC3 cell activity to produce IL-22, which protects mucosal barriers (Huang et al., 2013; Ji et al., 2015).

In light of the critical role DCs and macrophages play in regulating T-cell-mediated responses by presenting antigen to $CD4^+$ and $CD8^+$ T cells, as well as by providing co-stimulatory signals through surface expression of CD80 and CD86 to drive T-cell activation via CD28 (T-cell co-receptor), investigations have focused on characterizing the effects TCDD exerts on DC and macrophage function. Several studies have supported an effect of AhR ligands on the expression of surface markers associated with the differentiation and activation state of DCs and macrophages (i.e., CD80, CD86, and the integrin proteins CD1a, CD11c, and CD29), but the effects are dependent on ligand (i.e., TCDD vs. BaP and reactive metabolites) and the maturation or differentiation state at the time of exposure (van Grevenynghe et al., 2003, 2004; Lee et al., 2007; Monteiro et al., 2007; Vogel et al., 2008; Bankoti et al., 2010a, 2010b; Simones and Shepherd, 2011; Ilchmann et al., 2012; Jin et al., 2014). The functional impact of these changes is less clear. However, studies support altered responses to infection and generally an AhR-dependent increase in inflammatory cytokine expression (Lecureur et al., 2005; Vogel et al., 2005; N'Diaye et al., 2006; Cheon et al., 2007; Monteiro et al., 2008; Podechard et al., 2008; Sciullo et al., 2008; Bankoti et al., 2010b; Suen et al., 2013; Jaguin et al., 2015; Memari et al., 2015). Regarding animal models of infection, TCDD treatment decreased survival to influenza virus (Warren et al., 2000; Vorderstrasse et al., 2003) that corresponded to a decrease in two major classes of DCs ($CD11b^+$ and $CD103^+$) and a decrease in IFN-γ secretion and $CD8^+$ T-cell activation, which was shown to be dependent on the AhR and binding to dioxin response elements (DREs) (Jin et al., 2014). However, similar to the ligand-specific effects described above for EAE severity, FICZ did not increase the susceptibility to influenza (Wheeler et al., 2014). FICZ, in an AhR-dependent manner, also increased the survival of mice infected with *L. monocytogenes*, perhaps by increasing or maintaining ROS levels in infected macrophages and increased macrophage survival (Kimura et al., 2014).

Numerous investigations have demonstrated humoral immunity, and particularly the IgM AFC response, to be exquisitely sensitive to the toxic effects of TCDD. B cells are directly targeted by TCDD with most animal models showing an inhibition of Ig expression and secretion without much of an effect on proliferative capacity (reviewed by Sulentic and Kaminski [2011]). Studies have demonstrated an essential role of the AhR in these effects, the most direct evidence obtained using AhR antagonists and/or AhR knockout models (Ishikawa, 2009; Sulentic and Kaminski, 2011; Wourms and Sulentic, 2015; Kovalova et al., 2016; Phadnis-Moghe et al., 2016). Motif searches of regulatory regions of genes involved in Ig regulation lead to the identification of DREs in a transcriptional regulatory region (termed 3′*Igh*RR) of the Ig heavy chain gene. The 3′*Igh*RR plays a crucial role in mediating high-level Ig heavy-chain expression and Ig class switching (Pinaud et al., 2011). TCDD-induced AhR binding within the 3′*Igh*RR and in an AhR-dependent manner strongly suppressed luciferase reporter activity regulated by the 3′*Igh*RR in a cell line model of B-cell differentiation (Sulentic et al., 2000, 2004a, 2004b; Henseler et al., 2009; Salisbury and Sulentic, 2015; Wourms and Sulentic, 2015). However, suppression of humoral immune responses by TCDD cannot be completely reconciled by altered regulation of the 3′*Igh*RR and the Ig heavy chain as TCDD treatment also significantly suppresses expression of the Ig light chain and Ig J chain (Yoo et al., 2004; Fernando et al., 2012). Further studies revealed that a bistable biological switch controlling B cell to plasma cell differentiation is significantly altered in the presence of TCDD, resulting in elevated levels of the transcription factor paired box 5 (Pax5), which is highly expressed in resting B cells and represses the expression of Ig heavy chain, light chain, and J chain to prevent plasma cell differentiation and Ig production (Schneider et al., 2008, 2009; North et al., 2009; Bhattacharya et al., 2010; Zhang et al., 2010; De Abrew et al., 2011). Additionally, several studies support TCDD-induced inhibition of B-cell activation (North et al., 2010; Lu et al., 2011; Phadnis-Moghe et al., 2015, 2016).

There is little doubt that TCDD and related PCDDs are immunotoxic, particularly in mice. However, extrapolation to human exposure has proven to be difficult. There are a few instances (i.e., industrial accident in Seveso, Italy in 1976, contaminated dust control sprayed on roads in Times Beach, Missouri in 1971, and exposure during the Vietnam War to Agent Orange, a herbicide contaminated with TCDD) in which accidental human exposure to TCDD and related congeners has afforded the opportunity to study exposure-related human immunologic responses. For example, in children exposed to PCDDs in Seveso, nearly half of the exposed study group exhibited chloracne (a hallmark of high-level human exposure to PCDDs) 3 years after the accident. Immune parameters measured at that time were unaffected. In a second study conducted 6 years later on different subjects, there was an increase in complement, which correlated with the incidence of chloracne, an increase in circulating T and B cells, and an increase in PBMC mitogenic responses (reviewed by Baccarelli et al. [2002]). In 2002, follow-up studies in which the population in the most highly exposed zone was randomly sampled revealed modestly decreased median serum IgG levels but no effect on IgM, IgA, or complement concentrations as compared to human subjects in the surrounding noncontaminated area (Baccarelli et al., 2002). Studies have also been conducted to evaluate the in vitro effects of TCDD on human cells. Early studies demonstrated TCDD-induced suppression of IgM and IgG secretion by stimulated human B cells (Wood and Holsapple, 1993; Wood et al., 1993). More recently, human peripheral blood (HPB) B cells were found to possess similar sensitivity to TCDD, as quantified by suppression of cellular activation and IgM AFC response, as mouse B cells expressing the high affinity form of the AhR (C57BL/6) (Lu et al., 2010, 2011). However, the sensitivity of HPB B cells to inhibition by TCDD varied among donors (i.e., no effect or a greater, equal or lesser sensitivity compared to mice). Some donors even exhibited a TCDD-induced increase in IgM AFC response and IgM secretion (Lu et al., 2010; Dornbos et al., 2016). SNPs identified within the AhR transactivation domain have been associated with decreased TCDD-inducible CYP induction, but do not appear to explain the above donor variation in sensitivity (Lu et al., 2010;

Kovalova et al., 2016). Additionally, another study demonstrated an increase in IgE secretion (no effect on other isotypes) with TCDD treatment of B cells isolated from atopic patients compared to control patients (Kimata, 2003). Congruent with these effects, analysis of Korean Vietnam War veterans thought to be exposed to Agent Orange demonstrated increased plasma IgE and decreased plasma IgG1 compared to age-matched controls. Additionally, in vitro stimulation resulted in increased IL-4 secretion but decreased IFN-γ secretion from PBMC cells isolated from veterans versus control subjects (Kim et al., 2003). Besides accidental exposure to TCDD, there have been two cases of intentional poisoning involving three people; the most notable case is the politically motivated poisoning of Viktor Yushchenko (Geusau et al., 2001; Saurat et al., 2012). All three individuals exhibited chloracne, two of the patients also demonstrated a decreased percentage of NK cells 2 years after exposure, and Yushchenko exhibited chloracne from weeks to months after exposure (Geusau et al., 2001; Saurat et al., 2012).

Polychlorinated Dibenzofurans and Polychlorinated Biphenyls Like the PCDDs, polychlorinated dibenzofurans (PCDFs) are not produced commercially but are true environmental contaminants associated with the production of chlorophenoxy acids, pentachlorophenol, and polychlorinated biphenyl (PCB) mixtures. PCBs have seen extensive commercial use for over half a century. Their unique physical and chemical properties make PCB mixtures ideal for use in the production of plasticizers, adhesives, and as dielectric fluids in capacitors and transformers. Although higher concentrations are required to achieve observable effects, the immunotoxic profile of the PCDFs and a subset of PCBs (i.e., dioxin-like PCBs) is similar in nature to that described for TCDD (Johnson et al., 2000). Of note, most exposures to dioxins will be to mixtures of various dioxin congeners. Additionally, PCBs were made in mixtures (e.g., Aroclors) that included dioxin-like PCBs and PCBs that do not have dioxin-like effects and may even antagonize the effects of dioxin-like PCBs. Mixtures of dioxin-like PCDDs, PCDFs, and PCBs produced a similar inhibitory effect on the AFC response as compared to TCDD treatment alone when the mixture was adjusted to doses defined by the TCDD equivalent quantity (TEQ) (Smialowicz et al., 2008).

In terms of human exposure, mass exposures to rice oil contaminated with PCDDs, PCDFs, and PCBs occurred in Japan in 1968 (called Yusho) and later in Taiwan in 1979 (called Yucheng) (reviewed by Masuda and Schecter [2012]). There is evidence that the PCDFs were the primary contributors to the observed toxic effects (Nagayama et al., 2001a). However, the effects on immune parameters were less clear because there is little information regarding immune effects shortly after exposure, and studies evaluating just 16 Yusho patients 30 years later did not suggest a prolonged effect on serum Igs (IgM, IgG, IgA) or on lymphocyte populations, but there did appear to be an increase in rheumatoid factor with increased TEQ (Nagayama et al., 2001b). Occupational exposure of German workers to PCDDs also demonstrated no effect on serum Igs (Neubert et al., 2000). Interestingly, a study evaluating 40 Yusho patients 40 years after the initial exposure to the contaminated oil identified elevated serum levels of IL-17, IL-1β, and TNF-α and decreased serum levels of IL-22 compared to age-matched controls, which may have implications regarding Th17 cells and inflammatory diseases (Kuwatsuka et al., 2014). Several studies have evaluated the effect of PCDD, PCDFs, and PCBs on immune function in children (reviewed by Kimbrough and Krouskas [2001]). Infants exposed to PCDFs in utero and in breast milk exhibited an increased risk of infection (otitis media) but not allergies with increasing concentration of PCDF (Miyashita et al., 2011). Additionally, longitudinal studies in a cohort of Dutch children suggest that the developing human immune system may be susceptible to immunotoxic alterations from exposure to Western European environmental levels of dioxin-like compounds, primarily PCBs (Weisglas-Kuperus et al., 1995, 2000, 2004; ten Tusscher et al., 2003). Exposure to dioxin-like compounds was associated with immunological changes that included an increased number of lymphocytes, including T-cell subsets and lower antibody levels to mumps and measles vaccination at preschool age (Weisglas-Kuperus et al., 1995, 2000, 2004). In addition, an association was found between dioxin/PCB prenatal exposure and decreased shortness of breath with wheeze, and between the PCB burden and a higher prevalence of recurrent middle-ear infections and chicken pox, and a lower prevalence of allergic reactions (Weisglas-Kuperus et al., 2000). It is notable that although an association between dioxin/PCB exposure and changes in immune status was observed, all infants were found to be in the normal range. Perinatal exposure to dioxin-like compounds was also associated with a decrease in allergy and increased $CD4^+$ T cells (ten Tusscher et al., 2003). Additionally, occupational and environmental exposure to PCBs of a German population working in or living near a recycling plant was associated with altered immune parameters, which were correlated to specific PCB congeners; however, none of the changes were outside of normal reference ranges (Haase et al., 2016a).

Polybrominated Biphenyls The polybrominated biphenyls (PBBs) have been used primarily as flame-retardants (Firemaster BP-6 and FF-1). While it is assumed that their profile of activity is similar to that of the PCBs, few studies have actually evaluated the action of the PBBs on immunocompetence. In Michigan (in 1973) Firemaster BP-6 was inadvertently substituted for a nutrient additive in cattle feed, resulting in widespread exposure of animals and humans to PBBs. Studies conducted on livestock following the incident indicated little if any PBB-induced alterations in immunocompetence (Kately and Bazzell, 1978; Vos and Luster, 1989). In a birth cohort study of 384 mother–infant pairs in Eastern Slovakia, PBB concentrations for selected PBB congeners were quantified in maternal and cord serum and infant blood at 6 months of age. Although the PBB concentrations in the Eastern Slovakia birth cohort were several-fold higher than those found in the United States, no association was observed between PBB levels, sum total or congener specific, and total serum IgG, IgA, IgM, and IgE (Jusko et al., 2011).

Polycyclic Aromatic Hydrocarbons The polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of environmental contaminants. They enter the environment through many routes including the burning of fossil fuels and forest fires and are a major constituent of cigarette smoke. In addition to being carcinogenic and mutagenic, the PAHs have been found to be potent immune suppressants. Effects have been documented on immune system development, humoral immunity, CMI, and on host resistance (Dean et al., 1983; Davila et al., 1996; Gao et al., 2005a; Smith et al., 2010; Burchiel et al., 2016; Hong et al., 2016; Vrijheid et al., 2016; Zakharenko et al., 2017). The most extensively studied PAHs are 7,12-dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (BaP).

Early immunotoxicology studies of PAHs such as BaP, DMBA, and 3-methylcholanthrene demonstrated suppression of the antibody response to a variety of T-cell-dependent and T-cell-independent antigens. In addition, mice treated with BaP exhibit suppressed lymphoproliferative responses to mitogens, but not alloantigens (Dean et al., 1983; Davila et al., 1996; Gao et al., 2005a; Smith et al., 2010). In contrast to BaP, DMBA (the more potent PAH) significantly suppressed not only AFC responses, but

also NK cell activity, CTL responses, DTH responses, and alloantigen-induced lymphoproliferative responses. Therefore, DMBA exposure appears to result in long-lasting immune suppression of humoral immunity, CMI, and tumor resistance mechanisms in mice (Gao et al., 2005a, 2008). Suppression of the AFC response by various PAHs was greatly enhanced in vitro when combined with arsenic, another common environmental contaminant; PAH metabolites were more potent immunosuppressants than the parent compounds (Li et al., 2010). Additionally, arsenic in combinations with low levels of PAH target and inhibit lymphoid progenitor cells in mice (Ezeh et al., 2015). In addition, both BaP and DMBA produce significant effects on the bone marrow, as evidenced by suppression of pre-B-cell formation. Additionally, PAHs induce apoptosis of pre-B cells, which is dependent on AhR-mediated metabolism of PAHs into active metabolites in bone marrow stromal cells (Allan et al., 2003; Sherr and Monti, 2013).

Biologic and toxicologic activities of PAHs are generally dependent on AHR-induced metabolism of PAHs to active metabolites by cytochrome P-450 isozymes, CYP1A1, CYP1A2, and CYP1B1. Although leukocytes in general possess modest cytochrome P-450 drug metabolizing activity, studies support the presence and inducibility of CYP1A1, CYP1A2, and CYP1B1 in leukocytes (Ladics et al., 1991, 1992a, 1992b; Gao et al., 2005a). Macrophages also appear to be the primary cell type metabolizing BaP to 7,8-dihydroxy-9,10-epoxy-7,8,9,10-benzo[*a*]pyrene (BPDE), the carcinogenic and immunotoxic reactive metabolite (Ladics et al., 1992a, 1992b). PAHs, including BaP, have also been shown to induce AhR-independent biochemical changes in lymphocytes leading to oxidative stress, activation of tyrosine kinases, and increased intracellular calcium and proteins related to DNA damage (Mounho and Burchiel, 1998; Pessah et al., 2001; Gao et al., 2008). The elevation in intracellular calcium in B and T lymphocytes was shown to be mediated by BaP-7,8-dione and blocked by high concentrations of ryanodine, suggesting the involvement of ryanodine receptors (Gao et al., 2005b).

Regarding the effects of PAHs on humans, many studies support an increase in allergic inflammatory responses due to exposure to pollutants containing PAHs, such as cigarette and wood smoke and diesel exhaust (Klingbeil et al., 2014). Early in vitro studies with human PBMC demonstrated an increase in IgE following treatment with PAHs from diesel exhaust particles (DEPs) (Takenaka et al., 1995). DEPs also enhance IgE-mediated release of histamine and IL-4 from human basophils, perhaps mediated by increased ROS and tyrosine kinase activity (Kepley et al., 2003). Additionally, DEPs increase the basophil population in mice (Zakharenko et al., 2017). Analysis of PBMCs from humans exposed to hardwood smoke (contains PAHs along with many other chemicals) supports an increase in the production of pro-inflammatory cytokines (Burchiel et al., 2016). Furthermore, a study with asthmatic children identified an association between asthma and PAH exposure (Wang et al., 2017a).

Pesticides

Pesticides include all xenobiotics whose specific purpose is to kill another form of life, including insects (insecticides), small rodents (rodenticides), or even vegetation (herbicides). As such, these chemicals have clear biological activity and many pesticides have been studied for their effects on the immune system (Galloway and Handy, 2003). While there is much evidence that pesticides produce alterations in immune function in a variety of mammalian, amphibian, and fish animal models, the studies described here will focus on those that provide mechanistic information related to epidemiological effects in humans (reviewed by Colosio et al. [2005]). Exposure to pesticides occurs most often in occupational settings, in which manufacturers, pesticide applicators, or harvesters of treated agricultural products are exposed. In the United States, the most widely used class of pesticides (through 2007) were the organophosphates (Grube et al., 2011), although effects of organochlorines, organotins, carbamates, and the herbicide atrazine will also be discussed.

Organophosphates Organophosphates include malathion, parathion, methyl parathion, diazinon, and chlorpyrifos. Although the neurotoxic effects of organophosphates are well understood to occur via the inhibition of acetylcholinesterase (see Chap. 22, "Toxic Effects of Pesticides"), the mechanism by which these compounds suppress the immune system is not as well defined. A recent review suggests that in general, organophosphate immune suppression occurs via three mechanisms: inhibition of granular exocytosis affecting CTLs and NK cells, induction of apoptosis, and inhibition of serine hydrolase activity (Corsini et al., 2013).

Malathion exhibits both immune suppressive and immune enhancing effects (Rodgers and Ellefson, 1990). Malathion has been shown to suppress humoral immunity as measured by the AFC response to sRBC following a subacute exposure in mice (Casale et al., 1983). In vitro exposure of either human PBMC or murine splenocytes to malathion results in decreased lymphoproliferative responses, suppressed CTL generation, and a decrease in the stimulus-induced respiratory burst in peritoneal cells (Rodgers and Ellefson, 1990). The mechanism by which malathion is immunotoxic has been attributed to induction of oxidative stress in unstimulated mouse thymocytes (Olgun et al., 2004) or human PBMC (Ahmed et al., 2011). Parathion has attracted more attention than malathion, probably because it is more acutely toxic. This pesticide also produces mixed responses, which might depend on strain of animal or dose (Casale et al., 1983; Crittenden et al., 1998). A comprehensive screen of various classes of pesticides that exhibit binding affinity to, and activation of, the pregnane X receptor (PXR) demonstrated that parathion activates PXR (Kojima et al., 2011), which has been shown to suppress inflammatory responses through inhibition of NF-κB (Zhou et al., 2006).

Fewer studies exist that describe the immunotoxicity of diazinon. It has been shown that diazinon produces gross changes in the spleen and thymus in response to acute doses in mice (Handy et al., 2002; Neishabouri et al., 2004). Diazinon also suppresses humoral immunity as measured by the AFC response to sRBC, and CMI as measured by the DTH response following subchronic exposure to mice (Neishabouri et al., 2004).

Chlorpyrifos is one of the most widely used pesticides in the United States (Grube et al., 2011). Similar to other pesticides, its effects on immune function vary. An examination of humans exposed to chlorpyrifos demonstrated suppressed proliferative responses to ConA or PHA, but an increase in autoantibodies (Thrasher et al., 2002). Using human PBMC, it was demonstrated that chlorpyrifos modestly enhances or suppresses IFN-γ production in response to LPS at low (1 to 100 μg/mL) or high (1000 μg/mL) concentrations, respectively (Duramad et al., 2006). The mechanism by which chlorpyrifos modulates immunity likely involves ERα, PXR, and AhR as it induced reporter activity in receptor-activation assays (Kojima et al., 2004, 2011; Takeuchi et al., 2008). In mouse peritoneal macrophages, chlorpyrifos inhibits nitric oxide and pro-inflammatory cytokine production in response to LPS (Helali et al., 2016). Sex-specific effects were seen in female versus male offspring following gestational and postnatal chlorpyrifos exposure. The anti-sRBC AFC response, proliferative responses

to PHA, ConA, and LPS, and LPS-stimulated cytokine production are all suppressed by chlorpyrifos using splenocytes from either sex, but only females exhibit an increase in Tregs (Singh et al., 2013).

Organochlorines The organochlorines include chemicals such as chlordane, dichlorodiphenyltrichloroethane (DDT), mirex, pentachlorophenol, aldrin, dieldrin, and hexachlorobenzene. Although technically this class of compounds also includes the herbicide 2.4.5-T, the immunotoxicity of these compounds is likely primarily due to TCDD, a by-product formed in the manufacturing process (see the section "Halogenated Aromatic Hydrocarbons"). The organochlorines are among the longer-lived pesticides and they have an increased propensity for contamination of soil and ground water, thus providing an additional route of exposure to the general population. Many organochlorine compounds also have been shown to mediate their effects via glucocorticoid receptors (DDT and DDE) (Zhang et al., 2016a), estrogen receptors (DDT, dieldrin, and chlordane) (Kojima et al., 2004), or PXRs (DDT, dieldrin, and chlordane) (Kojima et al., 2011).

Although DDT has been banned in several countries, it is still used to control malaria, typhoid, and dengue infections in some places of the world. DDT suppresses both humoral immunity and CMI. Early studies demonstrated suppression of humoral immunity following oral gavage in mice (Wiltrout et al., 1978). More recently, DDT has been shown to suppress IL-2 production from a human T-cell line due to the suppression of the critical transcription factor NF-κB (Ndebele et al., 2004). Correlations were found between DDE (a primary metabolite of DDT) concentrations in breast milk and incidence of infant ear infections (Dewailly et al., 2000). Increased susceptibility to infection by DDT might also involve suppression of macrophage function (Nunez et al., 2002), increased apoptosis of macrophages, reduced complement function (Dutta et al., 2008), or suppression of NK cell function (Udoji et al., 2010). In another study in which occupationally exposed individuals were evaluated for cytokine levels, DDE was associated with suppression of IL-2 and IFN-γ, and enhancement of IL-4, suggesting an imbalance of Th1/Th2 populations (Daniel et al., 2002). A study in mice demonstrates macrophage polarization imbalance (Mangum et al., 2016), suggesting that alteration of immune cell populations might account for the immune suppression by DDE.

Dieldrin was used from the 1950s until 1987 when its use was banned due to environmental and human health concerns. The humoral immune response to both T-cell-dependent and T-cell-independent antigens is suppressed following exposure to dieldrin (Bernier et al., 1987). Macrophage functions are also depressed in dieldrin-exposed animals, which correlates with the increased susceptibility of dieldrin-exposed animals to murine hepatitis virus (a virus that targets macrophages) (Krzystyniak et al., 1985). CMI is also suppressed in mice following dieldrin exposure as measured by a suppression of the MLR and graft-versus-host disease (Hugo et al., 1988a, 1988b). Dieldrin also induces inflammation under certain conditions, as dieldrin enhances the pro-inflammatory cytokine IL-8 in vitro, increases superoxide production in vitro (Mangum et al., 2015), and induces neutrophilic inflammation in vivo (Pelletier et al., 2001).

Chlordane refers to a group of structurally related chemicals used to control termites. The principal constituents of chlordane are heptachlor, α-chlordane, γ-chlordane, α-nonachlor, β-nonachlor, α-chlordene, β-chlordene, and γ-chlordene. Definitive immune suppression produced by chlordane was first reported by Spyker-Cranmer et al. (1982). In utero exposure results in decreased DTH responses in mice with no deficit in antibody production to sRBC. This effect is correlated with an increase in resistance to influenza infection because DTH contributes to the pathology of the infection (Menna et al., 1985). In contrast to observations from mice exposed in utero, exposure of adult mice to chlordane does not result in any changes to several immune parameters, including AFC response to sRBC, MLR, DTH, or mitogenic lymphoproliferation. In rats, immunomodulatory effects of three constituents of the chlordane mixture (i.e. technical chlordane, cis-nonachlor, or trans-nonachlor) were analyzed following 28-day oral dosing. The most profound effect was increased lymphocyte numbers with decreased NK cell activity and proliferation in response to Salmonella typhimurium (Tryphonas et al., 2003). The increase in cell numbers is consistent with a study conducted in Japan in which high concentrations of chlordane in breast milk increased $CD3^+$ and $CD8^+$ T-cell percentages in infants at approximately 10 months of age (Nagayama et al., 2007).

Organotins Trisubstituted organotins such as tributyltin oxide are widely used as biocides and have recently been recognized as producing some immunotoxic effects. The most prominent action of tributyltin oxide is the induction of profound but reversible thymic atrophy. Studies by Vos et al. (1984) demonstrated a decrease in cellularity in the spleen, bone marrow, and thymus. The decrease in splenic cellularity was associated with a concomitant loss of T lymphocytes (Vos et al., 1984). In Jurkat T cells, tributyltin oxide increases intracellular calcium and NFAT translocation to the nucleus, but the increase in proliferation is not sustained, and at later times, there is decreased proliferation and increased expression of several apoptotic genes (Katika et al., 2011). In mouse thymocytes, there are increased annexin-V^+ and Fas ligand–expressing cells following oral administration of tributyltin chloride (Chen et al., 2011), providing additional evidence that thymic atrophy is due to apoptosis.

Another sensitive target of organotin compounds is the NK cell. Van Loveren et al. (1990) observed suppressed lung NK cytotoxicity in rats exposed orally to tributyltin oxide. The mechanism for suppressed NK cell activity was investigated using human NK cells, which demonstrates an increase in intracellular calcium and increased expression of various AP-1 transcription factors (i.e., jun and fos) (Lane et al., 2009; Person and Whalen, 2010). However, overall, there is decreased AP-1 binding activity, which the authors suggest is due to increased prevalence of jun–jun homodimers, which exhibit lower affinity for the AP-1 site than other complexes, such as jun–fos heterodimers (Person and Whalen, 2010). Moreover, the mechanism by which dibutyltin and tributyltin suppressed NK cell activity might be due to its ability to interact with sulfhydryl groups, because glutathione treatment reverses the suppression (Powell et al., 2009). Additional papers investigating direct effects of dibutyltin and tributyltin in different fractions of human PBMCs demonstrated differential effects of the toxicants depending on the cell composition (Hurt et al., 2013; Brown and Whalen, 2015; Lawrence et al., 2015). For instance, tributyltin robustly suppresses TNF-α production from highly purified NK cells, but this effect is lost in a T cell/NK cell mixed population (Hurt et al., 2013). These results demonstrate the importance of considering immune cell–cell interactions in understanding mechanisms of immunotoxicity. Recently, tributyltin was also demonstrated to impair stem cell to B-cell development in vitro through activation of apoptosis (Baker et al., 2017).

Carbamates Carbamates, which include carbaryl (Sevin), aldicarb, mancozeb, and sodium methyldithiocarbamate, are used primarily as insecticides. Similar to the organophosphates, the mechanism of action for the neurotoxic effects involves inhibition of acetylcholinesterase. In an evaluation of humoral immunity following a 2-week exposure to carbaryl in rats, suppression of

the IgM AFC response to sRBC is observed following inhalation exposure, but not oral or dermal exposure (Ladics et al., 1994). Following oral exposure in rats, carbaryl suppresses splenic proliferative responses, but enhances pulmonary inflammation and IgE, in response to house dust mite, suggesting that carbaryl exhibits tissue-specific effects (Dong et al., 1998). The mechanism by which carbaryl is suppressive involves inhibition of LPS-induced signaling in macrophages (Ohnishi et al., 2008).

Pruett et al. (1992a) evaluated the immunotoxicity of sodium methyldithiocarbamate, and observed decreased thymus weight, depletion of the $CD4^+/CD8^+$ population of thymocytes, and profound suppression of NK cell activity following both oral and dermal exposure. They also determined that the mechanism by which sodium methyldithiocarbamate alters cytokine production from peritoneal macrophages involves inhibition of MAP kinase and NF-κB activity via TLR4 (Pruett et al., 2005; Deng et al., 2013). The mechanism of cytokine alteration involves depletion of glutathione, alteration of copper-dependent proteins, induction of stress (Pruett et al., 2006), and suppression of interferon-related genes (Deng et al., 2013).

Atrazine Atrazine is a herbicide applied to various agricultural crops to control broad leaf weeds. It is widely used in the United States and it has been detected in soils and groundwater because of its resistance to degradation. Similar to other chemicals discussed, atrazine exhibits immunomodulatory effects. Using offspring of dams treated with atrazine and challenged with antigen, atrazine induces elevations in T-cell proliferation, cytolytic activity, and antigen-specific B cells in male offspring (Rowe et al., 2006). In contrast, young mice directly administered atrazine orally for 14 days exhibit suppressed thymic weight, spleen and thymic cellularity, and B-cell populations, although $CD4^+$ T-cell numbers increased (Filipov et al., 2005). Similarly, in adult mice, it was confirmed that atrazine suppresses thymic weight, and also suppresses splenic weight and decreases the host resistance of the mice to B16F10 melanoma tumors (Karrow et al., 2005). In vitro, atrazine suppresses cell surface expression of MHCII, CD86, CD11b, and CD11c using a mouse DC line (Pinchuk et al., 2007). Although the exact mechanism by which atrazine-induced immune suppression occurred is unclear; atrazine treatment of mice does induce corticosterone levels, indicating that activation of the hypothalamic-pituitary-adrenal axis might be involved (Pruett et al., 2003). Atrazine has also been shown to induce lymphocyte apoptosis in vivo (Zhang et al., 2011) and in vitro (Lee et al., 2016).

Metals

Generally speaking, metals target multiple organ systems and exert their toxic effects via an interaction of the free metal with targets, such as enzyme systems, membranes, or cellular organelles. In considering the immunotoxicity of most metals, early studies as reviewed by Koller (1980) established metals as immunomodulators, but not consistently immunosuppressive as will be explored below for specific metals. However, the most consistent finding in these studies is increased susceptibility to pathogens. Additionally, a consistent finding in human and rodent studies from exposure to the various metals found in tobacco smoke is the induction of pro-inflammatory cytokines (reviewed by Milnerowicz et al. [2015]). Furthermore, as with most immunotoxic agents, it is important to note that exposures to metals are likely not single exposures, although one metal might dominate depending on the exposure conditions (e.g., high levels of mercury in fish or high levels of lead from paint).

Similar to pesticides, there is certainly a large body of literature describing the metal-induced immune alterations in mammalian, amphibian, and fish animal models, but again, the studies described here will focus on those that provide mechanistic information related to epidemiological effects in humans and will focus on the four best studied immunotoxic metals: lead, arsenic, mercury, and cadmium. Moreover, there has been a substantial increase in efforts to understand immune alterations following exposure to metal-containing nanomaterials. For this, the reader is referred to Chap. 29, "Nanoparticle Toxicology" and several recent reviews (Zolnik et al., 2010; Kononenko et al., 2015; Luo et al., 2015).

Lead Studies support various immunotoxic effects of lead, ranging from decreased bacterial and viral resistance to increased hypersensitivity and autoimmunity (reviewed by Dietert and Piepenbrink [2006a], Mishra [2009], and Kasten-Jolly and Lawrence [2014]). Perhaps mediating the decreased resistance to pathogens, lead has been shown to decrease macrophage production of nitric oxide (Farrer et al., 2008) and to impair immune recognition by decreasing the ability of macrophages to process and present antigens to antigen-primed T cells (reviewed by Dietert and Piepenbrink [2006a] and Kasten-Jolly and Lawrence [2014]). Additionally, lead shifts the T-cell balance from Th1 to Th2 leading to increased Th2 and decreased Th1 cytokine expression profiles (i.e., increased IL-4 and decreased IFN-γ), which could promote M2 vs. M1 macrophages and contribute to a decreased ability to fight intracellular pathogens (reviewed by Dietert and Piepenbrink [2006a] and Kasten-Jolly and Lawrence [2014]). The mechanism by which lead induces a Th2 switch has been suggested to involve lead-induced alterations in bone marrow–derived DC populations that preferentially stimulate Th2 differentiation (Gao et al., 2007). A lead-induced Th2 shift may contribute to the consistent finding of increased IgE, whereas effects on the AFC response and other Ig isotypes are less consistent, perhaps due to differences in experimental conditions (reviewed by Dietert and Piepenbrink [2006a]). Correspondingly, it has been hypothesized that lead exposure might contribute to the development of asthma (Dietert and Piepenbrink, 2006a), a predominantly Th2-mediated disease. This theory is consistent with the observed increase in IgE production and asthma incidence in lead-exposed children (Lutz et al., 1999; Karmaus et al., 2005; Hsiao et al., 2011). Interestingly, Lutz et al. (2012) suggest that the increase in IgE in lead-exposed children is more related to a co-exposure with cigarette smoke exposure rather than due to the lead-exposure alone. Furthermore, children living near electronic waste sites in China exhibited decreased NK cell numbers correlated to blood lead levels (Zhang et al., 2016c). Corresponding to the decreases in Th1 responses and antigen recognition and processing, several studies have identified lead-induced inhibition of DTH responses (reviewed by Dietert and Piepenbrink [2006a]). Lead exposure is also associated with inflammation and increased expression of inflammatory cytokines, the generation of ROS, and tissue damage (reviewed by Dietert and Piepenbrink [2006a]). Specific to hematopoietic stem cells, lead-induced mitochondrial dysfunction and ROS generation resulted in apoptosis and loss of stem cells (Liu et al., 2015b). Additionally, in vitro and in vivo acute and subchronic (8 weeks) studies support an increase in neuroinflammation and neuronal death mediated by lead-induced activation of microglia and secretion of inflammatory cytokines (Liu et al., 2012; Kumawat et al., 2014). In contrast, subchronic (6 weeks) exposure to lead (or cadmium) in an animal model of colitis decreased inflammatory cytokines and signs of colitis (Breton et al., 2016). Furthermore, a cross-sectional analysis of data from the National Health and Nutrition Examination Survey (NHANES) found an association between blood lead levels and an increased risk of chronic infections (i.e., *Helicobacter pylori*, *Toxoplasma gondii*, and hepatitis B virus) (Krueger and Wade, 2016). In summary, lead clearly alters

immune function resulting mostly in increased pathogen susceptibility, tissue inflammation, and the potential for hypersensitivity and autoimmune reactions.

Arsenic A challenge in understanding the mechanisms by which arsenic is immunotoxic is that in addition to inconsistencies that arise from route of administration, concentrations/doses, and species/strain differences, the speciation of arsenic plays a significant role in arsenic toxicity (reviewed by Abdul et al. [2015] and Sattar et al. [2016]). Arsenic compounds affect human immune function. For instance, in environmentally exposed children, there was a correlation between total arsenic in urine (inorganic and arsenic metabolites) and superoxide anion production from stimulated monocytes (Luna et al., 2010) and in adult smelter workers, higher levels of urinary arsenic correlated with increased lipid peroxidation and lower vitamin E levels in blood (Escobar et al., 2010). These results suggest that arsenic exposure causes oxidative stress and in fact, using the THP-1 monocytic cell line, it was shown that arsenic trioxide decreased glutathione levels and induced heme oxygenase, which is often induced to combat oxidative stress (Wang et al., 2011). There are also several studies in which human PBMC from unexposed individuals were treated in vitro with arsenic compounds. The percentage of IL-2-producing T cells in human PBMC was suppressed following treatment with sodium arsenite (Galicia et al., 2003). An additional study showed that sodium arsenite suppressed IL-2 production while also stimulating heme oxygenase in purified T cells, indicating that the direct T-cell impairment by arsenic occurs in parallel with oxidative stress (Martin-Chouly et al., 2011). Additionally, arsenic trioxide decreased the proliferation of alloreactive $CD8^+$ memory T cells and prevented allograft rejection in an animal model (Li et al., 2015a). Humoral immune function is also compromised by arsenic compounds, as suggested by anti-sRBC AFC responses in mice (Sikorski et al., 1991; Burchiel et al., 2009). Interestingly, in spite of a fairly extensive database showing suppression of antibody responses in murine models, in purified human B cells stimulated with CD40L, sodium arsenite did not suppress IgM production (Lu et al., 2009). However, treatment of primary human macrophages with arsenic trioxide resulted in decreased expression of CD14 (a PRR and co-receptor of TLRs), altered morphology, and suppressed adhesion (Lemarie et al., 2006). The alterations in macrophage function were dependent on arsenic trioxide–induced activation of RhoA (small GTPase and Ras homolog) (Lemarie et al., 2006). Similar results (increased RhoA activation, altered morphology, and cell adhesion) were observed in macrophages isolated from humans chronically exposed to arsenic (Banerjee et al., 2009). At higher concentrations, arsenic trioxide also induced apoptosis of macrophages through inhibition of NF-κB signaling (Lemarie et al., 2006). Induction of macrophage apoptosis is the basis for the therapeutic use of arsenic trioxide to treat acute promyelocytic leukemia. Additionally, studies with both rodent and human macrophages treated with arsenic support decreased phagocytosis, NO and superoxide production, chemotaxis, and antigen presentation, which may account for the observed increases in susceptibility to infections and tumors (Bishayi and Sengupta, 2003; Arkusz et al., 2005; Banerjee et al., 2009). Correspondingly, an increased risk of fungal infections was associated with arsenic exposure in drinking water in two Taiwanese study cohorts (Hsu et al., 2016). In addition to altered macrophage function, a recent study evaluated the effects of sodium arsenite on porcine macrophage–derived DC and identified decreased phagocytosis and decreased ability to activate and induce T-cell proliferation; however, the pro-inflammatory cytokines IL-6 and IL-1β were increased (Mehrzad et al., 2017).

Mice exposed to arsenite in drinking water exhibited differences in speciation in the bone marrow, spleen, thymus, and plasma, whereas the bone marrow and thymus demonstrated increased and dose-dependent sensitivity to DNA damage as compared to the spleen (Xu et al., 2016a). Correspondingly, thymic evaluation following in vivo exposure to sodium arsenite in drinking water demonstrated increased oxidative stress and thymocyte apoptosis, which was associated with increased NF-κB nuclear localization and Cox-2 levels. Additionally, there appeared to be a shift toward the development of nTregs within the thymus (Choudhury et al., 2016). Interestingly, serum cytokines showed an increase in IL-2 (suggested to promote nTreg development), IL-17A, and IL-10, and a decrease in TNF-α and IL-6 with increased concentration of sodium arsenite (Choudhury et al., 2016). A second study demonstrated similar results in that sodium arsenite decreased $CD4^+$ T cells and increased oxidative stress as well as factors involved in the development of nTregs; but in contrast, this study also identified increased serum TNF-α, IL-6, and IL-1β, which appeared to be associated with increased activation of both the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways in the thymus and spleen (Duan et al., 2017). In a rat microglial cell line, arsenic trioxide induced the secretion of inflammatory cytokine IL-6 by activating the Akt and NF-κB pathways (Chen et al., 2016). Studies with arsenic trioxide demonstrated improved immune targeting against liver cancer by increasing effector T cells and decreasing Tregs in the tumor. Additionally, in vitro studies demonstrated an increase in the numbers of NKT, $CD8^+$, and NK cells and in vitro cytotoxicity by arsenic trioxide (Wang et al., 2017b). In human PBMCs stimulated in vitro and exposed to arsenic disulfide, there was a decreased frequency of $CD4^+$ and $CD8^+$ T cells but the frequency of Tregs within the $CD4^+$ cell population increased (Song et al., 2015). Arsenic increased the expression and secretion of pro-inflammatory cytokines (IL-1α, IL-1β, and IL-18) in a human keratinocyte cell line, and the increase in IL-1β and IL-18 secretion appears to be mediated by the induction of the inflammasome AIM2 (absent in melanoma 2) (Zhang et al., 2016b). In summary, arsenic alters inflammatory and adaptive immune processes but variations in the effects may be due to the speciation of arsenic and the target cell.

Mercury Mercury compounds include organic (i.e., methyl mercury) or inorganic forms. Mercury compounds suppress immunological responses, but also induce autoimmunity (see the section "Autoimmunity: Mercury"). Interestingly, in one genetically susceptible mouse model of autoimmunity, subcutaneous administration of methyl mercury reduced T- and B-cell numbers prior to autoimmunity induction, demonstrating the immunomodulatory actions of mercury in vivo (Haggqvist et al., 2005). Similar to arsenic, the mechanism by which mercury compounds are immunotoxic might be through induction of apoptosis (Yole et al., 2007). Mercury also induces oxidative stress as evidenced by glutathione depletion and impaired mitochondrial function (Mondal et al., 2005; Jorissen et al., 2013). Several studies have examined the effect of in vitro treatment of human PBMC with mercury compounds. Mercuric chloride treatment of PBMCs stimulated with either α-CD3/CD28/CD40 or bacterial antigen demonstrated that mercury differentially affects Th1/Th2 cytokines, depending on the mode of T-cell activation (Hemdan et al., 2007). Mercuric chloride enhanced an LPS-induced inflammatory response in PBMCs and increased cytokine secretion from human monocyte-derived DCs (Gardner et al., 2009; Rachmawati et al., 2015). The induction of pro-inflammatory cytokines was also observed in Brazilian gold miners using mercury as an amalgam. Mercury-exposed miners

demonstrated higher levels of IL-1β, TNF-α, and IFN-γ (Gardner et al., 2010). Increased pro-inflammatory cytokines were also identified in a Russian cohort occupationally exposed to mercury vapors; interestingly, there was an inverse relationship between serum cytokine concentrations and severity of neurological symptoms (Bodienkova et al., 2014). Additionally, environmental exposure to low levels of mercury was associated with increased atopic dermatitis in adults and asthma in children in two Korean cohorts (Park and Kim, 2011; Kim et al., 2015).

The mechanism for cytokine induction could be due to mercury-induced phosphorylation of ERK and p38 MAPKs, as demonstrated using mercuric chloride in a human T-cell line. This was due, in part, to ROS, because mercury-induced phosphorylation of ERK and p38 MAPKs was abrogated by *N*-acetyl cysteine (Haase et al., 2011). Similarly, treatment of a B-cell line with low levels of inorganic mercury induced tyrosine phosphorylation of proteins involved in the B-cell signaling pathway, whereas high levels induced cytotoxicity (Caruthers et al., 2014). Independent of an effect on MAPK signaling but dependent on ROS, organic and inorganic mercury induced the formation of extracellular traps by primary human neutrophils (neutrophil extracellular traps [NETs]) (Haase et al., 2016b). Therefore, depending on the concentration, mercury exposure could be cytotoxic or potentially inflammatory, and alter self-tolerance mechanisms.

Cadmium Like other metals, cadmium exhibits immunomodulatory effects. Early studies demonstrated that oral administration of cadmium to mice increased susceptibility to herpes simplex type 2 virus, suppressed T- and B-cell proliferation, but enhanced macrophage phagocytosis (Thomas et al., 1985b). As with many other immunotoxic agents, it has been suggested that the stress response, a shift to a Th2 cell population, induction of oxidative stress, and induction of apoptosis all contribute to the mechanism by which cadmium suppresses humoral immunity and CMI (Lall and Dan, 1999; Hemdan et al., 2006; Pathak and Khandelwal, 2006, 2008; Ebaid et al., 2014; ben Salah-Abbes et al., 2015; Milnerowicz et al., 2015). Interestingly, subchronic exposure to cadmium resulted in decreased inflammatory cytokines and demonstrated a protective role in an animal model of colitis, which appears to contrast with studies demonstrating an increase in inflammation, and may reflect differences in dosing and experimental models (Blum et al., 2014; Milnerowicz et al., 2015; Breton et al., 2016). Indeed in an animal model of arthritis, the inflammatory response and disease severity were decreased with lower doses of cadmium, but enhanced with higher doses of cadmium (Ansari et al., 2015).

A human study of workers occupationally and chronically exposed to cadmium identified an association between cadmium and lower percentages of neutrophils but higher percentages of lymphocytes (Ciarrocca et al., 2015). Using human HSCs isolated from umbilical cord blood, it was shown that in vitro treatment with cadmium chloride activated autophagy, an internal cellular degradation pathway allowing clearance of damaged cellular components (Di Gioacchino et al., 2008). Cadmium-induced oxidative stress was confirmed in PBMC isolated from a Japanese population of women living in a cadmium polluted area in which higher blood and urinary cadmium correlated with increased expression of several genes involved in oxidative stress signaling (Dakeshita et al., 2009). Furthermore, a cross-sectional analysis of data from NHANES found an association between blood cadmium levels and an increased risk of chronic infections (i.e., *H. pylori* and hepatitis B virus) (Krueger and Wade, 2016).

It has long been known that cadmium (and other metals, such as mercury) binds to a protein called metallothionein, which is a small, cysteine-rich protein that complexes normally with divalent cations, such as copper and zinc. The role of metallothionein in metal-induced immunotoxicity has been reviewed (Klaassen et al., 2009) and there are several mechanisms by which the cadmium–metallothionein complex might contribute to immune modulation. The binding of cadmium to metallothionein could displace copper or zinc, altering the availability of the latter cations for biochemical processes. Correspondingly, zinc treatment lessened the oxidative stress and the decreased lymphocyte survival induced by cadmium (Ebaid et al., 2014). Alternatively, metallothionein is induced in response to several stimuli, including metals, and it has been demonstrated that metallothionein influences lymphocyte proliferation, differentiation, and various effector functions. Finally, because metallothionein is a cysteine-rich protein, it also plays a role in the oxidative homeostasis of the cell, which could be compromised under conditions of oxidative stress.

Solvents and Related Chemicals

There is limited but substantive evidence that exposure to organic solvents and their related compounds can produce immune suppression. Chemicals included in this section are aromatic hydrocarbons, such as benzene, haloalkanes and haloalkenes, glycols and glycol ethers, and nitrosamines.

Aromatic Hydrocarbons By far the best characterized immunotoxic effects by an organic solvent are those produced by benzene. In animal models, benzene induces anemia, lymphopenia, and hypoplastic bone marrow. In addition, it has been suggested that this myelotoxicity may be a result of altered differentiative capacity in bone marrow–derived lymphoid cells. Benzene (oral and inhaled) exposure has been reported to alter both humoral and CMI parameters, including suppression of the anti-sRBC antibody response, decreased T- and B-cell lymphoproliferative responses (mitogens and alloantigens), and suppression of CTL activity. Pyatt et al. (1998) demonstrated that hydroquinone (HQ), a reactive metabolite of benzene, inhibits the activity of NF-κB. This HQ-induced inhibition of NF-κB has also been reported in monocytic cells and likely accounts for the HQ-induced inhibition of IL-12 in macrophages (Kim et al., 2005). The HQ-induced inhibition of IL-12, coupled with an HQ-induced increase in IL-4 in $CD4^+$ T cells (Lee et al., 2002), suggests that part of the mechanism by which benzene alters immune function involves metabolic activation to HQ, which promotes a Th2 phenotype. HQ induces apoptosis, which might also contribute to its reported decreases in human neutrophils, eosinophils (Yang et al., 2011), and naive $CD4^+$ T cells (Li et al., 2009). Interestingly, in chronic benzene-exposed workers, there is a decrease in $CD4^+$ cells as well as lower expression of genes encoding for the CD3 complex proteins, CD3γ, CD3δ, CD3ε, and CD3ξ (Li et al., 2012), which likely contributes to compromised T-cell responsiveness.

A number of compounds structurally related to benzene have also been studied for their potential effects on the immune system. For example, nitrobenzene (an oxidizing agent used in the synthesis of aniline and benzene compounds) has been reported to produce immunotoxic effects (Burns et al., 1994b), with the primary targets being erythrocytes and bone marrow. Immunomodulating activity has also been observed for toluene, although most effects occur at markedly high concentrations. When compared with benzene, toluene has little to no effect on immunocompetence. In contrast to the parent toluene, the monosubstituted nitrotoluenes (*para*- and *meta*-nitrotoluene) do significantly alter the immune system (Burns et al., 1994a, 1994c). Exposure to *p*-nitrotoluene suppresses the antibody response to sRBC, decreases the number of $CD4^+$ splenic

T cells, and suppresses the DTH response to KLH. In addition, host resistance to *L. monocytogenes* is impaired, suggesting the T cell as a primary target. Similarly, *m*-nitrotoluene suppresses the antibody response to sRBC, the DTH response to KLH, T-cell mitogenesis, and host resistance to *L. monocytogenes,* again suggesting the T cell as the cellular target. The di-substituted nitrotoluene (2,4-dinitrotoluene) is also immunosuppressive (Burns et al., 1994a), with exposure resulting in suppressed humoral immunity, NK cell activity, and phagocytosis by splenic macrophages.

Haloalkanes and Haloalkenes Carbon tetrachloride is widely recognized as hepatotoxic. Studies in mice revealed that carbon tetrachloride is also immunosuppressive. Mice exposed for 7 to 30 days to carbon tetrachloride (orally or intraperitoneally) exhibit a decreased TDAR (sRBC), suppressed MLR, and lower lymphoproliferative capacity (T and B cells) (Kaminski et al., 1989a). Ex vivo activation of splenic T cells isolated from carbon tetrachloride–treated mice reveal a marked enhancement in IL-2 production. Moreover the effect on IL-2 by carbon tetrachloride is associated with a serum factor in treated animals as direct addition of serum from carbon tetrachloride treated mice also produced strong enhancement of IL-2 in naive activated splenic T cells. Further characterization demonstrated that exposure to carbon tetrachloride causes the induction and release of TGF-β_1 from the liver, which is concomitant with the onset of immune suppression. Addition of anti-TGF-β_1 neutralizing antibodies abrogate IL-2 enhancement by serum isolated from carbon tetrachloride-treated mice, suggesting that the enhancing effects on IL-2 are mediated indirectly through TGF-β_1 released from the liver (Delaney et al., 1994; Jeon et al., 1997). Induction or inhibition of liver cytochrome P-450 activity augment and block, respectively, the immunotoxic actions of carbon tetrachloride, suggesting a requirement for metabolism in order for carbon tetrachloride to be immunosuppressive (Kaminski et al., 1990). Subsequently, it was demonstrated that TGF-β_1 produces profound effects on IL-2 regulation. Specifically, results in mouse spleen cells showed that TGF-β_1 exerts bifunctional effects on IL-2 and lymphoproliferation as evidenced by the fact that TGF-β_1 stimulates IL-2 production at low concentrations (0.1 to 1 pg/mL) and conversely suppresses IL-2 production at high concentrations (1 to 10 ng/mL), when activated using anti-CD3/CD28 (McKarns and Kaminski, 2000). Additionally, concentrations of TGF-β_1 that stimulate IL-2 production concomitantly suppress splenocyte proliferation under similar conditions. More recent studies have identified prostaglandin E2 (PGE2) as another soluble factor released following carbon tetrachloride liver injury (O'Brien et al., 2014). It was demonstrated that expression of cyclooxygenase-2 (COX-2), the enzyme that drives synthesis of PGE2, is upregulated in both liver and lung in carbon tetrachloride–treated mice. Plasma from carbon tetrachloride–treated mice suppresses TNF-α production in macrophages and this is not observed if the plasma was isolated from carbon tetrachloride–treated mice that had also received the nonselective COX inhibitor, indomethacin (O'Brien et al., 2014). Together these results suggest that the mechanism for compromised immunity in response to carbon tetrachloride is due, in part, to release of immune suppressive mediators, including TGF-β_1 and PGE2, from the liver.

There is relatively little information on solvents and other chemicals structurally related to carbon tetrachloride. Some early studies were conducted to assess the potential immunotoxicity of a number of drinking water contaminants. Exposure to dichloroethylene (in drinking water for 90 days) suppresses the anti-sRBC antibody response in male CD-1 mice and inhibits macrophage function in females (Shopp et al., 1985). Similarly, exposure to trichloroethylene (in the drinking water for 4 to 6 months) inhibits both humoral immunity and CMI and bone marrow colony-forming activity (Sanders et al., 1982). In those experiments, females were more sensitive than males. Exposure to 1,1,2-trichloroethane resulted in suppression of humoral immunity in both sexes. In addition, macrophage function is suppressed (males only) (Sanders et al., 1985). Inhalation studies with dichloroethane, dichloromethane, tetrachloroethane, and trichloroethene indicate that the pulmonary host resistance to *Klebsiella pneumoniae* is suppressed (Aranyi et al., 1986; Sherwood et al., 1987), suggesting that alveolar macrophages might be affected. More recent work with this series of solvents indicate that exposure to trichloroethylene might be an effective developmental immunotoxicant in B6C3F1 mice, suggesting that additional studies are required to determine the health risks associated with developmental exposure to this chemical (Peden-Adams et al., 2006).

Nitrosamines The nitrosamine family of chemicals comprises the nitrosamines, nitrosamides, and *C*-nitroso compounds. Exposure to nitrosamines, especially *N*-nitrosodimethylamine (e.g., also known as dimethylnitrosamine [DMN], and the most prevalent nitrosamine) comes primarily through industrial and dietary means, and minimally through environmental exposure. DMN is used commonly as an industrial solvent in the production of dimethylhydrazine. It has been used as an antioxidant, as an additive for lubricants and gasolines, and as a softener of copolymers. The toxicity and immunotoxicity of DMN have been extensively reviewed (Myers and Schook, 1996). Single or repeated exposure to DMN inhibits T-dependent humoral immune responses (IgM and IgG), but not T-independent responses. Other symmetrical nitrosamines, such as diethylnitrosamine (DEN), dipropylnitrosamine, and dibutylnitrosamine, demonstrated similar effects on humoral immunity but are not as potent as DMN (Kaminski et al., 1989b). T-cell-mediated lymphoproliferative responses (mitogens or MLR) and DTH response are also suppressed following DMN exposure. DMN induces apoptosis in human leukocytes, which could account for some of the compromised immune responses (Iwaniuk et al., 2015). In vivo exposure to DMN in mice followed by challenge with several pathogens does not produce a consistent pattern of effects: decreased resistance to *Streptococcus zooepidemicus* and influenza, no effects on resistance to herpes simplex types 1 or 2 or *Trichinella spiralis*, and increased resistance to *L. monocytogenes*. In contrast, anti-tumor activity in DMN-exposed animals is consistently enhanced (Thomas et al., 1985a). This could be related to enhanced macrophage activity, increased myelopoietic activity, and alterations in TNF-α transcriptional activity by DMN. DMN is also a potent liver carcinogen because it is metabolized by cytochrome P450s to a strong alkylating agent. There have been reports that DEN-induced hepatocarcinogenesis induces inflammatory responses (Schneider et al., 2012), which might explain enhanced macrophage activity following exposure to DMN.

Mycotoxins

Mycotoxins are structurally diverse secondary metabolites of fungi that grow on feed. This class of chemicals comprises such toxins as aflatoxin, ochratoxin, and the tricothecenes, notably T-2 toxin and deoxynivalenol (vomitoxin). As a class, these toxins can produce immune stimulation or immune suppression depending on dose, exposure frequency, or route of administration (Pestka et al., 2004). For the extensively studied trichothecenes, the mechanism of immune impairment is related in part to inhibition of protein synthesis. The mechanism for trichothecene-induced immune suppression at high doses also involves leukocyte apoptosis (Pestka

et al., 1994). Conversely, at low doses, trichothecenes promote expression of a diverse array of cytokines including IL-1, IL-2, IL-5, and IL-6. In addition, trichothecenes activate MAPKs in vivo and in vitro via a mechanism known as the ribotoxic stress response (Moon and Pestka, 2002; Chung et al., 2003; Zhou et al., 2003; Pan et al., 2013). Detailed studies in Jurkat cells have identified that the ribotoxic stress response to deoxynivalenol involves increased intracellular calcium (Katika et al., 2015). Prolonged consumption of deoxynivalenol by mice induces elevation of IgA and IgA immune complex formation, and kidney mesangial IgA deposition (Pestka, 2003). It has been postulated that the enhancement in IgA production induced by deoxynivalenol might be associated with the increase in cytokine production described above. The trichothecenes are currently considered among the most potent small-molecule inhibitors of protein synthesis in eukaryotic cells, which is dichotomous to the observed increase in IgA secretion.

Past studies with aflatoxin B1 suggest that CMI and phagocytic cell functions are affected as evidenced by decreased proliferative responses to PHA and suppression of DTH responses (Raisuddin et al., 1993). In addition, in vitro experiments demonstrate that aflatoxin B1 requires metabolic bioactivation in order to produce suppression of antibody responses and of mitogen-induced lymphoproliferation (Yang et al., 1986). Studies in laboratory animals have also shown increased risk to secondary infection after aflatoxin B1 treatment. The effects of aflatoxins on the human immune system have not been extensively characterized, but there is an association between high exposure to aflatoxins and lower T- and B-cell counts (Jiang et al., 2005).

Adverse health effects have been associated with damp indoor environments following building envelope breech resulting from heavy rains and/or flooding, as occurred during Hurricanes Katrina and Rita in the Gulf Coast of the United States. The adverse health effects have been attributed, at least in part, to the presence of molds, most notably *Stachybotrys chartarum*, also known as black mold. *Stachybotrys chartarum* produces the macrocyclic trichothecene toxin, satratoxin G, which like many of the trichothecenes is a potent inhibitor of protein synthesis. In one study, satratoxin G exposure of mice, 100 μg/kg for 5 consecutive days by intranasal instillation, induces apoptosis of olfactory sensory neurons and neutrophilic rhinitis (Islam et al., 2006). Elevated mRNA levels for pro-inflammatory cytokines TNF-α, IL-6, and IL-1, and the chemokine, MIP-2, are detected in nasal airways and the adjacent olfactory bulb of the brain. By day 7, marked atrophy of the olfactory nerve and glomerular layer of the olfactory bulb is detected. An additional study also showed that satratoxin G exposure produces epithelial injury and olfactory sensory neuron apoptosis in rhesus monkeys (Carey et al., 2012). These findings suggest that neurotoxicity and inflammation within the nose might be potential adverse health effects associated with *Stachybotrys* exposure in indoor air.

Natural and Synthetic Hormones

It is well established that a sexual dimorphism exists in the immune system. Females have higher levels of circulating Igs, a greater antibody response, and a higher incidence of autoimmune disease than do males. Males appear to be more susceptible to the development of sepsis and the mortality associated with soft tissue trauma and hemorrhagic shock. Specific natural sex hormones have been implicated to mediate this dichotomy with the evolutionary goal of increased mating success and species propagation (reviewed by Foo et al. [2017] and Roved et al. [2017]). A recent cross-species meta-analysis of immune function supports a general immunosuppressive effect of testosterone versus an immune-enhancing effect of estrogen on humoral-mediated immune function and cytokine levels, but estrogens also induced an immunosuppressive effect on cell-mediated immune function (Foo et al., 2017). Further analysis supports a decrease in Th2 and Th17 immune responses, a variable effect on Th1 immune responses, and increased wound healing by testosterone, whereas estrogen enhances Th2 immune responses and decreases Th1 and Th17 immune responses (reviewed by Roved et al. [2017]). Physiologically, immune effects of sex hormones appear to be very tightly controlled, and profound changes in immune activity can result for very slight changes in concentrations of hormones.

Estrogens Diethylstilbestrol is a synthetic nonsteroidal compound possessing estrogenic activity. Diethylstilbestrol was used in men to treat prostate cancer and in women as an estrogen replacement or contraceptive drug, and to prevent threatened abortions; however, use during pregnancy was later associated with development of genital adenocarcinoma in female offspring at the onset of puberty (Herbst et al., 1971). Extensive functional and host resistance studies on diethylstilbestrol (mg/kg/day dose range) have indicated that exposure to this chemical results in alterations in CMI and/or macrophage function that are thought to be mediated by the presence of the estrogen receptor in immune cells (Kalland, 1980; Luster et al., 1980, 1984; Holsapple et al., 1983; Lai et al., 2000; Brown et al., 2006a, 2006b). Targeted sites of action include the thymus (thymic depletion and alteration in T-cell maturation process), T cells (decreased MLR, DTH, and lymphoproliferative responses), and macrophages (enhanced phagocytic, anti-tumor, and suppressor function). Pre- and neonatal exposures (mg/kg/day dose range) also demonstrate immunotoxic effects related to T cell dysfunction. DTH and inflammatory responses associated with diethylstilbestrol exposure in adult mice have been shown to be reversible upon cessation of exposure (Luster et al., 1980; Holsapple et al., 1983). However, effects from in utero and neonatal exposures appear to have more lasting, possibly permanent effects on immune responses (Kalland et al., 1979; Luster et al., 1979; Ways et al., 1980).

Exposure of human PBMC from men and women to 17β-estradiol (E2) increases basal IgM, IgG, and IL-10 production with no effect on IL-1α, IL-1β, IL-2, IL-4, and IL-6. Interestingly, addition of IL10 enhances the effect of E2 on IgM and IgG production (Kanda et al., 1999). IL-10 appears to have both positive and negative effects on immune function, because in mouse models IL-10 is the mediator of a Breg subset (i.e., B10 or Br1). Similar to the above results, E2 enhances the induction of IgM and IgA AFCs from PBMC stimulated with pokeweed mitogen (testosterone had no effect) (Paavonen et al., 1981). In an autoimmune disease state, basal IgG production is more markedly induced by E2 in patients with active systemic lupus erythematosus (SLE) as compared to normal donors. In addition to an increase in total IgG, E2 also induces anti-dsDNA antibody (absent in normal donors). The profile is somewhat different for inactive SLE patients in that E2 increased total IgG production to a similar degree as for active SLE patients. Moreover, E2 does not induce anti-dsDNA antibody, which has a lower basal level in inactive SLE than active SLE (Kanda et al., 1999). Furthermore, microarray analysis of T cells purified from human PBMC demonstrated E2-altered gene expression for cellular signaling proteins in activated T cells from SLE patients versus controls (Walters et al., 2009). In endometriosis, estrogen levels, Tregs, and IL-10 are higher. Estrogen appears to induce IDO1 expression leading to increased Tregs and IL-10 levels as well as increase survival and invasion of endometrial stromal cells, perhaps by enhanced immune tolerance to these cells via Tregs (Wei et al., 2016). Interestingly, in an experimental mouse model of

drug-induced liver injury, E2 decreases Tregs and exacerbates hepatic injury, which is dependent on IL-6 (Cho et al., 2013). E2 therapy (1 mg) for 14 to 17 weeks in postmenopausal women has no effect on serum Ig levels or IL-4, IL-6, IL-10, and IL-7 levels compared to those before E2 therapy (Itaborahy and de Medeiros, 2016), but a review of studies evaluating immune response in postmenopausal women following hormone replacement therapy suggests immunological changes; the most consistent being a decrease in IL-6 levels (Abdi et al., 2016). ERβ activation in $CD4^+$ T cells is anti-inflammatory (induces Treg and inhibits Th17 polarization) in PBMCs from multiple sclerosis (MS) patients and in an EAE mouse model, as well as neuroprotective in the EAE mouse model (Aggelakopoulou et al., 2016).

Isoflavones ("isoflavone intervention") administered for 16 weeks to postmenopausal women through soymilk or supplemental tablets results in higher frequency of B cells in peripheral blood with no effect on the frequency of $CD4^+$, $CD8^+$, or NK subsets. The isoflavone intervention has no effect on IL-2 or TNF-α plasma levels, but IFN-γ trended toward an almost twofold nonsignificant increase (Ryan-Borchers et al., 2006). Other studies demonstrated an increase in plasma IL-6 with isoflavone diets (73 mg/day) (Jenkins et al., 2002) and increased cytotoxicity of human PBMC NK cells with isoflavone metabolites (0.1 μmol/L) (Zhang et al., 1999). In contrast to the human results with E2, in vivo administration of the phytoestrogen genistein suppresses anti-KLH IgG titers in mice (Yellayi et al., 2002). Genistein also inhibits cell-mediated immunity, but in an ER-independent manner. However, mouse cells treated with E2, diethylstilbestrol, or bisphenol A (discussed below) enhance IgG anti-DNA antibody production and IgG immune complex deposition in the kidney, which may be a result of increased autoantibody secretion from B-1 B cells (Yurino et al., 2004). Additionally, E2 has been shown to drive the expansion of the mouse Treg compartment and to increase Treg activity (Polanczyk et al., 2004; Luo et al., 2011). E2 also inhibits recruitment and activation of inflammatory cells resulting in inhibition of TNF-α and IFN-γ production and of the inflammatory response (Salem et al., 2000). These discrepancies in the effects of E2 (activation vs. inhibition) may be due to species differences, different cellular targets, or a lack of disease state. Taken together it is clear that E2 and phytoestrogens alter the human antibody response, an effect that is more marked in an autoimmune disease state.

A potential mechanism for the effects by estrogen and estrogenic compounds is activation of the nuclear estrogen receptor (ER, α or β isoform), which is ubiquitously expressed in most tissues. Binding of ligand stabilizes ER dimers, which then bind to estrogen response elements (ERE) in target genes, leading to transcriptional activation or inhibition. In-depth reviews outline studies evaluating ER expression and ER-mediated effects in innate and adaptive immunity from human and animal models as well as a role of ER in autoimmunity (Cunningham and Gilkeson, 2011; Kovats, 2015; Khan and Ahmed, 2016). In resting and activated human PBMC, $CD4^+$ T cells express higher levels of ERα than B cells, which express higher levels of ERβ. $CD8^+$ cells express ERα and ERβ equally, but at low levels (Phiel et al., 2005). ERα and ERβ are differentially expressed in APCs (Cunningham and Gilkeson, 2011) and in human secondary lymphoid tissues (Shim et al., 2006). Additionally, estrogen and the ER appear to play a significant role in autoimmunity. Ovariectomy before sexual maturation results in a significant suppression in a murine model of SLE, which can be reversed by supplementary estrogen treatment (Talal, 1981). ER knockout studies have primarily corroborated this finding with knockout of ERα in three different mouse models of SLE showing attenuation of the disease and prolonged survival (Bynote et al., 2008; Svenson et al., 2008). This effect is selective for ERα, because ERβ does not alter the disease state (Svenson et al., 2008). In contrast to these results, ERα (but not ERβ) knockout in a mildly autoimmune prone mouse strain leads to signs of autoimmunity and spontaneous (no antigen challenge) formation of germinal centers in the spleen (Shim et al., 2004). Indeed, unlike SLE, both ERα and ERβ exhibit a protective effect in MS and its experimental model (EAE), which may reflect differing functions of these receptors in different tissues and cell types (reviewed by Khan and Ahmed [2016]). An additional consideration in elucidating the mechanisms by which the ER mediates biological effects is the extensive cross-talk that can occur between the ER and other receptor signaling pathways (e.g., AhR and PPAR) and transcription factors (e.g., AP-1 and NF-κB) that may be independent of the ERE (Bonofiglio et al., 2005; Marino et al., 2006; Matthews and Gustafsson, 2006).

Bisphenol A, a monomer in polycarbonate plastics and a constituent of epoxy and polystyrene resins possessing weak binding affinity for the estrogen receptor, has been recently evaluated by a number of laboratories for its potential to affect various aspects of immune function (reviewed by Kharrazian [2014]). The majority of studies to date demonstrate that leukocytes cultured in the presence of very high concentrations (more than 1 μM) of bisphenol A exhibit a number of alterations primarily in innate immune function responses including suppression of LPS-induced nitric oxide production and TNF-α secretion by macrophages (Kim and Jeong, 2003). The effects on nitric oxide production correlate with a decrease in NF-κB DNA binding activity, a transcription factor critically involved in the regulation of inducible nitric oxide synthase and TNF-α. In this study, the estrogen receptor antagonist, ICI 182,780, blocks bisphenol A suppression of LPS-induced nitric oxide production. Bisphenol A (10 to 50 μM) has also been reported to enhance IL-4 production in a model of a secondary immune response (Lee et al., 2003). In vivo treatment of mice with bisphenol A (2.5 mg/kg) for 7 days produces a decrease in ex vivo Con A–induced proliferation and IFN-γ secretion, but has no effect on the number of $CD4^+$, $CD8^+$, and $CD19^+$ cells in the spleen (Sawai et al., 2003). Additional studies corroborate these findings by demonstrating an augmentation of Th1 immune responses (i.e., cytokine profile and increased expression of antigen-specific IgG2a and IgA) with one study showing increased Th1 and Th2 immune responses. In these studies, bisphenol A was administered either by i.p. injection (0.1 mg/g) four times every second day (Alizadeh et al., 2006), in drinking water (10 mg/L, ad libitum) for 2 weeks (Goto et al., 2007) or through maternal dosing (3 to 3000 μg/kg) for 18 days (Yoshino et al., 2004). Presently, the putative effects of bisphenol A on immune function are poorly defined and based on the current literature; it is unclear whether the majority of the immunomodulatory effects reported are mediated through an ER-dependent mechanism. Additionally, the relatively high potential for human exposure to bisphenol A due to its wide use in plastics and other products has been of considerable concern to the public and government regulators. Despite the large number of safety-related studies published on bisphenol A, there is considerable controversy regarding its safety and the current tolerable daily intake value (0.05 mg/kg body weight/day) (Sekizawa, 2008; Vandenberg et al., 2009; Hengstler et al., 2011). Therefore, the National Toxicology Program, National Institute of Environmental Health Sciences, and U.S. Food and Drug Administration jointly developed a consortium-based research program known as the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) to conduct hypothesis-driven research to address this controversy (Heindel et al., 2015). Results from the immunotoxicological evaluation of BPA from the CLARITY study are forthcoming.

Androgens Oxymetholone is a synthetic androgen structurally related to testosterone and was used in the past in the treatment of pituitary dwarfism and as an adjunctive therapy in osteoporosis. Its current use is limited to treatment of certain anemias. Oxymetholone administered orally to male mice daily for 14 consecutive days (50 to 300 mg/kg/day) (Karrow et al., 2000) results in a minimal decrease in CMI (MLR and CTL response) without altering the ability of the animals to resist infection in host resistance assays. In contrast, anabolic androgenic steroids have been shown to significantly suppress the sRBC AFC response and to increase the production of pro-inflammatory cytokines from human PBMC.

Testosterone through the activation of the cytosolic androgen receptor (AR) is capable of contributing to the suppression of immune function, particularly, macrophage activity, CMI responses, and the development of T and B cells (reviewed by Lai et al. [2012] and Trigunaite et al. [2015]). There are numerous reports in the clinical literature that males are more susceptible than females to infection following soft tissue trauma and hemorrhagic shock (reviewed by Catania and Chaudry [1999]). Treatment of males with chemicals that block testosterone (e.g., flutamide) can prevent the trauma- and hemorrhage-induced depression of immunity. Similarly, treatment of females with dihydrotestosterone prior to trauma hemorrhage results in depression of CMI similar to that of males. Furthermore, gonadectomized mice of either sex have elevated immune responses to endotoxin, which can be attenuated in either sex by the administration of testosterone. Studies with general and conditional AR knockout models underscore the role of the AR in regulating immune function, including supporting the generation and activation of neutrophils and the pro-inflammatory responses of macrophages but suppressing lymphocyte development and adaptive immune responses (reviewed by Lai et al. [2012] and Trigunaite et al. [2015]).

Glucocorticoids The immunosuppressive actions of corticosteroids have been known for years (reviewed by Oppong and Cato [2015]). Following binding to a cytosolic receptor, these chemicals produce profound lymphoid cell depletion in rodent models. In NHPs and humans, lymphopenia associated with decreased monocytes and eosinophils and increased neutrophils are seen. Corticosteroids induce apoptosis in rodents; T cells and B cells (pro-B to mature B-cell maturation states) are particularly sensitive (Gruver-Yates et al., 2014). In addition, these chemicals suppress macrophage function, the production of IL-1 from the macrophages, and the subsequent synthesis of IL-2 by T cells. In general, corticosteroids suppress the generation of CTL responses, MLR, NK cell activity, and lymphoproliferation. Glucocorticoids appear to induce DCs to promote the development of Tregs, favor a Th1 to Th2 shift, and induce the AFC response and secretion of IgE and IgG4. Increased IgE seems contrary to the use of glucocorticoids in allergy; however, these drugs have a strongly inhibitory effect on eosinophils and mast cells and appear to reduce antigen-specific IgE (reviewed by Franchimont [2004]).

In spite of the wide therapeutic use of glucocorticoids, the mechanism of action by which glucocorticoids mediate their anti-inflammatory/immunosuppressive activity is not well understood. Several mechanisms have been proposed, all of which involve activation of the glucocorticoid receptor (GR) (reviewed by Baschant and Tuckermann [2010]). Binding of glucocorticoids to the cytosolic GR (member of the nuclear receptor superfamily) activates the receptor to function as a ligand-activated transcription factor that undergoes homodimerization and DNA binding to glucocorticoid response elements (GRE) in the regulatory regions of glucocorticoid responsive genes. Depending on the gene, GRE can either positively or negatively regulate transcription. For example, glucocorticoids induce annexin 1 (lipocortin 1), a calcium and phospholipid binding protein, which acts to inhibit PLA2 (Goulding and Guyre, 1992; Taylor et al., 1997; Perretti and D'Acquisto, 2009). Inhibition of PLA2 results in a decrease in arachidonate formation, the precursor in the biosynthesis of inflammatory prostaglandins and leukotrienes. Similarly, GR activation induces transcription of IκB, which is the endogenous inhibitor of the transcription factor NF-κB (Auphan et al., 1995; Scheinman et al., 1995; Deroo and Archer, 2001). Because transcription of many key inflammatory cytokines is regulated positively by NF-κB, induction of IκB results in retention of NF-κB in the cytosol and thus suppression of inflammatory cytokine production. Ligand-activated GRs have also been found to physically interact with other transcription factors including AP-1 (Schule et al., 1990; Karmakar et al., 2013) and NF-κB (Ray and Prefontaine, 1994; Widen et al., 2003) to inhibit DNA binding and/or transcriptional activity. Alternatively, GRs can cooperatively interact with NF-κB in a negative feedback loop to induce the expression of anti-inflammatory genes (Altonsy et al., 2014). Cross-talk between the GR and other nuclear receptors may also play a role in mediating the effects of glucocorticoid ligands (Karmakar et al., 2013). Presently, it is thought that all of the above mechanisms contribute to the anti-inflammatory and immunosuppressive properties of glucocorticoids.

Drugs of Abuse

Drug abuse is a social issue with extensive pathophysiological effects on the abuser. Drugs of abuse exhibit immunosuppressive actions, and in fact it has been suggested that in addition to the direct risk of HIV contraction via needle sharing or judgment lapses, abuse of some drugs has been associated with disease progression to AIDS (Rogers, 2011). Several classes of drugs will be discussed, including cannabinoids, opioids, cocaine, methamphetamine, and ethanol. Reports regarding the immune system effects of many of these drugs are often contradictory, so it should be noted that the mechanisms by which drugs of abuse suppress immune function might depend on the development of tolerance or addiction to the drugs; the immune, withdrawal, and pain status of the individual; and levels of endogenous molecules (i.e., endorphins or endocannabinoids).

Cannabinoids Much attention has been focused on the immunomodulatory effects of the cannabinoids, which can be defined as plant-derived (i.e., from the marijuana plant), synthetic, or endogenous. Therapeutically, the primary psychoactive congener of marijuana, Δ^9-tetrahydrocannabinol, is federally approved in the United States in pill form as an antiemetic in patients undergoing cancer chemotherapy, and as an appetite stimulant for cachexia associated with advanced AIDS disease. Several states have also legalized marijuana for medical use over the last decade (Wilkinson et al., 2016), which is smoked in joints or bongs, ingested in brownies, cookies, or teas, or vaporized.

The mechanism by which Δ^9-tetrahydrocannabinol produces psychotropic effects is through a G protein–coupled cannabinoid receptor CB1 (Varvel et al., 2005). Peripheral tissues also express CB1, in addition to a second cannabinoid receptor CB2. Although both CB1 and CB2 are expressed on immune system cells (Galiegue et al., 1995), not all cannabinoid effects are mediated via the receptors. For instance, in T cells, many plant-derived compounds suppress IL-2 in a CB1/CB2-independent manner (Kaplan et al., 2003). IL-2 suppression by Δ^9-tetrahydrocannabinol is due, in part, to suppression of transcription factor activation (AP-1, NFAT, NF-κB) and ERK MAPK activity (Condie et al., 1996; Herring et al., 1998;

Faubert and Kaminski, 2000). In contrast, B-cell suppression by Δ^9-tetrahydrocannabinol is CB1 and/or CB2 receptor-dependent (Schatz et al., 1993; Springs et al., 2008). This is consistent with several studies identifying a critical role for CB2 in germinal centers, memory B cells, and T-cell-independent responses (Basu et al., 2011, 2013).

Cannabinoid suppression of other APCs, such as macrophages and DCs, is also mediated through the CB1 and/or CB2 receptors. Δ^9-Tetrahydrocannabinol exposure impairs lysosomal or cytochrome *c* processing in macrophages (McCoy et al., 1995; Matveyeva et al., 2000) likely via the CB2 receptor (Buckley et al., 2000; Chuchawankul et al., 2004). IL-12 production from DC and subsequent Th1 activation are suppressed by Δ^9-tetrahydrocannabinol in a CB1 and/or CB2 receptor-dependent manner (Lu et al., 2006a, 2006b). Overall, several mechanisms are involved in cannabinoid-mediated suppression of host resistance, including dysregulation of cytokine profiles (shifts in Th1/Th2/Th17 balance) (Newton et al., 2009; Sido et al., 2016), induction of apoptosis (McKallip et al., 2002), and iTregs (Rao et al., 2015; Dhital et al., 2017).

Opioids Similar to cannabinoids, opioids refer to plant-derived, synthetic, or endogenous (endorphins) compounds that bind opioid receptors. Although technically "opioid" refers to drugs derived from the poppy plant, and "opiate" refers to agonists and antagonists with morphine-like activity (including plant-derived and synthetic compounds), they are often used interchangeably. It is well established that opioids suppress immune responses and the mechanism often involves one of the G_i-coupled opioid receptors (μ, κ, and δ receptors), but there are opioid receptor–independent mechanisms as well (Roy et al., 2011).

Early studies evaluating the immune competence of heroin addicts revealed a decrease in total T cells, which was reversed with the general opioid receptor antagonist naloxone, suggesting a role for an opioid receptor in mediating immune suppression (McDonough et al., 1980). Later studies demonstrated that some of the effects of morphine were the result of increased circulating corticosteroids (Pruett et al., 1992b; LeVier et al., 1994). Both mechanisms can contribute to decreased host resistance to viral and bacterial infection by opioids.

For instance, morphine treatment of mice infected with *S. pneumoniae* increases bacterial burden in the lungs and increased mortality. The mechanism for compromised host resistance involves suppression of NF-κB gene transcription, which likely contributes to decreased expression of inflammatory mediators, such as chemokines, reducing recruitment of neutrophils to the infection site (Wang et al., 2005). Morphine also reactivates latent herpes simplex virus infection due to suppressed CTL activity and IFN-γ production (Mojadadi et al., 2009). Morphine suppression of cytokines, such as IL-2 and IFN-γ (Mojadadi et al., 2009), involves opioid-mediated suppression of many transcription factors, including NF-κB, AP-1, and CREB (Roy et al., 1997; Wang et al., 2003, 2005; Borner and Kraus, 2013).

Opioids also modulate innate immunity. NK cells are sensitive targets of suppression by opioids (Saurer et al., 2004, 2006). Interestingly, the NK effects might be centrally mediated as central administration of an opioid antagonist attenuates suppression by morphine (Shavit et al., 1986). Some studies demonstrate either suppression (Sacerdote, 2003) or enhancement (Peng et al., 2000) of cytokine production from macrophages. The differences might be due to the agonist used, in vitro versus in vivo administration, and the dosing regimen (i.e., whether tolerance was induced or not). Overall, it is clear that opioids suppress immune function and that the mechanism by which this occurs is complex and likely involves the CNS, the autonomic nervous system, the hypothalamic-pituitary-adrenal axis, and one or more opioid receptors (Alonzo and Bayer, 2002).

Cocaine Cocaine is a potent local anesthetic and CNS stimulant. This drug and its derivatives have been shown to alter several measures of immune competence, including humoral and cell-mediated immune responses and host resistance (Watson et al., 1983; Ou et al., 1989; Starec et al., 1991). Studies evaluating the effect of acute in vivo cocaine exposure on the generation of the anti-sRBC AFC determined that sex, strain, and age differences dictate differences in cocaine-induced immunomodulation (Matulka et al., 1996), and that immune suppression is due to a cytochrome P-450 metabolite of cocaine (Jeong et al., 1996). Similar to other immunotoxic chemicals, the mechanism by which cocaine alters immune function involves a disruption of the Th1/Th2 balance and the stress response (Stanulis et al., 1997a, 1997b; Jankowski et al., 2010). Cocaine also induces the secretion of TGF-β, which has been linked to the observation that cocaine exposure enhances replication of the HIV-1 virus in human PBMC (Chao et al., 1991; Peterson et al., 1991). A proteomic analysis conducted in human DCs treated ex vivo with cocaine for 48 hours demonstrated increased expression of NF-κB (Reynolds et al., 2009b), which could contribute to increased expression of various cytokines or chemokines. Cocaine causes an increased HIV viral burden in a human PBL-SCID animal model that is mediated through sigma 1 (σ1) receptors, which are informally referred to as psychoactive drug receptors (Roth et al., 2005). Although the function and role of σ1 receptors still remain to be elucidated, additional studies also suggest that cocaine effects are mediated through these receptors (Maurice and Romieu, 2004; Cabral, 2006). Cocaine, and particularly, crack, has been associated with HIV pathogenesis, which is likely due to cocaine/crack-induced suppression of T-cell function (Pandhare et al., 2014; Addai et al., 2015).

Methamphetamine Methamphetamine is a stimulant that is similar to amphetamine, although highly addictive and its use has been growing over the past several years. An investigation into the immunotoxicity of methamphetamine showed that methamphetamine suppresses the anti-sRBC AFC response, IgG production, and mitogenic stimulation of T-cell proliferation following oral administration in mice. Even more striking is the suppression of GM-CSF-stimulated bone marrow colony growth by methamphetamine (In et al., 2005). These results indicate suppression of both CMI and humoral immunity in vivo following methamphetamine administration.

In human T cells, methamphetamine treatment ex vivo induces ROS and increases intracellular calcium (Potula et al., 2010). Consistent with this, ex vivo treatment of human DCs with methamphetamine induces MEK, which activates MAPKs (Reynolds et al., 2009a). Both of these studies demonstrate that in the absence of immune stimulation, acute methamphetamine treatment causes biochemical changes consistent with cellular activation. A very detailed analysis of T-cell subsets altered by methamphetamine in LCMV-treated mice revealed that methamphetamine alters the co-expression of IFN-γ, TNF-α, and TGF-β, especially in $CD4^+$ cells (Sriram et al., 2016). In mice treated in vivo with methamphetamine or in methamphetamine-using patients, plasma IL-6 is suppressed and IL-10 is increased, suggesting immune suppression following chronic methamphetamine use (Loftis et al., 2011).

Ethanol Ethanol exposure has been studied both in alcoholic patients and in animal models of binge drinking. In humans, alcoholism is associated with an increased incidence of, and mortality from, pulmonary infection (reviewed by Simet and Sisson [2015]). There is also an increased incidence of bacterial infection and spontaneous bacteremia in alcoholics with cirrhosis of the liver

(reviewed by Szabo and Saha [2015]). A consistent finding in abusers of ethanol is the significant change in PBMC. In animal models, this is observed as depletion of T and B cells in the spleen and the T cells in the thymus, particularly $CD4^+$ $CD8^+$ cells. The latter effect may be related in part to increased levels of corticosteroids, particularly in females (Han et al., 1993; Glover et al., 2011). T-cell activation, as assessed by CD69 expression, was suppressed by alcohol treatment in a sepsis model (Margoles et al., 2016). In a binge-drinking animal model, ethanol suppresses innate immunity through impairment of TLR3 signaling in peritoneal macrophages, correlating with suppression of pro-inflammatory cytokines (Pruett et al., 2004b). In addition to suppression of TLR3, ethanol suppresses signaling through other TLRs, contributing to pleiotropic effects of ethanol on innate immunity (Pruett et al., 2004a). Moreover, signaling alterations via TLRs depends on whether the alcohol exposure is acute or chronic (Dai and Pruett, 2006). The latter might account for the paradoxical observation that alcohol produces alcoholic liver disease characterized by inflammation leading to steatosis, fibrosis, and cirrhosis (Neuman et al., 2015).

Ultraviolet Radiation

Ultraviolet radiation (UVR), especially mid-range UVB (290 to 340 nm), is an important environmental factor affecting human health with both beneficial effects including vitamin D production, tanning, and adaptation to UV, and adverse effects including sunburn, skin cancer, and ocular damage. Most, if not all, of the chemicals, drugs, and other materials discussed in this chapter were selected because they have been associated with immunotoxic effects, and because there is the chance for human exposure. Indeed, UVR has also been demonstrated to modulate immune responses in animals and humans (Garssen and van Loveren, 2001; Murphy, 2009; Ullrich and Byrne, 2012; Damiani and Ullrich, 2016; Gonzalez Maglio et al., 2016; Prasad and Katiyar, 2017). While UV-induced immunomodulation has been shown to have some beneficial effects on some skin diseases, such as psoriasis, and has been demonstrated to impair some allergic and autoimmune diseases in both animals and humans, UV-induced immunomodulation can also lead to several adverse health consequences, including a pivotal role during the process of skin carcinogenesis. Kripke (1981) provided the first evidence that UVR is immunosuppressive. UV-induced skin tumors were removed and transplanted into normal age- and sex-matched syngeneic recipient mice. Interestingly, the tumors did not grow in normal mice, and progressive tumor growth was only seen when the tumors were transplanted into recipient mice that were immunosuppressed. These results were explained by proposing that exposure to UVR had two effects: induction of skin tumors and induction of immune suppression. Studies have shown that these two events are related based on a series of observations (Ullrich, 2007b; Damiani and Ullrich, 2016; Prasad and Katiyar, 2017). First, UVR-induced keratinocyte-derived platelet-activating factor plays a role in the induction of immunosuppression. Second, *cis*-urocanic acid, a skin-derived immunosuppressive compound, mediates immune suppression by binding to serotonin receptors on target cells. Finally, studies showed that blocking the binding of these compounds to their receptors not only suppresses UVR-induced immune suppression, but also interferes with skin cancer induction (Sreevidya et al., 2008; Zhang et al., 2008).

There have been a number of studies to further characterize the mechanism of action for UV-induced immunomodulation. The first step is the absorption of UV photons by chromophores, so-called "photoreceptors," such as DNA and urocanic acid (Garssen et al., 1997). As a consequence of UV absorption by chromophores, epidermal and dermal cells, including keratinocytes, melanocytes, Langerhans cells, mast cells, dermal fibroblasts, endothelial cells, as well as skin-infiltrating cells (i.e., granulocytes and macrophages), produce and/or release many immunoregulatory mediators, including cytokines, chemokines, and neurohormones (Sleijffers et al., 2004). The mediators include both pro- and anti-inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-10, IL-12, and IL-17, which can modify directly or indirectly the function of APCs (Achachi et al., 2015; Li et al., 2015b; Prasad and Katiyar, 2017). Langerhans cells, the major APC in the skin, change phenotypically and functionally, which ultimately impacts the activity of T cells at the time of antigen presentation, both locally and systemically. One early explanation for UV-induced immunomodulation is that UVR induced a switch from a predominantly Th1 response (favoring DTH responses) to a Th2 response (favoring antibody responses). This hypothesis was supported by initial findings of altered cytokine secretion patterns indicative of a Th1 to Th2 switch (Araneo et al., 1989; Simon et al., 1990). Indeed, the majority of studies dealing with the effects of UVR indicate that Th1-mediated immune responses are especially sensitive to UV exposure. However, as noted above, UVR is associated with suppression of certain allergic and autoimmune reactions. Indeed, more recent studies demonstrate that Ig isotypes that are linked to either Th1 or Th2 cells can be suppressed by UVR and that UV exposure not only impairs Th1 responses, but also impairs humoral immunity by inhibiting T_{FH} and inducing Bregs that secrete IL-10 (Sleijffers et al., 2004; Byrne and Halliday, 2005; Gonzalez Maglio et al., 2016).

Schwartz (2008) summarized 25 years of studies by noting that in contrast to the general immunosuppression associated with the use of conventional immunosuppressive drugs, UVR suppresses the immune system in an antigen-specific fashion via the induction of tolerance. Several investigators have noted that the immunotolerance is mostly mediated via Tregs induced by UVR, and that induction of Tregs, expressing CD4 and CD25, is an active process, which requires antigen presentation by UV damage. Once activated in an antigen-specific manner, these Tregs can suppress immune responses in an IL-10-dependent manner. Ullrich (2007a) noted that this model is consistent with the fact that UVR is absorbed in the upper layers of the skin, does not penetrate into the underlying tissues and internal organs, and that T cells are not directly targeted by UVR in vivo because few T cells are found in normal skin (Ullrich, 2007a). Mast cells also appear to be key regulators of the systemic immunosuppression induced by UVR in that mast cells regulate both innate and adaptive immune responses through the cytokines they secrete and the generation of immunosuppressive cells (e.g., Treg and Breg) (Byrne et al., 2008; Chacon-Salinas et al., 2011; Ullrich and Byrne, 2012).

Therapeutic Drugs

The recent explosion of knowledge regarding the function and regulation of the immune system (at the cellular, biochemical, and molecular levels) has provided investigators with a relatively new avenue for specific drug development. The following discussion focuses on those small molecule drugs used primarily for modulating the immune system: anti-inflammatory drugs, immunosuppressants (corticosteroids are described in the section "Natural and Synthetic Hormones"), and AIDS therapeutics.

Anti-inflammatory Drugs Anti-inflammatory drugs include nonselective and selective nonsteroidal anti-inflammatory drugs (NSAIDs), which suppress the production of pro-inflammatory soluble factors, such as prostaglandins and thromboxanes. Nonselective NSAIDs are a large class of drugs that reversibly

inhibit both isoforms of cyclooxygenase (COX-1 and COX-2). The COX-2 enzyme, in particular, is induced in response to inflammatory cytokines and mediators and therefore represents an attractive target to combat inflammatory diseases. However, due to an increased risk of cardiovascular effects in some patients (reviewed by Grosser et al. [2006]), which is a side effect to varying degrees for all NSAIDs, the only COX-2 selective inhibitor currently approved for clinical use is celecoxib (celebrex). Aspirin, like nonselective NSAIDs, inhibits COX-1 and COX-2 enzymes but inhibition is irreversible due to covalent binding of aspirin by acetylation to a serine residue in the COX enzyme. Aspirin is especially effective as an antiplatelet because platelets possess little biosynthesizing capacity and therefore aspirin will inhibit COX for the life of the platelet (8 to 11 days).

Immunosuppressive Agents Several immunosuppressive drugs are efficacious simply due to their ability to impair cellular proliferation, because proliferation is required for lymphocyte clonal expansion and, subsequently, differentiation. More recently, drugs that inhibit specific intracellular proteins critical in the activation of the immune response have been approved.

Cyclophosphamide (Cytoxan, CTX) CTX, which was originally developed as an antineoplastic drug, is the prototypical member of a class of drugs known as alkylating agents. Upon entering the cell, the inactive drug is metabolically cleaved into phosphoramide mustard, a powerful DNA alkylating agent that leads to blockade of cell replication, and acrolein, a compound known to primarily bind to sulfhydral groups. Clinically, CTX has found use in reducing symptoms of autoimmune disease and in the pretreatment of bone marrow transplant recipients. Experimentally, this drug is often used as a positive immunosuppressive control in immunotoxicology studies because it can suppress both humoral and CMI responses. There appears to be preferential suppression of B-cell responses, possibly due to decreased production and surface expression of Igs. CMI activities that are suppressed include the DTH response, CTL, graft-versus-host disease, and the MLR. Administration of low doses of CTX prior to antigenic stimulation can produce immune enhancement of cell-mediated and humoral immune responses, which has been attributed, in part, to an inhibition of suppressor T-cell activity (Limpens et al., 1990; Limpens and Scheper, 1991). The immune-enhancing properties of CTX were demonstrated to be mediated by acrolein, but not by phosphoramide mustard (Kawabata and White, 1988).

Azathioprine (Imuran) Azathioprine is one of the antimetabolite drugs; it is a pro-drug that is metabolized to 6-mercaptopurine, which inhibits cell replication by interfering with purine synthesis. Immune suppression likely occurs because of the ability of the drug to inhibit purine biosynthesis for which lymphocytes are sensitive, due to their lack of a salvage pathway. In addition, it has been found that azathioprine blocks the downstream effects of CD28 costimulation via Rac1, resulting in an increase in apoptotic cells, and it reduces the number of neutrophils and monocytes (Maltzman and Koretzky, 2003). It has found widespread use in the suppression of allograft rejection and an array of autoimmune diseases. Clinical use of the drug is limited by bone marrow suppression and leukopenia. Azathioprine inhibits humoral immunity, but secondary responses (IgG) appear more sensitive than primary responses (IgM). Several CMI activities are also reduced by azathioprine treatment, including DTH response, MLR, and graft-versus-host disease. Although T-cell functions are the primary targets for this drug, inhibition of NK cell function and macrophage activities has also been reported.

Leflunomide (Arava) Leflunomide is an isoxazole derivative. It is another drug that suppresses cellular proliferation, which has been used in the treatment of rheumatic disease and transplantation (Xiao et al., 1994). Leflunomide inhibits de novo pathways of pyrimidine synthesis, thereby blocking progression from G_1 to S of the cell cycle. Thus, direct inhibition of B-cell proliferation may account for the drug's ability to inhibit both T-cell-dependent and T-cell-independent antibody production. Leflunomide can also directly inhibit T-cell proliferation induced by IL-2, mitogens, or antibodies directed against CD3.

Cyclosporin A (CsA, Sandimmune) CsA is a cyclic undecapeptide isolated from fungal organisms found in the soil. Important to its use as an immunosuppressant is the relative lack of secondary toxicity (e.g., myelotoxicity) at therapeutic concentrations (Calne et al., 1981). However, hepatotoxicity and nephrotoxicity are limiting side effects. CsA acts preferentially on T cells by inhibiting the biochemical signaling pathway emanating from the TCR (reviewed by Ho et al. [1996]). The result is inhibition of IL-2 gene transcription and subsequent inhibition of T-cell proliferation and clonal expansion of effector T cells. More specifically, CsA interacts with the intracellular molecule cyclophilin, an intracellular a protein with peptidyl proline isomerase activity (although this enzymatic activity has nothing to do with the immunosuppressive effect of CsA). The CsA–cyclophilin complex inhibits the serine/threonine phosphatase activity of a third molecule calcineurin. The function of calcineurin is to dephosphorylate the cytoplasmic form of the transcription factor, nuclear factor of activated T cells (NFAT), thereby facilitating the transport of NFAT into the nucleus, where it can couple with nuclear components and induce the transcription of the IL-2 gene. Inhibition of calcineurin phosphatase activity by the CsA–cyclophillin complex prevents nuclear translocation of NFAT and the resulting IL-2 gene transcription.

FK506 (Tacrolimus or Prograf) FK506 is a cyclic macrolide that is structurally distinct from CsA, but possesses a nearly identical mechanism of action (reviewed by Ho et al. [1996]). Like CsA, FK506 binds intracellularly to proteins with peptidyl proline isomerase activity, the most abundant of which is FK506 binding protein-12 (FKBP12). The FK506–FKBP12 complex also binds to and inhibits calcineurin activity, thereby inhibiting IL-2 gene transcription. Clinically, FK506 inhibits T-cell proliferation, lacks myelotoxicity (although, like CsA, it does cause nephrotoxicity), and induces transplantation tolerance. In addition, the minimum effective dose appears to be approximately one-tenth of CsA.

Rapamycin (RAP, Sirolimus or Rapamune) RAP is also a cyclic macrolide, which is structurally related to FK506. However, the mechanism by which it produces inhibition of proliferation is strikingly distinct. Unlike CsA and FK506, RAP does not inhibit TCR-dependent signaling events and IL-2 gene transcription. Rather, this compound inhibits IL-2-stimulated T-cell proliferation by blocking cell cycle progression from late G_1 into S phase (Morice et al., 1993; Terada et al., 1993). Like FK506, RAP binds to the intracellular protein FKbP12 but this RAP–FKBP12 complex does not bind calcineurin. Rather, the RAP–FKBP12 complex binds to the mechanistic target of rapamycin (mTOR) (Sabers et al., 1995), inhibiting its function. Inhibition of mTOR results in reduced cell growth and suppression of cell cycle progression and proliferation (reviewed by Fingar and Blenis [2004]). Unlike both CsA and FK506, RAP does not appear to be nephrotoxic. Mechanistically, a significant advantage of RAP over CsA and FK506 is that it is an effective immune suppressant even after T cells have been activated, due to the fact that it blocks signaling through the IL-2 receptor. Conversely, for CsA and FK506 to be effective, T cells must encounter the drug prior to activation because once IL-2 transcription begins, neither therapeutic will provide effective suppression of the already activated T cells and IL-2 production.

Tofacitinib (Xeljanz) Tofacitinib is a Janus kinase (JAK) inhibitor recently approved for treatment of rheumatoid arthritis and under evaluation for other autoimmune diseases and organ transplantation rejection. Cytokine signaling via JAK pathways leads to induction of inflammatory gene expression, which continues the loop of inflammatory signaling. Tofacitinib inhibits JAK1, JAK2, JAK3, and, to a lesser extent, TYK2, and thus modulates cytokines critical to the progression of immune and inflammatory responses. Tofacitinib affects both innate and adaptive immune responses. Specifically, tofacitinib inhibits the acute LPS-induced inflammatory response and the differentiation of Th1 and Th2 cells and interferes with the generation of pathogenic Th17 cells. Tofacitinib has a boxed warning on its label for injury or death due to serious infections, lymphoma, and other malignancies. Serious infections leading to hospitalization or death, including tuberculosis and bacterial, invasive fungal, viral, and other opportunistic infections, have occurred in patients receiving tofacitinib (Hodge et al., 2016). Treatment is also associated with decreases in lymphocytes and neutrophils.

Fingolimod (FTY720, Gilenya) Fingolimod is approved for the treatment of MS; it is a sphingosine-1-phosphate (S1P) receptor modulator, which blocks lymphocyte migration through sequestration of lymphocytes in the thymus and secondary lymphoid organs, leading to a marked lymphopenia. Fingolimod is a pro-drug; its phosphorylated form binds four out of the five S1P receptors (S1P-1, 3, 4, 5) and causes rapid internalization and subsequent degradation of the receptors. Among these four receptors, S1P-1 has been shown to be critically involved in lymphocyte trafficking. Beyond alteration of lymphocyte trafficking, fingolimod inhibits activation of DCs and macrophages as shown by reduced class II and CD86 surface expression and IL-12 production. Furthermore, fingolimod inhibits DC migration to the draining lymph nodes, thus suppressing T-cell priming and the overall immune response. Fingolimod decreased the TDAR to a neoantigen, but not a recall antigen in humans, indicating that immune memory function was not impaired (Boulton et al., 2012). Evidence of increased susceptibility to infections secondary to immunosuppression has been demonstrated in animal studies and humans have also shown an increased risk to some infections (e.g., herpes zoster), but immunosurveillance is mostly preserved with fingolimod treatment (Singer, 2014).

AIDS Therapeutics Traditionally, antiviral therapies have not been extremely successful in their attempt to rid the host of viral infection. This may be due to the fact that these organisms target the DNA of the host. Thus, eradication of the infection means killing infected cells, which for HIV are primarily $CD4^+$ T cells. Numerous strategies have been developed to combat HIV, including targeting viral reverse transcriptase (nucleoside and nonnucleoside reverse transcriptase inhibitors), viral protease, viral fusion and entry, virus–T-cell interaction proteins, and stimulating immune responses (reviewed by Broder [2010]). The multi-drug therapy used currently is referred to as HAART (highly active antiretroviral therapy) (reviewed by Este and Cihlar [2010]). However, eradication of this virus, and subsequently AIDS, remains a challenge because the very nature of the infection has significant immunosuppressive consequences. In addition, some of the current therapies also exhibit immunosuppressive actions. One such antiviral drug is zidovudine (Retrovir).

Zidovudine (3′-azido-3′-deoxythymidine) is a pyrimidine analog that inhibits viral reverse transcriptase. It was the first drug shown to have any clinical efficacy in the treatment of HIV-1 infection. Unfortunately, its use is limited by myelotoxicity (macrocytic anemia and granulocytopenia) (Luster et al., 1989). Early studies confirmed that the primary action of zidovudine is on innate immunity, although changes in both humoral immunity and CMI have also been observed (reviewed by Feola et al. [2006]). In addition, it was shown that oral administration of high doses of zidovudine causes thymic involution and decreased responsiveness of T cells to the HIV protein gp120 (McKallip et al., 1995). Clinically, zidovudine increases the number of circulating $CD4^+$ cells and can transiently stimulate cell-mediated immune responses (lymphoproliferation, NK cell activity, and IFN-γ production). A final consideration for the immunotoxicity associated with AIDS therapeutics such as zidovudine is that they are rarely administered alone and thus drug interactions likely contribute to various immune effects.

XENOBIOTIC-INDUCED HYPERSENSITIVITY AND AUTOIMMUNITY

When an individual's immune system responds in a manner producing tissue damage, it could result in hypersensitivity or autoimmunity, which could be exacerbated, or even induced by, a xenobiotic. Fig. 12-22 is a schematic delineating the possible cascade of effects that can occur when a chemical produces an immune-mediated disease.

Hypersensitivity

Polyisocyanates Polyisocyanates have a widespread use in industry and are responsible for occupationally related lung disease. These chemicals are used in the production of adhesives, paint hardeners, elastomers, and coatings. Occupational exposure is by inhalation and skin contact. Polyisocyanates are known to induce all four types of hypersensitivity responses.

Compounds in this group include toluene diisocyanate (TDI), methylene diphenyl diisocyanate (MDI), and hexamethylene diisocyanate (HDI). They are highly reactive compounds that readily conjugate with endogenous proteins, such as albumin, forming neoantigens responsible for hypersensitivity (Wisnewski et al., 2011). Pulmonary sensitization to these compounds can occur through either topical or inhalation exposure. In murine models employing intranasal or intratracheal sensitization and challenge with TDI, significant induction of Th2 cytokines, IgE, and eosinophilia has been demonstrated (Matheson et al., 2005; Plitnick et al., 2005; Ban et al., 2006; Johnson et al., 2007). Interestingly, although MDI is thought to be safer because it is less volatile, MDI and TDI both induce asthma-like symptoms, increase BALF neutrophils and eosinophils, and increase T cells and cytokine production in auricular lymph nodes in mice (Pollaris et al., 2016). Recently, Tregs were identified in TDI-mediated ear swelling. $CD4^+CD25^+$ Tregs were identified in the draining lymph nodes and the TDI-induced Tregs are more effective at suppressing target cell proliferation than Tregs induced with vehicle (Long et al., 2016). The exact role that Tregs play during TDI sensitization is not yet clear, although depletion of CD25 before TDI worsened the ear swelling and increased most immune endpoints, suggesting that Tregs likely temper the response to TDI (Long et al., 2016). A role for B cells was also recently identified in TDI-mediated ear swelling. B cells isolated from auricular lymph nodes following TDI sensitization are increased and express higher MHCII, CD80, and CD86, and produced IL-4, INF-γ, and IL-10 (De Vooght et al., 2013).

In humans diagnosed with occupational asthma associated with diisocyanate exposure, there is increased production of IL-8, MCP-1, TNF-α, and RANTES (chemokine ligand CCL5) in PBMC stimulated ex vivo with albumin-conjugated diisocyanate

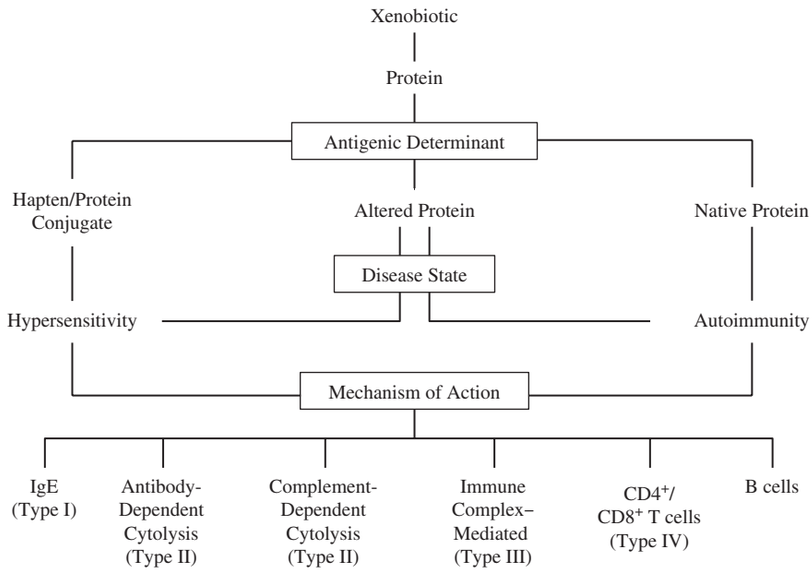

Figure 12-22. *Schematic diagram of xenobiotic induction of hypersensitivity or autoimmunity.* The mechanisms by which xenobiotics induce hypersensitivity or autoimmunity can overlap, although IgE production is most often associated with hypersensitivity.

(Lummus et al., 1998). Moreover, an examination of humans exposed to various diisocyanates demonstrated an association between incidence of occupational asthma from diisocyanate exposure and specific SNPs in genes encoding IL-4 receptor α, IL-13, CD14, TNF-α, TGF-β, PTGS1, PTGS2, and MHCII genes (Bernstein et al., 2006, 2011; Yucesoy et al., 2014, 2016). It has also been shown that diisocyanate-induced IgE is not as robust as that observed in allergic asthma, suggesting that distinct pathophysiological mechanisms control occupational versus allergic asthma (Bernstein et al., 2002).

Acid Anhydrides The acid anhydrides are reactive organic compounds used in the manufacturing of paints, varnishes, coating materials, adhesives, and casting and sealing materials. Trimellitic acid anhydride (TMA) is one of the most widely used compounds in this group and it causes all four hypersensitivity reactions. Similar to the diisocyanates, acid anhydrides bind to serum proteins, such as albumin (Valstar et al., 2006).

In an attempt to understand mechanisms by which TMA induced asthma following occupational exposure, a comparison between topical versus intranasal sensitization with TMA was conducted (Farraj et al., 2006). Intranasal challenge with TMA in mice that were either topically or intranasally sensitized with TMA produce a marked allergic rhinitis, of similar severity, characterized by an influx of eosinophils and lymphocytes. Both the topical and intranasal routes of sensitization also produce significant increases in total serum IgE after intranasal challenge with TMA. In addition, both the topical and intranasal routes of sensitization induce significant increases in the mRNA expression of the Th2 cytokines IL-4, IL-5, and IL-13 (Farraj et al., 2006). These findings are significant as they suggest that dermal exposure represents a potential route of sensitization of the respiratory tract to chemical allergens.

Toxicogenomic analyses have been conducted in an attempt to identify gene expression profiles for TMA-induced occupational asthma and/or contact dermatitis (Regal et al., 2007). Although overlap between genes induced in response to ovalbumin (allergic asthma model) or TMA (occupational asthma model) in mice occurred, over 100 unique TMA-induced genes were identified (Regal et al., 2007). Another study examined the gene expression profile in auricular lymph nodes following dermal exposure to TMA to determine changes in gene expression that correlated with the LLNA (Boverhof et al., 2009). Two additional studies have determined that Smad3 and Nrf2 are involved in TMA-induced inflammation using *Smad3*$^{-/-}$ and *Nrf2*$^{-/-}$ mice, respectively (Anthoni et al., 2010; El Ali et al., 2013).

Cellular assays are also being developed to identify respiratory sensitizers, particularly to determine if low dose exposures contribute to occupational asthma. For instance, in response to a low dose of intratracheal administration of TMA, IgE^{+} and MHCII^{+} B cells were identified in the lung-associated lymph nodes (Fukuyama et al., 2010). The increase in IgE^{+}MHCII^{+} B cells correlated with increased serum and BALF IgE and IgG1, and increased inflammatory cells in the BALF (eosinophils, neutrophils) (Fukuyama et al., 2010). At least two studies have identified a role for mast cells in TMA-mediated contact hypersensitivity (Chai and Song, 2015) and respiratory allergies (Nishino et al., 2016). An interesting human ex vivo model using precision cut lung slices from patients undergoing lung resection demonstrated that TMA induced robust levels of IL-1β at noncytotoxic concentrations (Lauenstein et al., 2014).

Metals Metals and metallic substances, including metallic salts and metal-containing nanomaterials, are responsible for producing contact and pulmonary hypersensitivity reactions. Metals typically produce a Type IV DTH response, which is mediated by metal-reactive T cells after initial sensitization. Sensitization to metals occurs mainly upon contact with skin (e.g., jewelry), contact with tissue (e.g., dental procedures or knee or hip prostheses), ingestion (metals in foods), or inhalation of metal dusts. Activation of PRRs has been suggested to play a role in sensitization, and may be the result of direct interaction between the metal and PRR, or metal-induced production of ROS or alarmins, or activation of the inflammasome (similar to nanomaterials as depicted in Fig. 12-23) (McKee and Fontenot, 2016). APC activation of

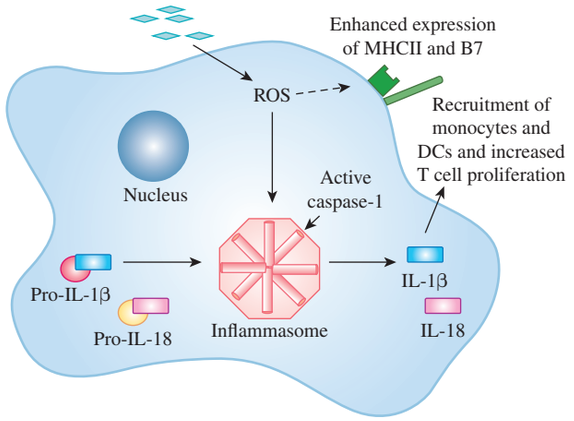

Figure 12-23. *Activation of the inflammasome by nanomaterials.* Nanomaterials can be adjuvants through effects in APCs. For example, in a macrophage, nanomaterials can induce reactive oxygen species (ROS) to enhance expression of MHC II or B7 proteins or activate the inflammasome. Upon activation, the inflammasome produces active IL-1β and IL-18, which can recruit other monocytes or DCs, or enhance T-cell proliferation.

T cells reactive to metals occurs by at least two mechanisms: metal ions become haptens by interacting with amino acid residues on MHC-bound peptides, or metal ions promote peptide-independent cross-linking (McKee and Fontenot, 2016). Hypersensitivity reactions to cobalt, chromium, nickel, and beryllium are summarized below.

Cobalt Cobalt exposure comes from metal-on-metal replacement prostheses, or occupationally in superalloy production and pigment manufacturing. Cobalt induces Type I and Type IV hypersensitivity reactions such as contact dermatitis, DTH in peri-implant areas in hip replacement patients, and occupational asthma (Thyssen and Menne, 2010).

Sensitization to cobalt involves activation of the inflammasome (Caicedo et al., 2009). There are several studies in which hip replacement patients who received metal-on-metal arthroplasty had increased metal-reactive T-cell responses. In one study, 7 out of 16 hip replacement patients demonstrated skin reactivity to cobalt, and of those 7 patients, 3 had elevated serum IgE (Thomas et al., 2009). Moreover, PBMC from 7 out of 16 patients proliferate ex vivo in response to cobalt chloride stimulation (Thomas et al., 2009). A more recent study examined leukocytes infiltrating a pseudotumor, a benign mass that forms near a failing prosthesis. Interestingly, macrophage- or T-cell-containing immune phenotypes are present. Those with the predominantly macrophage pseudotumors produce higher IL-6 and TNF-α, and also have higher blood cobalt and chromium levels (Paukkeri et al., 2016).

In workers exposed to hard metal dust (a cobalt-containing alloy), there was increased incidence of asthma and higher serum IgE levels (Shirakawa et al., 1992), indicating cobalt-induced occupational asthma. In a mouse model in which control or hypoxia inducible factor (HIF)-1α or HIF-2α is postnatally deleted in the lung, oropharyngeal aspiration of cobalt chloride induces a robust Th2-mediated asthma-like response characterized by eosinophilia, mucus cell metaplasia, and altered cytokine profile (Saini et al., 2010a, 2010b; Proper et al., 2014). These results suggest that hypoxia signaling might contribute to cobalt-induced occupational asthma.

Chromium Chromium is another metal in which exposure occurs either from metal-on-metal prosthesis, or occupationally in the paint, cement, and paper pulp production electroplating, leather tanning, industries. Chromium predominantly elicits contact dermatitis (Shelnutt et al., 2007), a Type IV hypersensitivity reaction, but can also induce hypersensitivity in patients with metal-on-metal hip arthroplasty, similar to those observed in response to cobalt (Thomas et al., 2009; Paukkeri et al., 2016). Chromium exists in several oxidation states, including chromium III or chromium VI, but chromium VI more readily permeates the skin barrier (Forte et al., 2008). However, once absorbed, chromium VI is reduced to chromium III, which is most likely responsible for chromium toxicity (Shelnutt et al., 2007). Upon the reduction of chromium VI to chromium III, ROS are produced. Production of ROS can activate the inflammasome, which for chromium is also dependent on potassium efflux (Adam et al., 2017).

Nickel Exposure to nickel occupationally occurs in the mining, milling, smelting, and refinishing industries. Consumers are exposed through clothing fasteners or body piercings, with adult women having the highest prevalence of nickel allergy (up to 17%) (Thyssen and Menne, 2010). Nickel causes both Type I and Type IV hypersensitivity. Similar to chromium, higher oxidation states of nickel have greater potential to sensitize individuals (Kasper-Sonnenberg et al., 2011), which could also be due to higher potential to generate ROS when reduced in vivo. Nickel has been shown to directly bind TLR4 (Schmidt et al., 2010; Raghavan et al., 2012), which likely contributes to sensitization.

Nickel sulfate treatment of human DC isolated from PBMC demonstrated robust induction of IL-12, which was due to nickel sulfate activation of NF-κB, p38 MAPK, and IFN regulatory factor-1 (IRF-1) (Antonios et al., 2010). Induction of IL-12 helps to stimulate T cells and, in fact, nickel-reactive T cells can be isolated from the PBMC of nickel-sensitized individuals (Moed et al., 2004). One possible source of IL-12 is dendritic cells and indeed nickel can induce $CD11c^{+}MHCII^{+}$ dendritic cells in the skin of ears isolated from nickel-treated mice (Watanabe et al., 2011). Nickel-treated dendritic cells express higher levels of thymic stromal lymphopoietin (TSLP) receptor, which interacts with TSLP on keratinocytes (Ashrin et al., 2014). The TSLP–TSLPR interaction is critical for nickel allergy as *Tslp* siRNA abrogates nickel-induced ear swelling (Ashrin et al., 2014).

Beryllium Beryllium exposure occurs most frequently in high-technology ceramics and dental alloy manufacturing, and in the electronics, nuclear, and aerospace industries. Although its use in the manufacturing of fluorescent bulbs has been discontinued, chronic beryllium disease (CBD) was originally identified in 1946 in a group of fluorescent lamp manufacturing workers (Forte et al., 2008). Beryllium produces Type IV hypersensitivity reactions. Skin contact has been found to produce lesions of contact hypersensitivity, whereas lesions produced by penetration of splinters of beryllium under the skin are granulomatous in nature. Inhalation of beryllium can result in acute beryllium disease (ABD; such as pneumonitis and tracheobronchitis), CBD, and increased risk of lung cancer (Cummings et al., 2009; McCleskey et al., 2009).

CBD is a pulmonary disease characterized by granulomas. It is well-established that a glutamic acid substitution in one of the MHCII molecules in humans is associated with increased risk of CBD following beryllium exposure (Richeldi et al., 1993) and has therefore been described as a classic gene-by-environment disease (Balmes et al., 2014). The granulomatous lesion is comprised of macrophages surrounded by a collar of T cells (Balmes et al., 2014). The mechanisms by which beryllium exhibits its adjuvant-like activity is not due to inflammasome activation, but does involve

IL-1-dependent neutrophil accumulation and IL-1-independent migration of DCs from the lung to lymph nodes (McKee et al., 2015). Finally, a role for Tregs has been suggested because severity of CBD in humans is inversely correlated with the presence of Tregs in the bronchoalveolar lavage fluid (Mack et al., 2014).

Latex Natural rubber latex is derived from the rubber tree *Hevea brasiliensis* and is used in the manufacture of over 40,000 products including examination and surgical gloves, among other medical products. Allergic reactions to natural rubber latex products have become an important occupational health concern with increased use of universal precautions, particularly latex gloves, to combat the spread of bloodborne pathogens. Hypersensitivity to latex usually occurs via a Type I or Type IV reaction. Dermatologic reactions to latex include irritant dermatitis due to chemical additives or mechanical abrasion and the occlusive conditions caused by wearing gloves; contact dermatitis due to the chemical additives used in the glove manufacturing (e.g., thiurams, carbamates, mercapto compounds, and phenylenediamines); and potentially more serious IgE-mediated responses due to residual latex proteins that remained in the finished products (reviewed by Shah and Chowdhury [2011]). The IgE responses may manifest as urticaria, asthma, or life-threatening anaphylaxis. At least 15 latex proteins have been identified (latex hevein proteins denoted as "hev") and antibodies to most can be detected in latex-allergic individuals (Cabanes et al., 2012; Vandenplas et al., 2016). In particular there is high prevalence of IgE reactivity to Hev b5 (Escobar et al., 2014). Natural rubber latex-specific T cells can also be detected in allergic individuals (Lehto et al., 2007; Escobar et al., 2014).

Food and Genetically Modified Organisms Awareness of hypersensitivity reactions to foods and genetically modified organisms or crops (GMOs) has increased in the last several years. The most common food allergens are milk, egg, peanuts, tree nuts, fish, shellfish, soy, and wheat. Peanut allergies are relatively common, and can be severe; thus, current information regarding the mechanism of peanut hypersensitivity is provided as an example. Hypersensitivity to peanuts occurs primarily via a Type I reaction and the IgE responses may include shortness of breath, asthma, and anaphylaxis. At least 12 peanut proteins have been identified and antibodies to most can be detected in peanut-allergic patients (reviewed by Bublin and Breiteneder [2014]). More recent studies have shown that peanut extract treatment of mouse or human plasma induced complement C3a, which could contribute to anaphylaxis (Khodoun et al., 2009; Kodama et al., 2013). In addition, peanut-reactive T cells have been isolated from the blood of peanut-allergic individuals (de Jong et al., 1996; DeLong et al., 2011; Wisniewski et al., 2015). A major breakthrough in peanut allergy prevention was made in the last several years as it was noted that the prevalence of peanut allergy was significantly lower in those who had consumed peanuts, regardless of whether they exhibited a positive skin prick test for peanut allergens, due to the development of oral tolerance (nonresponsiveness to peanut allergens following oral administration). Therefore, it is now recommended that high doses of age-appropriate peanut foods be given to infants, especially those without eczema or egg allergy (Togias et al., 2017).

Exposure to GMOs is becoming more widespread as biotechnological advances in food production are used, for example, to confer insect resistance, provide desired nutrients, or adapt to periods of drought. Allergenic determinants in GMOs result from the expression of novel proteins that might be recognized as nonself by the immune system. As an example, soybeans are representative of widely available genetically modified products. Of 15 putative soybean allergens, 8 have associated clinical data, including IgE binding and/or presentation of "allergic symptoms" after exposure (Ladics et al., 2014). However, there are several considerations in determining potential hypersensitivity to a GMO. First, it is important to note that many products, including soybeans, are potentially allergenic regardless of whether they are GM or non-GM. Second, standard methodology needs to be established, not only for detection of allergens in the product, but for allergenicity in workers or consumers (Ladics et al., 2014; Selb et al., 2017). Third, expression of proteins, whether natural or from the transgene, are influenced by environmental factors such as temperature, humidity, moisture, and nutrients (Stevenson et al., 2012). Fourth, an understanding of exposures is necessary. Some proteins, even those established as allergenic, are removed during processing so depending on the processing mechanism, exposures might be solely occupational or avoided entirely (Ladics et al., 2014).

Formaldehyde Formaldehyde is used as a preservative, sterilant, and fumigant. Additional exposures come from the textile industry, where it is used to improve wrinkle resistance, and in the furniture, auto upholstery, and resins industries. This low-molecular-weight compound is extremely soluble in water and forms haptens with human proteins easily (Maibach, 1983). Human predictive testing with 1% to 10% formalin (formalin is 37% formaldehyde) for induction and 1% formalin for challenge showed sensitization rates of 4.5% to 7.8% (Marzulli and Maibach, 1987), and Basketter et al. (2001) confirmed that formaldehyde is a contact sensitizer.

Occupational exposure to formaldehyde has been associated with the occurrence of asthma (Thompson and Grafstrom, 2008) and increased formaldehyde exposure has now also been associated with increased incidence of childhood asthma (McGwin et al., 2010). Formaldehyde can enhance the respiratory allergic response to other stimuli. For example, Sadakane et al. (2002) showed that formaldehyde exposure (0.5% mist once a week for 4 weeks) in ICR mice enhances the eosinophilic airway inflammation following the intratracheal instillation with a dust mite allergen. Fujimaki et al. (2004) showed that mice immunized with ovalbumin followed by exposure to formaldehyde (at the highest concentration of 2000 ppb for 12 weeks) increases the total number of BALF cells, macrophages, and eosinophils (Fujimaki et al., 2004). In response to a 2-week formaldehyde exposure in the absence of another stimulation, total IgE is increased in the serum of C57Bl/6 mice (Jung et al., 2007). Lung and liver IL-1β, IL-4, and IL-5 are also increased, and histopathology shows eosinophil infiltration, and inflammatory cell influx (Jung et al., 2007). Some of these results are confirmed using peripheral blood from formaldehyde-exposed workers, in which there is a higher proportion of $CD19^+$ B cells and increased serum IL-4 and IL-10 (Liu et al., 2014). A comprehensive evaluation of inhaled formaldehyde (2 ppb for 7 or 28 days) in rats demonstrated that formaldehyde produces changes in mRNA and miRNA expression in a tissue- and time-dependent manner (Rager et al., 2014). In particular, they demonstrated that cells in direct contact with formaldehyde exhibit more miRNA changes than those at distal sites (Rager et al., 2014), which could contribute to immune-mediated changes observed following formaldehyde exposure.

Inhaled Substances Pulmonary defenses against inhaled gases and particulates are dependent upon both physical and immunologic mechanisms. Immune mechanisms primarily involve the complex interactions between neutrophils and alveolar macrophages and their abilities to phagocytose foreign material and produce cytokines, which not only act as local inflammatory mediators, but also serve to attract other cells into the airways.

Oxidant Gases Exposure to oxidant gases, such as ozone (O_3), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), and phosgene, occurs

chronically over several days, most often in the summer months. Exposure can also come from ambient air in most buildings and in certain occupational settings. There are a few studies in which exposure to O_3 has been demonstrated to impair the phagocytic function of alveolar macrophages and to inhibit the clearance of bacteria from the lung (Gilmour et al., 1993a, 1993b), which could be attributed to an imbalance in Th1 and Th2 cytokines (Steerenberg et al., 1996, 2004). However, most reports demonstrate that ozone produces pulmonary inflammation and exacerbation of asthma. Specifically, three airway response phenotypes exist: pain-mediated nociceptive disease accompanied by chest discomfort; changes in airway reactivity (this is likely the most important mechanism for asthma exacerbation); and increases in airway inflammation (Peden, 2011). Interestingly, even in studies in which O_3 challenge produces a decrease in the number of macrophages, they exhibit evidence of activation and enhanced antigen-presenting capability as demonstrated by increased CD80, CD86, and HLA-DR (Lay et al., 2007). Alternatively, a decrease in percent of macrophages is due in part to the pronounced neutrophilia produced by O_3, either in normal volunteers or asthmatics (Hernandez et al., 2012). The allergic asthmatic volunteers challenged with O_3 are found to have increased levels of the inflammatory cytokines IL-1β and IL-6, while levels of the anti-inflammatory cytokine IL-10 are decreased in airway sputum (Hernandez et al., 2012). Enhanced immune responses were demonstrated in female mice in response to O_3 (Cabello et al., 2015), and similar effects were also suggested for human females, although more data are needed in humans (Stiegel et al., 2016).

Particles: Asbestos, Silica, and Nanoparticles Asbestos refers to a group of fibers that can be needle-like or serpentine. Likewise, silica can be crystalline or amorphous. Nanoparticles belong to a broader group of "nanomaterials," which are defined by the fact that they are less than 100 nm in size. Among these nanomaterials, nanosized silicas are members. Similar to asbestos and silica, exposure to nanoparticles can occur in the occupational setting. Nanoparticle exposure can also occur through environmental exposure to ultrafine particles, which are by-products of combustion engines and common constituents of urban air pollutants. Significant effort is currently being directed toward understanding the influence of shape, charge, composition, specific functional groups, catalytic activity, and other properties on the biological and toxicological potential of these nanomaterials (e.g., see Lee et al. [2013] and Kusaka et al. [2014]).

While there are reports that particles such as asbestos and silica produce immune suppression (Levy and Wheelock, 1975; Miller and Brown, 1985; Scheuchenzuber et al., 1985; Thurmond and Dean, 1988; Warheit and Hesterberg, 1994), most reports demonstrate marked inflammation (and even autoimmune disease) in response to asbestos, silica, and various nanoparticles. Specifically, asbestos and silica can produce pulmonary fibrosis known as asbestosis and silicosis. A common mechanism involved in inflammation induced by asbestos, silica, and nanoparticles is activation of the inflammasome (Sayan and Mossman, 2016) (Fig. 12-23). Specifically, caspase-1 is activated, which produces the mature forms of IL-1β and IL-18. While the production of pro-inflammatory mediators can contribute to an appropriate environment to sustain inflammation, some cells undergo cell death via apoptosis or pyroptosis, which might account for some of the reported immune suppression in response to particles.

Over the last few years, various studies have identified specific mechanisms involved in silica-induced pulmonary inflammation. In an overview of silica effects, it is demonstrated that silica-induced inflammation and fibrosis involve distinct signaling pathways: inflammation is dependent on IFN-γ while fibrosis is dependent on Th2 cytokines such as IL-4 and IL-13 (Pollard, 2016). Recently, a scavenger receptor (SR-B1) for silica has been identified (Tsugita et al., 2017). SR-B1 is reported to tether silica and activate the inflammasome, but SR-B1 is not involved in internalization of the silica particles. The authors suggested other integrin or tetraspanin proteins might be involved in internalization. In addition, silica recognition by SR-B1 is shown on mouse macrophages and HPB monocytes, but not on neutrophils (Tsugita et al., 2017).

ROS production is likely involved in the inflammatory state induced by any particle, including asbestos. Due to the size of asbestos particles, macrophages typically cannot ingest them. This produces "frustrated phagocytosis," characterized by sustained production of ROS following additional phagocytosis attempts. One recent study demonstrated that asbestos-induced oxidation of thioredoxin is involved in inflammasome activation (at least in a human mesothelial cell line) (Thompson et al., 2014). Another study demonstrated that nanoparticle or asbestos-induced inflammasome activation in human monocyte–derived macrophages could be blocked when the transmembrane ionotropic receptor P2X purinoreceptor 7 (P2X7 receptor) was antagonized with a chemical inhibitor or knocked down with siRNA (Palomaki et al., 2011).

Another concern with exposure to nanoparticles is the possibility they can enhance immune responses to airborne antigens due to their ability to induce pulmonary inflammation. Indeed, several recent studies have demonstrated with a number of different nanoparticles, including multi-walled carbon nanotubes and titanium oxide, that when administered to experimental animals in combination with ovalbumin, the inflammatory and immunological responses, as measured by cytokine production, cellular infiltrate, and ovalbumin specific antibodies, were significantly increased when compared to ovalbumin alone (Ryman-Rasmussen et al., 2009; Larsen et al., 2010).

Therapeutic Drugs Hypersensitivity responses to therapeutic drugs are among the major types of unpredictable drug reactions, accounting for up to 10% of all adverse effects. Drugs that commonly induce hypersensitivity include sulfa drugs, barbiturates, anticonvulsants, insulin, iodine (used in many x-ray contrast dyes), and platinum-containing chemotherapeutics. Penicillin and abacavir, drugs with different mechanisms of hypersensitivity, are discussed below.

Penicillin Penicillin is the most common drug involved in drug allergy. The high incidence of allergic reaction to penicillin is in part due to widespread exposure to the compound. Not only has there been indiscriminant use of the drug, but exposure occurs through food products, including milk from treated animals, and the use of penicillin as an antimicrobial in the production of vaccines. Efforts are still being made to reduce unnecessary exposure. The mechanism by which hypersensitivity to penicillin occurs is through the formation of a neoantigen. The formation of the primary penicillin neoantigen occurs during the breakdown of penicillin, in which the β-lactam ring opens, forming a reactive intermediate that reacts with other proteins. The resultant penicilloylated protein now acts as a hapten to which the immune system mounts a response. As is the case with other haptens, subsequent exposures to penicillin may not absolutely require the formation of penicilloylated proteins to elicit secondary responses. Reactions to penicillin are varied and may include any of the four types of hypersensitivity reactions (reviewed by Chang et al. [2012]). The most commonly seen clinical manifestation of Type I reactions is urticaria; however, anaphylactic reactions occur in about 10 to 40 of every 100,000 patients receiving injections. Clinical signs of

rhinitis and asthma are much less frequently observed. Blood dyscrasias can occur due to the production of IgG against penicillin metabolites bound to the surface of red blood cells (Type II reaction). Penicillin has also been implicated in Type III reactions leading to serum-sickness-like symptoms. Owing to the high frequency of Type IV reactions when penicillin is applied topically, especially to inflamed or abraded skin, penicillin products are no longer available for topical application. Type IV reactions generally result in an eczematous skin reaction, but a rare, life-threatening form of dermal necrosis may result. In these cases there is severe erythema and a separation of the epidermis at the basal layer. This reaction, which gives the clinical appearance of severe scalding, is thought to be a severe delayed reaction.

Abacavir Abacavir is a purine analog; it is an inhibitor of viral reverse transcriptase that has less adverse effects than zidovudine described above. However, in contrast to the immunosuppressive adverse effects of zidovudine, abacavir induces hypersensitivity reactions in approximately 5% to 9% of patients initiating antiretroviral therapy with abacavir. This adverse effect has been associated with the expression of the HLA haplotype HLA-B*5701 and elevated $CD8^+$ CTL at the initiation of abacavir treatment, as well as an increase in $CD8^+$ (but not $CD4^+$) T-cell proliferation in patients testing positive for abacavir hypersensitivity (Easterbrook et al., 2003; Phillips et al., 2005). Based on these studies, a large study (1956 patients) was conducted to determine the effectiveness of prospective HLA-B*5701 screening in preventing hypersensitivity reactions to abacavir (Mallal et al., 2008). Screening eliminated hypersensitivity reactions to abacavir and the subsequent introduction of screening prior to therapy has made a hypersensitivity reaction to abacavir a rare event. The mechanism underlying abacavir hypersensitivity syndrome has been determined to be due to the ability of abacavir to bind noncovalently with high specificity to the HLA-B*5701, changing the shape and chemistry of the antigen-binding cleft and thereby altering the repertoire of presented endogenous peptides that can bind. These altered self-peptides lead to the activation of abacavir-specific CTLs, which produce a systemic reaction known as abacavir hypersensitivity syndrome (Illing et al., 2012).

Autoimmunity

There are numerous reports of xenobiotics that have been associated with autoimmunity; however, firm evidence for their involvement is difficult to obtain. Presently there are very few instances of human autoimmune diseases for which an environmental trigger has been definitely identified (Rose, 2005). These relationships may be causative through direct mechanisms, or they may be indirect, acting as an adjuvant. In the area of xenobiotic-induced autoimmunity, exact mechanisms of action are not always known. Chemical exposure may also serve to exacerbate a preexisting autoimmune state (Coleman and Sim, 1994; Kilburn and Warshaw, 1994). Table 12-14 lists chemicals known to be associated with autoimmunity and identifies the proposed self-antigenic determinant for each chemical except for silica, which most likely acts as an adjuvant. The list includes both drug and nondrug chemicals. The heterogeneity of these structures and biological activities illustrate the breadth of potential for the induction of chemically mediated autoimmune disease. A number of papers have reviewed some specific examples of xenobiotics associated with autoimmune disease (Pollard et al., 2010), including drugs/immunotherapeutics (D'Cruz, 2000; Vial et al., 2002; Pichler, 2003), vaccines (Descotes et al., 2002; Vial and Descotes, 2004), environmental chemicals (D'Cruz, 2000; Hess, 2002), and pesticides (Holsapple, 2002a; Parks et al., 2011). Additionally, the National Institute of Environmental Health Sciences formed an interdisciplinary expert panel of scientists to conduct an extensive review of the literature regarding environmental exposures and autoimmunity. The findings of the expert panel are reviewed in several publications (Germolec et al., 2012; Miller et al., 2012; Selmi et al., 2012; Parks et al., 2014). Regarding mechanisms of autoimmunity, the review panel evaluated the likelihood of specific mechanistic paradigms in mediating environmental influences on human autoimmunity. The panel was confident that xenobiotics could influence the development of autoimmunity through the following mechanisms: (1) inducing innate immunity (i.e., TLR activation or adjuvant effects), (2) altered B-cell tolerance and increased B-cell activation, (3) increased development and activation of Th17 cells, (4) decreased development or activation of Tregs, (5) modification of self-antigens, and (6) modification of DNA methylation (reviewed by Selmi et al. [2012] and Parks et al. [2014]). Below is a brief discussion of selected drug and nondrug chemicals.

Vinyl Chloride and Organic Solvents Vinyl chloride, which is used in the plastics industry as a refrigerant and in the synthesis of organic chemicals, is a known carcinogen and is also associated with a scleroderma-like syndrome. The disease affects multisystemic collagenous tissues, manifesting itself as pulmonary

Table 12-14

Chemical Agents Known to be Associated with Autoimmunity

PROPOSED ANTIGENIC CHEMICAL	CLINICAL MANIFESTATIONS	DETERMINANT	ORIGINAL REFERENCE
Drugs			
Methyl dopa	Hemolytic anemia	Rhesus antigens	Murphy and Kelton (1991)
Hydralazine	SLE-like syndrome	Myeloperoxidase	Cambridge et al. (1994)
Isoniazid	SLE-like syndrome	Proteins targeted by myeloperoxidase metabolite of isoniazid	Jiang et al. (1994)
Procainamide	SLE-like syndrome	DNA	Totoritis et al. (1988)
Halothane	Autoimmune hepatitis	Liver microsomal proteins	Kenna et al. (1987)
Nondrug chemicals			
Vinyl chloride	Scleroderma-like syndrome	Abnormal protein synthesized in liver	Ward et al. (1976)
Mercury	Glomerular neuropathy	Glomerular basement membrane protein	Pelletier et al. (1994)
Silica	Scleroderma	Most likely acts as an adjuvant	Pernis and Paronetto (1962)

fibrosis, skin sclerosis, and/or fibrosis of the liver and spleen. Ward et al. (1976) reported on 320 exposed workers, showing that 58 (18%) had a scleroderma-like syndrome (Ward et al., 1976). The individuals who showed the disease were in a group genetically similar (i.e., HLA-DR5) to patients with classic idiopathic scleroderma. Although the exact mechanism whereby this chemical produces autoimmunity is unclear, it is presumed that vinyl chloride acts as an amino acid and is incorporated into protein. Because this would produce a structurally abnormal protein, which would be antigenic, an immune response would be directed against tissues with the modified protein present.

Other occupational exposures suspected to induce scleroderma-like and other autoimmune reactions include solvents, particularly organic solvents. Organic solvents may lead to autoimmune reactions by inducing oxidative stress and binding to proteins to generate neoantigens. For example, several epidemiological studies found an increased relative risk for scleroderma-like reactions following exposure to solvents when compared to the general population. However, the association was weak and not reproduced in other studies, and these studies frequently assessed exposure to solvents in general without providing details on specific solvents (Garabrant and Dumas, 2000; Garabrant et al., 2003). However, later literature reviews and meta-analysis of organic solvents as a group or trichloroethylene alone identified an increased risk for autoimmune disease in exposed populations and a consistent association between organic solvents and autoimmune-related effects in animal models (Cooper et al., 2009; Barragan-Martinez et al., 2012).

Mercury This widely used metal is known to have several target systems, including the CNS and renal system. Mercury also has two different actions with respect to the immune system. The first action is direct injury, described previously in the section "Immune Modulation by Xenobiotics: Metals." The second action is mercury-induced autoimmunity, which has been extensively reviewed and the highlights of these reviews are summarized below (Schiraldi and Monestier, 2009; Crowe et al., 2017). Although all forms of mercury can induce autoimmunity in genetically susceptible animals, only elemental and inorganic mercury, but not methylmercury, induce immune complex formation and glomerular nephropathy. Antibodies produced to laminin are believed to be responsible for damage to the basement membrane of the kidney. Mice and rats exposed to mercury also show antinuclear antibodies. The role of these antibodies in the autoimmune disease is not clear; however, they represent a known biomarker of autoimmunity. Studies in the brown Norway rat point to a mercury-induced autoreactive $CD4^+$ cell as being responsible for the polyclonal antibody response. Mercury chloride induces an increase in the expression of MHCII molecules on B cells as well as shifting the T helper cell population toward Th2. It is the Th2 cell that promotes antibody production. The imbalance between Th1 and Th2 cells may be caused by the depletion of cysteine and the reduced form of glutathione in Th1 cells. Reduced glutathione is known to be important in the synthesis of and responsiveness to IL-2 in T cells. Thus, Th1 cells that synthesize and respond to IL-2 would be at a greater risk than Th2 cells. Additionally, mercury-induced autoimmunity has a strong genetic component. This has been extensively studied in the rat and mouse. For example some strains of rats, such as the Lewis rat, are completely resistant, while others, such as the brown Norway rat, are exquisitely sensitive. Susceptibility appears to be linked to three or four genes, one of which is MHC. Furthermore, mercury treatment has been shown to exacerbate disease severity in a coxsackievirus-induced autoimmune myocarditis mouse model perhaps by inducing inflammation and acting as an adjuvant (Penta et al., 2015) as well as promoting the loss of self-tolerance (i.e., increased phosphorylation of signaling proteins and presentation of self-antigens in NETs) (see "Immune Modulation by Xenobiotics: Metals").

Silica Crystalline silica (silicon dioxide) is a primary source of elemental silicon and is used commercially in large quantities as a constituent of building materials, ceramics, concretes, and glasses. Experimental animals as well as humans exposed to silica may have perturbations in the immune system. Depending on the length of exposure, dose, and route of administration of silica, it may kill macrophages or may act as an immunostimulant. Silica has been shown to be associated with an increase in scleroderma in silica-exposed workers (Kilburn and Warshaw, 1994). A meta-analysis of occupational silica exposures identified an increased risk of developing scleroderma in silica-exposed males (McCormic et al., 2010). This effect may be mediated, at least in part, via an adjuvant mechanism (Parks et al., 2014). Adjuvancy as a mechanism of causing autoimmunity has been implicated with a number of other chemicals, including paraffin and silicones.

Brown et al. (2004) developed a model in which apoptosis plays a critical role in silica-induced autoimmune diseases. As described by the authors, inhalation of crystalline silica results in concurrent activation and apoptosis of the alveolar macrophage resulting in an environment of inflammation and apoptosis (Brown et al., 2004). This environment may provide excess self-antigen that is further ingested by activated macrophages or DCs, which are able to migrate to local lymph nodes. Within these local lymph nodes, these APCs, laden with apoptotic material, activate T cells and B cells thereby inducing an autoimmune response.

Hexachlorobenzene As noted above, autoimmunity induced by pesticides has been previously reviewed (Holsapple, 2002a). There is little doubt that the pesticide most extensively studied in the context of autoimmunity is hexachlorobenzene. Hexachlorobenzene is a low-molecular-weight compound that was used in the past as a fungicide for seed grains. Even though its use as a pesticide was prohibited in most countries in the 1970s, it is still generated as a by-product of several industrial processes and trace amounts of hexachlorobenzene are present as contaminants in some chlorine-containing pesticides. Although emissions of hexachlorobenzene have decreased dramatically compared to the 1970s, residues can still be found throughout the environment due to its stability and persistence.

One of the drivers for including hexachlorobenzene in this brief presentation of xenobiotics associated with autoimmune disease is based on an accidental poisoning incident that occurred in Turkey during 1955 to 1959. Approximately 3000 to 5000 people ingested seed grain contaminated with hexachlorobenzene. Patients developed a disease characterized by hepatic porphyria, called *porphyria turcica*, which was manifested as bullous skin lesions, mainly in sun-exposed skin that ultimately healed with severe scars. The skin lesions have been attributed to the phototoxicity associated with the elevated levels of porphyrins. In addition to the dermatological changes, other clinical manifestations included neurologic symptoms, hepatomegaly, enlarged thyroid, splenomegaly, hyperpigmentation, hirsutism, enlarged lymph nodes, and painful arthritis of the hands. For many of the clinical symptoms exhibited by the victims, an immune etiology was considered.

Indeed, the autoimmunogenic potential of hexachlorobenzene has been characterized in a number of laboratory studies, which have been reviewed (Michielsen et al., 1999; Ezendam et al., 2004). The former review emphasized a striking difference in the profile of

activity in rats, where the predominant effect was immune enhancement, and in mice, where the predominant effect was immune suppression (Michielsen et al., 1999). Only the former results will be discussed further in this section. Exposure to a variety of strains of rats produced increases in the following types of parameters: peripheral blood counts, serum IgM and IgG levels, autoantibodies, spleen and lymph node weights, marginal zones and follicles of spleens, and primary and secondary antibody responses to tetanus toxoid. Interestingly, exposure to hexachlorobenzene causes opposite effects on two induced autoimmune models in Lewis rats, causing an increase in the severity of experimental allergic encephalomyelitis, and a decrease in the severity of adjuvant arthritis (Michielsen et al., 1999). These findings suggest that comparative studies using different genetically autoimmune-prone models may be needed to investigate the role of xenobiotics in the onset and progression of autoimmunity.

Concerning a possible mechanism of action, Ezendam et al. (2005a, 2005b) proposed that after exposure to hexachlorobenzene, its deposition can directly induce cell damage or elicit damage by interfering with the integrity of cell membranes due to its lipophilic nature. Ultimately, hexachlorobenzene exposure triggers pro-inflammatory mediators, such as TNF-α, IL-1, IL-6, ROS, and chemokines. These pro-inflammatory mediators serve as adjuvant signals that induce a systemic inflammatory response with influxes of neutrophils and macrophages into various nonimmune and immune organs. Subsequently, this leads to polyclonal activation of T and B cells, eosinophilia, and eventually to visible clinical effects.

Therapeutic Drugs

Halothane Halothane, one of the most widely studied of the drugs inducing autoimmunity, was widely used as an inhalation anesthetic. Though used in developing countries, it is not used in the United States because of its side effects, such as autoimmune hepatitis, and the availability of safer alternatives (Reichle and Conzen, 2003). The incidence of this iatrogenic disease in humans is about 1 in 20,000. The pathogenesis of the hepatitis results from the chemical altering a specific liver protein to such a degree that the immune system recognizes the altered protein and antibodies are produced. Studies using rat microsomes show that halothane has to be oxidized by cytochrome P-450 enzymes to trifluoroacetylhalide before it binds to protein. Investigations indicate that in affected individuals antibodies to specific microsomal proteins are produced.

Methyldopa Methyldopa is a centrally acting sympatholytic drug that has been widely used for the treatment of essential hypertension; but with the advent of newer antihypertensive drugs, the use of methyldopa has declined. Platelets and erythrocytes are targeted by the immune system in individuals treated with this drug (Garratty, 2010). In the case of thrombocytopenia, antibodies are detected against platelets, which are indicative of immune recognition of a self-antigen or altered self-antigen. Hemolytic anemia occurs in at least 1% of individuals treated with methyldopa, and up to 30% of these individuals develop antibodies to erythrocytes as manifest in a positive Coombs test. Interestingly, the antibodies are not directed against the chemical or a chemical–membrane conjugate. Methyldopa, along with several other drugs or chemicals including hydralazine and trichloroethylene, is also associated with drug-induced liver injury with features of autoimmunity that appears to be mediated by metabolism into reactive metabolites that react with liver proteins (Gilbert, 2010; deLemos et al., 2014; de Boer et al., 2017).

Hydralazine, Isoniazid, and Procainamide Hydralazine is a direct-acting vasodilator drug used in the treatment of hypertension. Isoniazid is an antimicrobial drug used in the treatment of tuberculosis. Procainamide is a drug that selectively blocks sodium channels in myocardial membranes, making it useful in the treatment of cardiac arrhythmias. All three drugs produce autoimmunity, which is manifested as a systemic lupus erythematosus (SLE)-like syndrome. Notably, there is a long list of drugs associated with inducing SLE-like syndromes (Xiao and Chang, 2014). Procainamide represents one of the best examples of a clear association between exposure to a xenobiotic and the onset or progression of an autoimmune disease. The association between procainamide and the SLE-like condition is based on the finding that the disease remits when the drug is discontinued and recurs when the drug is readministered. Antibodies to DNA have been detected in individuals showing this syndrome. Studies with hydralazine indicate that an antigenic determinant is myeloperoxidase perhaps due to hydralazine-induced neutrophil cytotoxicity (Yokogawa and Vivino, 2009). Isoniazid and its reactive metabolites may induce liver injury by binding liver proteins and inducing an immune response (Wang et al., 2016). Although the actual mechanism by which these drugs induce autoimmunity requires further elucidation, studies suggest a breakdown of central tolerance to low-affinity self-antigens in the thymus and altered peripheral tolerance due to inhibition of DNA methylation (reviewed by Chang and Gershwin [2010]). In addition, these drugs have also been shown to produce hypersensitivity responses not associated with the SLE syndrome.

IMMUNOTOXICITY OF BIOLOGICS

Biologics refers to those therapies that are derived in some manner from living organisms and include monoclonal antibodies, recombinant proteins, and adoptive cell therapies. By its very nature, the immune system is often both the intended therapeutic target and unintended toxicologic target of various biologics. Overall, manifestations of toxicity may include exaggerated pharmacology, effects due to biochemical cross talk, and disruptions in immune regulation by cytokine networks. Many biologics are intended to treat indications such as cancer, inflammatory/autoimmune diseases, or transplantation and thus are designed to interact with and modulate the immune system. Therefore, they have the potential to induce immune-mediated toxicities due to immunosuppression that can lead to infections or malignancies or adverse immune stimulation that can cause infusion reactions, cytokine storms, or autoimmunity. In addition, due to their large size and/or foreignness to the host, biologics have the potential to be immunogenic and elicit an immune response to itself that can cause toxicities associated with hypersensitivity reactions or the neutralization of an endogenous protein due to cross-reactive antibodies. The adverse consequences of immunogenicity to therapeutic protein have been recently reviewed (Leach et al., 2014; Krishna and Nadler, 2016).

Many monoclonal antibodies have been designed in general to alter immune function, and include antibodies directed against certain molecules that are critical for inducing or sustaining an immune response and can be divided into the following mechanistic categories: (1) bind and neutralize specific cytokines (TNF-α, IL-6); (2) bind cell-surface molecules to trigger lysis by the adaptive immune response (CD3 on T cells; CD25, α subunit of IL-2 receptor on activated T cells; CD20 on mature B cells; CD52 on B cells, T cells, NK cells, monocytes, macrophages); (3) bind cell-surface molecules

and block costimulation signal from other cells (CD2 on T cells; CD80/CD86 on APCs, soluble BLyS/BAFF, CD40L on T cells); (4) bind cell-surface molecules and stimulate costimulatory receptors or block inhibitory receptors from other cells (CTLA4, PD1, CD40 on T cells and PDL1, CD137 on APCs); and (5) bind cell-adhesion molecules and block lymphocyte trafficking (LFA-1 or α4 on leukocytes).

While certainly many biologics are being utilized safely, the immunotoxicological aspects of TNF inhibitors, monoclonal antibodies (anti-CD3, anti-α4 integrin, and anti-CD40L), immune checkpoint inhibitors, and a recombinant protein (IFN-α) will be discussed. A summary of the mechanisms by which these biologics alter immunity, including a comparison between small molecule therapeutics and biologics, is provided in Table 12-15.

There are currently five TNF-α inhibitors approved to treat an array of autoimmune diseases: etanercept (Enbrel), a p75 TNF-receptor type II fused to the Fc region of IgG1 that binds both TNF-α and TNF-β; infliximab (Remicade), a chimeric (mouse/human) anti-TNF-α IgG4 mAb; adalimumab (Humira), a human anti-TNF-α IgG1 mAb; certolizumab pegol (Cimzia), a PEGylated anti-TNF-α antibody fragment; and golimumab (Simponi), a human anti-TNF-α IgG1 mAb. These agents block TNF to prevent (1) induction of other pro-inflammatory cytokines such as IL-1, IL-6, and IL-8; (2) macrophage and neutrophil activation; (3) fibroblast and endothelial activation; (4) induction of other acute-phase proteins; and (5) leukocyte migration to inflammation sites. TNF-α plays an essential role in host defense against tuberculosis (TB) and other granulomatous diseases, including protective granuloma formation and containment of disease. Consequently, patients treated chronically with anti-TNF-α biologics are at an increased risk for infection with *M. tuberculosis*. In addition, infections with *L. monocytogenes*, *Salmonella* and other facultative intracellular pathogens, and other rare opportunistic pathogens such as *P. carinii* have been reported. A meta-analysis of the incidence of malignancies in patients with RA treated with TNF-blocking drugs indicate that the most common tumor type was lymphoma. In this analysis, malignancies were more common in patients treated with, as compared with patients not treated with, TNF-blocking drugs (Bongartz et al., 2006). However, it is difficult to determine whether the increased incidence is related to the specific therapy or confounded by other factors, such as the severity of the autoimmune disease that may confer an intrinsically higher risk of lymphoma in those patients preferentially receiving TNF-blocking drugs (Thomas et al., 2000). Autoimmune-like symptoms have also been related to TNF-α blockers including the induction of antinuclear antibodies (ANAs) and anti-double-stranded DNA (anti-dsDNA) antibodies. Although ANAs are common among patients with autoimmunity, the prevalence significantly increases among those treated with TNF-α inhibitors. In addition, anti-dsDNA antibodies develop in patients treated with TNF-blocking drugs, which are not seen in TNF-naive patients

Table 12-15

Immunomodulatory Therapeutics and Biologics

AGENT	EFFECT	PRIMARY MECHANISM OF ACTION
Therapeutics		
NSAIDs	Anti-inflammatory	COX-1 and/or COX-2 inhibitor
Glucocorticoids	Anti-inflammatory	Phospholipase A2 inhibitor
	Immune suppressant	Induction of IκB (NF-κB inhibitor)
Cyclophosphamide	Immune suppressant	Inhibition of cell replication
Azathioprine		
Lefluonomide		
Cyclosporin A	Immune suppressant	Calcineurin and NFAT inhibitor
FK506		
Rapamycin	Immune suppressant	mTOR signaling inhibitor
Tofacitinib	Immune suppressant	Janus kinase inhibitor
Fingolimod	Immune suppressant	Sphingosine-1-phosphate modulator
Biologics		
Etanercept	Anti-inflammatory	TNF-α and TNF-β inhibitor
	Immune suppressant	
Infliximab	Anti-inflammatory	TNF-α inhibitor
Adalimumab	Immune suppressant	
Certolizumab		
Golimumab		
Muromonab	Immune suppressant	T-cell depletion following antibody opsonization
Natalizumab	Immune suppressant	α4 integrin inhibitor
Ipilimumab	Immune stimulant	CTLA-4 inhibitor
Nivolumab	Immune stimulant	PD1 or PDL-1 inhibitor
Pembrolizumab		
Atezolizumab		
Recombinant cytokines (IFN-α/IFN-γ/GM-CSF/IL-2/IL-12)	Immune stimulant	Similar mechanisms to cytokines as listed in Table 12-4

(De Rycke et al., 2005). Clinical sequelae that accompany the presence of these autoantibodies during TNF-α therapy are broad and varied, and despite the high prevalence of autoantibodies in patients who received TNF-α inhibitors, the frequency of patients developing systemic disease is relatively low.

Monoclonal antibodies (mAbs) directed against CD3 (muromonab-CD3, OKT3), part of the TCR complex, have been used for acute transplant rejection. All T cells express CD3 and binding by anti-CD3 Ig opsonizes T cells for depletion by complement activation and immune clearance. Because CD3 is part of the TCR signaling complex, binding of OKT3 to CD3 can acutely induce a "cytokine release syndrome" due to a transient activation and release of cytokines, soon after initial administration, which can lead to flu-like symptoms, pulmonary edema, and hematological disorders (reviewed by Sgro [1995]). Additionally, OKT3 is a murine monoclonal antibody; therefore, the therapeutic efficacy and duration of action may be decreased due to host recognition and clearance by antidrug antibodies. The establishment of immunological memory will lead to more rapid clearance on subsequent administration.

Natalizumab (Tysabri) is an anti-α4 integrin monoclonal antibody that inhibits cell trafficking into sites of inflammation and is used for the treatment of MS and Crohn's Disease. Natalizumab has been found to increase the risk of progressive multifocal leukoencephalopathy (PML), a rare but serious demyelinating disease of the brain due to infection of oligodendrocytes by JC virus, a human polyomavirus. (Berger and Koralnik, 2005; Berger, 2006). Consequently, natalizumab was initially withdrawn from the market, but subsequently reintroduced as a monotherapy available only through a special restricted distribution program. Despite a high seroprevalence in macaques of SV40, the simian counterpart to JC virus, and its association with PML in the context of simian immunodeficiency virus-induced immunosuppression, PML was not observed in preclinical studies of natalizumab in cynomolgus macaques (Sasseville and Diters, 2008). Other types of infections are uncommon with natalizumab treatment, suggesting a unique interaction between JC virus and natalizumab's mechanism of action (i.e., inhibition of lymphocyte trafficking and impacting the ability of virus-clearing T cells to home to the brain) (Stuve et al., 2006).

CD40 ligand (CD40L) blockade has demonstrated efficacy in experimental autoimmune and transplantation models. However, thromboembolism (TE), in the clinic and/or NHPs, has been reported for at least three anti-CD40L mAbs that were either in preclinical or clinical development (Hu5c8, IDEC131, and ABI1793). Hu5c8 caused TE in a clinical trial for lupus and IDEC131 caused TE in one patient in a Crohn's Disease trial, leading to termination of ongoing trials (Elgueta et al., 2009). Both hu5c8 and ABI1793 caused TE/thrombosis in renal transplantation studies in cynomolgus or rhesus monkeys (Kanmaz et al., 2004). TE by these antibodies is thought to result from the formation of higher-order immune complexes of the mAbs with membrane-bound CD40L on platelets or soluble CD40L shed from platelets that can ligate and thereby aggregate neighboring platelets via immune complex binding to FcγRIIα, an IgG Fc receptor, causing activation and resulting in thrombi formation (Mirabet et al., 2008). Therefore, anti-CD40L antibodies are being developed with an absent or inert Fc to mitigate this toxicity (Shock et al., 2015).

Several mAb that work by stimulating the immune response to tumors have been recently approved for the treatment of cancer. These drugs target the immune checkpoints CTLA4 (ipilimumab) and PD1/PDL-1 (nivolumab, pembrolizumab, and atezolizumab) (Topalian et al., 2015; Hoos, 2016). Immune checkpoints are off switches that regulate T-cell immune responses and protect the body from damaging immune responses. Tumor-specific T cells are one of the major arms for the immune system to combat tumor progression; thus, by blocking the negative regulatory signal, anti-tumor T-cell responses can be potentially augmented. While CTLA-4 serves to regulate early T-cell activation, PD-1 signaling functions in part to regulate T-cell activation in peripheral tissues. While breaking tolerance to the cancer, these drugs can also lead to immune-related adverse events (IRAEs) characterized by eliciting autoimmunity and inflammation such as dermatitis, colitis, hypophysitis, uveitis, pneumonitis, and hepatitis. Fortunately, these toxicities can generally be managed with steroids when identified early (Naidoo et al., 2014).

The majority of recombinant proteins have been used as immunostimulants, including IFN-α, IFN-γ, GM-CSF, IL-2, and IL-12. Recombinant proteins used as immunosuppressants include kineret, an IL-1 receptor antagonist (IL-1RA) and the earlier-mentioned etanercept, which is a hybrid of the extracellular ligand-binding portion of TNFR2 fused to the Fc portion of human IgG1. IFN-α is used to treat hepatitis C and other chronic viral illnesses as well as cancer. The mechanism of the antiviral action of IFN-α involves, in part, direct suppression of viral replication, activation of NK cells, and enhanced expression of MHCI on virally infected cells, thus increasing the likelihood of recognition by virus-specific T cells. Administration of IFN-α has been associated with adverse reactions such as flu-like symptoms, leukopenia, and thrombocytopenia stemming from bone marrow suppression and autoimmune diseases, including autoimmune hypothyroidism and lupus (Vial and Descotes, 1995).

NEW FRONTIERS AND CHALLENGES IN IMMUNOTOXICOLOGY

As noted throughout this chapter, the immune system has unquestionably been identified as a potential target organ for drugs and other chemicals. With the demonstration that (1) chemicals can perturb the immune system of animals, (2) perturbation of immune function is correlated with an increased risk of infectious disease, and (3) perturbations in immune function can occur in the absence of any clinically observable effect, attention has focused, and will continue to focus, on the risk to the human population following exposure to chemicals that can alter immune function in animals. In fact, the characterization of the risk associated with xenobiotic-induced immunotoxicity arguably represents one of the key challenges for this discipline in the immediate future.

Risk can be defined as the probability that an adverse event/effect will manifest itself. Risk must also incorporate the hazard, including dose–response relationships, and exposure. Exposure is a function of the amount of chemical involved and the time of its interaction with people and/or the environment. As such, assessment of risk is often an assessment of the probability for exposure. However, most papers in the immunotoxicology literature that are identified as "risk assessment" papers have focused on just one of the above components, most often, hazard identification. Thus, risk assessment in immunotoxicology remains a "New Frontier."

The science of immunotoxicology continues to evolve, and any overview, including this chapter, must consider the discipline as a "snapshot" in time. Just during the period since this chapter was last published, immunotoxicology has experienced significant advancement. This, in part, has been driven by the tremendous growth in knowledge within immunology and cell biology coupled

with an explosion in methodological and technological capabilities. New tests reflecting a variety of potential impacts of immunotoxicity have emerged, and traditional tests have been improved. In light of the aforementioned, there are several specific areas within immunotoxicology that are currently on the forefront that have the potential to significantly impact the ongoing evolution of this subdiscipline of toxicology and are briefly discussed below.

Arguably, some of the most significant advancements in immunotoxicology in the near future will likely be made with respect to approaches and strategies used to conduct mechanistic studies—specifically, the ability to make quantifiable measurements at the protein and mRNA level in individual cells. Historically, methodologies such as Western blotting, RT-PCR, and gene array approaches have used bulk cell preparations with the results representing an average of measurements across a heterogeneous sample containing many different cell types. One of the major strengths in immunology and also in immunotoxicology has been the application of flow cytometry to make measurements rapidly on individual cells. However, a major limitation in traditional flow cytometry has been the number of different measurements that can be made simultaneously on any one cell due to the use of fluorochromes for detection and their overlapping emission spectra, even with careful selection of fluorescent panels. A significant recent advancement in flow cytometry that has greatly increased the number of endpoint that can be quantified simultaneously is cytometry time of flight (CyTOF). CyTOF uses time-of-flight mass spectrometry in place of fluorescence for detection and quantifies ion mass to charge ratios. Therefore, antibodies are labeled with heavy metal ion tags in place of fluorochromes, which expand the repertoire of simultaneous measurements from approximately 15 to over 40. In addition, these heavy metal ion tags provide a unique signature with minimal overlap between mass channels. Early applications of CyTOF have, for example, allowed measurements of large swaths of signal transduction regulatory networks to investigate how individual cells respond to stimuli in their environment as well as immunophenotyping of complex mixtures of leukocytes (Mingueneau et al., 2014; Leipold et al., 2015). A second major recent advancement in flow cytometry using conventional fluorescent dye-based technology has been to measure changes in the full-emission spectra signature across all lasers for each fluorophore, hence mitigating the current detection limitations of spectral overlap. By utilizing the full spectral signatures, in combination with recently developed fluorophors, it is now possible to expand the repertoire of simultaneous measurements up to 50 with conventional fluorescent dyes, equaling the capabilities of CyTOF. Each of these technologies has its unique strengths and limitations, which are beyond the scope of this discussion, but exemplify the rapid advances taking place in flow cytometry.

Quantitative measurements of mRNA expression at the single cell level have also become possible on a routine basis. One approach, as discussed earlier in this chapter, is utilizing PrimeFlow®, which allows simultaneous measurements of gene-specific mRNA expression with quantitative measurements of extracellular and intracellular proteins using traditional flow cytometry. Albeit a major technological advancement, a significant limitation with PrimeFlow® is that a maximum of only two different genes can be quantified in any given sample. Another limitation is the detection of genes that are modestly expressed. Even with these limitations, the ability of quantifying changes in signal transducing protein in their active and inactive state (e.g., phosphorylated or unphosphorylated) simultaneously with one or two target genes in response to environmental stimuli in large numbers of cells rapidly is a very powerful approach. Another major breakthrough technology is single-cell RNA sequencing (RNA-seq), which allows quantification of all mRNA expressed at any given time in individual cells. RNA-seq has for the most part replaced microarray technology for transcriptomic analyses, but its application to single-cell analysis has been fairly recent. Using RNA-seq has been particularly insightful in heightening awareness concerning cellular heterogeneity. Although a very powerful tool, RNA-seq has a number of technical challenges, which include efficiency of cDNA synthesis and amplification bias (which decreases the accuracy of quantification). An article by Islam et al. (2014) discusses approaches on how to address both of these challenges.

Collectively, approaches for single-cell analysis will be critically important in the elucidation of molecular mechanisms by which xenobiotics modulate the immune system. As these new strategies become more widely used, many of the current technical challenges associated with their application will be resolved and their application will be incorporated more widely into safety testing and risk assessment. Equally important, as new insights into mechanisms of immune toxicity are gleaned so will the identification of new biomarkers of immunotoxicity that can be incorporated into testing strategies.

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REFERENCES

Abdi F, Mobedi H, Mosaffa N, et al. Effects of hormone replacement therapy on immunological factors in the postmenopausal period. *Climacteric*. 2016;19:234–239.

Abdul KS, Jayasinghe SS, Chandana EP, et al. Arsenic and human health effects: a review. *Environ Toxicol Pharmacol*. 2015;40:828–846.

Achachi A, Vocanson M, Bastien P, et al. UV radiation induces the epidermal recruitment of dendritic cells that compensate for the depletion of Langerhans cells in human skin. *J Invest Dermatol*. 2015;135:2058–2067.

Adam C, Wohlfarth J, Haussmann M, et al. Allergy-inducing chromium compounds trigger potent innate immune stimulation via ROS-dependent inflammasome activation. *J Invest Dermatol*. 2017;137:367–376.

Addai AB, Pandhare J, Paromov V, et al. Cocaine modulates HIV-1 integration in primary $CD4^+$ T cells: implications in HIV-1 pathogenesis in drug-abusing patients. *J Leukoc Biol*. 2015;97:779–790.

Aebersold R. Constellations in a cellular universe. *Nature*. 2003;422:115–116.

Aggelakopoulou M, Kourepini E, Paschalidis N, et al. ERbeta-dependent direct suppression of human and murine Th17 cells and treatment of established central nervous system autoimmunity by a neurosteroid. *J Immunol*. 2016;197:2598–2609.

Ahmed T, Pathak R, Mustafa M, et al. Ameliorating effect of *N*-acetylcysteine and curcumin on pesticide-induced oxidative DNA damage in human peripheral blood mononuclear cells. *Environ Monit Assess*. 2011;179:293–299.

Alizadeh M, Ota F, Hosoi K, et al. Altered allergic cytokine and antibody response in mice treated with bisphenol A. *J Med Invest*. 2006;53:70–80.

Allan LL, Mann KK, Matulka RA, et al. Bone marrow stromal–B cell interactions in polycyclic aromatic hydrocarbon-induced pro/pre-B cell apoptosis. *Toxicol Sci*. 2003;76:357–365.

Alonzo NC, Bayer BM. Opioids, immunology, and host defenses of intravenous drug abusers. *Infect Dis Clin North Am*. 2002;16:553–569.

Altonsy MO, Sasse SK, Phang TL, et al. Context-dependent cooperation between nuclear factor kappaB (NF-kappaB) and the glucocorticoid receptor at a TNFAIP3 intronic enhancer: a mechanism to maintain negative feedback control of inflammation. *J Biol Chem*. 2014;289:8231–8239.

Ansari MM, Neha, Khan HA. Effect of cadmium chloride exposure during the induction of collagen induced arthritis. *Chem Biol Interact*. 2015;238:55–65.

Anthoni M, Fyhrquist N, Lehtimaki S, et al. Smad3 regulates dermal cytokine and chemokine expression and specific antibody production in murine responses to a respiratory chemical sensitizer. *Int Arch Allergy Immunol*. 2010;151:155–167.

Antonios D, Rousseau P, Larange A, et al. Mechanisms of IL-12 synthesis by human dendritic cells treated with the chemical sensitizer $NiSO_4$. *J Immunol*. 2010;185:89–98.

Apetoh L, Quintana FJ, Pot C, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol*. 2010;11:854–861.

Araneo BA, Dowell T, Moon HB, et al. Regulation of murine lymphokine production in vivo. Ultraviolet radiation exposure depresses IL-2 and enhances IL-4 production by T cells through an IL-1-dependent mechanism. *J Immunol*. 1989;143:1737–1744.

Aranyi C, O'Shea WJ, Graham JA, et al. The effects of inhalation of organic chemical air contaminants on murine lung host defenses. *Fundam Appl Toxicol*. 1986;6:713–720.

Arkusz J, Stanczyk M, Lewiniska D, et al. Modulation of murine peritoneal macrophage function by chronic exposure to arsenate in drinking water. *Immunopharmacol Immunotoxicol*. 2005;27:315–330.

Ashrin MN, Arakaki R, Yamada A, et al. A critical role for thymic stromal lymphopoietin in nickel-induced allergy in mice. *J Immunol*. 2014;192:4025–4031.

Auphan N, DiDonato JA, Rosette C, et al. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science*. 1995;270:286–290.

Awasthi A, Kuchroo VK. Th17 cells: from precursors to players in inflammation and infection. *Int Immunol*. 2009;21:489–498.

Azizi G, Yazdani R, Mirshafiey A. Th22 cells in autoimmunity: a review of current knowledge. *Eur Ann Allergy Clin Immunol*. 2015;47:108–117.

Baccarelli A, Mocarelli P, Patterson DG Jr, et al. Immunologic effects of dioxin: new results from Seveso and comparison with other studies. *Environ Health Perspect*. 2002;110:1169–1173.

Bacher P, Scheffold A. New technologies for monitoring human antigen-specific T cells and regulatory T cells by flow-cytometry. *Curr Opin Pharmacol*. 2015;23:17–24.

Baker AH, Wu TH, Bolt AM, Gerstenfeld LC, Mann KK, Schlezinger JJ. Tributyltin alters the bone marrow microenvironment and suppresses B cell development. *Toxicol Sci*. 2017;158:63–75.

Balmes JR, Abraham JL, Dweik RA, et al. An official American Thoracic Society statement: diagnosis and management of beryllium sensitivity and chronic beryllium disease. *Am J Respir Crit Care Med*. 2014;190:e34–e59.

Baltimore D, Boldin MP, O'Connell RM, et al. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol*. 2008;9:839–845.

Ban M, Morel G, Langonne I, et al. TDI can induce respiratory allergy with Th2-dominated response in mice. *Toxicology*. 2006;218:39–47.

Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245–252.

Banerjee N, Banerjee S, Sen R, et al. Chronic arsenic exposure impairs macrophage functions in the exposed individuals. *J Clin Immunol*. 2009;29:582–594.

Bankoti J, Burnett A, Navarro S, et al. Effects of TCDD on the fate of naive dendritic cells. *Toxicol Sci*. 2010a;115:422–434.

Bankoti J, Rase B, Simones T, et al. Functional and phenotypic effects of AhR activation in inflammatory dendritic cells. *Toxicol Appl Pharmacol*. 2010b; 246:18–28.

Barnett JB, Brundage KM. Evaluating macrophages in immunotoxicity testing. *Methods Mol Biol*. 2010;598:75–94.

Barragan-Martinez C, Speck-Hernandez CA, Montoya-Ortiz G, et al. Organic solvents as risk factor for autoimmune diseases: a systematic review and meta-analysis. *PLoS One*. 2012;7:e51506.

Bartlett HS, Million RP. Targeting the IL-17-T(H)17 pathway. *Nat Rev Drug Discov*. 2015;14:11–12.

Baschant U, Tuckermann J. The role of the glucocorticoid receptor in inflammation and immunity. *J Steroid Biochem Mol Biol*. 2010;120:69–75.

Basketter DA, Wright ZM, Warbrick EV, et al. Human potency predictions for aldehydes using the local lymph node assay. *Contact Dermatitis*. 2001;45:89–94.

Basu R, O'Quinn DB, Silberger DJ, et al. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. *Immunity*. 2012;37:1061–1075.

Basu S, Ray A, Dittel BN. Cannabinoid receptor 2 is critical for the homing and retention of marginal zone B lineage cells and for efficient T-independent immune responses. *J Immunol*. 2011;187:5720–5732.

Basu S, Ray A, Dittel BN. Cannabinoid receptor 2 (CB2) plays a role in the generation of germinal center and memory B cells, but not in the production of antigen-specific IgG and IgM, in response to T-dependent antigens. *PLoS One*. 2013;8:e67587.

Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol*. 2011;11:34–46.

ben Salah-Abbes J, Abbes S, Zohra H, et al. Tunisian radish (*Raphanus sativus*) extract prevents cadmium-induced immunotoxic and biochemical alterations in rats. *J Immunotoxicol*. 2015;12:40–47.

Berger JR. Natalizumab and progressive multifocal leucoencephalopathy. *Ann Rheum Dis*. 2006;65(suppl 3):iii48–iii53.

Berger JR, Koralnik IJ. Progressive multifocal leukoencephalopathy and natalizumab—unforeseen consequences. *N Engl J Med*. 2005;353:414–416.

Bernier J, Hugo P, Krzystyniak K, et al. Suppression of humoral immunity in inbred mice by dieldrin. *Toxicol Lett*. 1987;35:231–240.

Bernstein DI, Cartier A, Cote J, et al. Diisocyanate antigen-stimulated monocyte chemoattractant protein-1 synthesis has greater test efficiency than specific antibodies for identification of diisocyanate asthma. *Am J Respir Crit Care Med*. 2002;166:445–450.

Bernstein DI, Kissling GE, Khurana Hershey G, et al. Hexamethylene diisocyanate asthma is associated with genetic polymorphisms of CD14, IL-13, and IL-4 receptor alpha. *J Allergy Clin Immunol*. 2011;128(2):418–20.

Bernstein DI, Wang N, Campo P, et al. Diisocyanate asthma and gene–environment interactions with IL4RA, CD-14, and IL-13 genes. *Ann Allergy Asthma Immunol*. 2006;97:800–806.

Bhattacharya S, Conolly RB, Kaminski NE, et al. A bistable switch underlying B-cell differentiation and its disruption by the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol Sci*. 2010;115:51–65.

Bishayi B, Sengupta M. Intracellular survival of *Staphylococcus aureus* due to alteration of cellular activity in arsenic and lead intoxicated mature Swiss albino mice. *Toxicology*. 2003;184:31–39.

Blum JL, Rosenblum LK, Grunig G, et al. Short-term inhalation of cadmium oxide nanoparticles alters pulmonary dynamics associated with lung injury, inflammation, and repair in a mouse model. *Inhal Toxicol*. 2014;26:48–58.

Bock KW. The human Ah receptor: hints from dioxin toxicities to deregulated target genes and physiological functions. *Biol Chem*. 2013;394:729–739.

Bodienkova GM, Alekseev R, Boklazhenko E, et al. Inflammation mediators in employees in chronic exposure to neurotoxicants. *Int J Occup Med Environ Health*. 2014;27:619–626.

Bongartz T, Sutton AJ, Sweeting MJ, et al. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *JAMA*. 2006;295:2275–2285.

Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol*. 2010;10:467–478.

Bonofiglio D, Gabriele S, Aquila S, et al. Estrogen receptor alpha binds to peroxisome proliferator-activated receptor response element and negatively interferes with peroxisome proliferator-activated receptor gamma signaling in breast cancer cells. *Clin Cancer Res*. 2005;11:6139–6147.

Borner C, Kraus J. Inhibition of NF-kappaB by opioids in T cells. *J Immunol*. 2013;191:4640–4647.

Bouchez C, Gervais F, Fleurance R, et al. Development of a delayed-type hypersensitivity (DTH) model in the cynomolgus monkey. *J Toxicol Pathol*. 2012;25:183–188.

Boulton C, Meiser K, David OJ, Schmouder, R. Pharmacodynamic effects of steady-state fingolimod on antibody response in healthy volunteers: A

4-week, randomized, placebo-controlled, parallel-group, multiple-dose study. *Journal of Clinical Pharmacology*. 2012; 52:1879–1890.

Boverhof DR, Gollapudi BB, Hotchkiss JA, et al. Evaluation of a toxicogenomic approach to the local lymph node assay (LLNA). *Toxicol Sci*. 2009;107:427–439.

Brembilla NC, Ramirez JM, Chicheportiche R, et al. In vivo dioxin favors interleukin-22 production by human $CD4^+$ T cells in an aryl hydrocarbon receptor (AhR)-dependent manner. *PLoS One*. 2011;6:e18741.

Breton J, Daniel C, Vignal C, et al. Does oral exposure to cadmium and lead mediate susceptibility to colitis? The dark-and-bright sides of heavy metals in gut ecology. *Sci Rep*. 2016;6:19200.

Broder S. The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. *Antiviral Res*. 2010;85:1–18.

Brown JM, Pfau JC, Pershouse MA, et al. Silica, apoptosis, and autoimmunity. *J Immunotoxicol*. 2004;1:177–188.

Brown N, Nagarkatti M, Nagarkatti PS. Diethylstilbestrol alters positive and negative selection of T cells in the thymus and modulates T-cell repertoire in the periphery. *Toxicol Appl Pharmacol*. 2006a;212:119–126.

Brown N, Nagarkatti M, Nagarkatti PS. Induction of apoptosis in murine fetal thymocytes following perinatal exposure to diethylstilbestrol. *Int J Toxicol*. 2006b;25:9–15.

Brown S, Whalen M. Tributyltin alters secretion of interleukin 1 beta from human immune cells. *J Appl Toxicol*. 2015;35:895–908.

Bublin M, Breiteneder H. Cross-reactivity of peanut allergens. *Curr Allergy Asthma Rep*. 2014;14:426.

Buchweitz JP, Karmaus PW, Harkema JR, et al. Modulation of airway responses to influenza A/PR/8/34 by Delta9-tetrahydrocannabinol in C57BL/6 mice. *J Pharmacol Exp Ther*. 2007;323:675–683.

Buckley NE, McCoy KL, Mezey E, et al. Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur J Pharmacol*. 2000;396:141–149.

Buehler EV. Delayed contact hypersensitivity in the guinea pig. *Arch Dermatol*. 1965;91:171–177.

Burchiel SW, Lauer FT, MacKenzie D, et al. Changes in HPBMC markers of immune function following controlled short-term inhalation exposures of humans to hardwood smoke. *Inhal Toxicol*. 2016;28:61–70.

Burchiel SW, Mitchell LA, Lauer FT, et al. Immunotoxicity and biodistribution analysis of arsenic trioxide in C57Bl/6 mice following a 2-week inhalation exposure. *Toxicol Appl Pharmacol*. 2009;241:253–259.

Burleson FG, Burleson GR. Host resistance assays including bacterial challenge models. *Methods Mol Biol*. 2010;598:97–108.

Burleson GR, Burleson FG, Dietert RR. The cytotoxic T lymphocyte assay for evaluating cell-mediated immune function. *Methods Mol Biol*. 2010;598:195–205.

Burns LA, Bradley SG, White KL Jr, et al. Immunotoxicity of mononitrotoluenes in female B6C3F1 mice: I. Para-nitrotoluene. *Drug Chem Toxicol*. 1994a;17:317–358.

Burns LA, Bradley SG, White KL Jr, et al. Immunotoxicity of nitrobenzene in female B6C3F1 mice. *Drug Chem Toxicol*. 1994b;17:271–315.

Burns LA, Sikorski EE, Saady JJ, et al. Evidence for arsenic as the immunosuppressive component of gallium arsenide. *Toxicol Applied Pharmacol*. 1991;110:157–169.

Burns LA, White KLJr, McCay JA, et al. Immunotoxicity of mono-nitrotoluenes in female B6C3F1 mice: II. Meta-nitrotoluene. *Drug Chem Toxicol*. 1994c;17:359–399.

Burns-Naas LA, Hastings KL, Ladics GS, et al. What's so special about the developing immune system? *Int J Toxicol*. 2008;27:223–254.

Bynote KK, Hackenberg JM, Korach KS, et al. Estrogen receptor-alpha deficiency attenuates autoimmune disease in (NZB x NZW)F1 mice. *Genes Immun*. 2008;9:137–152.

Byrne SN, Halliday GM. B cells activated in lymph nodes in response to ultraviolet irradiation or by interleukin-10 inhibit dendritic cell induction of immunity. *J Invest Dermatol*. 2005;124:570–578.

Byrne SN, Limon-Flores AY, Ullrich SE. Mast cell migration from the skin to the draining lymph nodes upon ultraviolet irradiation represents a key step in the induction of immune suppression. *J Immunol*. 2008;180:4648–4655.

Cabanes N, Igea JM, de la Hoz B, et al. Latex allergy: position paper. *J Investig Allergol Clin Immunol*. 2012;22:313–330; quiz follow 330.

Cabello N, Mishra V, Sinha U, et al. Sex differences in the expression of lung inflammatory mediators in response to ozone. *Am J Physiol Lung Cell Mol Physiol*. 2015;309:L1150–L1163.

Cabral GA. Drugs of abuse, immune modulation, and AIDS. *J Neuroimmune Pharmacol*. 2006;1:280–295.

Caicedo MS, Desai R, McAllister K, et al. Soluble and particulate Co-Cr-Mo alloy implant metals activate the inflammasome danger signaling pathway in human macrophages: a novel mechanism for implant debris reactivity. *J Orthop Res*. 2009;27:847–854.

Calne RY, Rolles K, White DJ, et al. Cyclosporin-A in clinical organ grafting. *Transplant Proc*. 1981;13:349–358.

Camacho IA, Singh N, Hegde VL, et al. Treatment of mice with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin leads to aryl hydrocarbon receptor–dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J Immunol*. 2005;175:90–103.

Cambridge G, Wallace H, Bernstein RM, et al. Autoantibodies to myeloperoxidase in idiopathic and drug-induced systemic lupus erythematosus and vasculitis. *Br J Rheumatol*. 1994;33:109–114.

Cappon GD, Bailey GP, Buschmann J, et al. Juvenile animal toxicity study designs to support pediatric drug development. *Birth Defects Res B Dev Reprod Toxicol*. 2009;86:463–469.

Carey SA, Plopper CG, Hyde DM, et al. Satratoxin-G from the black mold *Stachybotrys chartarum* induces rhinitis and apoptosis of olfactory sensory neurons in the nasal airways of rhesus monkeys. *Toxicol Pathol*. 2012;40:887–898.

Carfi M, Gennari A, Malerba I, et al. In vitro tests to evaluate immunotoxicity: a preliminary study. *Toxicology*. 2007;229:11–22.

Carleer J, Karres J. Juvenile animal studies and pediatric drug development: a European regulatory perspective. *Birth Defects Res B Dev Reprod Toxicol*. 2011;92:254–260.

Caruso C, Buffa S, Candore G, et al. Mechanisms of immunosenescence. *Immun Ageing*. 2009;6:10.

Caruthers NJ, Stemmer PM, Shin N, et al. Mercury alters B-cell protein phosphorylation profiles. *J Proteome Res*. 2014;13:496–505.

Casale GP, Cohen SD, DiCapua RA. The effects of organophosphate-induced cholinergic stimulation on the antibody response to sheep erythrocytes in inbred mice. *Toxicol Appl Pharmacol*. 1983;68:198–205.

Cassel SL, Eisenbarth SC, Iyer SS, et al. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A*. 2008;105:9035–9040.

Catania RA, Chaudry IH. Immunological consequences of trauma and shock. *Ann Acad Med Singapore*. 1999;28:120–132.

Chacon-Salinas R, Limon-Flores AY, Chavez-Blanco AD, et al. Mast cell–derived IL-10 suppresses germinal center formation by affecting T follicular helper cell function. *J Immunol*. 2011;186:25–31.

Chai OH, Song CH. Role of mast cell in the late phase of contact hypersensitivity induced by trimellitic anhydride. *Anat Cell Biol*. 2015;48:225–234.

Chang C, Gershwin ME. Drugs and autoimmunity—a contemporary review and mechanistic approach. *J Autoimmun*. 2010;34:J266–275.

Chang C, Mahmood MM, Teuber SS, et al. Overview of penicillin allergy. *Clin Rev Allergy Immunol*. 2012;43(1–2):84–97.

Chang ZL. Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflamm Res*. 2010;59:791–808.

Chao CC, Molitor TW, Gekker G, et al. Cocaine-mediated suppression of superoxide production by human peripheral blood mononuclear cells. *J Pharmacol Exp Ther*. 1991;256:255–258.

Chaplin DD. Overview of the immune response. *J Allergy Clin Immunol*. 2010;125:S3–23.

Chen G, Mao J, Zhao J, et al. Arsenic trioxide mediates HAPI microglia inflammatory response and the secretion of inflammatory cytokine IL-6 via Akt/NF-kappaB signaling pathway. *Regul Toxicol Pharmacol*. 2016;81:480–488.

Chen Q, Zhang Z, Zhang R, et al. Tributyltin chloride–induced immunotoxicity and thymocyte apoptosis are related to abnormal Fas expression. *Int J Hyg Environ Health*. 2011;214:145–150.

Cheon H, Woo YS, Lee JY, et al. Signaling pathway for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced TNF-alpha production in differentiated THP-1 human macrophages. *Exp Mol Med*. 2007;39:524–534.

Cho J, Kim L, Li Z, et al. Sex bias in experimental immune-mediated, drug-induced liver injury in BALB/c mice: suggested roles for Tregs, estrogen, and IL-6. *PLoS One*. 2013;8:e61186.

Choudhury S, Gupta P, Ghosh S, et al. Arsenic-induced dose-dependent modulation of the NF-kappaB/IL-6 axis in thymocytes triggers differential immune responses. *Toxicology*. 2016;357–358:85–96.

Chuchawankul S, Shima M, Buckley NE, et al. Role of cannabinoid receptors in inhibiting macrophage costimulatory activity. *Int Immunopharmacol*. 2004;4:265–278.

Chung YJ, Zhou HR, Pestka JJ. Transcriptional and posttranscriptional roles for p38 mitogen-activated protein kinase in upregulation of TNF-alpha expression by deoxynivalenol (vomitoxin). *Toxicol Appl Pharmacol*. 2003;193:188–201.

Ciarrocca M, Rosati MV, Tomei F, et al. Correlation between cadmium and blood counts in workers exposed to urban stressor. *Arch Environ Occup Health*. 2015;70:70–76.

Coleman JW, Sim E. Autoallergic responses to drugs: mechanistic aspects. In: Dean JH, Luster MI, Munson AE, et al. eds. *Immunotoxicology and Immunopharmacology*. New York, NY: Raven Press; 1994:553–572.

Collinge M, Cole SH, Schneider PA, et al. Human lymphocyte activation assay: an in vitro method for predictive immunotoxicity testing. *J Immunotoxicol*. 2010;7:357–366.

Colosio C, Birindelli S, Corsini E, et al. Low level exposure to chemicals and immune system. *Toxicol Appl Pharmacol*. 2005;207:320–328.

Condie R, Herring A, Koh WS, et al. Cannabinoid inhibition of adenylate cyclase-mediated signal transduction and interleukin 2 (IL-2) expression in the murine T-cell line, EL4.IL-2. *J Biol Chem*. 1996;271:13175–13183.

Coombs RRA, Gell PGH. Classification of allergic reactions responsible for clinical hypersensitivity and disease. In: Gell PGH, Coombs RRA, Lachmann PJ, eds. *Clinical Aspects of Immunology*. England: Blackwell Scientific Publications Oxford London; 1975:761.

Cooper GS, Makris SL, Nietert PJ, et al. Evidence of autoimmune-related effects of trichloroethylene exposure from studies in mice and humans. *Environ Health Perspect*. 2009;117:696–702.

Cooper RL, Lamb JC, Barlow SM, et al. A tiered approach to life stages testing for agricultural chemical safety assessment. *Crit Rev Toxicol*. 2006;36:69–98.

Corsini E, House RV. Evaluating cytokines in immunotoxicity testing. *Methods Mol Biol*. 2010;598:283–302.

Corsini E, Sokooti M, Galli CL, et al. Pesticide induced immunotoxicity in humans: a comprehensive review of the existing evidence. *Toxicology*. 2013;307:123–135.

Costes V, Magen V, Legouffe E, et al. The Mi15 monoclonal antibody (anti-syndecan-1) is a reliable marker for quantifying plasma cells in paraffin-embedded bone marrow biopsy specimens. *Hum Pathol*. 1999;30:1405–1411.

Crittenden PL, Carr R, Prutett SB. Immunotoxicological assessment of methyl parathion in female B6C3F1 mice. *J Toxicol Environ Health A*. 1998;54:1–20.

Crowe W, Allsopp PJ, Watson GE, et al. Mercury as an environmental stimulus in the development of autoimmunity—a systematic review. *Autoimmun Rev*. 2017;16:72–80.

Cummings KJ, Stefaniak AB, Virji MA, et al. A reconsideration of acute beryllium disease. *Environ Health Perspect*. 2009;117:1250–1256.

Cunningham M, Gilkeson G. Estrogen receptors in immunity and autoimmunity. *Clin Rev Allergy Immunol*. 2011;40:66–73.

D'Cruz D. Autoimmune diseases associated with drugs, chemicals and environmental factors. *Toxicol Lett*. 2000;112–113:421–432.

Dai Q, Pruett SB. Different effects of acute and chronic ethanol on LPS-induced cytokine production and TLR4 receptor behavior in mouse peritoneal macrophages. *J Immunotoxicol*. 2006;3:217–225.

Dakeshita S, Kawai T, Uemura H, et al. Gene expression signatures in peripheral blood cells from Japanese women exposed to environmental cadmium. *Toxicology*. 2009;257:25–32.

Damiani E, Ullrich SE. Understanding the connection between platelet-activating factor, a UV-induced lipid mediator of inflammation, immune suppression and skin cancer. *Prog Lipid Res*. 2016;63:14–27.

Daniel V, Huber W, Bauer K, et al. Associations of dichlorodiphenyltrichloroethane (DDT) 4.4 and dichlorodiphenyldichloroethylene (DDE) 4.4 blood levels with plasma IL-4. *Arch Environ Health*. 2002;57:541–547.

Davila DR, Romero DL, Burchiel SW. Human T cells are highly sensitive to suppression of mitogenesis by polycyclic aromatic hydrocarbons and this effect is differentially reversed by alpha-naphthoflavone. *Toxicol Appl Pharmacol*. 1996;139:333–341.

Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol*. 2011;29:707–735.

De Abrew KN, Phadnis AS, Crawford RB, et al. Regulation of Bach2 by the aryl hydrocarbon receptor as a mechanism for suppression of B-cell differentiation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol Appl Pharmacol*. 2011;252:150–158.

de Boer YS, Kosinski AS, Urban TJ, et al. Features of autoimmune hepatitis in patients with drug-induced liver injury. *Clin Gastroenterol Hepatol*. 2017;15:103–112, e102.

de Jong EC, Spanhaak S, Martens BP, et al. Food allergen (peanut)-specific TH2 clones generated from the peripheral blood of a patient with peanut allergy. *J Allergy Clin Immunol*. 1996;98:73–81.

De Rycke L, Baeten D, Kruithof E, et al. Infliximab, but not etanercept, induces IgM anti-double-stranded DNA autoantibodies as main antinuclear reactivity: biologic and clinical implications in autoimmune arthritis. *Arthritis Rheum*. 2005;52:2192–2201.

De Vooght V, Carlier V, Devos FC, et al. B-lymphocytes as key players in chemical-induced asthma. *PLoS One*. 2013;8:e83228.

Dean JH, Luster MI, Boorman GA, et al. Selective immunosuppression resulting from exposure to the carcinogenic congener of benzopyrene in B6C3F1 mice. *Clin Exp Immunol*. 1983;52:199–206.

Dearman RJ, Basketter DA, Blaikie L, et al. The mouse IgE test: interlaboratory evaluation and comparison of BALB/c and C57BL/6 strain mice. *Toxicol Meth*. 1998;8:69–85.

Dearman RJ, Betts CJ, Humphreys N, et al. Chemical allergy: considerations for the practical application of cytokine profiling. *Toxicol Sci*. 2003;71:137–145.

Dearman RJ, Spence LM, Kimber I. Characterization of murine immune responses to allergenic diisocyanates. *Toxicol Appl Pharmacol*. 1992;112:190–197.

Dearman RJ, Warbrick EV, Skinner R, et al. Cytokine fingerprinting of chemical allergens: species comparisons and statistical analyses. *Food Chem Toxicol*. 2002;40:1881–1892.

Delaney B, Strom SC, Collins S, et al. Carbon tetrachloride suppresses T-cell-dependent immune responses by induction of transforming growth factor-beta 1. *Toxicol Appl Pharmacol*. 1994;126:98–107.

deLemos AS, Foureau DM, Jacobs C, et al. Drug-induced liver injury with autoimmune features. *Semin Liver Dis*. 2014;34:194–204.

DeLong JH, Simpson KH, Wambre E, et al. Ara h 1-reactive T cells in individuals with peanut allergy. *J Allergy Clin Immunol*. 2011;127:1211–1218, e1213.

Demoly P, Michel F, Bousquet J. In vivo methods for study of allergy skin tests, techniques and interpretation. In: Middleton EJ, Reed C, Ellis E, eds. *Allergy Principles and Practice*. Philadelphia: Mosby; 1998:430–439.

Deng X, Nanduri B, Tan W, et al. Sodium methyldithiocarbamate exerts broad inhibition of cellular signaling and expression of effector molecules of inflammation. *Toxicol Sci*. 2013;136:430–442.

Deroo BJ, Archer TK. Glucocorticoid receptor activation of the I kappa B alpha promoter within chromatin. *Mol Biol Cell*. 2001;12:3365–3374.

Descotes J. *Immunotoxicology of Drugs and Chemicals: An Experimental and Clinical Approach*. New York: Elsevier; 2004.

Descotes J. Methods of evaluating immunotoxicity. *Expert Opin Drug Metab Toxicol*. 2006;2:249–259.

Descotes J, Ravel G, Ruat C. Vaccines: predicting the risk of allergy and autoimmunity. *Toxicology*. 2002;174:45–51.

Dewailly E, Ayotte P, Bruneau S, et al. Susceptibility to infections and immune status in Inuit infants exposed to organochlorines. *Environ Health Perspect*. 2000;108:205–211.

Dhital S, Stokes JV, Park N, et al. Cannabidiol (CBD) induces functional Tregs in response to low-level T cell activation. *Cell Immunol*. 2017;312:25–34.

Diana J, Lehuen A. NKT cells: friend or foe during viral infections? *Eur J Immunol*. 2009;39:3283–3291.

Dietert RR. *Immunotoxicity Testing: Methods and Protocols (Methods in Molecular Biology)*. New York, NY: Springer-Verlag; 2010.

Dietert RR, Bunn TL, Lee J-E. The delayed type hypersensitivity assay using protein and xenogenic cell antigens. In: Dietert RR, ed. *Immunotoxicity Testing: Methods and Protocols*. New York: Humana Press; 2009:185–194.

Dietert RR, Bunn TL, Lee JE. The delayed type hypersensitivity assay using protein and xenogeneic cell antigens. *Methods Mol Biol*. 2010;598:185–194.

Dietert RR, Burns-Naas LA. Developmental toxicity in rodents. In: Herzyk DJ, Bussiere JL eds. *Immunotoxicology Strategies for Pharmaceutical Safety Assessment*. Hoboken: Wiley; 2008:273–297.

Dietert RR, Etzel RA, Chen D, et al. Workshop to identify critical windows of exposure for children's health: immune and respiratory systems work group summary. *Environ Health Perspect*. 2000;108(suppl 3):483–490.

Dietert RR, Holsapple MP. Methodologies for developmental immunotoxicity (DIT) testing. *Methods*. 2007;41:123–131.

Dietert RR, Piepenbrink MS. Lead and immune function. *Crit Rev Toxicol*. 2006a;36:359–385.

Dietert RR, Piepenbrink MS. Perinatal immunotoxicity: why adult exposure assessment fails to predict risk. *Environ Health Perspect*. 2006b;114:477–483.

Di Gioacchino M, Petrarca C, Perrone A, et al. Autophagy as an ultrastructural marker of heavy metal toxicity in human cord blood hematopoietic stem cells. *Sci Total Environ*. 2008;392:50–58.

Dong W, Gilmour MI, Lambert AL, et al. Enhanced allergic responses to house dust mite by oral exposure to carbaryl in rats. *Toxicol Sci*. 1998;44:63–69.

Dornbos P, Crawford RB, Kaminski NE, et al. The influence of human interindividual variability on the low-dose region of dose–response curve induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in primary B cells. *Toxicol Sci*. 2016;153(2):352–360.

Duan X, Gao S, Li J, et al. Acute arsenic exposure induces inflammatory responses and $CD4^+$ T cell subpopulations differentiation in spleen and thymus with the involvement of MAPK, NF-κB, and Nrf2. *Mol Immunol*. 2017;81:160–172.

Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond)*. 2003;104:27–38.

Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res*. 2010;20:34–50.

Duramad P, Tager IB, Leikauf J, et al. Expression of Th1/Th2 cytokines in human blood after in vitro treatment with chlorpyrifos, and its metabolites, in combination with endotoxin LPS and allergen Der p1. *J Appl Toxicol*. 2006;26:458–465.

Durant L, Watford WT, Ramos HL, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity*. 2010;32:605–615.

Dutta R, Mondal AM, Arora V, et al. Immunomodulatory effect of DDT (bis[4-chlorophenyl]-1,1,1-trichloroethane) on complement system and macrophages. *Toxicology*. 2008;252:78–85.

Easterbrook PJ, Waters A, Murad S, et al. Epidemiological risk factors for hypersensitivity reactions to abacavir. *HIV Med*. 2003;4:321–324.

Ebaid H, Hassan I, Bashandy S, et al. Zinc improves the immune function and the proliferation of lymphocytes in cadmium-treated rats. *Cent Eur J Immunol*. 2014;39:441–448.

ECVAM. *EURL ECVAM Recommendation on the Direct Peptide Reactivity Assay (DRPA) for Skin Sensitization Testing*. Ispra, Italy: Joint Research Center; 2013.

ECVAM. *EURL ECVAM Recommendation on the KeratinoSENS Assay for Skin Sensitization Testing*. Ispra, Italy: Joint Research Center; 2014.

ECVAM. *EURL ECVAM Recommendation on the Human Cell Line Activation Test (h-CLAT) for Skin Sensitisation Testing*. Ispra, Italy: Joint Research Center; 2015.

Edwards CJ, Cooper C. Early environmental factors and rheumatoid arthritis. *Clin Exp Immunol*. 2006;143:1–5.

Eisenbarth SC, Colegio OR, O'Connor W, et al. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*. 2008;453:1122–1126.

El Ali Z, Gerbeix C, Hemon P, et al. Allergic skin inflammation induced by chemical sensitizers is controlled by the transcription factor Nrf2. *Toxicol Sci*. 2013;134:39–48.

Elgueta R, Benson MJ, de Vries VC, et al. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev*. 2009;229:152–172.

Elmore SA. Enhanced histopathology evaluation of lymphoid organs. *Methods Mol Biol*. 2010;598:323–339.

EMA. *ICH Topic S8: Immunotoxicity Studies for Human Pharmaceuticals*. European Medicines Agency, Committee for Human Medicinal Products; 2006.

EMA. *Guideline on the Need for Non-clinical Testing in Juvenile Animals of Pharmaceuticals for Paediatric Indications*. European Medicines Agency, Committee for Human Medicinal Products; London, England, 2008.

EPA. *EPA Health Effects Guidelines: OPPTS 870.3800—Reproduction and Fertility Effects*. Office of Prevention, Pesticides and Toxic Substances, Washington, DC, 1998.

Escobar A, Aguirre A, Guzman MA, et al. Tolerogenic dendritic cells derived from donors with natural rubber latex allergy modulate allergen-specific T-cell responses and IgE production. *PLoS One*. 2014;9:e85930.

Escobar J, Varela-Nallar L, Coddou C, et al. Oxidative damage in lymphocytes of copper smelter workers correlated to higher levels of excreted arsenic. *Mediators Inflamm*. 2010;2010:403830.

Este JA, Cihlar T. Current status and challenges of antiretroviral research and therapy. *Antiviral Res*. 2010;85:25–33.

Ezeh PC, Lauer FT, Liu KJ, et al. Arsenite interacts with dibenzo[def,*p*]chrysene (DBC) at low levels to suppress bone marrow lymphoid progenitors in mice. *Biol Trace Elem Res*. 2015;166:82–88.

Ezendam J, Kosterman K, Spijkerboer H, et al. Macrophages are involved in hexachlorobenzene-induced adverse immune effects. *Toxicol Appl Pharmacol*. 2005a;209:19–27.

Ezendam J, Vos JG, Pieters R. Mechanisms of hexachlorobenzene-induced adverse immune effects in brown Norway rats. *Journal Immunotoxicol*. 2004;1:167–176.

Ezendam J, Vos JG, Pieters R. Research articles mechanisms of hexachlorobenzene-induced adverse immune effects in brown Norway rats. *J Immunotoxicol*. 2005b;1:167–175.

Farraj AK, Harkema JR, Kaminski NE. Topical application versus intranasal instillation: a qualitative comparison of the effect of the route of sensitization on trimellitic anhydride-induced allergic rhinitis in A/J mice. *Toxicol Sci*. 2006;92:321–328.

Farrer DG, Hueber S, Laiosa MD, et al. Reduction of myeloid suppressor cell derived nitric oxide provides a mechanistic basis of lead enhancement of alloreactive CD4(+) T cell proliferation. *Toxicol Appl Pharmacol*. 2008;229:135–145.

Faubert BL, Kaminski NE. AP-1 activity is negatively regulated by cannabinol through inhibition of its protein components, *c*-fos and *c*-jun. *J Leukoc Biol*. 2000;67:259–266.

FDA. *Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs*. Center for Drug Evaluation and Research, Department of Health and Human Services, Washington, DC, 2002.

FDA. *Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products*. Office of Training and Communication, Division of Drug Information, Center for Drug Evaluation and Research, Department of Health and Human Services, Washington, DC, 2006.

Felter SP, Ryan CA, Basketter DA, et al. Application of the risk assessment paradigm to the induction of allergic contact dermatitis. *Regul Toxicol Pharmacol*. 2003;37:1–10.

Feola DJ, Thornton AC, Garvy BA. Effects of antiretroviral therapy on immunity in patients infected with HIV. *Curr Pharm Des*. 2006;12:1015–1022.

Fernando TM, Ochs SD, Liu J, et al. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induces transcriptional activity of the human polymorphic hs1,2 enhancer of the 3′Igh regulatory region. *J Immunol*. 2012;188:3294–3306.

Filipov NM, Pinchuk LM, Boyd BL, et al. Immunotoxic effects of short-term atrazine exposure in young male C57BL/6 mice. *Toxicol Sci*. 2005;86:324–332.

Finco D, Grimaldi C, Fort M, et al. Cytokine release assays: current practices and future directions. *Cytokine*. 2014;66:143–155.

Fingar DC, Blenis J. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene*. 2004;23:3151–3171.

Fischer A, Koeper LM, Vohr HW. Specific antibody responses of primary cells from different cell sources are able to predict immunotoxicity in vitro. *Toxicol In Vitro*. 2011;25:1966–1973.

Foo YZ, Nakagawa S, Rhodes G, et al. The effects of sex hormones on immune function: a meta-analysis. *Biol Rev Camb Philos Soc*. 2017;92:551–571.

Forte G, Petrucci F, Bocca B. Metal allergens of growing significance: epidemiology, immunotoxicology, strategies for testing and prevention. *Inflamm Allergy Drug Targets*. 2008;7:145–162.

Franchimont D. Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. *Ann N Y Acad Sci*. 2004;1024:124–137.

Freebern WJ. Viral host resistance studies. *Methods Mol Biol*. 2010;598:109–117.

Freebern WJ, Bigwarfe TJ, Price KD, et al. Methods: implementation of in vitro and ex vivo phagocytosis and respiratory burst function assessments in safety testing. *J Immunotoxicol*. 2013;10:106–117.

Fujimaki H, Kurokawa Y, Kunugita N, et al. Differential immunogenic and neurogenic inflammatory responses in an allergic mouse model exposed to low levels of formaldehyde. *Toxicology*. 2004;197:1–13.

Fukuyama T, Tajima Y, Ueda H, et al. A method for measuring mouse respiratory allergic reaction to low-dose chemical exposure to allergens: an environmental chemical of uncertain allergenicity, a typical contact allergen and a non-sensitizing irritant. *Toxicol Lett*. 2010;195:35–43.

Funatake CJ, Marshall NB, Kerkvliet NI. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin alters the differentiation of alloreactive $CD8^+$ T cells toward a regulatory T cell phenotype by a mechanism that is dependent on aryl hydrocarbon receptor in $CD4^+$ T cells. *J Immunotoxicol*. 2008;5:81–91.

Funatake CJ, Marshall NB, Steppan LB, et al. Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin generates a population of $CD4^+$ $CD25^+$ cells with characteristics of regulatory T cells. *J Immunol*. 2005;175:4184–4188.

Gad SC, Dunn BJ, Dobbs DW, et al. Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). *Toxicol Appl Pharmacol*. 1986;84:93–114.

Gagliani N, Amezcua Vesely MC, Iseppon A, et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature*. 2015;523:221–225.

Galbiati V, Mitjans M, Corsini E. Present and future of in vitro immunotoxicology in drug development. *J Immunotoxicol*. 2010;7:255–267.

Galicia G, Leyva R, Tenorio EP, et al. Sodium arsenite retards proliferation of PHA-activated T cells by delaying the production and secretion of IL-2. *Int Immunopharmacol*. 2003;3:671–682.

Galiegue S, Mary S, Marchand J, et al. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem*. 1995;232:54–61.

Galloway T, Handy R. Immunotoxicity of organophosphorous pesticides. *Ecotoxicology*. 2003;12:345–363.

Ganey PE, Luyendyk JP, Maddox JF, et al. Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. *Chem Biol Interact*. 2004;150:35–51.

Gao D, Mondal TK, Lawrence DA. Lead effects on development and function of bone marrow–derived dendritic cells promote Th2 immune responses. *Toxicol Appl Pharmacol*. 2007;222:69–79.

Gao J, Lauer FT, Dunaway S, et al. Cytochrome P450 1B1 is required for 7,12-dimethylbenz(*a*)-anthracene (DMBA) induced spleen cell immunotoxicity. *Toxicol Sci*. 2005a;86:68–74.

Gao J, Mitchell LA, Lauer FT, et al. p53 and ATM/ATR regulate 7,12-dimethylbenz[*a*]anthracene-induced immunosuppression. *Mol Pharmacol*. 2008;73:137–146.

Gao J, Voss AA, Pessah IN, et al. Ryanodine receptor–mediated rapid increase in intracellular calcium induced by 7,8-benzo(*a*)pyrene quinone in human and murine leukocytes. *Toxicol Sci*. 2005b;87:419–426.

Garabrant DH, Dumas C. Epidemiology of organic solvents and connective tissue disease. *Arthritis Res*. 2000;2:5–15.

Garabrant DH, Lacey JV Jr, Laing TJ, et al. Scleroderma and solvent exposure among women. *Am J Epidemiol*. 2003;157:493–500.

Gardner RM, Nyland JF, Evans SL, et al. Mercury induces an unopposed inflammatory response in human peripheral blood mononuclear cells in vitro. *Environ Health Perspect*. 2009;117:1932–1938.

Gardner RM, Nyland JF, Silva IA, et al. Mercury exposure, serum antinuclear/antinucleolar antibodies, and serum cytokine levels in mining populations in Amazonian Brazil: a cross-sectional study. *Environ Res*. 2010;110:345–354.

Garratty G. Drug-induced immune hemolytic anemia. *Clin Adv Hematol Oncol*. 2010;8:98–101.

Garssen J, van Loveren H. Effects of ultraviolet exposure on the immune system. *Crit Rev Immunol*. 2001;21:359–397.

Garssen J, Vandebriel RJ, van Loveren H. Molecular aspects of UVB-induced immunosuppression. *Arch Toxicol Suppl*. 1997;19:97–109.

Gasiewicz TA, Singh KP, Bennett JA. The Ah receptor in stem cell cycling, regulation, and quiescence. *Ann N Y Acad Sci*. 2014;1310:44–50.

Germolec D. Autoimmune disease, animal models. In: vohr H-W, ed. *Encyclopedic Reference of Immunotoxicology*. New York: Springer; 2005:75–79.

Germolec D, Kono DH, Pfau JC, et al. Animal models used to examine the role of the environment in the development of autoimmune disease: findings from an NIEHS Expert Panel Workshop. *J Autoimmun*. 2012;39:285–293.

Germolec DR, Kashon M, Nyska A, et al. The accuracy of extended histopathology to detect immunotoxic chemicals. *Toxicol Sci*. 2004;82:504–514.

Geusau A, Abraham K, Geissler K, et al. Severe 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) intoxication: clinical and laboratory effects. *Environ Health Perspect*. 2001;109:865–869.

Gilbert KM. Xenobiotic exposure and autoimmune hepatitis. *Hepat Res Treat*. 2010;2010:248157.

Gilmour MI, Park P, Doerfler D, et al. Factors that influence the suppression of pulmonary antibacterial defenses in mice exposed to ozone. *Exp Lung Res*. 1993a;19:299–314.

Gilmour MI, Park P, Selgrade MK. Ozone-enhanced pulmonary infection with *Streptococcus zooepidemicus* in mice. The role of alveolar macrophage function and capsular virulence factors. *Am Rev Respir Dis*. 1993b;147:753–760.

Glaser R, Sheridan J, Malarkey WB, et al. Chronic stress modulates the immune response to a pneumococcal pneumonia vaccine. *Psychosom Med*. 2000;62:804–807.

Glover M, Cheng B, Deng X, et al. The role of glucocorticoids in the immediate vs. delayed effects of acute ethanol exposure on cytokine production in a binge drinking model. *Int Immunopharmacol*. 2011;11:755–761.

Gonzalez Maglio DH, Paz ML, Leoni J. Sunlight effects on immune system: is there something else in addition to UV-induced immunosuppression? *Biomed Res Int*. 2016;2016:1934518.

Good RA. Organization and development of the immune system. Relation to its reconstruction. *Ann N Y Acad Sci*. 1995;770:8–33.

Goto M, Takano-Ishikawa Y, Ono H, et al. Orally administered bisphenol A disturbed antigen specific immunoresponses in the naive condition. *Biosci Biotechnol Biochem*. 2007;71:2136–2143.

Goulding NJ, Guyre PM. Regulation of inflammation by lipocortin 1. *Immunol Today*. 1992;13:295–297.

Greenlee WF, Dold KM, Irons RD, et al. Evidence for direct action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on thymic epithelium. *Toxicol Appl Pharmacol*. 1985;79:112–120.

Gribble EJ, Sivakumar PV, Ponce RA, et al. Toxicity as a result of immunostimulation by biologics. *Expert Opin Drug Metab Toxicol*. 2007;3:209–234.

Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest*. 2006;116:4–15.

Grube A, Donaldson D, Kiely T, Wu L. *Pesticide Industry Sales and Usage: 2006 and 2007 Market Estimates*. Washington, DC: Biological and Economic Analysis Division, Office of Pesticide Programs, Office of Chemical Safety and Pollution Prevention, U.S. EPA; 2011.

Gruver-Yates AL, Quinn MA, Cidlowski JA. Analysis of glucocorticoid receptors and their apoptotic response to dexamethasone in male murine B cells during development. *Endocrinology*. 2014;155:463–474.

Haase H, Engelhardt G, Hebel S, et al. Mercuric ions inhibit mitogen-activated protein kinase dephosphorylation by inducing reactive oxygen species. *Toxicol Appl Pharmacol*. 2011;250:78–86.

Haase H, Fahlenkamp A, Schettgen T, et al. Immunotoxicity monitoring in a population exposed to polychlorinated biphenyls. *Int J Environ Res Public Health*. 2016a;13(3). pii: E295.

Haase H, Hebel S, Engelhardt G, et al. Ethylmercury and Hg^{2+} induce the formation of neutrophil extracellular traps (NETs) by human neutrophil granulocytes. *Arch Toxicol*. 2016b;90:543–550.

Haggqvist B, Havarinasab S, Bjorn E, et al. The immunosuppressive effect of methylmercury does not preclude development of autoimmunity in genetically susceptible mice. *Toxicology*. 2005;208:149–164.

Han YC, Lin TL, Pruett SB. Thymic atrophy caused by ethanol in a mouse model for binge drinking: involvement of endogenous glucocorticoids. *Toxicol Appl Pharmacol*. 1993;123:16–25.

Handy RD, Abd-El Samei HA, Bayomy MF, et al. Chronic diazinon exposure: pathologies of spleen, thymus, blood cells, and lymph nodes are modulated by dietary protein or lipid in the mouse. *Toxicology*. 2002;172:13–34.

Hardy RR. B-1 B cell development. *J Immunol*. 2006;177:2749–2754.

Hawley TS, Hawley RG. *Methods in Molecular Biology: Flow Cytometry Protocols, 2nd edition*. New York: Humana Press; 2004.

Hawley TS, Hawley RG. *Methods in Molecular Biology: Flow Cytometry Protocols, 4th edition*. New York, NY: Springer-Verlag; 2016.

Hayes MD, Ovcinnikovs V, Smith AG, et al. The aryl hydrocarbon receptor: differential contribution to T helper 17 and T cytotoxic 17 cell development. *PloS One*. 2014;9:e106955.

Heindel JJ, Newbold RR, Bucher JR, et al. NIEHS/FDA CLARITY-BPA research program update. *Reprod Toxicol*. 2015;58:33–44.

Helali I, Ferchichi S, Maaouia A, et al. Modulation of macrophage functionality induced in vitro by chlorpyrifos and carbendazim pesticides. *J Immunotoxicol*. 2016;13:745–750.

Hemdan NY, Birkenmeier G, Wichmann G, et al. Interleukin-17-producing T helper cells in autoimmunity. *Autoimmun Rev*. 2010;9:785–792.

Hemdan NY, Emmrich F, Sack U, et al. The in vitro immune modulation by cadmium depends on the way of cell activation. *Toxicology*. 2006;222:37–45.

Hemdan NY, Lehmann I, Wichmann G, et al. Immunomodulation by mercuric chloride in vitro: application of different cell activation pathways. *Clin Exp Immunol*. 2007;148:325–337.

Hengstler JG, Foth H, Gebel T, et al. Critical evaluation of key evidence on the human health hazards of exposure to bisphenol A. *Crit Rev Toxicol*. 2011;41:263–291.

Henseler RA, Romer EJ, Sulentic CE. Diverse chemicals including aryl hydrocarbon receptor ligands modulate transcriptional activity of the 3′immunoglobulin heavy chain regulatory region. *Toxicology*. 2009;261:9–18.

Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med*. 1971;284:878–881.

Hernandez M, Brickey WJ, Alexis NE, et al. Airway cells from atopic asthmatic patients exposed to ozone display an enhanced innate immune gene profile. *J Allergy Clin Immunol*. 2012;129:259–261, e251–252.

Herring AC, Koh WS, Kaminski NE. Inhibition of the cyclic AMP signaling cascade and nuclear factor binding to CRE and kappaB elements by cannabinol, a minimally CNS-active cannabinoid. *Biochem Pharmacol*. 1998;55:1013–1023.

Hess EV. Environmental chemicals and autoimmune disease: cause and effect. *Toxicology*. 2002;181–182:65–70.

Higbee RG, Byers AM, Dhir V, et al. An immunologic model for rapid vaccine assessment—a clinical trial in a test tube. *Altern Lab Anim*. 2009;37(suppl 1):19–27.

Ho S, Clipstone N, Timmermann L, et al. The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol*. 1996;80:S40–S45.

Hodge JA, Kawabata TT, Krishnaswami S, et al. The mechanism of action of tofacitinib—an oral Janus kinase inhibitor for the treatment of rheumatoid arthritis. *Clin Exp Rheumatol*. 2016;34:318–328.

Holladay S, Smialowicz R. Development of the murine and human immune system: differential effects of immunotoxicants depend on time of exposure. *Environ Health*. 2000;108(suppl):463–473.

Holsapple MP. Immunotoxicity of halogenated aromatic hydrocarbons. In: Smialowicz RJ, Holsapple MP, eds. *Experimental Immunotoxicology*. Boca Raton, FL: CRC Press; 1996:257–297.

Holsapple MP. Autoimmunity by pesticides: a critical review of the state of the science. *Toxicol Lett*. 2002a;127:101–109.

Holsapple MP. Developmental immunotoxicology and risk assessment: a workshop summary. *Hum Exp Toxicol*. 2002b;21:473–478.

Holsapple MP, Burns-Naas LA, Hastings KL, et al. A proposed testing framework for developmental immunotoxicology (DIT). *Toxicol Sci*. 2005;83:18–24.

Holsapple MP, Dooley RK, McNerney PJ, et al. Direct suppression of antibody responses by chlorinated dibenzodioxins in cultured spleen cells from (C57BL/6 × C3H)F1 and DBA/2 mice. *Immunopharmacology*. 1986;12:175–186.

Holsapple MP, Jones D, Kawabata TT, et al. Assessing the potential to induce respiratory hypersensitivity. *Toxicol Sci*. 2006;91:4–13.

Holsapple MP, Morris DL, Wood SC, et al. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: possible mechanisms. *Annu Rev Pharmacol Toxicol*. 1991a;31:73–100.

Holsapple MP, Munson AE, Munson JA, et al. Suppression of cell-mediated immunocompetence after subchronic exposure to diethylstilbestrol in female B6C3F1 mice. *J Pharmacol Exp Ther*. 1983;227:130–138.

Holsapple MP, O'Lone R. Juvenile immunotoxicology. *Toxicol Pathol*. 2012;40:248–254.

Holsapple MP, Paustenbach DJ, Charnley G, et al. Symposium summary: children's health risk–what's so special about the developing immune system? *Toxicol Appl Pharmacol*. 2004;199:61–70.

Holsapple MP, Snyder NK, Wood SC, et al. A review of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: 1991 update. *Toxicology*. 1991b;69:219–255.

Holsapple MP, van der Laan JW, van Loveren H. Development of a framework for developmental immunotoxicity testing. In Luebke RW, House R, Kimber I, eds. *Immunotoxicology and Immunopharmacology*. Boca Raton, FL: CRC Press; 2007:347–361.

Holsapple MP, West LJ, Landreth KS. Species comparison of anatomical and functional immune system development. *Birth Defects Res B Dev Reprod Toxicol*. 2003;68:321–334.

Holt PG, Upham JW, Sly PD. Contemporaneous maturation of immunologic and respiratory functions during early childhood: implications for development of asthma prevention strategies. *J Allergy Clin Immunol*. 2005;116:16–24; quiz 25.

Hong CH, Lee CH, Yu HS, et al. Benzopyrene, a major polyaromatic hydrocarbon in smoke fume, mobilizes Langerhans cells and polarizes Th2/17 responses in epicutaneous protein sensitization through the aryl hydrocarbon receptor. *Int Immunopharmacol*. 2016;36:111–117.

Hoos A. Development of immuno-oncology drugs—from CTLA4 to PD1 to the next generations. *Nat Rev Drug Discov*. 2016;15:235–247.

Horwitz DA, Zheng SG, Gray JD. Natural and TGF-beta-induced Foxp3(+) CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol*. 2008;29:429–435.

House R. Regulatory guidance in immunotoxicology. In: Vohr H-W, ed. *Encyclopedic Reference of Immunotoxicology*. Springer; 2005:551–555.

Hsiao CL, Wu KH, Wan KS. Effects of environmental lead exposure on T-helper cell-specific cytokines in children. *J Immunotoxicol*. 2011;8:284–287.

Hsu LI, Cheng YW, Chen CJ, et al. Cumulative arsenic exposure is associated with fungal infections: two cohort studies based on southwestern and northeastern basins in Taiwan. *Environ Int*. 2016;96:173–179.

Huang Z, Jiang Y, Yang Y, et al. 3,3′-Diindolylmethane alleviates oxazolone-induced colitis through Th2/Th17 suppression and Treg induction. *Molecular Immunol*. 2013;53:335–344.

Huarte E, Jun S, Rynda-Apple A, et al. Regulatory T cell dysfunction acquiesces to $BTLA^{+}$ regulatory B cells subsequent to oral intervention in experimental autoimmune encephalomyelitis. *J Immunol*. 2016;196:5036–5046.

Hugo P, Bernier J, Krzystyniak K, et al. Transient inhibition of mixed lymphocyte reactivity by dieldrin in mice. *Toxicol Lett*. 1988a;41:1–9.

Hugo P, Bernier J, Krzystyniak K, et al. Abrogation of graft-versus-host reaction by dieldrin in mice. *Toxicol Lett*. 1988b;41:11–22.

Hurt K, Hurd-Brown T, Whalen M. Tributyltin and dibutyltin alter secretion of tumor necrosis factor alpha from human natural killer cells and a mixture of T cells and natural killer cells. *J Appl Toxicol*. 2013;33:503–510.

Hussain I, Piepenbrink MS, Fitch KJ, et al. Developmental immunotoxicity of cyclosporin-A in rats: age-associated differential effects. *Toxicology*. 2005;206:273–284.

Ichinohe T, Lee HK, Ogura Y, et al. Inflammasome recognition of influenza virus is essential for adaptive immune responses. *J Exp Med*. 2009;206:79–87.

Ilchmann A, Krause M, Heilmann M, et al. Impact of culture medium on maturation of bone marrow-derived murine dendritic cells via the aryl hydrocarbon receptor. *Mol Immunol*. 2012;51:42–50.

Illing PT, Vivian JP, Dudek NL, et al. Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature*. 2012;486:554–558.

In SW, Son EW, Rhee DK, et al. Methamphetamine administration produces immunomodulation in mice. *J Toxicol Environ Health A*. 2005;68:2133–2145.

Ishikawa S. Children's immunology, what can we learn from animal studies (3): impaired mucosal immunity in the gut by 2,3,7,8-tetraclorodibenzo-*p*-dioxin (TCDD): a possible role for allergic sensitization. *J Toxicol Sci*. 2009;34(suppl 2):SP349–SP361.

Islam S, Zeisel A, Joost S, et al. Quantitative single-cell RNA-seq with unique molecular identifiers. *Nat Methods*. 2014;11:163–166.

Islam Z, Harkema JR, Pestka JJ. Satratoxin G from the black mold *Stachbotrys chartarum* evokes olfactory sensory neuron loss and inflammation in the murine nose and brain. *Environ Health Perspect*. 2006;114:1099–1107.

Itaborahy RM, de Medeiros SF. Influence of estrogen therapy on immune markers in postmenopausal women. *Climacteric*. 2016;19:496–500.

Iwaniuk A, Jablonska E, Jablonski J, et al. Expression of selected proteins of the extrinsic and intrinsic pathways of apoptosis in human leukocytes exposed to *N*-nitrosodimethylamine. *Hum Exp Toxicol*. 2015;34:591–600.

Jaguin M, Fardel O, Lecureur V. Exposure to diesel exhaust particle extracts (DEPe) impairs some polarization markers and functions of human macrophages through activation of AhR and Nrf2. *PLoS One*. 2015;10:e0116560.

Jankowski MM, Ignatowska-Jankowska B, Glac W, et al. Cocaine administration increases CD4/CD8 lymphocyte ratio in peripheral blood despite lymphopenia and elevated corticosterone. *Int Immunopharmacol*. 2010;10:1229–1234.

Jenkins DJ, Kendall CW, Connelly PW, et al. Effects of high- and low-isoflavone (phytoestrogen) soy foods on inflammatory biomarkers and proinflammatory cytokines in middle-aged men and women. *Metabolism*. 2002;51:919–924.

Jeon YJ, Han SH, Yang KH, et al. Induction of liver-associated transforming growth factor beta 1 (TGF-beta 1) mRNA expression by carbon tetrachloride leads to the inhibition of T helper 2 cell–associated lymphokines. *Toxicol Appl Pharmacol*. 1997;144:27–35.

Jeong TC, Jordan SD, Matulka RA, et al. Immunosuppression induced by acute exposure to cocaine is dependent on metabolism by cytochrome P-450. *J Pharmacol Exp Ther*. 1996;276:1257–1265.

Jeuken A, Keser BJ, Khan E, et al. Activation of the Ah receptor by extracts of dietary herbal supplements, vegetables, and fruits. *J Agric Food Chem*. 2003;51:5478–5487.

Ji T, Xu C, Sun L, et al. Aryl hydrocarbon receptor activation down-regulates IL-7 and reduces inflammation in a mouse model of DSS-induced colitis. *Dig Dis Sci*. 2015;60:1958–1966.

Jiang X, Khursigara G, Rubin RL. Transformation of lupus-inducing drugs to cytotoxic products by activated neutrophils. *Science*. 1994;266:810–813.

Jiang Y, Jolly PE, Ellis WO, et al. Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanaians. *Int Immunol*. 2005;17:807–814.

Jin GB, Winans B, Martin KC, et al. New insights into the role of the aryl hydrocarbon receptor in the function of CD11c(+) cells during respiratory viral infection. *Eur J Immunol*. 2014;44:1685–1698.

Johnson CW, Williams WC, Copeland CB, et al. Sensitivity of the SRBC PFC assay versus ELISA for detection of immunosuppression by TCDD and TCDD-like congeners. *Toxicology*. 2000;156:1–11.

Johnson VJ, Yucesoy B, Reynolds JS, et al. Inhalation of toluene diisocyanate vapor induces allergic rhinitis in mice. *J Immunol*. 2007;179:1864–1871.

Jorissen A, Plum LM, Rink L, et al. Impact of lead and mercuric ions on the interleukin-2-dependent proliferation and survival of T cells. *Arch Toxicol*. 2013;87:249–258.

Jung WW, Kim EM, Lee EH, et al. Formaldehyde exposure induces airway inflammation by increasing eosinophil infiltrations through the regulation of reactive oxygen species production. *Environ Toxicol Pharmacol*. 2007;24:174–182.

Jusko TA, De Roos AJ, Schwartz SM, et al. Maternal and early postnatal polychlorinated biphenyl exposure in relation to total serum immunoglobulin concentrations in 6-month-old infants. *J Immunotoxicol*. 2011;8:95–100.

Jutel M, Akdis CA. T-cell subset regulation in atopy. *Curr Allergy Asthma Rep*. 2011;11:139–145.

Kalland T. Alterations of antibody response in female mice after neonatal exposure to diethylstilbestrol. *J Immunol*. 1980;124:194–198.

Kalland T, Strand O, Forsberg JG. Long-term effects of neonatal estrogen treatment on mitogen responsiveness of mouse spleen lymphocytes. *J Natl Cancer Inst*. 1979;63:413–421.

Kaminski NE, Barnes DW, Jordan SD, et al. The role of metabolism in carbon tetrachloride–mediated immunosuppression: in vivo studies. *Toxicol Appl Pharmacol*. 1990;102:9–20.

Kaminski NE, Jordan SD, Holsapple MP. Suppression of humoral and cell-mediated immune responses by carbon tetrachloride. *Fundam Appl Toxicol*. 1989a;12:117–128.

Kaminski NE, Jordan SD, Page D, et al. Suppression of humoral immune responses by dialkylnitrosamines: structure–activity relationships. *Fundam Appl Toxicol*. 1989b;12:321–332.

Kanda N, Tsuchida T, Tamaki K. Estrogen enhancement of anti-double-stranded DNA antibody and immunoglobulin G production in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Arthritis Rheum*. 1999;42:328–337.

Kane KL, Ashton FA, Schmitz JL, et al. Determination of natural killer cell function by flow cytometry. *Clin Diagn Lab Immunol*. 1996;3:295–300.

Kanmaz T, Fechner JJ Jr, Torrealba J, et al. Monotherapy with the novel human anti-CD154 monoclonal antibody ABI793 in rhesus monkey renal transplantation model. *Transplantation*. 2004;77:914–920.

Kaplan BL, Li J, LaPres JJ, et al. Contributions of nonhematopoietic cells and mediators to immune responses: implications for immunotoxicology. *Toxicol Sci*. 2015;145:214–232.

Kaplan BL, Rockwell CE, Kaminski NE. Evidence for cannabinoid receptor-dependent and -independent mechanisms of action in leukocytes. *J Pharmacol Exp Ther*. 2003;306:1077–1085.

Kaplan DH. In vivo function of Langerhans cells and dermal dendritic cells. *Trends Immunol*. 2010;31:446–451.

Karmakar S, Jin Y, Nagaich AK. Interaction of glucocorticoid receptor (GR) with estrogen receptor (ER) alpha and activator protein 1 (AP1) in dexamethasone-mediated interference of ERalpha activity. *J Biol Chem*. 2013;288:24020–24034.

Karmaus PW, Chen W, Crawford RB, et al. Deletion of cannabinoid receptors 1 and 2 exacerbates APC function to increase inflammation and cellular immunity during influenza infection. *J Leukoc Biol*. 2011;90:983–995.

Karmaus W, Brooks KR, Nebe T, et al. Immune function biomarkers in children exposed to lead and organochlorine compounds: a cross-sectional study. *Environ Health*. 2005;4:5.

Karol MH, Cormier Y, Donham KJ, et al. Animal models. *Am J Ind Med*. 1994;25:135–138.

Karrow NA, McCay JA, Brown R, et al. Oxymetholone modulates cell-mediated immunity in male B6C3F1 mice. *Drug Chem Toxicol*. 2000;23:621–644.

Karrow NA, McCay JA, Brown RD, et al. Oral exposure to atrazine modulates cell-mediated immune function and decreases host resistance to the B16F10 tumor model in female B6C3F1 mice. *Toxicology*. 2005;209:15–28.

Kasper-Sonnenberg M, Sugiri D, Wurzler S, et al. Prevalence of nickel sensitization and urinary nickel content of children are increased by nickel in ambient air. *Environ Res*. 2011;111:266–273.

Kasten-Jolly J, Lawrence DA. Lead modulation of macrophages causes multiorgan detrimental health effects. *J Biochem Mol Toxicol*. 2014;28:355–372.

Kately JR, Bazzell SJ. Immunological studies in cattle exposed to polybrominated biphenyl. *Environ Health Perspect*. 1978;23:750.

Katika MR, Hendriksen PJ, van Loveren H, et al. Exposure of Jurkat cells to bis (tri-*n*-butyltin) oxide (TBTO) induces transcriptomics changes indicative for ER- and oxidative stress, T cell activation and apoptosis. *Toxicol Appl Pharmacol*. 2011;254:311–322.

Katika MR, Hendriksen PJ, van Loveren H, et al. Characterization of the modes of action of deoxynivalenol (DON) in the human Jurkat T-cell line. *J Immunotoxicol*. 2015;12:206–216.

Kawabata TT, White KL Jr. Enhancement of in vivo and in vitro murine immune responses by the cyclophosphamide metabolite acrolein. *Cancer Res*. 1988;48:41–45.

Kawabata TT, White KL Jr. Benzo(*a*)pyrene metabolism by murine spleen microsomes. *Cancer Res*. 1989;49:5816–5822.

Kenna JG, Neuberger J, Williams R. Identification by immunoblotting of three halothane-induced liver microsomal polypeptide antigens recognized by antibodies in sera from patients with halothane-associated hepatitis. *J Pharmacol Exp Ther*. 1987;242:733–740.

Kepley CL, Lauer FT, Oliver JM, et al. Environmental polycyclic aromatic hydrocarbons, benzo(*a*) pyrene (BaP) and BaP-quinones, enhance IgE-mediated histamine release and IL-4 production in human basophils. *Clin Immunol*. 2003;107:10–19.

Kerkvliet NI. Recent advances in understanding the mechanisms of TCDD immunotoxicity. *Int Immunopharmacol*. 2002;2:277–291.

Kerkvliet NI, Burleson GR. Immunotoxicity of TCDD and related halogenated aromatic hydrocarbons. In: Dean JH, Luster MI, Munson AE, et al., eds. *Immunotoxicology and Immunopharmacology*. New York: Raven Press; 1994:97–121.

Kerkvliet NI, Shepherd DM, Baecher-Steppan L. T lymphocytes are direct, aryl hydrocarbon receptor (AhR)-dependent targets of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): AhR expression in both $CD4^+$ and $CD8^+$ T cells is necessary for full suppression of a cytotoxic T lymphocyte response by TCDD. *Toxicol Appl Pharmacol*. 2002;185:146–152.

Khan D, Ahmed SA. The immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases. *Front Immunol*. 2016;6:1056–1058.

Kharrazian D. The potential roles of bisphenol A (BPA) pathogenesis in autoimmunity. *Autoimmune Dis*. 2014;2014:743616.

Khodoun M, Strait R, Orekov T, et al. Peanuts can contribute to anaphylactic shock by activating complement. *J Allergy Clin Immunol*. 2009;123:342–351.

Kiecolt-Glaser JK, Glaser R, Gravenstein S, et al. Chronic stress alters the immune response to influenza virus vaccine in older adults. *Proc Natl Acad Sci U S A*. 1996;93:3043–3047.

Kilburn KH, Warshaw RH. Chemical-induced autoimmunity. In: Dean JH, Luster MI, Munson AE, et al., eds. *Immunotoxicology and Immunopharmacology*. New York: Raven Press; 1994:523–538.

Kim E, Kang BY, Kim TS. Inhibition of interleukin-12 production in mouse macrophages by hydroquinone, a reactive metabolite of benzene, via suppression of nuclear factor-kappaB binding activity. *Immunol Lett*. 2005;99:24–29.

Kim HA, Kim EM, Park YC, et al. Immunotoxicological effects of Agent Orange exposure to the Vietnam War Korean veterans. *Ind Health*. 2003;41:158–166.

Kim J, Jeong H. Suppression of inducible nitric oxide synthase and tumor necrosis alpha expression by bisphenol A via nuclear factor-kb inactivation in macrophages. *Toxicol Sci*. 2003;71:153–154.

Kim KN, Bae S, Park HY, et al. Low-level mercury exposure and risk of asthma in school-age children. *Epidemiology*. 2015;26:733–739.

Kimata H. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin selectively enhances spontaneous IgE production in B cells from atopic patients. *Int J Hyg Environ Health*. 2003;206:601–604.

Kimber I, Dearman RJ, Basketter DA, et al. The local lymph node assay: past, present and future. *Contact Dermatitis*. 2002;47:315–328.

Kimbrough RD, Krouskas CA. Polychlorinated biphenyls, dibenzo-*p*-dioxins, and dibenzofurans and birth weight and immune and thyroid function in children. *Regul Toxicol Pharmacol*. 2001;34:42–52.

Kimura A, Abe H, Tsuruta S, et al. Aryl hydrocarbon receptor protects against bacterial infection by promoting macrophage survival and reactive oxygen species production. *Int Immunol*. 2014;26:209–220.

Kimura A, Kishimoto T. Th17 cells in inflammation. *Int Immunopharmacol*. 2011;11:319–322.

Kiss EA, Vonarbourg C, Kopfmann S, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science (New York)*. 2011;334:1561–1565.

Klaassen CD, Liu J, Diwan BA. Metallothionein protection of cadmium toxicity. *Toxicol Applied Pharmacol*. 2009;238:215–220.

Klecak G: Identification of contact allergens: predicitive tests in animals. In: Marzulli FN, Maibach HI, eds. *Dermatotoxicology*. New York: Hemisphere; 1987:227–290.

Klingbeil EC, Hew KM, Nygaard UC, et al. Polycyclic aromatic hydrocarbons, tobacco smoke, and epigenetic remodeling in asthma. *Immunol Res*. 2014;58:369–373.

Kodama T, Sekine H, Takahashi M, et al. Role of complement in a murine model of peanut-induced anaphylaxis. *Immunobiology*. 2013;218:844–850.

Kojima H, Katsura E, Takeuchi S, et al. Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. *Environ Health Perspect*. 2004;112:524–531.

Kojima H, Sata F, Takeuchi S, et al. Comparative study of human and mouse pregnane X receptor agonistic activity in 200 pesticides using in vitro reporter gene assays. *Toxicology*. 2011;280:77–87.

Koller LD. Immunotoxicology of heavy metals. *Int J Immunopharmacol*. 1980;2:269–279.

Kononenko V, Narat M, Drobne D. Nanoparticle interaction with the immune system. *Arh Hig Rada Toksikol*. 2015;66:97–108.

Kooijman R, Devos S, Hooghe-Peters E. Inhibition of in vitro cytokine production by human peripheral blood mononuclear cells treated with xenobiotics: implications for the prediction of general toxicity and immunotoxicity. *Toxicol In Vitro*. 2010;24:1782–1789.

Korniotis S, Gras C, Letscher H, et al. Treatment of ongoing autoimmune encephalomyelitis with activated B-cell progenitors maturing into regulatory B cells. *Nat Commun*. 2016;7:12134.

Kovalova N, Manzan M, Crawford R, et al. Role of aryl hydrocarbon receptor polymorphisms on TCDD-mediated CYP1B1 induction and IgM suppression by human B cells. *Toxicol Appl Pharmacol*. 2016;309:15–23.

Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell Immunol*. 2015;294:63–69.

Kramer S, Hikel SM, Adams K, et al. Current status of the epidemiologic evidence linking polychlorinated biphenyls and non-hodgkin lymphoma, and the role of immune dysregulation. *Environ Health Perspect*. 2012;120:1067–1075.

Kripke ML. Immunologic mechanisms in UV radiation carcinogenesis. *Adv Cancer Res*. 1981;34:69–106.

Krishna M, Nadler SG. Immunogenicity to biotherapeutics : the role of anti-drug immune complexes. *Front Immunol*. 2016;7:21.

Krueger WS, Wade TJ. Elevated blood lead and cadmium levels associated with chronic infections among non-smokers in a cross-sectional analysis of NHANES data. *Environ Health*. 2016;15:16.

Krzystyniak K, Hugo P, Flipo D, et al. Increased susceptibility to mouse hepatitis virus 3 of peritoneal macrophages exposed to dieldrin. *Toxicol Appl Pharmacol*. 1985;80:397–408.

Kumawat KL, Kaushik DK, Goswami P, et al. Acute exposure to lead acetate activates microglia and induces subsequent bystander neuronal death via caspase-3 activation. *Neurotoxicology*. 2014;41:143–153.

Kusaka T, Nakayama M, Nakamura K, et al. Effect of silica particle size on macrophage inflammatory responses. *PLoS One*. 2014;9:e92634.

Kuwatsuka Y, Shimizu K, Akiyama Y, et al. Yusho patients show increased serum IL-17, IL-23, IL-1beta, and TNFalpha levels more than 40 years after accidental polychlorinated biphenyl poisoning. *J Immunotoxicol*. 2014;11:246–249.

Ladics GS, Budziszewski GJ, Herman RA, et al. Measurement of endogenous allergens in genetically modified soybeans—short communication. *Regul Toxicol Pharmacol*. 2014;70:75–79.

Ladics GS, Chapin RE, Hastings KL, et al. Developmental toxicology evaluations—issues with including neurotoxicology and immunotoxicology assessments in reproductive toxicology studies. *Toxicol Sci*. 2005;88:24–29.

Ladics GS, Kawabata TT, Munson AE, et al. Generation of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene by murine splenic macrophages. *Toxicol Appl Pharmacol*. 1992a;115:72–79.

Ladics GS, Kawabata TT, Munson AE, et al. Metabolism of benzo[*a*] pyrene by murine splenic cell types. *Toxicol Appl Pharmacol*. 1992b;116:248–257.

Ladics GS, Kawabata TT, White KL Jr. Suppression of the in vitro humoral immune response of mouse splenocytes by 7,12-dimethylbenz[*a*]anthracene metabolites and inhibition of immunosuppression by alpha-naphthoflavone. *Toxicol Appl Pharmacol*. 1991;110:31–44.

Ladics GS, Smith C, Elliott GS, et al. Further evaluation of the incorporation of an immunotoxicological functional assay for assessing humoral immunity for hazard identification purposes in rats in a standard toxicology study. *Toxicology*. 1998;126:137–152.

Ladics GS, Smith C, Heaps K, et al. Evaluation of the humoral immune response of CD rats following a 2-week exposure to the pesticide carbaryl by the oral, dermal, or inhalation routes. *J Toxicol Environ Health*. 1994;42:143–156.

Ladics GS, Smith C, Heaps K, et al. Possible incorporation of an immunotoxicological functional assay for assessing humoral immunity for hazard identification purposes in rats on standard toxicology study. *Toxicology*. 1995;96:225–238.

Lai JJ, Lai KP, Zeng W, et al. Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. *Am J Pathol*. 2012;181:1504–1512.

Lai ZW, Fiore NC, Hahn PJ, et al. Differential effects of diethylstilbestrol and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on thymocyte differentiation, proliferation, and apoptosis in bcl-2 transgenic mouse fetal thymus organ culture. *Toxicol Appl Pharmacol*. 2000;168:15–24.

Laiosa MD, Wyman A, Murante FG, et al. Cell proliferation arrest within intrathymic lymphocyte progenitor cells causes thymic atrophy mediated by the aryl hydrocarbon receptor. *J Immunol*. 2003;171:4582–4591.

Lall SB, Dan G. Role of corticosteroids in cadmium induced immunotoxicity. *Drug Chem Toxicol*. 1999;22:401–409.

Lane R, Ghazi SO, Whalen MM. Increases in cytosolic calcium ion levels in human natural killer cells in response to butyltin exposure. *Arch Environ Contam Toxicol*. 2009;57:816–825.

Lankveld DP, Van Loveren H, Baken KA, et al. In vitro testing for direct immunotoxicity: state of the art. *Methods Mol Biol*. 2010;598:401–423.

Larsen ST, Roursgaard M, Jensen KA, et al. Nano titanium dioxide particles promote allergic sensitization and lung inflammation in mice. *Basic Clin Pharmacol Toxicol*. 2010;106:114–117.

Lauenstein L, Switalla S, Prenzler F, et al. Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS). *Toxicol In Vitro*. 2014;28:588–599.

Lawrence S, Reid J, Whalen M. Secretion of interferon gamma from human immune cells is altered by exposure to tributyltin and dibutyltin. *Environ Toxicol*. 2015;30:559–571.

Lay JC, Alexis NE, Kleeberger SR, et al. Ozone enhances markers of innate immunity and antigen presentation on airway monocytes in healthy individuals. *J Allergy Clin Immunol*. 2007;120:719–722.

Leach M, Drummond M, Doig A, McKay P, Jackson B, Bain BJ. *Practical Flow Cytometry in Haematology: 100 Worked Examples*. Hoboken, NJ: Wiley; 2015.

Leach MW, Rottman JB, Hock MB, et al. Immunogenicity/hypersensitivity of biologics. *Toxicol Pathol*. 2014;42:293–300.

Lecureur V, Ferrec EL, N'Diaye M, et al. ERK-dependent induction of TNFalpha expression by the environmental contaminant benzo(*a*)pyrene in primary human macrophages. *FEBS Lett*. 2005;579:1904–1910.

Lee EJ, Jang Y, Kang K, et al. Atrazine induces endoplasmic reticulum stress-mediated apoptosis of T lymphocytes via the caspase-8-dependent pathway. *Environ Toxicol*. 31:998–1008, 2016.

Lee JA, Hwang JA, Sung HN, et al. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin modulates functional differentiation of mouse bone marrow–derived dendritic cells: Downregulation of RelB by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol Lett*. 2007;173:31–40.

Lee JS, Cella M, McDonald KG, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol*. 2011;13:144–151.

Lee MH, Chung SW, Kang BY, et al. Hydroquinone, a reactive metabolite of benzene, enhances interleukin-4 production in $CD4^+$ T cells and increases immunoglobulin E levels in antigen-primed mice. *Immunology*. 2002;106:496–502.

Lee MH, Chung SW, Kang BY, et al. Enhanced interleukin-4 production in $CD4^+$ T cells and elevated immunoglobulin E levels in antigen-primed mice by bisphenol A and nonylphenol, endocrine disruptors: involvement of nuclear factor-AT and Ca^{2+}. *Immunology*. 2003;109:76–86.

Lee S, Kim MS, Lee D, et al. The comparative immunotoxicity of mesoporous silica nanoparticles and colloidal silica nanoparticles in mice. *Int J Nanomedicine*. 2013;8:147–158.

Lehmann-Horn K, Sagan SA, Winger RC, et al. CNS accumulation of regulatory B cells is VLA-4-dependent. *Neurol Neuroimmunol Neuroinflamm*. 2016;3:e212.

Lehto M, Kotovuori A, Palosuo K, et al. Hev b 6.01 and Hev b 5 induce proinflammatory cytokines and chemokines from peripheral blood mononuclear cells in latex allergy. *Clin Exp Allergy*. 2007;37:133–140.

Leipold MD, Newell EW, Maecker HT. Multiparameter phenotyping of human PBMCs using mass cytometry. *Methods Mol Biol*. 2015;1343:81–95.

Lemarie A, Morzadec C, Bourdonnay E, et al. Human macrophages constitute targets for immunotoxic inorganic arsenic. *J Immunol*. 2006;177:3019–3027.

LeVier DG, McCay JA, Stern ML, et al. Immunotoxicological profile of morphine sulfate in B6C3F1 female mice. *Fundam Appl Toxicol*. 1994;22:525–542.

Levy MH, Wheelock EF. Effects of intravenous silica on immune and nonimmune functions of the murine host. *J Immunol*. 1975;115:41–48.

Li B, Li YQ, Yang LJ, et al. Decreased T-cell receptor excision DNA circles in peripheral blood mononuclear cells among benzene-exposed workers. *Int J Immunogenet*. 2009;36:107–111.

Li B, Niu Y, Liu S, et al. A change in CD3gamma, CD3delta, CD3, and CD3zeta gene expression in T-lymphocytes from benzene-exposed and benzene-poisoned workers. *J Immunotoxicol*. 2012;9:160–167.

Li C, Guan T, Gao C, et al. Arsenic trioxide inhibits accelerated allograft rejection mediated by alloreactive CD8(+) memory T cells and prolongs allograft survival time. *Transpl Immunol*. 2015a;33:30–36.

Li H, Prasad R, Katiyar SK, et al. Interleukin-17 mediated inflammatory responses are required for ultraviolet radiation-induced immune suppression. *Photochem Photobiol*. 2015b;91:235–241.

Li J, Phadnis-Moghe AS, Crawford RB, et al. Aryl hydrocarbon receptor activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin impairs human B lymphopoiesis. *Toxicology*. 2017;378:17–24.

Li Q. NK cell assays in immunotoxicity testing. *Methods Mol Biol*. 2010;598:207–219.

Li Q, Lauer FT, Liu KJ, et al. Low-dose synergistic immunosuppression of T-dependent antibody responses by polycyclic aromatic hydrocarbons and arsenic in C57BL/6J murine spleen cells. *Toxicol Appl Pharmacol*. 2010;245:344–351.

Limpens J, Garssen J, Scheper RJ. Local administration of cytostatic drugs enhances delayed-type hypersensitivity to Sendai virus in mice. *Clin Immunol Immunopathol*. 1990;54:161–165.

Limpens J, Scheper RJ. Inhibition of T suppressor cell function by local administration of an active cyclophosphamide derivative at the sensitization site. *Clin Exp Immunol*. 1991;84:383–388.

Lio CW, Hsieh CS. Becoming self-aware: the thymic education of regulatory T cells. *Curr Opin Immunol*. 2011;23:213–219.

Liu G, Asanoma K, Takao T, et al. Aryl hydrocarbon receptor SNP -130 C/T associates with dioxins susceptibility through regulating its receptor activity and downstream effectors including interleukin 24. *Toxicol Lett*. 2015a;232:384–392.

Liu J, Jia DY, Cai SZ, et al. Mitochondria defects are involved in lead-acetate-induced adult hematopoietic stem cell decline. *Toxicol Lett*. 2015b;235:37–44.

Liu J, Zhang L, Winterroth LC, et al. Epigenetically mediated pathogenic effects of phenanthrene on regulatory T cells. *J Toxicol*. 2013;2013:967029.

Liu MC, Liu XQ, Wang W, et al. Involvement of microglia activation in the lead induced long-term potentiation impairment. *PLoS One*. 2012;7:e43924.

Liu Y, She W, Wang F, et al. 3, 3′-Diindolylmethane alleviates steatosis and the progression of NASH partly through shifting the imbalance of Treg/Th17 cells to Treg dominance. *Int Immunopharmacol*. 2014;23:489–498.

Loftis JM, Choi D, Hoffman W, et al. Methamphetamine causes persistent immune dysregulation: a cross-species, translational report. *Neurotox Res*. 2011;20:59–68.

Long CM, Marshall NB, Lukomska E, et al. A role for regulatory T cells in a murine model of epicutaneous toluene diisocyanate sensitization. *Toxicol Sci*. 2016;152:85–98.

Lu H, Crawford RB, Kaplan BL, et al. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-mediated disruption of the CD40 ligand-induced activation of primary human B cells. *Toxicol Appl Pharmacol*. 2011;255(3):251–260.

Lu H, Crawford RB, North CM, et al. Establishment of an immunoglobulin m antibody-forming cell response model for characterizing immunotoxicity in primary human B cells. *Toxicol Sci*. 2009;112:363–373.

Lu H, Crawford RB, Suarez-Martinez JE, et al. Induction of the aryl hydrocarbon receptor-responsive genes and modulation of the immunoglobulin M response by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in primary human B cells. *Toxicol Sci*. 2010;118:86–97.

Lu T, Newton C, Perkins I, et al. Cannabinoid treatment suppresses the T helper cell polarizing function of mouse dendritic cells stimulated with *Legionella pneumophila* infection. *J Pharmacol Exp Ther*. 2006a;319(1):269–276.

Lu T, Newton C, Perkins I, et al. Role of cannabinoid receptors in Delta-9-tetrahydrocannabinol suppression of IL-12p40 in mouse bone marrow-derived dendritic cells infected with *Legionella pneumophila*. *Eur J Pharmacol*. 2006b;532:170–177.

Luebke RW. Parasite challenge as host resistance models for immunotoxicity testing. *Methods Mol Biol*. 2010;598:119–141.

Luebke RW, Chen DH, Dietert R, et al. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J Toxicol Environ Health B Crit Rev*. 2006a;9:1–26.

Luebke RW, Holsapple MP, Ladics GS, et al. Immunotoxicogenomics: the potential of genomics technology in the immunotoxicity risk assessment process. *Toxicol Sci*. 2006b;94(1):22–27.

Luebke RW, Parks C, Luster MI. Suppression of immune function and susceptibility to infections in humans: association of immune function with clinical disease. *J Immunotoxicol*. 2004;1:15–24.

Lummus ZL, Alam R, Bernstein JA, et al. Diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor-alpha by peripheral mononuclear cells of workers with occupational asthma. *J Allergy Clin Immunol*. 1998;102:265–274.

Luna AL, Acosta-Saavedra LC, Lopez-Carrillo L, et al. Arsenic alters monocyte superoxide anion and nitric oxide production in environmentally exposed children. *Toxicol Appl Pharmacol*. 2010;245:244–251.

Lundblad LK, Irvin CG, Adler A, et al. A reevaluation of the validity of unrestrained plethysmography in mice. *J Appl Physiol*. 2002;93:1198–1207.

Luo CY, Wang L, Sun C, et al. Estrogen enhances the functions of CD4(+) CD25(+)Foxp3(+) regulatory T cells that suppress osteoclast differentiation and bone resorption in vitro. *Cell Mol Immunol*. 2011;8:50–58.

Luo YH, Chang LW, Lin P. Metal-based nanoparticles and the immune system: activation, inflammation, and potential applications. *Biomed Res Int*. 2015;2015:143720.

Luster MI, Boorman GA, Dean JH. Effect of in utero exposure of diethylstilbestrol on the immune response in mice. *Toxicol Appl Pharmacol*. 1979;47:287–293.

Luster MI, Boorman GA, Dean JH, et al. The effect of adult exposure to diethylstilbestrol in the mouse: alterations in immunological functions. *J Reticuloendothel Soc*. 1980;28:561–569.

Luster MI, Boorman GA, Korach KS, et al. Mechanisms of estrogen-induced myelotoxicity: evidence of thymic regulation. *Int J Immunopharmacol*. 1984;6:287–297.

Luster MI, Dean JH, Germolec DR. Consensus workshop on methods to evaluate developmental immunotoxicity. *Environ Health Perspect*. 2003;111:579–583.

Luster MI, Germolec DR, White KL Jr, et al. A comparison of three nucleoside analogs with anti-retroviral activity on immune and hematopoietic functions in mice: in vitro toxicity to precursor cells and microstromal environment. *Toxicol Appl Pharmacol*. 1989;101:328–339.

Luster MI, Munson AE, Thomas PT, et al. Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. *Fundam Appl Toxicol*. 1988;10:2–19.

Luster MI, Portier C, Pait DG, et al. Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundam Appl Toxicol*. 1992;18:200–210.

Luster MI, Portier C, Pait DG, et al. Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. *Fundam Appl Toxicol*. 1993;21:71–82.

Lutz PM, Kelty EA, Brown TD, et al. Environmental cigarette smoke exposure modulates IgE levels of Pb-exposed children. *Toxicology*. 2012;291:43–50.

Lutz PM, Wilson TJ, Ireland J, et al. Elevated immunoglobulin E (IgE) levels in children with exposure to environmental lead. *Toxicology*. 1999;134:63–78.

Mack DG, Falta MT, McKee AS, et al. Regulatory T cells modulate granulomatous inflammation in an HLA-DP2 transgenic murine model of beryllium-induced disease. *Proc Natl Acad Sci U S A*. 2014;111:8553–8558.

Magnusson B, Kligman AM. The identification of contact allergens by animal assay. The guinea pig maximization test. *J Invest Dermatol*. 1969;52:268–276.

Maibach H. Formaldehyde: effects on animal and human skin. In Gibson J, ed. *Formaldehyde Toxicity*. New York: Hemisphere; 1983:166–174.

Mallal S, Phillips E, Carosi G, et al. HLA-B*5701 screening for hypersensitivity to abacavir. *N Engl J Med*. 2008;358:568–579.

Maltzman JS, Koretzky GA. Azathioprine: old drug, new actions. *J Clin Invest*. 2003;111:1122–1124.

Mangum LC, Borazjani A, Stokes JV, et al. Organochlorine insecticides induce NADPH oxidase–dependent reactive oxygen species in human monocytic cells via phospholipase A2/arachidonic acid. *Chem Res Toxicol*. 2015;28:570–584.

Mangum LH, Crow JA, Stokes JV, et al. Exposure to *p,p′*-DDE alters macrophage reactivity and increases macrophage numbers in adipose stromal vascular fraction. *Toxicol Sci*. 2016;150:169–177.

Margoles LM, Mittal R, Klingensmith NJ, et al. Chronic alcohol ingestion delays T cell activation and effector function in sepsis. *PLoS One*. 2016;11:e0165886.

Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. *Curr Genomics*. 2006;7:497–508.

Marnell L, Mold C, Du Clos TW. C-reactive protein: ligands, receptors and role in inflammation. *Clin Immunol*. 2005;117:104–111.

Marshall NB, Kerkvliet NI. Dioxin and immune regulation: emerging role of aryl hydrocarbon receptor in the generation of regulatory T cells. *Ann N Y Acad Sci*. 2010;1183:25–37.

Martin PL, Breslin W, Rocca M, et al. Considerations in assessing the developmental and reproductive toxicity potential of biopharmaceuticals. *Birth Defects Res B Dev Reprod Toxicol*. 2009;86:176–203.

Martin-Chouly C, Morzadec C, Bonvalet M, et al. Inorganic arsenic alters expression of immune and stress response genes in activated primary human T lymphocytes. *Mol Immunol*. 2011;48:956–965.

Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014;6:13.

Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol*. 2009;27:229–265.

Marzulli FN, Maibach HI. Contact allergy: predictive testing in humans. In: Marzulli FN, Maibach HI, eds. *Dermatotoxicology*. New York: Hemisphere; 1987:319–340.

Masuda Y, Schecter A. The yusho and yucheng rice oil poisoning incidents. In: Schecter A, ed. *Dioxins and Health: Including Other Persistent Organic Pollutants and Endocrine Disruptors*. Hoboken, NJ: Wiley; 2012:521–551.

Matheson JM, Johnson VJ, Vallyathan V, et al. Exposure and immunological determinants in a murine model for toluene diisocyanate (TDI) asthma. *Toxicol Sci*. 2005;84:88–98.

Matthews J, Gustafsson JA. Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nucl Recept Signal*. 2006;4:e016.

Matulka RA, Jordan SD, Stanulis ED, et al. Evaluation of sex- and strain-dependency of cocaine-induced immunosuppression in B6C3F1 and DBA/2 mice. *J Pharmacol Exp Ther*. 1996;279:12–17.

Matveyeva M, Hartmann CB, Harrison MT, et al. Delta(9)-tetrahydrocannabinol selectively increases aspartyl cathepsin D proteolytic activity and impairs lysozyme processing by macrophages. *Int J Immunopharmacol*. 2000;22:373–381.

Maurice T, Romieu P. Involvement of the sigma1 receptor in the appetitive effects of cocaine. *Pharmacopsychiatry*. 2004;37(suppl 3):S198–S207.

McCleskey TM, Buchner V, Field RW, et al. Recent advances in understanding the biomolecular basis of chronic beryllium disease: a review. *Rev Environ Health*. 2009;24:75–115.

McCormic ZD, Khuder SS, Aryal BK, et al. Occupational silica exposure as a risk factor for scleroderma: a meta-analysis. *Int Arch Occup Environ Health*. 2010;83:763–769.

McCoy KL, Gainey D, Cabral GA. delta 9-Tetrahydrocannabinol modulates antigen processing by macrophages. *J Pharmacol Exp Ther*. 1995;273:1216–1223.

McDonough RJ, Madden JJ, Falek A, et al. Alteration of T and null lymphocyte frequencies in the peripheral blood of human opiate addicts: in vivo evidence for opiate receptor sites on T lymphocytes. *J Immunol*. 1980;125:2539–2543.

McGwin G, Lienert J, Kennedy JI. Formaldehyde exposure and asthma in children: a systematic review. *Environ Health Perspect*. 2010;118:313–317.

McKallip RJ, Lombard C, Fisher M, et al. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood*. 2002;100:627–634.

McKallip RJ, Nagarkatti M, Nagarkatti PS. Immunotoxicity of AZT: inhibitory effect on thymocyte differentiation and peripheral T cell responsiveness to gp120 of human immunodeficiency virus. *Toxicol Appl Pharmacol*. 1995;131:53–62.

McKarns SC, Kaminski NE. TGF-beta 1 differentially regulates IL-2 expression and [3H]-thymidine incorporation in CD3 epsilon mAb- and CD28 mAb-activated splenocytes and thymocytes. *Immunopharmacology*. 2000;48:101–115.

McKee AS, Fontenot AP. Interplay of innate and adaptive immunity in metal-induced hypersensitivity. *Curr Opin Immunol*. 2016;42:25–30.

McKee AS, Mack DG, Crawford F, et al. MyD88 dependence of beryllium-induced dendritic cell trafficking and CD4(+) T-cell priming. *Mucosal Immunol*. 2015;8:1237–1247.

Mehrzad J, Mahmudy Gharaie MH, Taheri M. Effects of arsenic on porcine dendritic cells in vitro. *J Immunotoxicol*. 2017;14:1–8.

Memari B, Bouttier M, Dimitrov V, et al. Engagement of the aryl hydrocarbon receptor in *Mycobacterium tuberculosis*-infected macrophages has pleiotropic effects on innate immune signaling. *J Immunol*. 2015;195:4479–4491.

Menna JH, Barnett JB, Soderberg LS. Influenza type A virus infection of mice exposed in utero to chlordane; survival and antibody studies. *Toxicol Lett*. 1985;24:45–52.

Menon M, Blair PA, Isenberg DA, et al. A regulatory feedback between plasmacytoid dendritic cells and regulatory B cells is aberrant in systemic lupus erythematosus. *Immunity*. 2016;44:683–697.

Michielsen CC, van Loveren H, Vos JG. The role of the immune system in hexachlorobenzene-induced toxicity. *Environ Health Perspect*. 1999;107(suppl 5):783–792.

Miller FW, Pollard KM, Parks CG, et al. Criteria for environmentally associated autoimmune diseases. *J Autoimmun*. 2012;39:253–258.

Miller K, Brown RC. The immune system and asbestos-associated disease. In: Dean JH, Luster MI, Munson AE, et al. eds. *Immunotoxicology and Immunopharmacology*. New York: Raven Press; 1985:429–440.

Milnerowicz H, Sciskalska M, Dul M. Pro-inflammatory effects of metals in persons and animals exposed to tobacco smoke. *J Trace Elem Med Biol*. 2015;29:1–10.

Mingueneau M, Krishnaswamy S, Spitzer MH, et al. Single-cell mass cytometry of TCR signaling: amplification of small initial differences results in low ERK activation in NOD mice. *Proc Natl Acad Sci U S A*. 2014;111:16466–16471.

Mirabet M, Barrabes JA, Quiroga A, et al. Platelet pro-aggregatory effects of CD40L monoclonal antibody. *Mol Immunol*. 2008;45:937–944.

Mishell RI, Dutton RW. Immunization of dissociated mouse spleen cell culture from normal mice. *J Exp Med*. 1967;126:423–442.

Mishra KP. Lead exposure and its impact on immune system: a review. *Toxicol In Vitro*. 2009;23:969–972.

Mitzner W, Tankersley C. Interpreting Penh in mice. *J Appl Physiol*. 2003;94:828–831; author reply 831–822.

Miyashita C, Sasaki S, Saijo Y, et al. Effects of prenatal exposure to dioxin-like compounds on allergies and infections during infancy. *Environ Res*. 2011;111:551–558.

Miyawaki T, Moriya N, Nagaoki T, et al. Maturation of B-cell differentiation ability and T-cell regulatory function in infancy and childhood. *Immunol Rev*. 1981;57:61–87.

Moed H, Boorsma DM, Stoof TJ, et al. Nickel-responding T cells are $CD4^+$ CLA^+ $CD45RO^+$ and express chemokine receptors CXCR3, CCR4 and CCR10. *Br J Dermatol*. 2004;151:32–41.

Mojadadi S, Jamali A, Khansarinejad B, et al. Acute morphine administration reduces cell-mediated immunity and induces reactivation of latent herpes simplex virus type 1 in BALB/c mice. *Cell Mol Immunol*. 2009;6:111–116.

Mondal TK, Li D, Swami K, et al. Mercury impairment of mouse thymocyte survival in vitro: involvement of cellular thiols. *J Toxicol Environ Health A*. 2005;68:535–556.

Monteiro P, Gilot D, Le Ferrec E, et al. AhR- and c-maf-dependent induction of beta7-integrin expression in human macrophages in response to environmental polycyclic aromatic hydrocarbons. *Biochem Biophys Res Commun*. 2007;358:442–448.

Monteiro P, Gilot D, Le Ferrec E, et al. Dioxin-mediated upregulation of aryl hydrocarbon receptor target genes is dependent on the calcium/calmodulin/CaMKIalpha pathway. *Mol Pharmacol*. 2008;73:769–777.

Moon Y, Pestka JJ. Vomitoxin-induced cyclooxygenase-2 gene expression in macrophages mediated by activation of ERK and p38 but not JNK mitogen-activated protein kinases. *Toxicol Sci*. 2002;69:373–382.

Morandell S, Stasyk T, Grosstessner-Hain K, et al. Phosphoproteomics strategies for the functional analysis of signal transduction. *Proteomics*. 2006;6(14):4047–56.

Morice WG, Brunn GJ, Wiederrecht G, et al. Rapamycin-induced inhibition of p34cdc2 kinase activation is associated with G1/S-phase growth arrest in T lymphocytes. *J Biol Chem*. 1993;268:3734–3738.

Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol*. 1989;46:111–147.

Mosmann TR, Schumacher JH, Street NF, et al. Diversity of cytokine synthesis and function of mouse $CD4^+$ T cells. *Immunol Rev*. 1991;123:209–229.

Mounho BJ, Burchiel SW. Alterations in human B cell calcium homeostasis by polycyclic aromatic hydrocarbons: possible associations with cytochrome P450 metabolism and increased protein tyrosine phosphorylation. *Toxicol Appl Pharmacol*. 1998;149:80–89.

Murphy GM. Ultraviolet radiation and immunosuppression. *Br J Dermatol*. 2009;161(suppl 3):90–95.

Murphy K, Weaver C. *Janeway's Immunobiology*. 9th ed. New York: Garland Science, Taylor & Francis; 2016.

Murphy WG, Kelton JG. Immune haemolytic anaemia and thrombocytopenia: drugs and autoantibodies. *Biochem Soc Trans*. 1991;19:183–186.

Mydlarski P, Katz A, Sauder D. Contact dermatitis. In: Middleton EJ, Reed C, Ellis E, eds. *Allergy Principles and Practice*. Philadelphia: Mosby; 1998:1141–1143.

Myers MJ, Schook LB. Immunotoxicity of nitrosamines. In: Smialowicz RJ, Holsapple MP eds. *Exp Immunotoxicol*. Boca Raton, FL: CRC Press; 1996:351–366.

N'Diaye M, Le Ferrec E, Lagadic-Gossmann D, et al. Aryl hydrocarbon receptor- and calcium-dependent induction of the chemokine CCL1 by the environmental contaminant benzo[*a*]pyrene. *J Biol Chem*. 2006;281:19906–19915.

Nagayama J, Nagayama M, Iida T, et al. Comparison between "Yusho" patients and healthy Japanese in contamination level of dioxins and related chemicals and frequency of sister chromatid exchanges. *Chemosphere*. 2001a;43:931–936.

Nagayama J, Tsuji H, Iida T, et al. Effects of contamination level of dioxins and related chemicals on thyroid hormone and immune response systems in patients with "Yusho." *Chemosphere*. 2001b;43:1005–1010.

Nagayama J, Tsuji H, Iida T, et al. Immunologic effects of perinatal exposure to dioxins, PCBs and organochlorine pesticides in Japanese infants. *Chemosphere*. 2007;67:S393–S398.

Naidoo J, Page DB, Wolchok JD. Immune modulation for cancer therapy. *Br J Cancer*. 2014;111:2214–2219.

Nathan C: Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol*. 2006;6:173–182.

Ndebele K, Tchounwou PB, McMurray RW.Coumestrol, bisphenol-A, DDT, and TCDD modulation of interleukin-2 expression in activated $CD^{+}4$ Jurkat T cells. *Int J Environ Res Public Health*. 2004;1:3–11.

Neishabouri EZ, Hassan ZM, Azizi E, et al. Evaluation of immunotoxicity induced by diazinon in C57bl/6 mice. *Toxicology*. 2004;196:173–179.

Neubert R, Maskow L, Triebig G, et al. Chlorinated dibenzo-*p*-dioxins and dibenzofurans and the human immune system: 3. Plasma immunoglobulins and cytokines of workers with quantified moderately-increased body burdens. *Life Sci*. 2000;66:2123–2142.

Neuman MG, Maor Y, Nanau RM, et al. Alcoholic liver disease: role of cytokines. *Biomolecules*. 2015;5:2023–2034.

Neumann S, Burkert K, Kemp R, et al. Activation of the NLRP3 inflammasome is not a feature of all particulate vaccine adjuvants. *Immunol Cell Biol*. 2014;92:535–542.

Newton CA, Chou PJ, Perkins I, et al. CB(1) and CB(2) cannabinoid receptors mediate different aspects of delta-9-tetrahydrocannabinol (THC)-induced T helper cell shift following immune activation by *Legionella pneumophila* infection. *J Neuroimmune Pharmacol*. 2009;4:92–102.

Ng S, Yoshida K, Zelikoff JT. Tumor challenges in immunotoxicity testing. *Methods Mol Biol*. 2010;598:143–155.

Nikoopour E, Bellemore SM, Singh B. IL-22, cell regeneration and autoimmunity. *Cytokine*. 2015;74:35–42.

Nishino R, Fukuyama T, Watanabe Y, et al. Detection of respiratory allergies caused by environmental chemical allergen via measures of hyperactivation and degranulation of mast cells in lungs of NC/Nga mice. *J Immunotoxicol*. 2016;13:676–685.

Noh G, Lee JH. Regulatory B cells and allergic diseases. *Allergy Asthma Immunol Res*. 2011;3:168–177.

North CM, Crawford RB, Lu H, et al. Simultaneous in vivo time course and dose response evaluation for TCDD-induced impairment of the LPS-stimulated primary IgM response. *Toxicol Sci*. 2009;112:123–132.

North CM, Crawford RB, Lu H, et al. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated suppression of Toll-like receptor stimulated B-lymphocyte activation and initiation of plasmacytic differentiation. *Toxicol Sci*. 2010;116:99–112.

North CM, Ezendam J, Hotchkiss JA, et al. Developing a framework for assessing chemical respiratory sensitization: a workshop report. *Regul Toxicol Pharmacol*. 2016;80:295–309.

NTP. *Explanation of Levels of Evidence for Immune System Toxicity*. National Toxicology Program, National Institute of Environmental Health Sciences, National Institute of Health, Department of Health and Human Services; Washington, DC, 2009.

Nunez GM, Estrada I, Calderon-Aranda ES. DDT inhibits the functional activation of murine macrophages and decreases resistance to infection by *Mycobacterium microti*. *Toxicology*. 2002;174:201–210.

O'Brien AJ, Fullerton JN, Massey KA, et al. Immunosuppression in acutely decompensated cirrhosis is mediated by prostaglandin E2. *Nat Med*. 2014;20:518–523.

OECD. *Guideline for the Testing of Chemicals: Extended One-Generation Reproductive Toxicity Study*. Organization for Economic Cooperation and Development; Washington, DC, 2010.

Ohkura N, Hamaguchi M, Sakaguchi S. $FOXP3^{+}$ regulatory T cells: control of FOXP3 expression by pharmacological agents. *Trends Pharmacol Sci*. 2011;32:158–166.

Ohnishi T, Yoshida T, Igarashi A, et al. Effects of possible endocrine disruptors on MyD88-independent TLR4 signaling. *FEMS Immunol Med Microbiol*. 2008;52:293–295.

Olgun S, Gogal RM Jr, Adeshina F, et al. Pesticide mixtures potentiate the toxicity in murine thymocytes. *Toxicology*. 2004;196:181–195.

Ongradi J, Kovesdi V. Factors that may impact on immunosenescence: an appraisal. *Immun Ageing*. 2010;7:7.

Oppong E, Cato AC. Effects of glucocorticoids in the immune system. *Adv Exp Med Biol*. 2015;872:217–233.

Ou DW, Shen ML, Luo YD. Effects of cocaine on the immune system of Balb/C mice. *Clin Immunol Immunopathol*. 1989;52:305–312.

Paavonen T, Andersson LC, Adlercreutz H. Sex hormone regulation of in vitro immune response. Estradiol enhances human B cell maturation via inhibition of suppressor T cells in pokeweed mitogen-stimulated cultures. *J Exp Med*. 1981;154:1935–1945.

Palomaki J, Valimaki E, Sund J, et al. Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. *ACS Nano*. 2011;5:6861–6870.

Pan X, Whitten DA, Wu M, et al. Early phosphoproteomic changes in the mouse spleen during deoxynivalenol-induced ribotoxic stress. *Toxicol Sci*. 2013;135:129–143.

Pandhare J, Addai AB, Mantri CK, et al. Cocaine enhances HIV-1-induced CD4(+) T-cell apoptosis: implications in disease progression in cocaine-abusing HIV-1 patients. *Am J Pathol*. 2014;184:927–936.

Park H, Kim K: Association of blood mercury concentrations with atopic dermatitis in adults: a population-based study in Korea. *Environ Res*. 2011;111:573–578.

Park MK, Jung YO, Lee SY, et al. Amelioration of autoimmune arthritis by adoptive transfer of Foxp3-expressing regulatory B cells is associated with the Treg/Th17 cell balance. *J Transl Med*. 2016;14:191.

Parks CG, Miller FW, Pollard KM, et al. Expert panel workshop consensus statement on the role of the environment in the development of autoimmune disease. *Int J Mol Sci*. 2014;15:14269–14297.

Parks CG, Walitt BT, Pettinger M, et al. Insecticide use and risk of rheumatoid arthritis and systemic lupus erythematosus in the Women's Health Initiative Observational Study. *Arthritis Care Res (Hoboken)*. 2011;63:184–194.

Pathak N, Khandelwal S. Oxidative stress and apoptotic changes in murine splenocytes exposed to cadmium. *Toxicology*. 2006;220:26–36.

Pathak N, Khandelwal S. Impact of cadmium in T lymphocyte subsets and cytokine expression: differential regulation by oxidative stress and apoptosis. *Biometals*. 2008;21:179–187.

Paukkeri EL, Korhonen R, Hamalainen M, et al. The inflammatory phenotype in failed metal-on-metal hip arthroplasty correlates with blood metal concentrations. *PLoS One*. 2016;11:e0155121.

Pauluhn J. Animal models for respiratory hypersensitivity. In: Vohr H-W, ed. *Encyclopedic Reference of Immunotoxicology*. New York: Springer; 2005:15–21.

Pawelec G, Akbar A, Beverley P, et al. Immunosenescence and cytomegalovirus: where do we stand after a decade? *Immun Ageing*. 2010;7:13.

Pearson C, Uhlig HH, Powrie F. Lymphoid microenvironments and innate lymphoid cells in the gut. *Trends Immunol*. 2012;33:289–296.

Peden DB. The role of oxidative stress and innate immunity in O(3) and endotoxin-induced human allergic airway disease. *Immunol Rev*. 2011;242:91–105.

Peden-Adams MM, Eudaly JG, Heesemann LM, et al. Developmental immunotoxicity of trichloroethylene (TCE): studies in B6C3F1 mice. *J Environ Sci Health A Tox Hazard Subst Environ Eng*. 2006;41:249–271.

Pelletier L, Castedo M, Bellon B, et al. Mercury and autoimmunity. In: Dean JH, Luster MI, Munson AE, et al., eds. *Immunotoxicology and Immunopharmacology*. New York: Raven Press; 1994:539–552.

Pelletier M, Roberge CJ, Gauthier M, et al. Activation of human neutrophils in vitro and dieldrin-induced neutrophilic inflammation in vivo. *J Leukoc Biol*. 2001;70:367–373.

Peng X, Mosser DM, Adler MW, et al. Morphine enhances interleukin-12 and the production of other pro-inflammatory cytokines in mouse peritoneal macrophages. *J Leukoc Biol*. 2000;68:723–728.

Penta KL, Fairweather D, Shirley DL, et al. Low-dose mercury heightens early innate response to coxsackievirus infection in female mice. *Inflamm Res*. 2015;64:31–40.

Pernis B, Paronetto F: Adjuvant effect of silica (tridymite) on antibody production. *Proc Soc Exp Biol Med*. 1962;110:390–392.

Perretti M, D'Acquisto F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol*. 2009;9:62–70.

Person RJ, Whalen MM. Effects of butyltin exposures on MAP kinase–dependent transcription regulators in human natural killer cells. *Toxicol Mech Methods*. 2010;20:227–233.

Pesatori AC, Consonni D, Bachetti S, et al. Short- and long-term morbidity and mortality in the population exposed to dioxin after the "Seveso accident." *Ind Health*. 2003;41:127–138.

Pessah IN, Beltzner C, Burchiel SW, et al. A bioactive metabolite of benzo[*a*]pyrene, benzo[*a*]pyrene-7,8-dione, selectively alters microsomal Ca^{2+} transport and ryanodine receptor function. *Mol Pharmacol*. 2001;59:506–513.

Pestka JJ. Deoxynivalenol-induced IgA production and IgA nephropathy—aberrant mucosal immune response with systemic repercussions. *Toxicol Lett*. 2003;140–141:287–295.

Pestka JJ, Yan D, King LE. Flow cytometric analysis of the effects of in vitro exposure to vomitoxin (deoxynivalenol) on apoptosis in murine T, B and IgA$^+$ cells. *Food Chem Toxicol*. 1994;32:1125–1136.

Pestka JJ, Zhou HR, Moon Y, et al. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. *Toxicol Lett*. 2004;153:61–73.

Peterson PK, Gekker G, Chao CC, et al. Cocaine potentiates HIV-1 replication in human peripheral blood mononuclear cell cocultures. Involvement of transforming growth factor-beta. *J Immunol*. 1991;146:81–84.

Phadnis-Moghe A, Chen W, Li J, et al. Immunological characterization of the aryl hydrocarbon receptor (AHR) knockout rat in the presence and absence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Toxicology*. 2016;368–369:172–182.

Phadnis-Moghe AS, Crawford RB, Kaminski NE. Suppression of human B cell activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin involves altered regulation of B cell lymphoma-6. *Toxicol Sci*. 2015;144:39–50.

Phiel KL, Henderson RA, Adelman SJ, et al. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunol Lett*. 2005;97:107–113.

Phillips EJ, Wong GA, Kaul R, et al. Clinical and immunogenetic correlates of abacavir hypersensitivity. *AIDS*. 2005;19:979–981.

Pichler WJ. Drug-induced autoimmunity. *Curr Opin Allergy Clin Immunol*. 2003;3:249–253.

Pinaud E, Marquet M, Fiancette R, et al. The IgH locus 3′ regulatory region: pulling the strings from behind. *Adv Immunol*. 2011;110:27–70.

Pinchuk LM, Lee SR, Filipov NM. In vitro atrazine exposure affects the phenotypic and functional maturation of dendritic cells. *Toxicol Appl Pharmacol*. 2007;223:206–217.

Plitnick LM, Herzyk DJ. The T-dependent antibody response to keyhole limpet hemocyanin in rodents. *Methods Mol Biol*. 2010;598:159–171.

Plitnick LM, Loveless SE, Ladics GS, et al. Cytokine profiling for chemical sensitizers: application of the ribonuclease protection assay and effect of dose. *Toxicol Appl Pharmacol*. 2002;179:145–154.

Plitnick LM, Loveless SE, Ladics GS, et al. Identifying airway sensitizers: cytokine mRNA profiles induced by various anhydrides. *Toxicology*. 2003;193:191–201.

Plitnick LM, Loveless SE, Ladics GS, et al. Cytokine mRNA profiles for isocyanates with known and unknown potential to induce respiratory sensitization. *Toxicology*. 2005;207:487–499.

Podechard N, Lecureur V, Le Ferrec E, et al. Interleukin-8 induction by the environmental contaminant benzo(*a*)pyrene is aryl hydrocarbon receptor–dependent and leads to lung inflammation. *Toxicology Lett*. 2008;177:130–137.

Polanczyk MJ, Carson BD, Subramanian S, et al. Cutting edge: estrogen drives expansion of the CD4$^+$CD25$^+$ regulatory T cell compartment. *J Immunol*. 2004;173:2227–2230.

Pollard KM. Silica, silicosis, and autoimmunity. *Front Immunol*. 2016;7:97.

Pollard KM, Hultman P, Kono DH. Toxicology of autoimmune diseases. *Chem Res Toxicol*. 2010;23:455–466.

Pollaris L, Devos F, De Vooght V, et al. Toluene diisocyanate and methylene diphenyl diisocyanate: asthmatic response and cross-reactivity in a mouse model. *Arch Toxicol*. 2016;90:1709–1717.

Potula R, Hawkins BJ, Cenna JM, et al. Methamphetamine causes mitochondrial oxidative damage in human T lymphocytes leading to functional impairment. *J Immunol*. 2010;185:2867–2876.

Powell JJ, Davis MV, Whalen MM. Glutathione diminishes tributyltin- and dibutyltin-induced loss of lytic function in human natural killer cells. *Drug Chem Toxicol*. 2009;32:9–16.

Prasad R, Katiyar SK. Crosstalk among UV-induced inflammatory mediators, DNA damage and epigenetic regulators facilitates suppression of the immune system. *Photochem Photobiol*. 2017;93(4):930–936.

Price K. Cellular immune response in delayed-type hypersensitivity tests. In: Herzyk DJ, Bussiere JL, eds. *Immunotoxicology Strategies for Pharmaceutical Safety Assessment*. Hoboken, NJ: Wiley; 2008:87–103.

Proper SP, Saini Y, Greenwood KK, et al. Loss of hypoxia-inducible factor 2 alpha in the lung alveolar epithelium of mice leads to enhanced eosinophilic inflammation in cobalt-induced lung injury. *Toxicol Sci*. 2014;137:447–457.

Pruett SB, Barnes DB, Han YC, et al. Immunotoxicological characteristics of sodium methyldithiocarbamate. *Fundam Appl Toxicol*. 1992a;18:40–47.

Pruett SB, Fan R, Zheng Q. Involvement of three mechanisms in the alteration of cytokine responses by sodium methyldithiocarbamate. *Toxicol Appl Pharmacol*. 2006;213:172–178.

Pruett SB, Fan R, Zheng Q, et al. Modeling and predicting immunological effects of chemical stressors: characterization of a quantitative biomarker for immunological changes caused by atrazine and ethanol. *Toxicol Sci*. 2003;75:343–354.

Pruett SB, Han YC, Fuchs BA. Morphine suppresses primary humoral immune responses by a predominantly indirect mechanism. *J Pharmacol Exp Ther*. 1992b;262:923–928.

Pruett SB, Schwab C, Zheng Q, et al. Suppression of innate immunity by acute ethanol administration: a global perspective and a new mechanism beginning with inhibition of signaling through TLR3. *J Immunol*. 2004a;173:2715–2724.

Pruett SB, Zheng Q, Fan R, et al. Acute exposure to ethanol affects Toll-like receptor signaling and subsequent responses: an overview of recent studies. *Alcohol*. 2004b;33:235–239.

Pruett SB, Zheng Q, Schwab C, et al. Sodium methyldithiocarbamate inhibits MAP kinase activation through Toll-like receptor 4, alters cytokine production by mouse peritoneal macrophages, and suppresses innate immunity. *Toxicol Sci*. 2005;87:75–85.

Pyatt DW, Stillman WS, Irons RD. Hydroquinone, a reactive metabolite of benzene, inhibits NF-kappaB in primary human CD4$^+$ T lymphocytes. *Toxicol Appl Pharmacol*. 1998;149:178–184.

Qiu J, Guo X, Chen ZM, et al. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity*. 2013;39:386–399.

Qiu J, Heller JJ, Guo X, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity*. 2012;36:92–104.

Quintana FJ, Basso AS, Iglesias AH, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature*. 2008;453:65–71.

Rachmawati D, Alsalem IW, Bontkes HJ, et al. Innate stimulatory capacity of high molecular weight transition metals Au (gold) and Hg (mercury). *Toxicol In Vitro*. 2015;29:363–369.

Rager JE, Moeller BC, Miller SK, et al. Formaldehyde-associated changes in microRNAs: tissue and temporal specificity in the rat nose, white blood cells, and bone marrow. *Toxicol Sci*. 2014;138:36–46.

Raghavan B, Martin SF, Esser PR, et al. Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO Rep*. 2012;13:1109–1115.

Raisuddin S, Singh KP, Zaidi SI, et al. Immunosuppressive effects of aflatoxin in growing rats. *Mycopathologia.* 1993;124:189–194.

Rao R, Nagarkatti PS, Nagarkatti M. Delta(9) tetrahydrocannabinol attenuates staphylococcal enterotoxin B–induced inflammatory lung injury and prevents mortality in mice by modulation of miR-17–92 cluster and induction of T-regulatory cells. *Br J Pharmacol*. 2015;172:1792–1806.

Ravel G, Descotes J. Popliteal lymph node assay: facts and perspectives. *J Appl Toxicol*. 2005;25:451–458.

Ray A, Prefontaine KE. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci U S A*. 1994;91:752–756.

Regal JF, Greene AL, Regal RR. Mechanisms of occupational asthma: not all allergens are equal. *Environ Health Prev Med*. 2007;12:165–171.

Reichle FM, Conzen PF. Halogenated inhalational anaesthetics. *Best Pract Res Clin Anaesthesiol*. 2003;17:29–46.

Reynolds JL, Mahajan SD, Aalinkeel R, et al. Modulation of the proteome of peripheral blood mononuclear cells from HIV-1-infected patients by drugs of abuse. *J Clin Immunol*. 2009a;29:646–656.

Reynolds JL, Mahajan SD, Aalinkeel R, et al. Proteomic analyses of the effects of drugs of abuse on monocyte-derived mature dendritic cells. *Immunol Investig*. 2009b;38:526–550.

Rezaei N, Hedayat M, Aghamohammadi A, et al. Primary immunodeficiency diseases associated with increased susceptibility to viral infections

and malignancies. *J Allergy Clin Immunol*. 2011;127:1329–1341.e2; quiz 1342–1343.

Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol*. 2011;31:986–1000.

Richeldi L, Sorrentino R, Saltini C. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. *Science*. 1993;262:242–244.

Robinson MK, Babcock LS, Horn PA, et al. Specific antibody responses to subtilisin Carlsberg (Alcalase) in mice: development of an intranasal exposure model. *Fundam Appl Toxicol*. 1996;34:15–24.

Rodgers KE, Ellefson DD. Modulation of respiratory burst activity and mitogenic response of human peripheral blood mononuclear cells and murine splenocytes and peritoneal cells by malathion. *Fundam Appl Toxicol*. 1990;14:309–317.

Rogers TJ. Immunology as it pertains to drugs of abuse, AIDS and the neuroimmune axis: mediators and traffic. *J Neuroimmune Pharmacol*. 2011;6:20–27.

Rose NR. Autoimmunity, autoimmune diseases. In Vohr H-W, ed. *Encyclopedic Reference of Immunotoxicology*. New York: Springer; 2005:79–82.

Rosen F, Cooper M, Wedgewood R. The primary immunodeficiencies. *N Engl J Med*. 1995;333:431–440.

Roth MD, Whittaker KM, Choi R, et al. Cocaine and sigma-1 receptors modulate HIV infection, chemokine receptors, and the HPA axis in the huPBL-SCID model. *J Leukoc Biol*. 2005;78:1198–1203.

Roved J, Westerdahl H, Hasselquist D. Sex differences in immune responses: hormonal effects, antagonistic selection, and evolutionary consequences. *Horm Behav*. 2017;88:95–105.

Rowe AM, Brundage KM, Schafer R, et al. Immunomodulatory effects of maternal atrazine exposure on male Balb/c mice. *Toxicol Appl Pharmacol*. 2006;214:69–77.

Roy S, Chapin RB, Cain KJ, et al. Morphine inhibits transcriptional activation of IL-2 in mouse thymocytes. *Cell Immunol*. 1997;179:1–9.

Roy S, Ninkovic J, Banerjee S, et al. Opioid drug abuse and modulation of immune function: consequences in the susceptibility to opportunistic infections. *J Neuroimmune Pharmacol*. 2011;6(4):442–65.

Ryan-Borchers TA, Park JS, Chew BP, et al. Soy isoflavones modulate immune function in healthy postmenopausal women. *Am J Clin Nutr*. 2006;83:1118–1125.

Ryman-Rasmussen JP, Cesta MF, Brody AR, et al. Inhaled carbon nanotubes reach the subpleural tissue in mice. *Nat Nanotechnol*. 2009;4:747–751.

Sabers CJ, Martin MM, Brunn GJ, et al. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J Biol Chem*. 1995;270:815–822.

Sacerdote P: Effects of in vitro and in vivo opioids on the production of IL-12 and IL-10 by murine macrophages. *Ann N Y Acad Sci*. 2003;992:129–140.

Sachdeva N, Asthana V, Brewer TH, et al. Impaired restoration of plasmacytoid dendritic cells in HIV-1-infected patients with poor CD4 T cell reconstitution is associated with decrease in capacity to produce IFN-alpha but not proinflammatory cytokines. *J Immunol*. 2008;181:2887–2897.

Sadakane K, Takano H, Ichinose T, et al. Formaldehyde enhances mite allergen-induced eosinophilic inflammation in the murine airway. *J Environ Pathol Toxicol Oncol*. 2002;21:267–276.

Saini Y, Greenwood KK, Merrill C, et al. Acute cobalt-induced lung injury and the role of hypoxia-inducible factor 1alpha in modulating inflammation. *Toxicol Sci*. 2010a;116:673–681.

Saini Y, Kim KY, Lewandowski R, et al. Role of hypoxia-inducible factor 1{alpha} in modulating cobalt-induced lung inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2010b;298:L139–L147.

Sakaguchi S, Wing K, Onishi Y, et al. Regulatory T cells: how do they suppress immune responses? *Int Immunol*. 2009;21:1105–1111.

Salem ML, Hossain MS, Nomoto K. Mediation of the immunomodulatory effect of beta-estradiol on inflammatory responses by inhibition of recruitment and activation of inflammatory cells and their gene expression of TNF-alpha and IFN-gamma. *Int Arch Allergy Immunol*. 2000;121:235–245.

Salisbury RL, Sulentic CE. The AhR and NF-kappaB/Rel proteins mediate the inhibitory effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the 3′ immunoglobulin heavy chain regulatory region. *Toxicol Sci*. 2015;148:443–459.

Sanders VM, Tucker AN, White KL Jr, et al. Humoral and cell-mediated immune status in mice exposed to trichloroethylene in the drinking water. *Toxicol Appl Pharmacol*. 1982;62:358–368.

Sanders VM, White KL Jr, Shopp GM Jr, et al. Humoral and cell-mediated immune status of mice exposed to 1,1,2-trichloroethane. *Drug Chem Toxicol*. 1985;8:357–372.

Sandy P, Ventura A, Jacks T. Mammalian RNAi: a practical guide. *Biotechniques*. 2005;39:215–224.

Sarvaria A, Basar R, Mehta RS, et al. IL-10+ regulatory B cells are enriched in cord blood and may protect against cGVHD after cord blood transplantation. *Blood*. 2016;128:1346–1361.

Sasseville VG, Diters RW. Impact of infections and normal flora in nonhuman primates on drug development. *ILAR J*. 2008;49:179–190.

Satpathy AT, Wu X, Albring JC, et al. Re(de)fining the dendritic cell lineage. *Nat Immunol*. 2012;13:1145–1154.

Sattar A, Xie S, Hafeez MA, et al. Metabolism and toxicity of arsenicals in mammals. *Environ Toxicol Pharmacol*. 2016;48:214–224.

Saurat JH, Kaya G, Saxer-Sekulic N, et al. The cutaneous lesions of dioxin exposure: lessons from the poisoning of Victor Yushchenko. *Toxicol Sci*. 2012;125:310–317.

Saurer TB, Carrigan KA, Ijames SG, et al. Morphine-induced alterations of immune status are blocked by the dopamine D2-like receptor agonist 7-OH-DPAT. *J Neuroimmunol*. 2004;148:54–62.

Saurer TB, Carrigan KA, Ijames SG, et al. Suppression of natural killer cell activity by morphine is mediated by the nucleus accumbens shell. *J Neuroimmunol*. 2006;173:3–11.

Sawai C, Anderson K, Walser-Kuntz D. Effect of bisphenol A on murine immune function: modulation of interferon-gamma, IgG2a, and disease symptoms in NZB X NZW F1 mice. *Environ Health Perspect*. 2003;111:1883–1887.

Sayan M, Mossman BT. The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases. *Part Fibre Toxicol*. 2016;13:51.

Schatz AR, Koh WS, Kaminski NE. Delta 9-tetrahydrocannabinol selectively inhibits T-cell dependent humoral immune responses through direct inhibition of accessory T-cell function. *Immunopharmacology*. 1993;26:129–137.

Scheinman RI, Cogswell PC, Lofquist AK, et al. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science*. 1995;270:283–286.

Scheuchenzuber WJ, Eskew ML, Zarkower A. Effects of prolonged inhalation of silica and olivine dusts on immune functions in the mouse. *Environ Res*. 1985;38:389–399.

Schiraldi M, Monestier M. How can a chemical element elicit complex immunopathology? Lessons from mercury-induced autoimmunity. *Trends Immunol*. 2009;30:502–509.

Schmidt M, Raghavan B, Muller V, et al. Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol*. 2010;11:814–819.

Schneider C, Teufel A, Yevsa T, et al. Adaptive immunity suppresses formation and progression of diethylnitrosamine-induced liver cancer. *Gut*. 2012;61:1733–1743.

Schneider D, Manzan MA, Crawford RB, et al. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) –mediated impairment of B cell differentiation involves dysregulation of paired box 5 (Pax5) isoform, Pax5a. *J Pharmacol Exp Ther*. 2008;326:463–474.

Schneider D, Manzan MA, Yoo BS, et al. Involvement of Blimp-1 and AP-1 dysregulation in the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated suppression of the IgM response by B cells. *Toxicol Sci*. 2009;108:377–388.

Schule R, Umesono K, Mangelsdorf DJ, et al. Jun–Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell*. 1990;61:497–504.

Schwartz T. 25 Years of UV-induced immunosuppression mediated by T cells—from disregarded T suppressor cells to highly respected regulatory T cells. *Photochem Photobiol*. 2008;84:10–18.

Sciullo EM, Vogel CF, Li W, et al. Initial and extended inflammatory messages of the nongenomic signaling pathway of the TCDD-activated Ah receptor in U937 macrophages. *Arch Biochem Biophys*. 2008;480:143–155.

Sekizawa J. Low-dose effects of bisphenol A: a serious threat to human health? *J Toxicol Sci*. 2008;33:389–403.

Selb R, Wal JM, Moreno FJ, et al. Assessment of endogenous allergenicity of genetically modified plants exemplified by soybean : where do we stand? *Food Chem Toxicol*. 2017;101:139–148.

Selgrade MK. Immunotoxicity: the risk is real. *Toxicol Sci*. 2007;100:328–332.

Selgrade MK, Cooper GS, Germolec DR, et al. Linking environmental agents and autoimmune disease: an agenda for future research. *Environ Health Perspect*. 1999;107(suppl 5):811–813.

Selgrade MK, Lemanske RF Jr, Gilmour MI, et al. Induction of asthma and the environment: what we know and need to know. *Environ Health Perspect*. 2006;114:615–619.

Selmi C, Leung PS, Sherr DH, et al. Mechanisms of environmental influence on human autoimmunity: a National Institute of Environmental Health Sciences expert panel workshop. *J Autoimmun*. 2012;39:272–284.

Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. *Nat Immunol*. 2005;6:1191–1197.

Seubert A, Calabro S, Santini L, et al. Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88. *Proc Natl Acad Sci U S A*. 2011;108:11169–11174.

Sgro C. Side-effects of a monoclonal antibody, muromonab CD3/orthoclone OKT3: bibliographic review. *Toxicology*. 1995;105:23–29.

Shah D, Chowdhury MM. Rubber allergy. *Clin Dermatol*. 2011;29:278–286.

Shapiro HM. *Practical Flow Cytometry*. Hoboken, NJ: Wiley; 2003.

Shavit Y, Depaulis A, Martin FC, et al. Involvement of brain opiate receptors in the immune-suppressive effect of morphine. *Proc Natl Acad Sci U S A*. 1986;83:7114–7117.

Shelnutt SR, Goad P, Belsito DV. Dermatological toxicity of hexavalent chromium. *Crit Rev Toxicol*. 2007;37:375–387.

Sherr DH, Monti S. The role of the aryl hydrocarbon receptor in normal and malignant B cell development. *Semin Immunopathol*. 2013;35:705–716.

Sherwood RL, O'Shea W, Thomas PT, et al. Effects of inhalation of ethylene dichloride on pulmonary defenses of mice and rats. *Toxicol Appl Pharmacol*. 1987;91:491–496.

Shim GJ, Gherman D, Kim HJ, et al. Differential expression of oestrogen receptors in human secondary lymphoid tissues. *J Pathol*. 2006;208:408–414.

Shim GJ, Kis LL, Warner M, et al. Autoimmune glomerulonephritis with spontaneous formation of splenic germinal centers in mice lacking the estrogen receptor alpha gene. *Proc Natl Acad Sci U S A*. 2004;101:1720–1724.

Shimomura K. The value of juvenile animal studies: a Japanese industry perspective. *Birth Defects Res B Dev Reprod Toxicol*. 2011;92:266–268.

Shirakawa T, Kusaka Y, Morimoto K. Specific IgE antibodies to nickel in workers with known reactivity to cobalt. *Clin Exp Allergy*. 1992;22:213–218.

Shock A, Burkly L, Wakefield I, et al. CDP7657, an anti-CD40L antibody lacking an Fc domain, inhibits CD40L-dependent immune responses without thrombotic complications: an in vivo study. *Arthritis Res Ther*. 2015;17:234.

Shopp GM Jr, Sanders VM, White KL Jr, et al. Humoral and cell-mediated immune status of mice exposed to trans-1,2-dichloroethylene. *Drug Chem Toxicol*. 1985;8:393–407.

Sido JM, Jackson AR, Nagarkatti PS, et al. Marijuana-derived Delta-9-tetrahydrocannabinol suppresses Th1/Th17 cell-mediated delayed-type hypersensitivity through microRNA regulation. *J Mol Med (Berl)*. 2016;94:1039–1051.

Sikorski EE, Burns LA, Stern ML, et al. Splenic cell targets in gallium arsenide-induced suppression of the primary antibody response. *Toxicol Appl Pharmacol*. 1991;110:129–142.

Simet SM, Sisson JH. Alcohol's effects on lung health and immunity. *Alcohol Res*. 2015;37:199–208.

Simon JC, Cruz PD Jr, Bergstresser PR, et al. Low-dose ultraviolet B–irradiated Langerhans cells preferentially activate $CD4^+$ cells of the T helper 2 subset. *J Immunol*. 1990;145:2087–2091.

Simones T, Shepherd DM. Consequences of AhR activation in steady-state dendritic cells. *Toxicol Sci*. 2011;119:293–307.

Singer BA, Fingolimod for the treatment of relapsing multiple sclerosis. Expert Review of Neurotherapeutics. 2014;13:6, 589–602.

Singh AK, Parashar A, Singh AK, et al. Pre-natal/juvenile chlorpyrifos exposure associated with immunotoxicity in adulthood in Swiss albino mice. *J Immunotoxicol*. 2013;10:141–149.

Singh NP, Singh UP, Singh B, et al. Activation of aryl hydrocarbon receptor (AhR) leads to reciprocal epigenetic regulation of FoxP3 and IL-17 expression and amelioration of experimental colitis. *PLoS One*. 2011;6:e23522.

Sleijffers A, Garssen J, Vos JG, et al. Ultraviolet light and resistance to infectious diseases. *J Immunol*. 2004;1:3–14.

Smialowicz RJ, DeVito MJ, Williams WC, et al. Relative potency based on hepatic enzyme induction predicts immunosuppressive effects of a mixture of PCDDS/PCDFS and PCBS. *Toxicol Appl Pharmacol*. 2008;227:477–484.

Smith DC, Smith MJ, White KL. Systemic immunosuppression following a single pharyngeal aspiration of 1,2:5,6-dibenzanthracene in female B6C3F1 mice. *J Immunotoxicol*. 2010;7:219–231.

Song MM, Fang S, Tanaka S, et al. Effects of arsenic disulfide on proliferation, cytokine production, and frequencies of CD4(+), CD8(+), and regulatory T cells in mitogen-activated human peripheral blood mononuclear cells. *Int Immunopharmacol*. 2015;29:832–838.

Spits H, Artis D, Colonna M, et al. Innate lymphoid cells—a proposal for uniform nomenclature. *Nat Rev Immunol*. 2013;13:145–149.

Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol*. 2011;12:21–27.

Springs AE, Karmaus PW, Crawford RB, et al. Effects of targeted deletion of cannabinoid receptors CB1 and CB2 on immune competence and sensitivity to immune modulation by Delta9-tetrahydrocannabinol. *J Leukoc Biol*. 2008;84:1574–1584.

Spyker-Cranmer JM, Barnett JB, Avery DL, et al. Immunoteratology of chlordane: cell-mediated and humoral immune responses in adult mice exposed in utero. *Toxicol Appl Pharmacol*. 1982;62:402–408.

Sreevidya CS, Khaskhely NM, Fukunaga A, et al. Inhibition of photocarcinogenesis by platelet-activating factor or serotonin receptor antagonists. *Cancer Res*. 2008;68:3978–3984.

Sriram U, Hill BL, Cenna JM, et al. Impaired subset progression and polyfunctionality of T cells in mice exposed to methamphetamine during chronic LCMV infection. *PLoS One*. 2016;11:e0164966.

Stanulis ED, Jordan SD, Rosecrans JA, et al. Disruption of Th1/Th2 cytokine balance by cocaine is mediated by corticosterone. *Immunopharmacology*. 1997a;37:25–33.

Stanulis ED, Matulka RA, Jordan SD, et al. Role of corticosterone in the enhancement of the antibody response after acute cocaine administration. *J Pharmacol Exp Ther*. 1997b;280:284–291.

Starec M, Rouveix B, Sinet M, et al. Immune status and survival of opiate- and cocaine-treated mice infected with Friend virus. *J Pharmacol Exp Ther*. 1991;259:745–750.

Steerenberg P, Verlaan A, De Klerk A, et al. Sensitivity to ozone, diesel exhaust particles, and standardized ambient particulate matter in rats with a listeria monocytogenes-induced respiratory infection. *Inhal Toxicol*. 2004;16:311–317.

Steerenberg PA, Garssen J, van Bree L, et al. Ozone alters T-helper cell mediated bronchial hyperreactivity and resistance to bacterial infection. *Exp Toxicol Pathol*. 1996;48:497–499.

Stevenson SE, Woods CA, Hong B, et al. Environmental effects on allergen levels in commercially grown non-genetically modified soybeans: assessing variation across north america. *Front Plant Sci*. 2012;3:196.

Stewart J. Developmental toxicity testing of monoclonal antibodies: an enhanced pre- and postnatal study design option. *Reprod Toxicol*. 2009;28:220–225.

Stiegel MA, Pleil JD, Sobus JR, et al. Inflammatory cytokines and white blood cell counts response to environmental levels of diesel exhaust and ozone inhalation exposures. *PLoS One*. 2016;11:e0152458.

Stoermer KA, Morrison TE. Complement and viral pathogenesis. *Virology*. 2011;411:362–373.

Stuve O, Marra CM, Jerome KR, et al. Immune surveillance in multiple sclerosis patients treated with natalizumab. *Ann Neurol*. 2006;59:743–747.

Suen JL, Hsu SH, Hung CH, et al. A common environmental pollutant, 4-nonylphenol, promotes allergic lung inflammation in a murine model of asthma. *Allergy*. 2013;68:780–787.

Sulentic CE, Holsapple MP, Kaminski NE. Putative link between transcriptional regulation of IgM expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and the aryl hydrocarbon receptor/dioxin-responsive enhancer signaling pathway. *J Pharmacol Exp Ther*. 2000;295:705–716.

Sulentic CE, Kaminski NE. The long winding road toward understanding the molecular mechanisms for B-cell suppression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol Sci*. 2011;120(suppl 1):S171–S191.

Sulentic CE, Kang JS, Na YJ, et al. Interactions at a dioxin responsive element (DRE) and an overlapping kappaB site within the hs4 domain of the 3′alpha immunoglobulin heavy chain enhancer. *Toxicology*. 2004a;200:235–246.

Sulentic CE, Zhang W, Na YJ, et al. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, an exogenous modulator of the 3′alpha immunoglobulin heavy chain enhancer in the CH12.LX mouse cell line. *J Pharmacol Exp Ther*. 2004b;309:71–78.

Suntharalingam G, Perry MR, Ward S, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med*. 2006;355:1018–1028.

Svenson JL, EuDaly J, Ruiz P, et al. Impact of estrogen receptor deficiency on disease expression in the NZM2410 lupus prone mouse. *Clin Immunol*. 2008;128:259–268.

Szabo G, Saha B. Alcohol's effect on host defense. *Alcohol Res*. 2015;37:159–170.

Taggart BR, Harrington P, Hollingshead M. HIV hollow fiber SCID model for antiviral therapy comparison with SCID/hu model. *Antiviral Res*. 2004;63:1–6.

Takenaka H, Zhang K, Diaz-Sanchez D, et al. Enhanced human IgE production results from exposure to the aromatic hydrocarbons from diesel exhaust: direct effects on B-cell IgE production. *J Allergy Clin Immunol*. 1995;95:103–115.

Takeuchi S, Iida M, Yabushita H, et al. In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450–1A induction by propanil, diuron and linuron. *Chemosphere*. 2008;74:155–165.

Talal N. Sex steroid hormones and systemic lupus erythematosus. *Arthritis Rheum*. 1981;24:1054–1056.

Tartaro K, VanVolkenburg M, Wilkie D, et al. Development of a fluorescence-based in vivo phagocytosis assay to measure mononuclear phagocyte system function in the rat. *J Immunotoxicol*. 2015;12:239–246.

Tassinari MS, Benson K, Elayan I, et al. Juvenile animal studies and pediatric drug development retrospective review: use in regulatory decisions and labeling. *Birth Defects Res B Dev Reprod Toxicol*. 2011;92:261–265.

Taylor AD, Christian HC, Morris JF, et al. An antisense oligodeoxynucleotide to lipocortin 1 reverses the inhibitory actions of dexamethasone on the release of adrenocorticotropin from rat pituitary tissue in vitro. *Endocrinology*. 1997;138:2909–2918.

Taylor DD, Akyol S, Gercel-Taylor C. Pregnancy-associated exosomes and their modulation of T cell signaling. *J Immunol*. 2006;176:1534–1542.

ten Tusscher GW, Steerenberg PA, van Loveren H, et al. Persistent hematologic and immunologic disturbances in 8-year-old Dutch children associated with perinatal dioxin exposure. *Environ Health Perspect*. 2003;111:1519–1523.

Terada N, Lucas JJ, Szepesi A, et al. Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G1 phase of the cycle. *J Cell Physiol*. 1993;154:7–15.

Thomas E, Brewster DH, Black RJ, et al. Risk of malignancy among patients with rheumatic conditions. *Int J Cancer*. 2000;88:497–502.

Thomas P, Braathen LR, Dorig M, et al. Increased metal allergy in patients with failed metal-on-metal hip arthroplasty and peri-implant T-lymphocytic inflammation. *Allergy*. 2009;64:1157–1165.

Thomas P, Fugmann R, Aranyi C, et al. The effect of dimethylnitrosamine on host resistance and immunity. *Toxicol Appl Pharmacol*. 1985a;77:219–229.

Thomas PT, Ratajczak HV, Aranyi C, et al. Evaluation of host resistance and immune function in cadmium-exposed mice. *Toxicol Appl Pharmacol*. 1985b;80:446–456.

Thompson CM, Grafstrom RC. Mechanistic considerations for formaldehyde-induced bronchoconstriction involving *S*-nitrosoglutathione reductase. *J Toxicol Environ Health A*. 2008;71:244–248.

Thompson JK, Westbom CM, MacPherson MB, et al. Asbestos modulates thioredoxin–thioredoxin interacting protein interaction to regulate inflammasome activation. *Part Fibre Toxicol*. 2014;11:24.

Thomsen M, Yacoub-Youssef H, Marcheix B. Reconstitution of a human immune system in immunodeficient mice: models of human alloreaction in vivo. *Tissue Antigens*. 2005;66:73–82.

Thorne PS, Hawk C, Kaliszewski SD, et al. The noninvasive mouse ear swelling assay. I. Refinements for detecting weak contact sensitizers. *Fundam Appl Toxicol*. 1991;17:790–806.

Thrasher JD, Heuser G, Broughton A. Immunological abnormalities in humans chronically exposed to chlorpyrifos. *Arch Environ Health*. 2002;57:181–187.

Thurmond LM, Dean JH. Immunological responses following inhalation exposure to chemical hazards. In: Gardner DE, Crapo JD, Massaro EJ, eds. *Toxicology of the Lung*. New York: Raven Press; 1988:375–406.

Thyssen JP, Menne T. Metal allergy—a review on exposures, penetration, genetics, prevalence, and clinical implications. *Chem Res Toxicol*. 2010;23:309–318.

Togias A, Cooper SF, Acebal ML, et al. Addendum guidelines for the prevention of peanut allergy in the United States: report of the National Institute of Allergy and Infectious Diseases–sponsored expert panel. *J Allergy Clin Immunol*. 2017;139:29–44.

Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell*. 2015;27:450–461.

Totoritis MC, Tan EM, McNally EM, et al. Association of antibody to histone complex H2A-H2B with symptomatic procainamide-induced lupus. *N Engl J Med*. 1988;318:1431–1436.

Trifari S, Kaplan CD, Tran EH, et al. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol*. 2009;10:864–871.

Trigunaite A, Dimo J, Jorgensen TN. Suppressive effects of androgens on the immune system. *Cell Immunol*. 2015;294:87–94.

Tryphonas H, Bondy G, Hodgen M, et al. Effects of *cis*-nonachlor, *trans*-nonachlor and chlordane on the immune system of Sprague–Dawley rats following a 28-day oral (gavage) treatment. *Food Chem Toxicol*. 2003;41:107–118.

Tsugita M, Morimoto N, Tashiro M, et al. SR-B1 is a silica receptor that mediates canonical inflammasome activation. *Cell Rep*. 2017;18:1298–1311.

Udoji F, Martin T, Etherton R, et al. Immunosuppressive effects of triclosan, nonylphenol, and DDT on human natural killer cells in vitro. *J Immunotoxicol*. 2010;7:205–212.

Ullrich SE. Mechanisms by which ultraviolet radiation, a ubiquitous environmental toxin, suppresses the immune response. In: Luebke RW, House R, Kimber I, eds. *Immunotoxicology and Immunopharmacology*. Boca Raton, FL: CRC Press; 2007a.

Ullrich SE. Sunlight and skin cancer: lessons from the immune system. *Mol Carcinog*. 2007b;46:629–633.

Ullrich SE, Byrne SN. The immunologic revolution: photoimmunology. *J Invest Dermatol*. 2012;132:896–905.

Valstar DL, Schijf MA, Arts JH, et al. Alveolar macrophages suppress nonspecific inflammation caused by inhalation challenge with trimellitic anhydride conjugated to albumin. *Arch Toxicol*. 2006;80:561–571.

van Grevenynghe J, Rion S, Le Ferrec E, et al. Polycyclic aromatic hydrocarbons inhibit differentiation of human monocytes into macrophages. *J Immunol*. 2003;170:2374–2381.

van Grevenynghe J, Sparfel L, Le Vee M, et al. Cytochrome P450–dependent toxicity of environmental polycyclic aromatic hydrocarbons towards human macrophages. *Biochem Biophys Res Commun*. 2004;317:708–716.

Van Loveren H, Krajnc EI, Rombout PJ, et al. Effects of ozone, hexachlorobenzene, and bis(tri-*n*-butyltin)oxide on natural killer activity in the rat lung. *Toxicol Appl Pharmacol*. 1990;102:21–33.

Van Loveren H, Piersma A. Immunotoxicological consequences of perinatal chemical exposures. *Toxicol Lett*. 2004;149:141–145.

Van Loveren H, Van Amsterdam JG, Vandebriel RJ, et al. Vaccine-induced antibody responses as parameters of the influence of endogenous and environmental factors. *Environ Health Perspect*. 2001;109:757–764.

Vanaudenaerde BM, Verleden SE, Vos R, et al. Innate and adaptive interleukin-17-producing lymphocytes in chronic inflammatory lung disorders. *Am J Respir Crit Care Med*. 2011;183:977–986.

Vandenberg LN, Maffini MV, Sonnenschein C, et al. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev*. 2009;30:75–95.

Vandenplas O, Froidure A, Meurer U, et al. The role of allergen components for the diagnosis of latex-induced occupational asthma. *Allergy*. 2016;71:840–849.

Varvel SA, Bridgen DT, Tao Q, et al. Delta9-tetrahydrocannbinol accounts for the antinociceptive, hypothermic, and cataleptic effects of marijuana in mice. *J Pharmacol Exp Ther*. 2005;314:329–337.

Veldhoen M, Hirota K, Christensen J, et al. Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. *J Exp Med*. 2009;206:43–49.

Veldhoen M, Hirota K, Westendorf AM, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature*. 2008;453:106–109.

Veraldi A, Costantini AS, Bolejack V, et al. Immunotoxic effects of chemicals: a matrix for occupational and environmental epidemiological studies. *Am J Ind Med*. 2006;49:1046–1055.

Vial T, Choquet-Kastylevsky G, Descotes J. Adverse effects of immunotherapeutics involving the immune system. *Toxicology*. 2002;174:3–11.

Vial T, Descotes J. Autoimmune diseases and vaccinations. *Eur J Dermatol*. 2004;14:86–90.

Vial T, Descotes J. Immune-mediated side-effects of cytokines in humans. *Toxicology*. 1995;105:31–57.

Vinuesa CG, Linterman MA, Goodnow CC, et al. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunol Rev*. 2010;237:72–89.

Vivier E, Raulet DH, Moretta A, et al. Innate or adaptive immunity? The example of natural killer cells. *Science*. 2011;331:44–49.

Vogel CF, Goth SR, Dong B, et al. Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochem Biophys Res Commun*. 2008;375:331–335.

Vogel CF, Sciullo E, Wong P, et al. Induction of proinflammatory cytokines and C-reactive protein in human macrophage cell line U937 exposed to air pollution particulates. *Environ Health Perspect*. 2005;113:1536–1541.

Vogel R, Seidle T, Spielmann H. A modular one-generation reproduction study as a flexible testing system for regulatory safety assessment. *Reprod Toxicol*. 2010;29:242–245.

Vorderstrasse BA, Bohn AA, Lawrence BP. Examining the relationship between impaired host resistance and altered immune function in mice treated with TCDD. *Toxicology*. 2003;188:15–28.

Vos JG, de Klerk A, Krajnc EI, et al. Toxicity of bis(tri-*n*-butyltin)oxide in the rat. II. Suppression of thymus-dependent immune responses and of parameters of nonspecific resistance after short-term exposure. *Toxicol Appl Pharmacol*. 1984;75:387–408.

Vos JG, Luster MI. Immune alterations. In: Kimbrough RD, Jensen AA, eds. *Halogenated Biphenyls, Terphenyls, Napthalenes, Dibenzodioxins, and Related Products*. New York: Elsevier; 1989:295–322.

Vrijheid M, Casas M, Gascon M, et al. Environmental pollutants and child health—a review of recent concerns. *Int J Hyg Environ Health*. 2016;219:331–342.

Walters E, Rider V, Abdou NI, et al. Estradiol targets T cell signaling pathways in human systemic lupus. *Clin Immunol*. 2009;133:428–436.

Wang IJ, Karmaus WJ, Yang CC. Polycyclic aromatic hydrocarbons exposure, oxidative stress, and asthma in children. *Int Arch Occup Environ Health*. 2017a;90:297–303.

Wang J, Barke RA, Charboneau R, et al. Morphine negatively regulates interferon-gamma promoter activity in activated murine T cells through two distinct cyclic AMP-dependent pathways. *J Biol Chem*. 2003;278:37622–37631.

Wang J, Barke RA, Charboneau R, et al. Morphine impairs host innate immune response and increases susceptibility to *Streptococcus pneumoniae* lung infection. *J Immunol*. 2005;174:426–434.

Wang L, Wang R, Fan L, et al. Arsenic trioxide is an immune adjuvant in liver cancer treatment. *Mol Immunol*. 2017b;81:118–126.

Wang L, Weng CY, Wang YJ, et al. Lipoic acid ameliorates arsenic trioxide–induced HO-1 expression and oxidative stress in THP-1 monocytes and macrophages. *Chem Biol Interact*. 2011;190:129–138.

Wang P, Pradhan K, Zhong XB, et al. Isoniazid metabolism and hepatotoxicity. *Acta Pharm Sin B*. 2016;6:384–392.

Ward AM, Udnoon S, Watkins J, et al. Immunological mechanisms in the pathogenesis of vinyl chloride disease. *Br Med J*. 1976;1:936–938.

Warheit DB, Hesterberg TW. Asbestos and other fibers in the lung. In: Dean JH, Luster MI, Munson AE, et al., eds. *Immunotoxicology and Immunopharmacology*. New York: Raven Press; 1994:363–376.

Warren TK, Mitchell KA, Lawrence BP. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses the humoral and cell-mediated immune responses to influenza A virus without affecting cytolytic activity in the lung. *Toxicol Sci*. 2000;56:114–123.

Watanabe M, Ishimaru N, Ashrin MN, et al. A novel DC therapy with manipulation of MKK6 gene on nickel allergy in mice. *PLoS One*. 2011;6:e19017.

Watowich SS, Liu YJ. Mechanisms regulating dendritic cell specification and development. *Immunol Rev*. 2010;238:76–92.

Watson ES, Murphy JC, ElSohly HN, et al. Effects of the administration of coca alkaloids on the primary immune responses of mice: interaction with delta 9-tetrahydrocannabinol and ethanol. *Toxicol Appl Pharmacol*. 1983;71:1–13.

Ways SC, Blair PB, Bern HA, et al. Immune responsiveness of adult mice exposed neonatally to diethylstilbestrol, steroid hormones, or vitamin A. *J Environ Pathol Toxicol*. 1980;3:207–220.

Wei C, Mei J, Tang L, et al. 1-Methyl-tryptophan attenuates regulatory T cells differentiation due to the inhibition of estrogen-IDO1-MRC2 axis in endometriosis. *Cell Death Dis*. 2016;7:e2489.

Weinstock D, Lewis DB, Parker GA, et al. Toxicopathology of the developing immune system: investigative and development strategies. *Toxicol Pathol*. 2010;38:1111–1117.

Weisglas-Kuperus N, Patandin S, Berbers GA, et al. Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. *Environ Health Perspect*. 2000;108:1203–1207.

Weisglas-Kuperus N, Sas TC, Koopman-Esseboom C, et al. Immunologic effects of background prenatal and postnatal exposure to dioxins and polychlorinated biphenyls in Dutch infants. *Pediatr Res*. 1995;38:404–410.

Weisglas-Kuperus N, Vreugdenhil HJ, Mulder PG. Immunological effects of environmental exposure to polychlorinated biphenyls and dioxins in Dutch school children. *Toxicol Lett*. 2004;149:281–285.

Wheeler JL, Martin KC, Resseguie E, et al. Differential consequences of two distinct AhR ligands on innate and adaptive immune responses to influenza A virus. *Toxicol Sci*. 2014;137:324–334.

White KL Jr. Specific immune function assays. In: Miller K, Turk JL, Nicklin S, eds. *Principles and Practices of Immunotoxicology*. Oxford, England: Blackwell; 1992:304–323.

White KL, Musgrove DL, Brown RD. The sheep erythrocyte T-dependent antibody response (TDAR). *Methods Mol Biol*. 2010;598:173–184.

White SS, Birnbaum LS. An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2009;27:197–211.

WHO. *IPCS Harmonization Project Document No. 10: Guidance for Immunotoxicity Risk Assessment for Chemicals*. WHO Document Production Services. Washington, DC: WHO; 2012.

Widen C, Gustafsson JA, Wikstrom AC. Cytosolic glucocorticoid receptor interaction with nuclear factor-kappa B proteins in rat liver cells. *Biochem J*. 2003;373:211–220.

Wilkinson ST, Yarnell S, Radhakrishnan R, et al. Marijuana legalization: impact on physicians and public health. *Annu Rev Med*. 2016;67:453–466.

Wiltrout RW, Ercegovich CD, Ceglowski WS. Humoral immunity in mice following oral administration of selected pesticides. *Bull Environ Contam Toxicol*. 1978;20:423–431.

Winans B, Humble MC, Lawrence BP. Environmental toxicants and the developing immune system: a missing link in the global battle against infectious disease? *Reprod Toxicol*. 2011;31:327–336.

Wisnewski AV, Xu L, Robinson E, et al. Immune sensitization to methylene diphenyl diisocyanate (MDI) resulting from skin exposure: albumin as a carrier protein connecting skin exposure to subsequent respiratory responses. *J Occup Med Toxicol*. 2011;6:6.

Wisniewski JA, Commins SP, Agrawal R, et al. Analysis of cytokine production by peanut-reactive T cells identifies residual Th2 effectors in highly allergic children who received peanut oral immunotherapy. *Clin Exp Allergy*. 2015;45:1201–1213.

Wood SC, Holsapple MP. Direct suppression of superantigen-induced IgM secretion in human lymphocytes by 2,3,7,8-TCDD. *Toxicol Appl Pharmacol*. 1993;122:308–313.

Wood SC, Jeong HG, Morris DL, et al. Direct effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on human tonsillar lymphocytes. *Toxicology*. 1993;81:131–143.

Wourms MJ, Sulentic CE. The aryl hydrocarbon receptor regulates an essential transcriptional element in the immunoglobulin heavy chain gene. *Cell Immunol*. 2015;295:60–66.

Xiao F, Chong AS-F, Bartlett RR, et al. A promising immunosuppressant in transplantation. In: Thomas AW, Starzyl TE, eds. *Immunosuppressive Drugs: Developments in Anti-Rejection Therapy*. London, England: Edward Arnold; 1994:203–212.

Xiao X, Chang C. Diagnosis and classification of drug-induced autoimmunity (DIA). *J Autoimmun*. 2014;48–49:66–72.

Xu H, McClain S, Medina S, et al. Differential sensitivities of bone marrow, spleen and thymus to genotoxicity induced by environmentally relevant concentrations of arsenite. *Toxicol Lett*. 2016a;262:55–61.

Xu LZ, Yang LT, Qiu SQ, et al. Combination of specific allergen and probiotics induces specific regulatory B cells and enhances specific immunotherapy effect on allergic rhinitis. *Oncotarget*. 2016b;7(34):54360–54369.

Yang EJ, Lee JS, Yun CY, et al. The pro-apoptotic effect of hydroquinone in human neutrophils and eosinophils. *Toxicol In Vitro*. 2011;25:131–137.

Yang KH, Kim BS, Munson AE, et al. Immunosuppression induced by chemicals requiring metabolic activation in mixed cultures of rat hepatocytes and murine splenocytes. *Toxicol Appl Pharmacol*. 1986;83:420–429.

Yeatts K, Sly P, Shore S, et al. A brief targeted review of susceptibility factors, environmental exposures, asthma incidence, and recommendations for future asthma incidence research. *Environ Health Perspect*. 2006;114:634–640.

Yellayi S, Naaz A, Szewczykowski MA, et al. The phytoestrogen genistein induces thymic and immune changes: a human health concern? *Proc Natl Acad Sci U S A*. 2002;99:7616–7621.

Yeste A, Mascanfroni ID, Nadeau M, et al. IL-21 induces IL-22 production in $CD4^+$ T cells. *Nat Commun*. 2014;5:3753.

Yokogawa N, Vivino FB. Hydralazine-induced autoimmune disease: comparison to idiopathic lupus and ANCA-positive vasculitis. *Mod Rheumatol*. 2009;19:338–347.

Yole M, Wickstrom M, Blakley B. Cell death and cytotoxic effects in YAC-1 lymphoma cells following exposure to various forms of mercury. *Toxicology*. 2007;231:40–57.

Yoo BS, Boverhof DR, Shnaider D, et al. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) alters the regulation of Pax5 in lipopolysaccharide-activated B cells. *Toxicol Sci*. 2004;77:272–279.

Yoshino S, Yamaki K, Li X, et al. Prenatal exposure to bisphenol A upregulates immune responses, including T helper 1 and T helper 2 responses, in mice. *Immunology*. 2004;112:489–495.

Yucesoy B, Johnson VJ, Lummus ZL, et al. Genetic variants in the major histocompatibility complex class I and class II genes are associated with diisocyanate-induced Asthma. *J Occup Environ Med*. 2014;56:382–387.

Yucesoy B, Kashon ML, Johnson VJ, et al. Genetic variants in TNFalpha, TGFB1, PTGS1 and PTGS2 genes are associated with diisocyanate-induced asthma. *J Immunotoxicol*. 2016;13:119–126.

Yun AJ, Lee PY. The link between T helper balance and lymphoproliferative disease. *Med Hypotheses*. 2005;65:587–590.

Yurino H, Ishikawa S, Sato T, et al. Endocrine disruptors (environmental estrogens) enhance autoantibody production by B1 cells. *Toxicol Sci*. 2004;81:139–147.

Zakharenko AM, Engin AB, Chernyshev VV, et al. Basophil mediated pro-allergic inflammation in vehicle-emitted particles exposure. *Environ Res*. 2017;152:308–314.

Zhang J, Zhang J, Liu R, et al. Endocrine-disrupting effects of pesticides through interference with human glucocorticoid receptor. *Environ Sci Technol*. 2016a;50:435–443.

Zhang M, Qi Y, Li H, et al. AIM2 inflammasome mediates arsenic-induced secretion of IL-1 beta and IL-18. *Oncoimmunology*. 2016b;5:e1160182.

Zhang Q, Bhattacharya S, Kline DE, et al. Stochastic modeling of B lymphocyte terminal differentiation and its suppression by dioxin. *BMC Syst Biol*. 2010;4:40.

Zhang Q, Yao Y, Konger RL, et al. UVB radiation–mediated inhibition of contact hypersensitivity reactions is dependent on the platelet-activating factor system. *J Invest Dermatol*. 2008;128:1780–1787.

Zhang X, Wang M, Gao S, et al. Atrazine-induced apoptosis of splenocytes in BALB/C mice. *BMC Med*. 2011;9:117.

Zhang Y, Huo X, Cao J, et al. Elevated lead levels and adverse effects on natural killer cells in children from an electronic waste recycling area. *Environ Pollut*. 2016c;213:143–150.

Zhang Y, Song TT, Cunnick JE, et al. Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. *J Nutr*. 1999;129:399–405.

Zhou C, Tabb MM, Nelson EL, et al. Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest*. 2006;116:2280–2289.

Zhou HR, Islam Z, Pestka JJ. Rapid, sequential activation of mitogen-activated protein kinases and transcription factors precedes proinflammatory cytokine mRNA expression in spleens of mice exposed to the trichothecene vomitoxin. *Toxicol Sci*. 2003;72:130–142.

Zolnik BS, Gonzalez-Fernandez A, Sadrieh N, et al. Nanoparticles and the immune system. *Endocrinology*. 2010;151:458–465.

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chapter 13

Toxic Responses of the Liver

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INTRODUCTION

The liver performs many functions that are critical to life. Among these are the processing of foods and other substances absorbed from the intestinal tract and the subsequent delivery of processed nutrients to other organs in the body. The liver is also an integral contributor of immunity that protects mammals from harmful pathogens. In addition to its immunological roles, it is the main organ where exogenous chemicals are metabolized, a process that hastens their excretion into bile and urine. As a consequence, liver cells are exposed to significant concentrations of these chemicals and their metabolites, some of which can cause liver dysfunction. Many industrial chemicals, plant toxins, environmental pollutants, food-borne agents, herbal remedies, and drugs (both pharmaceutical and recreational) are known to be hepatotoxic. In the pharmaceutical industry, adverse effects on the liver are one of the most frequent reasons for discontinuing the development of drug candidates and withdrawal of drugs from the market (Temple and Himmel, 2002; Stickel et al., 2011).

Although many hepatotoxic chemicals have been identified in the last several decades and much has been learned about mechanisms by which liver toxicity occurs, liver injury from various chemicals continues to present major health concerns. The liver comprises several cell types, each with different functions, and it is nourished by a blood supply that is unique in the body. Knowledge of liver physiology and anatomy not only lends insight as to how the liver functions but also provides the underpinnings for understanding how hepatotoxicants cause liver dysfunction. Therefore, our journey begins with an overview of liver anatomy and physiology. Next, the repertoire of hepatic responses to toxic insult and the various ways of classifying and monitoring liver injury are discussed. Chemical-induced liver injury is typically initiated by one or more critical events, such as formation of a toxic metabolite, which trigger intracellular responses that can progress to dysfunction or death of hepatic parenchymal cells (i.e., HPCs, hepatocytes). These intrahepatocellular events can in turn prompt secondary events involving activation of nonparenchymal cells that magnify or attenuate the initial injury. These events typically determine whether injury

progresses to liver failure or to repair of damage. Mechanisms by which such intra- and extrahepatocellular events occur will be discussed in the context of examples of specific chemicals that cause liver injury. Finally, environmental and genetic factors that influence risk of hepatotoxicity from chemical exposure and how these factors determine individual sensitivity to intoxication will be considered.

In this chapter, reference is made to appropriate research and review articles that expand on specific topics that are discussed. To avoid unnecessary duplication, the reader is also referred to other chapters in this volume that treat liver-related topics such as biotransformation of chemicals (Chap. 6), hepatocarcinogenesis (Chap. 8), the importance of exposure and dose–response (Chap. 3), immune cells and mediators (Chap. 12), and pathways to cell death (Chap. 3). Drug- and chemical-induced liver injury has been discussed in detail in several monographs, reviews, and books that can provide interesting supplemental reading (Zimmerman, 1999; Jaeschke et al., 2002, 2012a, 2012b; Kaplowitz and DeLeve, 2002; Boyer et al., 2006b; Grattagliano et al., 2009; Jones et al., 2010; Roth and Ganey, 2010a).

LIVER ANATOMY AND PHYSIOLOGY

Hepatic Functional Anatomy

Livers of mammals typically have two (humans) or more (rodents) lobes into which blood vessels enter and exit. The liver is unusual among organs in that it has a dual blood supply (Fig. 13-1). As is typical of other organs, the liver has an arterial supply via the hepatic artery, which provides a minority of blood entering the liver (about 1/3 in humans, less in rodents). The major blood supply to the liver arises from the hepatic portal vein, which comprises venous drainage from the stomach and intestine. This unique anatomy positions the liver to have first contact with food-borne xenobiotic agents absorbed into the blood from the gastrointestinal (GI) tract, but it also means that the liver receives blood from which much oxygen has been removed after nourishing the GI tract.

These two blood supplies in part determine how liver is organized into smaller anatomical substructures within each lobe. Two concepts exist for organization of operational units, termed either "lobules" or "acini" (McCuskey, 2006b). According to the classical lobular concept, the liver is organized into hexagonal lobules that

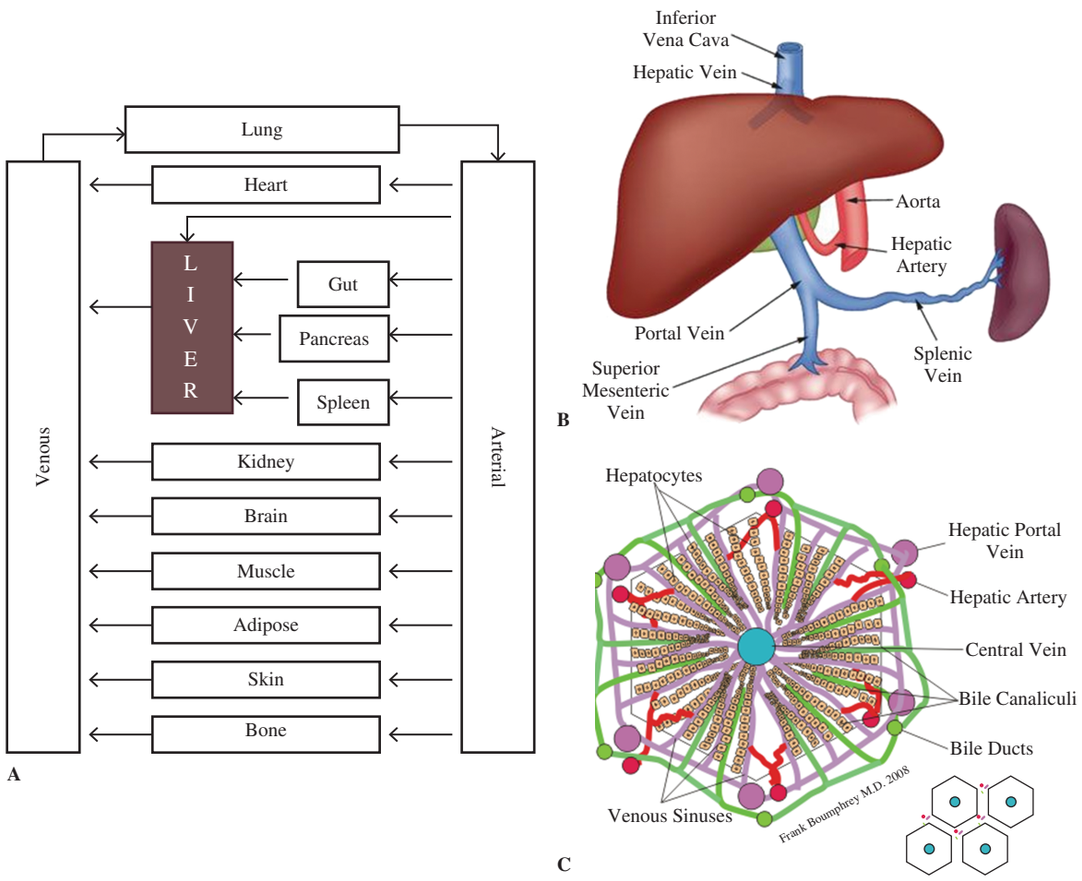


Figure 13-1. *Liver circulation and lobular organization.* (**A**) Physiologically based model of the body; the liver is unusual among organs in having a dual blood supply, one from arterial blood and the other from blood that drains the gastrointestinal tract. (Adapted from Kuo and Akpa [2013].) (**B**) Blood enters the liver from the hepatic artery and from the portal vein, which arises from gastrointestinal and splenic drainage. (From http://basicmedicalkey.com/gastrointestinal-physiology-2/.) (**C**) The structure of the liver lobule. Inflow blood from hepatic arterioles and portal venules mix as they enter the sinusoids of the lobule; blood leaving the sinusoids enters central veins that drain into the vena cava. (From http://amedleyofpotpourri.blogspot.com/2015/03/liver.html.)

are more or less apparent histologically. Each lobule is oriented around a central vein (also known as a terminal hepatic venule). At the corners of the lobule are portal triads (also known as portal tracts). As the name implies, each of these contains a branch of the portal vein (portal venule), a hepatic arteriole, and one or more small bile ducts (Figs. 13-1C and 13-2). The capillary equivalents in liver lobules are called "sinusoids." Blood entering the portal tract via the portal vein and hepatic artery is mixed in the penetrating vessels, enters the sinusoids, and percolates along the chords of parenchymal cells (HPCs). The blood then collects into terminal hepatic venules (central veins), which coalesce to form the hepatic vein that connects to the vena cava (Figs. 13-1B and 13-2). Keeping in mind this general structure, the lobule is viewed as having three regions known as periportal (nearest portal triad), centrilobular (surrounding the central vein), and midzonal (between periportal and centrilobular).

A somewhat different way of viewing the functional unit of the liver is the acinus. This concept is preferred from the standpoint that it better reflects the manner in which blood flows into the sinusoids; that is, some of the blood entering via the portal venule and hepatic arteriole mixes, then some flows laterally (between portal triads) before entering the sinusoid. The terminal branches of the portal vein and hepatic artery form the base of the acinus, which has three zones: zone 1 is closest to the entry of blood (i.e., cells near the portal triad), zone 3 abuts the central vein, and zone 2 is in between (Figs. 13-2 and 13-3). These zones correspond roughly to periportal, centrilobular, and midzonal areas of the classical lobule, respectively, but more closely align with the manner in which blood is delivered to the sinusoids.

Despite the greater functional accuracy of the acinar concept, lobular terminology is still used to describe location of pathological lesions of hepatic parenchyma. Acinar/lobular zonation is of considerable functional consequence regarding gradients of components both in blood and in HPCs (Jungermann and Kietzmann, 2000). For example, as noted above blood entering the acinus comprises mostly blood from the portal vein that is poorly oxygenated relative to the blood entering from the hepatic artery. Enroute to the central vein, oxygen rapidly leaves the blood to meet the high metabolic demands of the HPCs. Therefore, HPCs in zone 3 are exposed to substantially smaller concentrations of oxygen than those in zone 1. These anatomical substructures are also important because liver lesions caused by chemical exposure usually appear preferentially in one of them.

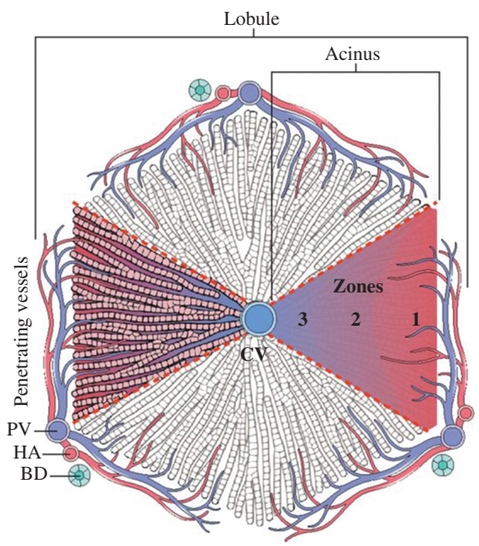


Figure 13-2. *Comparison of lobule and acinus models of liver functional units.* The acinar model reflects how blood enters the sinusoids. Zone 1 is closest to the inflow vessels, zone 3 is closest to the central venule drainage, and zone 2 is intermediate. (From https://www.slideshare.net/mannanrifat/histology-of-normal-liver; see this source for more on liver anatomy.)

The sinusoids are endothelium-lined channels between cords of HPCs through which blood flows on its way to the central vein. The sinusoidal microvasculature of the liver differs in important ways from capillaries in other organs. Sinusoids are larger and more irregular than typical capillaries. Three major types of cells in the sinusoids are sinusoidal endothelial cells (SECs), Kupffer cells, and stellate cells (Fig. 13-3) (McCuskey, 2006b, 2008). In addition, the liver contains dendritic cells and a substantial number of lymphocytes, especially natural killer (NK) and NKT cells (Gao et al., 2009). Each of these nonparenchymal cell types performs important functions (Heymann and Tacke, 2016; Xing et al., 2016).

Liver Cells and Their Functions

The liver's location in the circulation between the intestinal tract and the rest of the body facilitates its performance as a primary contributor to maintaining metabolic homeostasis of the body. Because venous blood from the stomach and intestine flows into the portal vein and then through the liver before entering the systemic circulation, it is the first organ to encounter ingested nutrients, vitamins, drugs, and environmental toxicants, as well as bacterial products that enter the portal blood after being absorbed in the gastrointestinal tract. Efficient scavenging or uptake processes extract these absorbed materials from the blood for processing, storage, and/or excretion into bile.

Hepatic Parenchymal Cells Hepatic parenchymal cells (HPCs), also known as hepatocytes, are large epithelial cells that account for about 60% of the cells in the liver and about 80% of the liver volume. These cells occur in cords along the microvasculature of the liver (Fig. 13-3), with microvilli on their basal surface in apposition with the endothelium (Fig. 13-4). The apical aspect of these cells is in contact with neighboring HPCs, which are joined by tight junctions. Here a channel, or canaliculus, is formed into which bile is secreted by HPCs (Fig. 13-3); this bile ultimately flows into bile ducts and from there into the small intestine.

HPCs perform most of the many critical metabolic functions of the liver (Table 13-1). They process dietary carbohydrates by converting monosaccharides into energy through glycolysis and mitochondrial metabolism. HPCs are also involved in anabolic processes, such as the synthesis of glycogen for carbohydrate storage. They respond to energy needs of other organs by releasing stored glucose into the circulation. Similarly, HPCs are a central hub for the regulation of lipids. They are capable of synthesizing lipids and removing them from the blood. HPCs can then process these lipids for energy production or for export into the circulation as lipoproteins for use by other organs. HPCs are also the source of many circulating proteins, such as plasma albumin and other binding proteins, coagulation factors, and complement proteins. In addition, HPCs detoxify circulating substances produced during metabolism by other organs. For example, ammonia is a toxic product of protein catabolism, and HPCs detoxify it by forming urea, which is eliminated by the kidneys. They also synthesize heme and are involved with the reutilization of iron. Additionally, HPCs synthesize bile acids that aid in absorption and processing of dietary lipids.

Another important function of these cells is clearing the circulation of many endogenous, biologically active substances

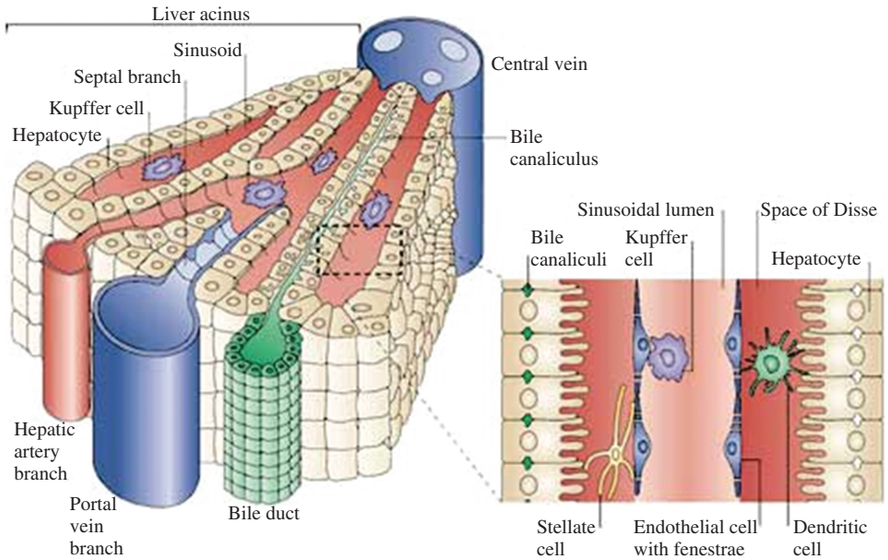


Figure 13-3. *Organization of cells in the hepatic acinus.* The insert on the right shows the position of major cells within the sinusoid. (From Adams and Eksteen [2006].)

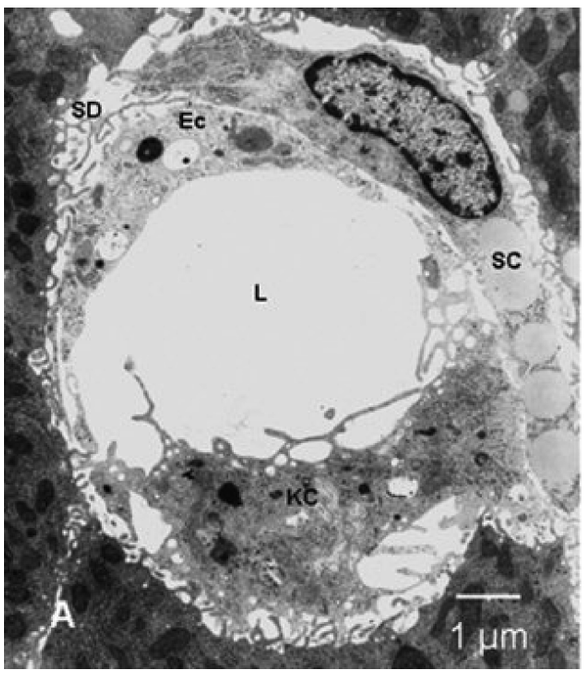


Figure 13-4. *Transmission electron micrograph of cross section of a liver sinusoid.* Hepatic parenchymal cells surround the sinusoid (A). Note the hepatocellular microvilli that form the space of Disse (SD) and contact the fenestrated sinusoidal endothelium (Ec). A Kupffer cell (KC) resides on the luminal (L) aspect of the endothelial cell and has projections that contact a hepatocyte. In the space of Disse at the top of the field is a stellate cell (SC) that contains lipid droplets. (From Wisse et al. [1996].)

(e.g., hormones, neurotransmitters, steroids) as well as xenobiotic agents such as drugs, environmental toxicants, and dietary toxins. This is accomplished primarily by oxidative and conjugative metabolism of such substances following uptake from plasma into the cells, sometimes by active transport processes (see Chaps. 5 and 6).

Table 13-1

General Functions of Liver

- Processing of foods
  - Monosaccharides → glycogen or energy
  - Gluconeogenesis
  - Lipids → processing, energy
- Synthesis of circulating lipids
- Uptake of dietary lipids (e.g., cholesterol) and vitamins from blood
- Degradation of cholesterol and steroids
- Protein synthesis—for intrinsic and extrinsic proteins (e.g., albumin, coagulation, and complement factors, lipoproteins)
- Ammonia detoxification (urea formation)
- Heme synthesis
- Elimination of bilirubin
- Iron reutilization
- Xenobiotic metabolism (drugs, food-borne agents, etc.)
- Excretion via biliary tract (drugs, metals, etc.)
- Elimination of particulates and bacterial products from blood

HPCs contain large concentrations of phase I enzymes, which typically produce metabolites that are more polar and easily excreted by the kidneys than the parent compound. They also have a wide variety of phase II enzymes that conjugate chemicals or their metabolites with endogenous molecules. Phase II metabolism typically reduces toxicity and markedly increases polarity, which facilitates excretion by biliary and renal routes. Hepatic metabolism of xenobiotic agents is a function of HPCs that has major toxicologic importance; for details about xenobiotic metabolism, see Chap. 6 of this volume.

Quantitative and qualitative differences in HPCs exist depending on their location along the sinusoid. The differences in exposure of HPCs to blood constituents from zone 1 to zone 3 may be one of several reasons why HPCs are functionally heterogeneous. For example, a gradient in bile acid concentration in plasma exists along sinusoids (Groothuis et al., 1982). Physiological concentrations of bile acids are efficiently extracted by zone 1 HPCs, leaving little

bile acid left in the blood that flows past zone 3 HPCs. Moreover, there is difference in bile acid transporter expression among different zones (Baier et al., 2006). Heterogeneities among HPCs along the acinus also generate gradients of metabolic functions. HPCs in the mitochondria-rich zone 1 predominate in fatty acid oxidation, gluconeogenesis, and ammonia detoxification to urea. Gradients of enzymes involved in the bioactivation and detoxification of xenobiotics have been observed along the acinus by immunohistochemistry (Jungermann and Katz, 1989; Gebhardt et al., 1994). Of toxicological importance are greater concentrations of glutathione (GSH) in zone 1 and of cytochrome P450 proteins in zone 3 (Tsutsumi et al., 1989; Shimizu and Morita, 1992; Gebhardt et al., 1994; Niemelä et al., 2000).

Some of the HPC functions described above render the liver a target organ for many toxicants. For example, metabolism of some xenobiotic agents by HPCs can result in toxic metabolites that directly injure these cells (see specific examples with acetaminophen and other chemicals below). In addition to HPCs, the liver contains other cell types (discussed in the next section) that perform important physiological functions but that also can participate in pathogenesis following exposure to toxicants. Importantly, the many functions of HPCs render the liver a critical organ for maintaining metabolic homeostasis of the entire organism. It follows that damage to HPCs and other liver cells from chemical exposure can lead not only to liver dysfunction but to dysfunction of other tissues (e.g., hepatic encephalopathy, coagulopathies [Lee, 2012; Kandiah and Kumar, 2016]).

Sinusoidal Endothelial Cells

Sinusoids are lined by thin, discontinuous endothelial cells with numerous fenestrae (or pores; Fig. 13-5) that allow molecules smaller than 250 kDa to cross the interstitial space (known as the space of Disse) between the endothelium and HPCs. In the space of Disse, sinusoidal endothelial cells (SECs) are separated from the HPCs by a basement membrane-like matrix, which is attenuated relative to basement membranes in other tissues (Friedman, 2000). However, this subendothelial extracellular matrix is important for the normal function of all resident liver cells (Friedman, 2000). The numerous fenestrae and the attenuated basement membrane facilitate exchange of fluids and molecules, such as albumin, between the sinusoid and HPCs, but hinder movement of particles larger than chylomicron remnants. Endothelial cells are important in the scavenging of lipoproteins via the apolipoprotein E receptor and of denatured proteins and advanced glycation endproducts by scavenger receptors (Enomoto et al., 2004; Elvevold et al., 2008). Hepatic endothelial cells also secrete biologically active molecules such as cytokines, prostanoids, nitric oxide, and endothelins and express intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the cell surface (Knolle and Wohlleber, 2016). These SEC-derived substances play important roles in liver injury and repair.

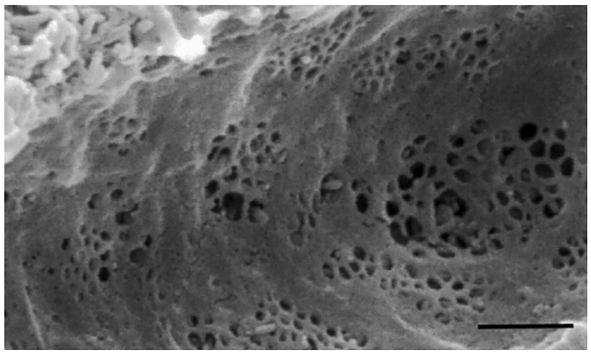


Figure 13-5. *Scanning electron micrograph of sinusoidal endothelial cells.* Note the many fenestrae. (From Fraser et al. [2003].)

Kupffer Cells Kupffer cells are the resident macrophages of the liver and constitute approximately 80% of the fixed macrophages in the body (McCuskey, 2006b). They are situated within the lumen of the sinusoid, in apposition to SECs and with processes that extend through fenestrae to contact HPCs (Figs. 13-3 and 13-4). A primary function of Kupffer cells is to ingest and degrade particulate matter (e.g., bacteria). Also, Kupffer cells are important players in immune responses; as such, they are a major source of cytokines, eicosanoids, and reactive oxygen species (ROS) and can act as antigen-presenting cells (APCs; Laskin, 2009).

Hepatic Stellate Cells Hepatic stellate cells (HSCs) are located between endothelial cells and HPCs (Figs. 13-3 and 13-4; Friedman, 2008a). They are also known as Ito cells or by the more descriptive term of fat-storing cells. Stellate cells are the major sites for vitamin A storage in the body (Friedman, 2008a). Indeed, early responses to high-dose vitamin A therapy are HSC engorgement, activation, increase in number, and protrusion into the sinusoid (Geubel et al., 1991). These cells express smooth muscle actin; they are contractile and appear to control local flow of blood in the sinusoids. When activated, especially during chronic injury to the liver, stellate cells can assume a myofibroblastic phenotype, synthesizing and secreting collagen and other extracellular matrix proteins and thereby can initiate liver fibrosis (Friedman, 2000; Lee and Friedman, 2011; Schon and Weiskirchen, 2014; Weiskirchen and Tacke, 2014).

Other Immune Cells of Liver Sinusoids In addition to resident liver macrophages (Kupffer cells), the liver is home to a population of immune cells of diverse lineage and function. Specific immune cell content and organization within the liver sinusoid makes the liver a true immune organ, capable of mounting both innate and adaptive immune responses both under normal conditions and during disease (Shuai et al., 2016). Immune cells contribute to liver homeostasis and immunity. Thus, these cells may become engaged in the progression of liver damage driven by inflammation and/or become activated in response to injury-associated changes, including inflammatory stimuli (McDonald and Kubes, 2016). An important role of various immune cells is to mount a specific response to injury or disease. For example, *dendritic cells* (DCs; Fig. 13-3) and *plasmacytoid DCs* play a key role in antigen processing and in presentation of antigens to other immune cells along with production of inflammatory cytokines, such as interferons (Chen et al., 2016). Notably, although the cell surface protein CD11c is often considered a DC marker, an increasing body of knowledge of DC and macrophage heterogeneity suggests more comprehensive cell surface marker analysis is required to identify DCs definitively. Interestingly, DCs may play a protective role in certain experimental settings, including in resolution of fibrosis (Jiao et al., 2012; Almeda-Valdes et al., 2015) and in acetaminophen hepatotoxicity (Connolly et al., 2011).

Natural Killer (NK) Cells and Natural Killer T (NKT) Cells These are subsets of lymphocytes that accumulate in liver in response to injury and inflammatory challenges; they produce a variety of cytokines that modify the activity of several other cell types in the liver. Both NK and NKT cells can display quite dichotomous functions in various disease or injury settings. Invariant NKT cells have been shown to drive alcohol-induced liver injury

in rodent models (Cui et al., 2015), and several studies showed that NK cells have both inflammatory and antifibrotic activity (Tian et al., 2013). Although some studies note a damaging role for NK and NKT cells in acetaminophen hepatotoxicity, other studies have challenged this finding (Masson et al., 2008). Indeed, other studies indicate that these same cell types play a protective role (Martin-Murphy et al., 2013); differences in these results might relate to differences in the solvents used for acetaminophen.

A set of recently described resident liver immune cells likely to emerge as significant contributors to toxicity are new members of the innate lymphoid cell (ILC) family (Eberl et al., 2015). Although functional overlap is observed between subtypes of ILCs and T lymphocytes, the stimuli required to activate and engage ILCs in local inflammatory responses are different. ILCs do not respond to specific antigens but rather lie waiting in the liver (and other tissues) to receive a variety of signals cued up by local inflammation or injury (Eberl et al., 2015). For example, IL-33 has been identified as an inflammatory cue released by injured HPCs that drives ILC activation and subsequent liver fibrosis (Tan et al., 2017). Notably, these observations represent just a few examples of an expanding literature on this topic. In addition to citations above, the reader is referred to recent reviews (Baeck and Tacke, 2014; Weiskirchen and Tacke, 2014).

Bile Formation

Bile is a yellow fluid comprising water that contains bile acids, GSH, phospholipids, cholesterol, bilirubin and other organic anions, proteins, metals, ions, and xenobiotic agents (Arrese and Trauner, 2003; Trauner and Boyer, 2003). Formation of bile is a specialized function of the liver. Adequate bile formation is essential for uptake of lipid nutrients from the small intestine, for protection of the small intestine from oxidative insults (Aw, 1994), and for excretion of endogenous and xenobiotic compounds. Bile is secreted into canaliculi by HPCs where it functions to transport various substances out of the liver and to help solubilize lipids in the intestine. As noted above, the canalicular lumen is a space formed by specialized regions of the plasma membrane between adjacent hepatocytes (Fig. 13-3). The canaliculi are separated from the intercellular space between HPCs by tight junctions, which form a barrier permeable only to water, electrolytes, and to some degree to small organic cations. Under physiological conditions, tight junctions are impermeable to organic anions, which allows high concentrations of bile acids, GSH, bilirubin diglucuronide, and other organic anions to accumulate in bile. The structure of the biliary tract is analogous to the roots and trunk of a tree, where the tips of the roots equate to the canalicular lumens. Canaliculi form channels between HPCs that connect to a series of increasingly larger channels (intrahepatic bile ducts) within the liver. The large extrahepatic bile ducts merge to form the common bile duct. Bile originating in the lobules travels from the intrahepatic bile ducts to the common bile duct and then into the gallbladder to be stored and concentrated before its release into the duodenum. However, the gallbladder is absent in several species, including the horse, whale, and rat, so that bile flows directly into the duodenum from the common bile duct in these species.

Bile Formation and Transport HPCs begin the process of bile formation by active transport into the canalicular lumen of bile acids, GSH, and other osmotically active compounds including xenobiotics and their metabolites (Klaassen and Watkins, 1984). These molecules are the major driving force for the passive movement of water and electrolytes across the HPC epithelium into the bile. Concentration of solutes in bile depends on their active transport at the locus of the canalicular membrane. With the identification of specific transporters, substantial progress has been made in the understanding of the molecular mechanisms of bile formation (reviewed by Jansen and Groen, 2006; Pauli-Magnus and Meier, 2006). On the basal (sinusoidal) side of the HPCs, there are sodium-dependent and sodium-independent uptake systems (Fig. 13-6). Most conjugated bile acids (taurine and glycine conjugates) and some unconjugated bile acids are transported into HPCs by sodium/taurocholate cotransporting polypeptide (NTCP) (Trauner and Boyer, 2003; Hagenbuch and Dawson, 2004; Stieger et al., 2007). Sodium-independent uptake of conjugated and unconjugated bile acids is performed by members of the organic anion–transporting polypeptides (OATPs) (Hagenbuch and Meier, 2004; Csanaky et al., 2011). OATP1B1 and OATP1B3 are predominantly expressed in liver and are capable of transporting conjugated and unconjugated bile acids, steroids, and many other organic anions. Furthermore, the OATPs transport numerous drugs and some hepatotoxicants such as phalloidin, microcystin, and amanitin (Hagenbuch and Gui, 2008; Roth et al., 2012). There also exist several organic-anion transporters (OATs, in humans). These transport small organic anions, the movement of which is coupled to exchange of another organic anion; this transport differs from that of OATPs, which tends to transport larger, more lipophilic anions in an ATP-dependent manner (Nigam et al., 2015). Organic cations are transported across the basolateral membrane by organic cation transporters (OCTs, especially OCT1 in humans). The driving force for transport by OCTs is the concentration gradient of the cation across the HPC membrane, so transport can be in either direction (Koepsell and Endou, 2004). In addition to the uptake or exchange systems, there are ATP-dependent efflux pumps located on the basolateral membrane of HPCs. These carriers are members of the multidrug resistance-associated proteins (MRPs; ABCC), which are multispecific transporters for many different anions (Homolya et al., 2003).

All unconjugated bile acids in HPCs are conjugated before being transported by the bile salt export pump (BSEP) across the canalicular (apical) membrane. Bile acid excretion is a major driving force of bile fluid formation (i.e., "bile salt-dependent bile flow"). Other solutes in bile are transported by members of the multidrug resistance (MDR) P-glycoprotein family such as MDR3 (ABCC2), which transports phospholipids, and the heterodimeric transporters ABCG5/ABCG8, which transport cholesterol and plant sterols into bile (Pauli-Magnus and Meier, 2006). In addition, MRP2 transports GSH, which is the main compound responsible for the bile salt-independent bile flow, as well as sulfated and glucuronidated bile acids, glutathione disulfide, glutathione conjugates, bilirubin diglucuronide, and many other conjugated drugs and chemicals (Borst et al., 2000; Gerk and Vore, 2002). Other transport systems of the canalicular membrane include the breast cancer resistance protein (BCRP; ABCG2), which can contribute to the biliary excretion of bile acids and xenobiotics.

Canalicular lumen bile is propelled forward into larger channels by dynamic, ATP-dependent contractions of the pericanalicular cytoskeleton (Watanabe et al., 1991). The biliary epithelial cells that comprise bile ducts, once regarded as passive conduits, modify bile by absorption and secretion of solutes (Lira et al., 1992). Bile acids are taken up into biliary epithelial cells (cholangiocytes) by OATP1A2 (sodium-independent uptake) and by the sodium-dependent bile acid transporter ASBT (Fig. 13-6; Obaidat et al., 2012; Roth et al., 2012). These bile acids are then secreted on the basolateral side mainly by MRP3 and heterodimeric organic solute transporter (OSTα/OSTβ) (Ballatori et al., 2009). The bile acids secreted from biliary epithelial cells return to the portal circulation

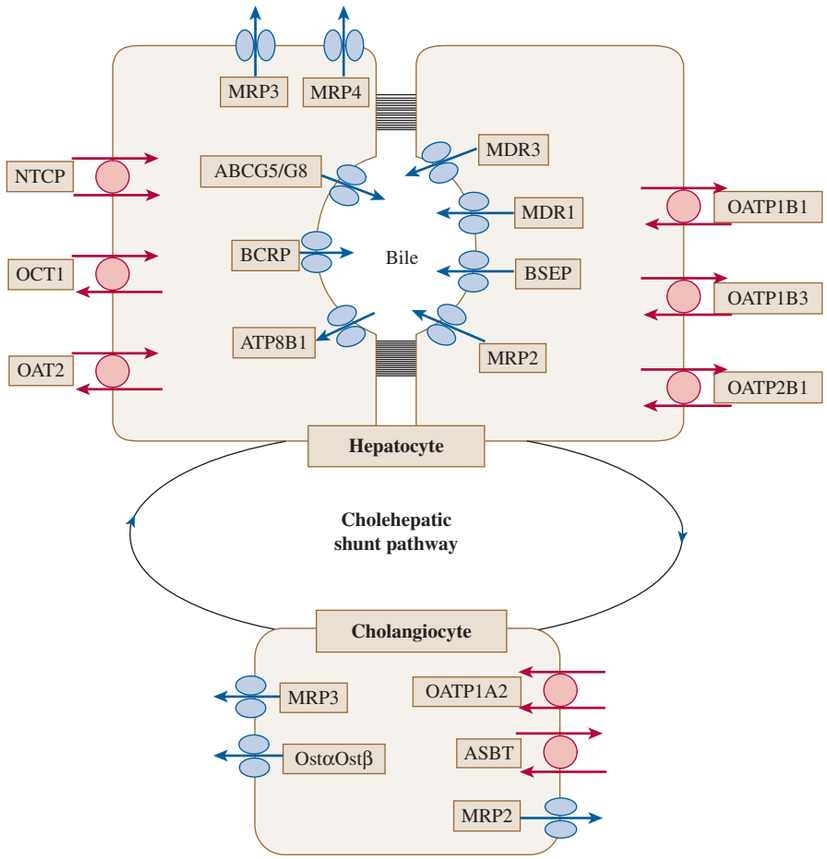


Figure 13-6. *Transport proteins in human hepatocytes and cholangiocytes.* Exporters of particular relevance to canalicular secretion of toxic chemicals and their metabolites are the canalicular multiple organic anion transporter (MOAT) system and the family of multiple-drug resistant (MDR) P-glycoproteins. Note: MDR3 (ABCB4) flops phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. Phosphatidylcholine can then be extracted by bile salts. ATP8B1 together with the accessory protein CDC50A flips phosphatidylserine from the outer to the inner membrane to maintain the lipid asymmetry of the canalicular membrane and to protect against bile acids (Groen et al., 2011).

Efflux transporters (blue symbols): BSEP, bile salt export pump; MDR, multidrug resistance protein; MRP, multidrug resistance–associated protein; ABCG5/8; BCRP, breast cancer resistance protein; Ostα/Ostβ.

Uptake transporters (red symbols): ASBT, apical sodium-dependent bile salt transporter; NTCP, sodium taurocholate cotransporting polypeptide; OATP, organic anion–transporting polypeptide; OCT, organic cation transporter; OAT, organic anion transporter. Transporters localized to the sinusoidal membrane extract solutes from the blood. Exporters localized to the canalicular membrane move solutes into the lumen of the canaliculus. (Adapted from Pauli-Magnus and Meier [2006]. Reprinted with permission of John Wiley & Sons, Inc.)

via the peribiliary plexus (cholehepatic shunt pathway). Biliary epithelial cells also express a variety of phase I and phase II xenobiotic metabolizing enzymes, which can contribute to the biotransformation of chemicals present in bile (Lakehal et al., 1999; Klaassen and Aleksunes, 2010).

Secretion into bile ducts is usually, but not always, a prelude to toxicant clearance by excretion in feces. Exceptions occur when compounds such as arsenic are repeatedly delivered into the intestinal lumen via bile, then efficiently reabsorbed from the intestinal lumen and redirected to the liver via portal blood, a process known as "enterohepatic cycling." A few compounds, such as methyl mercury, are absorbed from the biliary tract; the extensive reabsorption of methyl mercury from the gallbladder is thought to contribute to the long biological half-life and toxicity of this toxicant (Dutczak et al., 1991). Alternatively, secretion of toxic metabolites into the bile can be a prelude to the development of injury in extrahepatic tissues. A clinically relevant example of this is diclofenac, a widely prescribed nonsteroidal anti-inflammatory drug (NSAID) that causes small intestinal ulceration. Experiments with mutant rats lacking a functional MRP2 exporter showed that these mutants secrete little of the presumptive proximate toxicant metabolite into bile and are resistant to the intestinal toxicity of diclofenac (Seitz and Boelsterli, 1998).

LIVER PATHOPHYSIOLOGY: TYPES OF LIVER RESPONSES TO CHEMICALS

Liver injury can arise from exposure to many types of chemicals, including drugs, environmental pollutants, occupational chemicals, plant toxins, and others. Major adverse responses of the liver include steatosis (fatty liver), cell death, cholestasis, vascular damage/dysfunction, fibrosis, and cancer (Table 13-2). Inflammation and proliferative repair play important and sometimes dichotomous roles in determining outcome of exposure to hepatotoxicants. The specific response of the liver to a chemical insult depends on the intensity and duration of the exposure and the cell population(s) affected. Mild stresses may cause reversible cellular dysfunction

Table 13-2

Types of Liver Responses to Xenobiotic Agents

RESPONSE	REPRESENTATIVE TOXICANTS
Hepatocyte death	Acetaminophen, allyl alcohol, Cu, dimethylformamide, ethanol
Fatty liver (steatosis)	Amiodarone, CCl_4, ethanol, fialuridine, tamoxifen, valproic acid
Canalicular cholestasis	Chlorpromazine, cyclosporin A, 1,1-dichloroethylene, estrogens, Mn, phalloidin
Bile duct damage	Alpha-naphthylisothiocyanate, amoxicillin, methylenedianiline, sporidesmin
Sinusoidal endothelial damage	Cyclophosphamide, microcystin, pyrrolizidine alkaloids
Inflammation	Bacterial endotoxin, ethanol
Liver regeneration	Acetaminophen, ethanol, estrogens
Fibrosis and cirrhosis	CCl_4, ethanol, thioacetamide, vitamin A, vinyl chloride
Liver cancers	Aflatoxin, androgens, arsenic, thorium dioxide, vinyl chloride

and can prompt a reparative response. However, sufficient acute exposure to many chemicals can result in serious liver injury and irreversible dysfunction. The major responses of the liver to toxic insults are summarized below.

Cell Death

Cell death from chemical exposure is known to occur by several different molecular pathways. Resulting modes of cell death include oncotic necrosis, apoptosis, pyroptosis, necroptosis, etc. (Fig. 13-7). Differences among these are explained in Chap. 3, and therefore only some salient features of a couple of these will be covered here. *Oncotic necrosis*, often referred to simply as "necrosis," is characterized by cell swelling, leakage of cellular contents, nuclear disintegration (karyolysis), and an influx of inflammatory cells. Cell contents released during oncotic necrosis include intracellular enzymes such as alanine aminotransferase (ALT) and aspartate amino transferase (AST), which appear in the plasma and are used as biomarkers of hepatocellular injury (see below). In contrast to oncotic necrosis, *apoptosis* is characterized morphologically by cell shrinkage, chromatin condensation, nuclear fragmentation, and formation of membrane-bound cell fragments termed "apoptotic bodies." Because the latter are phagocytosed and digested by Kupffer cells or other neighboring cells, apoptosis is often not accompanied by an inflammatory response. The pathway to apoptosis involves activation of caspase enzymes that results in the cleavage of nuclear DNA. Oncotic necrosis and apoptosis as well as other modes of cell death typically occur as a result of activation of one or more intracellular molecular signaling pathways that result in cell death. These pathways are described in Chap. 3 and in many reviews (Jaeschke et al., 2004; Jaeschke and Bajt, 2006; Malhi et al., 2006; Schulze-Bergkamen et al., 2006; Luedde et al., 2014; Iorga et al., 2017). Liver injury from acetaminophen overdose is an example of a toxicant-induced cell death pathway in HPCs, and this is discussed below.

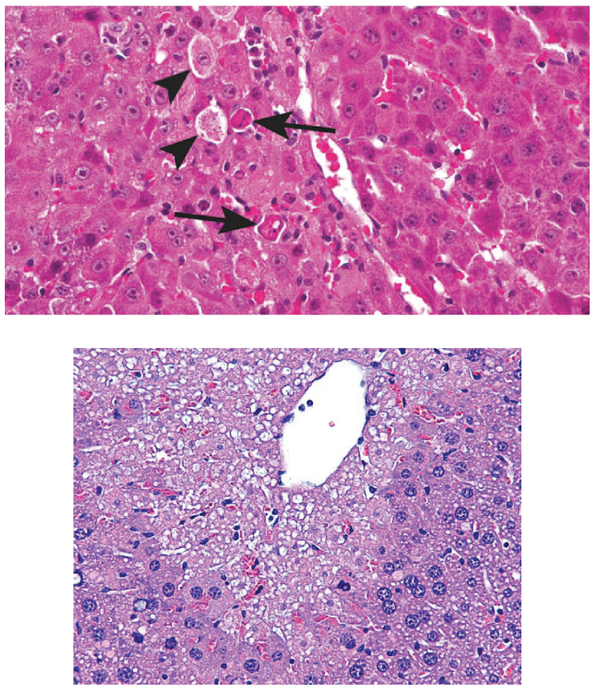


Figure 13-7. *Cell death in the liver: histopathology. Top*: Single cell apoptosis (arrows) and oncotic necrosis (arrowheads) occurring together in the liver. Apoptotic hepatocytes small with darker cytoplasm and nuclei that are absent or small, fragmented, and dark (pyknotic). The necrotic cells are enlarged with eosinophilic, pale cytoplasm and disappearing nuclei (karyolysis). Hematoxylin and eosin stain. (From http://focusontoxpath.com/recommendations-inhand-apoptosis-necrosis-working-group/). *Bottom*: Large area of necrosis surrounding the central vein in the liver of a mouse given a toxic dose of acetaminophen. Hepatocytes outside of the centrilobular area appear normal. (Absent in this photomicrograph is an influx of inflammatory cells that often accompanies larger areas of necrosis.) (From https://www.niehs.nih.gov/research/resources/visual-guides/liverpath/necrotic/inflammation_necrosis/. See this reference for more photomicrographs.)

Signaling mechanisms of apoptosis have been elucidated in detail (reviewed by Jaeschke and Bajt, 2006; Malhi et al., 2006; Schulze-Bergkamen et al., 2006). In the extrinsic pathway of apoptosis, ligands (e.g., Fas ligand, TNF-α) bind to their respective death receptor (Fas receptor, TNF receptor type I), which triggers the trimerization of the receptor that prompts the recruitment of various adapter molecules and procaspases to the cytoplasmic tail of the receptor. The assembly of this death-inducing signaling complex (DISC) leads to the activation of initiator caspases (caspase-8 or -10). In HPCs, the active initiator caspase cleaves Bid, a member of the Bcl-2 family of proteins, and the truncated Bid (tBid) translocates together with other Bcl-2 family members such as Bax to the mitochondria. These proteins form pores in the outer membrane of the mitochondria and cause the release of intermembrane proteins such as cytochrome *c* and the second mitochondria-derived activator of caspases (Smac). Cytochrome *c*, together with apoptosis protease activating factor-1 (APAF-1), ATP, and procaspase-9, form the apoptosome, causing the activation of caspase-9, which then processes (and activates) downstream effector caspases, for example caspase-3 and caspase-7. The effector caspases can propagate the apoptotic signal by activating caspase-activated DNase (CAD) to initiate nuclear DNA fragmentation and by cleaving numerous cellular proteins critical to cell function and to the structural integrity

of the cell and its nucleus (Fischer et al., 2003; Nagata et al., 2003). In addition to downstream substrates, caspase-3 can also process more procaspase-8 and thereby amplify the apoptotic signal. Although HPCs constitutively express Fas and TNF receptors, the death signal generated with ligation of the receptor is in most cases insufficient to trigger apoptosis in healthy cells. Inhibitor studies and experiments with gene-deficient mice support the hypothesis that only the amplification of the receptor-derived signal through multiple mitochondrial cycles can successfully induce apoptosis in HPDs (Yin et al., 1999a; Bajt et al., 2000). In addition to the direct propagation of the apoptosis signal by mitochondrial cytochrome *c* release, the simultaneous release of Smac ensures that the cytosolic inhibitors of apoptosis proteins (IAPs) are inactivated and do not interfere with the promotion of apoptosis (Li et al., 2002). Thus, mitochondria are an important part of the extrinsic (receptor-mediated) apoptotic signal transduction pathway in liver cells after most stimuli. However, under conditions of strong Fas receptor activation (MegaFas ligand), downstream caspases are activated without involvement of mitochondria (Schüngel et al., 2009).

In contrast to the extrinsic pathway, the intrinsic or mitochondrial pathway of apoptosis is initiated independent of the TNF receptor family, caspase-8 activation, and formation of the DISC. Despite the upstream differences, the postmitochondrial effects are largely similar to the extrinsic pathway. The intrinsic pathway is generally triggered by a cytotoxic stress or DNA damage, which activates the tumor suppressor p53 (Sheikh and Fornace, 2000). This protein acts as a transcription factor to promote the formation of proapoptotic Bcl-2 family members, for example, Bax. The increased Bax translocation to the mitochondria induces the release of mitochondrial intermembrane proteins including cytochrome *c*, Smac, endonuclease G, and apoptosis-inducing factor (AIF) (Saelens et al., 2004). An intrinsic mechanism of apoptosis has been discussed for cell death in aging livers (Zhang et al., 2002), prolonged treatment with alcohol (Ishii et al., 2003), and toxicity of benzo(*a*)pyrene and acetaminophen in hepatoma cells (Boulares et al., 2002; Ko et al., 2004). For other hepatotoxic chemicals, such as carbon tetrachloride (Cai et al., 2005), galactosamine (Gómez-Lechón et al., 2002), and microcystin (Ding et al., 2000a, 2000b), evidence for mitochondria-dependent apoptosis has been reported in cultured HPCs, and apoptotic cell death was observed after exposure to these chemicals in vivo (Shi et al., 1998; Hooser, 2000; Gujral et al., 2003b).

A special form of cell death is the more recently described type of programmed necrosis termed "necroptosis" (Vandenabeele et al., 2010; Grootjans et al., 2017). Necroptosis is generally initiated by death receptors, for example TNF receptor 1, and the formation of complex 1 with various adapter molecules including receptor-interacting protein kinases 1 and 3 (RIP1 and -3). If caspase-8 is activated, it will cleave RIP1 and -3, and apoptosis will be initiated. However, if caspase-8 is inhibited, RIP1 and RIP3 activate a caspase-independent execution mechanism involving the phosphorylation of mixed lineage kinase domain like pseudokinase (MLKL), which then translocates to the cell membrane and forms pores (Grootjans et al., 2017). Although there is an increasing number of papers published showing roles for RIP1, RIP3, and MLKL in the pathophysiology of many liver disease models in vivo, the results are variable, and it remains controversial whether some cell death is a classical necroptotic process or another form of programmed necrosis (Dara et al., 2016b).

Characterization of mode of cell death after chemical exposure has been based on morphology (Fig. 13-7) and more recently on activation of various molecular factors described above. Importantly, commonalities in signaling pathways in different modes of cell death can occur, and this has led to controversy about the mode of cell death that applies to particular hepatotoxicants. For example, common molecular changes in mitochondria are involved in pathways to apoptosis as well as to oncotic necrosis (Jaeschke et al., 2012a; Pessayre et al., 2012). Partly for this reason, histological examination of affected tissue remains the "gold standard" for characterizing cell death modes.

Fatty Liver (Steatosis)

Fatty liver (steatosis) is defined as an appreciable increase in lipid (mainly triglyceride) content of HPCs. Histologically, HPCs containing excess fat appear to have many round, empty vacuoles in standard, solvent-extracted and paraffin-embedded tissue sections. The accumulation of these vacuoles can displace the nucleus to the periphery of the cell. Use of frozen sections and special stains or biochemical analysis of lipids is needed to verify the contents of the vesicles as lipid (Fig. 13-8). Based on the size of the fat droplets, one can distinguish between macrovesicular (large droplets) and microvesicular (small droplets) steatosis.

Fatty acids of hepatic and extrahepatic origin can play integral roles in the generation of steatosis. Free fatty acids (FFAs) are synthesized in HPCs, mainly from carbohydrate-derived acetyl-coenzyme A. Also, FFAs can be generated in the liver from the hydrolysis of absorbed fat. Additionally, adipose tissue serves as an extrahepatic source of FFAs because adipocytes can release FFAs into the circulation. Once in the cytosol of HPCs, FFAs can be imported into mitochondria for degradation by β-oxidation or can be esterified into triglycerides for incorporation into very low density lipoproteins (VLDL), which transports FFAs to peripheral adipose tissue. Protein synthesis inhibitors can prompt the accumulation of lipids by inhibiting the protein component of VLDL needed for export of lipids from HPCs. FFA uptake into mitochondria depends on the activity of the mitochondrial enzyme, carnitine palmitoyl transferase 1, which can be downregulated by malonyl-coenzyme A, the first intermediate of FFA synthesis. Thus, FFA synthesis, consumption, and storage are in a well-balanced steady state, normally with little accumulation of triglycerides in the liver (Pessayre et al., 2002). However, under conditions of excess uptake into HPCs of FFAs derived from adipose tissue and/or food, FFAs can accumulate and become esterified to form triglycerides that appear as steatosis (Pessayre et al., 2002; Neuschwander-Tetri, 2010).

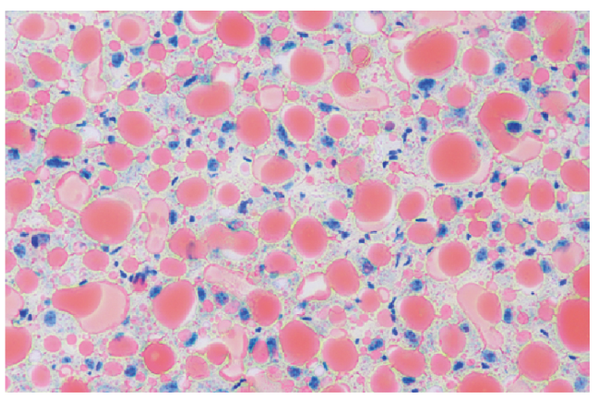


Figure 13-8. *Hepatocellular steatosis.* Lipid accumulation (macrovesicular steatosis) in a liver section stained with oil red O. Nuclei are stained blue. (From Maillo et al. [2017].)

Steatosis occurs commonly from moderate alcohol consumption and other factors and is reversible and probably harmless if the stimulus for it is temporary. However, livers with steatosis are more susceptible to additional insults, such as from hepatic ischemia (Selzner and Clavien, 2001) or hepatotoxicants (Donthamsetty et al., 2007). Perhaps more importantly, persistent steatosis appears to be a precursor to serious liver disease. When accompanied by inflammation (i.e., steatohepatitis), steatosis can progress to life-threatening chronic liver damage, fibrosis (e.g., cirrhosis), and hepatocellular carcinoma (Farrell and Larter, 2006; Ibrahim et al., 2011; Koo, 2013; Ferramosca and Zara, 2014; Angrish et al., 2016).

Although the most common cause of hepatic steatosis is insulin resistance associated with obesity and sedentary lifestyle, exposure to many hepatotoxicants can induce steatosis. Chemicals that produce steatosis associated with lethality include the antiepileptic drug valproic acid (Scheffner et al., 1988), the antiviral drug fialuridine (Honkoop et al., 1997), the solvent carbon tetrachloride (Zimmerman, 1999), and protein synthesis inhibitors such as ethionine, puromycin, and cycloheximide. Several drugs that accumulate in mitochondria and inhibit β-oxidation and mitochondrial respiration have been associated with steatosis. These include 4,4′-diethylaminoethoxyhexestrol, amiodarone, tamoxifen, perhexiline, amineptine, doxycycline, tetracycline, tianeptine, and pirprofen (Berson et al., 1998; Larosche et al., 2007, Pessayre et al., 2012). The effects on mitochondria not only lead to steatosis but also to increased reactive oxygen formation and lipid peroxidation. In addition, drugs such as amineptine, amiodarone, tetracycline, pirprofen, and tianeptine can directly inhibit microsomal triglyceride transfer protein, which lipidates apolipoprotein B to form triglyceride-rich VLDL particles (Lettéron et al., 2003). Drugs with this dual effect on β-oxidation and VLDL secretion are generally highly steatogenic. Ethanol is by far the steatosis-producing agent of most public health concern (Farrell, 2002; Pessayre et al., 2002; Stravitz and Sanyal, 2003; Saito et al., 2007; Neuschwander-Tetri, 2010) and is discussed as an example later in this chapter.

Canalicular Cholestasis

Cholestasis is characterized biochemically by elevated serum concentration of compounds normally concentrated in bile, particularly bile salts and bilirubin. This form of liver dysfunction is defined as a decrease in the rate of bile formation or an impaired secretion of specific solutes into bile (Padda et al., 2011). When biliary excretion of the yellowish bilirubin pigment is impaired, it accumulates in skin and eyes, producing jaundice. Additionally, excess bilirubin increases in urine, which becomes darker yellow or brown. The histological features of cholestasis can be subtle and include dilation of bile canaliculi and the presence of bile plugs in bile ducts and canaliculi. Toxicant-induced cholestasis can be transient or chronic; when substantial, it is associated with cell swelling, cell death, and inflammation. HPC injury is generally caused by the intracellular accumulation of chemicals normally secreted into the bile, such as drugs and their metabolites, other xenobiotic agents, cytotoxic bile acids, bilirubin, or other bile constituents. Many different types of chemicals, including metals, hormones, and drugs, can cause cholestasis (Zimmerman, 1999).

Molecular mechanisms of cholestasis are related to expression and function of transporter systems in the basolateral and canalicular membranes of HPCs described above (reviewed Wagner et al., 2009; Padda et al., 2011). In principle, increased hepatic uptake, decreased biliary excretion, and increased biliary reabsorption (cholehepatic shunting) of a xenobiotic agent can contribute to its accumulation in the liver. For example, basolateral uptake by OATPs can contribute to the liver injury from various toxins, including phalloidin, microcystin, and amanitin (Pauli-Magnus and Meier, 2006; Lu et al., 2008). Some drugs that cause cholestatic injury, such as rifampicin, bosentan, and troglitazone, directly inhibit BSEP and can thereby lead to accumulation of bile acids (Stieger et al., 2000; Fattinger et al., 2001). Estrogen and progesterone metabolites inhibit BSEP from the canalicular side after excretion by MRP2 (Stieger et al., 2000).

A substantial inhibition of excretion of bile salts can lead to their accumulation in HPCs and may thereby cause cell injury (Palmeira and Rolo, 2004). However, most bile acids that accumulate in rodent models are relatively nontoxic (Zhang et al., 2012); accordingly, it appears that inflammation caused by bile acid accumulation in the liver plays a major role in liver injury from some forms of cholestasis (Gujral et al., 2003a; Allen et al., 2011). In contrast, due to the increased accumulation of the more hydrophobic glycine-conjugated bile acids in humans, direct cytotoxicity can occur in human cholestasis (Woolbright et al., 2015).

Increased bile acid concentrations in liver can trigger compensatory mechanisms that limit cholestatic injury (Zollner et al., 2006; Zhang et al., 2012). These involve alterations in transporter expression. Bile acids bind to the nuclear receptor, farnesoid X receptor (FXR), the activation of which stimulates the small heterodimer partner (SHP) that downregulates NTCP expression and thereby limits bile acid uptake (Denson et al., 2001). In addition, FXR activation increases expression of BSEP and MDR3, which enhances the capacity for bile acids and phospholipids, respectively, to be transported into the canaliculus and excreted from the liver (Ananthanarayanan et al., 2001; Huang et al., 2003). Furthermore, the FXR-independent upregulation of the basolateral transporters MRP3 and MRP4 reduces intracellular bile acid and drug concentrations (Schuetz et al., 2001; Wagner et al., 2003; Fickert et al., 2006). Moreover, agonists of the nuclear xenobiotic receptors, constitutive androstane receptor (CAR) and pregnane X receptor (PXR), can induce MRP3 and MRP4 expression, resulting in enhanced export of bile acids during cholestasis (Wagner et al., 2005). In biliary epithelial cells, OSTα/OSTβ is upregulated at the basolateral membrane during cholestasis (Boyer et al., 2006a). This response, which is dependent on FXR, mediates the enhanced return of bile acids from bile to the plasma (Boyer et al., 2006a). Thus, such adaptation of transporter expression may counteract some of the detrimental effects of cholestasis (Zollner et al., 2006).

Bile Duct Damage

Another name for damage to the intrahepatic bile ducts is *cholangiodestructive cholestasis* (Cullen and Ruebner, 1991; Zimmerman, 1999). A useful biochemical index of bile duct damage is a sharp elevation in serum activities of enzymes localized to bile duct epithelial cells, particularly alkaline phosphatase (ALP). In addition, serum levels of bile acids and bilirubin are elevated, as observed with canalicular cholestasis. Initial lesions following a single exposure to cholangiodestructive chemicals include swollen biliary epithelium, debris of damaged cells within ductal lumens, and inflammatory cell infiltration of portal tracts. Chronic administration of toxicants that cause bile duct destruction can lead to bile duct proliferation and fibrosis resembling primary biliary cholangitis (PBC). Numerous drugs have been implicated in causing prolonged cholestasis with features of PBC (Zimmerman, 1999); however, only in rare cases does this result in permanent damage or even loss of bile ducts, a condition known as *vanishing bile duct syndrome*. Cases of this have been reported in patients receiving antibiotics, anabolic steroids, contraceptive steroids, or the anticonvulsant carbamazepine (Davies et al., 1994; Zimmerman, 1999).

Sinusoidal Endothelial Cell Damage

As noted earlier, the sinusoid is essentially a specialized capillary lined with endothelium with numerous fenestrae (holes) that allow for high permeability (Braet and Wisse, 2002). The functional integrity of the sinusoid can be compromised in two ways, by dilation or blockade of its lumen and by destruction of sinusoidal endothelial cells (SECs). Dilation of the sinusoid occurs when the downstream flow of blood is impeded. The rare condition of primary dilation, known as *peliosis hepatis*, has been associated with exposure to anabolic steroids and the drug danazol (Makdisi et al., 1995). Blockade can occur when the fenestrae enlarge to such an extent that red blood cells become caught in them or pass through with entrapment in the interstitial space of Disse. Gaps between endothelial cells and overt injury clearly affect SEC function. Such gaps occur after exposure to acetaminophen (Ito et al., 2005), galactosamine/endotoxin (Ito et al., 2006), or an anti-Fas antibody (Ogasawara et al., 1993). They can be caused by direct injury to SECs or by their detachment from the extracellular matrix (DeLeve et al., 1997; Bajt et al., 2000; Ito et al., 2006). In general, matrix metalloproteinase inhibitors prevent gap formation (McCuskey, 2006a). A consequence of SEC injury is the loss of barrier function with extensive blood accumulation in the liver parenchyma (i.e., hemorrhage).

Disruption of SECs is considered the early structural feature of the vascular disorder known as sinusoidal obstruction syndrome (formerly called veno-occlusive disease: DeLeve et al., 1999). Swelling and/or proliferation of SECs progresses to nonthrombotic occlusion of hepatic venules with cell debris and fibrotic material (DeLeve et al., 2003a). This results in congestion and medial hypertrophy of vessels and contributes to progressive necrosis of parenchymal tissue and fibrosis (Huxtable, 1989). Pyrrolizidine alkaloid plant toxins provide an example of a class of chemicals that cause pronounced SEC destruction and sinusoidal obstruction (discussed below).

Inflammation

Inflammation occurs in many organs as a response to injury that entails activation of the innate immune system. In the liver, the inflammatory response involves circulating blood cells as well as the resident cell types mentioned above (Fig. 13-9). Activated coagulation and complement cascades and alterations in microvascular function are also components of an acute inflammatory response. The activation of resident macrophages (Kupffer cells), NK, NKT cells, and innate lymphoid cells plays a major role in liver inflammation. Additionally, the accumulation and activation of blood-borne cells including platelets, neutrophils, lymphocytes, and monocytes within the damaged liver are well-recognized features of hepatotoxicity produced by many chemicals. Acute inflammatory responses participate in the removal of damaged tissue and in the initiation of liver repair. However, under certain circumstances, inflammatory cells and their products can aggravate existing liver injury by release of cytotoxic mediators or indirectly by

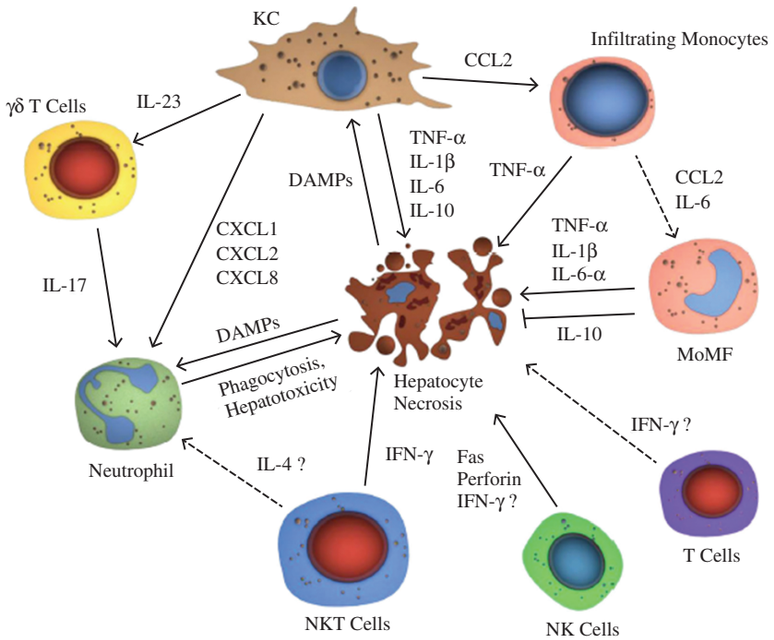


Figure 13-9. *Activation and crosstalk among resident and accumulating inflammatory cells in hepatotoxic responses.* Some immune cells such as Kupffer cells, natural killer (NK) cells, and stellate cells reside in healthy liver tissue. Others such as neutrophils, T cells, NK cells, and infiltrating monocytes (which differentiate in liver into monocyte-derived macrophages [MoMFs]) reach the liver via the blood and accumulate there upon exposure to inflammatory mediators such as chemokines (e.g., CXCLs, CCLs). Hepatocyte necrosis is associated with the release of damage-associated molecular patterns (DAMPs; e.g., HMGB1, Hsp70, free DNA), which are recognized by pattern recognition receptors (e.g., toll-like receptors) on Kupffer cells and neutrophils, resulting in activation of these cells and expression of cytokines and chemokines. Elaboration of the hepatic inflammatory response occurs through the recruitment and further activation of monocytes and other immune cells including various subsets of T cells, NK cells, and NKT cells. Coordinated amplification and tailoring of the hepatic inflammatory response is driven by cytokines and other inflammatory mediators produced by this collage of cell types. Depending on the context of hepatocellular injury and the timing, an inflammatory response can either exacerbate liver damage or contribute to repair processes. Used with permission from Krenkel O, Mossanen JC, Tacke F. Immune mechanisms in acetaminophen-induced acute liver failure. *Hepatobiliary Surg Nutr.* 2014;3(6):331-43. Review Article: Liver Immunology.

release of cytokines or other mediators that activate intracellular cell death signaling pathways in liver cells (Brenner et al., 2013).

The mechanisms responsible for triggering inflammation within the injured liver are not fully understood. However, the release of damage associated molecular pattern molecules (DAMPs; e.g., high mobility group box 1 [HMGB1]) from injured HPCs stimulates various receptors on inflammatory cells (e.g., toll-like receptors) (Schwabe et al., 2006; Bianchi, 2007; Kubes and Mehal, 2012; Woolbright and Jaeschke, 2017). Activation of inflammatory cells in liver can also occur independently of direct hepatocellular damage; rather, hepatic inflammation can be driven by infection or pathologic events transpiring in other tissues that result in circulating inflammatory triggers. For example, alterations in intestinal homeostasis can result in translocation of bacterial products such as lipopolysaccharide (LPS) from the intestine into the portal circulation entering the liver (Giannelli et al., 2014). LPS and other pathogen-associated molecular pattern molecules (PAMPs) act at cellular receptors (e.g., toll-like receptors) to activate Kupffer cells and other sinusoidal cells. PAMPs also arise from bacterial or viral infections.

Kupffer cells and neutrophils are phagocytes that have a vital function in host defense and removal of cell debris. Kupffer cells can be activated not only by bacterial products such as LPS, but also by circulating particles and activated complement factors (Bilzer et al., 2006). Production of reactive oxygen species by NADPH oxidase is a critical tool used by these cells for eliminating pathogens, but it can also contribute to damage of liver cells. Upon activation, Kupffer cells generate mainly hydrogen peroxide, which can diffuse into neighboring liver cells and create an intracellular oxidant stress, ultimately causing cellular injury (Bilzer et al., 1999). A detrimental role of Kupffer cells in the pathogenesis of toxicant-induced liver injury has been suggested for several chemicals including ethanol, acetaminophen, CCl_4, and 1,2-dichlorobenzene (Laskin, 2009), although the role of Kupffer cells in acetaminophen hepatotoxicity may be counterbalanced by their important role in liver repair (Ju et al., 2002; Holt et al., 2010; You et al., 2013; Krenkel and Tacke, 2017).

In addition to their capacity to cause oxidant-mediated cellular injury, Kupffer cells generate inflammatory mediators such as cytokines and prostanoids (Decker, 1990; Schwabe et al., 2006). These mediators can aggravate injury by recruiting neutrophils into the liver (Bajt et al., 2001); activation of neutrophils can cause cell death in susceptible HPCs (Jaeschke, 2006). However, Kupffer cells can also generate anti-inflammatory mediators, such as prostaglandin E_2 and interleukin-10 (Decker, 1990) that downregulate production of pro-inflammatory cytokines and thereby attenuate inflammatory liver injury (Bourdi et al., 2002; Ju et al., 2002). Thus, Kupffer cells can promote progression of injury or inhibit it by assisting in removal of cell debris and apoptotic bodies by phagocytosis. In addition, mononuclear cells (macrophages) recruited into liver from the blood can function in a similar way as Kupffer cells. Although there is a capacity for additional damage by these cells, their recruitment into the damaged liver and the mediators they release are important signals for inducing repair of damaged tissue (Holt et al., 2008; Laskin, 2009; Adams et al., 2010).

As mentioned before, the location of the liver downstream in the circulation from the intestine can result in exposure to microbes or microbial products that translocate into the portal circulation. Neutrophils arrest in the liver vasculature and can become activated in response to bacterial infection or extensive cell injury. These cells remove bacteria and cell debris at least in part through interactions with the resident macrophages (Gregory and Wing, 2002). Neutrophils generate the potent oxidant and chlorinating species, hypochlorous acid, through the combined activities of NADPH oxidase and myeloperoxidase (El-Benna et al., 2005). In addition, neutrophils can release proteolytic enzymes (e.g., elastase, cathepsin G) and bactericidal proteins (Wiedow and Meyer-Hoffert, 2005). The capability of neutrophils to migrate out of the vasculature, adhere to target cells and generate potent cytotoxic agents render this leukocyte an effective killer of invading microorganisms and a remover of dead or dying cells. However, if directed against still viable liver cells, these agents can promote additional tissue injury or even liver failure (Jaeschke, 2006). Notably, neutrophil-induced liver cell injury is a multistep process (Jaeschke and Hasegawa, 2006; Ramaiah and Jaeschke, 2007). It requires exposure to inflammatory mediators that upregulate adhesion molecules such as Mac-1 (CD11b/CD18) on the cell surface, which cause neutrophils to accumulate in liver sinusoids and prime them for reactive oxygen formation. If a chemotactic signal is received from the parenchyma, neutrophils will extravasate, adhere to the target, and become fully activated to release oxidants and proteolytic enzymes (Chosay et al., 1997; Jaeschke, 2006; Jaeschke and Hasegawa, 2006). These neutrophil products can contribute to liver injury through the destruction of HPCs and other cells compromised by toxicant exposure (Gujral et al., 2004; Jaeschke, 2006). Neutrophils are involved in liver injury from ischemia-reperfusion, alcohol, α-naphthylisothiocyanate, halothane, and other toxicants (see below and Ramaiah and Jaeschke, 2007).

Although this discussion has highlighted roles of Kupffer cells and neutrophils, several other cell types can contribute as well to acute and chronic inflammatory responses. A comprehensive accounting of the complexities of inflammation is beyond the scope of this chapter, but greater detail about various aspects of liver inflammation is available in several recent reviews (Byrne, 2010; Tilg and Moschen, 2010; Zhai et al., 2011; Liaskou et al., 2012; McDonald and Kubes, 2012; Brenner et al., 2013; Hoque et al., 2013; Bishayee, 2014; Ceccarelli et al., 2014; Yang et al., 2015; Zhu et al., 2016; Roth et al., 2017). The roles of inflammation in responses to specific hepatotoxicants are mentioned in sections below and in the section "Determinants of Susceptibility."

Regeneration and Repair

The liver has a high capacity to restore lost tissue and function by regeneration. Loss of HPCs due to hepatectomy, either after surgical resection in human patients or modeled by major removal of liver (e.g., 70%) in rodents, triggers proliferation of all mature liver cells. This process is capable of restoring the original liver mass (Fausto, 2000; Michalopoulos, 2017). The mechanisms critical for restoring liver mass are not entirely understood. HPCs are normally quiescent, that is, they are in G_0 phase of the cell cycle. In order to proliferate, they need to enter the cell cycle. This process is initiated by cytokines (TNF-α, IL-6), which prime HPCs to respond to essential growth factors such as HPC growth factor (HGF) and transforming growth factor-α (TGF-α) (Fausto, 2000). In contrast to Fausto's priming/progression hypothesis, Michalopoulos (2007) has argued that HPCs do not fit this two-stage model, rather there are primary direct mitogens for HPCs (e.g., HGF) and secondary proregenerative substances (TNF-α) that enhance the effect of the primary mitogen (Michalopoulos, 2007). Importantly, both cytokines and growth factors are involved in the activation of a number of transcription factors that drive expression of cell cycle-regulating proteins such as cyclins, the activators of cyclin-dependent kinases (CDKs), and CDK inhibitors such as p18, p21, and p27 (Trautwein, 2006). The coordinated expression of individual cyclins and inhibitors of CDKs guides the cell through the various phases of the

cell cycle, including DNA synthesis (S phase) and mitosis (M phase). If HPC replication is blocked, liver regeneration relies on hepatic stem cells or oval cells, which proliferate and differentiate to replace lost parenchyma (Michalopoulos and DeFrances, 1997; Fausto, 2000). This is allowed in part by the reduced sensitivity of oval cells to the proliferation-inhibiting cytokine, transforming growth factor-β (TGF-β) (Nguyen et al., 2007). For details of the intracellular signal mechanisms of HPC regeneration, the reader is referred to excellent reviews on this subject (Trautwein, 2006; Michalopoulos, 2010, 2014, 2017).

The partial hepatectomy animal model has provided abundant insights into the mechanisms of liver regeneration. It is important to note that although great similarity exists in the factors driving HPC proliferation after hepatectomy and after toxic liver injury, the stimuli for regeneration are distinct. As is the case for massive liver necrosis caused by acetaminophen, expansion of the HPC mass must be accompanied by removal of necrotic tissue, but the latter is not a key element of regeneration after partial hepatectomy. Thus, there is likely divergence in the precise role of different cell types, particularly macrophages, in repair of toxic responses in the liver compared to partial hepatectomy. The reader is referred to recent studies examining mechanisms of liver repair after acetaminophen overdose (Bhushan et al., 2014, 2016, 2017a, 2017b; Williams et al., 2014; Kopec et al., 2017).

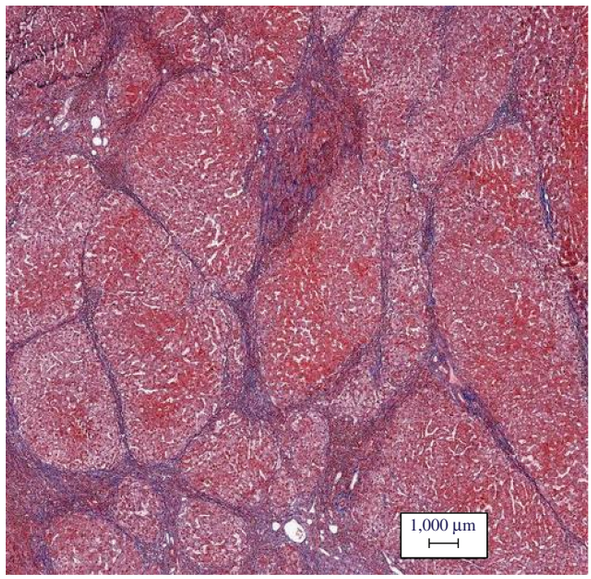


Figure 13-10. *Cirrhosis of the liver.* Fibrous bridges (stained blue) encase areas of liver tissue. (From https://casemed2013.wikispaces.com/Liver+Path.)

Fibrosis

Hepatic fibrosis (scarring) occurs in response to chronic liver injury that overwhelms the capacity of the organ to repair. It is characterized by the accumulation of excessive fibrous tissue, specifically fibril-forming collagen types I and III, and a decrease in normal plasma membrane collagen type IV (reviewed by Bataller and Brenner, 2005; Rockey and Friedman, 2006; Gutierrez-Ruiz and Gomez-Quiroz, 2007; Friedman, 2008b). Fibrosis can develop around central veins, portal tracts, or within the space of Disse. This progressive collagen deposition, marked by interconnecting fibrous scars, alters the architecture of the liver. When the fibrous scars subdivide the remaining liver mass into nodules of regenerating HPCs, fibrosis has progressed to cirrhosis (Fig. 13-10), and the liver has limited residual capacity to perform its essential functions. The excessive extracellular matrix protein deposition and loss of sinusoidal endothelial cell fenestrae and of HPC microvilli limit exchange of nutrients and waste material between HPCs and sinusoidal blood.

The primary cause of hepatic fibrosis/cirrhosis in humans worldwide is viral hepatitis; however, biliary obstruction and, in particular, alcoholic and nonalcoholic steatohepatitis are of growing public health importance for the development of hepatic fibrosis (Bataller and Brenner, 2005). In addition, fibrosis can be induced by chronic exposure to drugs and chemicals and by heavy metal overload (Gutierrez-Ruiz and Gomez-Quiroz, 2007). Repeated treatment with carbon tetrachloride, thioacetamide, dimethylnitrosamine, aflatoxin, or other chemicals has been associated with hepatic fibrosis in experimental animals and humans (Zimmerman, 1999). Indeed, just about any chronic insult to the liver that results in a rate of HPC death that exceeds the ability of the liver to repair itself can result in liver fibrosis.

Central to the development of fibrosis is the migration and activation of hepatic stellate cells and their differentiation into myofibroblast-like cells. Thus, stellate cells become the main cell type producing extracellular matrix proteins in the liver (Bataller and Brenner, 2005; Gressner and Weiskirchen, 2006; Rockey and Friedman, 2006). Clarifying the mechanisms underlying stellate cell activation is important to understanding the onset of fibrosis; however, it is important to note that other cell types (e.g., peribiliary fibroblasts, bone marrow-derived cells) can also become myofibroblastic. The increased accumulation of stellate cells at sites of injury is caused by their migration as well as proliferation. The proliferation of stellate cells is induced by the formation of mitogens such as platelet-derived growth factor (PDGF) and the upregulation of the PDGF receptor, which further enhances the responsiveness of stellate cells to this mitogen (Pinzani, 2002). PDGF, monocyte chemotactic protein 1 (MCP-1), and other chemokines are stellate cell chemoattractants (Marra, 2002).

Stellate cell activation is initiated by products formed during liver cell injury. For example, activating signals can be reactive oxygen species and lipid peroxidation products generated in injured HPCs. In addition, Kupffer cells can release reactive oxygen and pro-inflammatory cytokines during the phagocytosis of cell debris or apoptotic bodies, thereby recruiting more inflammatory cells and enhancing the injury and oxidant stress (Tsukamoto, 2002). Stellate cell activation is also driven by the excessive production of extracellular matrix proteins induced mainly by TGF-β1, which is generated to a large degree by this cell type. The effects of TGF-β1 are amplified by the increased expression of TGF-β receptors on stellate cells (Gressner and Weiskirchen, 2006). Damaged sinusoidal endothelial cells contribute to the activation of stellate cells by generating a splice variant of cellular fibronectin and by releasing urokinase-type plasminogen activator, which processes latent TGF-β1 (Friedman, 2000). Furthermore, platelets accumulated at the site of injury can produce TGF-β1 and growth factors such as PDGF (Rockey and Friedman, 2006).

Together, these and other stimuli cause the activation of stellate cells, which undergo phenotypic changes that involve proliferation, fibrogenesis, matrix remodeling, and pro-inflammatory mediator expression, including production of chemotaxins for inflammatory cells (Rockey and Friedman, 2006; Friedman, 2008b). Another hallmark of stellate cell activation is enhanced contractility due to the increased expression of α-smooth muscle actin. Increased expression

of endothelin-1 (ET-1) receptors on stellate cells together with the general imbalance between vasodilator (nitric oxide, carbon monoxide) and vasoconstrictor (endothelin-1) formation contributes to the development of portal hypertension during fibrosis (Rockey, 2003). During fibrogenesis there is not only an overall increase in extracellular matrix deposition, but also a qualitative change from the basement membrane-like matrix dominated by nonfibril-forming collagens (types IV, VI, and XIV) to one involving fibril-forming collagen types I and III. This effect involves the differential expression and release of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinase [TIMPs]) from stellate and Kupffer cells. TIMP1 and TIMP2 are upregulated and MMP1 (collagenase I) is downregulated during fibrogenesis, leading to the reduced degradation of fibril-forming collagens (e.g., type I collagen). At the same time, MMP2 and MMP9 (collagenase IV) are activated, causing the accelerated degradation of nonfibril-forming collagens (Arthur, 2000). The end result is enhanced deposition of fibril-forming collagens that characterize fibrosis.

Liver Cancers

Chemically induced neoplasia can involve tumors that are derived from HPCs as well as other cell types within the liver (Sell, 2002). A detailed discussion of chemical carcinogenesis is beyond the scope of this chapter but is discussed in Chap. 8. Hepatocellular cancer has been linked to chronic abuse of androgens, alcohol, and consumption of aflatoxin-contaminated diets. In addition, viral hepatitis, metabolic diseases such as hemochromatosis, α_1-antitrypsin deficiency, and nonalcoholic steatohepatitis are major risk factors for hepatocellular carcinoma (Zimmerman, 1999; McKillop et al., 2006; Wands and Moradpour, 2006; Aravalli et al., 2008). A pronounced synergy between dietary aflatoxin exposure and hepatitis B virus infection is now well recognized (Henry et al., 2002). The prevalence of hepatitis B and C viruses and environmental factors make hepatocellular carcinoma one of the most common malignant tumors worldwide (Bosch et al., 2005).

Nonparenchymal cells are the precursors for other types of cancers. Rare but highly malignant angiosarcomas are derived from sinusoidal lining cells. Angiosarcomas have been associated with occupational exposure to vinyl chloride and arsenic (Zimmerman, 1999). Exposure to radioactive thorium dioxide used as contrast medium for radiology (Thorotrast) has been linked to tumors derived from HPCs, sinusoidal cells, and bile duct cells (cholangiocarcinoma) (Zimmerman, 1999). The compound accumulates in Kupffer cells and emits radioactivity throughout its extended half-life. One study of Danish patients exposed to Thorotrast found that the risk for bile duct and gallbladder cancers was increased 14-fold and that for liver cancers more than 100-fold (Andersson and Storm, 1992). Furan is a chemical that causes cholangiocarcinomas experimentally in rats (Hickling et al., 2010).

CLASSIFICATION OF LIVER INJURY

Liver injury can be characterized in a number of different ways based on morphology (histopathology), serum or plasma biomarkers (clinical chemistry), or the nature of the toxic response (intrinsic vs. idiosyncratic). Each of these provides a different conceptual framework for classifying liver injury, which can be helpful in experimental and clinical settings.

Histopathology

In experimental studies with animals, histopathological evaluation remains the "gold standard" for characterizing liver injury. Histopathology of liver biopsies is sometimes used to aid in diagnosis of liver injury in human patients, but only under certain circumstances because the procedure involves some risk to the patient. The histological pattern of acute liver toxicity often follows the lobular zonation described above in section "Hepatic Functional Anatomy." Many toxicants, especially those that are metabolized to more toxic metabolites, cause necrosis of HPCs that occurs in a centrilobular distribution, that is, necrosis distributed around the central vein (Fig. 13-7, bottom panel). This is often because enzymes such various isoforms of cytochromes P450 (CYPs) that bioactivate many chemicals to toxic metabolites occur in greater concentration in HPCs residing in centrilobular (zone 3) regions than in periportal (zone 1) or midzonal (zone 2) areas. However, other determinants of zonal HPC heterogeneity such as supply of oxygen and concentration of cofactors for metabolism (e.g., NADPH, glutathione) can also contribute to distribution of lesions. Xenobiotic agents that cause centrilobular lesions include halothane anesthetic (Benjamin et al., 1985; Dugan et al., 2010), pyrrolizidine alkaloid plant toxins (Copple et al., 2002), solvents such as carbon tetrachloride (Brattin et al., 1985), acetaminophen (McGill et al., 2013), and other drugs and many other chemicals. In contrast, liver lesions from chemicals such as aflatoxin B1 and allyl alcohol occur in periportal regions, whereas midzonal lesions predominate after systemic exposure to bacterial endotoxin (Sneed et al., 1997; Barton et al., 2000).

Clinical Chemistry

When the liver is injured, various molecular constituents of damaged cells can be released into the blood. These constituents can be quantified in plasma or serum samples and serve as biomarkers that track with the severity of ongoing liver injury (Giannini et al., 2005). Examples that are used in both clinical and experimental quantification of hepatocellular injury are alanine aminotransferase (ALT) and aspartate aminotransferase (AST). These enzymes are normally contained selectively within HPCs but are released into the plasma when these cells are injured by chemicals or by liver diseases. Other enzymes that occur selectively in HPCs include mitochondrial glutamate dehydrogenase (GLDH), ornithine carbamoyltransferase (OCT), and various dehydrogenases (e.g., lactate dehydrogenase [LDH]). There are differences in these enzyme biomarkers of HPC injury with respect to their concentrations along the sinusoid, organellar associations, longevity in the plasma, occurrence in different species, and selective presence in liver relative to other organs. These differences determine specificity and sensitivity in detecting and quantifying injury caused by different mechanisms and arising from different lobular locations or species. For example, ALT occurs relatively selectively in liver, whereas AST occurs also in muscle and other tissues; therefore, AST is less selective as a biomarker of HPC injury (Vroon and Israili, 1990). In recent years, there has been interest in liver-selective microRNAs (e.g., miR-122), high mobility group box-1 (HMGB1) protein, and keratin-18 in plasma as HPC injury biomarkers, but these have yet to gain utility in clinical settings (Antoine et al., 2012, 2013; Hornby et al., 2014).

Other serum biomarkers are used to detect cholestatic injury. For example, bilirubin is a breakdown product of hemoglobin and is eliminated by uptake into HPCs, where it is conjugated with glucuronic acid and secreted into the bile by the MRP2 transporter (Zollner & Trauner, 2006). Reflux of conjugated bilirubin into the plasma ("direct bilirubin") occurs during HPC injury or biliary obstruction (Westwood, 1991). As noted above, bile acids are normally synthesized in HPCs and exported into the biliary system. Reflux of bile acids (e.g., cholic acid, chenodeoxycholic acid) into

the plasma can provide a biomarker of cholestasis (Burkard et al., 2005; Yamazaki et al., 2013).

Alkaline phosphatase is expressed on the canalicular membrane of HPCs and in biliary epithelium as well as in other tissues. Increased serum alkaline phosphatase (ALP) activity is used clinically as a biomarker of hepatobiliary injury. Such increases are thought to arise at least in part from enhanced accumulation of bile acids in HPCs that prompts increased ALP expression and release from HPCs into the plasma (Hatoff and Hardison, 1979; Kaplan, 1986). Gamma-glutamyl transferase (GGT) is localized to the plasma membrane of HPCs and biliary epithelial cells, and increases in plasma GGT activity are used in the diagnosis of biliary injury and to help confirm a liver source of ALP increase (Irie et al., 2007). 5′-Nucleotidase (5NT) is another such enzyme that is localized to the canalicular and sinusoidal membranes of HPCs and released into plasma during liver injury. Notably, all of these enzymes occur in other organs and are therefore not specific for detection of liver injury (Martin et al., 2007); nevertheless, they are widely used for the detection of cholestatic liver injury.

In addition to these constituents that are released into plasma, injection of certain dyes has been used to assess HPC and biliary injury. For example, indocyanine green (ICG) is rapidly excreted by HPCs into the bile and has been used to quantify hepatocellular function as well as liver blood flow (Schneider, 2004). Other tests of hepatic function rely on the synthesis of plasma proteins by the liver. HPCs synthesize and release into the circulation many coagulation factors, and coagulopathies therefore occur in patients with liver injury because they are less able to maintain adequate plasma concentrations of these proteins. Accordingly, increased tissue factor-induced clotting time (i.e., prothrombin time) indicates a high likelihood of hepatic failure (Stravitz et al., 2007).

Because values for enzyme activities in serum/plasma from normal (i.e., healthy) people can vary across clinical chemistry laboratories that perform the measurements, increases in the enzyme activities are typically expressed as a ratio normalized to the "upper limit of normal" (ULN) for the particular laboratory in which the measurement was performed. In humans, serum ALT activity that is 3 to 5 times or more above the ULN suggests liver injury. Clinically, drug-induced liver injury is often classified as hepatocellular, cholestatic, or mixed (i.e., both), depending on clinical chemistry results. For this classification, a so-called "R-value" is calculated from serum enzyme activities, where R = (ALT/ALT ULN)/(ALP/ALP ULN). If R is >/= 5, the injury is considered to be hepatocellular; if R </=2 cholestatic, and if R >2 but <5 mixed. This provides a conceptual framework for the diagnosis of liver injury (Table 13-3). Useful in predicting clinical prognosis in patients with liver injury is "Hy's Law." This is based on an observation by Dr Hyman Zimmerman that the combination of serum ALT > 3XULN and total bilirubin > 2XULN in patients with drug-induced liver injury portends a poor outcome (i.e., a risk of 10% mortality) (Chalasani et al., 2014).

Table 13-3

Classification of Drug-induced Liver Injury Pattern Based on Clinical Chemistry Measurements*

R = ALT/ALP	Pattern of injury
R >/= 5	Hepatocellular
R </= 2	Cholestatic
2 < R < 5	Mixed

**Serum ALT and ALP activity measurements are expressed as multiples of the upper limit of normal, then a ratio (R) of ALT/ALP is calculated.*

Finally, there are a couple of important points to be made about clinical chemistry biomarkers. First, neither they nor histopathological evaluation of liver injury can distinguish toxicant-induced liver injury in patients from liver disease caused by pathogens, genetic predisposition, or other etiologies. This can render diagnosing drug-induced liver injury a challenge for physicians, and consequently sets of criteria have been developed to aid in differential diagnosis (Corsini et al., 2012; Chalasani et al., 2014). Second, although it is common to refer to clinical chemistry biomarkers as "liver function tests," most of them are not measures of liver function. For example, increased serum ALT activity is a biomarker of HPC injury, but it does not necessarily reflect overall liver function; in contrast, an increase in serum bilirubin does reflect a dysfunction in the hepatobiliary disposition of bilirubin. This distinction becomes important in some clinical scenarios. For example, some patients with severe, drug-induced liver injury experience an early rise in serum ALT activity followed by an increase in serum bilirubin as the injured liver becomes dysfunctional. However, as injury progresses ALT activity can trend toward normal while bilirubin continues to rise, the latter marking a worsening of liver function and portending a poor outcome.

Intrinsic versus Idiosyncratic Responses

A commonly used classification of drug-induced liver injury (DILI) is based on the extent to which an individual's susceptibility enables the toxic response. "Intrinsic" toxic responses (aka, Type A responses or "toxic hepatitis") are those that have a well-defined relationship to the dose of the offending chemical and are capable of producing injury in all individuals (Licata, 2016; Iasella et al., 2017). There is often some difference in sensitivity among animals or among humans, but all individuals respond with toxicity at some dose. Intrinsic liver toxicity is associated with distinctive liver lesions that depend on the specific chemical and that occur after a predictable latent period. These reactions are initiated by a direct effect of the chemical or its metabolite(s) and are reproducible in experimental animals. Acetaminophen (APAP) is an example of a drug that causes intrinsic hepatotoxicity. When rodents or humans are exposed to APAP in overdose, centrilobular hepatocellular necrosis occurs in a predictable time period (e.g., within 6 hours in mice and within 48 hours in humans). Differences among individual human patients in the APAP dose needed to cause liver injury have been reported, and pronounced species and strain differences in sensitivity are evident in animals, but APAP is hepatotoxic at some dose in all animals (and probably in all humans). APAP is but one example of the many chemicals that cause intrinsic toxicity (see below for details on APAP hepatotoxicity).

"Idiosyncratic" drug reactions (Type B reactions) are adverse responses that occur in a minority of patients taking therapeutic doses of a drug, and the liver is frequently the target organ (Kaplowitz, 2005; Roth and Ganey, 2011). Large drug doses (i.e., overdoses) are not needed to elicit toxicity. Drugs from many pharmacological classes have caused idiosyncratic hepatotoxicity in human patients as have herbal medications, and it is likely that some environmental and occupational toxicants can precipitate idiosyncratic responses as well. Typically, these reactions occur only in a very small fraction of patients during drug therapy. Accordingly, individual susceptibility plays a major role in determining whether an idiosyncratic response occurs upon exposure to a particular drug. With these reactions, the relationship to dose is usually unclear. It was once thought that idiosyncratic responses had no relationship to

Table 13-4

Characteristics of Intrinsic and Idiosyncratic Hepatotoxicity

"INTRINSIC" TOXICITY (TYPE A)	"IDIOSYNCRATIC" TOXICITY (TYPE B)
– With drugs, usually occurs in overdose	– Occurs at pharmacologic treatment regimens
– Distinctive lesion	– Variable pathology
– Dose-related	– Dose relationship unclear
– Injury in all individuals	– Susceptible (small) fraction of people
– Predictable latent period	– Relationship of exposure to onset of toxicity variable
– Caused by "direct" effect of agent or its metabolite	– Highly influenced by host contribution (e.g., immune response, metabolism polymorphisms, etc.)
– Reproducible in animal models	– Not reproducible in typical preclinical animal tests

drug dose, but the observation that these reactions are more frequent for drugs that are consumed at doses greater than 50 mg/day suggests some relationship to dose (Chalasani and Björnsson, 2010). It is likely that they are dose-related within individuals but that a relationship with dose is not apparent in populations because of the wide disparity in susceptibility among individuals (Roth and Ganey, 2010b). Unlike intrinsic hepatotoxicity, idiosyncratic, drug-induced liver injury (IDILI) is associated with variable liver pathology, even for a single drug, and the relationship of exposure to the onset of liver injury is often variable; a reaction can occur within a few days of drug therapy or can reveal itself only after months of drug exposure. As in people, idiosyncratic reactions in animals are rare, and typical preclinical animal tests do not reveal the ability of drug candidates to cause IDILI. Table 13-4 contrasts the characteristics of intrinsic and idiosyncratic reactions.

Despite this classification into intrinsic and idiosyncratic toxicities, a question has been raised as to whether they actually fall into one spectrum (Roth and Ganey, 2010b; Funk and Roth, 2017). Importantly, the mechanisms by which idiosyncratic reactions arise remain unclear and largely unproven, and several hypotheses have arisen to explain the pathogenesis. Underlying all of the theories are various potential environmental and/or genetic determinants of individual susceptibility; a description of these and how they relate to idiosyncratic hepatotoxicity are described below (see sections "Determinants of Susceptibility" and "Idiosyncratic, Drug-induced Liver Injury").

INITIATION AND PROGRESSION OF HEPATOTOXICITY

The many molecular mechanisms by which toxicants can cause injury to cells and tissues are discussed in detail in Chap. 3 of this volume. The unique functions of the liver and its position in the circulation predispose it to toxicity-determining factors that do not apply to other organs. Depending on the chemical insult and the nature of the exposure, these can contribute to hepatotoxicity and can lead to one or more of the toxic manifestations described above in section "Liver Pathophysiology." The pathogenesis of chemical-induced liver injury can be viewed conceptually as comprising various molecular initiating events that trigger other events within and outside of HPCs that result in progression to HPC death and, consequently, to liver dysfunction (Fig. 13-11).

The initiation of injury typically begins with entry of a chemical into HPCs. Lipophilic compounds, particularly most drugs and environmental pollutants, readily diffuse into HPCs, and this is facilitated by the fenestrated endothelium in the sinusoids that enables close contact between circulating molecules and HPCs. Other toxicants are rapidly extracted from blood because they are substrates for uptake transporters located on the sinusoidal membrane of HPCs (Hagenbuch and Gui, 2008; Roth et al., 2012). Once inside the cell, some toxicants exert their effects directly by binding reversibly to receptors or other proteins, whereas others are bioactivated to toxic metabolites by CYPs or other enzymes (see Chaps. 3 and 6 and Park et al., 2011). It is usually oxidative (phase I) reactions that yield reactive, toxic metabolites, whereas conjugation (phase II) reactions generally (but not always) yield stable metabolites. If the rate of production of a toxic metabolite exceeds the capacity of the HPC to detoxify it, HPC injury can ensue (Park et al., 2011). Thus, the balance between rates of phase I reactions and conjugating phase II reactions can determine whether a toxic metabolite is detoxified or causes injury. Depending on the chemical and the relevant bioactivating enzyme(s), xenobiotic metabolism can result in production of damaging free radicals or reactive electrophiles that bind covalently to macromolecules such as DNA and intracellular proteins. Although there exists ample evidence that covalent binding of some xenobiotic metabolites to proteins initiates cell injury, the specific protein(s) that are critical to the response have not been identified for most toxicants that act by this mechanism. An exception is the binding of microcystins to cytoskeletal proteins (see below).

These various initiating events lead to the disruption of HPC homeostasis, often by activating intracellular cell death signaling pathways. This culminates in cellular ion imbalance, mitochondrial dysfunction, altered gene expression, and/or DNA damage through activation of caspases or other mechanisms. In addition to these intrahepatocellular events, injured HPCs can release various factors that promote progression of liver injury. For example, calpain is a calcium-activated protease that leaks from damaged HPCs, becomes activated in the extracellular milieu, and damages neighboring cells by cleaving membrane-associated proteins (Mehendale and Limaye, 2005; Mehendale, 2013). Many so-called damage-associated molecular pattern molecules (DAMPs) are released from injured HPCs. DAMPs activate pattern recognition receptors (e.g., toll-like receptors) present on nonparenchymal cells such as SECs, Kupffer cells, NK cells, and stellate cells. In addition, some hepatotoxic responses are associated with disturbance of the intestinal epithelium, which can result in the translocation into the portal circulation of pathogen-associated molecular pattern molecules (PAMPs; e.g., lipopolysaccharide, LPS) produced and released by intestinal flora. Like DAMPs, these PAMPs stimulate nonparenchymal cells, activation of which results in generation of ROS and NO and release of many inflammatory mediators including numerous cytokines, prostanoids, growth factors, proteases, etc. Expression of adhesion molecules and release of chemokines by activated nonparenchymal cells or by HPCs result in the infiltration into the liver of blood leukocytes and platelets. Activation of these cells prompts additional production of inflammatory mediators. In addition, the coagulation and complement cascades become activated. Together, these inflammatory events and other factors (e.g., SEC injury) can either promote or ameliorate the progression of HPC injury by activating various cell death or cell survival pathways, respectively.

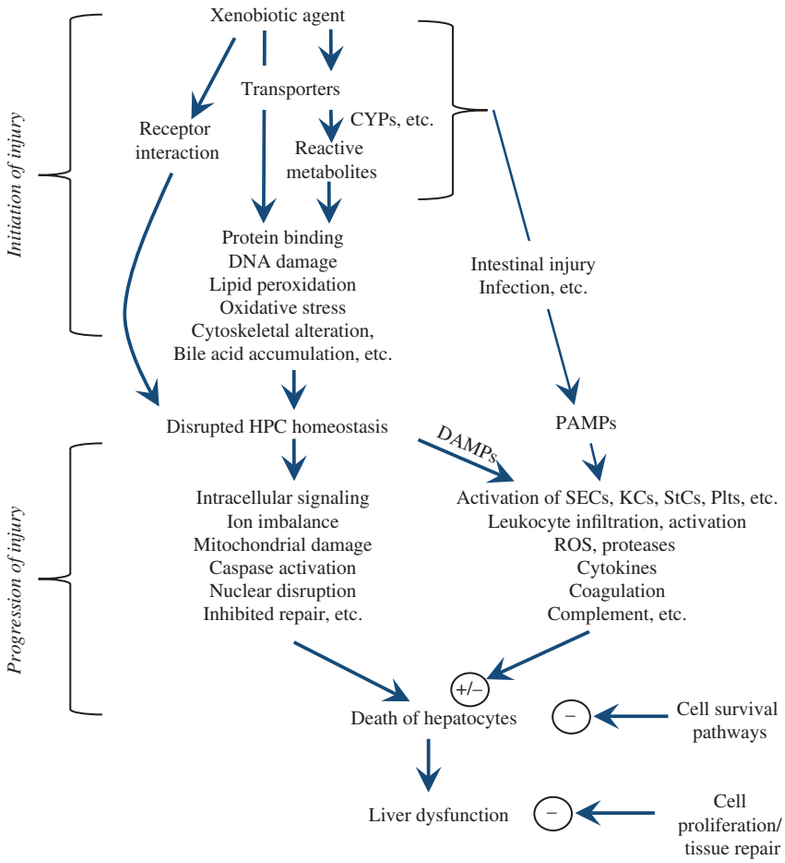


Figure 13-11. *Initiation and progression of hepatotoxicity.* A xenobiotic agent (drug, pollutant, etc.) or its metabolite(s) causes a molecular interaction with hepatocytes that is the initiating event in liver injury. This is followed by numerous events within hepatocytes (left) and/or within sinusoids (extrahepatocellular, right) that promote progression of injury. Accordingly, numerous mechanisms can contribute to hepatotoxicity from a single chemical. Cellular survival and repair mechanisms as well as extracellular events can modulate injury. Depending on the dose of the chemical, these complex and interdependent events can lead to recovery of tissue function or worsening of tissue injury. CYPs, cytochromes P450; HPC, hepatic parenchymal cell (hepatocyte); DAMP, damage-associated molecular pattern molecule; PAMP, pattern-associated molecular patternmolecule; SEC, sinusoidal endothelial cell; KC, Kupffer cell; StC, stellate cell; Plt, platelet; ROS, reactive oxygen species.

The ultimate outcome of these initiation and progression events is influenced by susceptibility factors, which vary among individuals (see below), and by mechanisms that oppose progression of injury. The latter include inactivation of ROS and other free radicals by enzymes or antioxidant molecules (see Chap. 3), elimination of toxic products by transport into bile or urine, and cell proliferation that replenishes the liver. Noteworthy is that failure of proliferative repair can lead to enhanced severity of chemical-induced liver injury. The next sections elaborate on these initiation and progression mechanisms as they relate to specific hepatotoxicants.

EXAMPLES OF HEPATOTOXICANTS AND THEIR MECHANISMS OF ACTION

In this section a few examples are presented of the many toxicants that injure the liver in people and animals. Xenobiotic agents were chosen for discussion based on their importance to human poisoning and/or their use in animal models of human disease. Acetaminophen and ethanol are emphasized because of their importance in causing liver injury in people and because their mechanisms of action have been extensively researched.

Acetaminophen

One of the most widely used analgesics, acetaminophen (*N*-acetyl-*p*-aminophenol; APAP) is a safe drug when used at therapeutically recommended doses. However, an overdose can cause severe liver injury and even liver failure in experimental animals and in humans (Lee, 2004, 2013). About half of all overdose cases are caused by suicide attempts, but an increasing number of cases are reported with unintentional overdosing (Lee, 2013). Although the toxicity is rare relative to the millions of patients taking the drug daily, APAP-mediated liver injury represents a significant clinical problem. During the last two decades, APAP-induced hepatotoxicity became the most frequent cause of acute, drug-induced liver failure in the United States and the United Kingdom (Lee, 2004, 2013).

APAP poisoning results in centrilobular hepatocellular necrosis (Fig. 13-12) in humans and in mice. The mouse model has been used extensively to study APAP metabolism and mechanisms of APAP hepatotoxicity. At therapeutic doses, approximately 90% of APAP is conjugated with sulfate or glucuronide and excreted. This limits formation by CYPs of a reactive, toxic metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). Most of the NAPQI is detoxified by conjugation with glutathione (GSH), thereby limiting its

covalent binding to cellular proteins, which is the initiating event for HPC damage (Jollow et al., 1973; Mitchell et al., 1973; Heard et al., 2011; McGill et al., 2013) (Fig. 13-13). In addition, the low levels of protein adducts formed after therapeutic doses are removed by autophagy (Ni et al., 2016). Thus, therapeutic doses of APAP pose minimal risk for liver injury. Consistent with these findings, long-term studies with APAP in osteoarthritis patients did not reveal evidence of liver dysfunction or cell injury even in patients consuming the maximal recommended daily dose of APAP for 12 months (Kuffner et al., 2006; Temple et al., 2006).

In contrast, after an overdose, overwhelmed sulfate and glucuronide conjugation pathways lead to break-through formation of large amounts of NAPQI, resulting in severe depletion of cellular GSH stores needed for NAPQI inactivation and thereby allowing extensive covalent binding of NAPQI to intracellular proteins

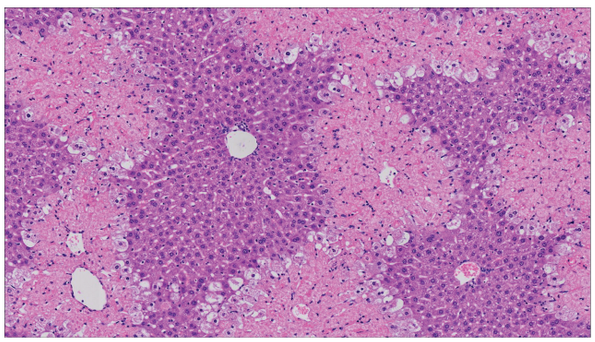


Figure 13-12. *Histopathology of acetaminophen-induced liver injury.* Liver section from a male, C57Bl/6J mouse treated 24 hours earlier with 300 mg/kg APAP. Liver was stained with hematoxylin and eosin. Note centrilobular necrosis and sparing of periportal areas. (Used with permission from Dr. Anna Kopec.)

(Jollow et al., 1973; Mitchell et al., 1973; McGill and Jaeschke, 2013) (Fig. 13-13). The generally greater concentration of bioactivating CYPs combined with the lesser GSH concentration in centrilobular HPCs are the main reasons for the predominantly centrilobular necrosis observed after APAP poisoning. Consistent with the critical role of protein binding for cell injury are the findings that APAP protein adducts are located mostly in centrilobular HPCs undergoing necrosis (Roberts et al., 1991) and that no APAP hepatotoxicity is observed without protein binding (Nelson, 1990). Because protein binding can be prevented by conjugation of NAPQI with GSH, any manipulation that reduces hepatic GSH levels, for example, fasting or protein malnutrition, enhances the toxicity of APAP. In contrast, interventions such as the supply of cysteine, the rate-limiting amino acid for GSH synthesis, promote the detoxification of NAPQI and limit cell injury (Mitchell et al., 1973). Based on this fundamental insight into the mechanism of APAP hepatotoxicity, *N*-acetylcysteine (NAC) was introduced into the clinic as intervention therapy (Prescott et al., 1977; Smilkstein et al., 1988). This highly successful therapeutic approach, which saved the lives of many patients who consumed an APAP overdose, is still the most effective treatment available (Rumack and Bateman, 2012; Waring, 2012). More recent evidence indicates that NAC treatment not only promotes cytosolic GSH synthesis to detoxify NAPQI but also replenishes the depleted mitochondrial GSH, which scavenges reactive oxygen and peroxynitrite (see below). In addition, excess NAC is degraded and supports mitochondrial ATP generation (Saito et al., 2010b).

Although the focus of early mechanistic investigations was on the role of covalent binding in APAP-induced hepatotoxicity, it became apparent that protein adduct formation is an important biomarker for APAP overdose (Davern et al., 2006); however, protein binding alone is not sufficient to explain cell injury. In fact, low levels of protein adducts are observed even after therapeutic doses

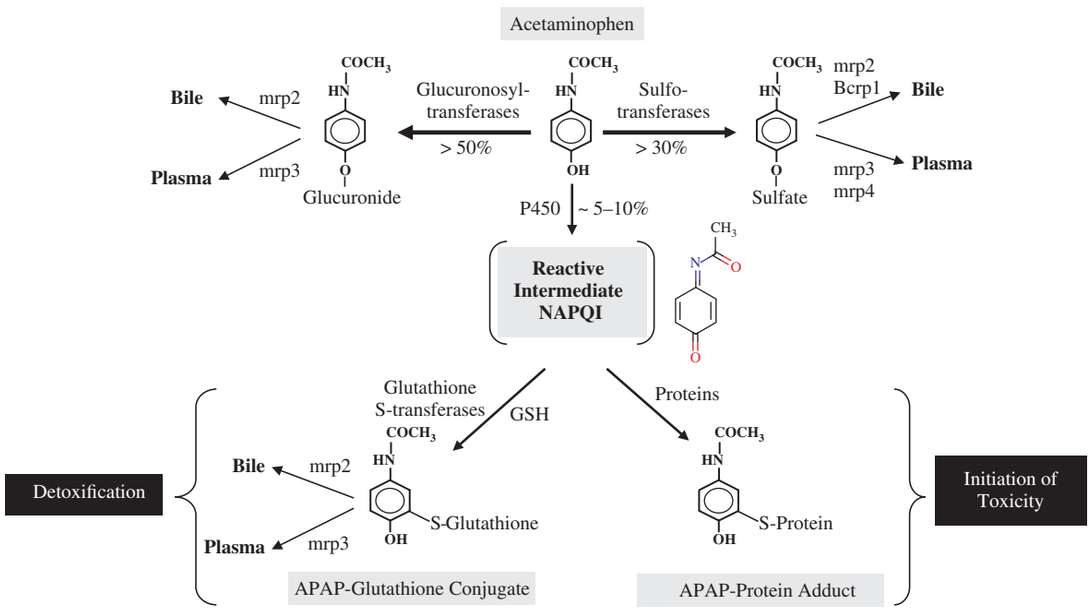


Figure 13-13. *Hepatic metabolism of acetaminophen.* At *therapeutic doses*, acetaminophen (*N*-acetyl-*p*-aminophenol) is metabolized in hepatocytes almost entirely by conjugation with sulfate and glucuronic acid (see percentages). These conjugates are transported into bile and into plasma for excretion by the kidneys. APAP is also deacetylated, but rapid reacetylation occurs in a "futile cycle," so that little *p*-aminophenol accumulates (not shown). A small fraction is metabolized by cytochromes P450 to *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is detoxified by conjugation with glutathione (GSH). At *toxic overdoses*, the sulfate and glucuronide pathways are overwhelmed, leading to greater NAPQI formation. If enough of this reactive metabolite is formed, GSH is depleted, then NAPQI binds to intracellular proteins to initiate an hepatotoxic response. (From Jaeschke and Bajt [2010].)

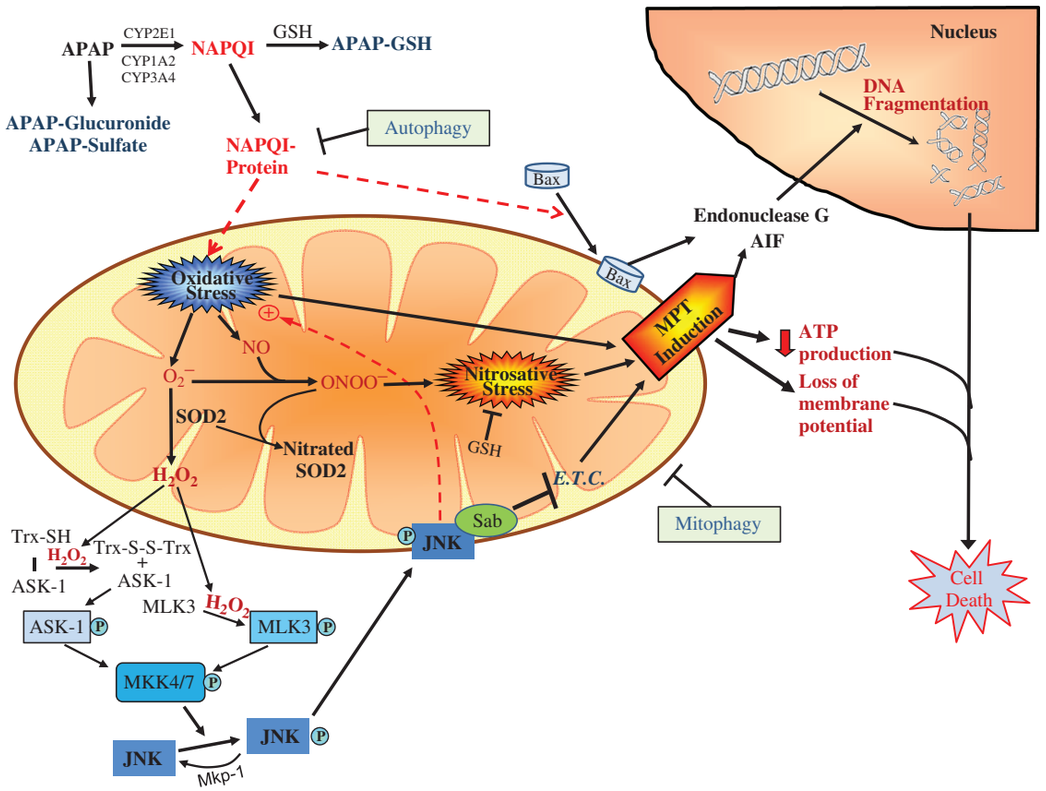


Figure 13-14. *Acetaminophen-induced cell death signaling in hepatocytes.* APAP is metabolized to reactive NAPQI, which binds to cellular proteins and initiates oxidative stress in mitochondria. This prompts activation of kinase signaling that results in phosphorylation of JNK, which translocates to the mitochondrial membrane where it interacts with Sab and inhibits the electron transport chain (E.T.C.) and enhances oxidative/nitrosative stress. The mitochondrial oxidative stress also results in the formation of peroxynitrite and consequent nitrosative stress. The combination of these events induces formation of the mitochondrial permeability transition (MPT) pore, which results in release of AIF and endonucleases that fragment nuclear DNA. These latter events are enhanced by the translocation of Bax to the outer mitochondrial membrane. In addition to DNA fragmentation, formation of the MPT pore results in reduced production of ATP and loss of mitochondrial membrane potential. Together, these events lead to compromised cell function and programmed necrosis. Depending on the magnitude of NAPQI-induced cell stress, autophagy and mitophagy can lead to cell survival, and GSH supplementation can also provide relief from the oxidative/nitrosative stresses. See text for abbreviations.

of APAP in mice (McGill et al., 2012a, 2013; McGill and Jaeschke, 2013) and humans (Heard et al., 2011). Because no APAP-induced cell injury is observed without covalent binding of NAPQI to cellular proteins, in particular mitochondrial proteins, it is considered a critical initiating event of the toxicity that requires additional intrahepatocellular events for progression to cell death (Jaeschke et al., 2012a; Du et al., 2016). Mitochondrial protein binding of NAPQI causes several untoward events, including inhibition of mitochondrial respiration, mitochondrial oxidant stress and peroxynitrite formation, and declining ATP concentration in the liver (Jaeschke et al., 2012a) (Fig. 13-14). Additional support for the central role of mitochondrial dysfunction comes from the observation that removal of damaged mitochondria by autophagy ("mitophagy") limits APAP-induced liver injury in vivo and in cultured cells (Ni et al., 2012). Mitophagy appears to be most relevant in preventing cell death at the periphery of centrilobular lesions (Ni et al., 2013).

The early mitochondrial translocation of Bax and Bid, members of the Bcl-2 family of proteins, triggers mitochondrial pore formation and the release of mitochondrial intermembrane proteins including endonuclease G and apoptosis-inducing factor (AIF) (Jaeschke et al., 2012a). These endonucleases, which translocate to the nucleus after APAP exposure, initiate nuclear DNA fragmentation (Jaeschke et al., 2012a). In addition, the continued exposure of GSH-depleted mitochondria to peroxynitrite results in nitration of mitochondrial proteins and mitochondrial DNA modifications (Cover et al., 2005). The prolonged oxidant stress eventually triggers opening of the mitochondrial permeability transition (MPT) pore with consequent breakdown of membrane potential, mitochondrial swelling, and rupture of the outer mitochondrial membrane (Kon et al., 2004; Ramachandran et al., 2011). These events lead to the loss of mitochondrial ATP synthesis capacity, more extensive nuclear DNA fragmentation due to the amplified release of intermembrane proteins after the MPT, and eventually oncotic necrotic cell death (Gujral et al., 2002). Because of the central role of mitochondrial oxidant stress in APAP hepatotoxicity, its regulation could be an important therapeutic target (Du et al., 2016).

Once initiated by early protein binding, the mitochondrial oxidant stress triggers activation (phosphorylation) of c-jun-N-terminal kinase (JNK), which then translocates to the mitochondria and amplifies the mitochondrial oxidant stress (Han et al., 2010; Jaeschke et al., 2012a; Du et al., 2015). JNK is phosphorylated by apoptosis signal regulating kinase-1 (ASK-1), which is liberated from the ASK-1-thioredoxin-1 complex in the cytosol after oxidation of thioredoxin-1 by the mitochondrial oxidant stress (Han et al., 2010; Jaeschke et al., 2012a; Du et al., 2015) (Fig. 13-14). Activated JNK is thereby an important contributor to APAP-induced hepatocellular necrosis.

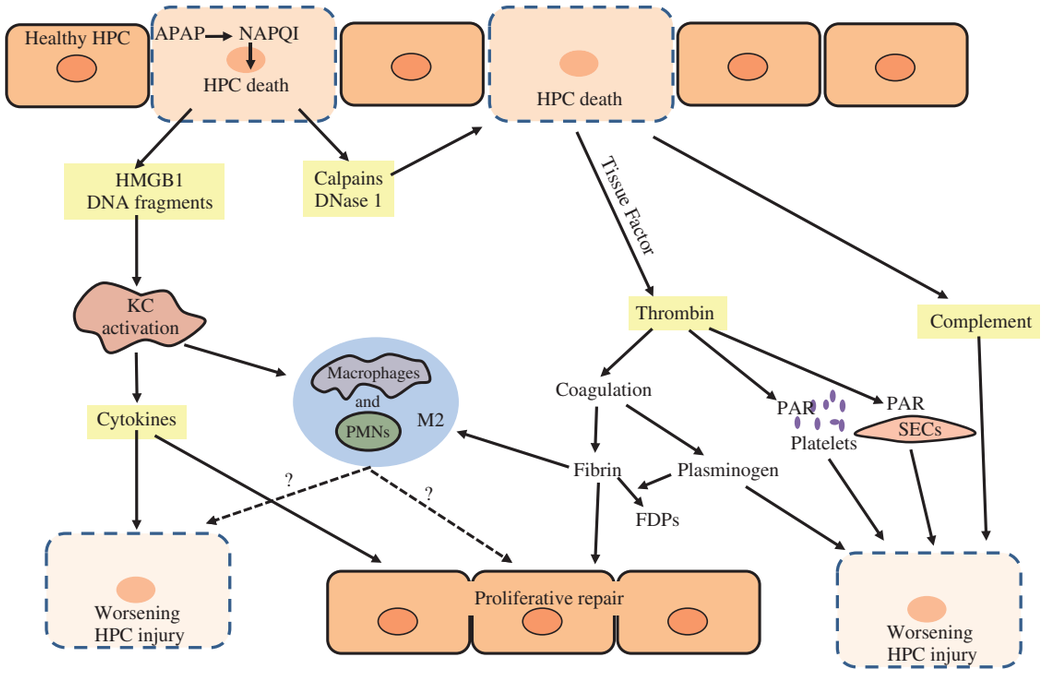


Figure 13-15. *Extrahepatocellular events in progression of APAP-induced liver injury.* Hepatocytes (HPCs) damaged directly by NAPQI release Ca^{2+}-activated proteases (calpains) and DNase that cause injury to neighboring HPCs. Release of DAMPs such as HMGB1 and DNA fragments activate Kupffer cells (KCs) and recruit additional leukocytes (neutrophils (PMNs) and monocyte-derived macrophages. Mediators such as cytokines released from these inflammatory cells can encourage either additional HPC death or proliferative repair of HPCs. HPC injury also exposes tissue factor that leads to thrombin activation in blood and consequent fibrin deposition that promotes repair through mechanisms involving αMβ2 integrin. Thrombin also activates protease-activated receptors (PARs) on platelets and on sinusoidal endothelial cells (SECs), and this, along with plasminogen and activated complement, contributes to worsening injury by unknown mechanisms. FDPs, fibrin degradation products.

Intrahepatocellular events leading to cell death from APAP are clearly complex, involving numerous factors and pathways. Even though some of these factors are involved in apoptotic pathways, the predominant histopathologic presentation is oncotic necrosis (Jaeschke and Bajt, 2006). Factors common to a "necroptotic" cell death, such as receptor interacting kinase protein 1 and 3 (RIP1/3), the critical executioner of necroptosis, participate in APAP-induced hepatocellular death; however, mixed lineage kinase domain like pseudokinase (MLKL), another necroptotic factor, is not involved. This suggests that APAP-induced cell death cannot be considered as necroptotic cell death but rather should be termed "programmed necrosis" (Ramachandran et al., 2013; Dara et al., 2015, 2016b).

In addition to these intracellular signaling mechanisms that are triggered directly by NAPQI and lead to cell death, secondary progression events can expand the area of necrosis (Fig. 13-15). For example, necrotic HPCs release Ca^{2+}-activated proteases (calpains) that can promote injury in nearby cells (Mehendale, 2012). Likewise, the release of DNase-1 enhances nuclear DNA fragmentation in adjacent cells and aggravates the injury after APAP overdose (Napirei et al., 2006). Some of the propagation of the injury might also progress through intercellular connexin channels (gap junctions) (Maes and Vinken, 2017).

Sinusoidal cells and blood elements can contribute as well to progression of hepatocellular injury and its resolution in APAP overdose. For example, during APAP-induced necrosis there is extensive release of various damage-associated molecular pattern molecules (DAMPs) including HMGB1, mitochondrial DNA, and nuclear DNA fragments (Antoine et al., 2012; McGill et al., 2012b). The release of these DAMPs from necrotic cells can activate resident macrophages (i.e., Kupffer cells) and recruit additional leukocytes (neutrophils, monocytes-derived macrophages) (Fig. 13-15). The precise role of the inflammatory response in APAP-induced liver injury is an area of intense investigation and continuing debate, for which consensus has not been reached. Likewise, deliberation is ongoing regarding soluble inflammatory mediators such as cytokines, studies of which employing similar approaches have produced different conclusions, as is the case for inflammasome involvement (e.g., see Williams et al., 2011, Xie et al., 2013; Woolbright and Jaeschke, 2017). Indeed, although strong evidence indicates that inflammatory cell activation plays a critical role in clearance of necrotic debris and repair of the injured liver (Jaeschke et al., 2012b; You et al., 2013; Woolbright and Jaeschke, 2017), other studies suggest that inhibition of these same cellular processes also reduces liver injury (Mossanen et al., 2016).

Toxic doses of APAP activate the hemostatic system and circulating complement (Fig. 13-15). Pharmacologic depletion of complement or genetic deficiency in complement factor C3 reduced APAP-induced liver injury in mice, suggesting a contribution of complement to the progression of hepatotoxicity (Singhal et al., 2012). Also, in mice given a toxic dose of APAP, tissue factor-mediated activation of the coagulation system led to thrombin activation and hepatic fibrin deposition, and anticoagulants reduced liver injury early (6 hours) but not at later times (24 hours) (Ganey et al., 2007; Sullivan et al., 2013; Kopec et al., 2017). These results suggest that an activated coagulation system contributes to both injury and repair. Thrombin not only leads to deposition of fibrin but also activates protease-activated receptors (PARs) present on SECs and platelets. Interestingly, studies in knockout mice suggested that the deposition of fibrin does not contribute to the progression of liver injury, rather activation of plasminogen and of PARs is involved

(Ganey et al., 2007; Sullivan et al., 2012; Miyakawa et al., 2015). Decreased concentration of blood platelets accompanied liver injury in APAP-treated mice, and a thrombin inhibitor prevented the thrombocytopenia and reduced liver injury. Moreover, antibody-mediated depletion of platelets prior to APAP administration reduced thrombin activation and injury to HPCs and endothelial cells, suggesting a role for platelets in the progression of liver injury (Miyakawa et al., 2015). In contrast, the coagulation-mediated deposition of fibrin appears to contribute to proliferative repair of livers injured by APAP by a mechanism that involves fibrin interaction with an integrin (αMβ2) on the surface of leukocytes and the consequent activation of matrix metalloproteinase 12 (MMP12) (Kopec et al., 2017). MMP12 is involved in the degradation of extracellular matrix and the migration of leukocytes that are needed for tissue repair. Together, these results indicate that the hemostatic system participates dichotomously in both liver injury and repair in the mouse model of APAP hepatotoxicity.

A treatise on the dichotomy in findings with respect to inflammation and hemostasis in APAP hepatotoxicity is beyond the scope of this chapter, but the reader is encouraged to recognize that inflammatory cells and hemostatic factors may not have singular roles in APAP-induced liver damage. The receipt of distinct extracellular cues can change the roles of these players from damaging to protective and vice versa. Additional understanding of how functions of inflammatory cells and blood elements can change under various circumstances might ultimately settle longstanding controversies about their roles in progression of injury and initiation of repair.

Although many details of mechanisms still remain to be elucidated, the newly gained knowledge into signaling events in response to APAP overdose suggests two fundamental insights. First, necrotic cell death is in most cases not caused by a single catastrophic event but can be the result of a cellular stress that is initiated by metabolic activation of APAP and which triggers sophisticated intra- and intercellular signaling mechanisms culminating in cell death. Second, the multitude of events following the initial stress offers many opportunities for therapeutic interventions at later times during injury progression. Because these events do not occur in all cells to the same degree and at the same time, delayed interventions might not completely prevent cell damage but might limit the area of necrosis enough to allow tissue regeneration and prevent liver failure. After APAP bioactivation in mice was complete, treatment with GSH or NAC to accelerate the recovery of mitochondrial GSH concentration effectively scavenged peroxynitrite, reduced the area of necrosis, and promoted regeneration, resulting in improved survival after APAP overdose (Bajt et al., 2003; Du et al., 2016). Equally effective in preventing APAP hepatotoxicity is the mitochondria-targeted superoxide dismutase-mimetic, Mito-TEMPO (Du et al., 2017). Overexpression of calpastatin, an inhibitor of calpains, also attenuated APAP-induced liver injury and enhanced survival (Limaye et al., 2006). Delayed treatment with inhibitors of JNK or of upstream MAP kinases such as apoptosis signal-regulating kinase 1 (ASK1) attenuated the mitochondrial oxidant stress and prevented the MPT and tissue injury in mice (Hanawa et al., 2008; Saito et al., 2010a; Du et al., 2015). Together these findings underscore the concept that the later stages of APAP-induced liver cell necrosis can be affected at the level of intracellular signaling in HPCs and during the propagation of the injury to neighboring cells.

A significant sensitivity factor in APAP hepatotoxicity can be the consumption of alcoholic beverages. In addition to potential malnutrition in alcoholic individuals, ethanol is a potent inducer of CYP2E1, which is the main enzyme responsible for the metabolic activation of APAP in humans (Gonzalez, 2007). Whereas simultaneous exposure to ethanol and APAP competitively inhibits NAPQI formation at the CYP2E1 locus and thereby can reduce APAP-induced toxicity (Sato and Lieber, 1981), the ethanol-induced increase in expression of CYP2E1 can enhance APAP toxicity after ethanol is eliminated (Gonzalez, 2007). Despite the clear evidence in experimental animals that alcohol consumption can increase the susceptibility to APAP (Sato et al., 1981; Sinclair et al., 2000) and the clinical observation of severe APAP hepatotoxicity in alcoholics, it remains controversial whether alcohol can actually induce hepatotoxicity at therapeutic doses of APAP in people, as suggested by some case reports (Zimmerman and Maddrey, 1995). An extensive review of the literature involving APAP consumption in alcoholics suggests no relevant risk for APAP hepatotoxicity at therapeutic levels in this patient population (Rumack et al., 2012). In addition, a randomized, double-blind, placebo-controlled trial with multiple therapeutic doses of APAP showed no evidence of liver dysfunction or cell injury in alcoholics (Dart et al., 2010). Thus, alcohol consumption does not appear to increase the risk for liver injury after therapeutic doses of APAP. Nevertheless, consistent with experimental data and clinical experience, inducers of CYPs can aggravate liver injury after a hepatotoxic dose of APAP. This finding may apply as well to the potential interactions of APAP with other drugs and dietary chemicals.

Much of the understanding of mechanisms of APAP toxicity has derived from studies in very useful murine models that recapitulate almost all aspects of APAP-induced liver injury in humans. Although the progress in understanding the complexity of APAP-induced liver injury during the last decade is encouraging, the critical question as to whether these mechanisms of cell death translate to human pathophysiology has only recently been addressed (Jaeschke et al., 2014). Studies employing metabolically competent human hepatoma cells (HepaRG cells) and primary human HPCs showed that key events in APAP toxicity occur also in human cells. These events include reactive metabolite formation with GSH depletion and mitochondrial protein adduct formation, oxidant stress, JNK activation and translocation of phospho-JNK to mitochondria, mitochondrial dysfunction, and necrotic cell death (Gunawan et al., 2006; McGill et al., 2011; Saberi et al., 2014; Xie et al., 2014). Moreover, assessments of serum biomarkers in humans who overdosed with APAP provide evidence for mitochondrial damage and nuclear DNA fragmentation similar to mice (McGill et al., 2012b). Importantly, the observation that poor survival correlates with release of mitochondrial biomarkers suggests that mitochondrial damage is critical for the injury process in humans (McGill et al., 2014). During APAP-induced necrosis in human patients, there is also extensive release of various DAMPs, including HMGB1, mitochondrial DNA, and nuclear DNA fragments (Antoine et al., 2012; McGill et al., 2012a, 2012b). Evaluation of peripheral blood neutrophils from patients demonstrated enhanced phagocytosis and priming for ROS generation during the recovery phase after injury (Williams et al., 2014). In addition, monocyte-derived macrophages recruited into the liver show an anti-inflammatory and proregenerative phenotype (Antoniades et al., 2012). Moreover, activation of coagulation occurs in APAP-poisoned people, and this might portend a poor outcome (Harrison et al., 1990; Kerr et al., 2003). Together, these results support the mouse model of APAP-induced liver injury as an appropriate reflection of the human pathophysiology.

Ethanol

Alcohol abuse is among the major causes of liver disease in Western countries (Crawford, 1999). The early stage of ethanol abuse is associated with hepatic lipid accumulation (steatosis).

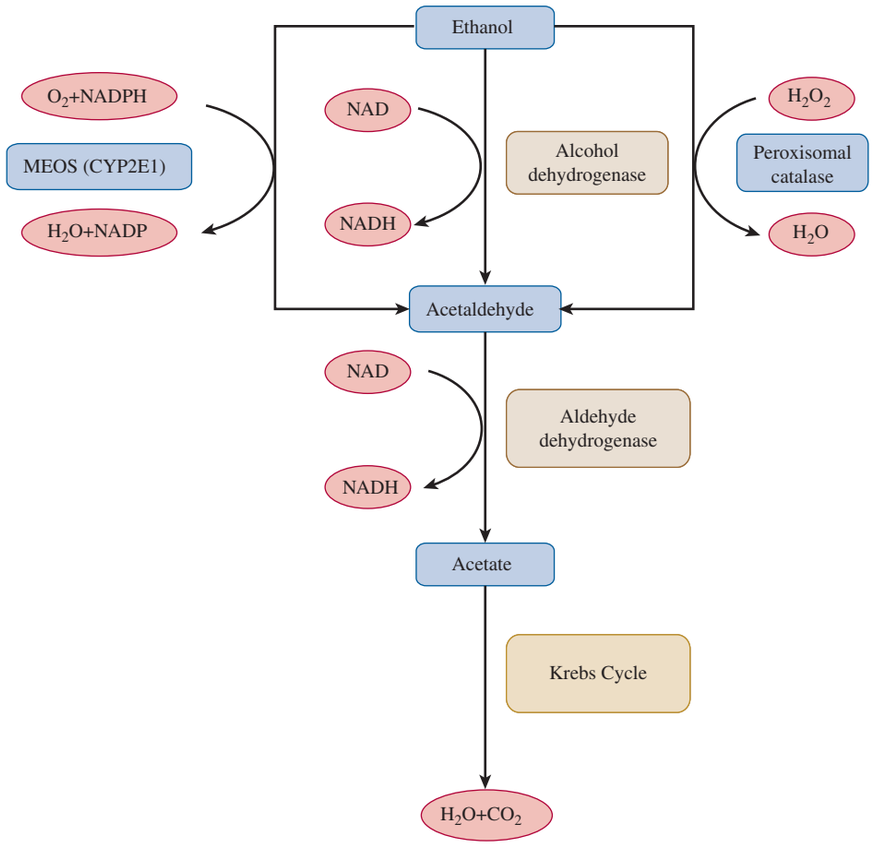


Figure 13-16. *Pathways of ethanol metabolism.* See text for abbreviations.

As alcohol-induced liver disease progresses, appreciable cell death occurs alongside increasing hepatic inflammation (i.e., steatohepatitis). If left unchecked, these pathologic processes drive replacement of functional liver mass with scar tissue (Bataller and Gao, 2015). This results in impairment of many functions of liver, including a progressive reduction in capacity for biotransformation of drugs. People with hepatic cirrhosis due to chronic alcohol abuse frequently become deficient at detoxifying both the ammonia formed by catabolism of amino acids and the bilirubin derived from breakdown of hemoglobin. Such hepatic dysfunction combined with defects in synthesis of key proteins, such as albumin and clotting factors, can ultimately drive multiple organ dysfunction and death (Gao and Bataller, 2011). Thus, ethanol provides a highly relevant example of a toxicant to which exposure contributes significantly to liver-related morbidity (Gao and Bataller, 2011).

Morbidity and mortality associated with the consumption of alcohol is mainly caused by the toxic effects of ethanol and its metabolites on the liver (Stewart and Day, 2006), although effects of alcohol on other tissues are increasingly appreciated (Yeligar et al., 2016). This targeted toxicity is observed because >90% of a dose of ethanol is metabolized in the liver. There are three principal pathways of ethanol metabolism (Fig. 13-16). Alcohol dehydrogenase (ADH) is quantitatively the most important pathway for alcohol metabolism. ADH oxidizes ethanol to acetaldehyde (Km of 1 mM); the electrons are transferred to NAD^+, which leads to the production of NADH. Acetaldehyde is further oxidized to acetate, also in a NAD^+-dependent reaction, by acetaldehyde dehydrogenase (ALDH). This pathway is mainly limited by the mitochondrial capacity to utilize NADH and regenerate NAD^+ (Stewart and Day, 2006).

Both ADH and ALDH exhibit genetic polymorphisms and ethnic variations that contribute to risk of alcoholism and ethanol-mediated liver damage (Agarwal, 2001; Day, 2006). One example of a toxicologically important polymorphism involves mitochondrial ALDH2, for which the ALDH2*2 form shows little or no catalytic activity. The resultant increased concentration of toxic acetaldehyde present in individuals who carry this polymorphism is thought to cause the uncomfortable "flushing" syndrome that accompanies ethanol exposure. This inactive form of ALDH is found in 50% of Asians but is absent in Caucasians. The discomfort experienced by people with this polymorphism might contribute the lower incidence of alcoholism in Asia compared to Europe and North America (Chen et al., 1999). However, heterozygotes of ALDH2*2 were found to develop more severe liver injury in response to lower alcohol consumption, suggesting a higher susceptibility to alcoholic liver disease (Enomoto et al., 1991). Polymorphisms also exist in ADH, some of which could lead to more rapid production of acetaldehyde (Wall, 2005). These findings underscore the importance of acetaldehyde in the pathophysiology of alcohol toxicity.

Although most ethanol is metabolized in the liver by ADH, a second pathway of importance involves the alcohol-inducible enzyme, CYP2E1, which, like ADH, oxidizes ethanol to acetaldehyde (Fig. 13-16). This enzyme is located predominantly in HPCs of the centrilobular region and requires both oxygen and NADPH. Because the Km of CYP2E1 for ethanol is >10 mM, this reaction is most relevant for large doses of ethanol, and because expression

of the enzyme can be induced by ethanol, it becomes relevant in patients with chronic alcoholism (Stewart and Day, 2006). The third pathway involves catalase contained in peroxisomes within HPCs. In this reaction, ethanol functions as an electron donor for the reduction of hydrogen peroxide to water, and in the process it is oxidized to acetaldehyde. Due to the small concentrations of hydrogen peroxide that are typically available within HPCs, the capacity of this pathway is limited; less than 2% of an ethanol dose is metabolized through this pathway (Stewart and Day, 2006).

The mechanisms of alcohol-induced liver disease are complex and still incompletely understood. Evolution of the rodent models used to mimic alcohol-induced liver damage in people has been reviewed elsewhere (Mathews et al., 2014; Gao et al., 2017); there may not be a single animal model of ethanol-induced liver damage that recapitulates the entire spectrum of pathologies observed in human chronic alcoholism. Hepatocellular steatosis is a common feature of chronic alcohol consumption. Alcohol-mediated steatosis is caused by the excessive supply of acetate and NADH, which increases fatty acid synthesis. In addition, ethanol and acetaldehyde disrupt constitutive regulation of fatty acid metabolizing enzymes by impairing DNA binding of the transcription factor, peroxisome proliferator-activated receptor-α (PPAR-α) (Aoyama et al., 1998). In addition to this imbalance favoring increased fatty acid synthesis, ethanol exposure inhibits the transfer of triglycerides from liver to adipose tissue. Acetaldehyde inhibits the microsomal triglyceride transfer protein, which incorporates triglycerides into VLDL (Lin et al., 1997) and reduces VLDL release from HPCs by interfering with microtubular function (Kannarkat et al., 2006). These effects of ethanol and its metabolites can be compounded in the presence of a high-fat diet. Although steatosis alone is not considered a form of severe liver disease, it represents an early and permissive phase in the spectrum of liver pathologies caused by alcohol exposure (Day and James, 1998a, 1998b; Tsukamoto et al., 2009). In the two-hit hypothesis of alcoholic liver disease, steatosis is considered the "first hit," which requires a "second hit" to drive simple steatosis to the necroinflammatory change observed in severe alcoholic liver injury (Day and James, 1998a, 1998b). This should not exclude the possibility that lipotoxicity itself is a critical determinant of disease progression (Neuschwander-Tetri, 2010). Moreover, results of recent studies suggest that inflammatory events triggered by ethanol can occur independently of steatosis (Wang et al., 2017).

CYP2E1 is a source of reactive oxygen formation during ethanol metabolism (Dey and Cederbaum, 2006). The resultant intracellular oxidant stress in HPCs can ultimately induce mitochondrial dysfunction and cell death of HPCs, but it also activates stellate cells and promotes fibrosis (Dey and Cederbaum, 2006). Direct evidence implicates CYP2E1 function in alcohol-induced pathologies, including gut leakiness and liver pathology (Abdelmegeed et al., 2013). In addition to the intracellular events, alcohol exposure causes an inflammatory response, which contributes to the oxidant stress (Arteel, 2003; Hines and Wheeler, 2004). Gut-derived endotoxin and other bacteria-derived products leaking into the portal circulation from a leaky gut can activate Kupffer cells through toll-like receptor activation to produce reactive oxygen species and cytokines, such as TNF-α (Seki and Schnabl, 2012). The formation of these mediators can be amplified by feedback loops, which enhance cytokine and chemokine formation through priming of the redox-sensitive transcription factor, NF-κB, in Kupffer cells (Arteel, 2003). In addition, TNF-α can increase expression of inducible nitric oxide synthase (iNOS, NOS2) leading to the formation of peroxynitrite, a potent oxidant species capable of nitrating proteins (Arteel, 2003). TNF-α can also directly promote cell death by acting on HPCs, which are rendered sensitive by ethanol-induced depletion of mitochondrial GSH (Colell et al., 1998; Nagai et al., 2002).

Inhibition of the proteasome pathway, a well-recognized feature of chronic alcohol exposure, can enhance chemokine formation in HPCs and promote inflammatory liver injury (McClain et al., 2005). Additional pro-inflammatory mediators and immune responses can be triggered by protein adducts of acetaldehyde and by lipid peroxidation products (Freeman et al., 2005) as well as by DAMPs released from HPCs and PAMPS (e.g., LPS) translocated from the intestine into the portal circulation (Miller et al., 2011) (Fig. 13-17). Interestingly, ethanol inhibits hepatic NK cells. Because NK cells can kill HSCs, the major cell type promoting hepatic fibrosis, ethanol may indirectly support fibrogenesis by preventing the elimination of activated stellate cells (Miller et al., 2011). More recently, studies indicate that invariant NKT cells contribute to alcohol-induced liver damage by recruiting inflammatory cells such as neutrophils (Cui et al., 2015; Maricic et al., 2015; Mathews et al., 2016). A defense mechanism that is activated during alcoholic liver disease is autophagy, which can remove damaged cell organelles and modified proteins (Ding et al., 2011) and thereby reduce the activation of the innate immune response. Thus, alcoholic liver disease is a complex interplay between the activation of pro-cell death mechanisms (reactive metabolite formation, oxidant stress, protein adducts, and stimulation of pro-inflammatory and profibrotic innate immune responses) and activation of defense mechanisms (antioxidants, autophagy, and NK cell activation) (Gao and Bataller [2011]).

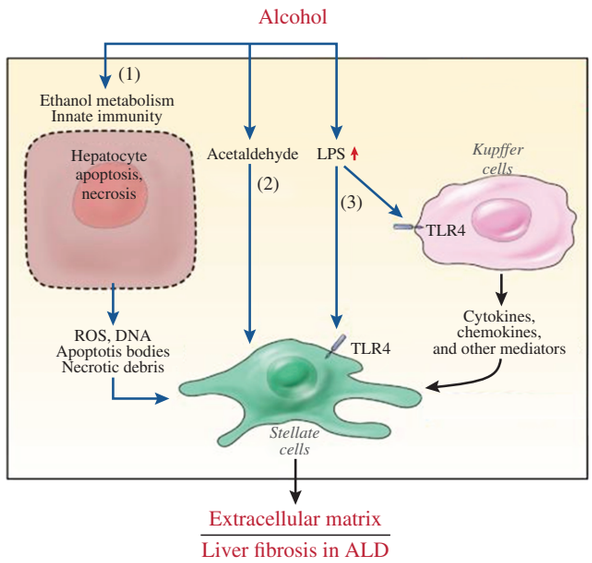


Figure 13-17. *Mechanisms of liver fibrosis in patients with alcoholic liver disease (ALD).* (1) Alcohol consumption causes hepatocyte damage, which leads to the release of a variety of mediators and the subsequent induction of stellate cell activation. (2) Acetaldehyde directly targets stellate cells and upregulates the expression of collagens by these cells. (3) Alcohol consumption results in elevation of gut-derived LPS levels in the liver. LPS can directly enhance stellate cell activation via upregulation of TGF-β signaling and can indirectly promote stellate cell activation via activation of Kupffer cells to release profibrotic cytokines and chemokines. (Modified from Gao and Bataller [2011].)

Allyl Alcohol

Allyl alcohol is an industrial chemical used in the production of resins, plastics, and fire retardants and has been used as a model hepatotoxicant due to its preferential periportal (zone 1) hepatotoxicity. The alcohol is metabolized by ADH to acrolein, a highly reactive aldehyde (Fig. 13-18), which is then further oxidized by ALDH to acrylic acid. The observations that the toxicity depends on depletion of hepatic GSH and is prevented by inhibitors of ADH but enhanced by inhibitors of ALDH suggest that acrolein formation is the critical event in liver injury (Jaeschke et al., 1987; Rikans, 1987). Age and gender differences in allyl alcohol hepatotoxicity can be explained by variations in the balance between ADH and ALDH expression (Rikans and Moore, 1987).

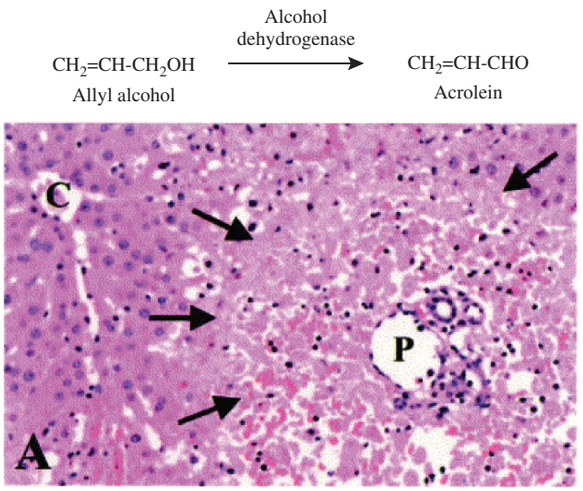


Figure 13-18. *Metabolism and liver injury from allyl alcohol.* Allyl alcohol is bioactivated to acrolein and produces periportal liver injury. Photomicrograph of a rat liver 24 hours after allyl alcohol administration. (From Yin et al. [1999b].)

The occurrence of allyl alcohol injury preferentially in zone 1 HPCs (Fig. 13-18) is caused by the predominant uptake of allyl alcohol in the periportal region and the oxygen dependence of the toxicity (Badr et al., 1986). Although protein binding of the acrolein and subsequent adduct formation appears to be the main cause of liver cell death (Kaminskas et al., 2004), lipid peroxidation can become an important mechanism of cell injury under conditions of a compromised antioxidant status or in the presence of excess iron (Jaeschke et al., 1987, 1992; Pompella et al., 1988; Knight et al., 2003). Lipid peroxidation is caused by a reductive stress during which excessive NADH formation produced by allyl alcohol metabolism leads to mobilization of redox-active iron from storage proteins (Jaeschke et al., 1992).

Carbon Tetrachloride

CCl_4 was once a widely used solvent, but human exposure to it has been restricted due to recognition that it is a potent hepatotoxicant. Acute exposure to CCl_4 causes centrilobular necrosis in animals and humans (Fig. 13-19). It has been used widely to model liver injury in animals, with its current use heavily weighted toward studying fibrosis (Fig. 13-19). Cytochrome P450-dependent conversion of CCl_4 to trichloromethyl free radical ($\bullet CCl_3$) and then to the trichloromethyl peroxyl radical ($CCl_3OO\bullet$) is a classic example of xenobiotic bioactivation to a free radical capable of initiating lipid peroxidation by abstracting hydrogen atoms from polyunsaturated fatty acids in phospholipid membranes (Fig. 13-19; Recknagel et al., 1989; Weber et al., 2003). Metabolic activation of CCl_4 in vivo involves primarily CYP2E1 as indicated by the absence of CCl_4 hepatotoxicity in CYP2E1 knockout mice (Wong et al., 1998). CCl_4-induced lipid peroxidation increases the permeability of the plasma membrane to Ca^{2+}, leading to severe disturbances in calcium homeostasis and consequent necrotic cell death (Weber et al., 2003). CCl_4 also induces significant mitochondrial damage, which is dependent on CYP2E-mediated metabolism and lipid peroxidation (Knockaert et al., 2012). In addition, the $\bullet CCl_3$ radical can bind directly to tissue macromolecules, as can some of the lipid peroxidation products such as 4-hydroxynonenal, which can form adducts with cellular proteins (Weber et al., 2003). In addition

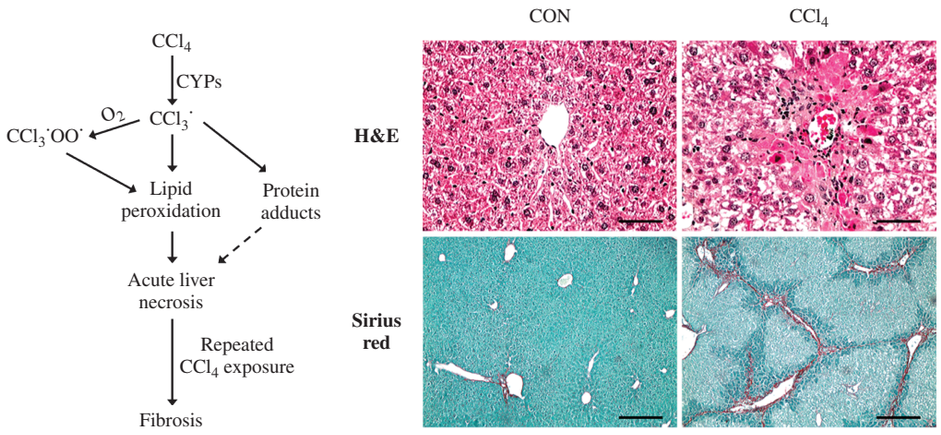


Figure 13-19. *Liver injury from carbon tetrachloride. Left*: CCl_4 is bioactivated by CYPs to the trichloromethyl free radical and in the presence of oxygen to trichloromethyl peroxyl radical. These radicals initiate lipid peroxidation of cell membranes and consequent hepatocellular necrosis. Protein adducts formed with radicals from CCl_4 and from lipid peroxidation also might contribute to liver injury. Repeated CCl_4 exposure leads to fibrosis. *Right*: Necrosis and fibrosis in livers of CCl_4-treated mice. Mice were treated twice per week for 10 weeks with CCl_4. *Top*: Hematoxylin and eosin staining of livers from control (CON) mice and mice treated with CCl_4. In the latter, note the centrilobular hepatocellular necrosis and presence of inflammatory cells within the lesion. *Bottom*: Sirius red staining. Note red staining of excess collagen in septa of lobules in the liver from the CCl_4-treated mouse. (Photomicrographs from Hyun et al. [2016].)

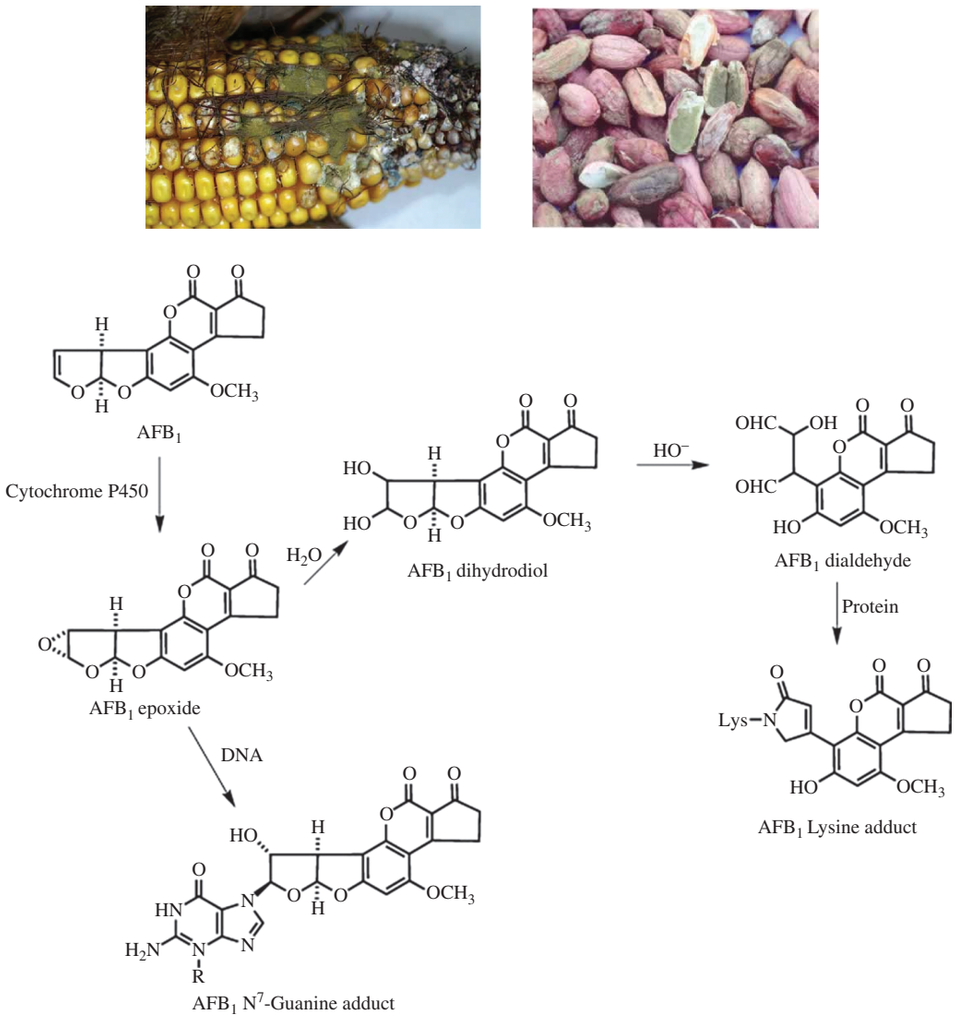


Figure 13-20. *Aspergillus flavus contamination and metabolic activation of aflatoxin B1.* Greenish *Aspergillus* mold contaminates corn, peanuts, and grains (top). Aflatoxin B1 is the most toxic of several, related structures produced by fungi; it is bioactivated by cytochromes P450 to an epoxide that gives rise to reactive species that bind to DNA or proteins to cause cancer or acute liver injury, respectively. (From Steyn et al. [2009]. Available at: https://www.researchgate.net/publication/229934927_Mycotoxins_with_a_Special_Focus_on_Aflatoxins_Ochratoxins_and_Fumonisins; pictures from http://www.ipm.iastate.edu/ipm/icm/2005/9-19/aflatoxin.html.)

to the intracellular events, Kupffer cell activation can contribute to liver injury (elSisi et al., 1993). Kupffer cells may enhance the injury by oxidant stress (elSisi et al., 1993) or TNF-α generation, which can lead to apoptosis (Shi et al., 1998). Supporting these different elements of the mechanisms of CCl_4-mediated cell and organ damage, protection from toxicity was conferred by inhibition of CYPs, preservation of Ca^{2+} homeostasis, antioxidants, and anoxia (Weber et al., 2003). In contrast, treatment with chemicals such as ethanol that induce CYP2E1 enhance injury. This was supported in humans by a case report showing greater vulnerability of workers with a history of alcohol abuse to CCl_4 vapors compared to similarly exposed, moderately drinking co-workers (Manno et al., 1996).

Aflatoxins

Fungi of various species synthesize metabolites known as mycotoxins of several types that cause injury to numerous organs. Aflatoxins are mycotoxins produced by *Aspergillus* molds that grow on nuts and crops such as corn, wheat, and rice (Fig. 13-20; Bennett and Klich, 2003; Bui-Klimke et al., 2014), and consumption of these foods results in exposure to humans and animals (Eaton and Groopman, 1994). Aflatoxins have also been detected in milk of animals that consume contaminated crops, and this can lead to exposure of young children (Montesano et al., 1997; Creppy, 2002). Exposure to aflatoxins is considered unavoidable, and the U.S. FDA has set tolerance limits in foods for human consumption (Groopman et al., 2008). Such regulations have typically resulted in minimal exposure from contaminated foods in most Western countries, but exposure is substantially greater in many developing countries, especially in Southeast Asia and Africa, where there is less regulation of aflatoxin concentrations in food (Schleicher et al., 2013).

Liver injury from acutely toxic exposures (i.e., aflatoxicosis) has happened rarely in humans, but sometimes with devastating consequences. One poisoning outbreak in Kenya in 2004 to 2005

resulted in over 300 cases of aflatoxicosis and 125 deaths (Azziz-Baumgartner et al., 2005; Probst et al., 2007; Groopman et al., 2008). Acute signs in humans include abdominal pain, emesis, steatosis, and necrosis. Hepatic biochemical alterations associated with necrosis of bile duct epithelial cells and parenchymal cells and bile duct proliferation have been documented in clinical and experimental studies in animals.

Chronic exposure to aflatoxins in feed causes hepatocellular carcinoma in animals, and numerous epidemiological studies have supported an association with cancer in humans (Ross et al., 1992; Qian et al., 1994; Liu et al., 2012). Although several aflatoxins occur in dietary sources, aflatoxin B1 (AFB1) occurs in the greatest concentration and in animal studies is one of the most potent mutagens and carcinogens known (Wang and Groopman, 1999; Creppy, 2002). Chronic exposure to 15ppb AFB1 in the diet of Fisher rats resulted in a high incidence of liver tumors (Wogan et al., 1974). Of the various aflatoxins, AFB1 is also the most hepatotoxic acutely, producing periportal necrosis in rats (Clifford and Rees, 1967; Barton et al., 2000). Notably, there are marked species differences in sensitivity to AFB1 (e.g., rats, trout>>mice), and sensitivity to hepatotoxicity is influenced within an animal species by nutritional status, strain, age, and sex (Cullen and Newberne, 1994).

All aflatoxins are metabolized in liver by oxidation, hydrolysis, reduction, and conjugation reactions, some of which lead to toxic products and others to inactive metabolites. AFB1 is oxidized by various isoforms of CYPs. Although most of the products lack significant toxicity, oxidation of the furan ring leads to a reactive epoxide that alkylates DNA (Swenson et al., 1977; Eaton et al., 1995; Lin et al., 2014) and that can be further metabolized to a dialdehyde that alkylates proteins (Fig. 13-20). The DNA adducts are thought to initiate liver cancer, whereas protein adducts appear to cause acute hepatotoxicity. Conjugation with GSH is an important pathway for detoxification of the reactive epoxide, which is also hydrolyzed both spontaneously and enzymically by epoxide hydrolases. The relative activities of the bioactivating and detoxifying enzymes appear to be an important determinant of DNA adduct formation and, by extension, to sensitivity to the mutagenic and carcinogenic effects of AFB1 (Buetler et al., 1992; Kew, 2013; Shen et al., 2014). In this regard, combinations of polymorphisms in several genes encoding enzymes that metabolize AFB1 are associated with increased risk of developing hepatocellular carcinoma (Kirk et al., 2005). In addition, there exists a pronounced synergy between AFB exposure and infection with hepatitis B virus in causing hepatocellular carcinoma in humans (Qian et al., 1994; Kew, 2013). Recent evidence suggests that hepatitis C virus might also synergize with AFB1 (Moudgil et al., 2013). Overall, it is clear that dietary AFB exposure causes acute hepatotoxicity and contributes significantly to relatively high rates of liver cancer in areas where exposure is high, especially in regions where hepatitis B infection is prevalent.

Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids are toxins produced by a wide variety of plant species around the world. They have been responsible for poisoning of livestock grazing on poor pastureland and have intoxicated people who consumed pyrrolizidine alkaloids in herbal teas, contaminated grains, or medicinal remedies (Huxtable, 1997; Edgar et al., 2011; Li et al., 2011; Stegelmeier et al., 2016). In the most toxic pyrrolizidine alkaloids, the pyrrolizidine nucleus is esterified to a branched-chained dicarboxylic acid (Fig. 13-21). Retrorsine, seneciphylline, and monocrotaline are examples of toxic pyrrolizidine alkaloids, the latter being the most well studied. Monocrotaline is taken into HPCs at least in part by OATP1 and is bioactivated by CYPs to monocrotaline pyrrole, which is a bifunctional alkylating

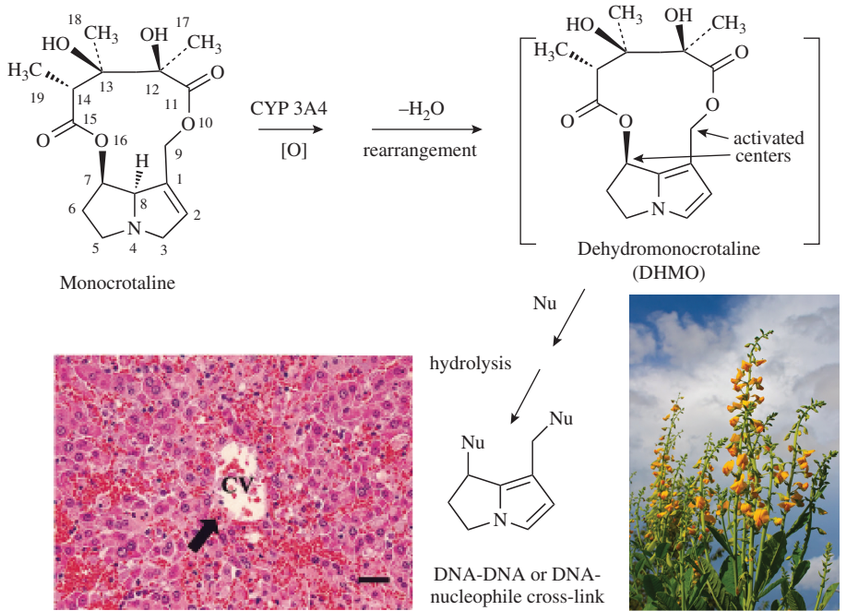


Figure 13-21. *Monocrotaline occurs in many plant species and is bioactivated by cytochromes (CYPs) P450.* Pictured is *Crotalaria spectabilis* ("rattlebox"); this and many other plant species contain hepatotoxic pyrrolizidine alkaloids such as monocrotaline. (Picture from http://majikphil.blogspot.com/2013/08/tropical-storm-erin-and-rattlebox.html.) Pyrrolizidine alkaloids such as monocrotaline are metabolized by several pathways, one of which results in reactive pyrroles (e.g., dehydromonocrotaline, DHMO) which can bind covalently to liver macromolecules such as DNA and proteins to initiate liver injury. (Pathway from Rieben and Coulombe [2004].) Photomicrograph shows hepatocellular necrosis and pronounced parenchymal hemorrhage in centrilobular region of liver (CV, central vein). (From Copple et al. [2002].)

agent capable of binding covalently to DNA and proteins to initiate hepatotoxicity (Hoorn et al., 1993; Reid et al., 1998; Lamé et al. 2000; Tu et al., 2013) (Fig. 13-21).

Acute administration of monocrotaline to rodents results in centrilobular megalocytosis (cell enlargement) and death of HPCs, which succumb from both apoptosis and oncotic necrosis (Fig. 13-21; Copple et al., 2004). The ability of monocrotaline pyrrole to cross-link DNA might be responsible for blockade of cell division at G2/M, failure to proliferate, and consequent increase in cell size (Hoorn et al., 1993; Lappin et al., 1998). A prominent feature of pyrrolizidine alkaloid hepatotoxicity is the pronounced destruction of SECs as well as endothelial cells of central venules (Copple et al., 2002). The SEC destruction leads to marked hemorrhage (Fig. 13-21) and is associated with centrilobular tissue hypoxia (Copple et al., 2006). Monocrotaline pyrrole is detoxified primarily by conjugation with GSH (Yan and Huxtable, 1996), and it is likely that monocrotaline bioactivation and profound GSH depletion in endothelial cells renders them highly sensitive to injury from monocrotaline (DeLeve et al., 1996; Wang et al., 2000).

Leukocytes such as Kupffer cells and neutrophils do not seem to play damaging roles in monocrotaline hepatotoxicity, but the SEC injury that occurs leads to activation of the coagulation system, which contributes to tissue hypoxia and is involved in the progression of liver injury (Copple et al., 2002, 2003, 2006). In addition, the activity of matrix metalloproteinases is increased in rodents after monocrotaline exposure, and inhibitors of these proteases prevented injury, suggesting that they contribute to the degradation of basement membrane and consequent loss of SECs that progresses to sinusoidal destruction (Hanumegowda et al., 2003; DeLeve et al., 2003a). A reduction in production of protective NO by SECs appears to contribute to the increase in matrix metalloproteinase activity (DeLeve et al., 2003b).

The swelling and destruction of SECs and their dissection from HPCs lead to emboli comprising SEC aggregates, red blood cells, and monocytes (DeLeve et al., 2003a). These events underlie progression into the sinusoidal obstruction syndrome (see section "Sinusoidal Endothelial Cell Damage") that characterizes chronic pyrrolizidine alkaloid toxicity. Although the sinusoidal obstruction syndrome can be produced experimentally in laboratory animals by a single administration of a pyrrolizidine alkaloid such as monocrotaline, human intoxication more typically results from subacute or chronic exposure to pyrrolizidine alkaloids (Huxtable, 1989). Clinically, human sinusoidal obstruction syndrome can be divided into acute, subacute, and chronic phases (Stuart and Bras, 1957). The acute phase is characterized by a rapid onset of nausea, emesis, abdominal pain, hepatomegaly, and ascites (Stillman et al., 1977; Sperl et al., 1995; Coulombe, 2003). This phase can be relatively short in duration, either resolving or progressing to the subacute phase, which involves persistent hepatomegaly, prominent megalocytosis, and recurrent ascites. The chronic phase consists of cirrhosis and liver failure, which can occur many months after pyrrolizidine alkaloid exposure (Stillman et al., 1977; Coulombe, 2003). This syndrome has a poor long-term prognosis, with death occurring up to 2 years after exposure (Coulombe, 2003).

Alpha-Naphthylisothiocyanate

Human exposure to alpha-naphthylisothiocyanate (ANIT; $C_{10}H_7N{=}C{=}S$) is effectively nonexistent, but it serves as a model chemical to recapitulate in rodents the features of both acute and chronic biliary disease in humans. Other available experimental models of acute and chronic cholestatic liver injury rely on investigator-imposed genetic mutations in transporters (e.g., Mdr2$^{-/-}$ mice) or surgically mediated cholestasis (i.e., bile duct ligation). Acutely, ANIT causes pronounced injury to intrahepatic bile ducts and mild periportal hepatocellular necrosis. The precise cellular mechanisms whereby ANIT damages the bile duct epithelium are unclear; what is known is that unique metabolism and transport of ANIT into the bile compartment renders biliary epithelium a proximal target of ANIT. GSH conjugation plays a permissive role in the biliary transport and accumulation of ANIT. GSH concentration in bile increases in parallel with ANIT after oral administration, and GSH depletion limits ANIT transport into bile (Jean and Roth, 1995). Additional studies revealed that an ANIT-GSH conjugate produced within the HPC is transported into bile by the canalicular transporter, Mrp2 (Dietrich et al., 2001). The GSH *S*-conjugate of ANIT is unstable at physiological pH and dissociates, resulting in large concentrations of free ANIT in the bile (Fig. 13-22). Depletion of GSH or Mrp2 deficiency reduced ANIT toxicity, highlighting the importance of biliary ANIT transport to its toxicity (Jean and Roth, 1995; Jean et al., 1995).

Accumulation of ANIT in bile damages intrahepatic bile ducts along with epithelial surfaces of extrahepatic tissues that contact bile, including gall bladder epithelium and extrahepatic bile ducts (McLean and Rees, 1958; Plaa and Priestly, 1976; Ungar and Popp, 1976). A single, oral administration of ANIT produces acute biliary injury, disruption of bile flow, compensatory biliary hyperplasia, and portal neutrophilic inflammation. As the injury progresses, periportal hepatocellular necrosis becomes evident. These lesions are formed as a consequence of neutrophil accumulation and activation and resemble so-called "bile infarcts" observed hours after experimental ligation of the common bile duct (Plaa and Priestly, 1976; Roth and Dahm, 1997; Woolbright and Jaeschke, 2012). In mice, either prior depletion of neutrophils or neutralization of IL-17, a neutrophil chemoattractant, suppressed the hepatotoxicity of ANIT (Dahm et al., 1991; Kobayashi et al., 2010). In mice lacking the leukocytic beta-2-integrin, CD18, ANIT-induced HPC injury was reduced (Kodali et al., 2006), and studies in vitro indicated that ANIT causes bile duct epithelial cells to release a factor(s) that attracts neutrophils and stimulates them to injure HPCs (Fig. 13-22; Hill et al., 1999).

Persistent exposure of mice to ANIT for >4 weeks effectively recapitulates biliary fibrosis associated with chronic cholestatic liver diseases in humans, including primary sclerosing cholangitis. Much like Mdr2$^{-/-}$ mice, "onion skin"-like layering of collagen is observed surrounding intrahepatic bile ducts after chronic ANIT challenge. Strong evidence indicates engagement of immune cells, biliary epithelium, and the hemostatic system in this model of biliary fibrosis (Tjandra et al., 2000; Sullivan et al., 2010; Golbar et al., 2013; Joshi et al., 2016; Joshi et al., 2017).

Cyclic Peptide Hepatotoxins: Microcystins, Nodularins, Phallotoxins, Amatoxins

Several cyclic peptides synthesized by fresh water cyanobacteria or by toxic mushrooms are highly potent hepatotoxins, producing liver injury in humans and/or animals at doses below 1 mg/kg body weight.

Cyanobacterial Toxins Microcystins are a group of more than 80 cyclic heptapeptide toxins that are produced by cyanobacteria ("blue-green algae"; e.g., *Microcystis aeruginosa*) that bloom in freshwater lakes and ponds (Fig. 13-23; Labine and Minuk, 2009). Consumption of contaminated water from these blooms in several countries has resulted in injury to liver and other organs in fish, domestic animals, and humans (Bischoff, 2001; Svircev et al.,

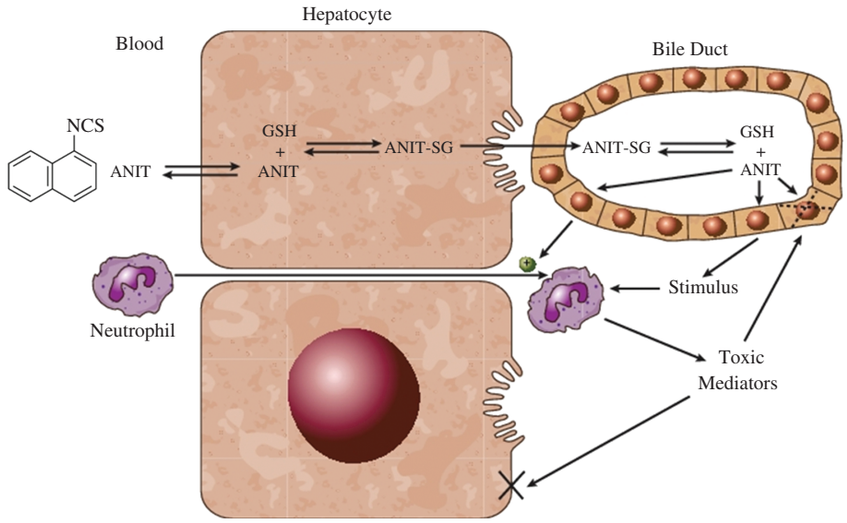
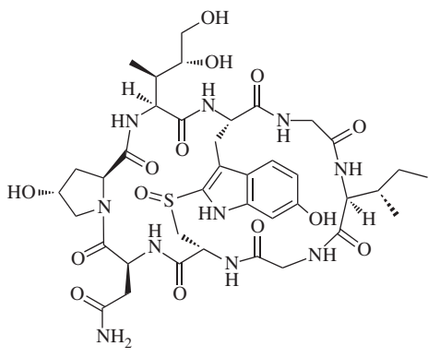


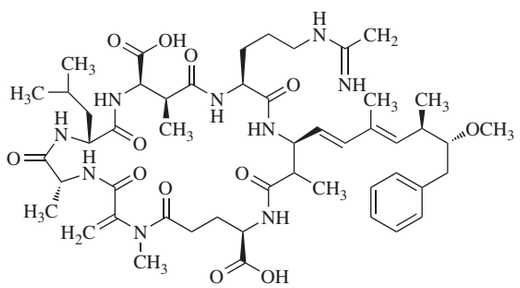
Figure 13-22. *Liver injury from α-naphthylisothiocyanate (ANIT).* Circulating ANIT enters hepatocytes where it forms a reversible conjugate with glutathione (GSH). The conjugate concentrates in bile, where it dissociates, leaving ANIT to injure bile duct epithelium and cause release of chemotactic stimuli that recruit neutrophils which become activated and release cytotoxic mediators that amplify the injury. (From Dahm et al. [2010].)

Amanita phalloides



Alpha-Amanitin

Bloom of blue-green algae



Microcystin LR

Figure 13-23. Sources and structures of two cyclic peptide hepatotoxins. *Top, left*: Poisonous amanita mushrooms (from Knoop KJ, et al., eds. *The Atlas of Emergency Medicine. 4th ed*. New York: McGraw-Hill, 2016. Used with permission from photo contributor: Edward J. Otten, MD); *top, right*: Structure of α-amanitin (from https://en.wikipedia.org/wiki/Alpha-Amanitin); *bottom, left*: Blue-green algal bloom in St. Lucie River, FL (Image used with permission from Jacqui Thurlow-Lippisch; https://jacquithurlowlippisch.com); *bottom, right*: Structure of microcystin LR (Reproduced with permission from Sigma-Aldrich Co. LLC; https://www.sigmaaldrich.com).

2010). An unusual episode of microcystin contamination of the water source used by a hemodialysis center in Brazil led to acute liver injury in 81% of 124 exposed patients and the death of 50 of these patients (Jochimsen et al., 1998). Depending on the nature of exposure, the microcystin toxins can cause liver failure or hepatocellular carcinoma.

Of the microcystins, microcystin LR (Fig. 13-23) is most commonly found and the first to be characterized chemically. It is

transported into HPCs by the basolateral organic anion transporters OATP1B2 in mice and OATP1B1 and OATP1B3 in humans (Fisher et al., 2005; Lu et al., 2008). Detoxification of microcystins occurs primarily via oxidation and conjugation with glutathione and cysteine (Brooks and Codd, 1987; Robinson et al., 1991). The conjugates are excreted in bile and urine.

The main toxicity target for microcystin LR is the liver, where acute injury is associated with blebbing and necrosis of HPCs, congestion, and hemorrhage (Hooser et al., 1989, 1990; Falconer and Yeung, 1992). These effects occur rapidly in rodents (Hooser et al., 1989). Microcystin dramatically deforms HPCs (Hooser et al., 1991), and these deformities produce a secondary change in the structural integrity of the sinusoid. Chronic exposure is associated with hypertrophy, karyomegaly, and multinucleation of HPCs (Clark et al., 2007) and can result ultimately in fibrosis and/or hepatocellular carcinoma (Frangez et al., 2000; Svircev et al., 2010; Fujiki and Suganuma, 2011). Microcystin LR affects hepatocellular microtubules, the function of which is critical to normal cell division and to maintaining normal cell structure. Acute exposure causes aggregation of cytokeratin-containing intermediate filaments and actin microfilaments, which detach from the plasma membrane, resulting in bleb formation (Hooser et al., 1990; Chorus and Bartram, 1999).

Mechanisms of acute microcytstin LR toxicity have been studied mostly in cultured cells and in rodents, in which centrilobular necrosis as well as injury to bile duct epithelial cells occurs (Solter et al., 1998; Lu et al., 2008). Microcystin LR binds to cysteine residues on protein phosphatase (PP)-2A and PP-1 and inhibits these enzymes, which are involved in dephosphorylation of many proteins critical for intracellular signaling that controls cellular functions such as expression of proteins, cell proliferation, cell differentiation, and cell death (Honkanen et al., 1990; Runnegar et al., 1993, 1995; Garcia et al., 2003; Van Hoof and Goris, 2003). Small doses of microcystin that are insufficient to produce cellular structural deformations diminish uptake and secretory functions of HPCs associated with hyperphosphorylation of the cytoplasmic motor protein, dynein (Runnegar et al., 1999). Dynein is a mechanochemical protein that drives vesicles along microtubules, using energy from ATP hydrolysis. Central to the hydrolysis of dynein-bound ATP is a cycle of kinase phosphorylation and phosphatase dephosphorylation. Thus, hyperphosphorylation of dynein freezes this motor pump. Microcystin LR also activates phosphorylase, which in turn can result in glycogen and glutathione depletion, oxidative stress, and increased intracellular free calcium that promote mitochondrial dysfunction and apoptosis in rat HPCs (Ding et al., 2000a, 2000b, 2001; Ding and Nam Ong, 2003; Bouaïcha et al., 2005). However, microcystin LR caused necrosis in primary human HPCs (Woolbright et al., 2017). Activation of NF-κB and MAPK pathways has also been reported in primary HPCs exposed to microcystin LR at low nM concentrations, suggesting an immunomodulatory action (Zhang et al., 2013). It is to be expected that exposure to a chemical that inhibits phosphatases involved in many cellular signaling pathways would demonstrate numerous adverse effects on cell biology, and that certainly is the case with microcystins.

Importantly, other hepatotoxins are produced during blooms of cyanobacteria (Dittmann and Wiegand, 2006; Chen et al., 2013). For example, nodularins are cyclic pentapeptides produced by *Nodularia spumigena* and are potent liver toxins (Chen et al., 2013). They cause many of the same molecular alterations as microcystins, such as phosphatase inhibition, oxidative stress, and intracellular calcium changes (Bouaïcha et al., 2005; Dittmann and Wiegand, 2006; Lankoff et al., 2006; Solstad et al., 2008; Fujiki and Suganuma, 2011; Ufelmann et al., 2012; Ufelmann and Schrenk, 2015). In addition, nodularin caused endoplasmic reticular stress, activation of MAPKs, and expression of TNF in HPCs in vitro (Meili et al., 2016). Thus, several hepatotoxins are produced by cyanobacterial blooms in freshwater sources. They disrupt numerous signaling pathways critical for maintenance of normal homeostasis and cause liver injury at very small doses. There is concern that global climate change will lead to more frequent and intense algal blooms and thereby enhance the risk of cyanobacterial poisoning in the future (Lürling et al., 2017).

Mushroom Toxins There are several toxic, cyclic peptides that occur in various species of wild mushrooms. These mushrooms have been mistaken for edible species, and consumption of them has been responsible for numerous poisonings and deaths over the years, especially in Europe but also in North America. Of these mushroom species, *Amanita phalloides* ("death cap") has been of greatest concern due to its ability to cause life-threatening toxicity (Fig. 13-23; Garcia et al., 2015). This mushroom produces several bicyclic heptapeptides known as phallotoxins and octapeptides known as amatoxins. Of these, phalloidin and α-amanitin (Fig. 13-23), respectively, have received the most attention as hepatotoxins.

Phalloidin is taken into HPCs by transporters of the OATP family (Lu et al., 2008) and causes cholestasis and hemorrhagic necrosis in livers after intraperitoneal administration to rodents (Vonk et al., 1982; Loranger et al., 1985; Ishizaki et al., 1997). Although phalloidin is a potent hepatotoxin, it is poorly absorbed after oral administration and might therefore contribute minimally to mushroom poisoning in humans (Wieland and Faulstich, 1978). Tight binding of phalloidin to actin filaments in HPCs prevents the disassembly phase of the normally dynamic rearrangement of the actin filament constituent of the cytoskeleton. This leads to striking alterations in the actin-rich web of cytoskeleton adjacent to the canalicular membrane of HPCs; the actin web becomes accentuated and the canalicular lumen dilates (Wieland, 1977; Phillips et al., 1986; Yamamoto et al., 1988; Frimmer, 1987). In isolated HPCs, phalloidin increases intracellular free calcium, which can lead to alterations in hepatocellular homeostasis (Kawaji et al., 1992).

Unlike phalloidin, α-amanitin is absorbed from the gastrointestinal tract and is rapidly eliminated in the urine (Homann et al., 1986; Jaeger et al., 1993). As with other cyclic peptides, α-amanitin enters HPCs via OATPs, especially OATP1B3, on the basal membrane (Letschert et al., 2006). Centrilobular hepatocellular necrosis and hemorrhage accompanied by a hepatorenal syndrome characterizes severe poisoning in humans, and the latter portends a poor prognosis for survival (Pond et al., 1986; Bonnet and Basson, 2002; Garcia et al., 2015). The lethal dose in humans has been estimated to be 0.1 mg/kg, an amount that can occur in one night cap mushroom (Karlson-Stiber and Persson, 2003).

A primary mechanism of toxicity of α-amanitin derives from its ability to bind to nuclear RNA polymerase II, thereby inhibiting RNA elongation with resultant impaired protein synthesis and cell death (Wang et al., 2006). In isolated HPCs, α-amanitin initiates apoptosis dependent on p53, and this might be connected to the inhibition of mRNA synthesis (Ljungman et al., 1999; Magdalan et al., 2011). In mice, knockout of p53 and BAK reduced α-amanitin hepatotoxicity, suggesting that a mitochondrial apoptotic pathway is important in death of HPCs in vivo (Leu and George, 2007). Leist et al. (1997) presented evidence that enhanced TNF expression occurs in mice treated with α-amanitin and is involved in the progression of injury. Some evidence suggests that oxidative stress is also involved, perhaps arising from free radical formation in α-amanitin itself (Zheleva, 2013).

Metals

Exposure to metals occurs via several routes, including diet and inhalation of metal-containing particulates from occupational exposure or cigarette smoke. Most metals target numerous organs, among them the liver, with a wide spectrum of consequences. A comprehensive treatise of the topic is beyond the scope of this chapter, but some salient points about the toxicities of iron, cadmium, and copper are mentioned here. For more information about metal toxicity, excellent reviews are available (Fowler, 2009; Goering, 2010; Corkins, 2011; Satarug, 2012; Patil et al., 2013; Rani et al., 2014; Sharma et al., 2014; Zischka and Lichtmannegger, 2014; Matović et al., 2015; Pietrangelo, 2016).

Metals are excreted into bile by a series of processes that include (1) uptake across sinusoidal membranes of HPCs by membrane transporters or receptor-mediated endocytosis; (2) storage in binding proteins or lysosomes; and (3) canalicular secretion via lysosomes, a GSH-coupled event, or by specific canalicular membrane transport, for example via MRP2 (Ballatori, 2002). Biliary excretion is important in the homeostasis of several metals, notably copper, manganese, cadmium, selenium, gold, silver, and arsenic (Klaassen, 1976; Gregus and Klaassen, 1986). Species differences are known for biliary excretion of several toxic metals; for example, dogs excrete arsenic into bile much more slowly than rats.

Inability to export *copper* into bile is a central problem in Wilson's disease, an autosomal recessive inherited disorder characterized by a defect in or the absence of a copper transporting P-type ATPase (ATP7B). This carrier is located in the trans-Golgi network within HPCs and transports copper into the secretory pathway for binding to ceruloplasmin and then excretion into bile (Loudianos and Gitlin, 2000). Because biliary excretion is the only way to eliminate copper, a defect in ATP7B results in excessive copper accumulation in HPCs, which causes chronic hepatitis and cirrhosis (Loudianos and Gitlin, 2000). Copper toxicity involves production of reactive oxygen species that initiate liver damage characterized by centrilobular necrosis and cholestasis. However, activation of Kupffer cells by copper exposure and a consequent inflammatory response contribute to liver injury (Videla et al., 2003).

Cadmium hepatotoxicity becomes manifest when cells exceed their capacity to sequester cadmium as a complex with the metal-binding protein, metallothionein (MT). This protective role for MT was documented by observations with MT transgenic and knockout mice. Overexpression of MT in the transgenic mice rendered them more resistant than wild-type animals to the hepatotoxicity and lethality of cadmium poisoning (Liu et al., 1995). In contrast, MT gene knockout mice were dramatically more susceptible to cadmium hepatotoxicity (Liu et al., 1996). Cadmium binding to sulfhydryl groups on proteins appears to be responsible for its toxic effect, but as with copper, activation of Kupffer cells and inflammation appear to play a role (Kyriakou et al., 2013).

Acute *iron* toxicity is most commonly observed in young children who accidentally ingest iron tablets (Chang and Rangan, 2011). Iron poisoning can result in severe liver damage and results when free iron concentration in extracellular fluid and within cells becomes elevated. HPCs contribute to the homeostasis of iron by extracting this essential metal from the sinusoidal blood by a receptor-mediated process and maintaining a reserve of iron bound to the storage protein, ferritin. The cytotoxicity of free iron is attributed to its capacity as an electron donor for the Fenton reaction, in which hydrogen peroxide is reductively cleaved to the highly reactive hydroxyl radical, an initiator of lipid peroxidation (see Chap. 3). Accumulation of excess iron beyond the capacity for its safe storage in ferritin is initially evident in zone 1 HPCs, which are closest to the blood entering the sinusoid. Thus, the zone 1 pattern of HPC damage after iron poisoning is attributable to the location of the preferential uptake of iron and to the greater oxygen concentration in zone 1 that facilitates the injurious process of lipid peroxidation. Chronic accumulation of excess iron in cases of hemochromatosis is associated with a spectrum of hepatic disease including a greater than 200-fold increased risk for liver cancer (Ramm and Ruddell, 2005).

DETERMINANTS OF SUSCEPTIBILITY

Pronounced variability in hepatotoxic response among individuals can occur upon exposure to xenobiotic agents. Underlying such differences in susceptibility and magnitude of response are many factors including differences in metabolism, hepatoprotective systems, age, sex, tissue reserve and regenerative capacity, and inflammatory response (Table 13-5). Interactions of hepatotoxicant exposure with various diseases or with other xenobiotic agents can also influence susceptibility of individuals to liver injury. Several factors that can affect either the initiation or progression of xenobiotic-induced liver injury within individuals are discussed in this section.

Xenobiotic Metabolism

Many chemicals are metabolized by the various CYP isoforms and conjugating enzymes to metabolites that are either more or less toxicologically active than the parent molecule. In humans, CYP3A4 is the CYP isoform most commonly involved in metabolism of drugs, but many other CYP isoforms participate too, depending on the chemical. It follows that differences among individuals in the activity of such enzymes can markedly affect sensitivity to intoxication, and these differences result from both genetic and environmental factors. Polymorphisms in genes encoding metabolizing enzymes occur among individuals and have a major impact on disposition of toxicants (Zanger and Schwab, 2013). In addition, genetic differences in receptors or transcription factors (e.g., AhR, PXR, CAR, Nrf2) that control the expression of these genes or epigenetic changes that regulate their expression can influence metabolic activity within individuals (Yokoi and Nakajima, 2011; Ishikawa et al., 2013; Smutny et al., 2013; Wallace and Redinbo, 2013; Wu et al., 2013; Elcombe et al., 2014; see also Chap. 6). Although there has been much emphasis over the years on how expression of CYPs influences bioactivation and toxicity, polymorphisms and expression differences in various xenobiotic conjugating enzymes also influence toxicity (for reviews, see Sim et al., 2008; Ginsberg

Table 13-5

Determinants of Individual Susceptibility to Hepatotoxicants

Xenobiotic metabolism
Hepatobiliary transporters
Protective enzymes
Age
Sex
Tissue reserve and regeneration
Inflammatory stress
Nutritional status
Chemical–chemical interaction
Adaptive immune system activation

et al., 2009, 2010; Crettol et al., 2010; Marques and Ikediobi, 2010; Sugatani, 2013).

The expression of many xenobiotic metabolizing enzymes and their activities is influenced by dietary constituents, exposure to drugs and environmental chemicals, nutritional status, liver diseases, inflammatory conditions, sex, and age (Merrell and Cherrington, 2011; Zanger and Schwab, 2013; Lu et al., 2014; Yeung et al., 2014; Yang, 2015; Guo et al., 2016). With some exceptions, inflammatory cytokines generally downregulate the expression of CYPs, conjugating enzymes, and transporters in HPCs and can thereby modify responses to chemicals that undergo metabolism (Renton, 2004; Fardel and Le Vée, 2009; Gandhi et al., 2012; Zanger and Schwab, 2013). Moreover, xenobiotic agents can influence the metabolism and toxicity of other chemicals by inhibiting or inducing metabolizing enzymes (Hu et al., 2012). Xenobiotic transporters are subject to similar effects (Kobori et al., 2013; Nigam et al., 2015). Together, these factors determine metabolic capability within an individual and, by extension, influence hepatotoxicity of chemicals that are metabolized. Because many chemicals are subject to metabolism by several pathways and because there are so many factors that can affect metabolic activity, how metabolism and hepatotoxicity are likely to be influenced in a particular individual is difficult to predict (Palatini and De Martin, 2016).

Hepatobiliary Transporters

Toxicant-related impairments of bile formation are more likely to have detrimental consequences in populations with other conditions in which biliary secretion is marginal. For example, neonates exhibit delayed development of multiple aspects of bile formation, including synthesis of bile acids and the expression of sinusoidal and canalicular transporters (Arrese et al., 1998). Neonates are more prone to develop jaundice when treated with drugs that compete with bilirubin for biliary clearance. Individuals with genetic deficiency of certain hepatobiliary transporters are not only at risk for chronic liver injury and fibrosis but may also be more susceptible to drugs and hepatotoxicants (Jansen and Sturm, 2003; Jansen and Groen, 2006). Patients with bacterial sepsis frequently develop cholestasis, which is mainly caused by downregulation of multiple canalicular transport systems (Geier et al., 2006). In addition, direct inhibition of the bile salt export pump (BSEP) (e.g., by the endothelin receptor antagonist, bosentan) can lead to retention of bile acids in the liver and consequent liver injury (Fattinger et al., 2001). Biliary excretion is important in the homeostasis of several metals, notably copper, manganese, cadmium, selenium, gold, silver, and arsenic (Klaassen, 1976; Gregus and Klaassen, 1986). Because biliary excretion is the only way to eliminate copper, a defect in the ATP7B carrier (located in the trans-Gogi network) results in excessive copper accumulation in HPCs, which causes chronic hepatitis and cirrhosis (Loudianos and Gitlin, 2000). Drug-induced inhibition of transporters also underlies some forms of toxic drug–drug interaction (Koepsell, 2015). Thus, the many transporters that occur in liver not only play important roles in elimination of xenobiotic and endogenous agents, but variation in their activities can be an important determinant of individual susceptibility to intoxication.

Protective Enzymes

As noted above, reactive oxygen species contribute to hepatotoxicity from certain xenobiotic agents. Accordingly, polymorphisms and differences in expression of genes which encode enzymes that provide protection from reactive oxygen species can influence susceptibility to intoxication. For example, DNA polymorphisms and/or variations in epigenetic or posttranslational control have been identified for GSH peroxidases (Hesketh, 2004; Lubos et al., 2011), superoxide dismutases (Miao and St. Clair, 2009; Becuwe et al., 2014), and other enzymes as well as transcription factors such as Nrf2 that regulate protective cellular responses to oxidants and other insults (Osburn and Kensler, 2008; Wu et al., 2013).

Development and Aging

Age-related differences in hepatotoxicity have been recognized for many years and appear to have many underlying causes. For example, drug metabolizing enzymes generally are poorly expressed in newborn animals and people, and the capacity to metabolize increases at various rates during maturity (Hines, 2013; Coughtrie, 2015). This results in differences in disposition of drugs and other xenobiotic agents, with consequent potential to affect toxicity (Rikans, 1989; Saghir et al., 2012). Elderly people typically have reduced liver blood flow, diminished xenobiotic metabolism, and differences in inflammatory responses, all of which have the potential to affect the toxicity of drugs (Tajiri and Shimizu, 2013). Importantly, elderly people also consume greater numbers of drugs than younger people, raising the risk of drug–drug interactions and hepatotoxicity (Adelizzi, 1994; Meier and Seitz, 2008; Stine et al., 2013).

Sex

Sex-related differences in hepatotoxic responses have been reported in many studies. For example, female sex is a risk factor for liver injury for several drugs and dietary supplements (Chalasani and Björnsson, 2010; Heidari et al., 2014; Korth, 2014; Kargar et al., 2014; Katarey and Verma, 2016). An example is halothane hepatitis, for which female sex is a risk factor in humans; female mice are also more sensitive to this anesthetic, and the sex difference appears to be related to a difference in immune response (Dugan et al., 2010). For most drugs, it remains unclear whether this predilection is due to differences in xenobiotic metabolizing activity, drug dosing, body composition (e.g., lipid composition tends to be greater in females than males), or other factors (Anderson, 2008).

Tissue Reserve and Regeneration

There is a large reserve capacity in the liver inasmuch as a large fraction of the liver volume can be lost without a serious decrement in function, and this organ also has a remarkable capacity for regeneration (Best and Coleman, 2011). Interindividual variations in either of these can affect the magnitude of hepatotoxic response. As noted above, proliferative repair is an important response of liver to injury (see section "Regeneration and Repair" on liver regeneration). In addition, such regeneration is not just a response to cell death but is a process that actively determines the magnitude of injury after exposure to many hepatotoxicants, such as thioacetamide, APAP, chloroform, bromobenzene, trichloroethylene, CCl_4, galactosamine, and allyl alcohol (Anand et al., 2005; Mehendale, 2005). Inhibition of mitosis with colchicine prevented tissue repair and aggravated liver injury after thioacetamide and other chemicals (Mangipudy et al., 1996; Mehendale, 2005, 2013). In contrast, stimulation of repair by exposure to a modest dose of a hepatotoxicant strongly attenuated tissue damage from a subsequent, larger dose of the same or a different chemical (autoprotection and heteroprotection, respectively) (Mehendale, 2005, 2013). Tissue repair is dose-responsive up to a threshold, above which the injury becomes too severe, and cell proliferation is inhibited (Mangipudy et al., 1995). In addition to the dose of the hepatotoxicant, other factors such as age, nutritional status, and disease state can influence tissue repair (Mehendale, 2005).

Inflammatory Stress

Inflammation is a common response to exposure to many hepatotoxicants, and genetic or environmental differences in one or more of the many factors involved in an inflammatory response occur commonly among individuals. Large exposures to some agents, such as bacterial lipopolysaccharide (LPS, endotoxin) can directly initiate an acute inflammatory response that can cause serious liver injury (Hewett and Roth, 1995; Han, 2002). More common is modest inflammation associated with alcohol consumption, pathogen infection, inflammatory diseases such as arthritis, surgery, etc., that by itself does not cause cell death or liver dysfunction. However, an independently initiated, modest inflammatory episode can qualitatively and/or quantitatively influence the magnitude of hepatotoxic response to drugs and other xenobiotic agents. As noted above, one way this can happen is via mediators such as TNF and other cytokines that regulate the expression of transporters and xenobiotic metabolizing enzymes which either bioactivate or inactivate chemicals.

An inflammatory stress can change the sensitivity of individuals to hepatotoxic chemicals independently of effects on metabolism. In human patients, inflammatory liver diseases such as hepatitis B and C have been linked to hepatotoxicity from antiviral drugs (Puoti et al., 2009), and these hepatitis viruses also synergize with aflatoxin exposure in causing liver cancer (see section "Aflatoxins"). Experimentally, exposure of rodents to a small dose of LPS that causes modest liver inflammation (e.g., cytokine release, neutrophil accumulation) but no hepatocellular injury markedly increases liver damage from a variety of xenobiotic agents that cause intrinsic hepatotoxicity, including aflatoxin B1 (Luyendyk et al., 2002), monocrotaline (Yee et al., 2000), carbon tetrachloride (Chamulitrat et al., 1995), galactosamine (Galanos et al., 1979), allyl alcohol (Sneed et al., 1997), cocaine (Labib et al., 2002), and APAP (Maddox et al., 2010) (reviewed in Ganey and Roth, 2001; Ganey et al., 2004). The hepatotoxic interaction of LPS with these xenobiotic agents depends on many inflammatory factors. For example, in rats the interaction of monocrotaline with LPS requires participation by Kupffer cells, neutrophils, TNF, and an activated coagulation system (Yee et al., 2003a, 2003b, 2003c). Interestingly, both dose and the timing of the exposures are critical to the type of response in these models. For example, when LPS and monocrotaline were given 4 hours apart, liver injury occurred, but when the same doses were given 1 hour apart, animals died rapidly from another cause, likely NO-mediated circulatory shock (Yee et al., 2002). In contrast, mice given a nontoxic dose of APAP 2 hours after a noninjurious dose of LPS experienced pronounced centilobuluar injury, whereas LPS given 24 hours prior to APAP afforded protection from liver toxicity (Liu et al., 2000; Maddox et al., 2010). The protection in the latter case was related to LPS-induced downregulation of CYPs that bioactivate acetaminophen and could be influenced also by the production of anti-inflammatory mediators that follow the flood of pro-inflammatory events that occurs shortly after LPS exposure. Collectively, these studies indicate the complexities in how inflammatory stress can act as a determinant of sensitivity to hepatotoxicants.

Nutritional Status

Nutritional status is yet another determinant of sensitivity to hepatotoxicants (Brautbar and Williams, 2002; Stickel et al., 2003; Bunchorntavakul and Reddy, 2013; Ingawale et al., 2014). For example, fasting decreases GSH concentration in liver; because detoxification of the toxic metabolite of APAP depends on GSH, fasting renders animals more sensitive to APAP hepatotoxicity (McClain et al., 1999). Deficiencies in many nutrients have been shown to enhance the toxicity of numerous hepatotoxicants, and dietary supplementation can sometimes improve liver function in affected individuals. Examples of such dietary nutrients include essential amino acids and lipids, *S*-adenosylmethionine, zinc, and antioxidants (Lieber, 2000; Basu, 2003; Stehbens, 2003). In contrast, excess consumption of some essential nutrients can exacerbate hepatotoxicity. An example of this is the hepatotoxic interaction of alcohol with iron overload (Tavill and Qadri, 2004).

The susceptibility factors discussed above are not meant to represent an exhaustive list. For example, interactions between chemicals can be an important determinant. Polymorphisms in genes controlling adaptive immune responses appear to be important for some drug-induced hepatotoxic reactions (see section below). Importantly, almost any genetic or environmental difference among individuals can affect sensitivity to hepatotoxicants if that difference alters an enzyme or another factor that is critical in the molecular pathway to liver injury for a particular chemical.

IDIOSYNCRATIC, DRUG-INDUCED LIVER INJURY (IDILI): OCCURRENCE AND MODES OF ACTION

As discussed above, idiosyncratic reactions occur in a minority of patients during drug therapy, and these reactions often target the liver. Although idiosyncratic, drug-induced liver injury (IDILI) occurs infrequently in human patients, it has importance because it is sometimes severe, requiring liver transplantation or causing death. Moreover, drugs that are associated with human IDILI are often restricted in use or withdrawn from the market, resulting in the loss of otherwise efficacious drugs (Kaplowitz, 2005; Raschi and DePonti, 2015; Licata, 2016; Roth and Lee, 2017). Conventional preclinical safety testing in animals and in vitro that occurs during drug development fails to detect IDILI liability of drug candidates. Because these reactions are typically rare, they are not usually exposed in clinical trials that employ too few volunteers to detect them. Accordingly, much remains unknown about why and how IDILI reactions occur.

Many pharmaceuticals as well as herbal remedies and food supplements have been associated with IDILI in humans (Stickel et al., 2011). Listings of drugs can be found in recent reviews (Björnsson, 2016; Björnsson and Hoofnagle, 2016), and NIH's LiverTox Web site provides a searchable database for summary information about many drugs (https://livertox.nih.gov/). Antibiotics are the drugs most commonly associated with human IDILI in Western countries, but drugs from many other pharmacologic classes are also culprits (Katarey and Verma, 2016; Bunchorntavakul and Reddy, 2017). In Asia, IDILI is caused more commonly by dietary and herbal supplements (Frenzel and Teschke, 2016; Ahmad and Odin, 2017).

DILI is difficult to diagnose because the clinical presentation is often indistinguishable from liver injury from other causes, so that diagnosis is largely by exclusion of other causes (Lewis, 2015; Raschi and DePonti, 2015). Hepatocellular injury may be the most common phenotype, but cholestatic and mixed injuries also occur. Many patients recover from liver injury when exposure to the offending drug is stopped, but others progress to acute liver failure culminating in death or a need for a liver transplant.

IDILI reactions doubtlessly depend on characteristics of the offending drug as well as susceptibility of individual patients, but the mechanisms underlying them are poorly understood. Consequently, many hypotheses about the modes of action of IDILI have arisen (Chen et al., 2015; Roth et al., 2017). Each of these hypotheses is

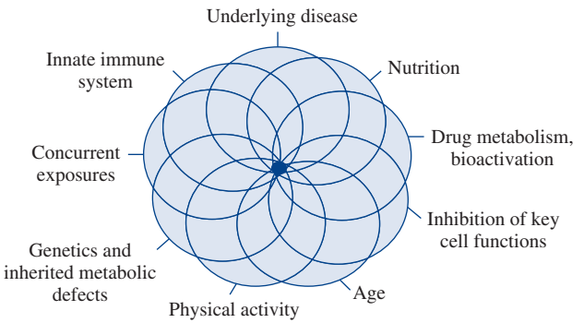


Figure 13-24. *Multiple determinant hypothesis of IDILI.* Several factors combine to render an individual sensitive to liver injury from a therapeutic dose of a drug that is normally innocuous. (From Ulrich [2007].)

currently supported by limited evidence from studies in humans and/or animals. A brief description of several of them follows; for greater detail, the reader is referred to the cited reviews.

According to the *multiple determinant hypothesis*, IDILI occurs from the intersection of several genetic and/or environmental susceptibility factors present simultaneously within a patient exposed to the offending drug. Such factors could be related to drug metabolism, transporters, nutrition, sex, age, concurrent exposure to other xenobiotic agents, physical activity, underlying disease, concurrent inflammation, etc. (Li, 2002; Ulrich, 2007) (Fig. 13-24). Each of the susceptibility factors is associated with a finite probability of occurrence (i.e., from 0 to 1), and the probability of an IDILI reaction would be related to the product of the individual probabilities. This would result in a small number and could explain why IDILI reactions are typically rare. Consistent with this hypothesis is the observation that severe liver injury occurred in halothane-treated mice subjected to a combination of known risk factors for human halothane hepatitis (female sex, genetics, age, fasting) (Dugan et al., 2010).

Numerous drugs that are associated with human IDILI can cause mitochondrial stress or mitochondrial toxicity in vitro (Boelsterli, 2003; Pessayre et al., 2012; Han et al., 2013; Boelsterli and Lee, 2014). Moreover, human polymorphisms in enzymes that either bioactivate or inactivate drugs have been identified, and some appear to be risk factors for IDILI (Andrade et al., 2009; Czaja, 2011; Markova et al., 2013; Ahmad and Odin, 2017). Similarly, many drugs that cause IDILI inhibit or downregulate expression of hepatocellular efflux transporters that move drugs and bile acids out of HPCs, and polymorphisms in genes that encode transporters have associations with toxicity from some drugs (Corsini and Bortolini, 2013; Pedersen et al., 2013; Ulzurrun et al., 2013; He et al., 2015; Guo et al., 2015). Although each of these qualities alone might be insufficient to explain IDILI, they are likely important risk factors for certain drugs and could act in combination with each other or with other factors to precipitate hepatotoxicity (Aleo et al., 2014; Köck et al., 2014; Ulzurrun et al., 2014; Zhou et al., 2017). Accordingly, these observations are consistent with the multiple determinant hypothesis. This hypothesis has raised the possibility that some idiosyncratic reactions are no different from intrinsic ones (e.g., dose-related) if viewed in the context of an individual's sensitivity to hepatotoxicity being heightened by the presence of one or more risk factors (Roth and Ganey, 2010b).

The *failure-to-adapt hypothesis* proposes that therapeutic doses of some drugs cause modest liver injury to which most patients adapt; those few who fail to do so progress to serious liver injury. For example, about 10% to 40% of patients taking the antibiotic, isoniazid, experience increases in serum ALT/AST that, in most patients, return to normal despite continued drug treatment (see Dara et al., 2016a for review). However, isoniazid is well known to cause life-threatening liver injury in a small fraction (about 1%) of patients taking the drug, so it seems plausible that the livers of these patients fail to adapt to the modest initial insult. This and other observations (Watkins, 2005) led to the hypothesis that many drugs can cause modest, subclinical injury to liver and that IDILI occurs in people whose livers fail to prevent the progression of injury. Support for this hypothesis is circumstantial, and mechanisms are unknown. For drugs that cause IDILI via adaptive immune system activation (see below), adaptation might involve tolerance mechanisms that prevent progression of liver injury (Dara et al., 2016a). Alternatively, the liver typically adapts to liver injury by proliferation and replacement of injured HPCs, and several drugs that cause IDILI also inhibit cell proliferation (Roth et al., 2017).

Many conditions such as inflammatory diseases, alcohol consumption, infection, etc., involve episodic exposure of the liver to PAMPs or DAMPs and consequent activation of the innate immune system (Fig. 13-25). According to the *inflammatory stress hypothesis*, drug exposure interacts with a modest, noninjurious inflammatory episode, resulting in liver damage. One can picture a patient on maintenance drug therapy progressing well until an inflammatory episode interacts with the drug, precipitating liver injury. Such a scenario can account for many of the bizarre characteristics of idiosyncratic reactions (see Table 13-4; Ganey et al., 2004; Shaw et al., 2010; Roth and Ganey, 2013). Almost all of the animal models of IDILI in which pronounced liver injury occurs from IDILI-associated drugs have been based on this hypothesis (Deng et al., 2009; Roth and Ganey, 2011). In these models, rodents are cotreated with an inflammagen such as LPS that is noninjurious by itself and with a nontoxic dose of a drug. Only the combination results in liver injury. For example, trovafloxacin is an antibiotic, the use of which was curtailed due to several cases of fatal liver injury. Nontoxic doses of trovafloxacin given to mice cotreated with a modest dose of LPS experienced liver necrosis (Shaw et al., 2007). The drug prolonged the LPS-induced appearance of TNF in the plasma, and neutralization of TNF or knockout of either of the TNF receptors eliminated the liver injury (Shaw et al., 2009e). Indeed, drug interaction with the TNF response appeared to be the initiator of a chain of events involving other cytokines (interferon-gamma, plasminogen activator inhibitor), neutrophils, and the coagulation system that contributed to the hepatotoxic drug–LPS interaction (Shaw et al., 2009a, 2009b, 2009c, 2009d). With several other IDILI-associated drugs (e.g., chlorpromazine [Buchweitz et al., 2002]; sulindac [Zou et al., 2009]; amiodarone [Lu et al., 2012]; halothane [Cheng et al., 2009; Dugan et al., 2010]; dicloxacillin [Higuchi et al., 2011]; diclofenac [Deng et al., 2006]; doxorubicin [Hassan et al., 2008]), cotreatment of rodents with an inflammatory stimulus led similarly to pronounced liver injury, consistent with this hypothesis.

The most widespread theory entails drug-induced activation of the adaptive immune system. According to the *adaptive immunity hypothesis* (or *hapten hypothesis*), IDILI reactions are initiated when drugs are metabolized to reactive metabolites that bind covalently to proteins (Fig. 13-25; Tailor et al., 2015; Thompson et al., 2016). These altered proteins are detected as antigens by antigen-presenting cells that process the antigens and present them to T-cells, which become activated, leading to an adaptive immune response that results in activation of inflammatory cells, release of cytokines, and other events (Fig. 13-25). Normally, this does not damage host tissue. However, in the presence of "danger signals" (e.g., DAMPs released from cells that are injured, perhaps by reactive drug metabolites) and elimination of "immune tolerance," the

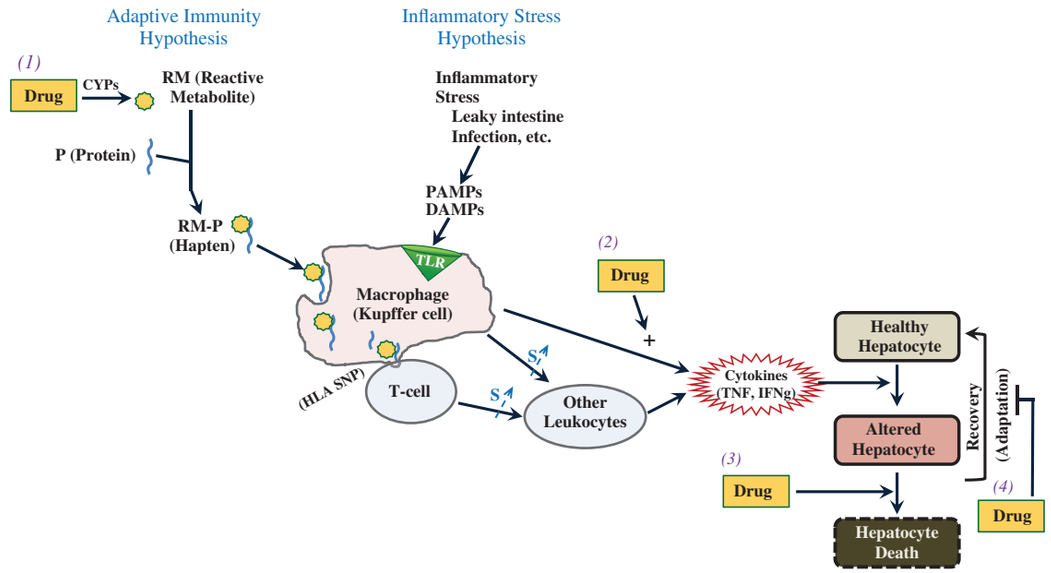


Figure 13-25. *Potential mechanisms by which drugs contribute to IDILI responses. (1)* Drugs can be metabolically bioactivated by cytochromes P450 (CYPs) to reactive metabolites that bind to cellular proteins to initiate an adaptive immune response. *(2)* Activation of adaptive or innate immune systems leads to the production of cytokines, which is enhanced by some IDILI-associated drugs. *(3)* One of the pleiotropic effects of cytokines is to activate cell death pathways, which is enhanced by numerous IDILI-associated drugs in vitro. *(4)* Some drugs can interfere with the ability of the liver to adapt to (recover from) modest injury, thereby leading to injury progression. (See Roth et al. [2017], for review.)

liver becomes poised for injury (Cho and Uetrecht, 2017). In a modification of the hapten hypothesis, the *pharmacological interaction (PI) hypothesis* proposes that certain drugs can activate lymphocytes directly to prompt an adaptive immune response without the need for haptenization (Wullemin et al., 2013).

Recent studies have demonstrated links between the adaptive immune system and drugs that cause IDILI. For example, for some IDILI-associated drugs (e.g., flucloxacillin), drug-specific T-cell responses have been identified in blood samples from IDILI patients (Ortega-Alonso et al., 2016; Usui and Naisbitt, 2017). Also, associations have been uncovered between certain polymorphisms in human leukocyte antigens (HLAs) and IDILI reactions to several drugs (e.g., flucloxacillin, ximelagatran, lapatinib, terbinafine, fenofibrate, ticlopidine, amoxicillin-clavulanate, abacavir) (Fontana, 2014; Yip et al., 2015; Nicoletti et al., 2017). In some of these studies, almost all patients who suffered IDILI from a specific drug had a specific HLA genotype; however, most people who were exposed to the drug and had the HLA polymorphism did not develop liver injury. This suggests that additional susceptibility factors are at play.

An unanswered question regarding immune-mediated IDILI is what causes the death of hepatocytes. Both innate and adaptive immune responses culminate in the cellular expression and release of various cytokines. TNF is important in animal models that have been developed with several drugs that cause IDILI (see Roth and Ganey, 2013, for partial listing). In vitro, TNF interacts with numerous IDILI-associated drugs to cause death of HPCs (Cosgrove et al., 2009; Zou et al., 2009; Lu et al., 2013; Maiuri et al., 2015, 2017). Various hepatocellular stresses initiated by drugs appear to be important in cytotoxic drug–TNF interactions in vitro. For example, amiodarone and sulindac sulfide, the active metabolite of sulindac, cause oxidative stress in vitro that underlies the interaction with TNF (Zou et al., 2010; Lu et al., 2013). Diclofenac and chlorpromazine cause endoplasmic reticulum stress that interacts with TNF to produce hepatocellular injury (Fredriksson et al., 2014), and cytotoxic interaction of trovafloxacin with TNF in vitro appears to depend on replication stress induced by drug-induced DNA damage (Beggs et al., 2014). For some drugs, interferon-gamma potentiates the drug–TNF interaction (Maiuri et al., 2015, 2017). Thus, there is mounting evidence that drug-induced, sublethal stress renders HPCs sensitive to the killing capacity of cytokines and that this might be important in IDILI reactions that are initiated by immune responses (Roth et al., 2017; Fig. 13-25).

In conclusion, IDILI likely occurs by several different mechanisms depending the particular drug and characteristics of the patient. It should be borne in mind that the hypotheses described above are not mutually exclusive and that supporting evidence for each of them is incomplete. Paramount needs in this area include a greater understanding of factors that render people susceptible to IDILI reactions and developing better strategies to predict the IDILI liability of compounds during drug development (Roth and Lee, 2017).

CONCLUSION

The liver comprises several cell types and has numerous functions, many of which relate to metabolism of endogenous and xenobiotic agents, elimination of toxic substances, immune surveillance, and supplying other organs with energy sources. Its blood supply arising predominately from intestinal drainage exposes the liver to large concentrations of components in the diet that are absorbed into the blood, and this in part renders the liver susceptible to toxicity. Responses to chemical insults are several and include steatosis, death of various cell types, inflammation, fibrosis, cancer, and proliferative repair. The mechanisms that result in liver injury are complex and, importantly, can be dose-related; that is, different mechanisms may apply at small and large exposures to the same toxicant. Toxic mechanisms typically begin with an insult to HPCs that initiates intracellular signaling which can lead to cell death. Activated sinusoidal cells and blood elements can encourage

progression of injury, and these extrahepatocellular factors add to the complexity of mechanisms. Injury initiation and progression are counterbalanced by adaptation and regeneration mechanisms, and together these determine the seriousness of the toxic outcome.

Progress in evaluating hepatotoxicity and its mechanisms has been aided by animal models with cell-specific genetic alterations made possible by selective breeding and recently by CRISPR-Cas9 and other technologies. Increasing availability of various types of human liver cells and emerging organotypic in vitro systems promise to contribute to understanding of cell–cell interactions important in injury progression (Guguen-Guillouzo et al., 2010; Godoy et al., 2013). Advances in genomic techniques (DNA and RNA sequencing, proteomics, metabonomics) have proven useful for hypothesis generation. These and other recently developed and emerging tools promise to contribute to improved detection of hepatotoxicity and greater understanding of mechanisms in the near future.

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REFERENCES

Abdelmegeed MA, Banerjee A, Jang S, et al. CYP2E1 potentiates binge alcohol-induced gut leakiness, steatohepatitis, and apoptosis. *Free Radical Biol Med*. 2013;65:1238–1245.

Adams DH, Eksteen B. Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel disease. *Nat Rev Immunol*. 2006;6(3):244–251.

Adams DH, Ju C, Ramaiah SK, Uetrecht J, Jaeschke H. Mechanisms of immune-mediated liver injury. *Toxicol Sci*. 2010 Jun;115(2):307–321.

Adelizzi RA. Clinical implications of NSAID pharmacokinetics: special populations, special considerations. *J Am Osteopath Assoc*. 1994;94(5):396–398, 401–403.

Agarwal DP. Genetic polymorphisms of alcohol metabolizing enzymes. *Pathol Biol (Paris)*. 2001;49:703–709.

Ahmad J, Odin JA. Epidemiology and genetic risk factors of drug hepatotoxicity. *Clin Liver Dis*. 2017;21(1):55–72.

Aleo MD, Luo Y, Swiss R, Bonin PD, Potter DM, Will Y. Human drug-induced liver injury severity is highly associated with dual inhibition of liver mitochondrial function and bile salt export pump. *Hepatology*. 2014;60(3):1015–1022.

Allen K, Jaeschke H, Copple BL. Bile acids induce inflammatory genes in hepatocytes: a novel mechanism of inflammation during obstructive cholestasis. *Am J Pathol*. 2011;178:175–186.

Almeda-Valdes P, Aguilar Olivos NE, Barranco-Fragoso B, Uribe M, Méndez-Sánchez N. The role of dendritic cells in fibrosis progression in nonalcoholic fatty liver disease. *Biomed Res Int*. 2015;2015:768071.

Anand SS, Mumtaz MM, Mehendale HM. Dose-dependent liver regeneration in chloroform, trichloroethylene and allyl alcohol ternary mixture hepatotoxicity in rats. *Arch Toxicol*. 2005;79:671–682.

Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J Biol Chem*. 2001 276:28857–28865.

Anderson GD. Gender differences in pharmacological response. *Int Rev Neurobiol*. 2008;83:1–10.

Andersson M, Storm HH. Cancer incidence among Danish Thorotrast exposed patients. *J Natl Cancer Inst*. 1992;84:1318–1325.

Andrade RJ, Robles M, Ulzurrun E, Lucena MI. Drug-induced liver injury: insights from genetic studies. *Pharmacogenomics*. 2009;10(9):1467–1487.

Angrish MM, Kaiser JP, McQueen CA, Chorley BN. Tipping the balance: hepatotoxicity and the 4 apical key events of hepatic steatosis. *Toxicol Sci*. 2016;150(2):261–268.

Antoine DJ, Dear JW, Lewis PS, et al. Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. *Hepatology*. 2013;58:777–787.

Antoine DJ, Jenkins RE, Dear JW, et al. Molecular forms of HMGB1 and keratin-18 as mechanistic biomarkers for mode of cell death and prognosis during clinical acetaminophen hepatotoxicity. *J Hepatol*. 2012;56:1070–1079.

Antoniades CG, Quaglia A, Taams LS, et al. Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology*. 2012;56(2):735–746.

Aoyama T, Peters JM, Iritani N, et al. Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). *J Biol Chem*. 1998;273:5678–5684.

Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. *Hepatology*. 2008;48:2047–2063.

Arrese M, Ananthanarayanan M, Suchy FJ. Hepatobiliary transport: molecular mechanisms of development and cholestasis. *Pediatr Res*. 1998;44:141–147.

Arrese M, Trauner M. Molecular aspects of bile formation and cholestasis. *Trends Mol Med*. 2003;9(12):558–564.

Arteel GE. Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology*. 2003;124:778–790.

Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. *Am J Physiol Gastrointest Liver Physiol*. 2000;279:G245–G249.

Aw TY. Biliary glutathione promotes the mucosal metabolism of lumenal peroxidized lipids by rat small intestine in vivo. *J Clin Invest*. 1994;94:1218–1225.

Azziz-Baumgartner E, Lindblade K, Gieseker K, et al. Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. *Environ Health Perspect*. 2005;113(12):1779–1783.

Badr MZ, Belinsky SA, Kauffman FC, Thurman RG. Mechanism of hepatotoxicity to periportal regions of the liver lobule due to allyl alcohol: role of oxygen and lipid peroxidation. *J Pharmacol Exp Ther*. 1986; 238:1138–1142.

Baeck C, Tacke F. Balance of inflammatory pathways and interplay of immune cells in the liver during homeostasis and injury. *EXCLI J*. 2014;13:67–81.

Baier PK, Hempel S, Waldvogel B, Baumgartner U. Zonation of hepatic bile salt transporters. *Dig Dis Sci*. 2006; 51:587–593.

Bajt ML, Farhood A, Jaeschke H. Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature. *Am J Physiol Gastrointest Liver Physiol*. 2001;281:G1188–G1195.

Bajt ML, Knight TR, Farhood A, Jaeschke H. Scavenging peroxynitrite with glutathione promotes regeneration and enhances survival during acetaminophen-induced liver injury in mice. *J Pharmacol Exp Ther*. 2003; 307:67–73.

Bajt ML, Lawson JA, Vonderfecht SL, Gujral JS, Jaeschke H. Protection against Fas receptor-mediated apoptosis in hepatocytes and nonparenchymal cells by a caspase-8 inhibitor in vivo: evidence for a postmitochondrial processing of caspase-8. *Toxicol Sci*. 2000;58:109–117.

Ballatori N. Transport of toxic metals by molecular mimicry. *Environ Health Perspect*. 2002;110(suppl 5):689–694.

Ballatori N, Li N, Fang F, Boyer JL, Christian WV, Hammond CL. OST alpha-OST beta: a key membrane transporter of bile acids and conjugated steroids. *Front Biosci (Landmark Ed)*. 2009;14:2829–2844.

Barton CC, Hill DA, Yee SB, Barton EX, Ganey PE, Roth RA. Bacterial lipopolysaccharide exposure augments aflatoxin B(1)-induced liver injury. *Toxicol Sci*. 2000;55(2):444–452.

Basu S. Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients. *Toxicology*. 2003;189(1–2):113–127.

Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest*. 2005;115:209–218.

Bataller R, Gao B. Liver fibrosis in alcoholic liver disease. *Semin Liver Dis*. 2015;35(2):146–156.

Becuwe P, Ennen M, Klotz R, Barbieux C, Grandemange S. Manganese superoxide dismutase in breast cancer: from molecular mechanisms of gene regulation to biological and clinical significance. *Free Radic Biol Med*. 2014;77:139–151.

Beggs KM, Fullerton AM, Miyakawa K, Ganey PE, Roth RA. Molecular mechanisms of hepatocellular apoptosis induced by trovafloxacin-tumor necrosis factor-alpha interaction. *Toxicol Sci*. 2014;137(1):91–101.

Benjamin SB, Goodman ZD, Ishak KG, Zimmerman HJ, Irey NS. The morphologic spectrum of halothane-induced hepatic injury: analysis of 77 cases. *Hepatology*. 1985;5:1163–1171.

Bennett JW, Klich M. Mycotoxins. *Clin Microbiol Rev*. 2003;16(3):497–516.

Berson A, De Beco V, Lettéron P, et al. Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes. *Gastroenterology*. 1998;114:764–774.

Best DH, Coleman WB. Activation and regulation of reserve liver progenitor cells. *Vitam Horm*. 2011;87:93–109.

Bhushan B, Chavan H, Borude P, et al. Dual role of epidermal growth factor receptor in liver injury and regeneration after acetaminophen overdose in mice. *Toxicol Sci*. 2017a;155(2):363–378.

Bhushan B, Edwards G, Desai A, Michalopoulos GK, Apte U. Liver-specific deletion of integrin-linked kinase in mice attenuates hepatotoxicity and improves liver regeneration after acetaminophen overdose. *Gene Expr*. 2016; 17(1): 35–45.

Bhushan B, Poudel S, Manley MW Jr, Roy N, Apte U. Inhibition of glycogen synthase kinase 3 accelerated liver regeneration after acetaminophen-induced hepatotoxicity in mice. *Am J Pathol*. 2017b;187(3):543–552.

Bhushan B, Walesky C, Manley M, et al. Pro-regenerative signaling after acetaminophen-induced acute liver injury in mice identified using a novel incremental dose model. *Am J Pathol*. 2014;184(11):3013–3025.

Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol*. 2007;81:1–5.

Bilzer M, Jaeschke H, Vollmar AM, Paumgartner G, Gerbes AL. Prevention of Kupffer cell-induced oxidant injury in rat liver by atrial natriuretic peptide. *Am J Physiol*. 1999;276(5 pt 1):G1137–G1144.

Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. *Liver Int*. 2006;26:1175–1186.

Bischoff K. The toxicology of microcystin-LR: occurrence, toxicokinetics, toxicodynamics, diagnosis and treatment. *Vet Hum Toxicol*. 2001;43(5):294–297.

Bishayee A. The role of inflammation and liver cancer. *Adv Exp Med Biol*. 2014;816:401–435.

Björnsson ES. Hepatotoxicity by drugs: the most common implicated agents. *Int J Mol Sci*. 2016;17(2):224.

Björnsson ES, Hoofnagle JH. Categorization of drugs implicated in causing liver injury: critical assessment based on published case reports. *Hepatology*. 2016;63(2):590–603.

Boelsterli UA. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Toxicol Appl Pharmacol*. 2003;192:307–322.

Boelsterli UA, Lee KK. Mechanisms of isoniazid-induced idiosyncratic liver injury: emerging role of mitochondrial stress. *J Gastroenterol Hepatol*. 2014;29(4):678–687.

Bonnet MS, Basson PW. The toxicology of Amanita phalloides. *Homeopathy*. 2002; 91:249–254.

Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst*. 2000;92:1295–1302.

Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. *Clin Liver Dis*. 2005;9:191–211.

Bouaïcha N, Maatouk I, Plessis MJ, Périn F. Genotoxic potential of Microcystin-LR and nodularin in vitro in primary cultured rat hepatocytes and in vivo in rat liver. *Environ Toxicol*. 2005;20(3):341–347.

Boulares AH, Zoltoski AJ, Stoica BA, Cuvillier O, Smulson ME. Acetaminophen induces a caspase-dependent and Bcl-XL sensitive apoptosis in human hepatoma cells and lymphocytes. *Pharmacol Toxicol*. 2002;90:38–50.

Bourdi M, Masubuchi Y, Reilly TP, et al. Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase. *Hepatology*. 2002;35:289–298.

Boyer JL, Trauner M, Mennone A, et al. Upregulation of a basolateral FXR-dependent bile acid efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents. *Am J Physiol Gastrointest Liver Physiol*. 2006a;290:G1124–G1130.

Boyer TD, Wright TL, Manns M. *Zakim and Boyer's Hepatology*. 5th ed. Philadelphia, PA: Saunders-Elsevier; 2006b.

Braet F, Wisse E. Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comp Hepatol*. 2002;1:1.

Brattin WJ, Glende EA Jr, Recknagel RO. Pathological mechanisms in carbon tetrachloride hepatotoxicity. *J Free Radic Biol Med*. 1985;1(1):27–38.

Brautbar N, Williams J 2nd. Industrial solvents and liver toxicity: risk assessment, risk factors and mechanisms. *Int J Hyg Environ Health*. 2002;205(6):479–491.

Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. *J Hepatol*. 2013;59(3):583–594.

Brooks WP, Codd GA. Distribution of Microcystis aeruginosa peptide toxin and interactions with hepatic microsomes in mice. *Pharmacol Toxicol*. 1987;60(3):187–191.

Buchweitz JP, Ganey PE, Bursian SJ, Roth RA. Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy? *J Pharmacol Exp Ther*. 2002;300(2):460–467.

Buetler TM, Slone D, Eaton DL. Comparison of the aflatoxin B1-8,9-epoxide conjugating activities of two bacterially expressed alpha class glutathione S-transferase isozymes from mouse and rat. *Biochem Biophys Res Commun*. 1992;188(2):597–603.

Bui-Klimke TR, Guclu H, Kensler TW, Yuan JM, Wu F. Aflatoxin regulations and global pistachio trade: insights from social network analysis. *PLoS One*. 2014;9(3):e92149.

Bunchorntavakul C, Reddy KR. Review article: herbal and dietary supplement hepatotoxicity. *Aliment Pharmacol Ther*. 2013;37(1):3–17.

Bunchorntavakul C, Reddy KR. Drug hepatotoxicity: newer agents. *Clin Liver Dis*. 2017;21(1):115–134.

Burkard I, von Eckardstein A, Rentsch KM. Differentiated quantification of human bile acids in serum by high-performance liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005;826(1–2):147–159.

Byrne CD. Fatty liver: role of inflammation and fatty acid nutrition. Prostaglandins *Leukot Essent Fatty Acids*. 2010;82(4–6):265–271.

Cai Y, Gong LK, Qi XM, Li XH, Ren J. Apoptosis initiated by carbon tetrachloride in mitochondria of rat primary cultured hepatocytes. *Acta Pharmacol Sin*. 2005 26:969–975.

Ceccarelli S, Nobili V, Alisi A. Toll-like receptor-mediated signaling cascade as a regulator of the inflammation network during alcoholic liver disease. *World J Gastroenterol*. 2014;20(44):16443–16451.

Chalasani N, Björnsson E. Risk factors for idiosyncratic drug-induced liver injury. *Gastroenterology*. 2010;138(7):2246–2259.

Chalasani NP, Hayashi PH, Bonkovsky HL, et al. ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. *Am J Gastroenterol*. 2014;109(7):950–966.

Chamulitrat W, Blazka ME, Jordan SJ, Luster MI, Mason RP. Tumor necrosis factor-alpha and nitric oxide production in endotoxin-primed rats administered carbon tetrachloride. *Life Sci*. 1995;57(24):2273–2280.

Chang TP, Rangan C. Iron poisoning: a literature-based review of epidemiology, diagnosis, and management. *Pediatr Emerg Care*. 2011;27:978–985.

Chen CC, Lu RB, Chen YC, et al. Interaction between the functional polymorphisms of the alcohol-metabolism genes in protection against alcoholism. *Am J Hum Genet*. 1999;65:795–807.

Cheng L, You Q, Yin H, Holt M, Franklin C, Ju C. Effect of polyI:C cotreatment on halothane-induced liver injury in mice. *Hepatology*. 2009;49(1):215–226.

Chen K, Wang JM, Yuan R, et al. Tissue-resident dendritic cells and diseases involving dendritic cell malfunction. *Int Immunopharmacol*. 2016;34:1–15.

Chen M, Suzuki A, Borlak J, Andrade RJ, Lucena MI. Drug-induced liver injury: interactions between drug properties and host factors. *J Hepatol*. 2015;63(2):503–514.

Chen Y, Shen D, Fang D. Nodularins in poisoning. *Clin Chim Acta*. 2013;425:18–29.

Cho T, Uetrecht J. How reactive metabolites induce an immune response that sometimes leads to an idiosyncratic drug reaction. *Chem Res Toxicol*. 2017;30(1):295–314.

Chorus I, Bartram J. *Toxic Cyanobacteria in Water. A Guide to their Public Health Consequences, Monitoring and Management*. London: WHO; 1999.

Chosay JG, Essani NA, Dunn CJ, et al. Neutrophil margination and extravasation in sinusoids and venules of liver during endotoxin-induced injury. *Am J Physiol*. 1997;272:G1195–G1200.

Clark SP, Davis MA, Ryan TP, Searfoss GH, Hooser SB. Hepatic gene expression changes in mice associated with prolonged sublethal Microcystin exposure. *Toxicol Pathol*. 2007;35(4):594–605.

Clifford JI, Rees KR. The action of aflatoxin B1 on the rat liver. *Biochem J*. 1967;102(1):65–75.

Colell A, García-Ruiz C, Miranda M, et al. Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor. *Gastroenterology*. 1998 115:1541–1551.

Connolly MK, Ayo D, Malhotra A, et al. Dendritic cell depletion exacerbates acetaminophen hepatotoxicity. *Hepatology*. 2011;54(3):959–968.

Copple BL, Banes A, Ganey PE, Roth RA. Endothelial cell injury and fibrin deposition in rat liver after monocrotaline exposure. *Toxicol Sci*. 2002;65(2):309–318.

Copple BL, Ganey PE, Roth RA. Liver inflammation during monocrotaline hepatotoxicity. *Toxicology*. 2003;190(3):155–169.

Copple BL, Rondelli CM, Maddox JF, et al. Modes of cell death in rat liver after monocrotaline exposure. *Toxicol Sci*. 2004;77(1):172–182.

Copple BL, Roth RA, Ganey PE. Anticoagulation and inhibition of nitric oxide synthase influence hepatic hypoxia after monocrotaline exposure. *Toxicology*. 2006;225(2–3):128–137.

Corkins MR. Copper metabolism and pediatric cholestasis. *Curr Opin Clin Nutr Metab Care*. 2011;14(6):642–646.

Corsini A, Ganey P, Ju C, et al. Current challenges and controversies in drug-induced liver injury. *Drug Saf*. 2012;35(12):1099–1117.

Corsini A, Bortolini M. Drug-induced liver injury: the role of drug metabolism and transport. *J Clin Pharmacol*. 2013;53(5):463–474.

Cosgrove BD, King BM, Hasan MA, et al. Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. *Toxicol Appl Pharmacol*. 2009;237(3):317–330.

Coughtrie MW. Ontogeny of human conjugating enzymes. *Drug Metab Lett*. 2015;9(2):99–108.

Coulombe RA Jr. Pyrrolizidine alkaloids in foods. *Adv Food Nutr Res*. 2003;45:61–99.

Cover C, Mansouri A, Knight TR, et al. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. *J Pharmacol Exp Ther*. 2005;315:879–887.

Crawford JM. The liver and the biliary tract. In: Cotran RS, Kumar V, Collins T, eds. *Robbins: Pathologic Basis of Disease*. 6th ed. Philadelphia, PA: WB Saunders; 1999:845–901.

Creppy EE. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol Lett*. 2002;127(1–3):19–28.

Crettol S, Petrovic N, Murray M. Pharmacogenetics of phase I and phase II drug metabolism. *Curr Pharm Des*. 2010;16(2):204–219.

Csanaky IL, Lu H, Zhang Y, Ogura K, Choudhuri S, Klaassen CD. Organic anion-transporting polypeptide 1b2 (Oatp1b2) is important for the hepatic uptake of unconjugated acids: studies in Oatp1b2-null mice. *Hepatology*. 2011;53:272–281.

Cui K, Yan G, Xu C, Chen Y, et al. Invariant NKT cells promote alcohol-induced steatohepatitis through interleukin-1beta in mice. *J Hepatol*. 2015;62(6):1311–1318.

Cullen JM, Newberne PM. Acute hepatotoxicity of aflatoxins. In: Eaton DL, Groopman JD, eds. *The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance*. New York: Academic Press; 1994:3–26.

Cullen JM, Ruebner BH. A histopathologic classification of chemical-induced injury of the liver. In: Meeks RG, Harrison SD, Bull RJ, eds. *Hepatotoxicology*. Boca Raton, FL: CRC Press; 1991:67–92.

Czaja AJ. Drug-induced autoimmune-like hepatitis. *Dig Dis Sci*. 2011;56(4):958–976.

Dahm LJ, Ganey PE, Roth RA. α-Naphthylisothiocyanate. In: C.A. McQueen, *Comprehensive Toxicology*. Vol 9. Elsevier; 2010:571–580.

Dahm LJ, Schultze AE, Roth RA. An antibody to neutrophils attenuates alpha-naphthylisothiocyanate-induced liver injury. *J Pharmacol Exp Ther*. 1991;256(1):412–420.

Dara L, Johnson H, Suda J, et al. Receptor interacting protein kinase 1 mediates murine acetaminophen toxicity independent of the necrosome and not through necroptosis. *Hepatology*. 2015;62(6):1847–1857.

Dara L, Liu ZX, Kaplowitz N. Mechanisms of adaptation and progression in idiosyncratic drug induced liver injury, clinical implications. *Liver Int*. 2016a;36(2):158–165.

Dara L, Liu ZX, Kaplowitz N. Questions and controversies: the role of necroptosis in liver disease. *Cell Death Discov*. 2016b;2:16089. eCollection 2016.

Dart RC, Green JL, Kuffner EK, et al. The effects of paracetamol (acetaminophen) on hepatic tests in patients who chronically abuse alcohol - a randomized study. *Aliment Pharmacol Ther*. 2010;32(3):478–486.

Davern TJ 2nd, James LP, Hinson JA, et al. Measurement of serum acetaminophen-protein adducts in patients with acute liver failure. *Gastroenterology*. 2006;130:687–694.

Davies MH, Harrison RF, Elias E, Hübscher SG. Antibiotic-associated acute vanishing bile duct syndrome: a pattern associated with severe, prolonged, intrahepatic cholestasis. *J Hepatol*. 1994 20:112–116.

Day CP. Genes or environment to determine alcoholic liver disease and non-alcoholic fatty liver disease. *Liver Int*. 2006;26:1021–1028.

Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology*. 1998a;114:842–845.

Day CP, James OF. Hepatic steatosis: innocent bystander or guilty party? *Hepatology*. 1998b;27:1463–1466.

Decker K. Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur J Biochem*. 1990;192:245–261.

DeLeve LD, Ito Y, Bethea NW, et al. Embolization by sinusoidal lining cells obstructs the microcirculation in rat sinusoidal obstruction syndrome. *Am J Physiol Gastrointest Liver Physiol*. 2003a;284(6):G1045–G1052.

DeLeve LD, McCuskey RS, Wang X, et al. Characterization of a reproducible rat model of hepatic veno-occlusive disease. *Hepatology*. 1999;29:1779–1791.

DeLeve LD, Wang X, Kanel GC, et al. Decreased hepatic nitric oxide production contributes to the development of rat sinusoidal obstruction syndrome. *Hepatology*. 2003b;38:900–908.

DeLeve LD, Wang X, Kaplowitz N, et al. Sinusoidal endothelial cells as a target for acetaminophen toxicity. Direct action versus requirement for hepatocyte activation in different mouse strains. *Biochem Pharmacol*. 1997;53:1339–1345.

DeLeve LD, Wang X, Kuhlenkamp JF, Kaplowitz N. Toxicity of azathioprine and monocrotaline in murine sinusoidal endothelial cells and hepatocytes: the role of glutathione and relevance to hepatic venoocclusive disease. *Hepatology*. 1996;23(3):589–599.

Deleve LD, Wang X, Tsai J, et al. Sinusoidal obstruction syndrome (veno-occlusive disease) in the rat is prevented by matrix metalloproteinase inhibition. *Gastroenterology*. 2003c;125:882–890.

Deng X, Stachlewitz RF, Liguori MJ, et al. Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. *J Pharmacol Exp Ther*. 2006;319(3):1191–1199.

Deng X, Luyendyk JP, Ganey PE, Roth RA. Inflammatory stress and idiosyncratic hepatotoxicity: hints from animal models. *Pharmacol Rev*. 2009;61(3):262–282.

Denson LA, Sturm E, Echevarria W, et al. The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, NTCP. *Gastroenterology*. 2001;121:140–147.

Dey A, Cederbaum AI. Alcohol and oxidative liver injury. *Hepatology*. 2006;43(2 suppl 1):S63–S74.

Dietrich CG, Ottenhoff R, de Waart DR, Oude Elferink RP. Role of MRP2 and GSH in intrahepatic cycling of toxins. *Toxicology*. 2001;167(1):73–81.

Ding WX, Manley S, Ni HM. The emerging role of autophagy in alcoholic liver disease. *Exp Biol Med (Maywood)*. 2011;236:546–556.

Ding WX, Nam Ong C. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol Lett*. 2003;220(1):1–7.

Ding WX, Shen HM, Ong CN. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. *Hepatology*. 2000a;32:547–555a.

Ding WX, Shen HM, Ong CN. Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. *Environ Health Perspect*. 2000b;108(7):605–609.

Ding WX, Shen HM, Ong CN. Pivotal role of mitochondrial Ca^{2+} in microcystine-induced mitochondrial permeability transition in rat hepatocytes. *Biochem Biophys Res Commun*. 2001;285: 1155–1161.

Dittmann E, Wiegand C. Cyanobacterial toxins—zzzoccurrence, biosynthesis and impact on human affairs. *Mol Nutr Food Res*. 2006;50(1):7–17.

Donthamsetty S, Bhave VS, Mitra MS, Latendresse JR, Mehendale HM. Nonalcoholic fatty liver sensitizes rats to carbon tetrachloride hepatotoxicity. *Hepatology*. 2007;45:391–403.

Du K, Farhood A, Jaeschke H. Mitochondria-targeted antioxidant Mito-Tempo protects against acetaminophen hepatotoxicity. *Arch Toxicol*. 2017;91(2):761–773.

Du K, Ramachandran A, Jaeschke H. Oxidative stress during acetaminophen hepatotoxicity: sources, pathophysiological role and therapeutic potential. *Redox Biol*. 2016;10:148–156.

Du K, Xie Y, McGill MR, Jaeschke H. Pathophysiological significance of c-jun N-terminal kinase in acetaminophen hepatotoxicity. *Expert Opin Drug Metab Toxicol*. 2015;11(11):1769–1779.

Dugan CM, MacDonald AE, Roth RA, Ganey PE. A mouse model of severe halothane hepatitis based on human risk factors. *J Pharmacol Exp Ther*. 2010;333(2):364–372.

Dutczak WJ, Clarkson TW, Ballatori N. Biliary-hepatic recycling of a xenobiotic: gallbladder absorption of methyl mercury. *Am J Physiol*. 1991;260:G873–G880.

Eaton DL, Gallagher EP, Bammler TK, Kunze KL. Role of cytochrome P4501A2 in chemical carcinogenesis: implications for human variability in expression and enzyme activity. *Pharmacogenetics*. 1995; 5(5):259–274

Eaton DL, Groopman JD, eds. *The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance*. New York: Academic Press; 1994.

Eberl G, Colonna M, Di Santo JP, McKenzie AN. Innate lymphoid cells. Innate lymphoid cells: a new paradigm in immunology. *Science*. 2015;348(6237):aaa6566.

Edgar JA, Colegate SM, Boppré M, Molyneux RJ. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2011;28(3):308–324.

El-Benna J, Dang PM, Gougerot-Pocidalo MA, Elbim C. Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp (Warsz)*. 2005;53:199–206.

Elcombe CR, Peffer RC, Wolf DC, et al. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: a case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. *Crit Rev Toxicol*. 2014;44(1):64–82.

elSisi AE, Earnest DL, Sipes IG. Vitamin A potentiation of carbon tetrachloride hepatotoxicity: role of liver macrophages and active oxygen species. *Toxicol Appl Pharmacol*. 1993;119:295–301.

Elvevold K, Smedsrød B, Martinez I. The liver sinusoidal endothelial cell: a cell type of controversial and confusing identity. *Am J Physiol Gastrointest Liver Physiol*. 2008;294:G391–G400.

Enomoto K, Nishikawa Y, Omori Y, et al. Cell biology and pathology of liver sinusoidal endothelial cells. *Med Electron Microsc*. 2004;37:208–215.

Enomoto N, Takase S, Takada N, Takada A. Alcoholic liver disease in heterozygotes of mutant and normal aldehyde dehydrogenase-2 genes. *Hepatology*. 1991; 13:1071–1075.

Falconer IR, Yeung DS. Cytoskeletal changes in hepatocytes induced by Microcystis toxins and their relation to hyperphosphorylation of cell proteins. *Chem Biol Interact*. 1992;81(1–2):181–196.

Fardel O, Le Vée M. Regulation of human hepatic drug transporter expression by pro-inflammatory cytokines. *Expert Opin Drug Metab Toxicol*. 2009;5(12):1469–1481.

Farrell GC. Drugs and steatohepatitis. *Semin Liver Dis*. 2002;22:185–194.

Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology*. 2006;43(suppl 1):S99–S112.

Fattinger K, Funk C, Pantze M, et al. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther*. 2001;69:223–231.

Fausto N. Liver regeneration. *J Hepatol*. 2000;32(1 suppl):19–31.

Ferramosca A, Zara V. Modulation of hepatic steatosis by dietary fatty acids. *World J Gastroenterol*. 2014;20(7):1746–1755.

Fickert P, Fuchsbichler A, Marschall HU, et al. Lithocholic acid feeding induces segmental bile duct obstruction and destructive cholangitis in mice. *Am J Pathol*. 2006;168:410–422.

Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ*. 2003;10: 76–100.

Fisher WJ, Altheimer S, Cattori V, Meier PJ, Dietrich DR, Hagenbuch B. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. *Toxicol Applied Pharmacol*. 2005;203(3):257–263.

Fontana RJ. Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives. *Gastroenterology*. 2014;146(4):914–928.

Fowler BA. Monitoring of human populations for early markers of cadmium toxicity: a review. *Toxicol Appl Pharmacol*. 2009;238(3):294–300.

Fredriksson L, Wink S, Herpers B, et al. Drug-induced endoplasmic reticulum and oxidative stress responses independently sensitize toward TNFα-mediated hepatotoxicity. *Toxicol Sci*. 2014;140(1):144–159.

Frangez R, Kosec M, Sedmak B, et al. Subchronic liver injuries caused by microcystins. *Pflugers Arch*. 2000;440(5 suppl):R103–R104.

Fraser R, Le Couteur DG, Warren A, et al. The liver sieve and gene therapy. *Blood*. 2003;101:3338.

Freeman TL, Tuma DJ, Thiele GM, et al. Recent advances in alcohol-induced adduct formation. *Alcohol Clin Exp Res*. 2005;29:1310–1316.

Frenzel C, Teschke R. Herbal hepatotoxicity: clinical characteristics and listing compilation. *Int J Mol Sci*. 2016;17(5).

Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem*. 2000;275:2247–2250.

Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev*. 2008a;88:125–172.

Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology*. 2008b;134:1655–1669.

Frimmer M. What we have learned from phalloidin. *Toxicol Lett*. 1987;35:169–182.

Fujiki H, Suganuma M. Tumor promoters–microcystin-LR, nodularin and TNF-α and human cancer development. *Anticancer Agents Med Chem*. 2011;11(1):4–18.

Funk C, Roth A. Current limitations and future opportunities for prediction of DILI from in vitro. *Arch Toxicol*. 2017;91(1):131–142.

Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc Natl Acad Sci U S A*. 1979;76:5939–5943.

Gandhi A, Moorthy B, Ghose R. Drug disposition in pathophysiological conditions. *Curr Drug Metab*. 2012;13(9):1327–1344.

Ganey PE, Luyendyk JP, Maddox JF, Roth RA. Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. *Chem Biol Interact*. 2004;150(1):35–51.

Ganey PE, Luyendyk JP, Newport SW, et al. Role of the coagulation system in acetaminophen-induced hepatotoxicity in mice. *Hepatology*. 2007;46(4):1177–1186.

Ganey PE, Roth RA. Concurrent inflammation as a determinant of susceptibility to toxicity from xenobiotic agents. *Toxicology*. 2001;169:195–208.

Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology*. 2011;141:1572–1585.

Gao B, Radaeva S, Park O. Liver natural killer and natural killer T cells: immunobiology and emerging roles in liver diseases. *J Leukoc Biol*. 2009;86:513–528.

Gao B, Xu MJ, Bertola A, Wang H, Zhou Z, Liangpunsakul S. Animal models of alcoholic liver disease: pathogenesis and clinical relevance. *Gene Expr*. 2017;17(3):173–186.

Garcia A, Cayla X, Guergnon J, et al. Serine/threonine protein phosphatases PP-1 and PP-2A are key players in apoptosis. *Biochimie*. 2003;85(8): 721–726.

Garcia J, Costa VM, Carvalho A, et al. Amanita phalloides poisoning: Mechanisms of toxicity and treatment. *Food Chem Toxicol*. 2015;86:41–55.

Gebhardt R, Alber J, Wegner H, Mecke D. Different drug metabolizing capacities in cultured periportal and pericentral hepatocytes. *Biochem Pharmacol*. 1994; 48:761–766.

Geier A, Fickert P, Trauner M. Mechanisms of disease: mechanisms and clinical implications of cholestasis in sepsis. *Nat Clin Pract Gastroenterol Hepatol*. 2006;3:574–585.

Gerk PM, Vore M. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J Pharmacol Exp Ther*. 2002;302:407–415.

Geubel AP, De Galocsy C, Alves N, Rahier J, Dive C. Liver damage caused by therapeutic vitamin A administration: estimate of dose-related toxicity in 41 cases. *Gastroenterology*. 1991;100:1701–1709.

Giannelli V, Di Gregorio V, Iebba V, et al. Microbiota and the gut-liver axis: bacterial translocation, inflammation and infection in cirrhosis. *World J Gastroenterol*. 2014;20(45):16795–16810.

Giannini EG, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. *CMAJ*. 2005;172(3):367–379.

Ginsberg G, Guyton K, Johns D, Schimek J, Angle K, Sonawane B. Genetic polymorphism in metabolism and host defense enzymes: implications for human health risk assessment. *Crit Rev Toxicol*. 2010;40(7):575–619.

Ginsberg G, Smolenski S, Hattis D, Guyton KZ, Johns DO, Sonawane B. Genetic Polymorphism in Glutathione Transferases (GST): population distribution of GSTM1, T1, and P1 conjugating activity. *J Toxicol Environ Health B Crit Rev*. 2009;12(5–6):389–439.

Godoy P, Hewitt NJ, Albrecht U, et al. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol*. 2013;87(8):1315–1530.

Goering PL. Hepatotoxicity of copper, iron, cadmium and arsenic. In: McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol. 9. Oxford: Academic Press; 2010:501–526.

Golbar HM, Izawa T, Ichikawa C, et al. Slowly progressive cholangiofibrosis induced in rats by alpha-naphthylisothiocyanate (ANIT), with particular references to characteristics of macrophages and myofibroblasts. *Exp Toxicol Pathol*. 2013;65(6):825–835.

Gómez-Lechón MJ, O'Connor E, Castell JV, Jover R. Sensitive markers used to identify compounds that trigger apoptosis in cultured hepatocytes. *Toxicol Sci*. 2002;65:299–308.

Gonzalez FJ. The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. *Drug Metab Dispos*. 2007;35:1–8.

Grattagliano I, Bonfrate L, Diogo CV, et al. Biochemical mechanisms in drug-induced liver injury: certainties and doubts. *World J Gastroenterol*. 2009;15(39):4865–4876.

Gregory SH, Wing EJ. Neutrophil–Kupffer cell interaction: a critical component of host defenses to systemic bacterial infections. *J Leukoc Biol*. 2002;72:239–248.

Gregus Z, Klaassen CD. Disposition of metals in rats: a comparative study of fecal, urinary, and biliary excretion and tissue distribution of eighteen metals. *Toxicol Appl Pharmacol*. 1986;85:24–38.

Gressner AM, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J Cell Mol Med*. 2006;10:76–99.

Groen A, Romero MR, Kunne C, et al. Complementary functions of the flippase ATP8B1 and the floppase ABCB4 in maintaining canalicular membrane integrity. *Gastroenterology*. 2011;141:1927–1937.

Groopman JD, Kensler TW, Wild CP. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. *Annu Rev Public Health*. 2008;29:187–203.

Groothuis GM, Hardonk MJ, Keulemans KP, Nieuwenhuis P, Meijer DK. Autoradiographic and kinetic demonstration of acinar heterogeneity of taurocholate transport. *Am J Physiol*. 1982;243:G455–G462.

Grootjans S, Vanden Berghe T, Vandenabeele P. Initiation and execution mechanisms of necroptosis: an overview. *Cell Death Differ*. 2017;24(7):1184–1195.

Guguen-Guillouzo C, Corlu A, Guillouzo A. Stem cell-derived hepatocytes and their use in toxicology. *Toxicology*. 2010;270:3–9.

Gujral JS, Farhood A, Bajt ML, Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. *Hepatology*. 2003a 38:355–363.

Gujral JS, Farhood A, Jaeschke H. Oncotic necrosis and caspase-dependent apoptosis during galactosamine-induced liver injury in rats. *Toxicol Appl Pharmacol*. 2003b;190:37–46.

Gujral JS, Hinson JA, Farhood A, Jaeschke H. NADPH oxidase-derived oxidant stress is critical for neutrophil cytotoxicity during endotoxemia. *Am J Physiol Gastrointest Liver Physiol*. 2004;287:G243–G252.

Gujral JS, Knight TR, Farhood A, Bajt ML, Jaeschke H. Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis? *Toxicol Sci*. 2002;67:322–328.

Gunawan BK, Liu ZX, Han D, et al. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology*. 2006;131(1):165–178.

Guo Y, Hu B, Xie Y, et al. Regulation of drug-metabolizing enzymes by local and systemic liver injuries. *Expert Opin Drug Metab Toxicol*. 2016;12(3):245–251.

Guo YX, Xu XF, Zhang QZ, et al. The inhibition of hepatic bile acids transporters Ntcp and Bsep is involved in the pathogenesis of isoniazid/rifampicin-induced hepatotoxicity. *Toxicol Mech Methods*. 2015;25(5):382–387.

Gutierrez-Ruiz MC, Gomez-Quiroz LE. Liver fibrosis: searching for cell model answers. *Liver Int*. 2007;27:434–439.

Hagenbuch B, Dawson P. The sodium bile salt cotransport family SLC10. *Pflugers Arch*. 2004;447:566–570.

Hagenbuch B, Gui C. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica*. 2008;38:778–801.

Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch*. 2004;447:653–665.

Han D, Dara L, Win S, et al. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. *Trends Pharmacol Sci*. 2013;34(4):243–253.

Han DW. Intestinal endotoxemia as a pathogenetic mechanism in liver failure. *World J Gastroenterol*. 2002;8(6):961–965.

Han D, Shinohara M, Ybanez MD, et al. Signal transduction pathways involved in drug-induced liver injury. *Handb Exp Pharmacol*. 2010;196:267–310.

Hanawa N, Shinohara M, Saberi B, et al. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J Biol Chem*. 2008;283:13565–13577.

Hanumegowda UM, Copple BL, Shibuya M, et al. Basement membrane and matrix metalloproteinases in monocrotaline-induced liver injury. *Toxicol Sci*. 2003;76(1):237–246.

Harrison PM, O'Grady JG, Keays RT, Alexander GJ, Williams R. Serial prothrombin time as prognostic indicator in paracetamol induced fulminant hepatic failure. *BMJ*. 1990;301(6758):964–966.

Hassan F, Morikawa A, Islam S, et al. Lipopolysaccharide augments the in vivo lethal action of doxorubicin against mice via hepatic damage. *Clin Exp Immunol*. 2008;151(2):334–340.

Hatoff DE, Hardison WG. Induced synthesis of alkaline phosphatase by bile acids in rat liver cell culture. *Gastroenterology*. 1979;77(5):1062–1067.

He K, Cai L, Shi Q, Liu H, Woolf TF. Inhibition of MDR3 activity in human hepatocytes by drugs associated with liver injury. *Chem Res Toxicol*. 2015;28(10):1987–1990.

Heard KJ, Green JL, James LP, et al. Acetaminophen-cysteine adducts during therapeutic dosing and following overdose. *BMC Gastroenterol*. 2011;11:20.

Heidari R, Niknahad H, Jamshidzadeh A, Abdoli N. Factors affecting drug-induced liver injury: antithyroid drugs as instances. *Clin Mol Hepatol*. 2014;20(3):237–248.

Henry SH, Bosch FX, Bowers JC. Aflatoxin, hepatitis and worldwide liver cancer risks. *Adv Exp Med Biol*. 2002;504:229–233.

Hesketh J. 3′-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. *Biochem Soc Trans*. 2004;32(pt 6):990–993.

Hewett JA, Roth RA. The coagulation system, but not circulating fibrinogen, contributes to liver injury in rats exposed to lipopolysaccharide from gram-negative bacteria. *J Pharmacol Exp Ther*. 1995;272(1):53–62.

Heymann F, Tacke F. Immunology in the liver--from homeostasis to disease. *Nat Rev Gastroenterol Hepatol*. 2016;13(2):88–110.

Hickling KC, Hitchcock JM, Chipman JK, Hammond TG, Evans JG. Induction and progression of cholangiofibrosis in rat liver injured by oral administration of furan. *Toxicol Pathol*. 2010;38:213–229.

Higuchi S, Kobayashi M, Yoshikawa Y, et al. IL-4 mediates dicloxacillin-induced liver injury in mice. *Toxicol Lett*. 2011;200(3):139–145.

Hill DA, Jean PA, Roth RA. Bile duct epithelial cells exposed to alpha-naphthylisothiocyanate produce a factor that causes neutrophil-dependent hepatocellular injury in vitro. *Toxicol Sci*. 1999;47(1):118–125.

Hines IN, Wheeler MD. Recent advances in alcoholic liver disease III. Role of the innate immune response in alcoholic hepatitis. *Am J Physiol Gastrointest Liver Physiol*. 2004;287:G310–G314.

Hines RN. Developmental expression of drug metabolizing enzymes: impact on disposition in neonates and young children. *Int J Pharm*. 2013;452(1–2):3–7.

Holt MP, Cheng L, Ju C. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J Leukoc Biol*. 2008;84:1410–1421.

Holt MP, Yin H, Ju C. Exacerbation of acetaminophen-induced disturbances of liver sinusoidal endothelial cells in the absence of Kupffer cells in mice. *Toxicol Lett*. 2010;194(1–2), 34–41.

Homann J, Rawer P, Bleyl H, Matthes KJ, Heinrich D. Early detection of amatoxins in human mushroom poisoning. *Arch Toxicol*. 1986;59(3):190–191.

Homolya L, Varadi A, Sarkadi B. Multidrug resistance-associated proteins: export pumps for conjugates with glutathione, glucuronate or sulfate. *Biofactors*. 2003;17:103–114.

Honkanen RE, Zwiller J, Moore RE, et al. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J Biol Chem*. 1990;265:19401–19404.

Honkoop P, Scholte HR, de Man RA, Schalm SW. Mitochondrial injury: lessons from the fialuridine trial. *Drug Saf*. 1997;17:1–7.

Hoorn CM, Wagner JG, Roth RA. Effects of monocrotaline pyrrole on cultured rat pulmonary endothelium. *Toxicol Appl Pharmacol*. 1993;120(2):281–287.

Hooser SB. Fulminant hepatocyte apoptosis in vivo following microcystin-LR administration to rats. *Toxicol Pathol*. 2000;28:726–733.

Hooser SB, Beasley VR, Basgall EJ, Carmichael WW, Haschek WM. Microcystin-LR-induced ultrastructural changes in rats. *Vet Pathol*. 1990;27(1):9–15.

Hooser SB, Beasley VR, Lovell RA, Carmichael WW, Haschek WM. Toxicity of microcystin LR, a cyclic heptapeptide hepatotoxin from Microcystis aeruginosa, to rats and mice. *Vet Pathol*. 1989;26(3):246–252.

Hooser SB, Beasley VR, Waite LL, et al. Actin filament alterations in rat hepatocytes induced in vivo and in vitro by microcystin-LR, a hepatotoxin from the blue-green alga, *Microcystis aeruginosa*. *Vet Pathol*. 1991;28(4):259–266.

Hoque R, Farooq A, Mehal WZ. Sterile inflammation in the liver and pancreas. *J Gastroenterol Hepatol*. 2013;28(suppl 1):61–67.

Hornby RJ, Starkey Lewis P, Dear J, Goldring C, Park BK. MicroRNAs as potential circulating biomarkers of drug-induced liver injury: key current and future issues for translation to humans. *Expert Rev Clin Pharmacol*. 2014;7(3):349–362.

Hu M, Wang DQ, Xiao YJ, Mak VW, Tomlinson B. Herb-drug interactions: methods to identify potential influence of genetic variations in genes encoding drug metabolizing enzymes and drug transporters. *Curr Pharm Biotechnol*. 2012;13(9):1718–1730.

Huang L, Zhao A, Lew JL, et al. Farnesoid X receptor activates transcription of the phospholipid pump MDR3. *J Biol Chem*. 2003;278:51085–51090.

Huxtable RJ. Human health implications of pyrrolizidine alkaloids and herbs containing them. In: Cheeke PR, ed. *Toxicants of Plant Origin*. Vol. 1. Boca Raton, FL: CRC Press; 1989:41–86.

Huxtable RJ. Pyrrolizidine alkaloids. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol 9. New York: Pergamon Press; 1997:423–431.

Hyun J, Park J, Wang S, et al. MicroRNA expression profiling in CCl_4-induced liver fibrosis of *Mus musculus*. *Int J Mol Sci*. 2016;17(6):961.

Iasella CJ, Johnson HJ, Dunn MA. Adverse drug reactions: type A (intrinsic) or type B (idiosyncratic). *Clin Liver Dis*. 2017;21(1):73–87.

Ibrahim SH, Kohli R, Gores GJ. Mechanisms of lipotoxicity in NAFLD and clinical implications. *J Pediatr Gastroenterol Nutr*. 2011;53:131–140.

Ingawale DK, Mandlik SK, Naik SR. Models of hepatotoxicity and the underlying cellular, biochemical and immunological mechanism(s): a critical discussion. *Environ Toxicol Pharmacol*. 2014;37(1):118–133.

Iorga A, Dara L, Kaplowitz N. Drug-induced liver injury: cascade of events leading to cell death, apoptosis or necrosis. *Int J Mol Sci*. 2017;18(5):pii: E1018.

Irie M, Suzuki N, Sohda T, et al. Hepatic expression of gamma-glutamyltranspeptidase in the human liver of patients with alcoholic liver disease. *Hepatol Res*. 2007;37:966–973.

Ishii H, Adachi M, Fernandez-Checa JC, et al. Role of apoptosis in alcoholic liver injury. *Alcohol Clin Exp Res*. 2003;27:1207–1212.

Ishikawa T, Kajimoto Y, Sun W, et al. Role of Nrf2 in cancer photodynamic therapy: regulation of human ABC transporter ABCG2. *J Pharm Sci*. 2013;102(9):3058–3069.

Ishizaki K, Kinbara S, Miyazawa N, et al. The biochemical studies on phalloidin-induced cholestasis in rats. *Toxicol Lett*. 1997;90(1):29–34.

Ito Y, Abril ER, Bethea NW, McCuskey RS. Inhibition of matrix metalloproteinases minimizes hepatic microvascular injury in response to acetaminophen in mice. *Toxicol Sci*. 2005;83:190–196.

Ito Y, Abril ER, Bethea NW, et al. Mechanisms and pathophysiological implications of sinusoidal endothelial cell gap formation following treatment with galactosamine/endotoxin in mice. *Am J Physiol Gastrointest Liver Physiol*. 2006;291:G211–G218.

Jaeger A, Jehl F, Flesch F, Sauder P, Kopferschmitt J. Kinetics of amatoxins in human poisoning: therapeutic implications. *J Toxicol Clin Toxicol*. 1993;31:63–80.

Jaeschke H. Mechanisms of liver injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. *Am J Physiol Gastrointest Liver Physiol*. 2006;290:G1083–G1088.

Jaeschke H, Bajt ML. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol Sci*. 2006;89:31–41.

Jaeschke H, Bajt ML. Mechanisms of Acetaminophen Hepatotoxicity. In: McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol 9. Oxford: Academic Press; 2010:421–436.

Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci*. 2002; 65:166–176.

Jaeschke H, Gujral JS, Bajt ML. Apoptosis and necrosis in liver disease. *Liver Int*. 2004;24:85–89.

Jaeschke H, Hasegawa T. Role of neutrophils in acute inflammatory liver injury. *Liver Int*. 2006;26:912–919.

Jaeschke H, Kleinwaechter C, Wendel A. The role of acrolein in allyl alcohol-induced lipid peroxidation and liver cell damage in mice. *Biochem Pharmacol*. 1987;36:51–57.

Jaeschke H, Kleinwaechter C, Wendel A. NADH-dependent reductive stress and ferritin-bound iron in allyl alcohol-induced lipid peroxidation in vivo: the protective effect of vitamin E. *Chem Biol Interact*. 1992;81:57–68.

Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. *Drug Metab Rev*. 2012a;44:88–106.

Jaeschke H, Williams CD, Ramachandran A. Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. *Liver Int*. 2012b;32:8–20.

Jaeschke H, Xie Y, McGill MR. Acetaminophen-induced liver injury: from animal models to humans. *J Clin Transl Hepatol*. 2014;2(3):153–161.

Jansen PL, Groen AK. Mechanisms of bile secretion. In: Boyer TD, Wright TL, Manns M, eds. *Zakim and Boyer's Hepatology*. 5th ed. Philadelphia, PA: Saunders-Elsevier; 2006:67–85.

Jansen PL, Sturm E. Genetic cholestasis, causes and consequences for hepatobiliary transport. *Liver Int*. 2003;23:315–322.

Jean PA, Roth RA. Naphthylisothiocyanate disposition in bile and its relationship to liver glutathione and toxicity. *Biochem Pharmacol*. 1995;50(9):1469–1474.

Jean PA, Bailie MB, Roth RA. 1-naphthylisothiocyanate-induced elevation of biliary glutathione. *Biochem Pharmacol*. 1995;49(2):197–202.

Jiao J, Sastre D, Fiel MI, Lee UE, et al. Dendritic cell regulation of carbon tetrachloride-induced murine liver fibrosis regression. *Hepatology*. 2012;55(1), 244–255.

Jochimsen EM, Carmichael WW, An JS, et al. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N Engl J Med*. 1998;338:873–878.

Jollow DJ, Mitchell JR, Potter WZ, et al. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding in vivo. *J Pharmacol Exp Ther*. 1973;187:195–202.

Jones DP, Lemasters JJ, Han D, Boelsterli UA, Kaplowitz N. Mechanisms of pathogenesis in drug hepatotoxicity putting the stress on mitochondria. *Mol Interv*. 2010;10:98–111.

Joshi N, Kopec AK, Cline-Fedewa H, Luyendyk JP. Lymphocytes contribute to biliary injury and fibrosis in experimental xenobiotic-induced cholestasis. *Toxicology*. 2017;377:73–80.

Joshi N, Kopec AK, Ray JL, et al. Fibrin deposition following bile duct injury limits fibrosis through an alphaMbeta2-dependent mechanism. *Blood*. 2016;127(22):2751–2762.

Ju C, Reilly TP, Bourdi M, et al. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol*. 2002; 15:1504–1513.

Jungermann K, Katz N. Functional specialization of different hepatocyte populations. *Physiol Rev*. 1989;69:708–764.

Jungermann K, Kietzmann T. Oxygen: modulator of metabolic zonation and disease of the liver. *Hepatology*. 2000;31:255–260.

Kaminskas LM, Pyke SM, Burcham PC. Strong protein adduct trapping accompanies abolition of acrolein-mediated hepatotoxicity by hydralazine in mice. *J Pharmacol Exp Ther*. 2004;310:1003–1010.

Kandiah PA, Kumar G. Hepatic Encephalopathy-the old and the new. *Crit Care Clin*. 2016;32(3):311–329.

Kannarkat GT, Tuma DJ, Tuma PL. Microtubules are more stable and more highly acetylated in ethanol-treated hepatic cells. *J Hepatol*. 2006;44:963–970.

Kaplan MM. Serum alkaline phosphatase—another piece is added to the puzzle. *Hepatology*. 1986;6(3):526–528.

Kaplowitz N. Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov*. 2005;4:489–499.

Kaplowitz N, DeLeve LD. *Drug-induced Liver Disease*. New York: Marcel Dekker, Inc.; 2002.

Kargar M, Mansouri A, Hadjibabaie M, et al. Anti-tuberculosis drugs adverse reactions: a review of the Iranian literature. *Expert Opin Drug Saf*. 2014;13(7):875–891.

Karlson-Stiber C, Persson H. Cytotoxic fungi–an overview. *Toxicon*. 2003;42:339–349.

Katarey D, Verma S. Drug-induced liver injury. *Clin Med (Lond)*. 2016;16(suppl 6):s104–s109.

Kawaji A, Yamauchi K, Fujii S, et al. Effects of mushroom toxins on glycogenolysis; comparison of toxicity of phalloidin, alpha-amanitin and DL-propargylglycine in isolated rat hepatocytes. *J Pharmacobiodyn*. 1992;15(3):107–112.

Kerr R, Newsome P, Germain L, et al. Effects of acute liver injury on blood coagulation. *J Thromb Haemost*. 2003; 1(4):754–759.

Kew MC. Aflatoxins as a cause of hepatocellular carcinoma. *J Gastrointestin Liver Dis*. 2013; 22(3):305–310.

Kirk GD, Turner PC, Gong Y, et al. Hepatocellular carcinoma and polymorphisms in carcinogen-metabolizing and DNA repair enzymes in a population with aflatoxin exposure and hepatitis B virus endemicity. *Cancer Epidemiol Biomarkers Prev*. 2005;14(2):373–379.

Klaassen CD. Biliary excretion of metals. *Drug Metab Rev*. 1976;5:165–193.

Klaassen CD, Aleksunes LM. Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol Rev*. 2010;62(1):1–96.

Klaassen CD, Watkins JB. Mechanisms of bile formation, hepatic uptake, and biliary excretion. *Pharmacol Rev*. 1984;36:1–67.

Knight TR, Fariss MW, Farhood A, Jaeschke H. Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. *Toxicol Sci*. 2003;76(1):229–236.

Knockaert L, Berson A, Ribault C, et al. Carbon tetrachloride-mediated lipid peroxidation induces early mitochondrial alterations in mouse liver. *Lab Invest*. 2012; 92:396–410.

Knolle PA, Wohlleber D. Immunological functions of liver sinusoidal endothelial cells. *Cell Mol Immunol*. 2016;13(3):347–353.

Ko CB, Kim SJ, Park C, et al. Benzo(a)pyrene-induced apoptotic death of mouse hepatoma Hepa1c1c7 cells via activation of intrinsic caspase cascade and mitochondrial dysfunction. *Toxicology*. 2004;199: 35–46.

Kobayashi M, Higuchi S, Mizuno K, et al. Interleukin-17 is involved in alpha-naphthylisothiocyanate-induced liver injury in mice. *Toxicology*. 2010;275(1–3):50–57.

Kobori T, Harada S, Nakamoto K, Tokuyama S. Functional alterations of intestinal P-glycoprotein under diabetic conditions. *Biol Pharm Bull*. 2013;36(9):1381–1390.

Köck K, Ferslew BC, Netterberg I, et al. Risk factors for development of cholestatic drug-induced liver injury: inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. *Drug Metab Dispos*. 2014;42(4):665–674.

Kodali P, Wu P, Lahiji PA, Brown EJ, Maher JJ. ANIT toxicity toward mouse hepatocytes in vivo is mediated primarily by neutrophils via CD18. *Am J Physiol Gastrointest Liver Physiol*. 2006;291(2):G355–G363.

Koepsell H. Role of organic cation transporters in drug-drug interaction. *Expert Opin Drug Metab Toxicol*. 2015;11(10):1619–1633.

Koepsell H, Endou H. The SLC22 drug transporter family. *Pflugers Arch*. 2004;447(5):666–676.

Kon K, Kim JS, Jaeschke H, Lemasters JJ. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. *Hepatology*. 2004;40:1170–1179.

Koo SH. Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis. *Clin Mol Hepatol*. 2013 Sep;19(3):210–215.

Kopec AK, Joshi N, Cline-Fedewa H, et al. Fibrin(ogen) drives repair after acetaminophen-induced liver injury via leukocyte alphaMbeta2 integrin-dependent upregulation of Mmp12. *J Hepatol*. 2017;66(4):787–797.

Korth C. Drug-induced hepatotoxicity of select herbal therapies. *J Pharm Pract*. 2014;27(6):567–572.

Krenkel O, Mossanen JC, Tacke F. Immune mechanisms in acetaminophen-induced acute liver failure. *Hepatobiliary Surg Nutr*. 2014;3(6):331–343.

Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol*. 2017;17(5):306–321.

Kubes P, Mehal WZ. Sterile inflammation in the liver. *Gastroenterology*. 2012;143(5):1158–1172.

Kuffner EK, Temple AR, Cooper KM, Baggish JS, Parenti DL. Retrospective analysis of transient elevations in alanine aminotransferase during long-term treatment with acetaminophen in osteoarthritis clinical trials. *Curr Med Res Opin*. 2006;22:2137–2148.

Kuo I, Akpa BS. Validity of the lipid sink as a mechanism for the reversal of local anesthetic systemic toxicity: a physiologically based pharmacokinetic model study. *Anesthesiology*. 2013;118(6):1350–1361.

Kyriakou LG, Tzirogiannis KN, Demonakou MD, Kourentzi KT, Mykoniatis MG, Panoutsopoulos GI. Gadolinium chloride pretreatment ameliorates acute cadmium-induced hepatotoxicity. *Toxicol Ind Health*. 2013;29:624–632.

Labib R, Turkall R, Abdel-Rahman MS. Endotoxin potentiates the hepatotoxicity of cocaine in male mice. *J Toxicol Environ Health A*. 2002;65(14):977–993.

Labine MA, Minuk GY. Cyanobacterial toxins and liver disease. *Can J Physiol Pharmacol*. 2009;87(10):773–788.

Lakehal F, Wendum D, Barbu V, et al. Phase I and phase II drug-metabolizing enzymes are expressed and heterogeneously distributed in the biliary epithelium. *Hepatology*. 1999;30:1498–1506.

Lamé MW, Jones AD, Wilson DW, Dunston SK, Segall HJ. Protein targets of monocrotaline pyrrole in pulmonary artery endothelial cells. *J Biol Chem*. 2000;275(37):29091–29099.

Lankoff A, Wojcik A, Fessard V, Meriluoto J. Nodularin-induced genotoxicity following oxidative DNA damage and aneuploidy in HepG2 cells. *Toxicol Lett*. 2006;164(3):239–248.

Lappin PB, Ross KL, King LE, Fraker PJ, Roth RA. The response of pulmonary vascular endothelial cells to monocrotaline pyrrole: cell proliferation and DNA synthesis in vitro and in vivo. *Toxicol Appl Pharmacol*. 1998;150(1):37–48.

Larosche I, Lettéron P, Fromenty B, et al. Tamoxifen inhibits topoisomerases, depletes mitochondrial DNA, and triggers steatosis in mouse liver. *J Pharmacol Exp Ther*. 2007;321:526–535.

Laskin DL. Macrophages and inflammatory mediators in chemical toxicity: a battle of forces. *Chem Res Toxicol*. 2009;22:1376–1385.

Lee WM. Acetaminophen and the U.S. Acute Liver Failure Study Group: lowering the risks of hepatic failure. *Hepatology*. 2004;40(1):6–9.

Lee WM. Acute liver failure. *Semin Respir Crit Care Med*. 2012;33(1):36–45.

Lee WM. Drug-induced acute liver failure. *Clin Liver Dis*. 2013;17(4):575–586.

Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. *Best Pract Res Clin Gastroenterol*. 2011;25:195–206.

Leist M, Gantner F, Naumann H, et al. Tumor necrosis factor-induced apoptosis during the poisoning of mice with hepatotoxins. *Gastroenterology*. 1997;112:923–934.

Letschert K, Faulstich H, Keller D, Keppler D. Molecular characterization and inhibition of amanitin uptake into human hepatocytes. *Toxicol Sci*. 2006;91:140–149.

Lettéron P, Sutton A, Mansouri A, Fromenty B, Pessayre D. Inhibition of microsomal triglyceride transfer protein: another mechanism for drug-induced steatosis in mice. *Hepatology*. 2003;38:133–140.

Leu JI, George DL. Hepatic IGFBP1 is a prosurvival factor that binds to BAK, protects the liver from apoptosis, and antagonizes the proapoptotic actions of p53 at mitochondria *Genes Dev*. 2007; 21:3095–3109.

Lewis JH. The art and science of diagnosing and managing drug-induced liver injury in 2015 and beyond. *Clin Gastroenterol Hepatol*. 2015;13:2173–2189.

Li AP. A review of the common properties of drugs with idiosyncratic hepatotoxicity and the "multiple determinant hypothesis" for the manifestation of idiosyncratic drug toxicity. *Chem Biol Interact*. 2002;142:7–23.

Li S, Zhao Y, He X, Kim TH, et al. Relief of extrinsic pathway inhibition by the Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocyte apoptosis. *J Biol Chem*. 2002;277:26912–26920.

Li N, Xia Q, Ruan J, Fu PP, Lin G. Hepatotoxicity and tumorigenicity induced by metabolic activation of pyrrolizidine alkaloids in herbs. *Curr Drug Metab*. 2011;12(9):823–834.

Liaskou E, Wilson DV, Oo YH. Innate immune cells in liver inflammation. *Mediators Inflamm*. 2012;2012:949157.

Licata A. Adverse drug reactions and organ damage: the liver. *Eur J Intern Med*. 2016;28:9–16.

Lieber CS. Alcohol: its metabolism and interaction with nutrients. *Annu Rev Nutr*. 2000;20:395–430.

Limaye PB, Bhave VS, Palkar PS, et al.Upregulation of calpastatin in regenerating and developing rat liver: role in resistance against hepatotoxicity. *Hepatology*. 2006;44:379–388.

Lin MC, Li JJ, Wang EJ, Princler GL, Kauffman FC, Kung HF. Ethanol down-regulates the transcription of microsomal triglyceride transfer protein gene. *FASEB J*. 1997;11:1145–1152.

Lin YC, Li L, Makarova AV, Burgers PM, Stone MP, Lloyd RS. Molecular basis of aflatoxin-induced mutagenesis-role of the aflatoxin B1-formamidopyrimidine adduct. *Carcinogenesis*. 2014;35(7):1461–1468.

Lira M, Schteingart CD, Steinbach JH, et al. Sugar absorption by the biliary ductular epithelium of the rat: evidence for two transport systems. *Gastroenterology*. 1992;102:563–571.

Liu J, Liu Y, Michalska AE, Choo KH, Klaassen CD. Metallothionein plays less of a protective role in cadmium-metallothionein-induced nephrotoxicity than in cadmium chloride-induced hepatotoxicity. *J Pharmacol Exp Ther*. 1996;276(3):1216–1223.

Liu J, Sendelbach LE, Parkinson A, Klaassen CD. Endotoxin pretreatment protects against the hepatotoxicity of acetaminophen and carbon tetrachloride: role of cytochrome P450 suppression. *Toxicology*. 2000;147(3):167–176.

Liu Y, Chang CC, Marsh GM, Wu F. Population attributable risk of aflatoxin-related liver cancer: systematic review and meta-analysis. *Eur J Cancer*. 2012;48(14):2125–2136.

Liu Y, Liu J, Iszard MB, et al. Transgenic mice that overexpress metallothionein-I are protected from cadmium lethality and hepatotoxicity. *Toxicol Appl Pharmacol*. 1995;135:222–228.

Ljungman M, Zhang F, Chen F, Rainbow AJ, McKay BC. Inhibition of RNA polymerase II as a trigger for the p53 response. *Oncogen*e. 1999; 18:583–592.

Loranger A, Tuchweber B, Gicquaud C, St-Pierre S, Côté MG. Toxicity of peptides of *Amanita virosa* mushrooms in mice. *Fundam Appl Toxicol*. 1985;5(6 pt 1):1144–1152.

Loudianos G, Gitlin JD. Wilson's disease. *Semin Liver Dis*. 2000;20: 353–364.

Lu H, Choudhuri S, Ogura K, et al. Characterization of organic anion transporting polypeptide 1b2-null mice: essential role in hepatic uptake/toxicity of phalloidin and microcystin-LR. *Toxicol Sci*. 2008;103(1):35–45.

Lu J, Jones AD, Harkema JR, Roth RA, Ganey PE. Amiodarone exposure during modest inflammation induces idiosyncrasy-like liver injury in rats: role of tumor necrosis factor-alpha. *Toxicol Sci*. 2012;125(1):126–133.

Lu J, Miyakawa K, Roth RA, Ganey PE. Tumor necrosis factor-alpha potentiates the cytotoxicity of amiodarone in Hepa1c1c7 cells: roles of caspase activation and oxidative stress. *Toxicol Sci*. 2013;131(1):164–178.

Lu L, Shi J, Li Q, Peng X, et al. Effects of liver diseases on drug-metabolizing enzymes: implications for drug fate alterations and nano-therapeutic openings. *Curr Med Chem*. 2014;21(22):2522–2541.

Lubos E, Loscalzo J, Handy DE. Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal*. 2011;15(7):1957–1997.

Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. *Gastroenterology*. 2014;147(4):765–783.

Lürling M, van Oosterhout F, Faassen E. Eutrophication and warming boost Cyanobacterial biomass and microcystins. *Toxins (Basel)*. 2017;9(2): pii: E64.

Luyendyk JP, Shores KC, Ganey PE, Roth RA. Bacterial lipopolysaccharide exposure alters aflatoxin B(1) hepatotoxicity: benchmark dose analysis for markers of liver injury. *Toxicol Sci*. 2002;68(1):220–225.

Maddox JF, Amuzie CJ, Li M, et al. Bacterial- and viral-induced inflammation increases sensitivity to acetaminophen hepatotoxicity. *J Toxicol Environ Health A*. 2010;73(1):58–73.

Maes M, Vinken M. Connexin-based signaling and drug-induced hepatotoxicity. *J Clin Transl Res*. 2017;3(S1): 189–198.

Magdalan J, Piotrowska A, Gomulkiewicz A, et al. Benzylpenicyllin and acetylcysteine protection from alpha-amanitin-induced apoptosis in human hepatocyte cultures. *Exp Toxicol Pathol*. 2011;63:311–315.

Maillo C, Martín J, Sebastián D, et al. Circadian- and UPR-dependent control of CPEB4 mediates a translational response to counteract hepatic steatosis under ER stress. *Nat Cell Biol*. 2017;19(2):94–105.

Maiuri AR, Breier AB, Gora LF, et al. Cytotoxic synergy between cytokines and NSAIDs associated with idiosyncratic hepatotoxicity is driven by mitogen-activated protein kinases. *Toxicol Sci*. 2015;146(2):265–280.

Maiuri AR, Wassink B, Turkus JD, et al. Synergistic cytotoxicity from drugs and cytokines in vitro as an approach to classify drugs according to their potential to cause idiosyncratic hepatotoxicity: a proof-of-concept study. *J Pharmacol Exp Ther*. 2017;362(3):459–473.

Makdisi WJ, Cherian R, Vanveldhuizen PJ, et al. Fatal peliosis of the liver and spleen in a patient with agnogenic myeloid metaplasia treated with danazol. *Am J Gastroenterol*. 1995;90(2):317–318.

Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology*. 2006;43(2 suppl 1):S31–S44.

Mangipudy RS, Chanda S, Mehendale HM. Tissue repair response as a function of dose in thioacetamide hepatotoxicity. *Environ Health Perspect*. 1995;103:260–267.

Mangipudy RS, Rao PS, Mehendale HM. Effect of an antimitotic agent colchicine on thioacetamide hepatotoxicity. *Environ Health Perspect*. 1996;104:744–749.

Manno M, Rezzadore M, Grossi M, Sbrana C. Potentiation of occupational carbon tetrachloride toxicity by ethanol abuse. *Hum Exp Toxicol*. 1996;15(4):294–300.

Maricic I, Sheng H, Marrero I, et al. Inhibition of type I natural killer T cells by retinoids or following sulfatide-mediated activation of type II natural killer T cells attenuates alcoholic liver disease in mice. *Hepatology*. 2015;61(4):1357–1369.

Markova SM, De Marco T, Bendjilali N, et al. Association of CYP2C9*2 with bosentan-induced liver injury. *Clin Pharmacol Ther*. 2013;94(6):678–686.

Marques SC, Ikediobi ON. The clinical application of UGT1A1 pharmacogenetic testing: gene-environment interactions. *Hum Genomics*. 2010;4(4):238–249.

Marra F. Chemokines in liver inflammation and fibrosis. *Front Biosci*. 2002;7:d1899–d1914.

Martin FP, Dumas ME, Wang Y, et al. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. *Mol Syst Biol*. 2007;3:112.

Martin-Murphy BV, Kominsky DJ, et al. Increased susceptibility of natural killer T-cell-deficient mice to acetaminophen-induced liver injury. *Hepatology*. 2013;57(4):1575–1584.

Masson MJ, Carpenter LD, Graf ML, Pohl LR. Pathogenic role of natural killer T and natural killer cells in acetaminophen-induced liver injury in mice is dependent on the presence of dimethyl sulfoxide. *Hepatology*. 2008;48(3):889–897.

Mathews S, Feng D, Maricic I, Ju C, Kumar V, Gao B. Invariant natural killer T cells contribute to chronic-plus-binge ethanol-mediated liver

injury by promoting hepatic neutrophil infiltration. *Cell Mol Immunol*. 2016;13(2):206–216.

Mathews S, Xu M, Wang H, Bertola A, Gao B. Animals models of gastrointestinal and liver diseases. Animal models of alcohol-induced liver disease: pathophysiology, translational relevance, and challenges. *Am J Physiol Gastrointest Liver Physiol*. 2014;306(10):G819–G823.

Matović V, Buha A, Đukić-Ćosić D, Bulat Z. Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. *Food Chem Toxicol*. 2015;78:130–140.

McClain C, Barve S, Joshi-Barve S, et al. Dysregulated cytokine metabolism, altered hepatic methionine metabolism and proteasome dysfunction in alcoholic liver disease. *Alcohol Clin Exp Res*. 2005;29(11 suppl):180S–188SS.

McClain CJ, Price S, Barve S, Devalarja R, Shedlofsky S. Acetaminophen hepatotoxicity: an update. *Curr Gastroenterol Rep*. 1999;1(1):42–49.

McCuskey RS. Sinusoidal endothelial cells as an early target for hepatic toxicants. *Clin Hemorheol Microcirc*. 2006a;34:5–10.

McCuskey RS. The hepatic microvascular system in health and its response to toxicants. *Anat Rec (Hoboken)*. 2008 Jun;291(6):661–671.

McCuskey RS. Anatomy of the liver. In: Boyer TD, Wright TL, Manns M, eds. *Zakim and Boyer's Hepatology*. 5th ed. Philadelphia, PA: Saunders-Elsevier; 2006b:3–21.

McDonald B, Kubes P. Neutrophils and intravascular immunity in the liver during infection and sterile inflammation. *Toxicol Pathol*. 2012;40(2):157–165.

McDonald B, Kubes P. Innate immune cell trafficking and function during sterile inflammation of the liver. *Gastroenterology*. 2016;151(6):1087–1095.

McGill MR, Jaeschke H. Metabolism and disposition of acetaminophen: recent advances in relation to hepatotoxicity and diagnosis. *Pharm Res*. 2013;30(9):2174–2187.

McGill MR, Lebofsky M, Norris HR, et al. Plasma and liver acetaminophen-protein adduct levels in mice after acetaminophen treatment: dose-response, mechanisms, and clinical implications. *Toxicol Appl Pharmacol*. 2013;269(3):240–249.

McGill MR, Sharpe MR, Williams CD, et al. Mechanisms of acetaminophen hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *J Clin Invest*. 2012a;122:1574–1583.

McGill MR, Staggs VS, Sharpe MR, Lee WM, Jaeschke H, Acute Liver Failure Study Group. Serum mitochondrial biomarkers and damage-associated molecular patterns are higher in acetaminophen overdose patients with poor outcome. *Hepatology*. 2014;60(4):1336–1345.

McGill MR, Williams CD, Xie Y, Ramachandran A, Jaeschke H. Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. *Toxicol Appl Pharmacol*. 2012b;264:387–394.

McGill MR, Yan HM, Ramachandran A, et al. HepaRG cells: a human model to study mechanisms of acetaminophen hepatotoxicity. *Hepatology*. 2011;53:974–982.

McKillop IH, Moran DM, Jin X, et al. Molecular pathogenesis of hepatocellular carcinoma. *J Surg Res*. 2006;136:125–135.

McLean MR, Rees KR. Hyperplasia of bile-ducts induced by alpha-naphthyl-iso-thiocyanate: experimental biliary cirrhosis free from biliary obstruction. *J Pathol Bacteriol*. 1958;76(1):175–188.

Mehendale HM. Tissue repair: an important determinant of final outcome of toxicant-induced injury. *Toxicol Pathol*. 2005;33:41–51.

Mehendale HM. Once initiated, how does toxic tissue injury expand? *Trends Pharmacol Sci*. 2012;33(4):200–206.

Mehendale HM. Role of tissue repair and death proteins in liver injury. In: Kaplowitz N, DeLeve LD, eds. *Drug-induced Liver Disease*. 3rd ed. London: Academic Press; 2013:195–214.

Mehendale HM, Limaye PB. Calpain: a death protein that mediates progression of liver injury. *Trends Pharmacol Sci*. 2005;26:232–236.

Meier P, Seitz HK. Age, alcohol metabolism and liver disease. *Curr Opin Clin Nutr Metab Care*. 2008;11(1):21–26.

Meili N, Christen V, Fent K. Nodularin induces tumor necrosis factor-alpha and mitogen-activated protein kinases (MAPK) and leads to induction of endoplasmic reticulum stress. *Toxicol Appl Pharmacol*. 2016;300:25–33.

Merrell MD, Cherrington NJ. Drug metabolism alterations in nonalcoholic fatty liver disease. *Drug Metab Rev*. 2011;43(3):317–334.

Miao L, St Clair DK. Regulation of superoxide dismutase genes: implications in disease. *Free Radic Biol Med*. 2009;47(4):344–356.

Michalopoulos GK. Liver regeneration. *J Cell Physiol*. 2007;213:286–300.

Michalopoulos GK. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am J Pathol*. 2010;176(1):2–13.

Michalopoulos GK. Advances in liver regeneration. *Expert Rev Gastroenterol Hepatol*. 2014;8(8):897–907.

Michalopoulos GK. Hepatostat: liver regeneration and normal liver tissue maintenance. *Hepatology*. 2017;65(4):1384–1392.

Michalopoulos GK, DeFrances MC. Liver regeneration. *Science*. 1997;276:60–66.

Miller AM, Horiguchi N, Jeong WI, Radaeva S, Gao B. Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines. *Alcohol Clin Exp Res*. 2011;35:787–793.

Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther*. 1973;187:211–217.

Miyakawa K, Joshi N, Sullivan BP, et al. Platelets and protease-activated receptor-4 contribute to acetaminophen-induced liver injury in mice. *Blood*. 2015;126(15):1835–1843.

Montesano R, Hainaut P, Wild CP. Hepatocellular carcinoma: from gene to public health. *J Natl Cancer Inst*. 1997;89(24):1844–1851.

Mossanen JC, Krenkel O, Ergen C, et al. Chemokine (C-C motif) receptor 2-positive monocytes aggravate the early phase of acetaminophen-induced acute liver injury. *Hepatology*. 2016;64(5):1667–1682.

Moudgil V, Redhu D, Dhanda S, Singh J. A review of molecular mechanisms in the development of hepatocellular carcinoma by aflatoxin and hepatitis B and C viruses. *J Environ Pathol Toxicol Oncol*. 2013;32(2):165–175.

Nagai H, Matsumaru K, Feng G, Kaplowitz N. Reduced glutathione depletion causes necrosis and sensitization to tumor necrosis factor-alpha-induced apoptosis in cultured mouse hepatocytes. *Hepatology*. 2002;36:55–64.

Nagata S, Nagase H, Kawane K, Mukae N, Fukuyama H. Degradation of chromosomal DNA during apoptosis. *Cell Death Differ*. 2003;10:108–116.

Napirei M, Basnakian AG, Apostolov EO, Mannherz HG. Deoxyribonuclease 1 aggravates acetaminophen-induced liver necrosis in male CD-1 mice. *Hepatology.* 2006;43(2):297–305.

Nelson SD. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. *Semin Liver Dis*. 1990;10:267–278.

Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology*. 2010;52:774–788.

Nguyen LN, Furuya MH, Wolfraim LA, et al. Transforming growth factor-beta differentially regulates oval cell and hepatocyte proliferation. *Hepatology*. 2007;45:31–41.

Ni HM, Bockus A, Boggess N, Jaeschke H, Ding WX. Activation of autophagy protects against acetaminophen hepatotoxicity in mice. *Hepatology*. 2012;55:222–231.

Ni HM, McGill MR, Chao X, et al. Removal of acetaminophen protein adducts by autophagy protects against acetaminophen-induced liver injury in mice. *J Hepatol*. 2016;65(2):354–362.

Ni HM, Williams JA, Jaeschke H, Ding WX. Zonated induction of autophagy and mitochondrial spheroids limits acetaminophen-induced necrosis in the liver. *Redox Biol*. 2013;1:427–432.

Nicoletti P, Aithal GP, Bjornsson ES, et al. Association of liver injury from specific drugs, or groups of drugs, with polymorphisms in HLA and other genes in a genome-wide association study. *Gastroenterology*. 2017;152(5):1078–1089.

Niemelä O, Parkkila S, Juvonen RO, Viitala K, Gelboin HV, Pasanen M. Cytochromes P450 2A6, 2E1, and 3A and production of protein–aldehyde adducts in the liver of patients with alcoholic and non-alcoholic liver diseases. *J Hepatol*. 2000;33:893–901.

Nigam SK, Bush KT, Martovetsky G, et al. The organic anion transporter (OAT) family: a systems biology perspective. *Physiol Rev*. 2015;95(1):83–123.

Obaidat A, Roth M, Hagenbuch B. The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. *Annu Rev Pharmacol Toxicol*. 2012;52:135–151.

Ogasawara J, Watanabe-Fukunaga R, et al. Lethal effect of the anti-Fas antibody in mice. *Nature*. 1993;364:806–809.

Ortega-Alonso A, Stephens C, Lucena MI, Andrade RJ. Case characterization, clinical features and risk factors in drug-induced liver injury. *Int J Mol Sci*. 2016;17(5):pii: E714.

Osburn WO, Kensler TW. Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. *Mutat Res*. 2008; 659(1–2):31–39.

Padda MS, Sanchez M, Akhtar AJ, Boyer JL. Drug-induced cholestasis. *Hepatology*. 2011;53:1377–1387.

Palatini P, De Martin S. Pharmacokinetic drug interactions in liver disease: an update. *World J Gastroenterol*. 2016;22(3):1260–1278.

Palmeira CM, Rolo AP. Mitochondrially-mediated toxicity of bile acids. *Toxicology*. 2004;203:1–15.

Park BK, Laverty H, Srivastava A, Antoine DJ, Naisbitt D, Williams DP. Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity. *Chem Biol Interact*. 2011;192:30–36.

Pauli-Magnus C, Meier PJ. Hepatobiliary transporters and drug-induced cholestasis. *Hepatology*. 2006;44:778–787.

Patil M, Sheth KA, Krishnamurthy AC, Devarbhavi H. A review and current perspective on Wilson disease. *J Clin Exp Hepatol*. 2013;3(4):321–336.

Pedersen JM, Matsson P, Bergström CA, et al. Early identification of clinically relevant drug interactions with the human bile salt export pump (BSEP/ABCB11). *Toxicol Sci*. 2013;136(2):328–343.

Pessayre D, Fromenty B, Berson A, et al. Central role of mitochondria in drug-induced liver injury. *Drug Metab Rev*. 2012;44:34–87.

Pessayre D, Mansouri A, Fromenty B. Nonalcoholic steatosis and steatohepatitis. V. Mitochondrial dysfunction in steatohepatitis. *Am J Physiol Gastrointest Liver Physiol*. 2002;282:G193–G199.

Pietrangelo A. Iron and the liver. *Liver Int*. 2016;36(suppl 1):116–123.

Phillips MJ, Poucell S, Oda M. Mechanisms of cholestasis. *Lab Invest*. 1986;54:593–608.

Pinzani M. PDGF and signal transduction in hepatic stellate cells. *Front Biosci*. 2002;7:d1720–d1726.

Plaa GL, Priestly BG. Intrahepatic cholestasis induced by drugs and chemicals. *Pharmacol Rev*. 1976;28(3):207–273.

Pompella A, Romani A, Fulceri R, Benedetti A, Comporti M. 4-Hydroxynonenal and other lipid peroxidation products are formed in mouse liver following intoxication with allyl alcohol. *Biochim Biophys Acta*. 1988;961(3):293–298.

Pond SM, Olson KR, Woo OF, et al. Amatoxin poisoning in northern California, 1982–1983. *West J Med*. 1986;145:204–209.

Prescott LF, Park J, Ballantyne A, Adriaenssens P, Proudfoot AT. Treatment of paracetamol (acetaminophen) poisoning with N-acetylcysteine. *Lancet*. 1977;2(8035):432–434.

Probst C, Njapau H, Cotty PJ. Outbreak of an acute aflatoxicosis in Kenya in 2004: identification of the causal agent. *Appl Environ Microbiol*. 2007;73(8):2762–2764.

Puoti M, Nasta P, Gatti F, et al. HIV-related liver disease: ARV drugs, coinfection, and other risk factors. *J Int Assoc Physicians AIDS Care (Chic)*. 2009;8(1):30–42.

Qian GS, Ross RK, Yu MC, et al. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol Biomarkers Prev*. 1994;3(1):3–10.

Ramachandran A, Lebofsky M, Baines CP, Lemasters JJ, Jaeschke H. Cyclophilin D deficiency protects against acetaminophen-induced oxidant stress and liver injury. *Free Radic Res*. 2011;45:156–164.

Ramachandran A, McGill MR, Xie Y, Ni HM, Ding WX, Jaeschke H. Receptor interacting protein kinase 3 is a critical early mediator of acetaminophen-induced hepatocyte necrosis in mice. *Hepatology*. 2013;58(6):2099–2108.

Ramaiah SK, Jaeschke H. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. *Toxicol Pathol*. 2007;35:757–766.

Ramm GA, Ruddell RG. Hepatotoxicity of iron overload: mechanisms of iron-induced hepatic fibrogenesis. *Semin Liver Dis*. 2005;25:433–449.

Rani A, Kumar A, Lal A, Pant M. Cellular mechanisms of cadmium-induced toxicity: a review. *Int J Environ Health Res*. 2014;24(4):378–399.

Raschi E, De Ponti F. Drug- and herb-induced liver injury: progress, current challenges and emerging signals of post-marketing risk. *World J Hepatol*. 2015;7(13):1761–1771.

Recknagel RO, Glende EA Jr, Dolak JA, Waller RL. Mechanisms of carbon tetrachloride toxicity. *Pharmacol Ther*. 1989;43:139–154.

Reid MJ, Lamé MW, Morin D, Wilson DW, Segall HJ. Involvement of cytochrome P450 3A in the metabolism and covalent binding of 14C-monocrotaline in rat liver microsomes. *J Biochem Mol Toxicol*. 1998;12(3):157–166.

Renton KW. Cytochrome P450 regulation and drug biotransformation during inflammation and infection. *Curr Drug Metab*. 2004;5(3):235–243.

Rieben WK Jr, Coulombe RA Jr. DNA cross-linking by dehydromonocrotaline lacks apparent base sequence preference. *Toxicol Sci*. 2004;82(2):497–503.

Rikans LE. The oxidation of acrolein by rat liver aldehyde dehydrogenases. Relation to allyl alcohol hepatotoxicity. *Drug Metab Dispos*. 1987;15:356–362.

Rikans LE. Influence of aging on chemically induced hepatotoxicity: role of age-related changes in metabolism. *Drug Metab Rev*. 1989;20(1):87–110.

Rikans LE, Moore DR. Effect of age and sex on allyl alcohol hepatotoxicity in rats: role of liver alcohol and aldehyde dehydrogenase activities. *J Pharmacol Exp Ther*. 1987;243:20–26.

Roberts DW, Bucci TJ, Benson RW, et al. Immunohistochemical localization and quantification of the 3-(cystein-S-yl)-acetaminophen protein adduct in acetaminophen hepatotoxicity. *Am J Pathol*. 1991;138:359–371.

Robinson NA, Matson CF, Pace JG. Association of microcystin-LR and its biotransformation product with a hepatic-cytosolic protein. *J Biochem Toxicol*. 1991; 6:171–180.

Rockey DC. Vascular mediators in the injured liver. *Hepatology*. 2003;37:4–12.

Rockey DC, Friedman SL. Hepatic fibrosis and cirrhosis. In: Boyer TD, Wright TL, Manns M, eds. *Zakim and Boyer's Hepatology*. 5th ed. Philadelphia, PA: Saunders-Elsevier; 2006:87–109.

Ross RK, Yuan JM, Yu MC, et al. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*. 1992;339(8799):943–946.

Roth RA, Dahm LJ. Neutrophil- and glutathione-mediated hepatotoxicity of alpha-naphthylisothiocyanate. *Drug Metab Rev*. 1997;29(1–2):153–165.

Roth AD, Lee MY. Idiosyncratic Drug-Induced Liver Injury (IDILI): potential mechanisms and predictive assays. *Biomed Res Int*. 2017;2017:9176937.

Roth M, Obaidat A, Hagenbuch B. OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br J Pharmacol*. 2012;165:1260–1287.

Roth RA, Ganey PE. Hepatic toxicology. In: McQueen CA, ed. *Comprehensive Toxicology*. 2nd ed. Vol 9. Oxford: Academic Press; 2010a:1–638.

Roth RA, Ganey PE. Intrinsic versus idiosyncratic drug-induced hepatotoxicity—two villains or one? *J Pharmacol Exp Ther*. 2010b;332(3):692–697.

Roth RA, Ganey PE. Animal models of idiosyncratic drug-induced liver injury—current status. *Crit Rev Toxicol*. 2011;41(9):723–739.

Roth RA, Ganey PE. Role of inflammation in drug-induced liver injury. In: Kaplowitz N, DeLeve LD, eds. *Drug-induced Liver Disease*. 3rd ed. London: Academic Press; 2013:157–174.

Roth RA, Maiuri AR, Ganey PE. Idiosyncratic Drug-Induced Liver Injury: Is Drug-Cytokine Interaction the Linchpin? *J Pharmacol Exp Ther*. 2017;360(2):461–470.

Rumack BH, Bateman DN. Acetaminophen and acetylcysteine dose and duration: past, present and future. *Clin Toxicol (Phila)*. 2012;50(2):91–98.

Rumack B, Heard K, Green J, et al. Effect of therapeutic doses of acetaminophen (up to 4 g/day) on serum alanine aminotransferase levels in subjects consuming ethanol: systematic review and meta-analysis of randomized controlled trials. *Pharmacotherapy*. 2012;32(9):784–791.

Runnegar MT, Berndt N, Kaplowitz N. Microcystin uptake and inhibition of protein phosphatases: effects of chemoprotectants and self-inhibition in relation to known hepatic transporters. *Toxicol Appl Pharmacol*. 1995;134:264–272.

Runnegar MT, Kong S, Berndt N. Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins. *Am J Physiol*. 1993 Aug;265(2 pt 1):G224–G230.

Runnegar MT, Wei X, Hamm-Alvarez SF. Increased protein phosphorylation of cytoplasmic dynein results in impaired motor function. *Biochem J*. 1999;342:1–6.

Saberi B, Ybanez MD, Johnson HS, et al. Protein kinase C (PKC) participates in acetaminophen hepatotoxicity through c-jun-N-terminal kinase (JNK)-dependent and -independent signaling pathways. *Hepatology*. 2014;59(4):1543–1554.

Saelens X, Festjens N, Vande Walle L, et al. Toxic proteins released from mitochondria in cell death. *Oncogene*. 2004;23:2861–2874.

Saghir SA, Khan SA, McCoy AT. Ontogeny of mammalian metabolizing enzymes in humans and animals used in toxicological studies. *Crit Rev Toxicol*. 2012;42(5):323–357.

Saito C, Lemasters JJ, Jaeschke H. c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. *Toxicol Appl Pharmacol*. 2010a;246(1–2):8–17.

Saito C, Zwingmann C, Jaeschke H. Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and N-acetylcysteine. *Hepatology*. 2010b;51:246–254.

Saito T, Misawa K, Kawata S. 1. Fatty liver and non-alcoholic steatohepatitis. *Intern Med*. 2007;46:101–103.

Satarug S. Long-term exposure to cadmium in food and cigarette smoke, liver effects and hepatocellular carcinoma. *Curr Drug Metab*. 2012;13(3):257–271.

Sato C, Lieber CS. Mechanism of the preventive effect of ethanol on acetaminophen-induced hepatoxicity. *J Pharmacol Exp Ther*. 1981;218:811–815.

Sato C, Matsuda Y, Lieber CS. Increased hepatotoxicity of acetaminophen after chronic ethanol consumption in the rat. *Gastroenterology*. 1981;80:140–148.

Scheffner D, Konig S, Rauterberg-Ruland I, et al. Fatal liver failure in 16 children with valproate therapy. *Epilepsia*. 1988;29:530–542.

Schleicher RL, McCoy LF, Powers CD, Sternberg MR, Pfeiffer CM. Serum concentrations of an aflatoxin-albumin adduct in the National Health and Nutrition Examination Survey (NHANES) 1999–2000. *Clin Chim Acta*. 2013;423:46–50.

Schneider PD. Preoperative assessment of liver function. *Surg Clin North Am*. 2004;84(2):355–373.

Schon HT, Weiskirchen R. Immunomodulatory effects of transforming growth factor-β in the liver. *Hepatobiliary Surg Nutr*. 2014;3(6):386–406.

Schuetz EG, Strom S, Yasuda K, et al. Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. *J Biol Chem*. 2001;276:39411–39418.

Schulze-Bergkamen H, Schuchmann M, Fleischer B, Galle PR. The role of apoptosis versus oncotic necrosis in liver injury: facts or faith? *J Hepatol*. 2006;44:984–993.

Schüngel S, Buitrago-Molina LE, Nalapareddy P, et al. The strength of the Fas ligand signal determines whether hepatocytes act as type 1 or type 2 cells in murine livers. *Hepatology*. 2009;50:1558–1566.

Schwabe RF, Seki E, Brenner DA. Toll-like receptor signaling in the liver. *Gastroenterology*. 2006;130:1886–1900.

Seitz S, Boelsterli UA. Diclofenac acyl glucuronide, a major biliary metabolite, is directly involved in small intestinal injury in rats. *Gastroenterology*. 1998;115:1476–1482.

Seki E, Schnabl B. Role of innate immunity and the microbiota in liver fibrosis—crosstalk between the liver and gut. *J Physiol*. 2012;590: 447–458.

Sell S. Cellular origin of hepatocellular carcinomas. *Semin Cell Dev Biol*. 2002;13:419–424.

Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. *Semin Liver Dis*. 2001;21:105–113.

Sharma B, Singh S, Siddiqi NJ. Biomedical implications of heavy metals induced imbalances in redox systems. *Biomed Res Int*. 2014;2014:640754.

Shaw PJ, Beggs KM, Sparkenbaugh EM, Dugan CM, Ganey PE, Roth RA. Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. *Toxicol Sci*. 2009e;111(2):288–301.

Shaw PJ, Ditewig AC, Waring JF, et al. Coexposure of mice to trovafloxacin and lipopolysaccharide, a model of idiosyncratic hepatotoxicity, results in a unique gene expression profile and interferon gamma-dependent liver injury. *Toxicol Sci*. 2009d;107(1):270–280.

Shaw PJ, Fullerton AM, Scott MA, Ganey PE, Roth RA. The role of the hemostatic system in murine liver injury induced by coexposure to lipopolysaccharide and trovafloxacin, a drug with idiosyncratic liability. *Toxicol Appl Pharmacol*. 2009c;236(3):293–300.

Shaw PJ, Ganey PE, Roth RA. Trovafloxacin enhances the inflammatory response to a Gram-negative or a Gram-positive bacterial stimulus, resulting in neutrophil-dependent liver injury in mice. *J Pharmacol Exp Ther*. 2009a;330(1):72–78.

Shaw PJ, Ganey PE, Roth RA. Tumor necrosis factor alpha is a proximal mediator of synergistic hepatotoxicity from trovafloxacin/lipopolysaccharide coexposure. *J Pharmacol Exp Ther*. 2009b;328(1):62–68.

Shaw PJ, Ganey PE, Roth RA. Idiosyncratic drug-induced liver injury and the role of inflammatory stress with an emphasis on an animal model of trovafloxacin hepatotoxicity. *Toxicol Sci*. 2010;118:7–18.

Shaw PJ, Hopfensperger MJ, Ganey PE, Roth RA. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. *Toxicol Sci*. 2007;100(1):259–266.

Sheikh MS, Fornace AJ Jr. Role of p53 family members in apoptosis. *J Cell Physiol*. 2000;182:171–181.

Shen YH, Chen S, Peng YF, et al. Quantitative assessment of the effect of glutathione S-transferase genes GSTM1 and GSTT1 on hepatocellular carcinoma risk. *Tumour Biol*. 2014;35(5):4007–4015.

Shi J, Aisaki K, Ikawa Y, et al. Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride. *Am J Pathol*. 1998;153:515–525.

Shimizu M, Morita S. Effects of feeding and fasting on hepatolobular distribution of glutathione and cadmium-induced hepatotoxicity. *Toxicology*. 1992;75(2):97–107.

Shuai Z, Leung MW, He X, et al. Adaptive immunity in the liver. *Cell Mol Immunol*. 2016;13(3):354–368.

Sim E, Walters K, Boukouvala S. Arylamine N-acetyltransferases: from structure to function. *Drug Metab Rev*. 2008;40(3):479–510.

Sinclair JF, Szakacs JG, Wood SG, et al. Acetaminophen hepatotoxicity precipitated by short-term treatment of rats with ethanol and isopentanol: protection by triacetyloleandomycin. *Biochem Pharmacol*. 2000;59:445–454.

Singhal R, Ganey PE, Roth RA. Complement activation in acetaminophen-induced liver injury in mice. *J Pharmacol Exp Ther*. 2012;341(2):377–385.

Smilkstein MJ, Knapp GL, Kulig KW, Rumack B. Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. Analysis of the national multicenter study (1976 to 1985). *N Engl J Med*. 1988;319:1557–1562.

Smutny T, Mani S, Pavek P. Post-translational and post-transcriptional modifications of pregnane X receptor (PXR) in regulation of the cytochrome P450 superfamily. *Curr Drug Metab*. 2013;14(10):1059–1069.

Sneed RA, Grimes SD, Schultze AE, Brown AP, Ganey PE. Bacterial endotoxin enhances the hepatotoxicity of allyl alcohol. *Toxicol Appl Pharmacol*. 1997;144(1):77–87.

Solstad T, Fismen L, Garberg H, Fladmark KE. Identification of a novel phosphorylation site of acyl-CoA binding protein (ACBP) in nodularin-induced apoptotic hepatocytes. *Exp Cell Res*. 2008;314(10):2141–2149.

Solter PF, Wollenberg GK, Huang X, Chu FS, Runnegar MT. Prolonged sublethal exposure to the protein phosphatase inhibitor microcystin-LR results in multiple dose-dependent hepatotoxic effects. *Toxicol Sci*. 1998;44:87–96.

Sperl W, Stuppner H, Gassner I, et al. Reversible hepatic veno-occlusive disease in an infant after consumption of pyrrolizidine-containing herbal tea. *Eur J Pediatr*. 1995;154(2):112–116.

Stegelmeier BL, Colegate SM, Brown AW. Dehydropyrrolizidine alkaloid toxicity, cytotoxicity, and carcinogenicity. *Toxins (Basel)*. 2016;8(12).

Stehbens WE. Oxidative stress, toxic hepatitis, and antioxidants with particular emphasis on zinc. *Exp Mol Pathol*. 2003;75(3):265–276.

Stewart SF, Day CP. Alcoholic liver disease. In: Boyer TD, Wright TL, Manns M, eds. *Zakim and Boyer's Hepatology*. 5th ed. Philadelphia, PA: Saunders-Elsevier; 2006:579–623.

Steyn PS, Gelderblom WCA, Shephard GS, van Heerden FR. Metabolic pathway from micotoxins with a special focus on aflatoxins, ochratoxins and fumonisins. In: B. Ballantyne, T.C. Marrs, T. Syversen. General, Applied and Systems Toxicology. West Sussex, UK: John Wiley and Sons; 2009: chap. 146, 3467–3528. Available at: https://www.researchgate.net/publication/229934927_Mycotoxins_with_a_Special_Focus_on_Aflatoxins_Ochratoxins_and_Fumonisins.

Stickel F, Hoehn B, Schuppan D, Seitz HK. Review article: nutritional therapy in alcoholic liver disease. *Aliment Pharmacol Ther*. 2003;18(4):357–373.

Stickel F, Kessebohm K, Weimann R, Seitz HK. Review of liver injury associated with dietary supplements. *Liver Int*. 2011;31:595–605.

Stieger B, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (BSEP) of rat liver. *Gastroenterology*. 2000;118:422–430.

Stieger B, Meier Y, Meier PJ. The bile salt export pump. *Pflugers Arch*. 2007;453:611–620.

Stine JG, Sateesh P, Lewis JH. Drug-induced liver injury in the elderly. *Curr Gastroenterol Rep*. 2013;15(1):299.

Stravitz RT, Kramer AH, Davern T, et al. Intensive care of patients with acute liver failure: recommendations of the U.S. Acute Liver Failure Study Group. *Crit Care Med*. 2007;35(11):2498–2508.

Stravitz RT, Sanyal AJ. Drug-induced steatohepatitis. *Clin Liver Dis*. 2003;7:435–451.

Stuart KL, Bras G. Veno-occlusive disease of the liver. *Q J Med*. 1957;26(103):291–315.

Stillman AS, Huxtable R, Consroe P, Kohnen P, Smith S. Hepatic veno-occlusive disease due to pyrrolizidine (Senecio) poisoning in Arizona. *Gastroenterology*. 1977;73(2):349–352.

Sugatani J. Function, genetic polymorphism, and transcriptional regulation of human UDP-glucuronosyltransferase (UGT) 1A1. *Drug Metab Pharmacokinet*. 2013;28(2):83–92.

Sullivan BP, Kassel KM, Jone A, Flick MJ, Luyendyk JP. Fibrin(ogen)-independent role of plasminogen activators in acetaminophen-induced liver injury. *Am J Pathol*. 2012;180(6):2321–2329.

Sullivan BP, Kopec AK, Joshi N, et al. Hepatocyte tissue factor activates the coagulation cascade in mice. *Blood*. 2013;121(10):1868–1874.

Sullivan BP, Weinreb PH, Violette SM, Luyendyk JP. The coagulation system contributes to alphaVbeta6 integrin expression and liver fibrosis induced by cholestasis. *Am J Pathol*. 2010;177(6):2837–2849.

Svircev Z, Baltić V, Gantar M, et al. Molecular aspects of microcystin-induced hepatotoxicity and hepatocarcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2010;28(1):39–59.

Swenson DH, Lin JK, Miller EC, Miller JA. Aflatoxin B1–2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B1 and B2 to rat liver DNA and ribosomal RNA in vivo. *Cancer Res*. 1977;37(1):172–181.

Tailor A, Faulkner L, Naisbitt DJ, Park BK. The chemical, genetic and immunological basis of idiosyncratic drug-induced liver injury. *Hum Exp Toxicol*. 2015;34(12):1310–1317.

Tajiri K, Shimizu Y. Liver physiology and liver diseases in the elderly. *World J Gastroenterol*. 2013;19(46):8459–8467.

Tan Z, Liu Q, Jiang R, et al. Interleukin-33 drives hepatic fibrosis through activation of hepatic stellate cells. *Cell Mol Immunol*. 2017;14:1–11.

Tavill AS, Qadri AM. Alcohol and iron. *Semin Liver Dis*. 2004; 24(3):317–325.

Tian Z, Chen Y, Gao B. Natural killer cells in liver disease. *Hepatology*. 2013;57(4):1654–1662.

Temple AR, Benson GD, Zinsenheim JR, Schweinle JE. Multicenter, randomized, double-blind, active-controlled, parallel-group trial of the long-term (6–12 months) safety of acetaminophen in adult patients with osteoarthritis. *Clin Ther*. 2006;28:222–235.

Temple RJ, Himmel MH. Safety of newly approved drugs: implications for prescribing. *J Am Med Assoc*. 2002;287:2273–2275.

Thompson RA, Isin EM, Ogese MO, Mettetal JT, Williams DP. Reactive metabolites: current and emerging risk and hazard assessments. *Chem Res Toxicol*. 2016; 29(4):505–533.

Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology*. 2010;52(5):1836–1846.

Tjandra K, Sharkey KA, Swain MG. Progressive development of a Th1-type hepatic cytokine profile in rats with experimental cholangitis. *Hepatology*. 2000;31(2):280–290.

Trauner M, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev*. 2003;83:633–671.

Trautwein C. Liver regeneration. In: Boyer TD, Wright TL, Manns M, eds. *Zakim and Boyer's Hepatology*. 5th ed. Philadelphia, PA: Saunders-Elsevier; 2006:23–36.

Tsukamoto H. Redox regulation of cytokine expression in Kupffer cells. *Antioxid Redox Signal*. 2002;4:741–748.

Tsukamoto H, Machida K, Dynnyk A, Mkrtchyan H. "Second hit" models of alcoholic liver disease. *Semin Liver Dis*. 2009;29(2):178–187.

Tsutsumi M, Lasker JM, Shimizu M, Rosman AS, Lieber CS. The intralobular distribution of ethanol-inducible P450IIE1 in rat and human liver. *Hepatology*. 1989;10:437–446.

Tu M, Sun S, Wang K, et al. Organic cation transporter 1 mediates the uptake of monocrotaline and plays an important role in its hepatotoxicity. *Toxicology*. 2013;311(3):225–230.

Ufelmann H, Krüger T, Luckas B, Schrenk D. Human and rat hepatocyte toxicity and protein phosphatase 1 and 2A inhibitory activity of naturally occurring desmethyl-microcystins and nodularins. *Toxicology*. 2012;293(1–3):59–67.

Ufelmann H, Schrenk D. Nodularin-triggered apoptosis and hyperphosphorylation of signaling proteins in cultured rat hepatocytes. *Toxicol In Vitro*. 2015;29(1):16–26.

Ulrich RG. Idiosyncratic toxicity: a convergence of risk factors. *Annu Rev Med*. 2007;58:17–34.

Ulzurrun E, Stephens C, Crespo E, et al. Role of chemical structures and the 1331T>C bile salt export pump polymorphism in idiosyncratic drug-induced liver injury. *Liver Int*. 2013;33(9):1378–1385.

Ulzurrun E, Stephens C, Ruiz-Cabello F, et al. Selected ABCB1, ABCB4 and ABCC2 polymorphisms do not enhance the risk of drug-induced hepatotoxicity in a Spanish cohort. *PLoS One*. 2014;9(4):e94675.

Ungar H, Popp JA. Acute cholecystitis and persistent liver necrosis in mice provoked by isothiocyanate. *Arch Pathol Lab Med*. 1976;100(3):127–131.

Usui T, Naisbitt DJ. Human leukocyte antigen and idiosyncratic adverse drug reactions. *Drug Metab Pharmacokinet*. 2017;32(1):21–30.

Vandenabeele P, Galluzzi L, Vanden Berghe T, et al. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol*. 2010;11:700–714.

Van Hoof C, Goris J. Phosphatases in apoptosis: to be or not to be, PP-2A is in the heart of the question. *Biochim Biophys Acta*. 2003;1640(2–3):97–104.

Videla LA, Fernandez V, Tapia G, Varela P. Oxidative stress-mediated hepatotoxicity of iron and copper: role of Kupffer cells. *Biometals*. 2003;16:103–111.

Vonk RJ, Yousef IM, Corriveau JP, Tuchweber B. Phalloidin-induced morphological and functional changes of rat liver. *Liver*. 1982;2(2):133–140.

Vroon DH, Israili Z. Aminotransferases. In: Walker HK, Hall WD, Hurst JW, eds. *Clinical Methods: The History, Physical, and Laboratory Examinations*. 3rd ed. Boston, MA: Buttersworths; 1990.

Wagner M, Fickert P, Zollner G, et al. Role of farnesoid X receptor in determining hepatic ABC transporter expression and liver injury in bile duct-ligated mice. *Gastroenterology*. 2003;125:825–838.

Wagner M, Halilbasic E, Marschall HU, et al. CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology*. 2005;42:420–430.

Wagner M, Zollner G, Trauner M. New molecular insights into the mechanisms of cholestasis. *J Hepatol*. 2009;51(3):565–580.

Wall TL. Genetic associations of alcohol and aldehyde dehydrogenase with alcohol dependence and their mechanisms of action. *Ther Drug Monit*. 2005;27(6):700–703.

Wallace BD, Redinbo MR. Xenobiotic-sensing nuclear receptors involved in drug metabolism: a structural perspective. *Drug Metab Rev*. 2013;45(1):79–100.

Wands JR, Moradpour D. Molecular pathogenesis of hepatocellular carcinoma. In: Boyer TD, Wright TL, Manns M, eds. *Zakim and Boyer's Hepatology*. 5th ed. Philadelphia, PA: Saunders-Elsevier; 2006:165–175.

Wang D, Bushnell DA, Westover KD, Kaplan CD, Kornberg RD. Structural basis of transcription: role of the trigger loop in substrate specificity and catalysis. *Cell*. 2006;127:941–954.

Wang JS, Groopman JD. DNA damage by mycotoxins. *Mutat Res*. 1999;424(1–2):167–181.

Wang X, Kanel GC, DeLeve LD. Support of sinusoidal endothelial cell glutathione prevents hepatic veno-occlusive disease in the rat. *Hepatology*. 2000;31:428–434.

Wang W, Xu MJ, Cai Y, et al. Inflammation is independent of steatosis in a murine model of steatohepatitis. *Hepatology*. 2017;66(1):108–123.

Waring WS. Criteria for acetylcysteine treatment and clinical outcomes after paracetamol poisoning. *Expert Rev Clin Pharmacol*. 2012;5(3):311–318.

Watanabe N, Tsukada N, Smith CR, Edwards V, Phillips MJ. Permeabilized hepatocyte couplets: adenosine triphosphate-dependent bile canalicular contractions and a circumferential pericanalicular microfilament belt demonstrated. *Lab Invest*. 1991;65:203–213.

Watkins PB. Idiosyncratic liver injury: challenges and approaches. *Toxicol Pathol*. 2005;33:1–5.

Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol*. 2003;33:105–136.

Weiskirchen R, Tacke F. Cellular and molecular functions of hepatic stellate cells in inflammatory responses and liver immunology. *Hepatobiliary Surg Nutr*. 2014;3(6):344–363.

Westwood A. The analysis of bilirubin in serum. *Ann Clin Biochem*. 1991;28(Pt 2):119–130.

Wiedow O, Meyer-Hoffert U. Neutrophil serine proteases: potential key regulators of cell signalling during inflammation. *J Intern Med*. 2005;257(4):319–328.

Wieland T, Faulstich H. Amatoxins, phallotoxins, phallolysin, and antamanide: the biologically active components of poisonous Amanita mushrooms. *CRC Crit Rev Biochem*. 1978;5:185–260.

Wieland T. Modification of actins by phallotoxins. *Naturwissenschaften*. 1977;64(6):303–309.

Williams CD, Antoine DJ, Shaw PJ, et al. Role of the Nalp3 inflammasome in acetaminophen-induced sterile inflammation and liver injury. *Toxicol Appl Pharmacol*. 2011;252:289–297.

Williams CD, Bajt ML, Sharpe MR, et al. Neutrophil activation during acetaminophen hepatotoxicity and repair in mice and humans. *Toxicol Appl Pharmacol*. 2014;275(2):122–133.

Wisse E, Braet F, Luo D, et al. Structure and function of sinusoidal lining cells in the liver. *Toxicol Pathol*. 1996; 24:100–111.

Wogan GN, Paglialunga S, Newberne PM. Carcinogenic effects of low dietary levels of aflatoxin B1 in rats. *Food Cosmet Toxicol*. 1974;12(5–6):681–685.

Wong FW, Chan WY, Lee SS. Resistance to carbon tetrachloride-induced hepatotoxicity in mice which lack CYP2E1 expression. *Toxicol Appl Pharmacol*. 1998;153:109–118.

Woolbright BL, Dorko K, Antoine DJ, et al. Bile acid-induced necrosis in primary human hepatocytes and in patients with obstructive cholestasis. *Toxicol Appl Pharmacol*. 2015;283:168–177.

Woolbright BL, Jaeschke H. Novel insight into mechanisms of cholestatic liver injury. *World J Gastroenterol*. 2012;18(36):4985–4993.

Woolbright BL, Jaeschke H. Role of the inflammasome in acetaminophen-induced liver injury and acute liver failure. *J Hepatol*. 2017;66(4):836–848.

Woolbright BL, Williams CD, Ni H, et al. Microcystin-LR induced liver injury in mice and in primary human hepatocytes is caused by oncotic necrosis. *Toxicon*. 2017;125:99–109.

Wu TY, Khor TO, Lee JH, et al. Pharmacogenetics, pharmacogenomics and epigenetics of Nrf2-regulated xenobiotic-metabolizing enzymes and transporters by dietary phytochemical and cancer chemoprevention. *Curr Drug Metab*. 2013;14(6):688–694.

Wullemin N, Adam J, Fontana S, et al. HLA haplotype determines hapten or p-i T cell reactivity to flucloxacillin. *J Immunol*. 2013;190(10):4956–4964.

Xie Y, McGill MR, Dorko K, et al. Mechanisms of acetaminophen-induced cell death in primary human hepatocytes. *Toxicol Appl Pharmacol*. 2014;279(3):266–274.

Xie Y, Williams CD, McGill MR, et al. Purinergic receptor antagonist A438079 protects against acetaminophen-induced liver injury by inhibiting p450 isoenzymes, not by inflammasome activation. *Toxicol Sci*. 2013;131(1):325–335.

Xing XK, Wu HY, Feng HG, Yuan ZQ. Immune function of nonparenchymal liver cells. *Genet Mol Res*. 2016;15(1).

Yamamoto K, Makino Y, Itoshima T, Kobayashi T, Tsuji T. Phalloidin-induced alterations of bile canaliculi. *Acta Med Okayama*. 1988;42(4):207–213.

Yamazaki M, Miyake M, Sato H, et al. Perturbation of bile acid homeostasis is an early pathogenesis event of drug induced liver injury in rats. *Toxicol Appl Pharmacol*. 2013;268(1):79–89.

Yan CC, Huxtable RJ. Effects of monocrotaline, a pyrrolizidine alkaloid, on glutathione metabolism in the rat. *Biochem Pharmacol*. 1996;51(3):375–379.

Yang CS. Influences of dietary and other factors on xenobiotic metabolism and carcinogenesis—a review article in memory of Dr Allan H. Conney (1930–2013). *Nutr Cancer*. 2015;67(8):1207–1213.

Yang Q, Shi Y, Yang Y, Lou G, Chen Z. The sterile inflammation in the exacerbation of HBV-associated liver injury. *Mediators Inflamm*. 2015;2015:508681.

Yee SB, Copple BL, Ganey PE, Roth RA. The temporal relationship between bacterial lipopolysaccharide and monocrotaline exposures influences toxicity: shift in response from hepatotoxicity to nitric oxide-dependent lethality. *J Toxicol Environ Health A*. 2002;65(14): 961–976.

Yee SB, Ganey PE, Roth RA. The role of Kupffer cells and TNF-alpha in monocrotaline and bacterial lipopolysaccharide-induced liver injury. *Toxicol Sci*. 2003a;71(1):124–132.

Yee SB, Hanumegowda UM, Hotchkiss JA, Ganey PE, Roth RA. Role of neutrophils in the synergistic liver injury from monocrotaline and bacterial lipopolysaccharide exposure. *Toxicol Sci*. 2003b;72(1):43–56.

Yee SB, Harkema JR, Ganey PE, Roth RA. The coagulation system contributes to synergistic liver injury from exposure to monocrotaline and bacterial lipopolysaccharide. *Toxicol Sci*. 2003c;74(2):457–469.

Yee SB, Kinser S, Hill DA, et al. Synergistic hepatotoxicity from coexposure to bacterial endotoxin and the pyrrolizidine alkaloid monocrotaline. *Toxicol Appl Pharmacol*. 2000;166(3):173–185.

Yeligar SM, Chen MM, Kovacs EJ, et al. Alcohol and lung injury and immunity. *Alcohol*. 2016; 55: 51–59.

Yeung CK, Shen DD, Thummel KE, Himmelfarb J. Effects of chronic kidney disease and uremia on hepatic drug metabolism and transport. *Kidney Int*. 2014;85(3):522–528.

Yin L, Lynch D, Sell S. Participation of different cell types in the restitutive response of the rat liver to periportal injury induced by allyl alcohol. *J Hepatol*. 1999b;31(3):497–507.

Yin XM, Wang K, Gross A, et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature*. 1999a;400:886–891.

Yip VL, Alfirevic A, Pirmohamed M. Genetics of immune-mediated adverse drug reactions: a comprehensive and clinical review. *Clin Rev Allergy Immunol*. 2015;48(2–3):165–175.

Yokoi T, Nakajima M. Toxicological implications of modulation of gene expression by microRNAs. *Toxicol Sci*. 2011;123(1):1–14.

You Q, Holt M, Yin H, et al. Role of hepatic resident and infiltrating macrophages in liver repair after acute injury. *Biochem Pharmacol*. 2013; 86(6):836–843.

Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*. 2013;138(1):103–141.

Zhai Y, Busuttil RW, Kupiec-Weglinski JW. Liver ischemia and reperfusioninjury: new insights into mechanisms of innate-adaptive immune-mediated tissue inflammation. *Am J Transplant*. 2011;11(8):1563–1569.

Zhang J, Chen J, Xia Z. Microcystin-LR exhibits immunomodulatory role in mouse primary hepatocytes through activation of the NF-κB and MAPK signaling pathways. *Toxicol Sci*. 2013;136(1):86–6.

Zhang Y, Chong E, Herman B. Age-associated increases in the activity of multiple caspases in Fisher 344 rat organs. *Exp Gerontol*. 2002;37:777–789.

Zhang Y, Hong JY, Rockwell CE, et al. Effect of bile duct ligation on bile acid composition in mouse serum and liver. *Liver Intern*. 2012;32:58–69.

Zheleva A. Phenoxyl radicals formation might contribute to severe toxicity of mushroom toxin alpha-amanitin- an electron paramagnetic resonance study. *Trakia J Sci*. 2013;11:33–38.

Zhou L, Pang X, Jiang J, Zhong D, Chen X. Nimesulide and 4'-Hydroxynimesulide as bile acid transporters inhibitors are contributory factors for drug-induced cholestasis. *Drug Metab Dispos*. 2017;45(5):441–448.

Zhu C, Fuchs CD, Halilbasic E, Trauner M. Bile acids in regulation of inflammation and immunity: friend or foe? *Clin Exp Rheumatol*. 2016;34(4 suppl 98):25–31.

Zimmerman HJ. *Hepatotoxicity*. 2nd ed. Philadelphia, PA: Lippincott, Williams & Wilkins; 1999.

Zimmerman HJ, Maddrey WC. Acetaminophen (paracetamol) hepatotoxicity with regular intake of alcohol: analysis of instances of therapeutic misadventure. *Hepatology*. 1995;22:767–773.

Zischka H, Lichtmannegger J. Pathological mitochondrial copper overload in livers of Wilson's disease patients and related animal models. *Ann N Y Acad Sci*. 2014;1315:6–15.

Zollner G, Marschall HU, Wagner M, Trauner M. Role of nuclear receptors in the adaptive response to bile acids and cholestasis: pathogenetic and therapeutic considerations. *Mol Pharm*. 2006;3:231–251.

Zollner G, Trauner M. Molecular mechanisms of cholestasis. *Wien Med Wochenschr*. 2006;156(13–14):380–385.

Zou W, Beggs KM, Sparkenbaugh EM, et al. Sulindac metabolism and synergy with tumor necrosis factor-alpha in a drug-inflammation interaction model of idiosyncratic liver injury. *J Pharmacol Exp Ther*. 2009;331(1):114–121.

Zou W, Roth RA, Younis HS, Burgoon LD, Ganey PE. Oxidative stress is important in the pathogenesis of liver injury induced by sulindac and lipopolysaccharide cotreatment. *Toxicology*. 2010;272(1–3):32–38.

chapter 14

Toxic Responses of the Kidney

Rick G. Schnellmann

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Acknowledgments

References

The functional integrity of the mammalian kidney is vital to total body homeostasis because the kidney plays a principal role in the excretion of metabolic wastes and in the regulation of extracellular fluid volume, electrolyte composition, and acid–base balance. In addition, the kidney synthesizes and releases hormones such as renin and erythropoietin and metabolizes vitamin D_3 to the active 1,25-dihydroxy vitamin D_3 form. A toxic insult to the kidney therefore could disrupt any or all of these functions and could have profound effects on total body metabolism. Fortunately, the kidneys are equipped with a variety of detoxification mechanisms and have considerable functional reserve and regenerative capacities. Nonetheless, the nature and severity of the toxic insult may be such that these detoxification and compensatory mechanisms are overwhelmed, and kidney injury ensues. The outcome of renal failure can be profound; permanent renal damage may result, requiring chronic dialysis treatment or kidney transplantation.

FUNCTIONAL ANATOMY

Gross examination of a sagittal section of the kidney reveals three clearly demarcated anatomic areas: the cortex, medulla, and papilla (Figs. 14-1 and 14-2). The cortex constitutes the major portion of the kidney and receives a disproportionately higher percentage (90%) of blood flow compared to the medulla (about 6% to 10%) or papilla (1% to 2%). Thus, when a blood-borne toxicant is delivered to the kidney, a high percentage of the material will be delivered to the cortex and will have a greater opportunity to influence cortical rather than medullary or papillary functions. However, medullary and papillary tissues are exposed to higher luminal concentrations of toxicants for prolonged periods of time, a consequence of the more concentrated tubular fluid and the more sluggish flow of blood and filtrate in these regions.

The functional unit of the kidney, the nephron, may be considered in three portions: the vascular element, the glomerulus, and the tubular element.

Renal Vasculature and Glomerulus

The renal artery branches successively into interlobar, arcuate, and interlobular arteries (Fig. 14-1). The last of these give rise to the afferent arterioles, which supply the glomerulus; blood then leaves the glomerular capillaries via the efferent arteriole. Both the afferent and efferent arterioles, arranged in a series before and after the glomerular capillary tuft, respectively, are ideally situated to control glomerular capillary pressure and glomerular plasma flow rate. Indeed, these arterioles are innervated by the sympathetic nervous system and contract in response to nerve stimulation, angiotensin II, vasopressin, endothelin, adenosine, and norepinephrine decreasing glomerular filtration rate and renal blood blow. The efferent arterioles draining the cortical glomeruli branch into a peritubular capillary network, whereas those draining the juxtamedullary glomeruli

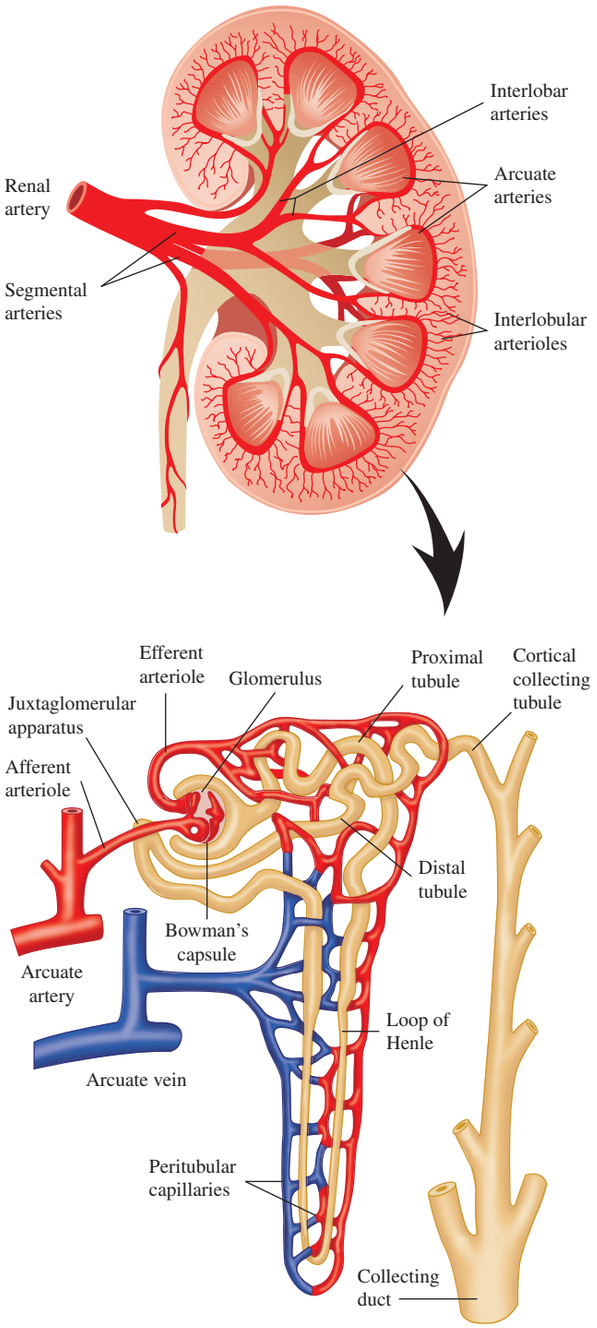

Figure 14-1. *Schematic of the human kidney showing the major blood vessels and the microcirculation and tubular components of each nephron.* (From Guyton and Hall [1996, p. 318], with permission from Elsevier.)

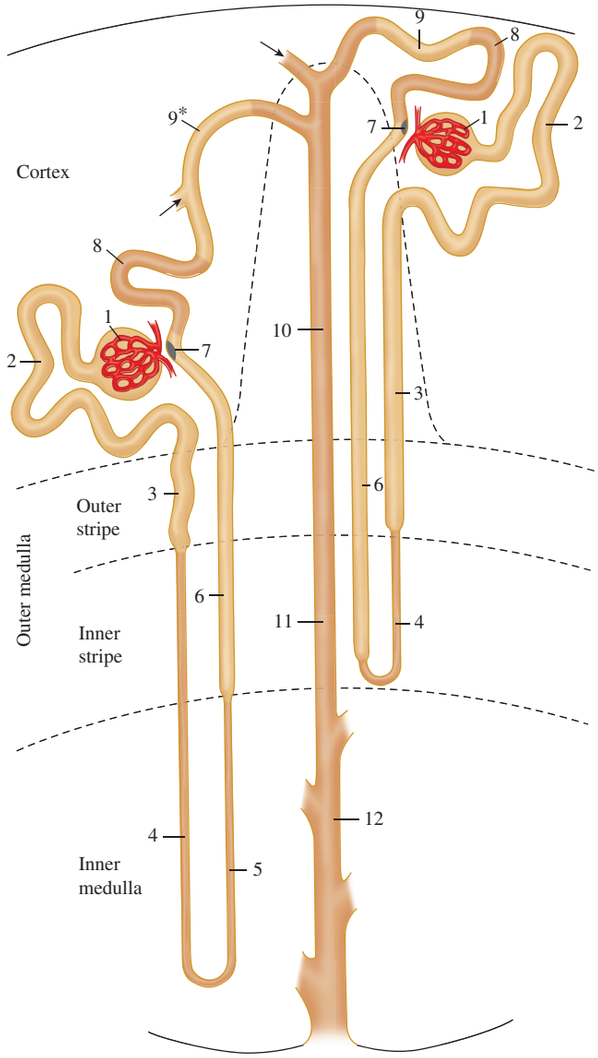

Figure 14-2. *Schematic of short- and long-looped nephrons and the collecting system.* A medullary ray is delineated by a dashed line within the cortex. (1) Renal corpuscle including Bowman's capsule and the glomerulus; (2) proximal convoluted tubule; (3) proximal straight tubule; (4) descending thin limb; (5) thin ascending limb; (6) thick ascending limb; (7) macula densa, located within the final portion of the thick ascending limb; (8) distal convoluted tubule; (9) connecting tubule; (9*) connecting tubule of the juxtamedullary nephron, which forms an arcade; (10) cortical collecting duct; (11) outer medullary collecting duct; (12) inner medullary collecting duct. (From Kriz [1988], with permission.)

form a capillary loop, the vasa recta, supplying the medullary structures. These postglomerular capillary loops provide an efficient arrangement for delivery of nutrients to the postglomerular tubular structures, delivery of wastes to the tubule for excretion, and return of reabsorbed electrolytes, nutrients, and water to the systemic circulation.

The glomerulus is a complex, specialized capillary bed composed primarily of endothelial cells that are characterized by an attenuated and fenestrated cytoplasm, visceral epithelial cells characterized by a cell body (podocyte) from which many trabeculae and pedicles (foot processes) extend, and a glomerular basement membrane (GBM), which is a trilamellar structure sandwiched between the endothelial and epithelial cells (Fig. 14-3). A portion of the blood entering the glomerular capillary network is fractionated into a virtually protein-free and cell-free ultrafiltrate, which passes through Bowman's space and into the tubular portion of the nephron. The formation of such an ultrafiltrate is the net result of the Starling forces that determine fluid movement across capillary beds, that is, the balance between transcapillary hydrostatic pressure and colloid oncotic pressure (Maddox and

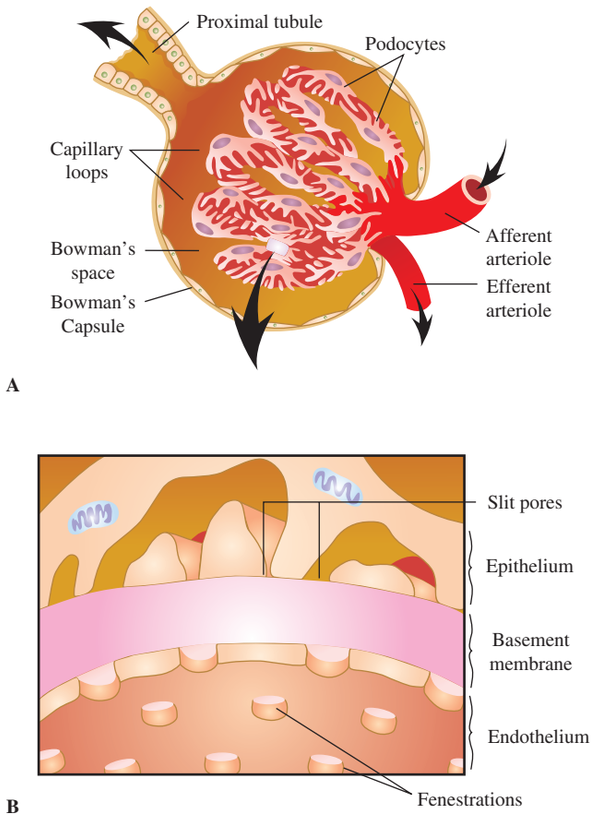

Figure 14-3. *(**A**) Schematic of the ultrastructure of the glomerular capillaries. (**B**) Cross-section of the glomerular capillary membrane with the capillary endothelium, basement membrane, and epithelium podocytes.* (From Guyton and Hall [1996, p. 32], with permission from Elsevier.)

Brenner, 1991). Filtration is therefore favored when transcapillary hydrostatic pressure exceeds plasma oncotic pressure. An additional determinant of ultrafiltration is the effective hydraulic permeability of the glomerular capillary wall, in other words, the ultrafiltration coefficient (K_f), which is determined by the total surface area available for filtration and the hydraulic permeability of the capillary wall. Consequently, chemically induced decreases in glomerular filtration rate (GFR) may be related to decreases in transcapillary hydrostatic pressure and glomerular plasma flow due to increased afferent arteriolar resistance or to decreases in the surface area available for filtration, resulting from decreases in the size and/or number of endothelial fenestrae or detachment or effacement of foot processes.

Although the glomerular capillary wall permits a high rate of fluid filtration (approximately 20% of blood entering the glomerulus is filtered), it provides a significant barrier to the transglomerular passage of macromolecules. Experiments using a variety of charged and neutral tracers have established that this barrier function is based on the ability of the glomerulus to act as a size-selective and charge-selective filter (Brenner et al., 1977). In general, the filtration of macromolecules is inversely proportional to the molecular weight of a substance; thus, small molecules, such as inulin (molecular weight [MW] ~5500), are freely filtered, whereas large molecules, such as albumin (MW 56,000 to 70,000), are restricted. Filtration of anionic molecules tends to be restricted compared to that of neutral or cationic molecules of the same size. These permselective properties of the glomerulus appear to be directly related to the physicochemical properties of the different cell types within the glomerulus (Kanwar et al., 1991). In particular, charge-selective properties of the glomerulus appear to be related to the anionic groups of the GBM coupled with the anionic coating of the epithelial and endothelial cells (Fig. 14-3). These highly anionic components produce electrostatic repulsion and hinder the circulation of polyanionic macromolecules, thereby markedly retarding passage of these molecules across the filtration barrier. Toxicants that neutralize or reduce the number of fixed anionic charges on glomerular structural elements, therefore, will impair the charge- and/or size-selective properties of the glomerulus, resulting in urinary excretion of polyanionic and/or high-molecular-weight proteins.

Proximal Tubule

The proximal tubule consists of three discrete segments: the S_1 (pars convoluta), S_2 (transition between pars convoluta and pars recta), and S_3 (the pars recta) segments (Fig. 14-2). The S_1 segment is the initial portion of the proximal convoluted tubule and is characterized by a tall brush-border and a well-developed vacuolar lysosomal system. The basolateral membrane is extensively interdigitated and many long mitochondria fill the basal portion of the cell, characteristic of Na^+-transporting epithelia. The S_2 segment comprises the end of the convoluted segment and the initial portion of the straight segment. These cells possess a shorter brush-border, fewer apical vacuoles and mitochondria, and less basolateral interdigitation compared to the S_1 cells. The S_3 segment comprises the distal portion of proximal segments and extends to the junction of the outer and inner stripe of the outer medulla. The S_3 cells have a well-developed brush border but fewer and smaller lysosomes and mitochondria than S_1 and S_2 cells.

The formation of urine is a highly complex and integrated process in which the volume and composition of the glomerular filtrate are progressively altered as fluid passes through each of the different tubular segments. The proximal tubule is the workhorse of the nephron, as it reabsorbs approximately 60% to 80% of solute and water filtered at the glomerulus. Toxicant-induced injury to the proximal tubule, therefore, will have major consequences for water and solute balance. Water reabsorption is through a passive iso-osmotic process, driven primarily by Na^+ reabsorption, mediated by the Na^+, K^+-ATPase localized in the basolateral plasma membrane. In addition to active Na^+ reabsorption, the proximal tubule reabsorbs other electrolytes, such as K^+, HCO_3^-, Cl^-, PO_4^{3-}, Ca^{2+}, and Mg^{2+}. The proximal tubule contains numerous transport systems capable of driving concentrative transport of many metabolic substrates, including amino acids, glucose, and citric acid cycle intermediates. The proximal tubule also reabsorbs virtually all the filtered low-molecular-weight proteins by specific endocytotic protein reabsorption processes. In addition, small linear peptides may be hydrolyzed by peptidases associated with the proximal tubular brush border. An important excretory function of the proximal tubule is secretion of weak organic anions and cations by specialized transporters that drive concentrative movement of these ions from postglomerular blood into proximal tubular cells, followed by secretion into tubular fluid. Toxicant-induced interruptions in the production of energy for any of these active transport mechanisms or the function of critical membrane-bound enzymes or transporters can profoundly affect proximal tubular and whole-kidney function.

The various segments of the proximal tubule exhibit marked biochemical and physiologic heterogeneity (Goldstein, 1993).

For example, filtered HCO_3^-, low-molecular-weight proteins, amino acids, and glucose are primarily reabsorbed by the S_1 segment. Transport capacities for these substances in the S_2 and S_3 segments are appreciably less; for example, glucose reabsorption in the S_2 and S_3 segments is about 50% and 10% of that in the S_1 segment, respectively. In contrast, the principal site of organic anion and cation secretion is in the S_2 and S_1/S_2 segments, respectively. Oxygen consumption, Na^+, K^+-ATPase activity, and gluconeogenic capacity are greater in the S_1 and S_2 segments than in the S_3 segment. Catabolism and apical transport of glutathione (GSH) occur to a much greater extent in the S_3 segment, where the brush-border enzyme γ-glutamyl transpeptidase is present in greater amounts. Chemically induced injury to distinct proximal tubular segments, therefore, may be related in part to their segmental differences in biochemical properties (see the section "Site-Selective Injury").

Loop of Henle

The thin descending and ascending limbs and the thick ascending limb of the loop of Henle are critical to the processes involved in urinary concentration (Fig. 14-2). Approximately 25% of the filtered Na^+ and K^+ and 20% of the filtered water are reabsorbed by the segments of the loop of Henle. The tubular fluid entering the thin descending limb is iso-osmotic to the renal interstitium; water is freely permeable and solutes, such as electrolytes and urea, may enter from the interstitium. In contrast, the thin ascending limb is relatively impermeable to water and urea, and Na^+ and Cl^- are reabsorbed by passive diffusion. The thick ascending limb is impermeable to water, and active transport of Na^+ and Cl^- is mediated by the $Na^+/K^+–2Cl^-$ cotransport mechanism, with the energy provided by the Na^+, K^+-ATPase. The relatively high rates of Na^+, K^+-ATPase activity, and oxygen demand, coupled with the meager oxygen supply in the medullary thick ascending limb, are thought to contribute to the vulnerability of this segment of the nephron to hypoxic injury. The close interdependence between metabolic workload and tubular vulnerability has been demonstrated, revealing that selective damage to the thick ascending limb in the isolated perfused kidney can be blunted by reducing tubular work and oxygen consumption (via inhibition of the Na^+, K^+-ATPase with ouabain) or by increasing oxygen supply (via provision of an oxygen carrier, hemoglobin) (Brezis and Epstein, 1993). Conversely, increasing the tubular workload (via the ionophore amphotericin B) exacerbates hypoxic injury in this segment (Brezis et al., 1984).

Distal Tubule and Collecting Duct

The macula densa comprises specialized cells located between the end of the thick ascending limb and the early distal tubule, in close proximity to the afferent arteriole (Fig. 14-2). This anatomic arrangement is ideally suited for a feedback system whereby a stimulus received at the macula densa is transmitted to the arterioles of the same nephron. Under normal physiologic conditions, increased solute delivery or concentration at the macula densa triggers a signal resulting in afferent arteriolar constriction leading to decreases in GFR (and hence decreased solute delivery). Thus, increases in fluid/solute out of the proximal tubule, due to impaired tubular reabsorption, will activate this feedback system, referred to as tubuloglomerular feedback (TGF) and resulting in decreases in the filtration rate of the same nephron. This regulatory mechanism is viewed as a powerful volume-conserving mechanism, designed to decrease GFR in order to prevent massive losses of fluid/electrolytes due to impaired tubular reabsorption. Humoral mediation of TGF by the renin-angiotensin system has been proposed, and evidence suggests that other substances may be involved. The distal tubular cells contain numerous mitochondria but lack a well-developed brush border and an endocytotic apparatus characteristic of the pars convoluta of the proximal tubule. The early distal tubule reabsorbs most of the remaining intraluminal Na^+, K^+, and Cl^- but is relatively impermeable to water.

The late distal tubule, cortical collecting tubule, and medullary collecting duct perform the final regulation and fine-tuning of urinary volume and composition. The remaining Na^+ is reabsorbed in conjunction with K^+ and H^+ secretion in the late distal tubule and cortical collecting tubule. The combination of medullary and papillary hypertonicity generated by countercurrent multiplication and the action of antidiuretic hormone (ADH, vasopressin) serve to enhance water permeability of the medullary collecting duct. Chemicals that interfere with ADH synthesis, secretion, or action, therefore, may impair concentrating ability. Additionally, because urinary concentrating ability is dependent upon medullary and papillary hypertonicity, chemicals that increase medullary blood flow may impair concentrating ability by dissipating the medullary osmotic gradient.

Table 14-1 illustrates the efficiency of the nephrons in the conservation of electrolytes, substrates, and water and excretion of nitrogenous wastes (urea). The reader may refer to *Brenner and Rector's The Kidney* (Skorecki et al., 2015) and *Schrier's Diseases of the Kidney* (Schrier et al., 2012) for further review of renal physiology.

Table 14-1

Filtration, Reabsorption, and Excretion Rates of Different Substances by the Kidneys*

	FILTERED (MEQ/24 H)	REABSORBED (MEQ/24 H)	EXCRETED (MEQ/24 H)	REABSORBED (%)
Glucose (g/day)	180	180	0	100
Bicarbonate (mEq/day)	4320	4318	2	>99.9
Sodium (mEq/day)	25,560	25,410	150	99.4
Chloride (mEq/day)	19,440	19,260	180	99.1
Water (L/day)	169	167.5	1.5	99.1
Urea (g/day)	48	24	24	50
Creatinine (g/day)	1.8	0	1.8	0

**Glomerular filtration rate: 125 mL/min = 180 L/24 h.*

PATHOPHYSIOLOGIC RESPONSES OF THE KIDNEY

Acute Kidney Injury

One of the most common manifestations of nephrotoxic damage is acute renal failure or acute kidney injury (AKI). AKI is a group of syndromes that comprises multiple causative factors and occurs in a variety of settings with varied clinical manifestations ranging from a minimal elevation in serum creatinine to anuric renal failure. Considering that AKI is a complex disorder, the Acute Dialysis Quality Initiative developed the RIFLE (Risk, Injury, Failure, Loss, and End-stage renal disease) classification of AKI (Bellomo et al., 2004) and the AKI Network proposed minor modifications (Mehta et al., 2007; Ricci et al., 2011; Kellum et al., 2012; Lopes and Jorge, 2013). In both cases, AKI classification is based on the extent of serum creatinine increases or changes in urine output.

Any decline in GFR is complex and may result from prerenal factors (renal vasoconstriction, intravascular volume depletion, and insufficient cardiac output), postrenal factors (ureteral or bladder obstruction), and intrarenal factors (glomerulonephritis, tubular cell injury, death, and loss resulting in back leak; renal vasculature damage; interstitial nephritis) (Fig. 14-4). Fig. 14-5 illustrates the pathways that lead to diminished GFR following chemical exposure. As discussed above, pre- and postrenal factors can lead to decreased GFR. If a chemical causes tubular damage directly, then tubular casts can cause tubular obstruction, increased tubular pressure, and decreased GFR. The tubular damage may result in epithelial cell death/loss, leading to back leak of glomerular filtrate and a decrease in GFR. If a chemical causes intrarenal vascular damage with hemodynamic alterations that lead to vasoconstriction, the resulting medullary hypoxia may cause tubular damage and/or decreases in perfusion pressure, glomerular hydrostatic pressure, and GFR. If a chemical causes intrarenal inflammation, then tubular and vascular damage may follow with decreases in GFR. Finally, a chemical may disrupt glomerular function, resulting in decreased glomerular ultrafiltration and GFR. It has been estimated that prerenal factors are responsible for AKI in 55% to 60% of patients, intrarenal factors are responsible for AKI in 35% to 40% of patients, and postrenal factors are responsible for AKI in less than 5% of patients (Brady et al., 2004). Further, it is thought that more than 90% of AKI mediated by intrarenal factors is the result of ischemia/reperfusion injury or nephrotoxicity. Table 14-2 provides a partial list of chemicals that produce AKI through these different mechanisms.

The maintenance of tubular integrity is dependent on cell-to-cell and cell-to-matrix adhesion; these interactions are mediated in part by integrins and cell adhesion molecules (Fig. 14-6). After a chemical or hypoxic insult, adhesion of nonlethally damaged, apoptotic, and oncotic cells to the basement membrane is compromised, leading to their detachment from the basement membrane and appearance in the tubular lumen (Goligorsky et al., 2010). Morphologically, such an event would lead to gaps in the epithelial cell lining, potentially resulting in back-leak of filtrate and diminished GFR. These detached cells may aggregate in the tubular lumen (cell-to-cell adhesion) and/or adhere or reattach to adherent epithelial cells downstream, resulting in tubular obstruction. Furthermore, the loss of expression of integrins on the basolateral membrane may be responsible for the exfoliation of tubular cells, and the redistribution of integrins from the basolateral to the apical membrane facilitates adhesion of detached cells to the in situ epithelium.

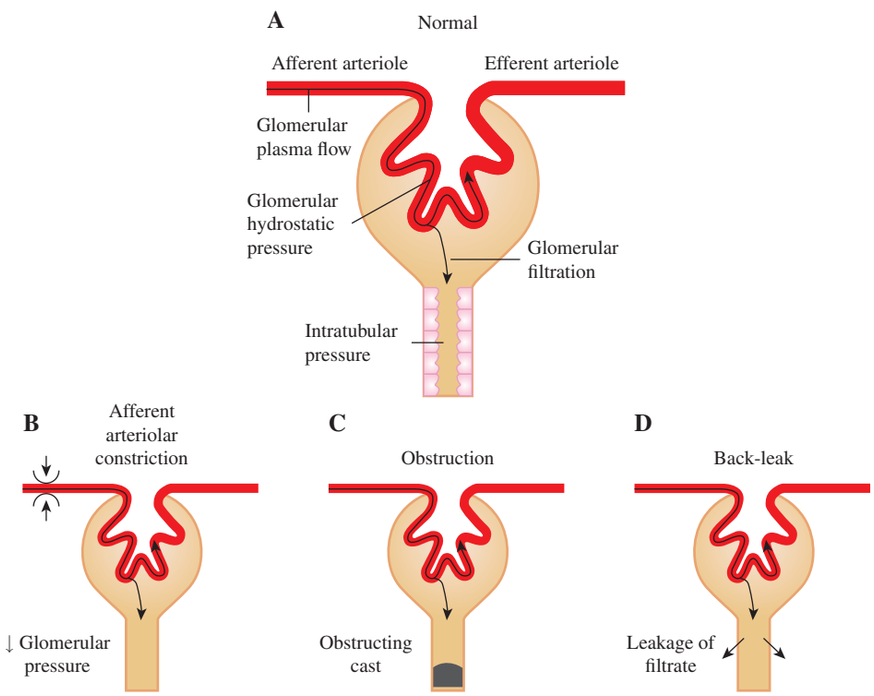

Figure 14-4. *Mechanisms of reduction of the GFR.* (**A**) GFR depends on four factors: (1) adequate blood flow to the glomerulus, (2) adequate glomerular capillary pressure, (3) glomerular permeability, and (4) low intratubular pressure. (**B**) Afferent arteriolar constriction decreases GFR by reducing blood flow, resulting in diminished capillary pressure. (**C**) Obstruction of the tubular lumen by cast formation increases tubular pressure; when tubular pressure exceeds glomerular capillary pressure, filtration decreases or ceases. (**D**) Back-leak occurs when the paracellular space between cells increases and the glomerular filtrate leaks into the extracellular space and bloodstream. (From Molitoris and Bacallao [1999, p. 13.5], with permission.)

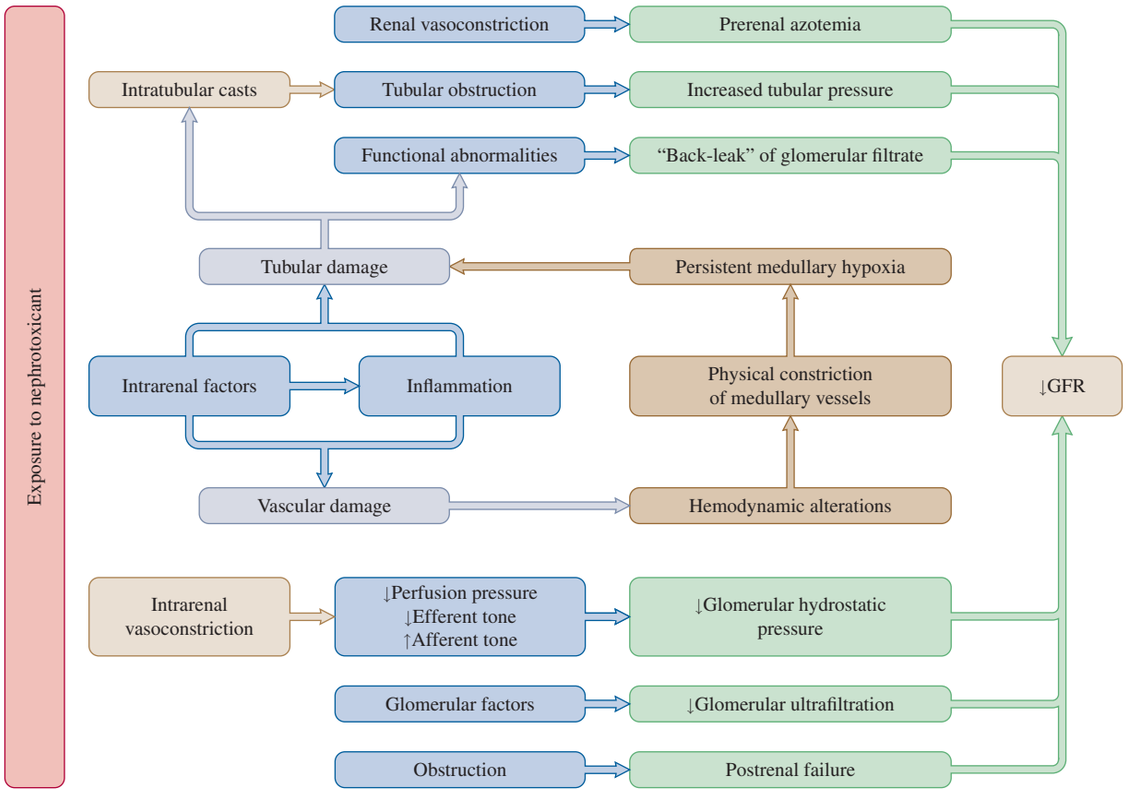

Figure 14-5. *Mechanisms that contribute to decreased GFR in acute renal failure.* After exposure to a nephrotoxicant, one or more mechanisms may contribute to a reduction in the GFR. These include renal vasoconstriction resulting in prerenal azotemia and obstruction due to precipitation of a drug or endogenous compound within the kidney. Intrarenal factors include direct tubular obstruction and dysfunction, with or without inflammation, resulting in tubular back-leak and increased tubular pressure. Vascular damage, with or without inflammation, leading to hemodynamic changes. Alterations in the levels of a variety of vasoactive mediators may result in decreased renal perfusion pressure or efferent arteriolar tone and increased afferent arteriolar tone, leading to decreased glomerular hydrostatic pressure. (Modified from Schnellmann and Kelly [1999, p. 15.4], with permission.)

Table 14-2

Mechanisms of Chemically Induced Acute Renal Failure

PRERENAL	VASOCONSTRICTION	CRYSTALLURIA	TUBULAR TOXICITY	ENDOTHELIAL INJURY	GLOMERULOPATHY	INTERSTITIAL NEPHRITIS
Diuretics	Nonsteroidal anti-inflammatory drugs	Sulfonamides	Aminoglycosides	Cyclosporine	Gold	Antibiotics
Angiotensin receptor antagonists	Radiocontrast agents	Methotrexate	Cisplatin	Mitomycin C	Penicillamine	Nonsteroidal anti-inflammatory drugs
Angiotensin-converting enzyme inhibitors	Cyclosporine	Acyclovir	Vancomycin	Tacrolimus	Nonsteroidal anti-inflammatory drugs	Diuretics
Antihypertensive agents	Tacrolimus	Triamterene	Pentamidine	Cocaine		
	Amphotericin B	Ethylene glycol	Radiocontrast agents	Conjugated estrogens		
		Protease inhibitors	Heavy metals	Quinine		
			Haloalkane- and haloalkene-cysteine conjugates			

Extensive evidence supports the idea that renal endothelial injury and inflammatory cells play a critical role in ischemia-induced AKI (Rabb, 2012). Injury to the renal vasculature endothelium results in chemokine and pro-inflammatory cytokine production and neutrophil adhesion (Fig. 14-7). Adhesion of neutrophils to the vascular endothelium leads to capillary damage/leakage and vascular congestion.

Renal dendritic cells, a leukocyte subset found in the interstitial extracellular space, are involved in the initiation of innate immunity after renal injury (Bonventre and Yang, 2011; Kinsey and

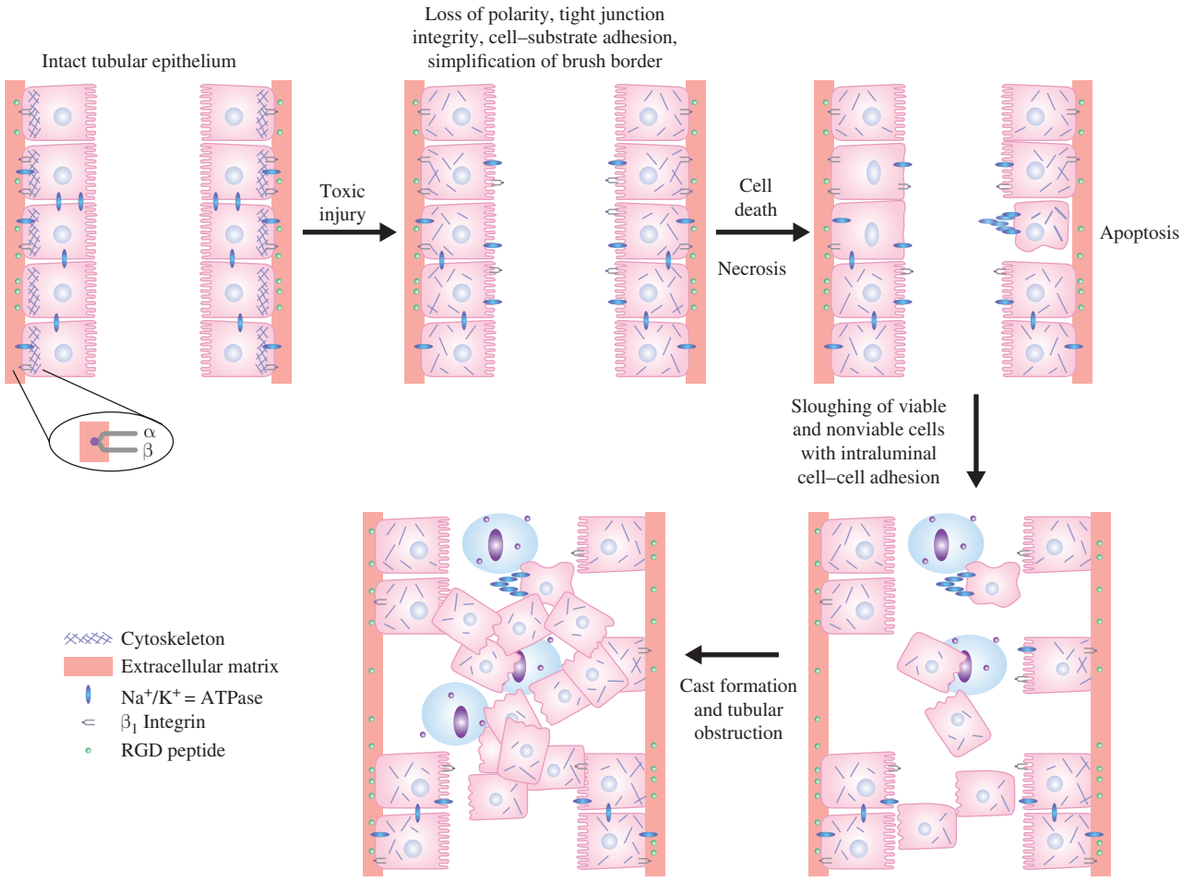

Figure 14-6. *After injury, alterations can occur in the cytoskeleton and in the normal distribution of membrane proteins such as Na^+, K^+-ATPase, and β_1 integrins in sublethally injured renal tubular cells.* These changes result in loss of cell polarity, tight junction integrity, and cell–substrate adhesion. Lethally injured cells undergo oncosis or apoptosis, and both dead and viable cells may be released into the tubular lumen. Adhesion of released cells to other released cells and to cells remaining adherent to the basement membrane may result in cast formation, tubular obstruction, and further compromise the GFR. (From Schnellmann and Kelly [1999, p. 15.5], with permission.)

Okusa, 2011; Rabb, 2012; Rabb et al., 2016). Subsequent influx of macrophage and dendritic cell subpopulations, neutrophils, and lymphocytes (e.g., $CD4^+$ B and T cells) is mediated by cytokines and chemokines produced by the resident dendritic cells and macrophages. These various cells can contribute to kidney injury and promote inflammation. In addition, once the injury has occurred, regulatory T cells and macrophage subsets can decrease the injury and/or promote repair processes. Although significant advances have been made in the past few years concerning the roles of inflammatory cells in AKI, fewer studies have explored their role in chemical-induced AKI.

Adaptation Following Toxic Insult

Fortunately, the kidney has a remarkable ability to compensate for a loss of renal functional mass. Micropuncture studies have revealed that following unilateral nephrectomy, GFR of the remnant kidney increases by approximately 40% to 60%, an effect associated with early compensatory increases in glomerular plasma flow rate and glomerular hydraulic pressure. Compensatory increases in single-nephron GFR are accompanied by proportionate increases in proximal tubular water and solute reabsorption; the glomerulotubular balance is therefore maintained and overall renal function appears normal by standard clinical tests. Consequently, chemically induced changes in renal function may not be detected until these compensatory mechanisms are overwhelmed by significant nephron loss and/or damage.

There are a number of cellular and molecular responses to a nephrotoxic insult. After a population of tubular epithelial cells is exposed to a toxicant, a fraction of the cells will be severely injured and will undergo cell death by apoptosis or oncosis. Those cells that are nonlethally injured may undergo cell repair and/or adaptation, and contribute to the recovery of the nephron (Fig. 14-8). In addition, there is a population of cells that are uninjured and may undergo compensatory hypertrophy, adaptation, and proliferation. Current evidence supports the hypothesis that tubular epithelial cells are primarily responsible for the structural and functional recovery of the nephron following injury and that bone marrow-derived stem cells do not play a significant role (see Humphreys et al. [2008, 2011]). Surviving tubular epithelial cells replace dead and detached cells through dedifferentiation, proliferation, migration, and redifferentiation. Growth factors delivered to tubular epithelial cells from local and systemic sources may help orchestrate the proliferative response of the nephron. Several growth factors

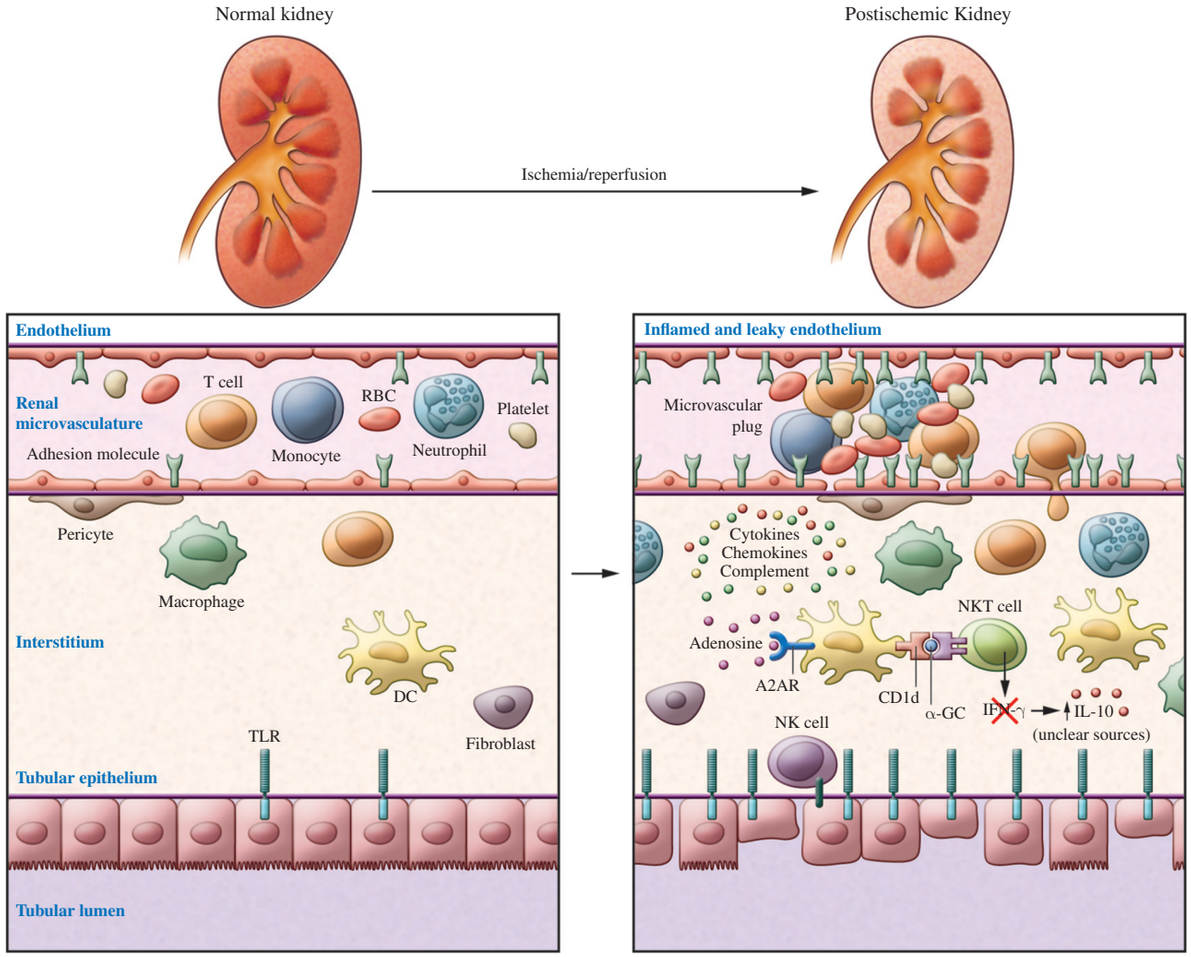

Figure 14-7. *Immune cells mediate AKI.* Immune cells likely mediate AKI while in circulation and when localized in the renal microvasculature, renal interstitium, and lymphoid tissue. While in the renal microcirculation during reperfusion, these cells increase their adhesiveness and adhere to activated endothelium and other cells, accentuating the "plug" that contributes to the no-reflow phenomenon. Some immune cells migrate into the interstitium, but there are also well-described resident renal immune cells. T and B cells, DC, NK and NKT cells, macrophage, and neutrophil crosstalk accentuate the postischemic inflammation. These cells produce and respond to cytokines, chemokines, oxygen-free radicals, complement, coagulant factors, and other mediators. Adenosine, acting via $A_{2A}R$, activates DCs, which in turn modulate NKT cell function by decreasing IFN-γ secretion. This triggers increased IL-10 levels, which subsequently down-regulate postischemic inflammation. Panels depict the outer medulla. (From Rabb [2012], with permission.)

such as epidermal growth factor (EGF) and heparin-binding epidermal growth factor (HB-EGF) have been implicated in proximal tubular regeneration (Hammerman and Miller, 1994), and the EGF receptor is required for this process (Wang et al., 2003; Zhuang et al., 2004; Hallman et al., 2008). Interestingly, exogenous administration of EGF, HGF, or IGF-1 accelerates renal repair following ischemic-, gentamicin-, bromohydroquinone-, and/or $HgCl_2$-induced AKI.

Two of the most notable cellular adaptation responses are metallothionein induction (see the section "Cadmium") and stress protein induction. Heat-shock proteins (HSPs) and glucose-regulated proteins are two examples of stress protein families that are induced in response to a number of pathophysiologic states such as heat shock, anoxia, oxidative stress, toxicants, heavy metal exposure, and tissue trauma (Kelly, 2005; van de Water et al., 2006). The distribution of individual stress proteins varies between different cell types in the kidney and within subcellular compartments (Goering et al., 2000). These proteins play important roles in protein folding, translocation of proteins across organelle membranes, prevention of aggregation of damaged proteins, and repair and degradation of damaged proteins, and thereby provide a defense mechanism against toxicity and/or for the facilitation of recovery and repair. HSP induction in renal tissue has been demonstrated following treatment with nephrotoxicants such as gentamicin (Komatsuda et al., 1993), haloalkane cysteine conjugates (Chen et al., 1992), and $HgCl_2$ (Goering et al., 1992).

Chronic Kidney Disease

It is generally thought that progression to chronic kidney disease (CKD) and end-stage renal failure is not simply a function of a primary renal insult per se, but rather is related to secondary pathophysiologic processes triggered by the initial injury. Animal studies have begun to define a relationship between AKI and CKD. For example, Basile et al. (2001) reported that rats subjected to ischemia

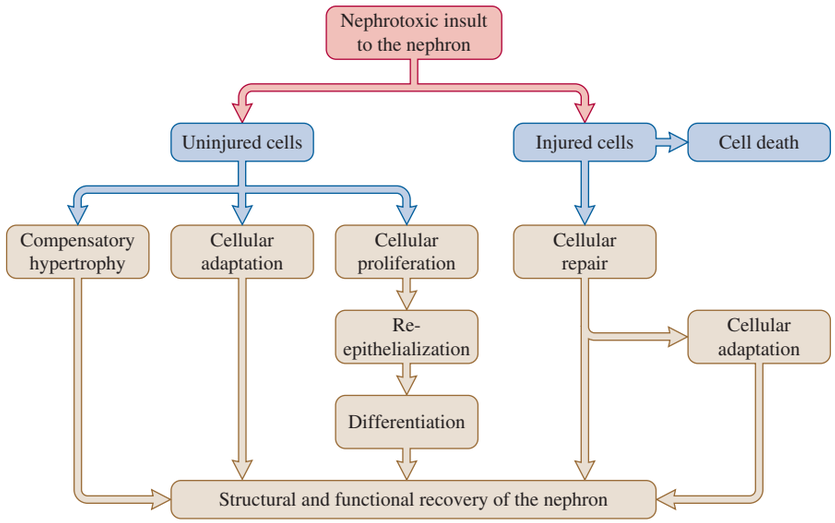

Figure 14-8. *The response of the nephron to a nephrotoxic insult.* After a population of cells is exposed to a nephrotoxicant, the cells respond; ultimately the nephron recovers function or, if cell death and loss are extensive, nephron function ceases. Terminally injured cells undergo cell death through oncosis or apoptosis. Cells injured sublethally undergo repair and adaptation in response to the nephrotoxicant. Cells not injured and adjacent to the injured area may undergo dedifferentiation, proliferation, migration or spreading, and differentiation. Cells not injured may also undergo compensatory hypertrophy in response to the cell loss and injury. Finally, the uninjured cells also may undergo adaptation in response to a nephrotoxicant exposure. (From Schnellmann and Kelly [1999, p. 15.4], with permission.)

undergo renal dysfunction followed by morphologic recovery and return to normal serum creatinine levels. However, 16 weeks following ischemia, protein and albumin excretion increased and microvessel density decreased, markers of CKD. Consequently, a low level of injury or inflammation may exist following AKI that ultimately leads to CKD and/or may sensitize the kidney to a second insult.

Deterioration of renal function may occur with long-term exposure to a variety of chemicals (e.g., analgesics, lithium, and cyclosporine). The progression of chronic renal disease, for example, may be a consequence of the glomerular hemodynamic response to renal injury (Brenner et al., 1982). That is, following nephron loss, there are adaptive increases in glomerular pressures and flows that increase the single-nephron GFR of remnant viable nephrons. Although these compensatory mechanisms serve to maintain whole-kidney GFR, evidence has accumulated to suggest that, with time, these alterations are maladaptive and foster the progression of renal failure. Focal glomerulosclerosis eventually develops and may lead to tubular atrophy and interstitial fibrosis. Consequently, glomerulosclerosis in these nephrons will perpetuate the cycle of triggering further compensatory increases in the hemodynamics of less damaged nephrons, contributing, in turn, to their eventual destruction. Although the underlying mechanisms are not precisely known, compensatory increases in glomerular pressures and flows of the remnant glomeruli may result in mechanical damage to the capillaries due to increased shear stress on the endothelium and damage to the glomerular capillary wall, leading to altered permeabilities, and mesangial thickening due to increased transcapillary flux and local deposition of macromolecules (Dunn et al., 1986). Other factors likely to play a role in the pathogenesis of chronic renal failure include growth promoters and inhibitors, increased extracellular matrix deposition, reactive oxygen species (ROS), lipid accumulation, and tubulointerstitial injury.

SUSCEPTIBILITY OF THE KIDNEY TO TOXIC INJURY

Incidence and Severity of Toxic Nephropathy

A wide variety of drugs, environmental chemicals, and metals can cause nephrotoxicity (Table 14-2). As stated above, it has been estimated that ischemia/reperfusion and nephrotoxicants are responsible for 35% of AKI. Nephrotoxicity is a recognized clinical liability of certain classes of drugs, in particular, antibiotics, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers, analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs), radiocontrast media, and anticancer agents. Approximately 70% of the patients presenting with drug-induced AKI were nonoliguric; the pathologic findings revealed acute tubular necrosis in 60%. Approximately 50% recovered completely. A myriad of risk factors appears to contribute to the incidence/severity of AKI, including genetic/hereditary factors, volume depletion, septic shock, hypotension, multiple chemical insults, age, diabetes, and preexisting renal disease. The consequences of AKI can be profound, as permanent renal damage may result and dialysis or renal transplantation may be required.

Chronic renal failure leading to end-stage renal failure has been associated with long-term abuse of analgesics. The incidence of analgesic nephropathy has been reported to be as high as 20% to 25% in certain countries (e.g., Switzerland). Other chemicals, such as lithium, cyclosporine, NSAIDs, lead, and cadmium, may produce chronic tubulointerstitial nephropathy with progressive loss of renal function.

Reasons for the Susceptibility of the Kidney to Toxicity

The unusual susceptibility of the mammalian kidney to the toxic effects of noxious chemicals can be attributed in part to the unique

physiologic and anatomic features of this organ. Although the kidneys constitute only 0.5% of total body mass, they receive about 20% to 25% of the resting cardiac output. Consequently, any drug or chemical in the systemic circulation will be delivered to these organs in relatively high amounts. The processes involved in forming concentrated urine also serve to concentrate potential toxicants in the tubular fluid. As water and electrolytes are reabsorbed from the glomerular filtrate, chemicals in the tubular fluid may be concentrated, thereby driving passive diffusion of toxicants into tubular cells. Therefore, a nontoxic concentration of a chemical in the plasma may reach toxic concentrations in the kidney. Progressive concentration of toxicants along the nephron may result in intraluminal precipitation of relatively insoluble compounds, causing AKI secondary to tubular obstruction. Finally, renal transport, accumulation, and metabolism of xenobiotics contribute significantly to the susceptibility of the kidney (and specific nephron segments) to toxic injury (see the section "Site-Selective Injury").

In addition to intrarenal factors, the incidence and/or severity of chemically induced nephrotoxicity may be related to the sensitivity of the kidney to circulating vasoactive substances. Under these conditions, vasoconstrictors such as angiotensin II or vasopressin are increased. Normally, the actions of high circulating levels of vasoconstrictor hormones are counterbalanced by the actions of increased vasodilatory prostaglandins; thus, renal blood flow (RBF) and GFR are maintained. However, when prostaglandin synthesis is suppressed by NSAIDs, RBF declines markedly and AKI ensues, due to the unopposed actions of vasoconstrictors. Another example of predisposing risk factors relates to the clinical use of ACE inhibitors. ACE inhibitors have been reported to produce AKI in patients with severe hypertension, due to either bilateral renal artery stenosis or renal artery stenosis in a solitary kidney. Under these conditions, glomerular filtration pressure is dependent on angiotensin II-induced efferent arteriolar constriction. ACE inhibitors will block this vasoconstriction, resulting in a precipitous decline in filtration pressure and AKI.

Site-Selective Injury

Many nephrotoxicants have their primary effects on discrete segments or regions of the nephron. For example, the proximal tubule is the primary target for most nephrotoxic antibiotics, antineoplastics, halogenated hydrocarbons, mycotoxins, and heavy metals, whereas the glomerulus is the primary site for immune complexes, the loop of Henle/collecting ducts for fluoride ions, and the medulla/papilla for chronically consumed analgesic mixtures. The reasons underlying this site-selective injury are complex but can be attributed in part to site-specific differences in blood flow, transport, and accumulation of chemicals, physicochemical properties of the epithelium, reactivity of cellular/molecular targets, balance of bioactivation/detoxification reactions, cellular energetics, and/or regenerative/repair mechanisms. See Sweet (2010) and Lock (2010) for extensive reviews of the transporters and xenobiotic metabolizing enzymes in the kidney.

Glomerular Injury

The glomerulus is the initial site of chemical exposure within the nephron, and a number of nephrotoxicants produce structural injury to this segment. In certain instances, chemicals alter glomerular permeability to proteins by altering the size- and charge-selective functions. Both puromycin aminonucleoside and doxorubicin target glomerular epithelial cells, resulting in changes in size and charge selectivity and proteinuria. The decrease in charge selectivity is thought to result from a decrease in negatively charged sites, while the loss of size selectivity is thought to result from focal detachment of podocytes from the GBM.

Cyclosporine, amphotericin B, and gentamicin are examples of chemicals that impair glomerular ultrafiltration without significant loss of structural integrity and decrease GFR. Amphotericin B decreases GFR by causing renal vasoconstriction and decreasing the glomerular capillary ultrafiltration coefficient (K_f), an effect probably mediated through the endothelial cells. Because of its polycationic nature, the aminoglycoside gentamicin interacts with the anionic sites on the endothelial cells, decreasing K_f, and GFR. Finally, cyclosporine not only causes renal vasoconstriction and vascular damage but also is injurious to the glomerular endothelial cell.

Chemically induced glomerular injury may also be mediated by extrarenal factors. Circulating immune complexes may be trapped within the glomeruli; binding of complement, attraction of neutrophils, and phagocytosis may result. Neutrophils and macrophages are commonly observed within glomeruli in membranous glomerulonephritis, and the local release of cytokines and ROS may contribute to glomerular injury. Heavy metals (e.g., $HgCl_2$, gold, and cadmium), hydrocarbons, penicillamine, and captopril can produce this type of glomerular injury. A chemical may function as a hapten attached to some native protein (e.g., tubular antigens released secondary to toxicity) or as a complete antigen—particularly if it is sequestered within the glomerulus via electrostatic interactions—and elicit an antibody response. Antibody reactions with cell-surface antigens (e.g., GBM) lead to immune deposit formation within the glomeruli, mediator activation, and subsequent injury to glomerular tissue. Volatile hydrocarbons, solvents, and $HgCl_2$ have been implicated in this type of glomerulonephritis. See Bikbov et al. (2010) for more information on glomerular injury.

Proximal Tubular Injury

The proximal tubule is the most common site of toxicant-induced renal injury. The reasons for this relate in part to the selective accumulation of xenobiotics into this segment of the nephron. For example, in contrast to the distal tubule, which is characterized by a relatively tight epithelium with high electrical resistance, the proximal tubule has a leaky epithelium, favoring the flux of compounds into proximal tubular cells. More importantly, tubular transport of organic anions and cations, low-molecular-weight proteins and peptides, GSH conjugates, and heavy metals is localized primarily if not exclusively to the proximal tubule. Thus, transport of these molecules will be greater in the proximal tubule than in other segments, resulting in proximal tubular accumulation and toxicity. Indeed, segmental differences in transport and accumulation appear to play a significant role in the onset and development of proximal tubular toxicity associated with certain drugs such as aminoglycosides, β-lactam antibiotics, and cisplatin; environmental chemicals such as ochratoxin, haloalkene *S*-conjugates, D-limonene, and 2,4,4-trimethylpentane; and metals such as cadmium and mercury. Although correlations between proximal tubular transport, accumulation, and toxicity suggest that the site of transport is a crucial determinant of the site of toxicity, transport is unlikely to be the sole criterion. For example, the S_2 segment is the primary site of transport and toxicity of cephaloridine, and several lines of evidence suggest a strong correlation between the transport, accumulation, and nephrotoxicity of this antibiotic. However, when a variety of cephalosporins are considered, the rank order of accumulation does not follow the rank order of nephrotoxicity; for example, renal cortical concentrations of the potent nephrotoxicant cephaloglycin are comparable to those of the relatively nontoxic cephalexin. Thus,

site-specific transport and accumulation are necessary but not sufficient to cause proximal tubular toxicity of cephalosporins. Once taken up and sequestered by the proximal tubular cell, the nephrotoxic potential of these drugs ultimately may be dependent upon the intrinsic reactivity of the drug with subcellular or molecular targets.

In addition to segmental differences in transport, segmental differences in cytochrome P450 and cysteine conjugate β-lyase activity also are contributing factors to the enhanced susceptibility of the proximal tubule. Both enzyme systems are localized almost exclusively in the proximal tubule, with negligible activity in the glomerulus, distal tubules, or collecting ducts. Thus, nephrotoxicity requiring P450 and β-lyase-mediated bioactivation will most certainly be localized in the proximal tubule. Indeed, the site of proximal tubular bioactivation contributes at least in part to the proximal tubular lesions produced by chloroform (via cytochrome P450) and by haloalkene *S*-conjugates (via cysteine β-lyase).

Finally, proximal tubular cells appear to be more susceptible to ischemic injury than distal tubular cells. Therefore, the proximal tubule will likely be the primary site of toxicity for chemicals that interfere with RBF, cellular energetics, and/or mitochondrial function.

Loop of Henle/Distal Tubule/Collecting Duct Injury

Chemically induced injury to the more distal tubular structures, compared to the proximal tubule, is an infrequent occurrence. Functional abnormalities at these sites manifest primarily as impaired concentrating ability and/or acidification defects. Drugs that have been associated with acute injury to the more distal tubular structures include amphotericin B, cisplatin, and methoxyflurane. Each of these drugs induces an ADH-resistant polyuria, suggesting that the concentrating defect occurs at the level of the medullary thick ascending limb and/or the collecting duct. However, the mechanisms mediating these drug-induced concentrating defects appear to be different. Amphotericin B is highly lipophilic and interacts with lipid sterols such as cholesterol, resulting in the formation of transmembrane channels or pores and disrupting membrane permeability (Bernardo and Branch, 1997). Thus, amphotericin effectively transforms the tight distal tubular epithelium into one that is leaky to water and ions and impairs reabsorption at these sites. The mechanisms mediating cisplatin-induced polyuria are not completely understood, but the first phase is responsive to vasopressin and inhibitors of prostaglandin synthesis (Safirstein and Deray, 1998). The second phase is not responsive to vasopressin or prostaglandin synthesis inhibitors but is associated with decreased papillary solute content. Methoxyflurane nephrotoxicity is associated with the inhibitory effects of the metabolite fluoride on solute and water reabsorption (Jarnberg, 1998). Fluoride inhibits sodium chloride reabsorption in the thick ascending limb and inhibits ADH-mediated reabsorption of water, possibly due to disruption in adenylate cyclase.

Papillary Injury

The renal papilla is susceptible to the chronic injurious effects of abusive consumption of analgesics. The initial target is the medullary interstitial cells, followed by degenerative changes in the medullary capillaries, loops of Henle, and collecting ducts (Bach, 1997). Although the exact mechanisms underlying selective damage to the papilla by analgesics are not known, the intrarenal gradient for prostaglandin H synthase activity has been implicated as a contributing factor. This activity is highest in the medulla and least in the cortex, and the prostaglandin hydroperoxidase component metabolizes phenacetin to reactive intermediates capable of covalent binding to cellular macromolecules. Other factors may contribute to this site-selective injury, including high papillary concentrations of potential toxicants and inhibition of vasodilatory prostaglandins, compromising RBF to the renal medulla/papilla, and resulting in tissue ischemia. The lack of animal models that mimic the papillary injury observed in humans has limited mechanistic research in this area (Schnellmann, 1998).

ASSESSMENT OF RENAL FUNCTION

Evaluation of the effects of a chemical on the kidney can be accomplished using a variety of both in vivo and in vitro methods. Initially, nephrotoxicity can be assessed by evaluating serum and urine chemistries following treatment with the chemical in question. The standard battery of noninvasive tests includes measurement of urine volume and osmolality, pH, and urinary composition (e.g., electrolytes, glucose, and protein). Although specificity is often lacking in such an assessment, urinalysis provides a relatively easy and noninvasive assessment of overall renal functional integrity and can provide some insight into the nature of the nephrotoxic insult. For example, chemically induced increases in urine volume accompanied by decreases in osmolality may suggest an impaired concentrating ability, possibly via a defect in ADH synthesis, release, and/or action. To determine whether the impaired concentrating ability is due to an altered tubular response to ADH, concentrating ability can be determined before and after an exogenous ADH challenge. Glucosuria may reflect chemically induced defects in proximal tubular reabsorption of sugars; however, because glucosuria also may be secondary to hyperglycemia, measurement of serum glucose concentrations also must be evaluated. Urinary excretion of high-molecular-weight proteins, such as albumin, is suggestive of glomerular damage, whereas excretion of low-molecular-weight proteins, such as β_2-microglobulin, suggests proximal tubular injury. Urinary excretion of enzymes localized in the brush border (e.g., alkaline phosphatase and γ-glutamyl transpeptidase) may reflect brush-border damage, whereas urinary excretion of other enzymes (e.g., lactate dehydrogenase) may reflect more generalized cell damage. Enzymuria is often a transient phenomenon, as chemically induced damage may result in an early loss of most of the enzyme available. Thus, the absence of enzymuria does not necessarily reflect an absence of damage. In vivo methodologies used to assess renal function and injury have recently been reviewed (Hart, 2010).

The simultaneous analysis of cellular metabolites in sera and urine using nuclear magnetic analysis (metabonomics) has matured over the past few years and may provide an additional technology to identify and monitor nephrotoxicity (Coen et al., 2008). For example, rats treated with the nephrotoxicant $HgCl_2$ exhibited increased levels of threonine, isobutyric acid, glutamate, and lysine in renal cortical tissue (Wang et al., 2006) and increased levels of isoleucine and lysine and decreased levels of fumarate in the urine. These changes were associated with renal dysfunction (Holmes et al., 2006).

GFR can be quantified directly by determining creatinine or inulin clearance. Creatinine is an endogenous compound released from skeletal muscle at a constant rate under most circumstances. Further, it is completely filtered with limited secretion. Inulin is an exogenous compound that is completely filtered with no reabsorption or secretion. Following the injection of inulin, inulin serum and urinary concentrations and urine volume are determined over time. If creatinine is being used, then serum and urinary creatinine concentrations and

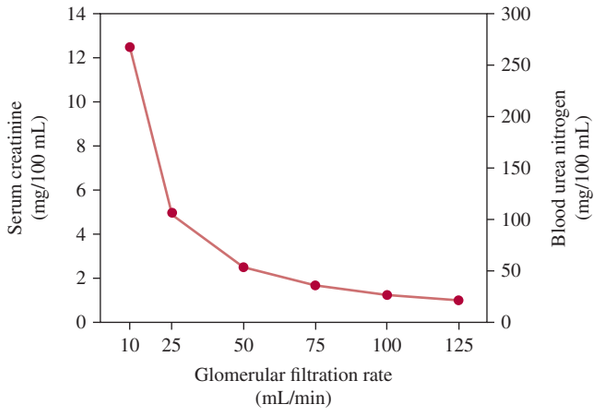

Figure 14-9. *Relationships among GFR, serum creatinine, and BUN concentrations in the determination of renal function.* In general, approximately 50% of renal function must be lost before serum creatinine or BUN increases. (From Tarloff and Kinter [1997], with permission.)

urine volume are determined over time. Creatinine or inulin clearance is determined by the following formula:

$$\text{Inulin clearance (mL/min)} = \frac{\text{Inulin concentration in urine (mg/L)} \times \text{Urine volume (mL/min)}}{\text{Inulin concentration in serum (mg/L)}}$$

Indirect markers of GFR are serial blood urea nitrogen (BUN) and serum creatinine concentrations. However, both serum creatinine and BUN are rather insensitive indices of GFR; a 50% to 70% decrease in GFR must occur before increases in serum creatinine and BUN develop (Fig. 14-9). Chemically induced increases in BUN and/or serum creatinine may not necessarily reflect renal damage but rather may be secondary to dehydration, hypovolemia, and/or protein catabolism. These extrarenal events should be taken into consideration in evaluating BUN/serum creatinine as potential endpoints of renal toxicity and/or when correlating these endpoints with renal histopathology.

Cystatin C, a 13-kDa endogenous protein that inhibits cysteine proteases, is a viable candidate to replace creatinine in the measurement of GFR (Filler et al., 2005; Hart and Kinter, 2005; Stevens and Levey, 2005). It is produced at a constant rate by all tissues, freely filtered by the glomerulus and catabolized by the tubular epithelial cells; thus, its use is limited to serum levels and not urinary levels. Serum cystatin C levels appear to be independent of height, gender, age, muscle mass, and coexisting diseases and are more sensitive than creatinine in mildly impaired GFR.

The lack of sensitivity of current biomarkers of renal dysfunction has resulted in extensive searches for better biomarkers of AKI. Better biomarkers would not only advance the timely diagnosis of AKI but would also allow for a better prediction of severity and greatly advance safety assessment during drug development. Vaidya et al. (2010) have reviewed site-specific biomarkers for common nephrotoxicants and mechanisms of injury (Fig. 14-10). At this time kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), *N*-acetyl-β-glucosaminidase (NAG), fatty acid–binding protein (FABP), hepatocyte growth factor (HGF), and albumin are promising biomarkers of AKI.

Histopathologic evaluation of the kidney following treatment is crucial in identifying the site, nature, and severity of a nephrotoxic lesion. Assessment of chemically induced nephrotoxicity, therefore, should include urinalysis, serum clinical chemistry, and histopathology to provide a reasonable profile of the functional and morphologic effects of a chemical on the kidney. Further, information on the biotransformation and toxicokinetics of the chemical should be used to direct further in vivo and in vitro studies, particularly, what metabolites are found in the kidney and what are the concentrations of parent compound and metabolites in the kidney over time.

Once a chemical has been identified as a nephrotoxicant in vivo, a variety of in vitro techniques may be used to elucidate underlying mechanisms (Table 14-3). Tissue obtained from naive animals may be used in the preparation of isolated perfused kidneys, kidney slices, isolated suspensions of renal tubules, cells, or subcellular organelles, primary cultures of renal cells, and established renal cell lines. For example, freshly prepared isolated perfused kidneys, kidney slices, and renal tubular suspensions and cells exhibit the greatest degree of differentiated functions and similarity to the in vivo situation. However, these models have limited lifespans of 2 to 24 hours. In contrast, primary cultures of renal cells and established renal cell lines exhibit longer lifespans (more than 2 weeks), but—by comparison to the in vivo condition—exhibit less differentiated functions and similarity; this is particularly true of immortalized renal cell lines. The reader is referred to several excellent reviews for further details on the utility and limitations of these preparations (Ford, 1997, 2005; Tarloff and Kinter, 1997; Hart and Kinter, 2005; Kirkpatrick and Gandolfi, 2005). Such approaches may be used to distinguish between an effect on the kidney due to a direct chemical insult and one caused by extrarenal effects, such as extrarenally generated metabolites, hemodynamic effects, immunologic effects, and so forth. Care must be taken to ensure that the cell type affected in the in vitro model is the same as that affected in vivo. In addition, concentrations of the nephrotoxicant to be used in the in vitro preparations must be comparable to those observed in vivo, as different mechanisms of toxicity may be operative at concentrations that saturate metabolic pathways or overwhelm detoxification mechanisms. Once a mechanism has been identified in vitro, the postulated mechanism must be tested in vivo. Thus, appropriately designed in vivo and in vitro studies should provide a complete characterization of the biochemical, functional, and morphologic effects of a chemical on the kidney and an understanding of the underlying mechanisms in the target cell population(s).

BIOCHEMICAL MECHANISMS/MEDIATORS OF RENAL CELL INJURY

Cell Death

In many cases, renal cell injury may culminate in cell death. In general, cell death is thought to occur through either oncosis or apoptosis (Levin et al., 1999). The morphologic and biochemical characteristics of oncosis ("necrotic cell death") and apoptosis are very different. For example, apoptosis is a tightly controlled, organized process that usually affects scattered individual cells. The organelles retain integrity while cell volume decreases. Ultimately, the cell breaks into small fragments that are phagocytosed by adjacent cells or macrophages without producing an inflammatory response. Caspases, a class of cysteine proteases, are primary mediators of apoptosis. In contrast, oncosis often affects many contiguous cells; the organelles swell, cell volume increases, and the cell ruptures with the release of cellular contents, followed by inflammation. The receptor interacting protein (RIP) kinase family has recently been reported to be mediators of oncosis (Declercq et al., 2009). The reader is encouraged to see Chap. 3, "Mechanisms of Toxicity," for additional details of apoptosis and oncosis. With many toxicants, lower but injurious concentrations produce cell death through apoptosis. As the concentration of the toxicant

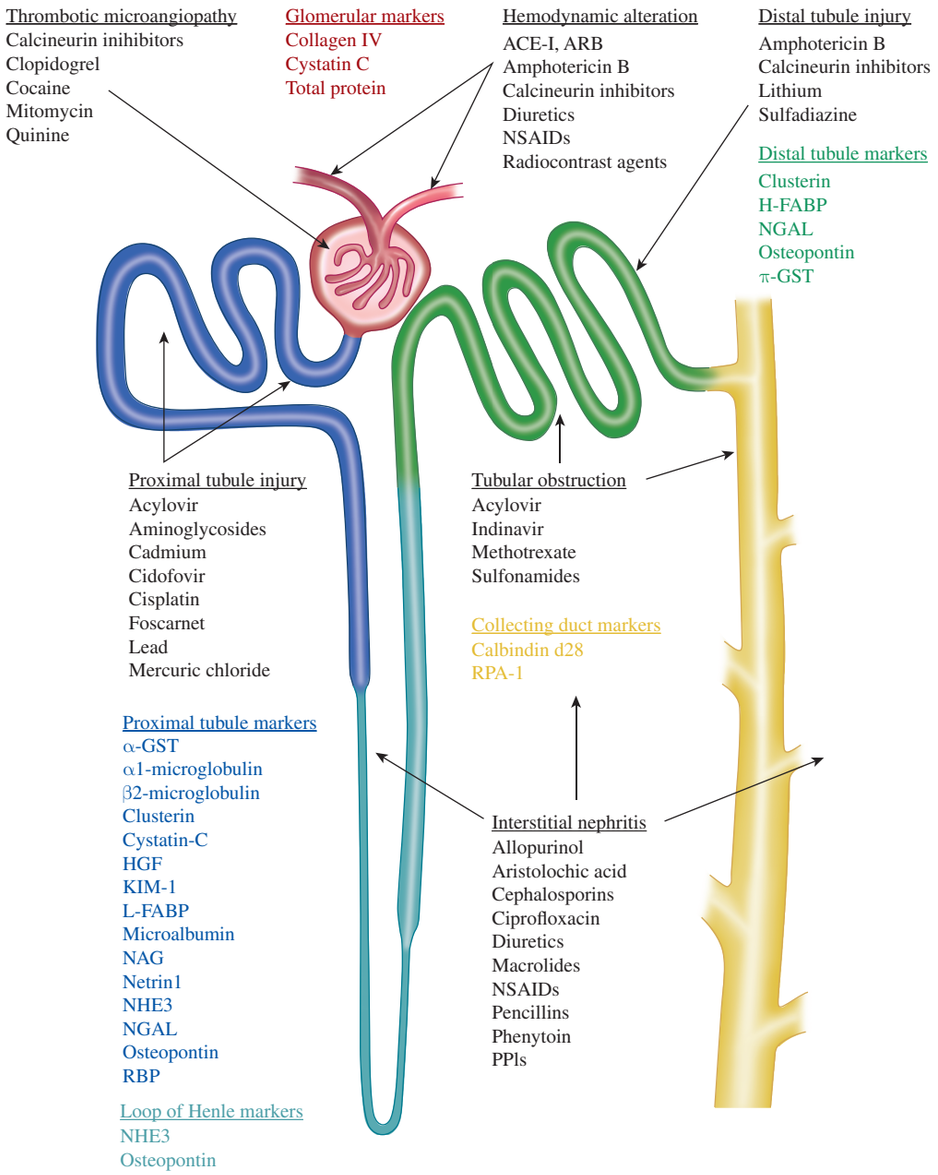

Figure 14-10. *Site-specific biomarkers, common nephrotoxicants, and mechanisms of injury.* (From Vaidya et al. [2010].)

increases, oncosis plays a predominant role. However, because apoptosis is an ATP-dependent process, for those toxicants that target the mitochondrion, oncosis may be the predominant pathway with only limited apoptosis occurring. In general, nephrotoxicants produce cell death through apoptosis and oncosis, and it is likely that both forms of cell death contribute to AKI.

Mediators of Toxicity

A chemical can initiate cell injury by a variety of mechanisms. In some cases, the chemical may initiate toxicity due to its intrinsic reactivity with cellular macromolecules. For example, amphotericin B reacts with plasma membrane sterols, increasing membrane permeability; fumonisin B_1 inhibits sphinganine (sphingosine) *N*-acyltransferase; and Hg^{2+} binds to sulfhydryl groups on cellular proteins. In contrast, some chemicals are not toxic until they are biotransformed to a reactive intermediate. Biologically reactive intermediates, also known as alkylating agents, are electron-deficient compounds (electrophiles) that bind to cellular nucleophiles (electron-rich compounds), such as proteins and lipids. For example, acetaminophen and chloroform are metabolized in the mouse kidney by cytochrome P450 to the reactive intermediates, *N*-acetyl-*p*-benzoquinoneimine, and phosgene, respectively (see the sections "Chloroform" and "Acetaminophen"). The covalent binding of the reactive intermediate to critical cellular macromolecules is thought to interfere with the normal biological activity of the macromolecule and thereby initiate cellular injury. In other instances, extrarenal biotransformation may be required prior to the delivery of the penultimate nephrotoxic species to the proximal tubule, where it is metabolized further to a reactive intermediate.

Finally, chemicals may initiate injury indirectly by inducing oxidative stress via increased production of ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. ROS can react with a variety of cellular constituents to induce toxicity. For

Table 14-3

Models to Study Renal Function

In Vivo
Continuous microperfusion
Stopped-flow microperfusion
Free-flow micropuncture
Confocal microscopy
Ex Vivo
Isolated perfused kidney
Isolated perfused tubules
In Vitro
Renal slices
Freshly isolated and purified glomeruli
Freshly isolated and purified tubular segments
Freshly isolated and purified proximal tubular epithelial cells
Primary cultures of renal cells
Tubular epithelial cells
Glomerular cells
Fibroblasts
Immortalized renal epithelial cell lines
LLC-PK1
MDCK
NRK-52E
OK
HK-2

example, ROS are capable of inducing lipid peroxidation, which may result in altered membrane fluidity, enzyme activity, and membrane permeability and transport characteristics; inactivating cellular enzymes by directly oxidizing critical protein sulfhydryl or amino groups; depolymerizing polysaccharides; and inducing DNA strand breaks and chromosome breakage. Each of these events could lead to cell injury and/or death. Oxidative stress has been proposed to contribute, at least in part, to the nephrotoxicity associated with ischemia/reperfusion injury, gentamicin, cyclosporine, cisplatin, and haloalkene cysteine conjugates.

Although nitric oxide is an important second messenger in a number of physiologic pathways, recent studies suggest that in the presence of oxidative stress, nitric oxide can be converted into reactive nitrogen species that contribute to cellular injury and death. For example, in the presence of superoxide anion, nitric oxide can be transformed into peroxynitrite ($ONOO^-$), a strong oxidant and nitrating species. Proteins, lipids, and DNA are all targets of peroxynitrite. The primary evidence for a role of peroxynitrite in renal ischemia/reperfusion injury is the formation of nitrotyrosine–protein adducts, and the attenuation of renal dysfunction through the inhibition of the inducible form of nitric oxide synthase.

Cellular/Subcellular and Molecular Targets

A number of cellular targets have been identified to play a role in cell death. It is generally thought that an intracellular interaction (e.g., an alkylating agent or ROS with a macromolecule) initiates a sequence of events that leads to cell death. In the case of oncosis, a "point of no return" is reached in which the cell will die regardless of any intervention. The idea of a single sequence of events is probably simplistic for most toxicants, given the extensive number of targets available for alkylating species and ROS. Rather, multiple pathways, with both distinct and common sequences of events, may lead to cell death. In the following paragraphs, examples of molecular targets will be discussed and a more conclusive list can be found in Lash and Cummings (2010).

Many cellular processes depend on mitochondrial ATP and thus become compromised simultaneously with inhibition of respiration. Conversely, mitochondrial dysfunction may be a consequence of some other cellular process altered by the toxicant. Numerous nephrotoxicants cause mitochondrial dysfunction (Schnellmann and Griner, 1994). For example, following an in vivo exposure, $HgCl_2$ altered isolated renal cortical mitochondrial function and mitochondrial morphology prior to the appearance of tubular necrosis (Weinberg et al., 1982b). Furthermore, $HgCl_2$ produced similar changes in various respiratory parameters when added to isolated rat renal cortical mitochondria (Weinberg et al., 1982a). Various toxicants also produce different types of mitochondrial dysfunction. For example, pentachlorobutadienyl-L-cysteine initially uncouples oxidative phosphorylation in renal proximal tubular cells by dissipating the proton gradient, whereas TFEC does not uncouple oxidative phosphorylation but rather inhibits state 3 respiration by inhibiting sites I and II of the electron transport chain (Schnellmann et al., 1987, 1989; Wallin et al., 1987; Hayden and Stevens, 1990).

Whether toxicants target mitochondria directly or indirectly, it is clear that mitochondria play a critical role in determining whether cells die by apoptosis or oncosis. The mitochondrial permeability transition (MPT) is characterized by the opening of a high-conductance pore that allows solutes of less than 1500 molecular weight to pass (Lemasters et al., 1999). It is thought that the MPT occurs during cell injury and ultimately progresses to apoptosis if sufficient ATP is available, or oncosis if ATP is depleted. Further, the release of apoptotic proteins, such as apoptosis-inducing factor, cytochrome *c*, Smac/Diablo, Omi, and endonuclease G following MPT play a key role in activating downstream caspases and executing apoptosis.

Ca^{2+} is a second messenger and plays a critical role in a variety of cellular functions. Sustained elevations or abnormally large increases in cytosolic free Ca^{2+} can exert a number of detrimental effects on the cell. For example, an increase in cytosolic free Ca^{2+} can activate a number of degradative Ca^{2+}-dependent enzymes, such as phospholipases and proteinases (e.g., calpains), and can produce aberrations in the structure and function of cytoskeletal elements. Release of endoplasmic reticulum (ER) Ca^{2+} stores may be a key step in initiating the injury process and increasing cytosolic free Ca^{2+} concentrations, because prior depletion of ER Ca^{2+} stores protects renal proximal tubules from extracellular Ca^{2+} influx and cell death produced by mitochondrial inhibition and hypoxia (Waters et al., 1997b; Harriman et al., 2002). Further, the release of ER Ca^{2+} activates calpains that leads to further disruption of ion homeostasis, cleavage of cytoskeleton proteins, cell swelling, and, ultimately oncosis (Waters et al., 1997a; Liu and Schnellmann, 2003). Mitochondria are known to accumulate Ca^{2+} in lethally injured cells through a low-affinity, high-capacity Ca^{2+} transport system. Although this system plays a minor role in normal cellular Ca^{2+} regulation, under injurious conditions the uptake of Ca^{2+} may facilitate ROS formation and damage.

Caspases are another class of cysteine proteinases that play a role in the initiation and execution of renal cell apoptosis. A number of caspases have been identified in the rat kidney (e.g., caspases 1, 2, 3, 6, 9), and rat kidneys subjected to ischemia/reperfusion injury exhibit differential expression of caspases with marked increases in caspases 1 and 3 (Kaushal et al., 1998, 2004). The administration of a pancaspase inhibitor blocked the increase in caspase activities and renal injury following ischemia/reperfusion injury (Daemen et al., 1999).

Table 14-4

Selected Signaling Kinases Involved in Renal Cell Injury, Survival, or Repair

KINASE	LOCATION	NEPHROTOXICANT	REFERENCES
Protein kinase C (PKC)			
Conventional PKC			
PKCα	Proximal tubules	Cisplatin, DCVC*	Nowak (2002), Liu et al. (2004)
Novel PKC			
PKCε	Proximal tubules	TBHP†	Nowak et al. (2004, 2011)
Mitogen-activated protein kinase (MAPK)			
ERK1/2	Proximal tubules	Cisplatin, H_2O_2, TBHP, TGHQ‡	Nowak (2002), Ramachandiran et al. (2002), Arany et al. (2004), Dong et al. (2004), Zhuang et al. (2004), Nowak et al. (2006), Zhuang et al. (2007, 2008)
JNK/SAPK*	Proximal tubules	Cisplatin	Arany et al. (2004)
P38	Proximal tubules	Cisplatin, H_2O_2, TGHQ‡	Ramachandiran et al. (2002), Arany et al. (2004), Dong et al. (2004), Zhuang et al. (2005)
Other kinases			
Protein kinase B	LLC-PK1, Proximal tubules	Cisplatin, mechanical injury, H_2O_2, DCVC	Zhuang et al. (2004), Shaik et al. (2007, 2008)

* *S-(1,2)-Dichlorovinyl-L-cysteine.*
† *t-Butylhydroperoxide.*
‡ *2,3,5-tris-(Glutathion-S-yl)hydroquinone.*
Protein kinase B is also known as AKT.

Signaling kinases such as protein kinase C, mitogen-activated protein kinases (e.g., ERK1/2, p38, and JNK/SAPK), protein kinase B (Akt), src, and phosphoinositide-3-kinase phosphorylate other proteins and, thereby, alter their activity, expression, or localization. Numerous recent studies reveal critical roles for signaling kinases in renal cell death and in the recovery of renal cells after toxicant injury (Table 14-4). For example, PKC mediates fumonisin-B1- and TNF-induced toxicity in human renal cells and in LLC-PK1 cells (Woo et al., 1996; Nowak, 2002; Gopee et al., 2003). However, the exact role of PKC in renal cell death depends on the toxicant and the specific isoform(s) involved (Nowak, 2002, 2003; Nowak et al., 2004, 2011). Activation of PKCα contributes to mitochondrial dysfunction and cell death in rabbit RPTC exposed to cisplatin, whereas PKCε mediates mitochondrial dysfunction and cell death after exposure. Interestingly, cisplatin- and oxidant-induced PKC activation was followed by ERK1/2 activation, which decreased mitochondrial membrane potential and caused caspase 3 activation and apoptosis. Experiments using LLC-PK1 cells exposed to 2,3,5-tris-(glutathione-*S*-yl)hydroquinone (TGHQ) and in vivo studies in mice exposed to cisplatin both support a role for ERK in renal cell death (Ramachandiran et al., 2002; Arany et al., 2004; Dong et al., 2004; Sheikh-Hamad et al., 2004; van de Water et al., 2006). Other examples of signaling kinases that play a role in nephrotoxicant injury are found in Table 14-4.

Cell volume and ion homeostasis are tightly regulated and are critical for the reabsorptive properties of the tubular epithelial cells. Toxicants can disrupt cell volume and ion homeostasis by interacting directly with the plasma membrane and increasing ion permeability or by inhibiting energy production. The loss of ATP during oncosis, for example, results in the inhibition of membrane transporters that maintain the internal ion balance and drive transmembrane ion movement. Following ATP depletion, Na^+, K^+-ATPase activity decreases, resulting in K^+ efflux, Na^+ and Cl^- influx, cell swelling, and ultimately cell membrane rupture (Miller and Schnellmann 1993, 1995). In contrast, the cell shrinkage that occurs during apoptosis is mediated by K^+ and Cl^- efflux through respective channels and inhibition of these channels is cytoprotective (Okada et al., 2004).

SPECIFIC NEPHROTOXICANTS

Heavy Metals

Many metals, including cadmium, chromium, lead, mercury, platinum, and uranium, are nephrotoxic (Fowler, 2010; Zalups, 2010). It is important to recognize that the nature and severity of metal nephrotoxicity vary with respect to its form. For example, salts of inorganic mercury produce a greater degree of renal injury and a lesser degree of neurotoxicity than do organic mercury compounds; the neurotoxicity is associated with the higher lipophilicity of organic mercury compounds (Conner and Fowler, 1993; Zalups and Lash, 1994). In addition, different metals have different primary targets within the kidney. For example, potassium dichromate and cadmium primarily affect the S_1 and S_2 segments of the proximal tubule, whereas mercuric chloride affects the S_2 and S_3 segments (Zalups and Lash, 1994; Zalups and Diamond, 2005).

Metals may cause toxicity through their ability to bind to sulfhydryl groups. For example, the affinity of mercury for sulfhydryl groups is very high and is about 10 orders of magnitude higher than the affinity of mercury for carbonyl or amino groups (Ballatori, 1991). Thus, metals may cause renal cellular injury through their ability to bind to sulfhydryl groups of critical proteins within the cells and thereby inhibit their normal function.

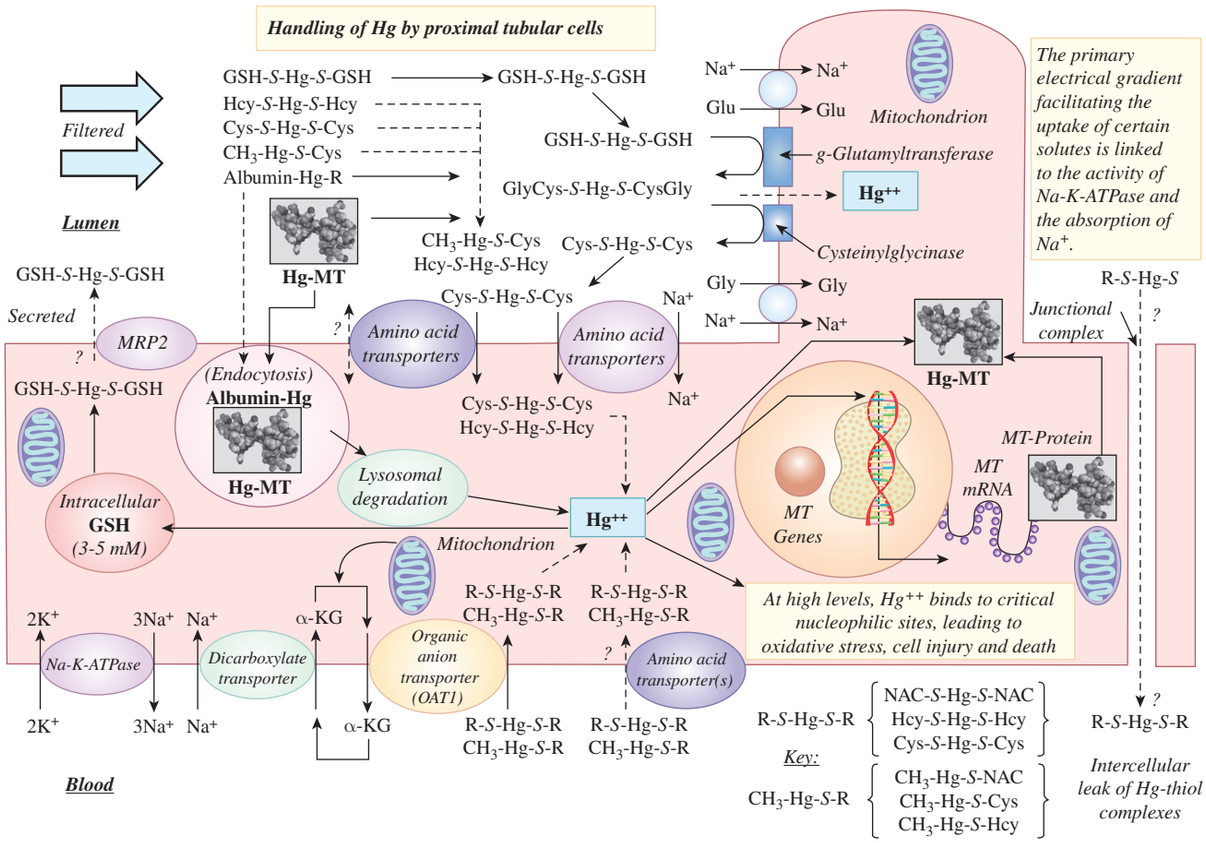

Figure 14-11. *Cellular transport of Hg^{2+}.* Proximal tubular uptake of inorganic mercury is thought to be the result of the transport of Hg^{2+} conjugates (e.g., diglutathione-Hg^{2+} conjugate [GSHHg-GSH], dicysteine-Hg^{2+} conjugate [CYS-HG-CYS]). At the luminal membrane, GSH-Hg-GSH is metabolized by γ-GT and a dipeptidase to form CYS-HG-CYS. CYS-HG-CYS may be taken up by amino acid transporters. It is not clear whether albumin-Hg-R conjugates are transported across the liminal membrane in vivo. At the basolateral membrane, Hg^{2+}-conjugates appear to be transported by organic anion transporters OAT1 and OAT3. (Used with permission from Zalups RK, Diamond GL. Nephrotoxicology of metals. In: Tarloff JB, Lash LH, eds. Toxicology of the Kidney. 3rd ed. CRC Press; 2005:954.)

Mercury Humans and animals are exposed to elemental mercury vapor, inorganic mercurous and mercuric salts, and organic mercuric compounds through the environment. Administered elemental mercury is rapidly oxidized in erythrocytes or tissues to inorganic mercury, and thus the tissue distribution of elemental and inorganic mercury is similar. Due to its high affinity for sulfhydryl groups, virtually all of the Hg^{2+} found in blood is bound to cells—albumin, other sulfhydryl-containing proteins, glutathione, and cysteine.

The kidneys are the primary target organs for accumulation of Hg^{2+}, and the S_3 segment of the proximal tubule is the initial site of toxicity. As the dose or duration of treatment increases, the S_1 and S_2 segments may be affected. Renal uptake of Hg^{2+} is very rapid with as much as 50% of a nontoxic dose of Hg^{2+} found in the kidneys within a few hours of exposure. Considering the fact that virtually all of the Hg^{2+} found in blood is bound to an endogenous ligand, it is likely that the luminal and/or basolateral transport of Hg^{2+} into the proximal tubular epithelial cell is through cotransport of Hg^{2+} with an endogenous ligand such as glutathione, cysteine, or albumin, or through some plasma membrane Hg^{2+}-ligand complex (Zalups and Diamond, 2005). Current evidence indicates that at least two mechanisms are involved in the proximal tubular uptake of Hg^{2+} (Fig. 14-11) (Zalups and Diamond, 2005). One mechanism appears to involve the apical activity of γ-glutamyl transpeptidase, cysteinylglycinase, and the transport of Cys–S–Hg–S–Cys through one or more amino acid transporters. Basolateral membrane transport is likely to be mediated by the organic anion transport system.

The acute nephrotoxicity induced by $HgCl_2$ is characterized by proximal tubular necrosis and AKI within 24 to 48 hours after administration (Zalups, 1997). Early markers of $HgCl_2$-induced renal dysfunction include an increase in the urinary excretion of brush-border enzymes such as alkaline phosphatase and γ-GT, suggesting that the brush border may be an initial target of $HgCl_2$. As injury progresses, tubular reabsorption of solutes and water decreases and there is an increase in the urinary excretion of glucose, amino acids, albumin, and other proteins. Associated with the increase in injured proximal tubules is a decrease and progressive decline in the GFR. For example, GFR was reduced 35% in rats within 6 hours of $HgCl_2$ administration and continued to decline to 32% and 16% of controls at 12 and 24 hours, respectively (Eknoyan et al., 1982). The reduction in GFR results from the glomerular injury, tubular injury, and/or vasoconstriction. Interestingly, there is an early decrease in RBF secondary to the vasoconstriction. RBF may return to normal within 24 to 48 hours, whereas GFR continues to decline. If the decline in renal function is not too severe, the remaining proximal tubular cells undergo a proliferative response and renal function returns over time. Chelation therapy with 2,3-dimercaptopropane-1-sulfonate or 2,3-mesodimercaptosuccinic acid is used for the treatment of mercury-induced nephrotoxicity (Zalups and Diamond, 2005).

As stated above, inorganic mercury has a very high affinity for protein sulfhydryl groups, and this interaction is thought to play an important role in the toxicity of mercury at the cellular level. Changes in mitochondrial morphology and function are very early events following $HgCl_2$ administration, supporting the hypothesis that mitochondrial dysfunction is an early and important contributor to inorganic mercury–induced cell death along the proximal tubule. Other studies have suggested that oxidative stress and dysregulation of Ca^{2+} homeostasis plays an important role in $HgCl_2$-induced renal injury (Fukino et al., 1984; Smith et al., 1987; Lund et al., 1993).

Several animal studies have shown that chronic exposure to inorganic mercury results in an immunologically mediated membranous glomerular nephritis secondary to the production of antibodies against the GBM and the deposition of immune complexes (Zalups and Diamond, 2005). See Zalups (2010) for additional information.

Cadmium Chronic exposure of nonsmoking humans and animals to cadmium is primarily through food and results in nephrotoxicity (Kido and Nordberg, 1998; Zalups and Diamond, 2005; Byber et al., 2016). In the workplace, inhalation of cadmium-containing dust and fumes is the major route of exposure. Cadmium has a half-life of greater than 10 years in humans and thus accumulates in the body over time. Approximately 50% of the body burden of cadmium can be found in the kidney and nephrotoxicity can be observed when Cd concentrations exceed 50 μg/g kidney wet weight (Zalups and Diamond, 2005). Lauwerys et al. (1994) suggested that tubular dysfunction is associated with urinary cadmium concentrations greater than 5 and 2 nmol/mmol creatinine in adult male workers and in the general population, respectively, are associated with tubular dysfunction. Cadmium produces proximal tubule dysfunction (S_1 and S_2 segments) and injury characterized by increases in urinary excretion of glucose, amino acids, calcium, and cellular enzymes (Prozialeck and Edwards, 2012). This injury may progress to a chronic interstitial nephritis.

A very interesting aspect of cadmium nephrotoxicity is the role of metallothioneins (Klaassen et al., 1999). Metallothioneins are a family of low-molecular-weight, cysteine-rich metal-binding proteins that have a high affinity for cadmium and other heavy metals. In general, the mechanism by which metallothionein is thought to play a role in cadmium and heavy metal toxicity is through its ability to bind to a heavy metal and thereby render it biologically inactive. This assumes that the unbound or "free" concentration of the metal is the toxic species. Metallothionein production can be induced by low, nontoxic concentrations of metals. Subsequently, animals challenged with a higher dose of the metal will not exhibit toxicity compared to naive animals.

Following an oral exposure to $CdCl_2$, Cd^{2+} is thought to reach the kidneys both as Cd^{2+} and as a Cd^{2+}–metallothionein complex formed and released either from intestinal cells or hepatocytes. The Cd^{2+}–metallothionein complex is freely filtered by the glomerulus and reabsorption by the proximal tubule is probably by endocytosis and is limited (Zalups and Diamond, 2005). Inside the tubular cells, it is thought that lysosomal degradation of the Cd^{2+}–metallothionein results in the release of "free" Cd^{2+}, which, in turn, induces renal metallothionein production. Once the renal metallothionein pool is saturated, "free" Cd^{2+} initiates injury. It is also likely that Cd is reabsorbed luminally and basolaterally as a cysteine conjugate (Zalups and Diamond, 2005). The mechanism by which Cd^{2+} produces injury at the cellular level is not clear; however, low concentrations of Cd^{2+} have been shown to interfere with the normal function of several cellular signal transduction pathways (Prozialeck and Edwards, 2012).

Chemically Induced α_{2u}-Globulin Nephropathy

A diverse group of chemicals, including unleaded gasoline, jet fuels, D-limonene, 1,4-dichlorobenzene, tetrachloroethylene, decalin, and lindane, cause α_{2u}-globulin nephropathy or hyaline droplet nephropathy (Lehman-McKeeman, 2010). This nephropathy occurring in male rats is characterized by the accumulation of protein droplets in the S_2 segment of the proximal tubule and results in single-cell necrosis, the formation of granular casts at the junction of the proximal tubule and the thin loop of Henle, and cellular regeneration. Chronic exposure to these compounds results in progression of these lesions and ultimately in chronic nephropathy. With compounds such as unleaded gasoline, chronic exposure results in an increased incidence of renal adenomas/carcinomas by non-genotoxic mechanisms.

As the name implies, the expression of this nephropathy requires the presence of the α_{2u}-globulin protein. α_{2u}-Globulin is synthesized in the liver of male rats and is under androgen control. Due to its low molecular weight (18.7 kDa), α_{2u}-globulin is freely filtered by the glomerulus with approximately half being reabsorbed via endocytosis in the S_2 segment of the proximal tubule. Many of the compounds that cause α_{2u}-globulin nephropathy bind to α_{2u}-globulin in a reversible manner and decrease the ability of lysosomal proteases in the proximal tubule to breakdown α_{2u}-globulin. This results in the accumulation of α_{2u}-globulin in the proximal tubule with an increase in the size and number of lysosomes and the characteristic protein-droplet morphology. A proposed mechanism of α_{2u}-globulin nephropathy is that cellular necrosis secondary to lysosomal overload leads to a sustained increase in cell proliferation, which, in turn, results in the promotion of spontaneously or chemically initiated cells to form preneoplastic and neoplastic foci (Melnick, 1992; Lehman-McKeeman, 2010).

α_{2u}-Globulin nephropathy appears to be sex- and species-specific. That is, it occurs in male rats but not female rats and in male or female mice, rabbits, or guinea pigs because they do not produce α_{2u}-globulin (Lehman-McKeeman, 2010). Furthermore, it does not occur in male Black Reiter rats that lack α_{2u}-globulin. Considering the diversity of compounds that cause α_{2u}-globulin nephropathy and renal tumors and the fact that humans are exposed to these compounds regularly, the question arises whether humans are at risk for α_{2u}-globulin nephropathy and renal tumors when exposed to these compounds. Current data suggest that humans are not at risk because (1) humans do not synthesize α_{2u}-globulin, (2) humans secrete fewer proteins in general and in particular less low-molecular-weight proteins in urine than the rat, (3) the low-molecular-weight proteins in human urine are either not related structurally to α_{2u}-globulin, do not bind to compounds that bind to α_{2u}-globulin, or are similar to proteins in female rats, male Black Reiter rats, rabbits, or guinea pigs that do not exhibit α_{2u}-globulin nephropathy, and (4) mice excrete a low-molecular-weight urinary protein that is 90% homologous to α_{2u}-globulin, but they do not exhibit α_{2u}-globulin nephropathy and renal tumors following exposure to α_{2u}-globulin-nephropathy-inducing agents.

Halogenated Hydrocarbons

Halogenated hydrocarbons are a diverse class of compounds and are used extensively as chemical intermediates, solvents, and pesticides. Consequently, humans are exposed to these compounds not only in the workplace but also through the environment. Numerous toxic effects have been associated with acute and chronic exposure to halogenated hydrocarbons, including nephrotoxicity (Mehendale,

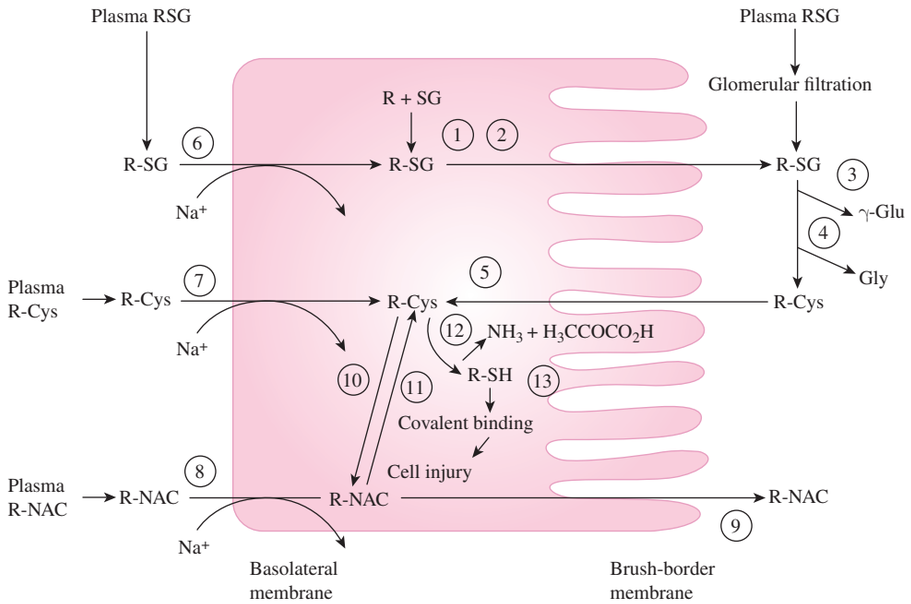

Figure 14-12. *Renal tubular uptake and metabolism of GSH conjugates.* (1) Intracellular formation of GSH conjugates (R-SG) catalyzed by renal GSH *S*-transferase(s); (2) secretion of the R-SG into the lumen; (3) γ-GT-mediated catabolism of R-SG and formation of the corresponding cysteinylglycine conjugate; (4) formation of the corresponding cysteine conjugate (R-Cys); (5) Na^+-coupled transport of R-Cys into the renal proximal tubular cell; (6) Na^+-coupled transport of RSG across the basolateral membrane; (7) Na^+-coupled transport of R-Cys across the basolateral membrane; (8) Na^+-coupled and probenecid-sensitive transport of the mercapturate (RNAC) across the basolateral membrane; (9) secretion of R-NAC into the lumen; (10) deacetylation of R-NAC to R-Cys; (11) acetylation of RCys to R-NAC; (12) reactive thiol formation via β-lyase; and (13) binding of the reactive thiol to cellular macromolecules and initiation of cell injury. (From Monks and Lau [1987], with permission.)

2010). The two examples provided below illustrate the importance of biotransformation in the nephrotoxicity of halogenated hydrocarbons (Dekant, 2005; Rankin and Valentovic, 2005).

Chloroform Chloroform produces nephrotoxicity in a variety of species, with some species being more sensitive than others. The primary cellular target is the proximal tubule, with no primary damage to the glomerulus or the distal tubule. Proteinuria, glucosuria, and increased BUN levels are all characteristic of chloroform-induced nephrotoxicity. The nephrotoxicity produced by chloroform is linked to its metabolism by renal cytochrome P450 and the formation of a reactive intermediate that binds covalently to nucleophilic groups on cellular macromolecules (Liu et al., 2013). Cytochrome P450 biotransforms chloroform to trichloromethanol, which is unstable and releases HCl to form phosgene. Phosgene can react with (1) water to produce $2HCl + CO_2$, (2) two molecules of glutathione to produce diglutathionyl dithiocarbonate, (3) cysteine to produce 2-oxothizolidine-4-carboxylic acid, or (4) cellular macromolecules to initiate toxicity. The sex differences observed in chloroform nephrotoxicity appear to be related to differences in renal cytochrome P450 isozyme contents. For example, castration of male mice decreased renal cytochrome P450 and chloroform-induced nephrotoxicity (Smith et al., 1984). Likewise, testosterone pretreatment of female mice increased cytochrome P450 content and rendered female mice susceptible to the nephrotoxic effects of chloroform. Cytochrome P450 isozyme 2E1 is present in male mice and expressed in female mice treated with testosterone (Lock and Reed, 1997). Thus, these isozymes may play a role in chloroform-induced nephrotoxicity.

Tetrafluoroethylene Tetrafluoroethylene is metabolized in the liver by GSH-*S*-transferases to *S*-(1,1,2,2-tetrafluoroethyl)-glutathione. The GSH conjugate is secreted into the bile and small intestine where it is degraded to the cysteine *S*-conjugate (TFEC), reabsorbed, and transported to the kidney. The mercapturic acid may also be formed in the small intestine and reabsorbed. Alternatively, the glutathione conjugate can be transported to the kidney and biotransformed to the cysteine conjugate by γ-GT and a dipeptidase located on the brush border (Fig. 14-12). The mercapturic acid is transported into the proximal tubule cell by the organic anion transporter, whereas cysteine conjugates are transported by the organic anion transporter and the sodium-independent L and T transport systems. The cysteine *S*-conjugate of these compounds is thought to be the penultimate nephrotoxic species. Following transport into the proximal tubule, which is the primary cellular target for haloalkenes and haloalkanes, the cysteine *S*-conjugate is a substrate for the cytosolic and mitochondrial forms of the enzyme cysteine conjugate β-lyase. In the case of the *N*-acetyl-cysteine *S*-conjugate, the *N*-acetyl group must be removed by a deacetylase for it to be a substrate for cysteine conjugate β-lyase. The products of the reaction are ammonia, pyruvate, and a reactive thiol that is capable of binding covalently to cellular macromolecules. There is a correlation between the covalent binding of the reactive thiol of the cysteine conjugate with renal protein and nephrotoxicity. Hayden et al. (1991) and Bruschi et al. (1993) have shown that biotransformation of TFEC results in difluorothioamidyl-L-lysine-protein adducts in mitochondria and that two of the targeted proteins may belong to the heat shock family of proteins. Hayden et al. (1992) have also shown that halogenated thioamide adducts of phosphatidylethanolamine are formed in mitochondria following cysteine conjugate β-lyase biotransformation of TFEC.

The nephrotoxicity produced by haloalkenes is characterized morphologically by proximal tubular necrosis, primarily affecting the S_3 segment, and functionally by increases in urinary glucose,

protein, cellular enzymes, and BUN. Following in vivo and in vitro exposures to TFEC, the mitochondrion appears to be a primary target. In rabbit renal proximal tubules and isolated mitochondria, there is a marked decrease in state 3 respiration (respiration associated with maximal ATP formation) following TFEC exposure (Groves et al., 1993). Furthermore, the decrease in mitochondrial function occurs prior to the onset of cell death. Oxidative stress may also play a contributing role in TFEC-induced cell death because lipid peroxidation products were formed prior to the onset of cell death, and antioxidants and iron chelators decreased cytotoxicity (Chen et al., 1990; Groves et al., 1991).

Aristolochic Acid and Fungal Toxins

Mycotoxins are products of molds and fungi that produce nephrotoxicity and these include aflatoxin B_1, citrinin, ochratoxins, fumonisins, and patulin (Dickman and Grollman, 2010). Three examples of nephrotoxic mycotoxins will be discussed. Citrinin nephrotoxicity is characterized by decreased urine osmolality, GFR and RBF, glycosuria, and increased urinary enzyme excretion. Interestingly, the location of citrinin-induced tubular vacuolization and necrosis (proximal, distal) varies among species. Whereas the mechanism of citrinin toxicity to the tubules remains unresolved, citrinin enters the cells through the organic anion transporter and causes mitochondrial dysfunction.

Fumonisins B_1 and B_2 are commonly found on corn and corn products and produce nephrotoxicity in numerous species, some species are very sensitive (e.g., rabbits) whereas others are more resistant (e.g., mice) (Bucci et al., 1998). Histologic examination of the kidney revealed disruption of the basolateral membrane, mitochondrial swelling, increased numbers of clear and electron-dense vacuoles, and apoptosis in proximal tubular cells at the junction of the cortex and medulla. Changes in renal function included increased urine volume, decreased osmolality, and increased excretion of low- and high-molecular-weight proteins. The fumonisins are structurally similar to sphingoid bases and are thought to produce their toxicity through the inhibition of sphinganine (sphingosine) *N*-acyltransferase. Inhibition of this enzyme results in an increase in the ratio of free sphinganine to free sphingosine and a decrease in complex sphingolipids. The toxicity of fumonisins may be through increased sphinganine, reactive oxygen species, and apoptosis.

Aristolochic acids (AAs) and aristolactams are natural products found in the *Aristolochia* and *Asarum* genera (Dickman and Grollman, 2010). Despite the extensive use of *Aristolochia* as a herbal remedy for thousands of years, its human renal toxicity was not reported until 1993 (Vanherweghem et al., 1993). Since then AAs have been shown to play a role in Balkan endemic nephropathy and Chinese herb nephropathy, now known as AA nephropathy (Stiborová et al., 2016). In both cases, the renal dysfunction is characterized by tubular dysfunction, proteinuria, and interstitial fibrosis.

AAs are a mixture of compounds that form covalent DNA adducts, and are genotoxic and carcinogenic (Sidorenko et al., 2014). Renal uptake of the penultimate toxicant, AA-I, is through the mouse OAT-mediated transporter, and is bioactivated through nitroreduction to produce DNA and protein adducts (Fig. 14-13). The nephrotoxicity of AA has been studied in rabbits, rats, and mice, and in cell culture. Shibutani et al. (2007) reported that AA-I produced renal proximal tubule necrosis and interstitial fibrosis whereas AA-II did not. Interestingly, both AA-I and AA-II produced similar levels of DNA adducts. Thus, the nephrotoxicity of AA-I may not be due to DNA adducts and damage.

Therapeutic Agents

Acetaminophen Large doses of the antipyretic and analgesic acetaminophen (APAP) are commonly associated with hepatotoxicity (Tarloff, 2010). However, large doses of APAP can also cause nephrotoxicity in humans and laboratory animals. APAP nephrotoxicity is characterized by proximal tubular necrosis with increases in BUN and plasma creatinine; decreases in GFR and clearance of *para*-aminohippurate; increases in the fractional excretion of water, sodium, and potassium; and increases in urinary glucose, protein, and brush-border enzymes. There appears to be a marked species difference in the nature and mechanism of APAP nephrotoxicity (Emeigh Hart et al., 1994; Tarloff, 1997). Morphologically, the primary targets in the mouse kidney are the S_1 and S_2 segments of the proximal tubule, whereas in the rat kidney the S_3 segment is the target. In the mouse, renal cytochrome P450 2E1 has been associated with APAP biotransformation to a reactive intermediate, *N*-acetyl-*p*-amino-benzoquinoneimine, that arylates proteins in the proximal tubule and initiates cell death. Two of the proteins that are targets of *N*-acetyl-*p*-amino-benzoquinoneimine are a selenium-binding protein and a glutamine synthetase (Emeigh Hart et al., 1994; Tarloff, 1997). However, the mechanism by which protein adducts initiate proximal tubular cell death and ultimately nephrotoxicity remains to be determined. Although renal cytochrome P450 plays a role in APAP activation and nephrotoxicity, glutathione conjugates of APAP may also contribute to APAP nephrotoxicity. Evidence for this pathway was provided by experiments in which γ-GT or organic anion transport was inhibited and APAP-induced nephrotoxicity decreased (Emeigh Hart et al., 1990). In contrast to its effects in the mouse, a critical and early step in APAP nephrotoxicity in the rat is the conversion of APAP to *para*-aminophenol (PAP) (Tarloff, 1997). The steps after PAP formation and the expression of nephrotoxicity are less clear.

Nonsteroidal Anti-Inflammatory Drugs NSAIDs such as aspirin, ibuprofen, naproxen, indomethacin, and cyclooxygenase-2 inhibitors (e.g., celecoxib) are extensively used as analgesics and anti-inflammatory drugs and produce their therapeutic effects through the inhibition of prostaglandin synthesis. At least three different types of nephrotoxicity have been associated with NSAID administration (Tarloff, 2010). AKI may occur within hours of a large dose of an NSAID, is usually reversible upon withdrawal of the drug, and is characterized by decreased RBF and GFR and by oliguria. When the normal production of vasodilatory prostaglandins (e.g., PGE_2 and PGI_2) is inhibited by NSAIDs, vasoconstriction induced by circulating catecholamines and angiotensin II is unopposed, resulting in decreased RBF and ischemia. A number of risk factors (e.g., renal insufficiency, congestive heart failure, hepatic cirrhosis, hemorrhage, hypertension, sepsis, and diabetes) are known to facilitate the development of AKI after NSAIDs consumption.

In contrast, chronic consumption of combinations of NSAIDs and/or APAP (more than 3 years) results in an often irreversible form of nephrotoxicity known as analgesic nephropathy (Palmer and Heinrich, 2004; De Broe, 2005; Tarloff, 2010). The incidence of analgesic nephropathy varies widely in the Western world, ranging from less than 2% to 5% of all end-stage renal disease patients in countries where analgesic consumption is low (e.g., United States and Canada), and up to 20% of all end-stage renal disease patients in countries with the highest analgesic consumption (e.g., Australia and Sweden). Impaired urinary concentration and acidification are the earliest clinical manifestations. The primary lesion in this nephropathy is papillary necrosis with chronic interstitial nephritis. Initial changes are to the medullary interstitial cells and

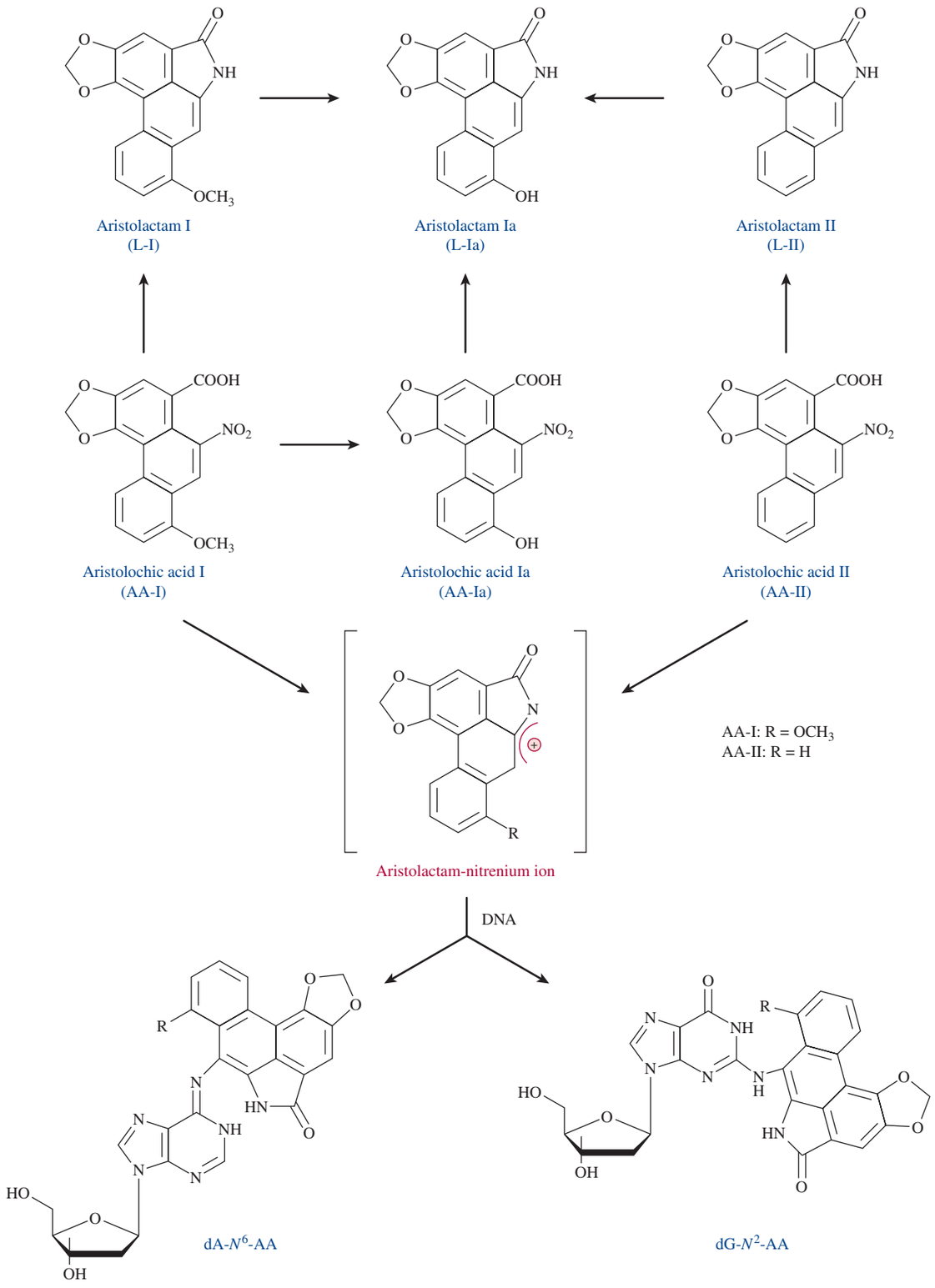

Figure 14-13. *Scheme outlines the metabolism of aristolochic acids and formation of AA-derived DNA adducts.* (From Shibutani et al. [2007].)

are followed by degenerative changes to the medullary loops of Henle and medullary capillaries. Well-defined clinical signs have been associated with analgesic nephropathy and are helpful in the diagnosis thereof. De Broe (2005) has developed an effective computed tomography (CT) protocol that does not use contrast media to diagnose analgesic nephropathy. Although analgesic nephropathy is associated with a number of well-defined effects, the mechanism by which NSAIDs produce analgesic nephropathy is not known, but may result from chronic medullary/papillary ischemia secondary to renal vasoconstriction. Other studies have suggested that a reactive intermediate is formed in the cells that, in turn, initiates an oxidative stress, or binds covalently to critical cellular macromolecules.

The third, even though rare, type of nephrotoxicity associated with NSAIDs is an interstitial nephritis with a mean time of NSAID exposure to development of approximately 5 months (Tarloff, 2010; Raghavan and Shawar, 2017). This nephrotoxicity is characterized by a diffuse interstitial edema with mild-to-moderate infiltration of inflammatory cells. Patients normally present with elevated serum creatinine, proteinuria, and nephritic syndrome. If NSAIDs are discontinued, renal function improves in 1 to 3 months.

Aminoglycosides The aminoglycoside antibiotics are so named because they consist of two or more amino sugars joined in a glycosidic linkage to a central hexose nucleus. Although they are drugs of choice for many gram-negative infections, their use is primarily limited by their nephrotoxicity. The incidence of renal dysfunction following aminoglycoside administration ranges from 0% to 50% but seldom leads to a fatal outcome (Palmer and Heinrich, 2004; Servais et al., 2005; Decker and Molitoris, 2010).

Renal dysfunction by aminoglycosides is characterized by a nonoliguric renal failure with reduced GFR and an increase in serum creatinine and BUN. Polyuria is an early event following aminoglycoside administration and may be due to inhibition of chloride transport in the thick ascending limb (Kidwell et al., 1994). Within 24 hours, increases in urinary brush-border enzymes, glucosuria, aminoaciduria, and proteinuria are observed. Histologically, lysosomal alterations are noted initially, followed by damage to the brush border, ER, mitochondria, and cytoplasm, ultimately leading to tubular cell necrosis. Interestingly, proliferation of renal proximal tubule cells can be observed early after the onset of nephrotoxicity.

Aminoglycosides are highly polar cations; they are almost exclusively filtered by the glomerulus and excreted unchanged. Filtered aminoglycosides undergo proximal tubular reabsorption by binding to anionic phospholipids in the brush border, followed by endocytosis and sequestration in lysosomes of the S_1 and S_2 segments of proximal tubules (Fig. 14-14). Basolateral membrane binding and uptake also may occur, but this is a minor contribution to the total proximal tubular uptake of aminoglycosides. The earliest lesion observed following clinically relevant doses of aminoglycosides is an increase in the size and number of lysosomes. These lysosomes contain *myeloid bodies*, which are electron-dense lamellar structures containing undergraded phospholipids. The renal phospholipidosis produced by the aminoglycosides is thought to occur through their inhibition of lysosomal hydrolases, such as sphingomyelinase and phospholipases. Although phospholipidosis plays an important role in aminoglycoside nephrotoxicity, the steps between the phospholipid accumulation in the lysosomes and tubular cell death are less clear. One hypothesis suggests that the lysosomes become progressively distended until they rupture, releasing lysosomal enzymes and high concentrations of aminoglycosides into the cytoplasm (Fig. 14-14). The released lysosomal contents can interact with various membranes and organelles and trigger cell death. Another mechanism of aminoglycoside nephrotoxicity includes a decrease in K_f and GFR (see the section "Glomerular Injury").

Amphotericin B Amphotericin B is a very effective antifungal drug whose clinical utility is limited by its nephrotoxicity (Bernardo and Branch, 1997; Palmer and Heinrich, 2004; Ulozas, 2010). Renal dysfunction associated with amphotericin B treatment is dependent on cumulative dose and is due to both hemodynamic and tubular effects. With respect to hemodynamics, amphotericin B administration is associated with decreases in RBF and GFR secondary to renal arteriolar vasoconstriction or

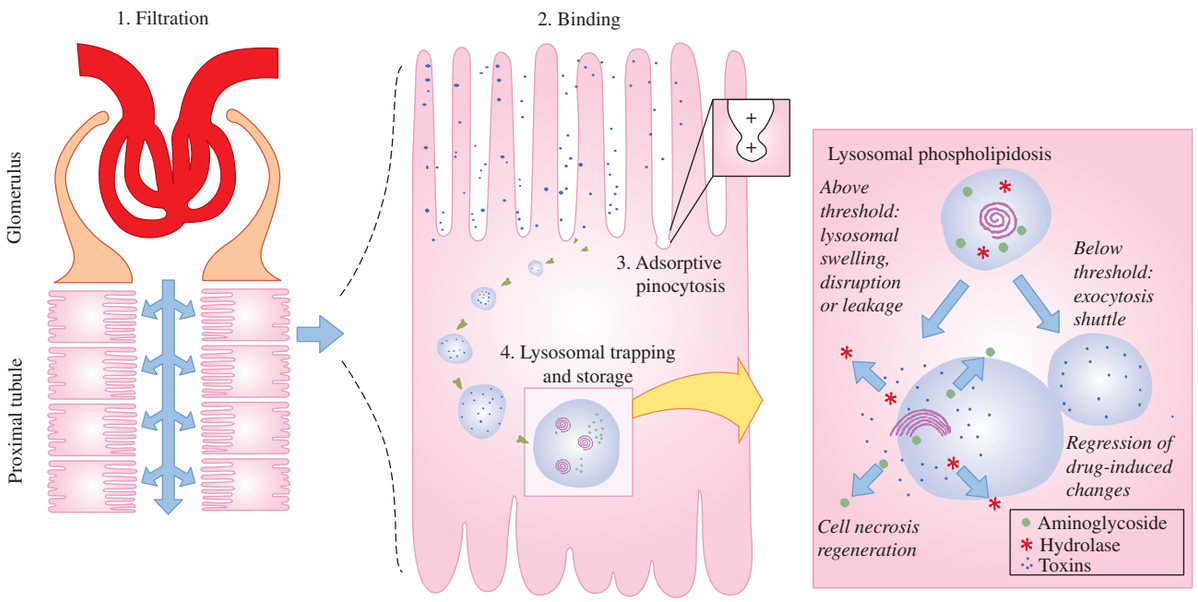

Figure 14-14. *Renal handling of aminoglycosides: (1) glomerular filtration, (2) binding to the brush-border membranes of the proximal tubule, (3) pinocytosis, and (4) storage in the lysosomes.* (From De Broe [1999, p. 11.4], with permission.)

activation of TGF. Amphotericin B nephrotoxicity is characterized by ADH-resistant polyuria, renal tubular acidosis, hypokalemia, and either acute or chronic renal failure. Amphotericin B nephrotoxicity is unusual in that it impairs the functional integrity of the glomerulus and of the proximal and distal portions of the nephron.

Some of the renal tubular cell effects of amphotericin B are due to the ability of this polyene to bind to cholesterol in the plasma membrane and form aqueous pores. In the presence of amphotericin B, cells of the turtle and rat distal tubule do not produce a normal net outward flux of protons, due to an increase in proton permeability (Steinmetz and Husted, 1982; Gil and Malnic, 1989). This results in impaired proton excretion and renal tubular acidosis. The hypokalemia observed with amphotericin B may be due to an increase in luminal potassium ion permeability in the late distal tubule and the cortical collecting duct and the loss of potassium ions in the urine.

Cyclosporine Cyclosporine is an important immunosuppressive drug and is widely used to prevent graft rejection in organ transplantation (Palmer and Heinrich, 2004; Charney et al., 2005; Naesens et al., 2009). Cyclosporine is a fungal cyclic polypeptide and acts by selectively inhibiting cyclophylin and, in turn, calcineurin and T-cell activation. Nephrotoxicity is a critical side effect of cyclosporine, with nearly all patients who receive the drug exhibiting some form of nephrotoxicity. Clinically, calcineurin inhibitor (CNI)-induced nephrotoxicity may manifest as (1) acute reversible renal dysfunction, (2) acute vasculopathy, and (3) chronic CNI nephrotoxicity with interstitial fibrosis.

Acute renal dysfunction is characterized by dose-related decreases in RBF and GFR and increases in BUN and serum creatinine. These effects are lessened by reducing the dosage or by cessation of therapy. The decrease in RBF and GFR is related to marked vasoconstriction induced by cyclosporine, and it is probably produced by a number of factors, including an imbalance in vasoconstrictor and vasodilatory prostaglandin production, increased production of the vasoconstrictor thromboxane and endothelin, and activation of the renin-angiotensin system. Endothelin may contribute to constriction of the afferent arteriole because endothelin receptor antagonists inhibit cyclosporine-induced vasoconstriction (Lanese and Conger, 1993). Although cyclosporine can produce proximal tubular epithelial changes (many small equally sized vacuoles in the cytosol), it is still not clear whether a direct effect of cyclosporine on tubular cells plays a significant role in the nephrotoxicity.

Acute vasculopathy or thrombotic microangiopathy is a rather unusual nephrotoxic lesion that affects arterioles and glomerular capillaries, without an inflammatory component, following cyclosporine treatment. The lesion consists of fibrin-platelet thrombi and fragmented red blood cells occluding the vessels (Charney et al., 2005). The pathogenesis of this lesion is poorly understood. Although the characteristics of this lesion differ from the vascular changes of acute rejection, a variety of factors may contribute to this lesion in the clinical transplant setting.

Long-term treatment with cyclosporine can result in chronic nephropathy with interstitial fibrosis and tubular atrophy. Modest elevations in serum creatinine and decreases in GFR occur along with hypertension, proteinuria, and tubular dysfunction. Histologic changes are profound; they are characterized by arteriolopathy, global and segmental glomerular sclerosis, striped interstitial fibrosis, and tubular atrophy. These lesions may not be reversible if cyclosporine therapy is discontinued and may result in end-stage renal disease. Although the mechanism of chronic cyclosporine nephropathy is not known, vasoconstriction probably plays a contributing role. Studies by Wang and Salahudeen (1994, 1995) indicated that rats treated with cyclosporine and a lazaroid antioxidant for 30 days exhibited increased GFR and RBF and less tubulointerstitial fibrosis and lipid peroxidation than rats treated with cyclosporine alone, suggesting that oxidative stress plays a role in cyclosporine nephrotoxicity in rats. The marked interstitial cell proliferation and increased procollagen secretion that occur following cyclosporine administration may contribute to the interstitial fibrosis (Racusen and Solez, 1993).

Tacrolimus (FK-506) is a newer CNI immunosuppressive drug that also exhibits nephrotoxicity. However, recent data have provided evidence that chronic CNI nephrotoxicity in the transplanted kidney is not the major cause of renal dysfunction and graft failure (Issa et al., 2013). Rather, the chronic renal changes that have been attributed to chronic CNI may be the result of previously unrecognized immunologic injury.

Cisplatin Cisplatin is a valuable drug in the treatment of solid tumors, with nephrotoxicity limiting its clinical use (Manohar and Leung, 2018). The kidney is not only responsible for the majority of cisplatin excreted but also is the primary site of accumulation. The effects of cisplatin on the kidney are several, including acute and chronic renal failure, renal magnesium wasting, and polyuria, and patients treated with cisplatin regimens permanently lose 10% to 30% of their renal function (Bonegio and Lieberthal, 2005; Sanchez-Gonzalez et al., 2011). The nephrotoxicity of cisplatin can be grouped as (1) tubular toxicity, (2) vascular damage, (3) glomerular injury, and (4) interstitial injury.

Early effects of cisplatin are decreases in RBF and polyuria that is concurrent with increased electrolyte excretion (Clifton et al., 1982). GFR produced by vasoconstriction and is followed by tubular injury with enzymuria. Although the primary cellular target associated with AKI is the proximal tubule S_3 segment in the rat, in humans the S_1 and S_2 segments, distal tubule, and collecting ducts can also be affected.

The mechanism by which cisplatin produces cellular injury is not known but may involve metabolites of cisplatin. For example, in a mouse model of cisplatin-induced nephrotoxicity, the inhibition of γ-glutamyl transpeptidase or cysteine *S*-conjugate β-lyase blocked toxicity, suggesting that cisplatin–glutathione conjugates may be important in targeting cisplatin to the kidney and its resulting nephrotoxicity (Townsend and Hanigan, 2002). Uptake of cisplatin into tubular cells is thought to be mediated by organic cation transporter 2. Interestingly, the *trans* isomer of cisplatin is not nephrotoxic even though similar concentrations of platinum are observed in the kidney after dosing. Thus, it is not the platinum atom per se that is responsible for the toxicity but rather the geometry of the complex or a metabolite. The antineoplastic and perhaps the nephrotoxic effects of cisplatin may be due to its intracellular hydrolysis to the reactive mono-chloro-mono-aquodiammine-platinum or diaquodiammine-platinum species and the ability of these metabolites to alkylate purine and pyrimidine bases.

Extensive in vitro and in vivo studies have been conducted in which single large concentrations/doses are given and endpoints are measured over several days. Cisplatin produces inter and intrastrand cross-links in renal genomic DNA. Using rabbit renal proximal tubule cells, Courjault et al. (1993) showed that while DNA synthesis, protein synthesis, glucose transport, Na^+, K^+-ATPase activity, and cell viability were all inhibited by cisplatin, DNA synthesis was the most sensitive. These results suggest that cisplatin may produce acute nephrotoxicity through its ability to inhibit DNA synthesis as well as transport functions. However, the previous in vivo studies

do not mimic the human cisplatin dose nor dosing protocol during cancer chemotherapy. Sharp et al. (2016) developed a repeated dosing model in which low-dose cisplatin was administered weekly for 4 weeks, similar to that used in humans. Under these conditions, markers (e.g., BUN, serum creatinine, and KIM-1) of AKI did not increase. In contrast, renal inflammatory markers (e.g., TNF-α, IL-6, and IL-1-β) and renal fibrosis markers (e.g., fibronectin and transforming growth factor β) increased. These results provide strong evidence that the mechanism of cisplatin nephrotoxicity is different using an acute large-dose versus a low-dose, repeated-dosing exposure model, similar to that used in humans.

Radiocontrast Agents Iodinated contrast media are used for the imaging of tissues, with two major classes of compounds currently in use. The ionic compounds, diatrizoate derivatives, are (1) ionized at physiologic pH, (2) not significantly bound to protein, (3) restricted to the extracellular space, (4) almost entirely eliminated by the kidney, and (5) freely filtered by the glomerulus and neither secreted nor reabsorbed. These agents have a very high osmolality (more than 1200 mOsm/L) and are potentially nephrotoxic, particularly in patients with existing renal impairment, diabetes, or heart failure or who are receiving other nephrotoxic drugs. The newer contrast agents (e.g., iotrol and iopamidol) are nonionic, owing to the addition of an organic side chain, with lower osmolality, and less nephrotoxicity. The nephrotoxicity of these agents is due to both hemodynamic alterations (vasoconstriction) and proximal tubular injury (Koyner et al., 2010; Chalikias et al., 2016). The vasoconstriction is prolonged and is probably produced by more than one mediator while ROS are thought to play a role in the proximal tubular injury.

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REFERENCES

Arany I, Megyesi JK, Kaneto H, et al. Cisplatin-induced cell death is EGFR/src/ERK signaling dependent in mouse proximal tubule cells. *Am J Physiol Renal Physiol*. 2004;87: F543.

Bach PH. The renal medulla and distal nephron toxicity. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 1997:279–298.

Ballatori N. Mechanisms of metal transport across liver cell plasma membrane. *Drug Metab Rev*. 1991;23:83–132.

Basile DP, Donohoe D, Roethe K, Osborn JL. Renal ischemic injury results in permanent damage to peritubular capillaries and influences long-term function. *Am J Physiol Renal Physiol*. 2001;281:F887–F899.

Bellomo R, Ronco C, Kellum J, et al. Acute renal failure—definition, outcome measures, animal models, fluid therapy and information technology needs the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. *Crit Care*. 2004;8(4): R204–R212.

Bernardo JF, Branch RA. Amphotericin B. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 1997:475–494.

Bikbov B, Perico N, Abbate M, et al. The glomerulus: mechanisms of injury. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:245–261.

Bonegio R, Lieberthal W. Cisplatin-induced nephrotoxicity. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:779–815.

Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest*. 2011;121:4210–4221.

Brady HR, Clarkson W, Lieberthal W. Acute renal failure. In: Brenner BM, ed. *Brenner and Rector's The Kidney*. 7th ed. Philadelphia, PA: WB Saunders; 2004:1215–1292.

Brenner BM, Bohrer MP, Baylis C, Deen WM. Determinants of glomerular permselectivity: insights derived from observations in vivo. *Kidney Int*. 1977;12:229–237.

Brenner BM, Meyer TH, Hotstetter TH. Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of glomerular sclerosis in angina, renal ablation and intrinsic renal disease. *N Engl J Med*. 1982;307:652–659.

Brezis M, Epstein FH. Pathophysiology of acute renal failure. In: Hook JB, Goldstein RS, eds. *Toxicology of the Kidney*. 2nd ed. New York: Raven Press; 1993:129–152.

Brezis M, Rosen S, Silva P. Transport activity modifies thick ascending limb damage in isolated perfused kidney. *Kidney Int*. 1984;25:65–72.

Bruschi SA, West K, Crabb JW, et al. Mitochondrial HSP60 (P1 protein) and a HSP-70 like protein (mortalin) are major targets for modification during *S*-(1,1,2,2-tetrafluorethyl)-L-cysteine induced nephrotoxicity. *J Biol Chem*. 1993;268:23157–23161.

Bucci TJ, Howard PC, Tolleson WH, et al. Renal effects of fumonisin mycotoxins in animals. *Toxicol Pathol*. 1998;26:160–164.

Byber K, Lison D, Verougstraete V, et al. Cadmium or cadmium compounds and chronic kidney disease in workers and the general population: a systematic review. *Crit Rev Toxicol*. 2016;46:191–240.

Chalikias G, Drosos I, Tziakas DN. Contrast-induced acute kidney injury: an update. *Cardiovasc Drugs Ther*. 2016;30:215–228.

Charney D, Solez K, Racusen LC. Nephrotoxicity of cyclosporine and other immunosuppressive and immunotherapeutic agents. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:687–777.

Chen Q, Jones TW, Brown PC, Stevens JL. The mechanism of cysteine conjugate cytotoxicity in renal epithelial cells. *J Biol Chem*. 1990;265:21603–21611.

Chen Q, Yu K, Stevens JL. Regulation of the cellular stress response by reactive electrophiles: the role of covalent binding and cellular thiols in transcriptional activation of the 70-kDa heat shock protein gene by nephrotoxic cysteine conjugates. *J Biol Chem*. 1992;267:24322–24327.

Clifton GG, Pearce C, O'Neill WM Jr, Wallin JD. Early polyuria in the rat following single-dose *cis*-dichlorodiammineplatinum (II): effects on plasma vasopressin concentration and posterior pituitary function. *J Lab Clin Med*. 1982;100:659–670.

Coen M, Holmes E, Lindon JC, Nicholson JK. NMR-based metabolic profiling and metabonomic approaches to problems in molecular toxicology. *Chem Res Toxicol*. 2008;21(1):9–27.

Conner EA, Fowler BA. Mechanisms of metal-induced nephrotoxicity. In: Hook JB, Goldstein RS, eds. *Toxicology of the Kidney*. 2nd ed. New York: Raven Press; 1993:437–457.

Courjault F, Leroy D, Coquery I, Toutain H. Platinum complex–induced dysfunction of cultured renal proximal tubule cells. *Arch Toxicol*. 1993;67:338–346.

Daemen MARC, van 't Veer C, Denecker G, et al. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest*. 1999;104:541–549.

De Broe ME. Renal injury due to environmental toxins, drugs, and contrast agents. In: Berl T, Bonventre JV, eds. *Atlas of Diseases of the Kidney*. Philadelphia, PA: Current Medicine; 1999:11.1–11.16.

De Broe ME. Analgesic nephropathy. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:619–634.

Decker B, Molitoris BA. Aminoglycoside-induced nephrotoxicity. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:329–346.

Declercq W, Vanden Berghe T, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. *Cell*. 2009;138(2):229–232.

Dekant W. Chemical-induced nephrotoxicity mediated by glutathione *S*-conjugate formation. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:995–1020.

Dickman KG, Grollman AP. Nephrotoxicity of natural products: aristolochic acid and fungal toxins. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010: 433–458.

Dong J, Ramachandiran S, Tikoo K, Jia Z, Lauu SS, Monks TJ. EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death. *Am J Physiol Renal Physiol*. 2004;287:F1049–F1058.

Dunn RB, Anderson S, Brenner B. The hemodynamic basis of progressive renal disease. *Semin Nephrol*. 1986;6:122–138.

Eknoyan G, Bulger RE, Dobyan DC. Mercuric chloride-induced acute renal failure in the rat: I. Correlation of functional and morphologic changes and their modification by clonidine. *Lab Invest*. 1982;46:613–620.

Emeigh Hart SGE, Beierschmitt WP, Wyand DS, et al. Acetaminophen nephrotoxicity in CD-1 Mice. I. Evidence of a role for in situ activation in selective covalent binding and toxicity. *Toxicol Appl Pharmacol*. 1994;126:267–275.

Emeigh Hart SGE, Wyand DS, Khairallah EA, Cohen SD. A role for the glutathione conjugate and renal cytochrome P450 in acetaminophen (APAP) induced nephrotoxicity in the CD-1 mouse. *Toxicologist*. 1990;11:57.

Filler G, Bokenkamp A, Hofmann W, Le Bricon T, Martinez-Bru C, Grubb A. Cystatin C as a maker of GFR-history, indications and future research. *Clin Biochem*. 2005;38:1–8.

Fowler BA. Other nephrotoxic metals and nanometallic particles. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:495–506.

Ford SM. In vitro toxicity systems. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 1997:121–142.

Ford SM. In vitro techniques in screening and mechanistic studies: cell culture, cell-free systems, and molecular and cell biology. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:191–213.

Fukino H, Hirai M, Hsueh YM, Yamane Y. Effect of zinc pretreatment on mercuric chloride–induced lipid peroxidation in the rat kidney. *Toxicol Appl Pharmacol*. 1984;73:395–401.

Gil FZ, Malnic G. Effect of amphotericin B on renal tubular acidification in the rat. *Pflugers Arch*. 1989;413:280–286.

Goering PL, Fisher BR, Chaudhary PP, Dick CA. Relationship between stress protein induction in rat kidney by mercuric chloride and nephrotoxicity. *Toxicol Appl Pharmacol*. 1992;113:184–191.

Goering PL, Fisher BR, Noren BT, et al. Mercury induces regional and cell-specific stress protein expression in rat kidney. *Toxicol Sci*. 2000;53:447–457.

Goldstein RS. Biochemical heterogeneity and site-specific tubular injury. In: Hook JB, Goldstein RS, eds. *Toxicology of the Kidney*. 2nd ed. New York: Raven Press; 1993:201–248.

Goligorsky MS, Patschan D, Kuo M-C, et al. Cell adhesion molecules in renal injury. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:213–244.

Gopee NV, He Q, Sharma RP. Fumonisin B1-induced apoptosis is associated with delayed inhibition of protein kinase C, nuclear factor-kappaB and tumor necrosis factor alpha in LLC-PK1 cells. *Chem Biol Interact*. 2003;146:131–145.

Groves CE, Hayden PJ, Lock EA, Schnellmann RG. Differential cellular effects in the toxicity of haloalkene and haloalkane cysteine conjugates to rabbit renal proximal tubules. *J Biochem Toxicol*. 1993;8:49–56.

Groves CE, Lock EA, Schnellmann RG. The role of lipid peroxidation in renal proximal tubule cell death induced by haloalkene cysteine conjugates. *J Toxicol Appl Pharmacol*. 1991;107:54–62.

Guyton AC, Hall JE, eds. *Textbook of Medical Physiology*. Philadelphia, PA: WB Saunders; 1996.

Hallman MA, Zhuang S, Schnellmann RG. Regulation of dedifferentiation and redifferentiation in renal proximal tubular cells by the epidermal growth factor receptor. *J Pharmacol Exp Ther*. 2008;325(2):520–528.

Hammerman MR, Miller SB. Therapeutic use of growth factors in renal failure. *J Am Soc Nephrol*. 1994;5:1–11.

Harriman JF, Liu XL, Aleo MD, Machaca K, Schnellmann RG. Endoplasmic reticulum Ca^{2+} signaling and calpains mediate renal cell death. *Cell Death Differ*. 2002;9:734–741.

Hart SE, Kinter LB. Assessing renal effects of toxicants in vivo. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:81–147.

Hart SGE. In vivo methodologies used to assess renal function and injury. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:263–303.

Hayden PJ, Stevens JL. Cysteine conjugate toxicity, metabolism and binding to macro-molecules in isolated rat kidney mitochondria. *Mol Pharmacol*. 1990;37:468–476.

Hayden PJ, Welsh CJ, Yang Y, et al. Formation of mitochondrial phospholipid adducts by nephrotoxic cysteine conjugate metabolites. *Chem Res Toxicol*. 1992;5:231–237.

Hayden PJ, Yang Y, Ward AJ, et al. Formation of diflourothionoacetyl-protein adducts by *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine metabolites: nucleophilic catalysis of stable lysyl adduct formation by histidine and tyrosine. *Biochemistry*. 1991;30:5935–5943.

Holmes E, Cloarec O, Nicholson JK. Probing latent biomarker signatures and in vivo pathway activity in experimental disease states via statistical total correlation spectroscopy (STOCSY) of biofluids: application to $HgCl_2$ toxicity. *J Proteome Res*. 2006;5:1313–1320.

Humphreys BD, Czerniak S, DiRocco DP, Hasain W, Cheema R, Bonventre JV. Repair of injured proximal tubule does not involve specialized progenitors. *Proc Natl Acad Sci U S A*. 2011;108(22):9226–9231.

Humphreys BD, Valerius MT, Kibayashi A, et al. Intrinsic epithelial cells repair the kidney after injury. *Cell Stem Cell*. 2008;2(3):284–291.

Issa N, Kukla A, Ibrahim HN. Calcineurin inhibitor nephrotoxicity: a review and perspective of the evidence. *Am J Nephrol*. 2013;37:602–612.

Jarnberg P. Renal toxicity of anesthetic agents. In: de Broe ME, Porter GA, Bennett AM, Verpooten GA, eds. *Clinical Nephrotoxins: Renal Injury from Drugs and Chemicals*. The Netherlands: Kluwer; 1998:413–418.

Kanwar YS, Liu ZZ, Kashihara N, Wallner EI. Current status of the structural and functional basis of glomerular filtration and proteinuria. *Semin Nephrol*. 1991;11:390–413.

Kaushal GP, Basnakian AG, Shah SV. Apoptotic pathways in ischemic acute renal failure. *Kidney Int*. 2004;66(2):500–506.

Kaushal GP, Singh AB, Shah SV. Identification of gene family of caspases in rat kidney and altered expression in ischemia-reperfusion injury. *Am J Physiol*. 1998;274:F587–F595.

Kellum JA, Lameire N, Aspelin P, et al. Kidney disease: improving global outcomes (KDIGO) acute kidney injury work group. KDIGO clinical practice guideline for acute kidney injury. *Kidney Int Suppl*. 2012;2:1–138.

Kelly KJ. Heat shock (stress response) proteins and renal ischemia/reperfusion injury. *Contrib Nephrol*. 2005;148:86–106.

Kido T, Nordberg G. Cadmium-induced renal effects in the general environment. In: de Broe ME, Porter GA, Bennett AM, Verpooten GA, eds. *Clinical Nephrotoxins: Renal Injury from Drugs and Chemicals*. The Netherlands: Kluwer; 1998:345–362.

Kidwell DT, KcKeown JW, Grider JS, et al. Acute effects of gentamicin on thick ascending limb function in the rat. *Eur J Pharmaco Environ Toxicol Pharmacol Section*. 1994;270:97–103.

Kinsey GR, Okusa MD. Pathogenesis of acute kidney injury: foundation for clinical practice. *Am J Kidney Dis*. 2011;58:291-301.

Kirkpatrick DS, Gandolfi AJ. In vitro techniques in screening and mechanistic studies: organ perfusion, slices, and nephron components. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:149–189.

Klaassen CD, Liu J, Choudhuri S. Metallothionein: an intracellular protein to protect cadmium toxicity. *Ann Rev Pharmacol Toxicol*. 1999;39:267–294.

Komatsuda A, Wakui H, Satoh K, et al. Altered localization of 73-kilodalton heat shock protein in rat kidneys with gentamicin-induced acute tubular injury. *Lab Invest*. 1993;68:687–695.

Koyner JL, Murray PT, Bakris GK. The pathogenesis and prevention of radiocontrast medium–induced renal dysfunction. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:359–386.

Kriz W. Standard nomenclature for structures of the kidney. *Am J Physiol*. 1988;254:F1–F8.

Lanese DM, Conger JD. Effects of endothelin receptor antagonist on cyclosporine-induced vasoconstriction in isolated rat renal arterioles. *J Clin Invest*. 1993;91:2144–2149.

Lash LH, Cummings BS. Mechanisms of toxicant-induced acute kidney injury. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:81–115.

Lauwerys RR, Bernard AM, Roels HA, Buchet JP. Cadmium: exposure markers as predictors of nephrotoxic effects. *Clin Chem*. 1994;40:1391–1394.

Lehman-McKeeman LD. α_{2u}-Globulin nephropathy. In: McQueen CA, Schnellmann RG. eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:507–521.

Lemasters JJ, Qian T, Bradham CA, et al. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *J Bioenerg Biomembr*. 1999;31:305–319.

Levin S, Bucci TJ, Cohen SM, et al. The nomenclature of cell death: recommendations of an *ad hoc* Committee of the Society of Toxicologic Pathologists. *Toxicol Pathol*. 1999;27:484–490.

Liu S, Yao Y, Lu S, et al. The role of renal proximal tubule P450 enzymes in chloroform-induced nephrotoxicity: utility of renal specific P450 reductase knockout mouse models. *Toxicol Appl Pharmacol*. 2013;272:230–237.

Liu X, Godwin ML, Nowak G. Protein kinase C-alpha inhibits the repair of oxidative phosphorylation after S-(1,2-dichlorovinyl)-L-cysteine injury in renal cells. *Am J Physiol Renal Physiol*. 2004;287:F64–F73.

Liu X, Schnellmann RG. Calpain mediates progressive plasma membrane permeability and proteolysis of cytoskeleton-associated paxillin, talin, and vinculin during renal cell death. *J Pharmacol Exp Ther*. 2003;304:63–70.

Lock EA. Renal xenobiotic metabolism. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:55–79.

Lock EA, Reed CJ. Renal xenobiotic metabolism. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 1997:77–98.

Lopes JA, Jorge S. The RIFLE and AKIN classifications for acute kidney injury: a critical and comprehensive review. *Clin Kidney J*. 2013;6(1):8–14.

Lund BO, Miller DM, Woods JS. Studies in Hg(II)-induced H_2O_2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria. *Biochem Pharmacol*. 1993;45:2017–2024.

Maddox DA, Brenner BM. Glomerular ultrafiltration. In: Brenner BM, Rector FC, eds. *The Kidney*. 4th ed. Philadelphia, PA: WB Saunders; 1991:205–244.

Manohar S, Leung N. Cisplatin nephrotoxicity: a review of the literature. *J Nephrol*. 2018;31:15–25.

Mehendale HM. Halogenated hydrocarbons. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. 2nd ed. Vol 7. Oxford, UK: Elsevier; 2010:459–474.

Mehta RL, Kellum JA, Shah SV, et al. Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. *Crit Care*. 2007;11(2):R31.

Melnick R. An alternative hypothesis on the role of chemically induced protein droplet (α_{2u}-globulin) nephropathy in renal carcinogenesis. *Regul Toxicol Pharmacol*. 1992;16:111–125.

Miller GW, Schnellmann RG. Cytoprotection by inhibition of chloride channels: the mechanism of action of glycine and strychnine. *Life Sci*. 1993;53:1211–1215.

Miller GW, Schnellmann RG. Inhibitors of renal chloride transport do not block toxicant-induced chloride influx in the proximal tubule. *Toxicol Lett*. 1995;76:179–184.

Molitoris BA, Bacallao R. Pathophysiology of ischemic acute renal failure: cytoskeletal aspects. In: Berl T, Bonventre JV, eds. *Atlas of Diseases of the Kidney*. Philadelphia, PA: Current Medicine; 1999:13.1–13.8.

Monks TJ, Lau SS. Renal transport processes and glutathione conjugate-mediated nephrotoxicity. *Drug Metab Dispos*. 1987;15:437–441.

Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. *Clin J Am Soc Nephrol*. 2009;4(2):481–508.

Nowak G. Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na^+ transport, and cisplatin-induced apoptosis in renal cells. *J Biol Chem*. 2002;277:43377–43388.

Nowak G. Protein kinase C mediates repair of mitochondrial and transport functions after toxicant-induced injury in renal cells. *J Pharmacol Exp Ther*. 2003;306:157–165.

Nowak G, Bakajsova D, Clifton GL. Protein kinase C-epsilon modulates mitochondrial function and active Na^+ transport after oxidant injury in renal cells. *Am J Physiol Renal Physiol*. 2004;286:F307.

Nowak G, Clifton GL, Godwin ML, Bakajsova D. Activation of ERK1/2 pathway mediates oxidant-induced decreases in mitochondrial function in renal cells. *Am J Physiol Renal Physiol*. 2006;291:F840–F55.

Nowak G, Bakajsova D, Samarel AM. Protein kinase C-varepsilon activation induces mitochondrial dysfunction and fragmentation in renal proximal tubules. *Am J Physiol Renal Physiol*. 2011;301:F197–F208.

Okada Y, Maeno E, Shimizu T, Manabe K, Mori S, Nabekura T. Dual roles of plasmalemmal chloride channels in induction of cell death. *Pflugers Arch*. 2004;448:287–295.

Palmer BF, Heinrich WL. Toxic nephropathy. In: Brenner BM, ed. *Brenner and Rector's The Kidney*. 7th ed. Philadelphia, PA: WB Saunders; 2004:1625–1658.

Prozialeck WC, Edwards JR. Mechanisms of cadmium-induced proximal tubule injury: new insights with implications for biomonitoring and therapeutic interventions. *J Pharmacol Exp Ther*. 2012;343:2–12.

Rabb H. The promise of immune cell therapy for acute kidney injury. *J Clin Invest*. 2012;122:3852–3854.

Rabb H, Griffin MD, McKay DB. Inflammation in AKI: current understanding, key questions, and knowledge gaps. *J Am Soc Nephrol*. 2016;27:371–379.

Racusen LC, Solez K. Nephrotoxicity of cyclosporine and other immunotherapeutic agents. In: Hook JB, Goldstein RS, eds. *Toxicology of the Kidney*. 2nd ed. New York: Raven Press; 1993:319–360.

Raghavan R, Shawar S. Mechanisms of drug-induced interstitial nephritis. *Adv Chronic Kidney Dis*. 2017;24:64–71.

Ramachandiran S, Huang Q, Dong J, Lau SS, Monks TJ. Mitogen-activated protein kinases contribute to reactive oxygen species-induced cell death in renal proximal tubule epithelial cells. *Chem Res Toxicol*. 2002;15:1635.

Rankin GO, Valentovic MA. Role of xenobiotic metabolism. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:217–243.

Ricci Z, Cruz DN, Ronco C. Classification and staging of acute kidney injury: beyond the RIFLE and AKIN criteria. *Nat Rev Nephrol*. 2011;7:201–208.

Safirstein R, Deray G. Anticancer: cisplatin/carboplatin. In: de Broe ME, Porter GA, Bennett AM, Verpooten GA, eds. *Clinical Nephrotoxins: Renal Injury from Drugs and Chemicals*. The Netherlands: Kluwer; 1998:261–272.

Sanchez-Gonzalez PD, Lopez-Hernandez FJ, Lopez-Novoa JM, Morales AI. An integrative view of the pathophysiological events leading to cisplatin nephrotoxicity. *Crit Rev Toxicol*. 2011;41:803–821.

Schnellmann RG. Analgesic nephropathy in rodents. *J Toxicol Environ Health, Part B*. 1998;1:81–90.

Schnellmann RG, Cross TJ, Lock EA. Pentachlorabutadienyl-L-cysteine uncouples oxidative phosphorylation by dissipating the proton gradient. *Toxicol Appl Pharmacol*. 1989;100:498–505.

Schnellmann RG, Griner RD. Mitochondrial mechanisms of tubular injury. In: Goldstein RS, ed. *Mechanisms of Injury in Renal Disease and Toxicity*. Boca Raton, FL: CRC Press; 1994:247–265.

Schnellmann RG, Kelly KJ. Pathophysiology of nephrotoxic acute renal failure. In: Berl T, Bonventre JV, eds. *Atlas of Diseases of the Kidney*. Philadelphia, PA: Current Medicine; 1999:15.1–15.14.

Schnellmann RG, Lock EA, Mandel LJ. A mechanism of *S* -(1,2,3,4,4-pentachloro-1,3-butadienyl)-L-cysteine toxicity to rabbit renal proximal tubules. *Toxicol Appl Pharmacol*. 1987;90:513–521.

Schrier RW, Coffman TM, Falk RJF, et al. *Schrier's Diseases of the Kidney*. 9th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2012.

Servais H, Mingeot-Leclercq M-P, Tulkens PM. Antibiotic-induced nephrotoxicity. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:635–685.

Shaik ZP, Fifer EK, Nowak G. Protein kinase B/Akt modulates nephrotoxicant-induced necrosis in renal cells. *Am J Physiol Renal Physiol*. 2007;292:F292–F303.

Shaik ZP, Fifer EK, Nowak G. Akt activation improves oxidative phosphorylation in renal proximal tubular cells following nephrotoxicant injury. *Am J Physiol Renal Physiol*. 2008;294:F423–F432.

Sharp CN, Doll MA, Dupre TV, et al. Repeated administration of low-dose cisplatin in mice induces fibrosis. *Am J Physiol Renal Physiol*. 2016;310:F560–F568.

Sheikh-Hamad D, Cacini W, Buckley AR, et al. Cellular and molecular studies on cisplatin-induced apoptotic cell death in rat kidney. *Arch Toxicol*. 2004;78:147–155.

Shibutani S, Dong H, Suzuki N, et al. Selective toxicity of aristolochic acids I and II. *Drug Metab Dispos*. 2007;35(7):1217–1222.

Sidorenko VS, Attaluri S, Zaitseva I, et al. Bioactivation of the human carcinogen aristolochic acid. *Carcinogenesis*. 2014;35(8):1814-22.

Skorecki K, Chertow GM, Marsden PA, et al. *Brenner and Rector's The Kidney*. 10th ed. Philadelphia, PA: Elsevier; 2015.

Smith JH, Maita K, Sleight SD, Hook JB. Effect of sex hormone status on chloroform nephrotoxicity and renal mixed function oxidases in mice. *Toxicology*. 1984;30:305–316.

Smith MW, Ambudkar IS, Phelps PC, et al. $HgCl_2$-induced changes in cytosolic Ca^{2+} of cultured rabbit renal tubular cells. *Biochem Biophys Acta*. 1987;931:130–142.

Steinmetz PR, Husted RF. Amphotericin B toxicity for epithelial cells. In: Porter GA, ed. *Nephrotoxic Mechanisms of Drugs and Environmental Toxins*. New York: Plenum Press; 1982:95–98.

Stevens LA, Levey AS. Measurement of kidney function. *Med Clin North Am*. 2005;89:457–473.

Stiborová M, Arlt VM, Schmeiser HH. Balkan endemic nephropathy: an update on its aetiology. *Arch Toxicol*. 2016;90:2595–2615.

Sweet DH. Renal organic cation and anion transport: from physiology to genes. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:23–53.

Tarloff JB. Analgesics and nonsteroidal anti-inflammatory drugs. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol. 7. Oxford, UK: Elsevier; 1997:583–600.

Tarloff JB. Analgesics and nonsteroidal anti-inflammatory drugs. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol. 7. Oxford, UK: Elsevier; 2010:387–403.

Tarloff JB, Kinter LB. *In vivo* methodologies used to assess renal function. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol. 7. Oxford, UK: Elsevier; 1997:99–120.

Townsend DM, Hanigan MH. Inhibition of γ glutamyl transpeptidase or cysteine *S*-conjugate β-lyase activity blocks the nephrotoxicity of cisplain in mice. *J Pharmacol Exp Ther*. 2002;300:142–148.

Ulozas E. Amphotericin B–induced nephrotoxicity. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:347–357.

Vaidya VS, Bonventre JV, Ferguson MA. Biomarkers of acute kidney injury. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:197–211.

van de Water B, de Graauw M, Le Devedec S, Alderliesten M. Cellular stress responses and molecular mechanisms of nephrotoxicity. *Toxicol Lett*. 2006;162:83–93.

Vanherweghem JL, Depierreux M, Tielemans C, et al. Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *Lancet*. 1993;341(8842):387–391.

Wallin A, Jones TW, Vercesi AE, et al. Toxicity of *S*-pentachlorobutadienyl-L-cysteine studies with isolated rat renal cortical mitochondria. *Arch Biochem Biophys*. 1987;258:365–372.

Wang C, Salahudeen AK. Cyclosporine nephrotoxicity: attenuation by an antioxidant-inhibitor of lipid peroxidation in vitro and in vivo. *Transplantation*. 1994;58:940–946.

Wang C, Salahudeen AK. Lipid peroxidation accompanies cyclosporine nephrotoxicity: effects of vitamin E. *Kidney Int*. 1995;47:927–934.

Wang Y, Bollard ME, Nicholson JK, Holmes E. Exploration of the direct metabolic effects of mercury II chloride on the kidney on Sprague–Dawley rats using high-resolution magic angle spinning 1H NMR spectroscopy of intact tissue and patter recognition. *J Pharm Biomed Anal*. 2006;40:375–381.

Wang Z, Chen JK, Moeckel G, Harris RC. Importance of functional EGF receptors in recovery from acute nephrotoxic injury. *J Am Soc Nephrol*. 2003;14(12):3147–3154.

Waters SL, Sarang SS, Wang KKW, Schnellmann RG. Calpains mediate calcium and chloride influx during the late phase of cell injury. *J Pharmacol Exp Ther*. 1997a;283:1177–1184.

Waters SL, Wong JK, Schnellmann RG. Depletion of endoplasmic reticulum calcium stores protects against hypoxia- and mitochondrial inhibitor–induced cellular injury and death. *Biochem Biophys Res Commun*. 1997b;240:57–60.

Weinberg JM, Harding PG, Humes HD. Mitochondrial bioenergetics during the initiation of mercuric chloride–induced renal injury: I. Direct effects of in vitro mercuric chloride on renal cortical mitochondrial function. *J Biol Chem*. 1982a;257:60–67.

Weinberg JM, Harding PG, Humes HD. Mitochondrial bioenergetics during the initiation of mercuric chloride–induced renal injury: II. Functional alterations of renal cortical mitochondria isolated after mercuric chloride treatment. *J Biol Chem*. 1982b;257:68–74.

Woo KR, Shu WP, Kong L, Liu BC. Tumor necrosis factor mediates apoptosis via Ca^{2+}/Mg^{2+} dependent endonuclease with protein kinase C as a possible mechanism for cytokine resistance in human renal carcinoma cells. *J Urol*. 1996;155:1779–1783.

Zalups RK. Renal toxicity of mercury. In: Sipes IG, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 1997:633–652.

Zalups RK. Renal handling and toxicity of mercury. In: McQueen CA, Schnellmann RG, eds. *Comprehensive Toxicology*. Vol 7. Oxford, UK: Elsevier; 2010:475–493.

Zalups RK, Diamond GL. Nephrotoxicology of metal. In: Tarloff JB, Lash LH, eds. *Toxicology of the Kidney*. 3rd ed. Boca Raton, FL: CRC Press; 2005:937–994.

Zalups RK, Lash LH. Advances in understanding the renal transport and toxicity of mercury. *J Toxicol Environ Health*. 1994;42:1–44.

Zhuang S, Dang Y, Schnellmann RG. Requirement of the epidermal growth factor receptor in renal epithelial cell proliferation and migration. *Am J Physiol Renal Physiol*. 2004;287(3):F365–F372.

Zhuang S, Kinsey GR, Yan Y, Han J, Schnellmann RG. Extracellular signal-regulated kinase activation mediates mitochondrial dysfunction and necrosis induced by hydrogen peroxide in renal proximal tubular cells. *J Pharmacol Exp Ther*. 2008;325:732–740.

Zhuang S, Yan Y, Daubert RA, Han J, Schnellmann RG. ERK promotes hydrogen peroxide-induced apoptosis through caspase-3 activation and inhibition of Akt in renal epithelial cells. *Am J Physiol Renal Physiol*. 2007;292:F440–F447.

Zhuang S, Yan Y, Han J, Schnellmann RG. p38 kinase-mediated transactivation of the epidermal growth factor receptor is required for dedifferentiation of renal epithelial cells after oxidant injury. *J Biol Chem*. 2005;280:21036–21042.

chapter 15

Toxic Responses of the Respiratory System

George D. Leikauf

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Acknowledgments

References

"Since the time of Hippocrates the growth of scientific medicine has in reality been based on the study of the manner in which what he called 'Nature' of the living body expresses itself in response to changes in the environment, and reasserts itself in face of disturbances and injury"

—John Scott Haldane (Haldane, 1922)

HISTORICAL PERSPECTIVE

Toxic substances can disrupt the respiratory system and distant organs after chemicals enter the body by means of inhalation. Blood-borne agents also can cause pathological changes in the respiratory tract. Inhalation toxicology refers to the route of exposure, whereas respiratory toxicology refers to target organ toxicity. This chapter reviews the toxic responses of the respiratory system.

Historically, respiratory toxicology is a keystone of medicine, dating back to Hippocrates. In his medical thesis *On Airs, Waters, and Places*, Hippocrates recommended that physicians evaluate local atmospheres to discover the causes of diseases (Adams, 1849). In 1661, John Evelyn appealed to the English King and Parliament for relief from the poor air quality of London that was a result of the burning of "sea-coale" (a brown coal likely enriched in sulfur that washed up on the banks of the River Thames (Evelyn, 1661). This situation continued and became worse in the 19th century when the Industrial Revolution quickened awareness of respiratory toxicology due to air pollutions (see Chap. 31).

Later, Bernardino Ramazzini proposed that clinicians evaluate the relationships between occupational atmospheres and disease pathogenesis, starting a long history of respiratory toxicology in occupational medicine. He observed that "corruption of the atmosphere" can be at the origin of many respiratory diseases. In his work *De Morbis Artificum Diatriba* (*Diseases of Workers*) (1713), he writes: "Miners who maintain an almost daily contact with evil powders in the earth's depths … have lungs which absorb mineral exhalations and must be the first to suffer the attack of poisonous fumes …. The mortality rate of miners is very high and, as a proof of this, we remember that their wives re-marry many times." Supporting the concept of exposure, he continues: "It is not only miners working in mines who run the risk of dying from diseases due to metals: so do many others working around mines." He also supported the concept of prevention over treatment stating "Prevention is better than cure, just as it is better, on seeing storm arrive, to get under cover than to suffer its damages" (Bisetti, 1988).

During the 19th century the relationship between dusty trades and bronchitis (Thackrah, 1832), and silica dust and pneumoconiosis (Holland, 1843) became well recognized. In 1873, excessive bronchitis deaths were attributed to London fogs (smog) (British Medical Journal, 1880). In a report on London fogs (smog) in 1880 that lead to 1817 excessive deaths, Russell (Russel, 1880) states "And smoke in London has continued probably for many years to shorten the lives of thousands, but only lately has the sudden, palpable rise of the death-rate in an unusually dense and prolonged fog attracted much attention to the depredations of this quiet and despised destroyer." He goes on to note: "A London fog is brown, reddish-yellow, or greenish, darkens more than a white fog, has a smoky, or sulphurous smell, is often somewhat dryer than a country fog, and produces, when thick, a choking sensation. Instead of diminishing while the sun rises higher, it often increases in density, and some of the most lowering London fogs occur about midday or late in the afternoon. Sometimes the brown masses rise and interpose a thick curtain at a considerable elevation between earth and sky. A white cloth spread out on the ground rapidly turns dirty, and particles of soot attach themselves to every exposed object." In 1884, John Aitken proposed that particles contribute to the haze and alter the color of the sunset (Aitken, 1884).

Also toward the end of the 19th century, John Scott Haldane identified carbon monoxide as the lethal constituent of "afterdamp," a gas mixture created by combustion in mines, after examining many bodies of miners killed in pit explosions (Haldane, 1896). He noted the skin was colored cherry-pink from carboxyhemoglobin and studied carbon monoxide's ability to displace oxygen. An experimentalist, he had investigated the effect of carbon monoxide on his own breathing in a chamber (Haldane, 1895). In the late 1800s, he supported efforts to improved mine safety by introducing gas masks for rescue workers and the use of small animals (canaries and white mice) to detect dangerous levels of carbon monoxide.

By the start of the 20th century, respiratory toxicology became even more inseparable from occupational medicine. Coal workers' pneumoconiosis (black lung), silicosis, and byssinosis were noted in specific trades, even in the absence of bacterial infection (tuberculosis) (Oliver, 1902; Hoffman, 1918; Blanc, 2005).

During World War I, respiratory toxicology turned to the dark side. Efforts of Fritz Haber (Germany) (Witschi, 2000) and Victor Grignard (France) (Hodson, 1987) led to the development and use of chlorine, phosgene, and other gases in chemical warfare. This was accompanied by toxicological studies of poison gases in laboratory animals, in which Haber noted that exposure to a low concentration of a poisonous gas for an extended time could produce the same effect (death) as exposure to a high concentration for a short time. The so-called "Haber's rule" is $C \times t = k$, where C is the gas concentration (mass/volume), t is the exposure time producing a given toxic effect, and k is a constant. Although sometimes useful, this rule has many exceptions and should be applied with caution.

In the 1920s, Yandell Henderson and Howard Haggard began testing numerous noxious chemicals and culminated a compendium of dose–response analysis and median lethal concentration (LC_{50}) of a number of noxious chemicals (Henderson and Haggard, 1943). Although a simple test of lethality clearly has limitation in understanding the mechanism of toxicity, it still has value today. The results from Henderson and Haggard's efforts are often referred to in assessments of Immediately Dangerous to Life or Health concentrations, which can be informative following accidental inhalation exposures.

From the 1920s to 1950, CN Davies (Davies, 1949), Lucien Dautrebande (Dautrebande et al., 1948), Phillip Drinker (Drinker et al., 1928), Lars Friberg (Friberg, 1948), Theodore Hatch (Hatch, 1937; Hatch and Hemeon, 1948), Earl King (Robson et al., 1934), Frank Patty (Patty, 1949), HD Landahl (Landahl and Black, 1947), Leslie Silverman (Silverman and Lee, 1946; Silverman and Whittenberger, 1949), Henry Smyth Jr (Smyth, 1946), Herbert Stokinger (Stokinger, 1949; Stokinger et al., 1950), and many others advanced exposure science (i.e., measure of concentrations of gas and particles in workplace and ambient atmospheres), began controlled inhalation exposure of laboratory animals, and made recommendations for human occupational exposure limits (threshold limit values [TLV]).

These investigations provided the foundation for the modern era of respiratory toxicology led by Yves Alarie (Alarie et al., 1961), Roy Albert (Albert and Arnett, 1955), Mary Amdur (Amdur et al., 1952), David Bates (Young et al., 1964), Eula Bingham (Bingham et al., 1968), Joe Brain (Brain, 1970), Louis Casarett (Casarett, 1960), David Coffin (Coffin et al., 1968), John Craighead (Tegtmeyer and Craighead, 1968), Carol Cross (DeLucia et al., 1972), Tore Dalhamn (Dalhamn and Rodin, 1956), Robert Drew (Drew and Eisenbud, 1970), Juraj Ferin (Ferin et al., 1965), Robert Frank (Frank, 1970), Gustav Freeman (Freeman and Haydon, 1964), Donald Gardner (Gardner et al., 1969), Bernard Goldstein (Goldstein and Balchum, 1967), Elliot Goldstein (Goldstein et al., 1969), Gareth Green (Green and Carolin, 1967), Paul Gross (Gross et al., 1952), Theodore Hatch (Palm et al., 1956), Harold Hodge (Hodge et al., 1956), Sidney Laskin (Laskin et al., 1963), Morton Lippmann (Lippmann and Albert, 1968), Paul Morrow (Morrow et al., 1958), J. Brian Mudd (Mudd et al., 1969), Sheldon Murphy (Murphy et al., 1963), Jay Nadel (Nadel and Comroe, 1961), Norton Nelson (Nelson, 1955), Ed Palmes (Palmes et al., 1959), Otto Raabe (Raabe, 1967), Verald Rowe (Rowe et al., 1952), Ragnar Rylander (Rylander, 1968), Irving Selikoff (Selikoff et al., 1965), Norman Staub (Kato and Staub, 1966), Walter Tyler (Tyler and Pearse, 1965), and Hanspeter Witschi (Witschi and Aldridge, 1968). These individuals and many others have enabled a better understanding of the deposition and effects of gases and particles on the respiratory tract.

RESPIRATORY TRACT STRUCTURE AND FUNCTION

Oronasal Passages

Structure The respiratory tract is divided into the upper respiratory tract (extrathoracic airway passages above the neck) and lower respiratory tract (airway passages and lung parenchyma below the pharynx) (Harkema et al., 2006) (Fig. 15-1). The upper respiratory tract reaches from the nostril or mouth to the pharynx and functions to conduct, heat, humidify, filter, and chemosense incoming air. Leaving the nasal passage, air is warmed to about 33°C and

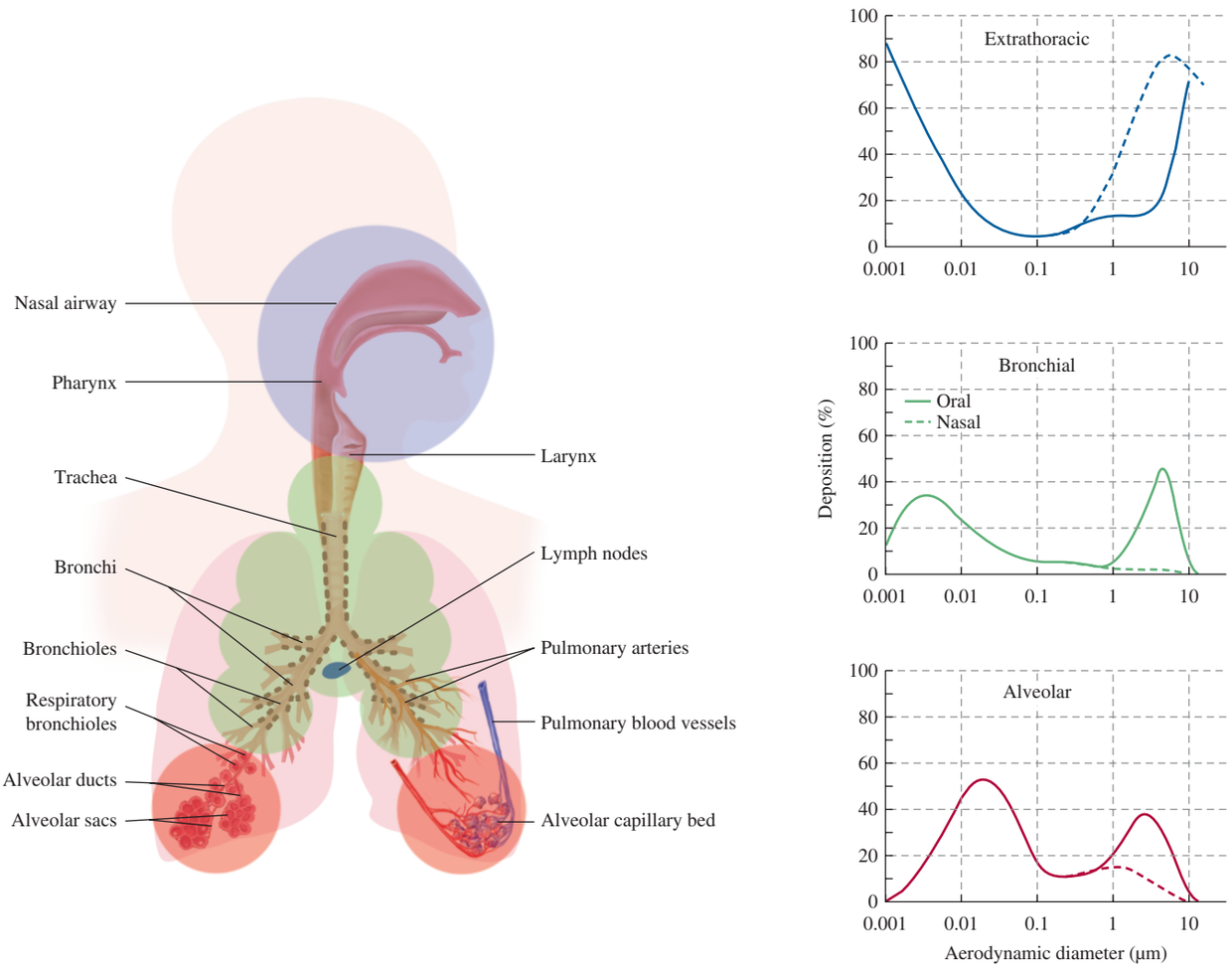

Figure 15-1. *Major regions of the respiratory tract and predicted fractional deposition of inhaled particles in the extrathoracic, bronchial, and alveolar region of the human respiratory tract during (solid line) oral or (dashed) nasal breathing.* (Adapted from Figure 15-8 in Oberdörster et al. (2005) with drawing courtesy of J. Harkema and data from ICRP [1994].)

humidified to about 98% water saturation. Air is filtered in the nasal passages with highly water-soluble gases being absorbed efficiently. The nasal passages also filter particles, which may be deposited by impaction or diffusion on the nasal mucosa. Many species, particularly mice and rats, are obligate nose breathers in which air passes almost exclusively through the nasal passages. Other species, including humans, monkeys, and dogs, inhale air through both the nose and the mouth (oronasal breathers).

The surface area of the oronasal region in humans has been estimated to be 4700 cm^2. In mammals, the nasal passages are separated by a cartilaginous septum and the hard and soft palates form the base. Filtration, heating, and humidification are greatly aided by aqueous layer lining the mucosa and turbinates, which are perturbed from the lateral nasal walls. To warm the air, blood flow in the turbinates is retrograde to the inward direction of the air and can be modulated by pterygopalatine ganglion innervation of the venous plexus.

Turbinates vary in size and shape with the anterior being simple and the posterior being more complex. The airflow through the nasal passage is complex, and the narrowest region (smallest cross-sectional area) is located in the anterior aspect of the anterior turbinate. This region has the highest airflow and can be viewed as a nasal valve (ostium internum). The resistance of this region limits the amount of air that can be inhaled through the nose. In oronasal breathers, oral breathing can be initialed and will vary based on the workload, speech, and nasal congestion.

In humans, three flat turbinates are fairly simple structures. The inferior turbinates are the largest (~8-cm long) and are responsible for the majority of the control of airflow direction, humidification, heating, and filtering of air inhaled through the nose. The smaller middle turbinates (~4 to 6 cm) project downward over the openings of the maxillary and ethmoid sinuses, and protect the sinuses from pressurized nasal airflow. Most of the inhaled air travels between the inferior turbinate and the middle turbinate. The superior turbinates are smaller structures and serve to protect the olfactory bulb. In rodents, the anterior portion of the nasal cavity contains a dorsal nasal turbinate and a ventral maxilloturbinate, both with simple scroll structures. Posterior to these turbinates are complex multiscrolled ethmoturbinates, which contain ~50% of the surface area of the rodent nasal passage. However, the amount of airflow over the ethmoturbinates region has been estimated to be only 10% to 15% of the air passing through the nose, and thus the complexity of this structure in rodents may contribute little additional risk of contact or damage.

The nasal passages are lined with stratified squamous epithelium in the anterior vestibule, nonciliated cuboidal/columnar

epithelium in the anterior chamber, and ciliated pseudostratified respiratory epithelium in the remainder of the passage including the turbinates. The cell types of the nasal respiratory epithelium are similar to the cell types of the conducting airways. The turbinates also contain airflow pressure- and temperature-sensing neural receptors linked to the trigeminal nerve.

Sensory Functions In addition to conducting, conditioning, and filtering air flowing to the lower respiratory tract, a major function of the oronasal passage is chemosensory (Feron et al., 2001; Morris, 2001). Nasal epithelia can metabolize many foreign compounds by cytochrome P450 and other enzymes. Humans can distinguish between more than 5000 odors. The detection of odor can be protective and can induce avoidance behaviors. Odorant can be added to the otherwise colorless and almost odorless gas used by consumers (e.g., mercaptans to methane), to assist in detecting leaks and thereby preventing fires or explosions.

Although the detection threshold concentrations can be low, a concentration only 10 to 50 times above the detection threshold value often is the maximum intensity that can be detected by humans. In contrast, the maximum intensity of sight or hearing is about 500,000 times and 1 to 10 trillion times that of the threshold intensity. For this reason, smell often identifies the presence or absence of odor rather than quantifies concentration. In addition, odor thresholds vary greatly between individuals (>1000 fold) and can be altered by allergies or nasal infections, and individuals can acclimate to odors. Some individuals cannot smell certain odors. For example, 0.1% of the population cannot detect mercaptans in natural gas. Olfactory acuity also decreases with age (decreasing by 20%, 60%, and 70% at age of 20, 60, and 80 years, respectively). Therefore, about 30% of the elderly cannot detect mercaptans in natural gas and are at risk of leaving the gas on but the burner unlit. Lastly, odor thresholds for many compounds (e.g., chlorinated solvents) are often higher than the Occupational Safety and Health Administration (OSHA) Permissible Exposure Limits (PELs). Therefore, odor should not be used as a measure of safety. The chemosensory functions of the nasal passages are accomplished by a wide variety of specialized receptors in major subtypes including (1) olfactory, (2) trace amine–associated receptors (TAARs), (3) membrane guanylyl cyclase GC-D, (4) vomeronasal, and (5) formyl peptide receptors (FPRs) (Table 15-1).

The olfactory epithelium contains specialized chemosensory olfactory neurons located above the superior turbinates. Airflow in this region of the nasal passage is typically low, thus sniffing can increase perception. This may enable the assessment of multiple odors and strength of a smell through intermediate sampling. Capable of regeneration, olfactory neurons form the first cranial nerve and directly lead to the olfactory bulb in the brain. These cells have surface olfactory receptor proteins in cilia that interact with odorant molecules (DeMaria and Ngai, 2010). Olfactory receptors are 7-transmembrane domain G-protein–coupled receptors that mediate transduction of odorant signals through formation of cyclic adenosine monophosphate (cAMP) (Fleischer et al., 2009). The olfactory receptor gene family is one of the largest in the genome, with over 400, 850, 1100, and 1200 members in humans, dogs, mice, and rats, respectively. Olfactory receptors are also involved in developmental events, including the patterning of the olfactory sensory neuron synaptic connections in the brain.

Also in the olfactory region and originally identified because of activation by amines, TAARs detect trace amine (including 2-phenylethylamine, tyramine, tryptamine, and octopamine) and other substances (Fleischer et al., 2009). Low-molecular-weight amines have a fishy or putrid odor. These odorants can be found in foods (including fish, chocolate, alcoholic beverages, cheese, soy sauce, sauerkraut, and processed meat) and can be generated during fermentation or decay. Certain trace amines are neurotransmitters and are found in the brain. Compared to olfactory receptors, the number of distinct TAAR subtypes is low (15 in mice and 6 in humans). In mouse urine, trace amine concentration varies by gender or during stress, suggesting that TAARs might be involved in the detection of "urine-borne" signals.

Table 15-1

Oronasal Sensory Receptors I

RECEPTOR PROTEIN FAMILY	SYMBOL	LIGAND	HUMAN	MOUSE
Olfactory receptor	OR	Odorants	>400	>1200
Trace amine–associated receptor	TAAR	Amines	6	15
Guanylate cyclase 2D	GUCY	Natriuretic peptides	1	2
Vomeronasal receptor	VN1R/ VN2R	Pheromones	2*	226
Formyl peptide receptors	FPR	*N*-formyl-peptides	3	8

**Only expressed in fetus and may be vestigial.*

Another olfactory sensory neuron receptor is the membrane guanylyl cyclase receptor, GUCY2D, which contains a cyclic guanosine monophosphate (cGMP)-dependent phosphodiesterase PDE2A and a cGMP-sensitive cyclic nucleotide-gated ion channel (Fleischer et al., 2009). In mice, this receptor is localized to olfactory sensory neuron apical cilia and detects the natriuretic peptides: uroguanylin (which is also found in urine) and guanylin. In mice, GUCY2D also can detect carbon dioxide by conversion into bicarbonate via carbonic anhydrase (Kuhn, 2016). In contrast to rodents, carbon dioxide is odorless to humans, and the homologous gene, *GUSY2EP* is a pseudogene (i.e., a gene that is present but does not yield a functional protein) in humans and other primates (Young et al., 2007). The main olfactory bulb is accompanied by the accessory olfactory bulb. Neurons from these two systems do not interconnect and the two systems function separately in the integration of specific chemicals. In rodents, the accessory olfactory bulb contains olfactory neurons that lead to the vomeronasal organ in the nose. Vomeronasal neurons can respond to olfactory stimuli that can be of higher molecular weight including nonvolatile chemicals (Touhara and Vosshall, 2009). Vomeronasal receptors exist in two protein families, VMN1R and VMN2R. These receptors are similar to pheromone receptors. Pheromones are chemical signals that elicit specific physiological and behavioral responses in recipients of the same species. Similar to olfactory receptor genes, several vomeronasal genes exist in rodents, with few in humans, that is, 2, 8, 163, and 286 members in human, dogs, rats, and mice, respectively. Humans have not been demonstrated to generate or respond to pheromones. Expressed only during fetal gestation, vomeronasal receptors are thought to be merely vestigial in humans.

In addition, the vomeronasal organ contains FPRs that are activated by bacterial or mitochondrial formylated peptides (Fleischer et al., 2009). These receptors were initially identified in leukocytes in which *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) mediates chemotaxis and cell activation. In the mouse vomeronasal epithelium, FPRs are activated by fMLP and other compounds (including

Table 15-2

Oronasal Irritant, Thermo- and Mechanosensory Receptors

RECEPTOR PROTEIN FAMILY	SYMBOL	LIGAND
Transient receptor potential channels	TRP	
Subfamily A (ANKTM1)	TRPA	Natural ingredients: Allyl isothiocyanate (wasabi), cinnamaldehyde, allicin and allyl sulfides (garlic), carvacrol, isovelleral, and polygodial
		Pain
		Cold (<17ºC)
		Mechosenstory (stretch)
		Irritants (Acrolein, isocyanates, tear gas, ozone, etc.)
Subfamily C (Canonical)	TRPC	Mechanosensory
Subfamily M (Melastain)	TRPM2	Hydrogen peroxide
		Heat (>38ºC)
	TRPM8	Menthol, eucalyptol
		Cold (<17ºC)
Subfamily ML (Mucolipins)	MCOLN	Acid (low pH)
Subfamily P (Polycystic kidney disease)	PKD	Mechanosensory
		Acid (low pH)
Subfamily V (Vanilloid)	TRPV1	Capsaicin, allicin, and allyl sulfides (garlic)
		Moderate heat (≥43ºC)
	TRPV2	High heat (>52ºC)
Taste receptors	TAS	
Subfamily 1R	TAS1R	Umami (glutamate)
		Irritants (Acrolein, isocyanates, tear gas, ozone, etc.)
Subfamily 2R	TAS2R	Bitter
		Irritants (Acrolein, isocyanates, tear gas, ozone, etc.)
Subfamily 3R	TAS3R	Umami (glutamate)

lipoxin A4 and cathelicidin antimicrobial peptide) indicating that vomeronasal cells are likely to perform olfactory functions associated with the identification of pathogens or of pathogenic states, thereby enhancing detection of infected cells or contaminated food.

Two evolutionary hypotheses have been proposed to explain the large interspecies difference in the number of chemosensory receptor genes (Nei et al., 2008). One states that humans have developed full trichromatic vision and therefore do not need as many chemosensory receptor genes for finding food, mates, or supportive environments (Gilad et al., 2004). The other is that the number of chemosensory receptor genes has expanded in the rodent lineage because rodents probably need a higher level of olfaction to survive in heterogeneous environments (Niimura and Nei, 2006). However, dogs known for a good sense of smell have a smaller number of functional chemosensory receptor genes than mice or rats. This suggests that the relationship between the number of olfactory receptor genes and the sense of smell may not be straightforward. Even more complexity is suggested in that humans can detect certain odors at concentrations equal to or even below those detected by dogs or mice. One reason for this may be that olfactory perception also involves the brain (Liman, 2012). With a better memory, humans may have better olfactory ability from the small number of genes, particularly in detecting fine differences in food flavors.

Irritant, Thermosensory, and Mechanosensory Functions In addition to the detection of odor, the detection of irritant chemicals, cold and hot temperatures, or mechanical stress can be a protective mechanism that may limit exposure. The main nerve endings that perceive irritants, the chemical nociceptors, also discern temperature and mechanical stress. Two protein families, the transient receptor potential (TRP) channels and the taste (TAS) receptors, perform these functions in the upper respiratory tract (Table 15-2).

TRP channels are ion channels that are permeable to cations, including calcium, magnesium, and sodium. In mammals, 28 genes encode the TRP ion channel proteins that are divided into six subfamilies including TRPA (ANKTM1), TRPC (canonical), TRPM (melastatin), mucolipins (TRPML also known as [aka] MCOLN), polycystic kidney disease (autosomal dominant) (PCK or TRPP), and TRPV (vanilloid) families. TRPA1 and TRPV1 are the major irritant receptors in the nasal passage and are primarily within the trigeminal nerve (Bessac and Jordt, 2008). TRPA1 is responsive to a variety of natural ingredients including allyl isothiocyanate (in mustard and wasabi), cinnamaldehyde (in cinnamon), allicin and allyl sulfides (in garlic and onion), carvacrol (in oregano), isovelleral (a fungal deterrent), and polygodial (in Dorrigo pepper). TRPA1 is also responsive to pain stimuli, cold (≤17°C), stretch, and a wide range of chemical irritants. TRPV1 is responsive to capsaicin (in chili pepper) or moderate heat (≥43°C), whereas TRPV2 is responsive to higher heat (≥52°C). TRPM8 is responsive to menthol (in peppermint and cigarettes) and cold (≤28°C). Mucolipins are involved in the late endocytic pathway and in the regulation of lysosomal exocytosis. TRPC proteins are located mainly in the central nervous system and to a lesser extent in peripheral tissues. PCK1, TRPV5, and TRPV6 are calcium entry channels mainly found in the kidney and intestine.

Other chemosensory receptors are taste receptors (TAS), which are divided into two types (Chandrashekar et al., 2006). Taste buds determine salt, sour, sweet, umami (glutamates and nucleotides), and bitter. In the mouth, salt may be perceived by sodium ion channels, but this is controversial. Sour also may be perceived by hydrogen ion channels and possibly a TRP channel (polycystic kidney disease 2-like 1). Sweet and umami are perceived by type 1 receptors, which consist of three members (TAS1R1, TAS1R2, and TAS1R3). Taste variety is achieved by formation heterodimers of these proteins. For example, umami is detected by TAS1R1 and TAS1R3

heterodimers and through metabotropic glutamate receptors 1 and 4. Bitter taste is detected by type 2 receptors (TAS2Rs), which is a larger subfamily having over 35 members. Single solitary chemosensory cells (SCCs) are present in the nasal cavity and throughout the airways. In the mouse nose, SCCs contain both TAS1R and TAS2R, which can detect irritants and foreign substances that trigger trigeminally mediated protective airway reflexes.

Conducting Airways

Structure At the beginning of the lower respiratory tract is the larynx, which is responsible for speech (phonation). The conducting airways of the lower respiratory tract can be divided into proximal (trachea and bronchi) and distal regions (bronchioles). Conducting airways have a bifurcating structure, with successive airway generations containing about twice the number of bronchi progressively decreasing in internal diameter. In humans, this branching pattern is referred to as irregular dichotomous (because some branches have more or less than two daughters) and resembles the pattern of an oak tree. In laboratory animals, the branching pattern is more monopodial and resembles the pattern of a pine tree. Successive branching has two consequences—it increases total surface area of the airway epithelium, and it increases the cumulative cross-section diameter of the airways. Thus, airflow is faster in the larger diameter proximal airways, whereas airflow is slower in the smaller distal airways. The latter is somewhat counterintuitive because flow through a smaller diameter increases in many incidences (as in a weir), but flow is slower because the larger number of small airways have a much larger cumulative diameter.

The bifurcations of proximal airways are flow dividers. As airway bending points, they serve as sites of impaction for particles. The airflow is also altered by airway smooth muscle that surrounds the airways and is under autonomic innervation via the vagus nerve. Vagal nerve endings release acetylcholine, which contracts smooth muscle and induces mucus secretion. Contraction of the smooth muscle leads to bronchoconstriction that increases particle deposition in the proximal airway upon inhalation, and can reduce airflow upon exhalation. The contraction of airway smooth muscle can be reversed by adrenergic stimulation either by circulating epinephrine or by inhaled bronchodilators that stimulate adrenergic receptors beta 2 on airway smooth muscle.

Eventually a transition zone is reached where cartilaginous airways (bronchi) give way to noncartilaginous airways (bronchioles), which in turn give way to gas exchange regions, respiratory bronchioles, and alveoli. Although the airways are successively narrower in diameters, the cumulative diameter of the airways becomes very large ultimately leading to very slow or no net airflow. This favors the collection of gases and particles on airway walls by radial diffusion or sedimentation. In the bronchiolar epithelium, mucus-producing cells and glands give way to bronchiolar secretoglobin cells (BSCs).

Mucociliary Clearance and Antimicrobial Functions In humans, the proximal airway and a portion of the nasal passage are covered by a pseudostratified respiratory epithelium that contains a number of specialized cells including ciliated, mucous, and basal cells (Fig. 15-2). These cells work together to form a mucous layer that traps and removes inhaled material via mucociliary clearance (Fahy and Dickey, 2010). The epithelial cells are covered by an upper mucus layer (a gel-like polymer network of high-molecular-weight mucins) and a lower periciliary liquid layer that separates the epithelial cell surface from the mucus layer. For mucociliary clearance in the airways to function optimally, regulation of ion transport, fluid, and mucus must be coordinated. To move fluid into the airway lumen, the large diameter airway epithelium can secrete chloride

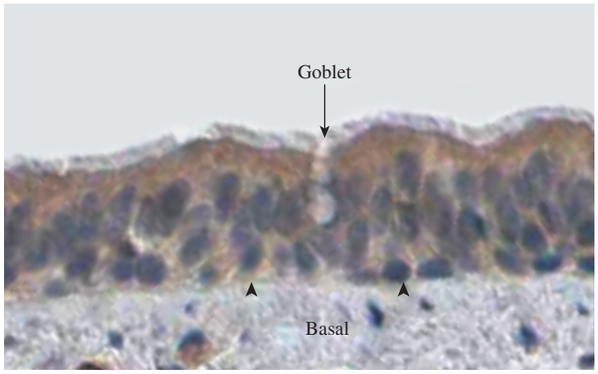

Figure 15-2. *Pseudo stratified respiratory epithelium lines the nasal cavity, trachea, and bronchi.* The surface includes mainly ciliated epithelial cells that may or may not touch the basement membrane, (arrow) surface mucous (goblet) cell, and (arrowhead) basal cells. (Photomicrograph modified from the Human Protein Atlas [www.proteinatlas.org] [Uhlen et al., 2010].)

ions via chloride channels (Patel et al., 2009) and the cystic fibrosis transmembrane regulator (Chen et al., 2010). To move water out of the lumen or alveolus, sodium ions are absorbed via sodium channels. These ionic gradients permit water movement that can travel pericellulary or through specialized proteins called aquaporins.

Ciliated cells have cilia that are microtubule-based, apical membrane protrusions (Sanderson and Sleigh, 1981; Salathe, 2007). There are two general types of cilia: motile and primary. Motile cilia exert mechanical force through continuous motion to propel harmful inhaled material out of the nose and lung. Primary cilia often serve as sensory organelles. Motile cilia are ~6 to 10 μm in length with a tubulin-based axoneme motor. The axoneme of each cilia consists of nine outer doublets of microtubules and a single central pair of microtubules (9 + 2 structure) formed by heterodimers of α and β tubulin. In motile cilium, dynein heavy chains on one microtubule interact with an adjacent microtubule that enables ciliary movement through energy generated by ATPase. Primary cilium lacks dynein and has 9 + 0 structure.

Ciliary beat frequency is about 12 to 15 Hz (720 to 900 beats/minute), which can change in response to cholinergic (acetylcholine) or purinergic (adenosine or ATP) stimuli that alter the phosphorylation state of ciliary targets, intracellular [Ca^{2+}] or intracellular pH. In addition to controlling ciliary beat frequency, calcium is also involved in synchronizing the beat among cilia of a single cell and between cilia on different cells (Schmid and Salathe, 2011). Adenosine acts through the adenosine A2b receptor (ADORA2B) (Allen-Gipson et al., 2011). Motile cilia of the mammalian respiratory epithelium also exhibit both mechanosensory (via TRPV4) and chemosensory (via TAS2Rs) functions. TRPV4 channels respond to mechanical stress, heat, acidic pH, endogenous and synthetic agonists, and activation leads to increases in intracellular calcium and ciliary beat frequency (Lorenzo et al., 2008). In response to bitter compounds, TAS2Rs also increase the intracellular calcium and stimulate ciliary beat frequency (Shah et al., 2009).

Mucus cells are full of lucid mucus granules. These granules increase in size as they move toward the apical cytoplasm, which produces a goblet shape and thus surface mucus cells are also called goblet cells. Mucus consists mainly of water (95%) combined with salts, lipids, proteins, and mucin glycoproteins (Kesimer et al., 2009; Ali et al., 2011). Mucin glycoproteins provide the gel-like viscoelastic properties of mucus. Of the 20 identified membrane-associated or secretory mucin gene products, 16 have been identified in the airways (Leikauf, 2002; Ali and Pearson, 2007). Of these proteins, mucin 5,

subtypes A and C (MUC5AC), and MUC5B are the predominant mucins and to a lesser extent MUC2, 7, 8, 11, 13, 19, and 20 are produced by goblet cells on the surface epithelium and mucus cells from submucosal glands (Rose and Voynow, 2006). Membrane-associated mucins in the airways include MUC1, MUC4, and MUC16.

Surface and submucosal epithelial cells can secrete antimicrobial peptides/proteins (AMPs) including bactericidal permeability increasing (BPI) protein, BPI fold containing family A, member 1 (BPIFA1 aka short palate, lung, and nasal epithelium associated 1, SPLUNC1), and BPIFB1 (aka long palate, lung, and nasal epithelium associated 1, LPUNC1) (Bingle and Bingle, 2011). Other AMPs include cathelicidin antimicrobial peptide (cAMP aka. LL37), defensin beta 1 (DEFB1), lysozyme (LYZ), secretory leukocyte proteinase inhibitor (SLPI), WAP four-disulfide core domain 2 (WFDC2), lactotransferin (LTF), lipocalin 2 (LCN2 aka neutrophil gelatinase-associated lipocalin), phospholipid transfer protein (PLTP), and peptidase inhibitor 3 (PI3 aka elafin). Leukocytes can also contribute to the antimicrobial response with alpha defensins, S100 calcium-binding proteins (e.g., S100A8), and additional LCN2, and LTF. Mice have a second lysozyme, LYZ2, which is highly abundant in the lung (Ganz et al., 2003).

The pseudostratified epithelium also contains a basal cell with an apical membrane that does not make contact with the airway lumen (Evans et al., 2001). These cells have desmosomal and hemidesmosomal attachments to other columnar cells and thereby anchor the respiratory epithelium. Positioned on basal lamina, basal cells can also interact with neurons, basement membrane, underlying mesenchymal cells, lymphocytes, and dendritic cells. Moreover, they can divide and differentiate into ciliated, goblet, or BC cells (Rock and Hogan, 2011).

Serous cells contain and secrete a less viscous fluid, and are enriched in antimicrobial proteins including LYZ, LTF, and BPIFA2 (aka SPLUNC2). In addition to surface epithelial cells, mucus and serous cells are contained in the submucosal glands limited mainly to the cartilaginous airways. The glands contain multiple branching tubules arranged with the proximal tubules containing mucus cells and the distal ascini containing serous cells. Submucosal glands secrete MUC5B and MUC8, with MUC5B being predominate in submucosal glands, whereas surface mucus cells secrete mainly MUC5AC. Submucosal glands are contained in the cartilaginous airways (bronchi) in humans, but are minimal in rodents (especially mice).

SLPI is a serine proteinase inhibitor produced locally in the lung by cells of the submucosal bronchial glands and by nonciliated epithelial cells. One function of SLPI is the inhibition of neutrophil elastase and other proteinases, along with antimicrobial functions. Neutrophil elastase (ELANE) enhances SLPI mRNA levels while decreasing SLPI protein release in airway epithelial cells. In addition, glucocorticoids (used to treat airway inflammation) increase both constitutive and ELANE-induced SLPI mRNA levels (Abbinante-Nissen et al., 1993; Sallenave, 2010). Other submucosal gland/nonciliated epithelial cell antiproteinase/antimicrobial proteins include PI3 (aka elafin) and WFDC2.

Another airway secretory cell is the bronchiolar secretoglobin cell (BSC), previously called the Club or Clara cell (Winkelmann and Noack, 2010). BSCs have an extensive endoplasmic reticulum and secretory granules containing secretoglobins including SCGB1A1 (aka CCSP or CC10). The roles of secretoglobins are not fully understood, but in the lung, SCGB1A1 can inhibit phospholipase A2 (Levin et al., 1986), and may limit inflammation (Mukherjee et al., 1999; Watson et al., 2001) and polychlorinated biphenyl accumulation (Stripp et al., 1996). In humans, BSCs are found mainly in the distal airways and can act as tissue stem cells (Rock and Hogan, 2011). In mice, BSCs are found throughout the airways and can become ciliated cells (Rawlins et al., 2009) or mucus-producing cells (Chen et al., 2009) and can express chitinases following inflammation (Homer et al., 2006).

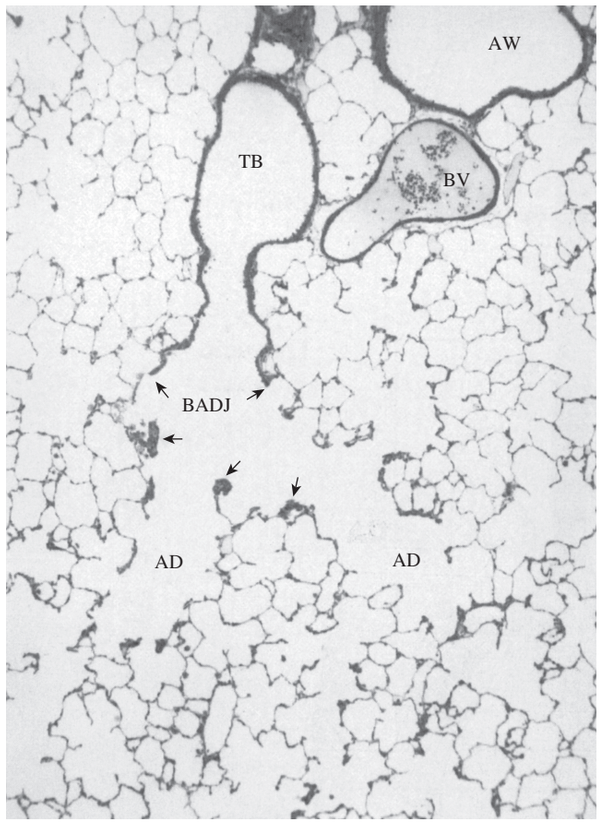

Figure 15-3. *Centriacinar region (ventilatory unit) of the lung.* An airway (AW) and a blood vessel (BV) (arteriole) are in close proximity to the terminal bronchiole (TB). The terminal bronchiole leads to the bronchiole–alveolar duct junction (BADJ) the alveolar duct (AD). A number of the (arrows) alveolar septal tips close to the BADJ are thickened after a brief (4-hour) exposure to asbestos fibers, indicating localization of fiber deposition. Other inhalants, such as ozone, produce lesions in the same locations. (Photograph courtesy of Dr Kent E. Pinkerton, University of California, Davis.)

Neuroendocrine cells are contained in neuroepithelial bodies or separately in the proximal airways (Van Lommel, 2001) and contact can stimulate underlying sensory nerve fibers. They synthesize, store, and release bioactive substances including 5-hydroxytryptamine (aka serotonin), calcitonin-related polypeptide α (aka calcitonin), and gastrin-releasing peptide (aka bombesin). These cells express cholinergic receptor, nicotinic, α polypeptide 7 (CHRNA7) and release serotonin in response to nicotine. Serotonin can also be released following hypoxia or mechanical strain. The release of these bioactive substances can redistribute pulmonary blood flow, and alter bronchomotor tone and immune responses (Cutz et al., 2007). Pulmonary neuroendocrine cells and neuroepithelial bodies in the fetal and neonatal lung modulate airway development and these cells are linked to specific types of lung cancer.

Gas Exchange Region

Structure The gas exchange region consists of terminal bronchioles, respiratory bronchioles, alveolar ducts, alveoli, blood vessels, and lung interstitium (Fig. 15-3). Human lung has five lobes: the superior and inferior left lobes and the superior, middle, and

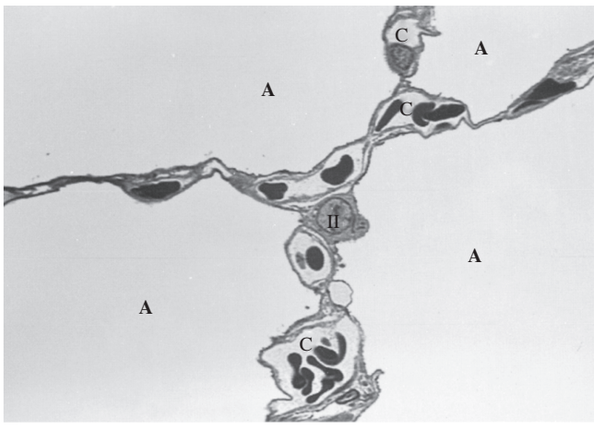

Figure 15-4. *Alveolar region of the lung.* The (A) alveolus is separated by the thin air-to-blood tissue barrier of the alveolar septal wall, which is composed of flat alveolar type I cells and occasional rounded (II) alveolar type II cells. A small interstitial space separates the epithelium and endothelium that form the (C) capillary wall. During lung injury the interstitial space enlarges and interferes with gas exchange. (Photograph courtesy of Dr Kent E. Pinkerton, University of California, Davis.)

inferior right lobes. In rats, mice, and hamsters, the left lung consists of a single lobe and the right lung consists of four lobes: cranial, middle, caudal, and ancillary. A ventilatory unit is defined as an anatomical region that includes all alveolar ducts and alveoli distal to each bronchiolar–alveolar duct junction (Mercer and Crapo, 1991). Gas exchange occurs in the alveoli, which comprise ~85% of the total parenchymal lung volume. Adult human lungs contain an estimated 300 to 500 million alveoli. The ratio of total capillary surface to total alveolar surface is slightly less than one. Capillaries, blood plasma, and formed blood elements are separated from the air space by a thin layer of tissue formed by epithelial, interstitial, and endothelial components.

The alveolar epithelium consists of two cells, the alveolar type I and type II cell (Fig. 15-4). Alveolar type I cells cover ~95% of the alveolar surface and therefore are susceptible to damage by noxious agents that penetrate to the alveolus (Williams, 2003). Alveolar type I cells have an attenuated cytoplasm to enhance gas exchange. Alveolar type II cells are cuboidal and have abundant perinuclear cytoplasm, extensive secretory capacity, and contain secretory vesicles called lamellar bodies (Whitsett et al., 2010). They produce surfactant, a mixture of lipids, and four surfactant associated proteins and can undergo mitotic division and replace damaged type I cells (Rock and Hogan, 2011). Surfactant protein B and C are amphipathic and aide in spreading secreted lipids that form a monolayer that reduces surface tension. Surfactant protein A1, A2, and D are members of the subfamily of C-type lectins called collectins, which defend against pathogens. Surfactant protein A1 and A2 do not alter lipid structure but bind lipopolysaccharides (LPS) and various microbial pathogens, enhancing their clearance from the lung. Surfactant protein D is also necessary in the suppression of pulmonary inflammation and in host defense against viral, fungal, and bacterial pathogens. Like surfactant protein B and C, surfactant protein D does influence the structural form of pulmonary surfactant. Surfactant protein D also influences alveolar surfactant pool sizes and reuptake. The shape of type I and type II cells is independent of alveolar size and is remarkably similar in different species. A typical rat alveolus (14,000-μm^2 surface area) contains two type I cells and three type II cells, whereas a human alveolus with a surface area of 300,000 μm^2 contains ~30 type I cells and ~50 type II cells (Pinkerton et al., 1991).

The mesenchymal interstitial cell population consists of fibroblasts and myofibroblasts that produce collagen and elastin as well as other cell matrix components and various effector molecules. Pericytes, monocytes, and lymphocytes also reside in the interstitium, as do macrophages before they enter the alveoli. Endothelial cells have a thin cytoplasm and cover about one-fourth of the area covered by type I cells.

Ventilation The principal function of the lung is gas exchange, which consists of ventilation, diffusion, and perfusion. The lung is superbly equipped to handle its main task: bringing essential oxygen to the organs and tissues of the body and eliminating its most abundant waste product, CO_2 (Weibel, 1983).

During inhalation, fresh air moves into the lung through the upper respiratory tract and conducting airways and into the terminal respiratory units when the thoracic cage enlarges and the diaphragm moves downward; the lung passively follows this expansion. The thoracic cage enlarges mainly by the constriction of external intercostal and internal (interchondral part) intercostal muscles, which elevate the sternum and ribs and thus increase the width of the thoracic cavity. When the parenchyma of the lung expands during inhalation, force is transferred to the airways (especially the small diameter distal airways), which increases the airway diameter and diminishes obstruction to airflow. After diffusion of oxygen into the blood and that of CO_2 from the blood into the alveolar spaces, the air (now enriched in CO_2) is expelled by exhalation. Relaxation of the chest wall and diaphragm diminishes the internal volume of the thoracic cage, the elastic fibers of the lung parenchyma recoil, and air is expelled from the alveolar zone through the airways. Any interference with the elastic properties of the lung, for example, the alteration of elastic fibers that occurs in emphysema, adversely affects ventilation, as do the decrease in the diameters of, or blockage of, the conducting airways, as in asthma.

Lung function changes with age and disease and can be measured with a spirometer (Fig. 15-5). The total lung capacity (TLC) is the total volume of air in an inflated human lung, 4 to 5 L (women) and 6 to 7 L (men) (American Thoracic Society [ATS], 1991). After a maximum expiration, the lung retains 1.1 L (women) and 1.2 L (men), which is the residual volume (RV). The functional residual capacity and residual volume cannot be measured with spirometry. These are determined by several other methods including nitrogen washout, in which the concentration of nitrogen is measured in expired air following inhalation of 100% oxygen. The vital capacity (VC) is the air volume moved into and out of the lung during maximal inspiratory and expiratory movement and is about 3.1 L (women) and 4.8 L (men). Only a small fraction of the VC, the tidal volume (TV), is typically moved into and out of the lung during quiet breathing. In resting humans, the TV measures ~0.5 L with each breath. The respiratory frequency, or the number of breaths per minute, is 12 to 20 (thus the resting ventilation is about 6 to 8 L/min). During exercise, both the TV and the respiratory rate can increase markedly. The amount of air moved into and out of the human lung may increase from 12 to 15 L/min to 40 to 60 L/min with light and moderate exercise, respectively. Increased ventilation in a polluted atmosphere increases the deposition of inhaled toxic material. Thus, susceptible individuals, particularly children and the elderly, should not exercise during episodes of heavy air pollution.

Lung function changes with age and disease and can be quantified by a forced expiratory maneuver with a spirometer. In this test, an individual first inhales maximally and then exhales as rapidly as possible. The volume of air expired in 1 second is the forced expiratory volume in 1 second (FEV1), and the total amount expired is the forced vital capacity (FVC). Along with the ratio of FEV1/FVC, these are good measures of the recoil capacity and

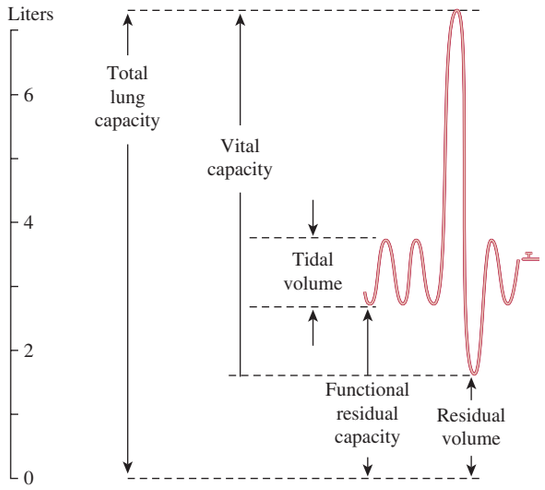

Figure 15-5. *A spirometer reading of lung volumes.* The total lung capacity is the total volume of air in an inflated human lung. After a maximum expiration, the lung retains a small volume of air, which is the residual volume. The vital capacity is the air volume moved into and out of the lung during maximal inspiratory and expiratory movement. The tidal volume is typically moved into and out of the lung during each breathe. The functional residual capacity and residual volume cannot be measured with a spirometer.

airway obstruction of the lung. In a healthy individual the FEV1/FVC = ~80%. In chronic obstructive pulmonary disease (COPD), the parenchyma recoil is compromised, small airways close during exhalation obstructing airflow, and more air is trapped in the lung (Fletcher and Peto, 1977). Although the FVC may stay the same or may even increase slightly, narrowed small airways slow airflow at low lung volumes and thereby decrease the FEV1. Thus, the FEV1/FVC is also decreased and airflow is considered obstructed when FEV1/FVC is >70% of the predicted value (based on sex, height, and age). The decreased FVC is accompanied by an increase in RV. If part of the lung collapses, becomes filled with edema fluid, or is restricted due to altered lung collagen (fibrosis), FEV1 and FVC can be reduced equally. Thus, FEV1/FVC is unaltered.

Diffusion Gas exchange takes place across the entire alveolar surface. Contact with an airborne toxic chemical thus occurs over a surface of ~140 m^2. This surface area is second only to the small intestine (~250 m^2) and is considerably larger than the skin (~2 m^2), two other organs that are in direct contact with the outside world. A variety of abnormal processes may severely compromise the unhindered diffusion of oxygen to the erythrocytes. Acute events may include collection of liquid in the alveolar or interstitial space and disruption of the pulmonary surfactant system. Chronic toxicity can impair diffusion due to abnormal alveolar architecture or abnormal formation and deposition of extracellular substances such as collagen in the interstitium.

Perfusion The lung receives the entire output from the right ventricle, ~75 mL of blood per heartbeat. Blood with high CO_2 and low O_2 travels to the lung via the pulmonary artery and leaves the lung with high O_2 and low CO_2 via the pulmonary vein. The bronchi also have independent circulation with O_2-enriched blood supplied by an artery. Substantial amounts of toxic chemicals carried in the blood can be delivered to the lung. A chemical placed onto or deposited under the skin (subcutaneous injection) or introduced directly into a peripheral vein (intravenous injection) travels through the venous system to the right ventricle and then to the pulmonary capillary bed before distribution to other organs or tissues in the body.

BIOTRANSFORMATION IN THE RESPIRATORY TRACT

Often overlooked as an organ involved in metabolism of chemicals, in favor of the liver, the lung has substantial capabilities for biotransformation (see Chap. 6). Total lung cytochrome P450 (CYP) activity is roughly one-tenth to one-third of that in the liver. However, when specific activity in a few cell types is considered, the difference is only twofold for many enzymes. On a per cell basis, the specific activity of nasal mucosa even may be greater than that of the liver (Buckpitt and Cruikshank, 1997). Metabolic competence in the lung and nasal tissues is concentrated in a few cell types that have a defined, and sometimes limited, distribution in the respiratory tract, which can vary substantially by species (Table 15-3). The balance of activation and inactivation is a critically important determinant of lung protection from injury. Protection from oxidation is another important function because oxygen concentrations that occur in the respiratory tract are high relative to other organs. Other factors that can influence the role of phase I and phase II systems in lung toxicity include age, sex, diet, local inflammation, and the history of prior exposure (Plopper et al., 2001). Interestingly, many xenobiotic metabolizing enzymes have different patterns of induction (less) in the respiratory tract than in the liver, leading to the concept that regulation of these systems may be different depending on where they are located (Buckpitt and Cruikshank, 1997).

The major phase I enzyme system, the CYP monooxygenases (Nebert and Dalton, 2006), is concentrated into a few lung cells: BSCs, alveolar type II cells, macrophages, and endothelial cells. Of these cell types, BSCs have the most CYP, followed by the type II cells. The amount of total lung CYP contributed by BSCs is species-dependent, with humans having less CYP in their lungs from BSCs than mice or rats. Furthermore, the CYP isoforms present and their location in the respiratory tract also vary by species. For an extrahepatic tissue, CYPs are expressed at high levels in the nasal mucosa and this pattern of expression varies by nasal region and cell type (Thorton-Manning and Dahl, 1997; Ding and Kaminsky, 2003; Harkema et al., 2006). Most species have CYPs in nasal tissue and some are predominantly expressed in the olfactory mucosa (e.g., CYP2G1, CYP2A3, CYP2A10, CYP2A11, and CYP2A13) (Ling et al., 2004). Metabolism by the olfactory epithelium may play a role in providing or preventing access of inhalants directly to the brain; for example, inhaled xylene may be converted into metabolites that move to the brain by axonal transport (Ghantous et al., 1990). The presence of the following CYP isozymes in the respiratory tract of at least one species has been reported: CYP1A1, CYP1A2, CYP1B1, CYP2A4, CYP2A5, CYP2B6, CYP2E1, CYP2F2, CYP2J2, CYP2J6, CYP2J9, CYP2S1, CYP2U1, CYP3A7, CYP4B1, CYP4F2, CYP4V2, CYP27A1, CYP51A1 (Hukkanen et al., 2002; Anttila et al., 2011).

In some cases, species differences in expression of particular CYP isozymes can result in higher toxicity to the respiratory epithelium. An example includes CYP2F2 that can activate naphthalene and is highly enriched in bronchiolar secretoglobin cells. This explains the high toxicity of naphthalene to these cells in the lungs of mice (Warren et al., 1982). Another example is CYP4B1 that bioactivates 4-ipomeanol, which induces pneumonia in livestock and several other species following ingestion of spoiled sweet potatoes (Plopper et al., 1994; Lo-Guidice et al., 2002; Parkinson et al., 2013).

Other phase I enzymes found in lung tissue include epoxide hydrolases, flavin monooxygenases, prostaglandin (PG)-endoperoxide synthases, carbonyl reductases, and NAD(P)H:quinone oxidoreductase 1 (NQO1). The constant features of the

Table 15-3

Distribution of Xenobiotic Metabolizing Enzymes in the Respiratory Tract

		EPITHELIUM				
ENZYME	NASAL TISSUE	PROXIMAL AIRWAY	DISTAL AIRWAY	ALVEOLAR	MACROPHAGE	ENDOTHELIUM
Cytochrome P450 monooxygenases	+++	++	+++	++	++	++
Microsomal epoxide hydrolases	+++	++	+++	++	+	++
Flavin monooxygenases	+	++	+++	++	−	+
Prostaglandin-endoperoxide synthase 1	++	++	++	+/−	++	−
Prostaglandin-endoperoxide synthase 2	++	+	++	+/−	+++	++
Glutathione *S*-transferases	+	++	+++	+	Unknown	Unknown
Glucuronosyl transferases	+	Unknown	++	+	Unknown	Unknown
Sulfotransferases	++	+	+	+/−	Unknown	Unknown

Code: +++, most isoforms highly expressed; ++, some isoforms highly expressed; +, low expression; +/−, low expression found in some studies but not others.

expression of these enzymes are lack of uniformity in their expression by cell type and region throughout the lung and their tendency to concentrate in epithelia. Both microsomal (EPHX1) and cytosolic (EPHX2) epoxide hydrolases are found in the lung and nasal tissues, and the activity of EPHX1 can be higher in the distal airways of the lung than in the liver (Bond et al., 1988). Functional variants of EPHX1 have been associated with respiratory diseases including lung cancer (Kiyohara et al., 2006), childhood asthma (Salam et al., 2007), and possibly COPD (Hu et al., 2008; Lee et al., 2011). Flavin monooxygenase activity (FMO1 and FMO2) is found in rodent and human lung and nasal tissue. The isoforms present in the lung (FMO2) are different from those found in the liver (FMO1). FMO1 is the predominant isoform in the nasal mucosa (Shehin-Johnson et al., 1995; Henderson et al., 2008). The gene for FMO2 in human lung contains a premature stop codon encoding production of an inactive protein, but some ethnic groups have at least one copy of an allele that expresses the full-length protein (Whetstine et al., 2000). PG-endoperoxide synthases (aka cyclooxygenases) oxidize substrates at a much lower rate than CYP monooxygenases but may have a role in human pulmonary metabolism due to the relatively lower CYP activity in human lung tissue compared to rodents (Smith et al., 1991).

Carbonyl reductase (especially carbonyl reductase 2, which is abundant in mice but is not present in the human genome) enables pulmonary metabolism of endogenous carbonyl compounds, such as aliphatic aldehydes and ketones, 3-ketosteroids, fatty aldehydes, and PGs (converting PGE2 to PGF2α). NQO1 reduces quinones to hydroquinones, which then can be acted upon by NADH CYP reductase to generate semiquinone free radicals. In the lung, NQO1 activates carcinogenic heterocyclic amines found in cigarette smoke (De Flora et al., 1994), whereas NQO1 prevents formation of benzo[*a*] pyrene–quinone DNA adducts (Joseph and Jaiswal, 1994). Genetic polymorphisms in *NQO1* are associated with lung cancer (Kiyohara et al., 2005) and susceptibility to ozone (Minelli et al., 2011).

Phase II enzymes include glutathione-*S*-transferases (GSTs) (alpha, mu, and pi), glucuronosyl transferases, and sulfotransferases (SULTs). GSTs and glutathione play a major role in the modulation of both acute and chronic chemical toxicity in the lung (West et al., 2000). A key point to keep in mind is that these enzyme systems work in concert with one another (i.e., a decrease in one enzyme may result in a concomitant increase in another) and it is the combined action of all of these enzymes, and their location, that determines toxicity. The regulation of many of these enzymes is under coordinated control of the transcription factor nuclear factor, erythroid derived 2, like 2 (NFE2L2, aka NRF2) (Slocum and Kensler, 2011).

A major determinant of the potential for detoxification may also be the cellular localization of, and ability to synthesize, glutathione in the lung. Pulmonary GST activity is 5% to 15% that of the liver in rodents and about 30% of that in human liver (Buckpitt and Cruikshank, 1997). The distribution of the isoforms of glutathione *S*-transferase varies by lung region with the alpha, mu, and pi isoforms. The alpha and pi classes are the predominate isoforms in the airway epithelium of human lung. In nasal tissue, glutathione *S*-transferases are found in the olfactory mucosa. The mu isoform has a zonal pattern of expression increased in the lateral olfactory turbinates of the mouse (Whitby-Logan et al., 2004). Polymorphisms in glutathione *S*-transferases genes have been associated with a possible increase in risk of developing lung cancer, particularly in smokers (Jourenkova-Mironova et al., 1998). In lung cancer, the GSTM1 genotype is one of the most widely analyzed genetic variants, and an increased risk has been noted among null carriers (Shi et al., 2008).

The activity of glucuronosyl transferase has been reported in both rodent and human nasal and pulmonary tissue. Glucuronosyl transferase (UGT2A1) is thought to have a role in termination of odorant signals (Lazard et al., 1991). The detoxification enzyme in the metabolism of polycyclic aromatic hydrocarbons (PAHs) within target tissues for tobacco carcinogens and functional polymorphisms in UGT2A1 may play a role in tobacco-related cancer risk (Bushey et al., 2011). Sulfotransferase activity has been demonstrated in human bronchoscopy samples (Gibby and Cohen, 1984) and SULT2B1 is immunochemically localized to the olfactory epithelium and conducting airway epithelium (He et al., 2005). Sulfotransferases have been localized to the sustentacular cells of the olfactory epithelium and some isoforms may be specific to the

olfactory epithelium (Tamura et al., 1998). In lung endothelial and epithelial cells, SULT1A3 could play a role in the inactivation and/or disposal of excess chlorotyrosine (Yasuda et al., 2011).

GENERAL PRINCIPLES IN THE PATHOGENESIS OF LUNG DAMAGE CAUSED BY CHEMICALS

Toxic Inhalants, Gases, and Dosimetry

In inhalation toxicology, exposure is measured as a concentration (compound mass per unit of air). Typically highly toxic compounds can produce adverse effects in a concentration of mg/m^3 or $\mu g/m^3$. A m^3 is 1000 L. For gases, concentration may also be expressed as volume to volume of air, that is, parts per million (ppm) or parts per billion (ppb). This can be calculated from the mass per unit air by using the ideal gas law to determine the gas's volume. Concentration is useful because it can be quantified by many air-sampling methods that rely on many chemical analytical methods. Large volumes of air can be collected so that low levels (i.e., ppb) can be detected. Exposure does not equate to dose (compound mass per unit), which requires a measure of mass of the organ, cell, or subcellular target.

The sites of deposition of gases in the respiratory tract define the pattern of toxicity of those gases. Solubility, diffusivity, and metabolism/reactivity in respiratory tissues and breathing rate are the critical factors in determining how deeply a given gas penetrates into the lung (Asgharian et al., 2011; Gloede et al., 2011). Highly soluble gases such as SO_2 or formaldehyde do not penetrate farther than the nose (during nasal breathing) unless doses are very high, and are therefore relatively nontoxic to the lung of rats (which are obligatory nasal breathers). However, formaldehyde causes cancer in the rat nasal passages (Albert et al., 1982). Relatively insoluble gases such as ozone and NO_2 penetrate deeply into the lung and reach the smallest airways and the alveoli (centriacinar region), where they can elicit toxic responses. Mathematical models of gas entry and deposition in the lung predict sites of lung lesions fairly accurately. These models may be useful for extrapolating findings made in laboratory animals to humans (Asgharian et al., 2011; Gloede et al., 2011). Very insoluble gases such as CO and H_2S efficiently pass through the respiratory tract and are taken up by the pulmonary blood supply to be distributed throughout the body.

Regional Particle Deposition

Because of the architecture of the airways that modulate airflow, particle size is a critical factor in determining the region of the respiratory tract in which a particle will be deposited. Deposition of particles on the airway mucosal surface is brought about by a combination of aerodynamic forces and particle characteristics (Lippmann et al., 1980; International Commission on Radiological Protection [ICRP], 1994; Lippmann and Leikauf, 2009). The efficiency of particle deposition in various regions of the respiratory tract depends mainly on particle size.

Aerosols are dispersed solids or liquids. Particles in air are classified by particle size (Lippmann and Leikauf, 2009). Size controls particle shape and thus influences light-scattering properties or deposition by interception. Size also controls particle mass and thus influences the probability for coagulation, dispersion, sedimentation, and impaction. Aerosols are a population of particles that can be monodispersed (essentially of one size like pollens) or, more typically, heterodispersed (many difference sizes). Particles generated from a single source typically have diameters that are lognormal (Poisson) distributed. This distribution will become Gaussian when plotted on a log scale and the distribution's central tendency is expressed as the mass median diameter (MMD). This measure of central tendency is accompanied by the measure of variability called the geometric standard deviation (σ_g). Monodispersed aerosols are typically defined as having a low σ_g (typically <1.2). Because the density (e.g., plutonium) and the shape (e.g., asbestos fibers) of aerosols can vary greatly, MMD is normalized to a unit-density sphere and is referred to as the mass median aerodynamic diameter (MMAD). In the following discussion, the particle size distributions are MMADs. Particle surface area is of special importance when the surface is reactive with biomolecular targets or when toxic materials are adsorbed on particles and thus are carried into the lung.

In respiratory toxicology, aerosols (particles dispersed into air) include any of the following: (1) dusts ($\geq$1.0-μm particles generated by mechanical division as in grinding), (2) fumes ($\leq$0.1-μm particles generated by condensation of vapors as in heat metals or oils), (3) smoke ($\leq$0.5-μm complex carbon particles generated condensation of products from combustion), (4) mists (2- to 50-μm water droplet or solutions generated by mechanical shearing of bulk liquid as in spraying), (5) fog ($\leq$1.0-μm water droplets generated by water vapor condensation on atmospheric nuclei), or (6) smog ($\geq$0.01- to 50-μm air pollution generated by stationary and mobile pollution source) (Lippmann and Leikauf, 2009). Haze has been used to describe either a dilute fog (at lower humidity) or smog. Smaller aerosols include submicrometer particles ($0.1 \leq x \leq 1.0$ μm), nanometer particles or nanoparticles ($\leq$0.1 μm). All these distinguishing forms are included in the term "aerosol" or "particle."

Aerosols are typically dynamic and change by processes including dilution, dispersion, coagulation, and chemical reaction (Pandis, 2004). Atmospheric particles originate either as primary particles—by direct emission from a source—or as secondary particles—through atmospheric formation from the gas phase constituents (nucleation) (Fig. 15-6). Atmospheric particles are typically distributed into two modes and five submodes (John et al., 1990). The two modes are the accumulation and coarse mode, which dominate the particle volume (and therefore mass) distribution.

Dominating the particle number distribution are the nucleation and Aitken submodes. The smallest nucleation submode particles are generated from gas phase emissions. This mode is dominated by a large number of nanoparticles $\leq$0.01 μm, but because they are so small they do not add much to the cumulative volume and therefore have little mass. For example, typical urban atmospheres can have 100,000 nucleation submode particles per cubic meter of air, but the total weight of all these particles is only about 50 ng (which is less than 0.5% of a typical urban atmosphere). The second submode is the Aitken nucleus submode, which also consists of nanoparticles ($0.01 \leq x \leq 0.1$ μm). Like the nucleation submode, particles in the Aitken nucleus submode can be formed by chemical conversion of gases from combustion processes or can be freshly generated as primary particles. This mode is named after John Aitken, an atmospheric scientist interested in cloud physics (Aitken, 1880). Most Aitken particles are primary particles that have grown due to material condensing on their surface as they move through the atmosphere. Condensation can occur as plumes cool and particles and gases emitted together interact. Particles in the first two submodes typically have very short half-lives as singlet primary particles. This is because their motion is influenced by collision with gas molecules and other particles that lead them to coagulate, especially around larger, slower moving particles.

The next largest submode is the condensation submode ($0.05 \leq x \leq 0.5$ μm). These particles are formed from the coagulation of smaller solid particles and condensation of gases including sulfates, nitrates, and organics on the particles' surface. They can be formed from chain aggregates of numerous particles of smaller diameter

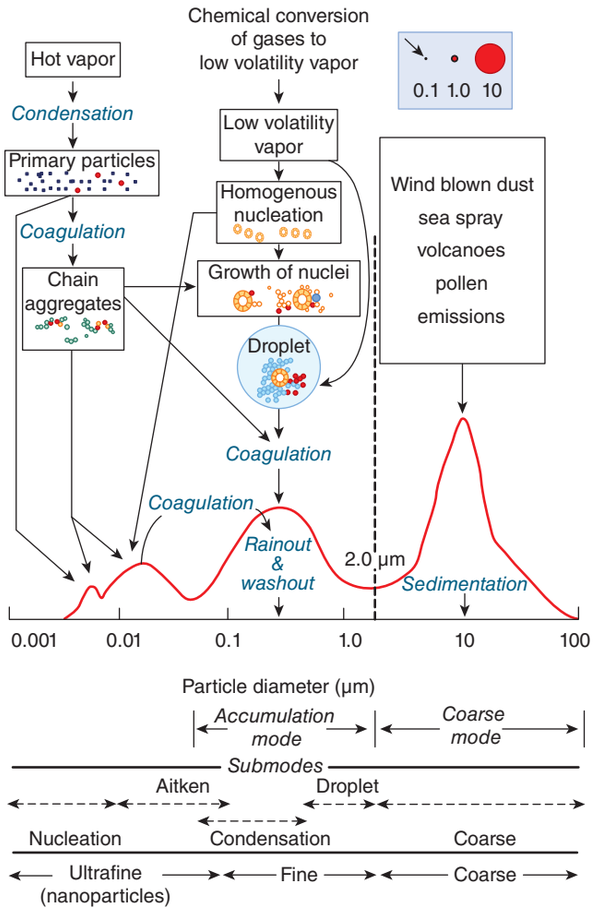

Figure 15-6. *Typical distribution of atmospheric particles consists of two major modes and five submodes.* The Accumulation and Coarse modes dominate the particle mass and Nucleation and Aitken submodes dominate the particle number. Nucleation particles are generated from gas phase emissions. Condensation can occur as plumes cool and particles and gases emitted together interact. Particles in the first two submodes typically have very short half-lives as singlet primary particles. The condensation submode is formed from the coagulation of smaller solid particles and condensation of gases including sulfates, nitrates, and organics on the particles' surface. They can be formed from chain aggregates of numerous particles of smaller diameter or also can be spherical with liquid surfaces. Particles move from the smaller submodes into the accumulation mode and these larger particles can have very long half-lives (hours to days) in the atmosphere and can travel over long distances. When the atmospheric relative humidity is very high (near 100%), particles in the accumulation mode seed rain droplets and are removed from the atmosphere. The coarse particles consist of particles >2.0 µm that are generated by mechanical processes or suspension of surface dust. Many of the particles in this range can be from natural sources (e.g., windblown desert sand). Particles larger than 50 µm readily settle and are removed from the atmosphere within minutes. Human exposure to these large particles is typically occupational (e.g., dust from grinding wheels or wood sanding that is inhaled due to proximity to the source). Inset: The volume, and therefore mass, is to the cubed root of the radius. The size and the physical forces that maintain particles in the atmosphere are why the total mass is mainly in the accumulation and coarse modes.

or also can be spherical with liquid surfaces. When these particles are hydroscopic and in a humid atmosphere, the size can increase to about 0.5 to 2.0 µm and become the droplet submode. Particles in the condensation and droplet submode contribute to the accumulation mode. These particles can have very long half-lives (hours to days) in the atmosphere and can travel over long distances from sources because they are too few in number for rapid coagulation and too small for gravitational sedimentation. Particles in this mode can travel from one state to another (e.g., from the Ohio River Valley to the eastern seaboard), or even across oceans (from China to California). When the atmospheric relative humidity is very high (near 100%), particles in the accumulation mode seed rain droplets and are removed from the atmosphere. In addition, submicrometer particles have high light scattering properties that contribute to low visibility during pollution episodes. The second mode is the coarse particle and contains the coarse submode, which are particles >2.0 µm that are generated by mechanical processes or suspension of surface dust. Many of the particles in this range can be from natural sources (e.g., windblown desert sand). Particles larger than 50 µm settle readily and are removed from the atmosphere within minutes. Human exposure to these large particles is typically occupational (e.g., dust from grinding wheels or wood sanding that is inhaled due to proximity to the source).

The upper respiratory tract is very efficient in removing particles that are very large (>10 µm) or very small (<0.01 µm) (Fig. 15-1). During nasal breathing, 1- to 10-µm particles are usually deposited in the upper nasopharyngeal region or the first five generations of large conducting airways. During oral breathing, deposition of these particles can increase in the tracheobronchial airways and alveolar region. Smaller particles (0.001 to 0.1 µm) can also be deposited in the tracheobronchial region. Particles ranging from 0.003 to 5 µm can be transported to the smaller airways and deposited in the alveolar region. Patterns of breathing can change the site of deposition of a particle of a given size. It must be kept in mind that the size of a particle may change during inspiration before deposition in the respiratory tract. Materials that are hygroscopic, such as sodium chloride, sulfuric acid, and glycerol, take on water and grow in size in the warm, saturated atmosphere of the upper and lower respiratory tract. Because adverse health effects of ambient particles have been associated with particles that were <10 µm, and subsequently <2.5 µm particles, the United States Environmental Protection Agency standard for ambient particulate matter (PM) is set at <2.5-µm particles, which is called $PM_{2.5}$ (see Chap. 29).

Deposition Mechanisms

In the respiratory tract, particles deposit by impaction, interception, sedimentation, diffusion (Brownian movement), and electrostatic deposition (for positively charged particles only) (Lippmann et al., 1980; ICRP, 1994; Lippmann and Leikauf, 2009) (Fig. 15-7). Impaction occurs in the upper respiratory tract and large proximal airways where the airflow is faster than in the small distal airways because the cumulative diameter is smaller in the proximal airways. Fast airflow imparts momentum to the inhaled particle, and it is the particle's inertia that causes it to continue to travel along its original path. In airstream bends, such as an airway bifurcation, larger diameter particles deviate from the airflow and impact on the surface. In humans, most >10 µm particles are deposited in the nose or oral pharynx and cannot penetrate tissues distal to the larynx. For 2.5- to 10-µm particles, impaction continues to be the mechanism of deposition in the first generations of the tracheobronchial region.

Interception occurs when the trajectory of a particle brings it near enough to a surface so that an edge of the particle contacts the airway surface. Interception is important for all particles but is particularly important in the deposition of fibers. Although fiber diameter determines the probability of deposition by impaction and sedimentation, interception is dependent on fiber length. Thus, a fiber with a diameter of 1 µm and a length of 200 µm will be deposited in the bronchial tree primarily by interception rather than impaction. Interception is also important for submicrometer particles in

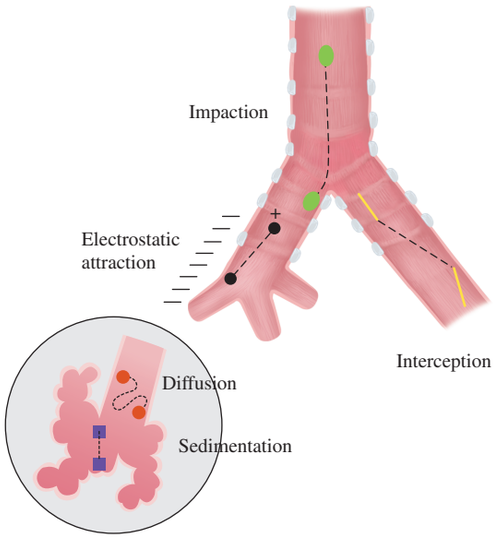

Figure 15-7. *Mechanism of particle deposition in the respiratory tract.* Impaction occurs in the upper respiratory tract and large proximal airways where fast airflow imparts momentum to the inhaled particle. The particle's inertia causes it to continue to travel along its original path and deposit on the airway surface. Interception occurs when the trajectory of a particle brings it near enough to a surface so that an edge of the particle contacts the airway surface. Sedimentation controls deposition in the smaller bronchi, the bronchioles, and the alveolar spaces, where the airways are small and the velocity of airflow is low. Sedimentation is dependent on the time a particle is in a compartment (i.e., an alveolus) and can be increased by breath holding. Diffusion is an important factor in the deposition of submicrometer particles. Electrostatic deposition is a minor deposition mechanism for positively charged particles. The surface of the airways is negatively charged and attracts positively charged particles. (Adapted from Lippmann and Leikauf [2009].)

the tracheobronchial region where inertial airflow directs a disproportionately large fraction of the flow volume toward the surface of small airway bifurcations.

Sedimentation controls deposition in the smaller bronchi, the bronchioles, and the alveolar spaces, where the airways are small and the velocity of airflow is low. Indeed, in the alveoli, there is no bulk airflow. As a particle moves through air, buoyancy and the resistance of air act on the particle in an upward direction while gravitational force acts on the particle in a downward direction. Eventually, the gravitational force equilibrates with the sum of the buoyancy and the air resistance, and the particle continues to settle with a constant velocity known as the terminal settling velocity. Sedimentation is not a significant route of particle deposition when the aerodynamic diameter is ≤0.5 μm. Sedimentation is dependent on the time a particle is in a compartment (i.e., an alveolus) and can be increased by breath holding.

Diffusion is an important factor in the deposition of submicrometer particles. A random motion is imparted to these particles by collisions with gas molecules. Thus, the ratio of a particle's mass to the momentum of the colliding gas molecules controls the distance the particle will travel. Larger particles are hardly moved by a gas molecule, whereas nanometer particles can be moved extensively. Diffusion is an important deposition mechanism in the nose, airways, and alveoli for particles ≤0.5 μm. Nanometer particles (0.1 μm and smaller) are also trapped relatively efficiently in the upper airways by diffusion. Particles that penetrate beyond the upper airways are available to be deposited in the bronchial region and the deep-lying airways. Therefore, the alveolar region has significant deposition efficiencies for particles smaller than 5 μm and larger than 0.01 μm.

During quiet breathing, in which the TV is only two to three times the volume of the anatomic dead space (i.e., the volume of the conducting airways where gas exchange does not occur), a large proportion of the inhaled particles may be exhaled. During exercise, when larger volumes are inhaled at higher velocities, impaction in the large airways and sedimentation and diffusion in the smaller airways and alveoli increase. Breath holding also increases deposition from sedimentation and diffusion. Cigarette smoke is a hydroscopic aerosol of nicotine-laden particles that grow to a median diameter of about 0.5 to 1.0 μm. Thus, a smoker's respiratory pause at the end of inhalation increases alveolar sedimentation and thereby nicotine delivery to the alveolar surface and to the blood upon absorption.

Electrostatic deposition is a minor deposition mechanism for positively charged particles. The surface of the airways is negatively charged and attracts positively charged particles. Freshly fractured mineral dust particles and laboratory-generated aerosols from evaporation of aqueous droplets can have substantial electrostatic mobilities.

Factors that modify the diameter of the conducting airways can alter particle deposition. In patients with chronic bronchitis or pneumonia, the airway lining fluid can greatly thicken and may partially block the airways in some areas. Sonic jets (e.g., during wheezing and rales) formed by high air flowing through such partially occluded airways have the potential to increase the deposition of particles by impaction and diffusion in the small airways. Irritant materials that produce bronchoconstriction tend to increase the proximal tracheobronchial deposition of particles.

Nanoparticles are particles with at least one dimension of ≤100 nm. They are made from a variety of materials including carbon (e.g., 60 carbon [C_{60}] fullerenes, nanotubes, or nanowires), metals (e.g., gold, silver, or quantum dots), or metal oxides (e.g., cerium oxide, titanium dioxide, or zinc oxide). Engineered biological nanoparticles include liposomes and viruses designed for gene or drug delivery. These particles can be generated in a wide array of dimensions and physicochemical properties. For example, carbon nanotubes can be single-walled (i.e., SWCNT) or multiwalled (i.e., MWCNT) and generated at various lengths (>1.0 μm). Carbon nanotubes can have many surface modifications (e.g., metal coat) or can be core loaded. In addition, nanoparticles make up a portion (small by mass, but large by number and surface area) of emissions from vehicle engines, especially diesel engines, and industrial furnaces and burners. The toxicity of nanoparticles may be enhanced over large particles because of an increased surface area that can provide a biological reactive surface that can generate secondary reaction products (e.g., reactive oxygen species) or may provide an inert surface that carries adsorbed copollutants (Oberdörster et al., 2005). Commercial nanoparticles are often rod shaped with lengths of 5 to 10 μm and thereby share aspect properties with asbestos (see below) (Donaldson et al., 2010). Additional concern about potential toxicity of nanoparticles is triggered by epidemiological studies that associate increased mortality in sensitive populations with increased ambient PM exposure (see Chap. 29). Lastly, nanoparticles may have increased toxicity because normal host defenses may have limited effectiveness against these particles. These particles may be too small to be recognized by macrophage and because nanoparticles can move through membranes, these particles can escape from phagosomes.

Particle Clearance

Lung defense is dependent on particle clearance (Table 15-4). Rapid removal lessens the time available to cause damage to the pulmonary tissues or permit local absorption (Lippmann et al., 1980). However, it is important to remember that particle clearance is not equivalent to complete protection for many reasons.

Table 15-4

Respiratory System Structures, Histology, Functions, and Particle Deposition and Clearance

REGION	HISTOLOGY	CELL TYPES	FUNCTIONS	NUMBER	SURFACE AREA EXPOSED TO AIR (M^2)	VOLUME OF BREATH (L)	DEPOSITION MECHANISM	CLEARANCE MECHANISM
Nares and anterior nasal passage	Anterior vestibule: Stratified squamous epithelium Anterior chamber: Nonciliated cuboidal/ columnar epithelium with nasal hair	Anterior vestibule: Squamous epithelium Anterior chamber: Cuboidal epithelium, ciliated cells, mucous cells, serous cells, brush cells, neuroendocrine cells, basal cells, subcutaneous hair follicles	Conduct, heat, humidify, and filter air	2	2×10^{-2}	0.175	Impaction and interception	Sneeze
Posterior nasal passage	Ciliated pseudostratified respiratory epithelium, submucosal glands	Ciliated cells, mucous cells, serous cells, brush cells, neuroendocrine cells, basal cells, smooth muscle cells, submucosal glands (mucous and serous cells)	Conduct, heat, humidify, and filter air	2	4.5×10^{-2}			Sneeze and mu-cociliary Clearance
Olfactory bulb	Olfactory epithelium, Bowman glands	Olfactory cells, supporting cells, basal cells, brush cells	Smell	2	2×10^{-4}			Sneeze
Mouth	Nonkeratinized stratified squamous epithelium	Stratified squamous epithelium	Conduct, heat, humidify, and filter air	1	2.2×10^{-2}			Cough, swallowing
Teeth and gingiva	Tooth: Bone with enamel, dentin, cementum, pulp Gingiva: Gingival, junctional and sulcular epithelium	Tooth: Odontoblasts, fibroblasts, preodontoblasts Gingiva: Nonkeratinized stratified squamous epithelium	Mastication	20 (Primary) 32 (Permanent)	5×10^{-3}			Swallowing
Tongue	Fungiform, filiform, foliate, circumvallate papillae	Supporting cells, gustatory cells	Taste, food manipulation	1	1×10^{-2}			Cough, Swallowing
Salivary glands (Parotid, submaxillary, sublingual, and minor)	Lobular secretory glands with adenomeres	Serous and mucous secretory epithelial cells	Moisten food, amylase diges-tion, lysozyme secretion	6 major 600 minor	*			Saliva Secretion

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Pharynx	Nonkeratinized stratified squamous epithelium, adenoid tissue, tonsils	Nonkeratinized stratified squamous epithelium, adenoid and tonsils: Mucosal associated lymphoid tissue	Conduct, heat, humidify, and filter air Adenoid, Immunoglobin A secretion	1	1×10^{-2}			Sneeze, Cough, and Mucociliary Clearance
Larynx	Vocal folds (cords), 3 single (thyroid, cricoid, and epiglottic) and 3 paired (arytenoid, corniculate, and cuneiform) cartilages	Stratified squamous epithelium (true folds), respiratory epithelium (false fold), chondroblasts (cartilage)	Phonation	1	5×10^{-4}			Cough and Mucociliary Clearance
Trachea	Ciliated pseudostratified respiratory epithelium, submucosal glands, cartilage rings	Ciliated cells, mucous cells, serous cells, brush cells, neuroendocrine cells, basal cells, smooth muscle cells, submucosal glands (mucous and serous cells)	Conduct, heat, humidify, and filter air	1	7.5×10^{-3}			
Bronchi	Ciliated pseudostratified respiratory epithelium, cartilage plates, airway smooth muscle	Ciliated cells, mucous cells, serous cells, brush cells, neuroendocrine cells, basal cells, smooth muscle cells, submucosal glands (mucous and serous cells)	Conduct and filter air	510	0.26			
Bronchioles	Transition from ciliated pseudostratified to cuboidal ciliated respiratory epithelium (No cartilage and no submucosal glands)	Ciliated cells, Clara cells, few or no mucus cells	Conduct and filter air	6.5×10^{4}	7.5			Mucociliary clearance
Respiratory bronchioles	Cuboidal nonciliated respiratory epithelium with alveoli in a portion of the wall	Cuboidal epithelium and alveolar epithelium	Conduct and filter air	4.6×10^{5}	*	0.2	Sedimentation, diffusion, and electrostatic attraction	Macrophage phagocytosis and migration
Alveolar duct and sac	Cuboidal nonciliated respiratory epithelium with alveoli in a portion of the wall	Some low cuboidal cells and mostly type I (squamous non-secretory), type II (secretory cuboidal) alveolar epithelial cells, macrophage	Conduct and filter air, gas exchange	$3–5 \times 10^{8}$	140	4.5		
Pleural cavity and diaphragm	Mesothelium and skeletal muscle	Mesothelial cells, skeletal muscle cells, nerves	Generation of negative pleural pressure for inhalation	1	1.8	0	Translocation	Macrophage lymphatic migration

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An inert particle that can penetrate to the alveolar region can be a vehicle carrying adsorbed toxic gases. Once deposited in the lung, the adsorbed materials may dissolve from the surfaces of particles and enter the epithelium, endothelium, bloodstream, or lymphatics. Small particles (e.g., nanoparticles) may directly penetrate cell membranes and evade clearance. Moreover, particle clearance from the respiratory tract is not equivalent to clearance from the body. The only mechanisms by which deposited particles can be removed from the body are nasal wiping and coughing.

Nasal Clearance Particles deposited in the nose are cleared depending on their site of deposition and solubility in mucus. Particles deposited in the anterior portion of the nose are removed by extrinsic actions such as wiping and blowing. Particles deposited in the posterior portion of the nose are entrapped in mucus and removed by mucociliary clearance that propels mucus toward the glottis, where the particles are swallowed. Insoluble particles are generally cleared from this region in healthy adults and swallowed within an hour of deposition. Particles that are soluble in mucus may dissolve and enter the epithelium and/or blood before they can be mechanically removed.

Tracheobronchial Clearance Particles deposited in the tracheobronchial tree are also removed by mucociliary clearance. In addition to deposited particles, particle-laden macrophages are also moved upward to the oropharynx, where they are swallowed. Mucociliary clearance is relatively rapid in healthy individuals and is completed within 24 to 48 hours for particles deposited in the lower airways. Infection and other injuries can greatly impair clearance.

Alveolar Clearance Particles deposited in the alveolar region are removed by specialized cells, the alveolar macrophage. Lung defense involves both the innate and adaptive immune systems. The innate immune system confers immediate recognition, phagocytosis, and killing of bacteria and microbes that are in the airway or alveolus. It is nonspecific and does not confer longer-term memory of the invading pathogenic stimuli. Alveolar macrophages are major effectors of innate immunity in the lung. Other innate immune cells include polymorphonuclear leukocytes (aka neutrophils) that can augment this capacity but are typically present only when the lung is inflamed. The adaptive immune system confers long-lasting or protective immunity to the host that is specific to a foreign microbe or material (antigen). Adaptive immunity involves dendritic cells that take up and present antigens to T lymphocytes (T cells) or antibody-producing B lymphocytes (B cells). Dendritic cells are derived from monocytes and reside in the airway epithelium. Lymphocytes reside in the hilar or mediastinal lymph nodes, lymphoid aggregates, and lymphoepithelial nodules, as well as in aggregates or as single cells throughout the airways. Ideally, the antibody generated by the B cell recognizes a single molecular signature on the antigen and initiates other cells to evoke responses that protect the host (see Chap. 12).

Macrophage phagocytosis depends on the recognition of foreign or damaged cells by a variety of macrophage surface macromolecules and receptors. Phagocytosis requires (1) particle binding to the membrane specifically via recognition molecule–receptor interactions or nonspecifically by electrostatic forces (inert materials), (2) receptor activation that initiates cell signaling, (3) actin polymerization and coordinated cytoskeletal movements that leads to extension of membranes, and (4) vesicular membrane closure closely apposed to the particle or the fiber ingested forming a phagosome shaped by the material ingested (Bowden, 1987).

Particles in the formed phagosome fuse with a lysosome to create a phagolysosome, where the ingested material is eventually degraded. Degradation can be oxygen-dependent (involving the respiratory burst) or oxygen-independent. Oxygen-independent degradation requires the fusion of granules containing proteolytic enzymes such as defensins, lysozyme, and cationic proteins. Additional antimicrobial peptides are present in these granules, including lactoferrin, which sequesters iron to provide unfavorable growth conditions for bacteria. Inhaled nanoparticles deposit along the entire respiratory tract, but are not efficiently engulfed by surface macrophages or may escape from phagosomes (Geiser, 2010).

Following alveolar deposition, macrophages rapidly engulf particles (≥50% within 3 hours and nearly 100% by 24 hours) (Alexis et al., 2006). After several days or weeks, most macrophages then move to the airways and are removed mainly by mucociliary clearance. A small portion of insoluble particles may be phagocytized by alveolar macrophages and removed via lymphatic drainage. A portion of deposited particles not cleared by macrophages can be found in epithelial and interstitial cells or may be found in the lymphatic system with clearance time of months or years. Insoluble particles, especially long narrow fibers, may be sequestered in the lung for very long periods, often in macrophages located in the interstitium.

ALVEOLAR MACROPHAGE RECEPTORS

Alveolar Macrophage Receptors and Innate Immunity

Several receptors are involved in the phagocytic uptake of micrometer-sized particles (Geiser, 2010). Among these are Fc receptor and pattern-recognition receptors (PRRs) including complement, mannose, scavenger receptors (Table 15-5), and other PRRs (Table 15-6). These receptors differ in pathogen recognition motifs and are opsonin-dependent or -independent. Opsonins are binding enhancers (e.g., antibodies), which coat the negatively charged molecules, especially those on bacterial membranes. Molecules that activate the complement system also are considered opsonins.

Phagocytosis of particles is mediated by various Fc receptors, which recognize immunoglobulin-coated particles. Macrophage Fc receptors recognize Fc fragments of the antibodies immunoglobin (Ig) A, E, and G. Fc receptors are classified based on the type of antibody they recognize. For example, Fc fragment of IgA, receptor for (FCAR aka CD89) recognizes IgA. Other macrophage Fc receptors include FCER1A/G, FCER2 (aka CD23) that recognize IgE, and FCGR1A/B/C (aka CD64), FCGR2A/B/C (aka CD32), and FCGR3A/B (aka CD16) that recognize IgG. The main receptors associated with alveolar macrophage phagocytosis are the IgA (FCAR) and IgG (FCGR1, 2, 3 types) receptors. Phagocytic cells do not have a FC receptor for IgM, making IgM ineffective in assisting phagocytosis. However, IgM efficiently activates complement and is therefore considered an opsonin. Clustering and activation of the low-affinity receptor FCGR2 by uncoated quartz has been demonstrated in vitro (Haberzettl et al., 2008).

Once activated, FCAR modulates the respiratory burst (Ouadrhiri et al., 2002). The respiratory burst involves the freshly generated reactive oxygen species produced by the phosphorylation, assembly, and activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Iles and Forman, 2002). Assembly of the NADPH oxidase complex that includes cytochrome b-245 and β polypeptide (CYBB aka NADPH oxidase 2 [NOX2]) involves Ras-related C3 botulinum toxin substrate 1 (RAC1), neutrophil cytosolic factors (NCFs) 1, 2, and 4 (aka p47PHOX, p67PHOX, and p40PHOX, respectively), and CYBA (aka p22PHOX). Activation requires phosphorylation of the NCF2 subunit of the NADPH oxidase complex that activates the oxidase,

Table 15-5

Major Alveolar Macrophage Receptors

RECEPTOR PROTEIN FAMILY	SYMBOL	SYNONYMS	LIGAND
Fc receptors			
Subfamily A (FCAR)			
Fc fragment of IgA, receptor for	FCAR	CD89	Immunoglobin A
Subfamily E (FCER)			
Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	FCER1A		Immunoglobin E
Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	FCER1G		Immunoglobin E
Fc fragment of IgE, low affinity II, receptor for (CD23)	FCER2	CD23	
Subfamily G (FCGR)			
Fc fragment of IgG, high affinity Ia, receptor (CD64)	FCGR1A	CD64a	Immunoglobin G
Fc fragment of IgG, high affinity Ib, receptor (CD64)	FCGR1B	CD64b	Immunoglobin G
Fc fragment of IgG, high affinity Ic, receptor (CD64)	FCGR1C	CD64c	Immunoglobin G
Fc fragment of IgG, low affinity IIa, receptor (CD32)	FCGR2A	CD32A	Immunoglobin G, quartz
Fc fragment of IgG, low affinity IIb, receptor (CD32)	FCGR2B	CD32B	Immunoglobin G, quartz
Fc fragment of IgG, low affinity IIc, receptor (CD32)	FCGR2C	CD32C	Immunoglobin G, quartz
Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	FCGR3A	CD16a	Immunoglobin G
Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	FCGR3B	CD16b	Immunoglobin G
Complement receptors			
Integrin, alpha M (complement component 3 receptor 3 subunit)	ITGAM as a dimer with ITGBM	CD11b	Complement component 3 (iC3b), beta glucan, intercellular adhesion molecule I (ICAM1 aka CD54)
Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	ITGBM as a dimer with ITGAM	CD18	Complement component 3 (iC3b), beta glucan, intercellular adhesion molecule I (ICAM1 aka CD54)
Mannose receptor			
Mannose receptor, C type 1	MRC1	CD206	Microbial mannose structures
Scavenger receptors			
Macrophage scavenger receptor 1	MSR1	CD204	Acetylated low density lipoprotein (LDL), oxidized LDL, advanced glycation end products-modified, maleylated-bovine serum albumin, lipoteichoic acid (LTA), lipid A component of lipopolysaccharide (LPS)
Macrophage receptor with collagenous structure	MARCO	SCARA2	Titanium dioxide, iron oxide and polystyrene latex particles. Acetylated low density lipoprotein (LDL), oxidized LDL, advanced glycation end products-modified, maleylated-bovine serum albumin, lipoteichoic acid (LTA), lipid A component of lipopolysaccharide (LPS)

Table 15-6

Major Alveolar Pattern Recognition Receptors (PRR)

RECEPTOR PROTEIN FAMILY	SYMBOL	SYNONYMS	LIGAND
Toll-like receptors(TLRs)			
Toll-like receptor 1	TLR1		Bacterial PAMPs (lipoproteins, lipoteichoic acid [LTA], peptidoglycan), fungal PAMPs, beta-glucan, zymosan, mannan
Toll-like receptor 2	TLR2		Bacterial PAMPs (lipoproteins, lipoteichoic acid [LTA], peptidoglycan), fungal PAMPs, beta-glucan, zymosan, mannan
Toll-like receptor 3	TLR3		Viral PAMPs (double-stranded RNA)
Toll-like receptor 4	TLR4		Gram negative bacterial lipopolysaccharides (LPS), mannan
Toll-like receptor 5	TLR5		Bacterial flagellin
Toll-like receptor 6	TLR6		Bacterial PAMPs [Lipoproteins, lipoteichoic acid (LTA), peptidoglycan] Fungal PAMPs, beta-glucan, zymosan
Toll-like receptor 7	TLR7		Bacterial or viral PAMPs (single-stranded RNA)
Toll-like receptor 8	TLR8		Bacterial or viral PAMPs (single-stranded RNA)
Toll-like receptor 9	TLR9		Bacterial, viral, fungal, or parasitic DNA, unmethylated CpG motifs
Toll-like receptor 10	TLR10		Lipoproteins, lipoteichoic acid (LTA), peptidoglycan (possibly)
Toll-like receptor 11	TLR11		Parasitic PAMPs (profilin)
C-type lectin receptors (CLRs)			
C-type lectin domain family 4, member E	CLEC4E	Mincle	Cord factor (trehalose-6,6′-dimycolate (tuberculosis)
C-type lectin domain family 7, member A	CLEC7A	Dectin-1	Bacterial PAMPs (beta-glucans, zymosan)
Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs)			
Caspase activation and recruitment domain (CARD) subtype			
Nucleotide-binding oligomerization domain containing 1	NOD1	CARD4	*Legionella pneumophilia, Pseudomonas aeruginosa*, and *Staphylococcus aureus*, and other gram negative and some Gram-positive bacterial peptidoglycan: muropeptides (iE-DAPs)
Nucleotide-binding oligomerization domain containing 2	NOD2	CARD15	Muramyl dipeptide in all peptidoglycan types
Pyrin domain (PYD) (aka death fold domain) subtype			
NLR family, pyrin domain containing 1	NLRP1	CARD7	Inflammasome initiators
NLR family, pyrin domain containing 3	NLRP3	NALP3	Inflammasome initiators
NLR family, CARD domain containing 4	NLRC4	IPAF	Inflammasome initiators
PYD and CARD domain containing	PYCARD	ASC	Inflammasome initiators
RNA helicase retinoic acid-inducible gene I-like receptors (RLRs)			
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	DDX58	RIG-I	Shorter double-stranded RNA fragments, influenza A/B, respiratory syncytial virus, and Sendai virus
Interferon induced with helicase C domain 1	IFIH1	MDA5	Longer nucleotide fragments poly(I:C) motifs and picoaviral ssRNA
DEXH (Asp-Glu-X-His) box polypeptide 58	DHX58	LPG2	Regulator of DDX58/IFIH signaling

which utilizes cytosolic NADPH to reduce extracellular oxygen to produce superoxide. Superoxide is converted to hydrogen peroxide by superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), and diatomic oxygen. H_2O_2 can be combined with chlorine by myeloperoxidase to produce hypochlorite (chlorine bleach), which plays a role in destroying bacteria. There are three types of SODs: SOD1 (containing Cu/Zn) is the cytoplasmic isoform, SOD2 (containing Mn/Fe) is the mitochondrial isoform, and SOD3 (containing Cu/Zn) is the extracellular isoform (Zelko et al., 2002). The latter is particularly abundant in the lung's extracellular matrix (ECM).

Under ideal conditions, superoxide anion is released totally into the phagosome and thereby held inside the cell. However, it can be released and react with NO. The oxidation of L-arginine produces NO endogenously by a reaction catalyzed by NO synthase. The enzyme exists in constitutive neuronal (NOS1), inducible (NOS2), and endothelial (NOS3) isoforms. In aqueous solution, freshly generated NO persists for several minutes in micromolar concentrations before it can react with O_2 to form much stronger oxidants such as NO_2. Nonetheless, in the body, the half-life of NO is only seconds because NO diffuses (>100 μm) from the tissue and enters red blood cells and reacts with oxyhemoglobin. The direct toxicity of endogenous NO is modest but is greatly enhanced by reacting with superoxide to form peroxynitrite ($ONOO^-$) (Beckman, 2009; Ferrer-Sueta and Radi, 2009). Most NO formed in the lung is excreted as nitrate in the urine within 48 hours, or NO can be exhaled (Meyer and Piiper, 1989). Other metabolites include nitrogen gas, ammonia, and urea (Yoshida and Kasama, 1987; Kosaka et al., 1989). Nonetheless, NO is produced in sufficiently high concentrations to outcompete SOD for superoxide. Compared to other reactive oxygen and nitrogen species, peroxynitrite reacts relatively slowly with most biological molecules, making peroxynitrite a selective oxidant. Peroxynitrite mainly modifies tyrosine in proteins to create nitrotyrosines, leaving a biological signature. Nitration of structural proteins, including neurofilaments and actin, can disrupt filament assembly with major pathological consequences. Nitrotyrosines have been detected in acute lung injury and several other inflammatory diseases (Beckman et al., 1990; Freeman et al., 1995; Squadrito and Pryor, 1998).

In addition, H_2O_2 produced by the respiratory burst also functions as a second messenger and activates signaling pathways in alveolar macrophages (Iles and Forman, 2002; Gwinn and Vallyathan, 2006). A major event is the activation of nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (NFKB1 aka NF–κB) by H_2O_2. Activated NFKB1 translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions, especially cytokines and chemokines that modulate the inflammatory response. Cytokines are intercellular mediators of inflammation that activate other inflammatory cells. Chemokines are intercellular mediators that attract other leukocytes through chemotaxis. The main cytokines generated by classically activated macrophages (aka M1 macrophages) include tumor necrosis factor (TNF), and interleukin (IL) 1, β (IL1B), IL6, and IL12. Interferon-γ (IFNG) released from activated macrophages or T cells (especially the Th1 subtype lymphocyte) drive immature macrophages toward the classically activated phenotype. Classically activated macrophages also express increased nitric oxide synthase 2, inducible (NOS2), which generate nitric oxide that can react with superoxide to form the more stable peroxynitrite, a reactive nitrogen species (Pacher et al., 2007). H_2O_2 also activates the mitogen-activated protein kinase (MAPK) pathways that phosphorylate transcription factors including MAPK1/3 (aka extracellular-regulated kinase [ERK]) and the MAPK8 (aka c-Jun N-terminal kinase [JNK]). Phosphorylation of MAPK1/3 also modulates the expression of genes via phosphorylation of the transcription factor ELK1, member of ETS oncogene family (ELK1) that controls the production of the FBJ osteosarcoma oncogene (FOS) transcription factor.

Complement receptors recognize complement-coated particles (van Lookeren Campagne et al., 2007). Macrophage complement receptors include integrin alpha M (complement component 3 receptor 3 subunit) (ITGAM aka CD11b) and integrin beta 2 (complement component 3 receptor 3 and 4 subunit) (ITGB2 aka CD18). Complement component C3 is central to opsonization and its first cleavage product, C3b, forms the multisubunit enzyme, C3bBb, that proteolytically cleaves additional C3 molecules on the pathogen surface. C3b is further degraded to iC3b, C3c, and C3dg. These receptors dimerize to form the ITGAM/ITGB2 (aka CD11b/CD18) complex, which recognizes multiple ligands including iC3b, β-glucans, and intercellular adhesion molecule 1 (ICAM1 aka CD54). β-Glucans are glucose polymers found in the cell walls of plants, fungi, and some bacteria (Palma et al., 2006). Because cell walls are actively remodeled during bacterial cell growth and division, the constant release of these ligands from bacteria allows the innate immune system to survey its surroundings for the presence of active bacteria. ICAM1 is also a ligand for integrin α L (ITGAL aka CD11A) when complexed with ITGBM.

Macrophage mannose receptor, namely mannose receptor C type 1 (MRC1 aka CD206), is a C-type lectin receptor (CLR) that recognizes high-mannose structures on the surface of potentially pathogenic viruses, bacteria, and fungi (Gazi and Martinez-Pomares, 2009). Unlike other CLRs that elicit microbicidal effector functions (see below), MRC1 expression and its endocytic function are selectively decreased by IFNG, but increased by IL4 and IL13. IL4 and IL13 are cytokines generated by Th2 lymphocytes that increase in parasitic infections (e.g., helminthic infections) or acquired allergies (e.g., allergic asthma).

The induction of MRC1 is a marker of the alternatively activated macrophage (aka M2 macrophage) (Gordon and Martinez, 2010). Alternatively activated macrophages are thought to be immunosuppressive because they release IL10, do not produce IL12, and have decreased Th1-attracting chemokine, chemokine (C–C motif) ligand 3 (CCL3 aka MIP1A). However, alternatively activated macrophages express increased levels of arginase 1 (ARG1), resistin-like alpha (RETNLA), and chitinase 3-like 3 and 4 (CHI3L3 and CHI3L4). ARG1 can reduce NO production. RETNLA can modulate inflammation. CHI3L3/4 can bind chitins in insects, helminth eggs, and nematode pharynx. MRC1 also can interact with tumor cell mucin glycoproteins (e.g., MUC16 aka CA125 and TAG-72), which induces MRC1 internalization and thereby modulates (perhaps suppresses) cytokine production (Allavena et al., 2010). It should be noted that phenotypes of M1 or M2 macrophages can overlap and are controlled by the source of macrophage, activators, and markers that depend on activation (Murray et al., 2014).

Macrophage scavenger receptors, macrophage scavenger receptor 1 (MSR1 aka CD204), and macrophage receptor with collagenous structure (MARCO) recognize a range of modified host polyanionic molecules and apoptotic cells (Bowdish and Gordon, 2009). Ligands for MSR1 are shared for the most part with other scavenger receptors, include acetylated low-density lipoprotein (LDL), oxidized LDL, advanced glycation end products (AGEs)-modified, and maleylated-bovine serum albumin. Other ligands include lipoteichoic acid (LTA) and the lipid A component of LPS. Three MSR1 protein isoforms can be generated from the *MSR1* gene. The isoform type 3 does not internalize modified LDL (acetyl-LDL) despite having the domain that mediates this function in type 1 and 2 isoforms. Isoform 3 has an altered intracellular processing and is trapped within the endoplasmic reticulum, rendering the macrophage unable to complete endocytosis. The isoform type 3 can inhibit the function of isoforms type 1 and type 2 when coexpressed, indicating

a dominant negative effect and suggesting a mechanism for regulation of scavenger receptor activity in macrophages. MARCO is also involved in uptake of unopsonized particles such as titanium dioxide (TiO_2), iron oxide (Fe_2O_3), and polystyrene latex (PSL) (Arredouani et al., 2005). The particles tested in this study were about 1 μm in diameter.

Alveolar Macrophage Pattern-Recognition Receptors

Pattern-recognition receptors (PRRs) have various ectodomains that recognize pathogen-associated molecular patterns (PAMPs) present on microbial surfaces. PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the RNA helicase retinoic acid-inducible gene I (RIG-I) (aka DExD/H-box helicase 58) (Table 15-6).

TLR proteins contain three major domains, a leucine-rich repeat motif containing ectodomain, a transmembrane region, and a cytosolic Toll-IL1 receptor (TIR) domain (Kawai and Akira, 2011). Expressed either on the cell surface or associated with intracellular vesicles, 10 and 13 functional TLRs have been identified in the human and mouse genome, respectively. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are expressed on the plasma membrane and mainly recognize microbial membrane components; TLR3, TLR7, TLR8, and TLR9 are expressed in the endosome and mainly recognize nucleic acids. Membrane-associated TLRs detect distinct PAMPs with the leading example being LPS, an endotoxin in the cell membrane of gram-negative bacteria, which is specifically recognized by TLR4. Other bacterial PAMPs include lipoproteins, LTA from gram-positive bacteria, and peptidoglycan (PGN) that are recognized by heterodimeric TLR2/TLR1 or TLR2/TLR6 and possibly TLR10, whereas bacterial flagellin is recognized by TLR5. Fungal PAMPs, β-glucan, and zymosan are recognized by TLR2 and TLR6, and mannan is recognized by TLR2 and TLR4. Parasitic PAMPs include profilin of *Toxoplasma gondii* recognized by TLR11. Viral PAMPs include nucleic acid variants of double-stranded (ds) RNA recognized by endosomal TLR3 with bacterial or viral single-stranded (ss) RNA being recognized by endosomal TLR7 and TLR8. Bacterial, viral, fungal, or parasitic DNA and unmethylated CpG motifs are recognized by endosomal TLR9. Alveolar macrophages express all the known TLRs, but most prominently TLR2, TLR3, TLR4, TLR5, TLR6 (Maris et al., 2006), and TLR9 (Juarez et al., 2010). In addition to TLRs, other membranes and cytosolic PRRs are found in the lung.

Other macrophage membrane PRRs include CLRs that recognize β-glucan and mannose structural molecules (Osorio and Reis e Sousa, 2011). For example, C-type lectin domain family 7, member A (CLEC7A aka Dectin-1) binds β-glucans and zymosan present in lung bacterial pathogens such as *Mycobacterium tuberculosis*, *Aspergillus* spp, and *Pneumocystis* spp. Another important macrophage CLR is CLEC4E, which recognizes trehalose-6,6′-dimycolate (aka cord factor), a major cell wall glycolipid of *M. tuberculosis* (Ishikawa et al., 2009). *M. tuberculosis* produces several other molecules that are recognized via MRC1, TLR2, and TLR9.

Other cytosolic PRRs are NOD-like receptors (NLRs). NLR proteins are characterized by a shared domain architecture that includes a nucleotide-binding domain (NBD) and, like TLRs, a leucine-rich repeat (LRR) domain (Elinav et al., 2011). NLRs can be grouped into two subfamilies based on additional domains, which can be either a caspase activation and recruitment domain (CARD) or a pyrin domain (PYD aka death-fold domain). The major CARD NLRs are NOD containing 1 (NOD1) and NOD2. NOD1 and NOD2 are cytosolic receptors that recognize distinct PGN subunits contained in gram-positive bacteria cell walls. NOD1 recognizes muropeptides (iE-DAPs) that are found in the PGN of gram-negative bacteria and only some gram-positive bacteria, whereas NOD2 recognizes a minimal motif of muramyl dipeptide that is found in all PGNs. In the lung, NOD1 is important because it recognizes and participates in regulating inflammatory responses to *Legionella pneumophila* (Berrington et al., 2010), *Staphylococcus aureus* (Travassos et al., 2004), and *Pseudomonas aeruginosa* (Travassos et al., 2005).

PYD NLR proteins along with other members of the NLR protein family can form multiprotein complexes called inflammasomes (Sutterwala et al., 2007). Inflammasomes are composed of one of several NLR and Pyhin proteins (Schattgen and Fitzgerald, 2011), including NLR family, pyrin domain containing 1 (NLRP1), NLRP3, and NLR family, CARD domain containing 4 (NLRC4 aka IPAF) sense endogenous or exogenous PAMPs (Sutterwala et al., 2007). Inflammasomes are assembled through homophilic CARD–CARD and PYD–PYD interactions between NLRs, PYD, and CARD domains containing (PYCARD aka apoptosis-associated speck-like protein containing a CARD [ASC]) (Elinav et al., 2011). Inflammasome assembly initiates the activation of inflammatory caspases, cysteine proteases that are synthesized as inactive prozymogens. Upon activation, caspases trigger cellular programs that lead to inflammation or cell death. Caspase 1, apoptosis-related cysteine peptidase (IL1, β, convertase (CASP1), which joins the inflammasome and is regulated in a signal-dependent manner, is the most prominent member of pro-inflammatory caspases. Other pro-inflammatory caspases include CASP4, CASP5, CASP11, and CASP12. CASP1 catalytic activity is tightly regulated and essential for pro-IL1B and pro-IL18 processing and secretion. Inflammasomes require two signals to accomplish their biological function. Signal I initiates transcriptional activation of inflammasome components and is often provided through TLR and NFKB1 signaling, whereas signal II is required to initiate inflammasome assembly. For example, during influenza infection, signal I requires TLR7 recognition of viral RNA and signal II activation of inflammasomes requires an acidified Golgi compartment (Ichinohe et al., 2010).

Additional cytosolic PRRs are the RIG-I–like receptors (RLRs), a 3-protein family of DExD/H box RNA helicases that function as cytoplasmic sensors of viral PAMPs. The RLRs signal downstream transcription factor activation to drive type 1 IFN production and antiviral gene expression that elicits responses to control virus infection (Loo and Gale, 2011). The founding member of the family is DEAD (Asp–Glu–Ala–Asp) box polypeptide 58 (DDX58 aka RIG-I: retinoic acid-inducible gene I), which recognizes viral dsRNA. Lung pathogens recognized by DDX58 include influenza A, influenza B, respiratory syncytial virus, and Sendai virus. The second member is IFN induced with helicase C domain 1 (IFIH1 aka MDA5 melanoma differentiation associated factor 5). IFIH was originally named MDA5 because it increases in response to β-IFN, β1, fibroblast (IFNB), and mezerein, a protein kinase C-activating compound, which causes melanoma differentiation. These proteins contain an N-terminal region consisting of tandem CARD domains, a DExD/H box RNA helicase domain with the capacity to hydrolyze ATP and to bind and possibly unwind RNA, and a C-terminal repressor domain embedded within the C-terminal domain that in the case of RIG-I is involved in autoregulation. DDX58 preferentially associates with influenza's shorter RNA fragments (e.g., 50 triphosphorylated ends-RNA sequence motifs) and some dsRNA regions (Baum et al., 2010). In contrast, IFIH interacts with and preferentially

recognizes high-molecular-weight fragments (e.g., picornaviral ssRNA or longer synthetic poly(I:C) motif). The third member is DExH (Asp–Glu–X–His) box polypeptide 58 (DHX58 aka LGP2: laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2). Although similar to DDX58 and IFIH in structure, DHX58 lacks the N-terminal CARD domains and may function as a regulator of DDX58/IFIH signaling.

Upon PAMP recognition, PRR proteins recruit a specific set of adaptor molecules that share a cytoplasmic domain that helps to initiate the cell signaling that activates antimicrobial responses and transcription of intra- and intercellular signaling molecules. For example, the TIR domains of TLR4 interact with the TIR-domain containing adaptor proteins, including the myeloid differentiation primary response gene (88) (MyD88) or TLR adaptor molecule 1 (TICAM1 aka TRIF), and initiate downstream signaling events (Kawai and Akira, 2010). The TLR4–TICAM1–MyD88 complex activates the IL1-receptor–associated kinase 1 (IRAK1) and TNF-receptor–associated factor 6 (TRAF6) complex, which in turn, activates transforming growth factor (TGF)-beta–activated kinase 1 (TAK1). This leads to the activation of NFKB1 by removal of the inhibitory protein, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, α (NFKBIA), and other members of an inhibitory complex that prevents NFKB1 movement to the nucleus. Nuclear NFKB1 increases transcription of a wide array of cytokines and chemokines. The secreted chemokines attract, and cytokines activate other leukocytes and structural cells. Alternatively, when TLR4 is internalized within the phagosome, it can form a complex with RAB11A, member RAS oncogene family (RAB11A), TICAM1, and TICAM2 (aka TRAM). This initiating step can either activate TRAF6 or add to a late NFKB1 response that causes additional recruitment of neutrophils and activation of macrophages. In addition, phagosomal TLR4 can activate TRAF3 that leads to IFN regulatory factor 3 (IRF3) activation. IRF3 translocates to the nucleus and activates the transcription of type I IFN (e.g., *IFNA1* and *IFNB1*) genes, as well as other IFN-induced genes. This can result in direct killing of infected pathogens. Moreover, activation of TLR signaling leads to maturation of dendritic cells, a macrophage that presents antigens to lymphocytes, contributing to the induction of adaptive immunity (see below).

ACUTE RESPONSES OF THE RESPIRATORY TRACT TO INJURY

Trigeminally Mediated Airway Reflexes to Inhaled Chemicals

Inhaled toxic chemicals or particles can come into contact with cells lining the respiratory tract from the nostrils to the gas-exchanging region. The sites of deposition in the respiratory tract have important implications in evaluating the risks posed by inhalants. For example, rats have more nasal surface on a per body weight basis than do humans. Measurement of DNA–protein cross-links formed in nasal tissue by formaldehyde has demonstrated that rats, which readily develop nasal tumors, have many more DNA cross-links per unit of exposure (concentration of formaldehyde × duration of exposure) than monkeys. Because the breathing pattern of humans resembles that of monkeys more than that of rats, extrapolation of tumor data from rats to humans on the basis of formaldehyde concentration may overestimate the nasal dose of formaldehyde to humans. Patterns of animal activity also can affect dose to the lung. Nocturnally active animals such as rats receive a greater dose per unit of exposure at night than during the day, whereas humans show the opposite diurnal relationships of exposure concentration to dose.

Nasal and airway irritation represents a common response to inspired toxic compounds (Lanosa et al., 2010). Nasal irritation is mediated by irritant receptors (e.g., TRPA1) that trigger trigeminal nerve responses characterized by tickling, itching, and painful nasal sensations (Alarie, 1973; Nielsen et al., 2007). TRPA1 is sensitive to several irritants including acrolein, allyl isothiocyanate, chlorine, 4-hydroxynonenal, and hydrogen peroxide (Bautista et al., 2006; Bessac and Jordt, 2008; Bessac et al., 2009). Nasal tissues of rodents express high levels of cytochromes P450 (CYP450) (Morris, 2000; Ding and Kaminsky, 2003), and biotransformation enzymes that can result in the formation of electrophilic metabolites from volatile organic compound vapors that activate TRPA1 (Lanosa et al., 2010). For example, styrene (Morris, 2000) and naphthalene (Morris and Buckpitt, 2009) are extensively metabolized by CYP2F isozymes within nasal mucosa. When the concentration of an inhaled substance exceeds the biotransformation capacity of the nasal passages, it can penetrate to the lower respiratory tract (Morris, 2001).

Nasal irritation has been used as a basis for occupational exposure levels and is a common component in sick building syndrome resulting from poor indoor air quality (Hall et al., 1993; Hodgson, 2002). One of the consequences of nasal irritation is nasal mucus secretion that can dilute the irritant. Some chemicals can produce severe irritation at concentrations below those that induce a toxic response upon chronic exposure. Under these circumstances, nasal irritation could be a limiting factor in human exposures, and thus the chronic effects are less likely to occur. This can be viewed as a protective mechanism. However, because the threshold dose of an irritant response can vary greatly among individuals (much like olfactory acuity), nasal irritation is not a reliable method for occupational safety. In mice, sensory irritation can lead to decreased breathing frequency during irritant exposure or increased pause at the onset of expiration, which can be highly quantifiable (Alarie, 1973; Vijayaraghavan et al., 1993; Morris et al., 2003). If continued exposure cannot be avoided, many irritants will produce cell necrosis and increase permeability of the airway epithelium.

Bronchoconstriction, Airway Hyperreactivity, and Neurogenic Inflammation

Substances that penetrate the nasal passage or are inhaled orally (with less efficient deposition) can trigger irritant receptors in the airways. Large diameter airways are surrounded by collagen and bronchial smooth muscle, which helps maintain airway tone and diameter during expansion and contraction of the lung. Bronchoconstriction can be evoked by irritants (acrolein, etc.), cigarette smoke, or air pollutants, and by cholinergic drugs such as acetylcholine. Bronchoconstriction causes a decrease in airway diameter and a corresponding increase in resistance to airflow. Bronchoconstriction can also be accompanied by an accumulation of thick mucus in the airway lumen. Characteristics of bronchoconstriction include the associated signs of coughing, wheezing, and rapid shallow breathing, and the associated symptoms of a sensation of chest tightness, substernal pain, and dyspnea (a feeling of breathlessness). Dyspnea is a normal consequence of exercise, which potentiates these problems as well as leads to more mouth breathing. Because the major component of airway resistance usually is contributed by large bronchi, inhaled chemicals that cause bronchoconstriction are generally irritant gases with moderate solubility.

Bronchial smooth muscle tone is regulated by the autonomic nervous system. Postganglionic parasympathetic fibers, when stimulated, will release acetylcholine to the smooth muscle layer surrounding the bronchi. These smooth muscle cells have membrane cholinergic receptors, muscarinic 3 (CHRM3). The activation of these receptors by acetylcholine activates an intracellular G protein domain, which increases cGMP. The increase in cGMP in turn activates a phospholipase C (PLC) pathway that increases intracellular calcium concentrations $[Ca^{2+}]_i$. PLC catalyzes phosphatidylinositol 4,5-bisphosphate leading to the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 initiates calcium release from the smooth endoplasmic reticulum. Increased $[Ca^{2+}]_i$ leads to contraction of the smooth muscle cells. The actions of cGMP can be antagonized by increased cAMP evoked by protein kinase A (PRKA). Increased cAMP can be accomplished by ligands that bind to β-adrenergic receptors on the cell surface. The latter can be stimulated by inhaled bronchodilators (β-adrenergic agonists such as albuterol) or by injected epinephrine (adrenaline).

In addition to inducing an acute bronchoconstriction, irritants can prime the autonomic response by lowering the threshold dose of acetylcholine needed to induce bronchoconstriction. A lower threshold of acetylcholine-mediated bronchoconstriction is called airway hyperreactivity (or hyperresponsiveness). This response serves as the basis for a sensitive measure of whether a toxicant can cause bronchoconstriction in animals or humans primed by a prior dose of an acetylcholine-like chemical (bronchoprovocation testing). These tests are performed by measuring airway resistance following inhalation of increasing doses of a methacholine aerosol. Methacholine is used because it is more stable than acetylcholine. Other important bronchoconstrictive substances include histamine, various eicosanoids (including PGs [mainly $PGF_2\alpha$ and PGD_2] thromboxane A_2, leukotrienes C_4 and D_4], and adenosine. The bronchial smooth muscles of individuals with asthma contract at a lower threshold dose during provocation than do those of individuals without asthma (see the section "Asthma").

In addition to bronchoconstriction, cough, and airway hyperreactivity, irritants can stimulate TRP channels (especially TRPA1 and TRPV1) that cause neurogenic inflammation. Mediated by neuropeptides (including tachykinins) released from nociceptive nerve terminals, neurogenic inflammation also includes vasodilatation, plasma protein extravasation, and leukocyte adhesion to the vascular endothelium (Geppetti et al., 2006). Tachykinins including substance P (aka neurokinin 1) activate tachykinin receptors (TACRs) on airway tissues. Tachykinins stimulate airway smooth muscle TACR2s and TACR1s to mediate bronchoconstriction, submucosal gland TACR1s to mediate mucin secretion, and cholinergic nerve TACR3s to mediate terminal stimulation. TRPV1 mediates the tussive action (cough) of capsaicin, which is widely used in cough provocation studies. TRPA1 is targeted by acrolein, 4-hydroxy-2-nonenal, and hydrogen peroxide. TRPV1 and TRPA1 antagonists may therefore represent potential anti-tussive and anti-inflammatory therapeutics for respiratory airway diseases.

Acute Lung Injury and Pulmonary Edema

Initiated by numerous factors, acute lung injury (aka adult or infant respiratory distress syndrome) is marked by alveolar epithelial and endothelial cell perturbation and inflammatory cell influx that leads to surfactant disruption, pulmonary edema, and atelectasis (Ware and Matthay, 2000). Pulmonary edema produces a thickening of the alveolar capillary barrier and thereby limits O_2 and CO_2 exchange. Matching ventilation to vascular perfusion is critical to efficient gas exchange and is disrupted during acute lung injury. Alterations in coagulation and fibrinolysis accompany lung injury. Pulmonary edema may not only induce acute compromise of lung structure and function but also cause abnormalities that remain after resolution of the edematous process. The outcome may be beneficial or damaging to the lung. To resolve the alveolar and interstitial exudates, inflammatory cells accumulate and release factors critical to wound repair. The turnover of inflammatory cells and related immune responses play a role in eliciting both mitogenic activity and fibrogenic responses. During acute lung injury, profibrotic growth factors, TGFB1 (Wesselkamper et al., 2005) and platelet-derived growth factor (PDGF) (Bellomo, 1992), are activated and can initiate epithelial–mesenchymal transition (EMT) (see the section "Pulmonary Fibrosis").

When inhaled in high concentrations, acrolein, HCl, NO_2, NH_3, or phosgene may produce immediate alveolar damage leading to a rapid death. However, these gases inhaled in lower concentrations may produce very little apparent damage in the respiratory tract. After a latency period of several hours, exposure to these compounds may compromise alveolar barrier function that leads to delayed pulmonary edema that is often fatal. In addition, acute lung injury can result from systemic effects including sepsis, transfusion, and blunt trauma to other organs. In several animal studies, neutrophil depletion can be protective; however, acute lung injury can develop in the absence of circulating neutrophils.

During acute lung injury, innate immunity is activated through activation of PRRs (Xiang and Fan, 2010). In addition, a number of damage-associated molecular pattern proteins (DAMPs) are formed and include AGEs, high-mobility group protein B1 (HMGB1), amyloid β-peptide, and members of the S100 calcium binding protein (especially S100A8 [aka calgranulin A], S100A9 [aka calgranulin B], and S100A12 [aka ENRAGE]). These ligands bind to the ectodomain of the AGE receptor (AGER aka RAGE) and initiate intracellular signaling leading to NFKB1-mediated induction of inflammatory cytokines and through positive feedback, AGER expression. In particular, HMGB1 is a potent mediator of acute lung injury and can induce PMN accumulation, edema formation, and production of pro-inflammatory mediators in the lung. HMGB1 is also a mediator of lethality during endotoxemia and sepsis in mice. In humans with severe trauma, plasma HMGB1 levels correlate positively with severity of injury and progression to acute lung injury. Activated macrophages and other inflammatory cells produce excessive reactive oxygen (superoxide anion, hydroxyl radicals, hydrogen peroxide) and nitrogen (nitric oxide, peroxynitrate) species. Because these oxidant species are potentially cytotoxic, they may mediate or promote the actions of various respiratory toxicants. Such mechanisms have been proposed for paraquat- and nitrofurantoin-induced lung injury. In this regard, the lung is well equipped with antioxidant enzymes, especially SOD3, which converts superoxide anion to hydrogen peroxide. Hydrogen peroxide is the mediator of the extracellular cytotoxic mechanism of activated phagocytes. In addition, hydrogen peroxide is a potent intracellular signaling molecule that readily crosses cell membranes, and can thereby amplify cell damage. Phagocytic production of reactive oxygen species causes activation of proteinase enzyme and inactivation of proteinase inhibitors. Platelets (and platelet microthrombi) also have the ability to generate activated O_2 species.

Pulmonary edema is customarily quantified in experimental animals by measurement of lung water content. Lung water content can be expressed as the wet (undesiccated) weight of the whole lung or that of a single lung lobe. This value is often normalized to the weight of the animal or to the weight of the lung after complete drying in a desiccator or oven. The latter is typically expressed as lung wet weight:dry weight ratio.

CHRONIC RESPONSES OF THE LUNG TO INJURY

Asthma

Asthma is defined by sporadic bouts of airflow obstruction, leading to dyspnea. Airflow obstruction is measured as increased airway resistance (or decreased predicted FEV1). In asthma, resting levels of airway resistance can be normal or slightly increased, which typically is reversible with bronchodilators. The hallmark of asthma is a persistent or recurrent airway hyperreactivity. As noted above, airway hyperreactivity is defined by a lower threshold to acetylcholine- or methacholine-mediated increased airway resistance. Airway hyperreactivity can be specific, as in allergic asthma, and induced by exposure to a single known irritant or antigen. Knowledge of the causal trigger is extremely valuable because avoidance can reduce the frequency of or even eliminate bronchospasm. Airway hyperreactivity can also be nonspecific, and bronchoconstriction can result from a wide range of triggers including irritants, cold-dry air, or exercise. Therapy for nonspecific airway hyperreactivity is therefore more difficult.

Responses to a methacholine or histamine challenge can vary with severity of the disease. The response in mild asthma is marked by a greater sensitivity (i.e., the dose–response curve is shifted to the left) but may not include a greater maximal airway resistance (Woolcock et al., 1984). This may be caused by altered neural control, inflammation, mediator metabolism, or epithelial damage (Sterk and Bel, 1989). In more severe asthma, greater sensitivity is accompanied by a greater maximal response than observed in healthy subjects. This may be due to additional increases in airway smooth contractility, thickening of the airway wall, and decreased lung elastic load. Airway inflammation is typically marked by activation of receptor and epithelial injury that leads to an influx of eosinophils, which are rarely present in a healthy lung. During exacerbations, the inflammation involves eosinophils, mast cells, lymphocytes, and neutrophils.

The pathogenesis of asthma involves the adaptive and innate immune systems. In allergic asthma, previous exposure to an antigen typically leads to the generation of immunoglobin E (IgE) molecules that have molecular recognition sites specific to the antigen. Upon reexposure, the antigen causes cross-linking of IgE molecules and activation of lymphocytes, eosinophils, macrophages, and mast cells, with elaboration and release of an array of cytokines, chemokines, eicosanoids, histamine, tachykinins, and other mediators. Together, these mediators induce smooth muscle constriction, vascular leakage, mucus secretion, and inflammatory cell recruitment. These processes lead to airway obstruction, airway edema, formation of intraluminal mucus and plugging, and neutrophil recruitment with additional mediator release, respectively. Severe airway obstruction and ventilation-vascular perfusion mismatching lead to impaired gas exchange and hypoxemia. In irritant-induced (nonallergic) asthma, the inflammatory cascade is not initiated by an inhaled antigen but many of the same mediators and effector pathways are activated. Persistent inflammation and epithelial damage contribute to airway hyperreactivity. In laboratory animals, short-term airway hyperreactivity can be induced by many irritant stimuli. However, this response is likely to resolve within a few hours or days after a single exposure. It can also be induced by antigen sensitization (typically ovalbumin injections) and subsequent antigen challenges (typically an aerosol of albumin in saline). This is followed by a determination of airway resistance following increasing doses of methacholine or histamine.

Asthma has been associated with a number of occupations (Malo and Chan-Yeung, 2009). Work-related asthma is a general term that covers occupational asthma, wherein new-onset disease is caused by a sensitizing agent or airway irritant present in the workplace. In addition, it covers work-exacerbated asthma, wherein factors at work can trigger or worsen the symptoms of an existing disease. Occupational asthma can involve adaptive immunity induced by high-molecular-weight and some low-molecular-weight substances. High-molecular-weight agents, including flour-, cereals-, latex-, or animal-derived proteins and enzymes, cause sensitization through an IgE-mediated mechanism, such as in common atopic asthma. Occupational asthma, for example, is common among laboratory technicians and veterinarians who become sensitized to proteins excreted in rodent urine. It also has been associated with the use of proteolytic enzymes in the manufacture of detergents (Schweigert et al., 2000). Low-molecular-weight agents include chemicals like acid anhydrides and platinum salts that induce asthma through an IgE mechanism. However, most low-molecular-weight agents involve an uncertain mechanism of induction. A portion of low-molecular-weight chemicals can cross-link biological macromolecules (Jarvis et al., 2005). The latter chemicals include metals (e.g., nickel, vanadium, chromium, cobalt, zinc, cadmium, or aluminum), diisocyanates (e.g., toluene diisocyanate), cleaning agents, wood dusts, soldering fluxes, pesticides, pharmaceuticals, and reactive dyes. Low-molecular-weight chemicals may act as haptens that combine with endogenous proteins to form a complex that is recognized as an antigen by the immune system.

A subtype of asthma-like respiratory dysfunction (aka reactive airways dysfunction syndrome) or persistent cough can be a sequela to accidental high-level irritant (e.g., ammonia, glacial acetic acid, chlorine, or toluene diisocyanate) exposure that can also induce acute lung injury (Brooks et al., 1985; Kern, 1991; Kivity et al., 1994; Shakeri et al., 2008). In some cases, it becomes a chronic illness with airway hyperreactivity that develops shortly after a single, brief inhalation exposure. However, it is unclear whether the acute exposure alone causes asthma or uncovers existing asthma. Nonetheless, it is clear that persons with asthma may be susceptible to lower doses of irritants (such as sulfur dioxide) (Sheppard et al., 1980). In addition, because ambient particle matter and other air pollutants contain many metals and other chemicals that are associated with asthma in the workplace, it is likely that these irritants can exacerbate asthma (Leikauf, 2002).

Bronchiolitis Obliterans

Bronchiolitis obliterans is marked by fibrotic obstruction of the small diameter airways (bronchioles) (Weigt et al., 2013). The bronchiolar epithelium becomes damaged by inhaled chemicals or respiratory infections, particularly after organ transplants, leading to extensive fibro-proliferative, subepithelial thickening that blocks the airways. Chemicals associated with this condition include diacetyl (Kreiss et al., 2002), sulfur mustard (Khateri et al., 2003; Ghanei et al., 2004), and chemicals and incinerator aerosol released during "burn pit" combustion (King et al., 2011). It also has been associated with rheumatoid arthritis and graft-versus-host disease following a lung or hematopoietic cell transplantation. The respiratory infections linked to this condition include cytomegalovirus, respiratory syncytial virus, adenovirus, *Pseudomonas aeruginosa* or *Mycoplasma pneumonia* (Aguilar et al., 2016).

Like COPD, bronchiolitis obliterans begins with a cough, wheezing, dyspnea (shortness of breath), and fatigue (Barker et al., 2014). These symptoms usually progresses slowly, but severe symptoms can develop without warning. Other symptoms that appear in some individuals include fever, weight loss, and night sweats. The diagnosis of bronchiolitis obliterans is defined by a *sustained* (≥3 weeks) decline in FEV1 provided alternative causes of pulmonary

dysfunction (e.g., COPD or asthma) have been excluded. The pathology is marked by subepithelial fibrosis causing partial or complete luminal occlusion of the bronchioles. The fibrotic lesions may be concentric or eccentric and are often associated with disorganization of elastic tissue of the airway wall. The presence of lymphocytic bronchiolitis or intraluminal granulation tissue can be present but is not sufficient to diagnose this condition. Masson's trichrome (collagen) and Verhoeff–Van Gieson (elastic tissue) staining of lung biopsy specimens may facilitate identification of damaged or obliterated airways.

The fibrotic response is mediated by several factors including TGFB1 (El-Gamel et al., 1999), platelet-derived growth factor (PDGF) (Hertz et al., 1992), hepatocyte growth factor (HGF) (Aharinejad et al., 2004), and insulin-like growth factor-1 (IGF1) (Charpin et al., 2000). Chemokines and their interaction with specific cell receptors are essential components of inflammatory and immune responses via recruitment of specific leukocyte subpopulations. In bronchiolitis obliterans, inflammation is driven by the following ligand/receptor interactions: CCL5/CCR1 and CCR5 (Belperio et al., 2000), CCL2/CCR2 (Belperio et al., 2001), IL8/CXCR1 and CXCR2 (DiGiovine et al., 1996; Belperio et al., 2005), and CXCL9, CXCL10, and CXCL11/CXCR3 (Belperio et al., 2003).

Studies of lung transplant rejection suggest that airway wound repair becomes abnormal leading to fibrosis. Critical to airway wound repair is a delicate balance between effector and regulatory T cell immune responses (Weigt et al., 2013). These responses are modulated by cytokines and other mediators that can be detected in the bronchoalveolar lavage fluid (BALF) from patients with bronchiolitis obliterans. The effector immune responses are of three types: Th1, Th2, and Th17. The type Th1 immune response is mainly associated with a cytotoxic T lymphocyte (CTL) and delayed type hypersensitivity (DTH) response, and is characterized by the production of interleukin IL2, IL12, IFNG, and lymphotoxins. Classically, Th1 cytokines have been associated with acute cellular rejections including bronchiolitis obliterans. The Th2 type immune response is characterized by the production of IL4, IL5, and IL13, which promote mucosal, allergic, and humoral immunity. Although a type 2 immune profile has favored the acquisition of tolerance in some animal models, the type 2 response often has a role in rejection, especially chronic rejection. The Th17 type immune response is characterized by the production of IL17 and IL23 and is associated with autoimmunity. Type 17 T cell skewing is induced by TGFB1, IL6, and IL23 and the type 17 effector cytokine IL17. Treg cells are CD4+ T cells characterized by constitutively high expression of the transcription factor FOXP3 and the secretion of IL10, which can inhibit effector type immune responses. Studies suggest that prevention of bronchiolitis obliterans may depend on the downregulation of all three effector immune responses and preserving or augmenting the regulatory immune response.

Chronic Obstructive Pulmonary Disease

Characterized by a progressive airflow obstruction, COPD involves an airway (bronchitis) and an alveolar (emphysema) pathology. With more than 200 million cases worldwide, COPD is the fourth leading cause of death (Pauwels and Rabe, 2004). The major risk factor for COPD is tobacco smoking and about 20% of smokers will develop COPD. Indoor air pollution from burning biomass fuels is associated with an increased risk of COPD in developing countries (Salvi and Barnes, 2009).

Chronic bronchitis is defined by the presence of sputum production and cough for at least 3 months in each of 2 consecutive years. Bronchitis in COPD involves airway inflammation with excessive mucus production from surface epithelial (goblet) cells and submucosal glands (Leikauf et al., 2002; Rogers, 2007). The number of goblet cells increases, the number of ciliated cells decreases, and the size of the submucosal glands increases markedly. The latter are the major source of mucus and thereby sputum in the disease (Reid, 1960). The associated decreased mucociliary clearance and mucus retention may obstruct the airways and contribute to COPD exacerbations and possibly mortality (Miravitlles, 2011). Acrolein can increase mucin overproduction and can be formed endogenously in the airways during COPD (Bein and Leikauf, 2011).

COPD almost always is characterized by chronic cough. A cough starts with activation of the sensory terminals of cough receptors located in the airway mucosa that triggers a deep inhalation and a forced respiratory exhalation against a closed glottis. It ends with the opening of the glottis that produces a rapid acceleration of air from the lungs accompanied by a distinct sound (Chung and Pavord, 2008). Frequent coughing in COPD can be exacerbated by respiratory infections, and many viruses and bacteria induce cough to move from host to host. Respiratory mechanical and ligand-gated cough receptors on rapidly adapting receptors, C fibers, and slowly adapting fibers provide input to the brainstem medullary central cough generator through the intermediary relay neurons in the nucleus tractus solitarius. As noted above, cough can be evoked by thermal, osmotic, and chemical (especially capsaicin) stimuli that engage the TRP channel (especially TRPV1) protein family. In addition, respiratory ligand-gated receptors are the degenerin/epithelial sodium channel proteins that include nonvoltage-gated sodium channels (e.g., SCNN1A) and amiloride-sensitive cation channels (ACCN2 aka acid-sensing ion channel 1) (Kollarik and Undem, 2006). Members of both families have been implicated in the transduction of mechanical and acidic stimuli. Cough can also be evoked by stimuli of other ligand-gated receptors including 5-hydroxytryptamine activation of 5-hydroxytryptamine (serotonin) receptor 3A (HTR3A), ATP activation of purinergic receptor P2X, ligand-gated ion channels, and nicotine activation of cholinergic receptors, nicotinic subtypes. The central cough generator then establishes and coordinates the output to the muscles that cause cough, bronchoconstriction, and mucus secretion. Cough is effective in removing mucus in the first five to eight bronchial generations but as the cumulative diameter of the airways increases the acceleration of airflow is diminished.

Emphysema is physiologically defined by airflow obstruction that leads to dyspnea (especially on exertion) (Fig. 15-8) accompanied by diminished FEV1. The diagnosis of COPD is made by decreased FEV1:FVC ratio (which is not reversible by administration of a bronchodilator) and includes mild (<80% of normal; GOLD: I), moderate (50% to 57%; GOLD: II), severe (30% to 49%; GOLD: III), and very severe (<30% or chronic respiratory failure; GOLD: IV) (Rabe et al., 2007). Emphysema is pathologically defined by an abnormal enlargement of the airspaces distal to the terminal bronchiole accompanied by destruction of the walls without obvious fibrosis (Snider et al., 1985). Destruction of the gas-exchanging surface area results in a distended, hyperinflated lung that no longer effectively exchanges oxygen and carbon dioxide as a result of both loss of tissue and air trapping. Pathologically, emphysema can be (a) centriacinar emphysema that begins in the respiratory bronchioles and spreads peripherally (typically starting in the apical lung and is associated with cigarette smoking), (b) panacinar emphysema that destroys the entire alveolus uniformly (typically in the lower half of the lungs), and (c) paraseptal emphysema that forms giant bullae.

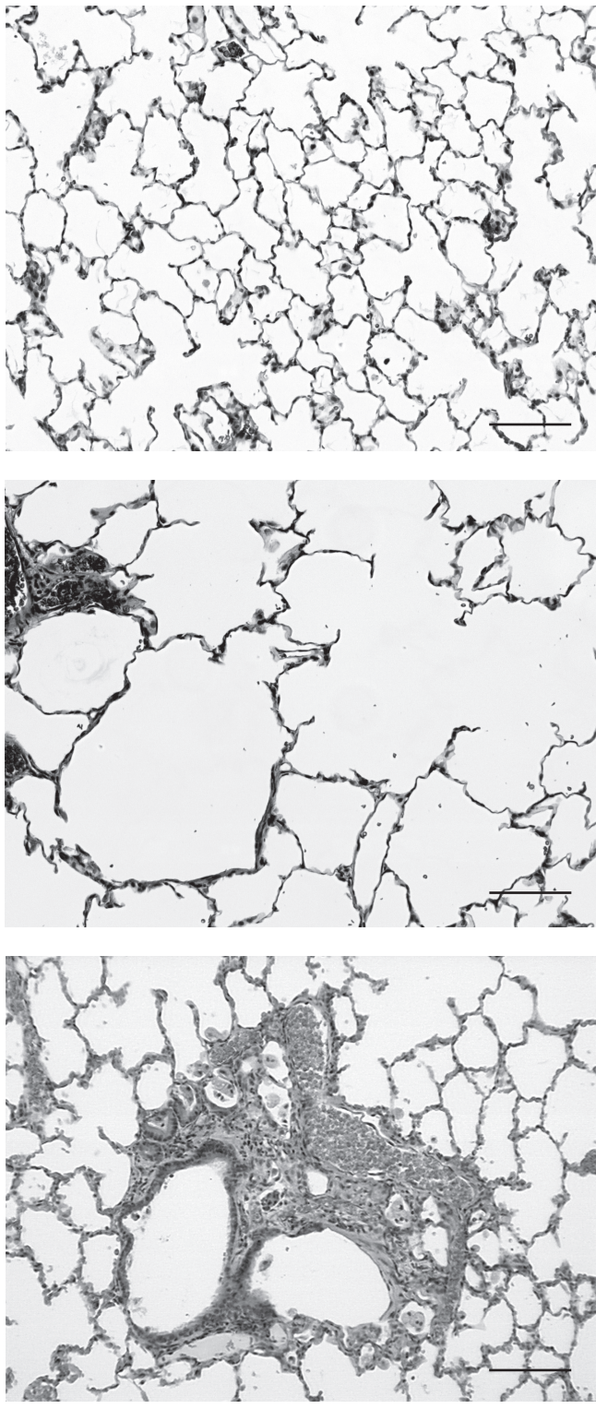

Figure 15-8. *Airspace enlargement induced by tobacco smoke and pulmonary fibrosis induced by asbestos in rat lung.* Top panel: Normal rat lung. Middle panel: Extensive distention of the alveoli (emphysema) in rat lung following inhalation of tobacco smoke (90 mg/m^3 of total suspended particulate material). Bottom panel: Lung of a rat 1 year after exposure to chrysotile asbestos. Note accumulation of connective tissue around blood vessel and airways (fibrosis). Bar length: 100 μm. (Photograph courtesy of Dr Kent E. Pinkerton, University of California, Davis.)

The pathogenesis of emphysema involves a proteinase–antiproteinase imbalance that leads to the remodeling of the supportive connective tissue in the parenchyma and separate lesions that coalesce to destroy lung tissue (Shapiro and Ingenito, 2005). This mechanism was proposed because of the association of serpin peptidase inhibitors, clade A (α-1 antiproteinase, antitrypsin), member 1 (SERPINA1) deficiency with early-onset emphysema (Laurell and Eriksson, 1963) and the ability to induce emphysema in rodents instilled with proteinase, for example, papain (Gross et al., 1965) or neutrophil elastase (ELANE) (Janoff et al., 1977). SERPINA1 inhibits serine proteinases including trypsin and ELANE and protects against cigarette smoke–induced alveolar enlargement in mice (Churg, 2003; Churg et al., 2003). ELANE-deficient mice are partially protected against emphysema (Shapiro et al., 2003) and macrophage matrix metalloproteinase 12 (MMP12 aka macrophage elastase)–deficient mice do not develop cigarette smoke–induced alveolar enlargement (Hautamaki et al., 1997). In addition to MMP12, MMP1 (D'Armiento et al., 1992), MMP2, MMP9 (Senior et al., 1991), proteinase 3 (PRTN3) (Kao et al., 1988), and plasminogen activator tissue (PLAT) (Chapman et al., 1984) can mediate alveolar enlargement. In addition, MMP14 and ADAM metallopeptidase domain 17 (ADAM17 aka TACE) can mediate mucus overproduction. Both conditions have been demonstrated in laboratory animals or human cells in culture. These changes can be accompanied by decreases in antiproteinases, for example, tissue inhibitor of metalloproteinase 3 (TIMP3) decreases when MMP9 increases in mucus overproduction (Deshmukh et al., 2008).

In all individuals, elastolytic events accumulate with time and can cause a decline in lung function that is normally associated with aging. Toxicants that activate macrophages or cause inflammatory cell influx (which increases the burden of ELANE) can accelerate this process. Macrophages also can be activated by endogenous intracellular DAMPs released by activated or necrotic cells and degraded ECM molecules that increase following tissue damage. For example, ECM elastin fragments have been implicated in cigarette smoke–induced emphysema in mice (Houghton et al., 2006).

In addition to macrophage and neutrophil activation, COPD involves cytotoxic T-cell lymphocytes (CTLs) that express a killer cell lectin-like receptor subfamily K, member 1 (KLRK1 aka natural killer cell group 2D) receptor, which recognizes MHC class I polypeptide-related sequence A (MICA) and MICB, and the related UL16-binding proteins (ULBP1, ULBP2, and ULBP3) (Borchers et al., 2009). Respiratory epithelial cells undergoing toxicant stress (e.g., caused by cigarette smoke or infection) express MICA on the cell surface and emphysema results from epithelial expression of the murine homolog (retinoic acid early transcript 1, α). These receptors are recognized by KLRK1 expressing CTLs, which then selectively perform cell cytolysis on the targeted cell and thereby remove damaged or infected cells.

Lung Cancer

At the beginning of the 20th century, lung cancer was a rare disease. Because of cigarette smoking, lung cancer is now the leading cause of death from cancer among men and women in the United States (Siegel et al., 2011) (Fig. 15-9). In addition to inducing COPD, retrospective and, more conclusively, prospective epidemiological studies unequivocally associated tobacco smoking with this epidemic in lung cancer. Currently, ~75% to 85% of lung cancer cases (and many cases of cancer of the bladder, esophagus, oral cavity, and pancreas) are caused by cigarette smoking. The main factor responsible for smoking dependence is nicotine, a ligand for the nicotinic acetylcholine receptor α subunit, which makes up 0.3%

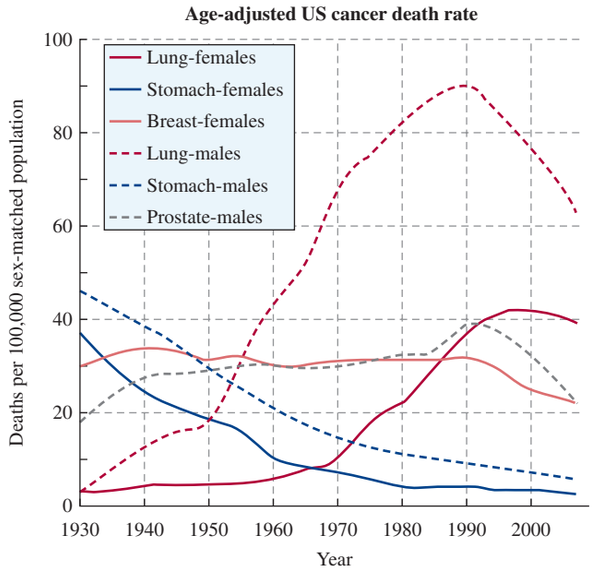

Figure 15-9. *Trends in age-adjusted United States cancer death rates for stomach, lung, breast (females), and prostate (males) cancers.* Solid line: females; dashed line: males. (From US Mortality Volumes 1930 to 1959, US Mortality Data, 1960 to 2007. National Center for Health Statistics, Centers for Disease Control and Prevention.)

to 5% of tobacco plant and accumulates in the tobacco leaf during curing. The increased risk of developing lung cancer for average smokers compared with nonsmokers is 8- to 10-fold and for heavy smokers about 20- to 40-fold (Pope et al., 2011). Stopping smoking reduces the risk of developing lung cancer or COPD (International Agency for Research on Cancer [IARC], 2004). The global burden is ~1.2 million cancer deaths per year, with 85% of lung cancer cases in men and 47% of lung cancer cases in women being attributable to tobacco use.

The development of lung cancer is likely to be a result of multiple genetic and gene–environment interactions because not all smokers develop lung cancer and nonsmokers develop lung cancer (Sun et al., 2007). Single-nucleotide polymorphisms (SNPs) located on chromosomal regions 5p15.33 and 15q25.1 have been consistently identified by genome-wide association studies (GWAS) as significant predictors of lung cancer risk, and a SNP in 6p22.1 has been associated with survival time in small-cell lung cancer (SCLC) patients (Xun et al., 2011). Others have used transcription signatures to predict prognosis and therapeutic options in lung cancer (Chen et al., 2007; Kadara et al., 2011).

Many chemical exposures encountered in industrial settings also pose a lung cancer risk. Arsenic, asbestos, beryllium, cadmium, chromium, and nickel have been associated with cancer of the respiratory tract (IARC, 1993). Workers exposed to chloromethyl ether, mustard gas, or effluent gases from coke ovens have an increased lung cancer risk. First noted among radium miners and then in homes, radon gas is a known human lung carcinogen. Formaldehyde is a probable human respiratory carcinogen. Silica, human-made fibers, and welding fumes are suspected carcinogens. Smokers who inhale radon or asbestos fibers increase their risk for developing lung cancer severalfold, suggesting a synergistic interaction between the carcinogens. For example, working with asbestos increases the lung cancer risk about 2-fold. As noted above, the lung cancer risk for persons who smoke moderately in the general population is 8- to 10-fold. However, asbestos workers who smoke moderately have an associated lung cancer risk of 16-fold.

Lung cancer in never-smokers is the seventh leading cause of cancer death worldwide (Sun et al., 2007). In addition to radon, indoor environmental tobacco smoke increases the risk of developing lung cancer in nonsmokers (IARC, 2004). In addition, biomass cooking, high-temperature oil cooking, and coal heating especially in poor-ventilated households are associated with lung cancer, COPD, and pneumonia, together leading to over 2 million deaths per year worldwide (World Health Organization [WHO], 2006). Ambient $PM_{2.5}$ has been associated with increased lung cancer risk (Beeson et al., 1998; Pope et al., 2002).

Human lung cancers may have a latency period of 20 to 40 years, making the relationship to specific exposures difficult to establish. The two major forms of lung cancer are non–small-cell lung cancer (NSCLC; which accounts for ~85% of all lung cancer cases) and SCLC (which accounts for ~15% of all lung cancer cases) (Herbst et al., 2008). NSCLC can be subdivided into three major histological subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer. Smoking causes all types of lung cancer but is most strongly linked with SCLC and squamous-cell carcinoma. Adenocarcinoma is the most common type in patients who have never smoked (Sun et al., 2007). Large-cell lung cancer is really rare and makes up less than 10% of the NSCLC cases.

Compared with lung cancer, cancer in the upper respiratory tract is less common in humans but can occur frequently in experimental animals exposed to inhaled carcinogens (Harkema et al., 2006). Nonetheless, head and neck cancers are still a major cause of cancer deaths worldwide. Upper respiratory cancers are associated with occupational exposures to chromium, nickel, mustard gas, and isopropyl alcohol. Nasal cancers also are associated with wood dust, textile dust, and possibly leather dust or formaldehyde. Buccal carcinogens are associated with tobacco smoke, smokeless tobacco, betel quid chewing, and alcohol consumption.

Epithelial lung cancers develop in a sequence of distinct morphological changes. Initially, cell numbers in the epithelium lining the airways increase (hyperplasia) and eventually display abnormal nuclei and changes in shape (dysplasia), often assuming squamous-cell characteristics (squamous metaplasia). The lesions then progress to first carcinoma in situ, an accumulation of cancerous cells in small foci, and then into large tumor masses. Eventually tumor cells invade adjacent local tissues, blood vessels, and lymphatics, leading to the formation of distant metastases. The histologically visible sequential development is accompanied by multiple molecular lesions (Wistuba and Gazdar, 2003).

The potential mechanisms of lung carcinogenesis have been studied extensively by means of analysis of tumors and human bronchial cells maintained in culture. A key component is DNA damage induced by reactions with an activated carcinogen or its metabolic product, such as PAHs (e.g., benzo[*a*]pyrene: BaP) or alkyldiazonium ions derived from tobacco specific *N*-nitrosamines (TSNAs). Formed from nicotine in the tobacco curing process, TNSAs include a potent carcinogen, nitrosoaminoketone (NNK) (i.e., 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) (Ter-Minassian et al., 2011). Found in unburned tobacco and tobacco smoke, NNK is metabolized to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide metabolites (NNAL-Gluc). NNK and NNAL form DNA adducts, which are found in lung tumors in mice treated with NNK. Urinary levels of total NNAL (NNAL plus NNAL-Gluc) are associated with lung cancer in a dose-dependent manner in cigarette smokers (Yuan et al., 2009).

Persistence of O^6-alkyl-deoxyguanosine mutations in DNA appears to correlate with carcinogenicity (Hecht, 1999). However, tumors do not always develop when adducts are present, and adduct formation may be a necessary but not sufficient condition

for carcinogenesis. DNA damage caused by reactive oxygen species is another potentially important mechanism. Ionizing radiation leads to the formation of superoxide, which is converted through the action of SOD to hydrogen peroxide. In the presence of Fe and other transition metals, hydroxyl radicals may be formed, which then cause DNA strand breaks. Cigarette smoke contains high quantities of reactive oxygen species (Pryor, 1997) and acrolein (Bein and Leikauf, 2011). Additional oxidative stress may be placed on the lung tissue of smokers by the release of reactive oxygen/nitrogen species formed by activated macrophages and other inflammatory leukocytes. In addition, diminished DNA repair and loss of enzymes that maintain chromosomal integrity (e.g., aberrant telomere homeostasis that evades senescence) are critical events in many cancers, including lung cancer (Buch et al., 2012).

Genomic instability is a hallmark of lung cancer (Sato et al., 2007). Critical genetic and epigenetic changes include DNA mutations, loss of heterozygosity, and promoter methylation and global transcriptome changes that can include stimulation of mitogenic pathways and suppression of apoptosis pathways. Loss of heterozygosity is the loss of normal function of one allele of a gene in which the other allele was already inactivated. In the context of lung cancer, an inactivated allele of a tumor suppressor gene could be inherited from a parent's germline cell that was passed on to the offspring. The offspring would thereby be heterozygous for that allele and subsequent exposure to a mutagen during the offspring's life could then inactivate the functional allele. Genetic and epigenetic changes can persist over years and eventually lead to aberrant cellular function to produce premalignant changes, including dysplasia and clonal patches.

Many molecular changes in earliest-stage cancer also occur in advanced disease (Herbst et al., 2008). Premalignant patches contain clones and subclones, which often involve mutations in tumor protein p53 (TP53 aka p53) and epidermal growth factor receptor (EGFR). Lung cancers unrelated and related to smoking have different molecular profiles, mutation in EGFR being more common in never-smokers, and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) being more common in smokers. Mutations in TP53 are found in both smokers and nonsmokers; however, they are more frequent in never-smokers (Sun et al., 2007). In addition, the TP53 mutational signature (i.e., relative amounts of specific transitions, transversions, or deletions), and spectrum (i.e., specific mutation within the gene) differ between smokers and never-smokers.

In response to diverse cellular stresses, TP53 regulates target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. TP53 is a DNA-binding protein containing transcription activation, DNA-binding, and oligomerization domains. Normally, TP53 can bind to a consensus DNA-binding site and activate expression of downstream genes that inhibit growth and/or invasion, and thus it functions as a tumor suppressor. Found in both NSCLCs and SCLCs, mutant TP53 protein fails to bind these sites, and hence lacks tumor suppressor activity. Acrolein (Tang et al., 2011) and BaP (Denissenko et al., 1996) adducts of TP53 are common in lung cancers.

A receptor tyrosine kinase (RTK), EGFR is a receptor for a number of growth factor ligands (including EGF and transforming growth factor α) and subsequently can stimulate downstream signaling pathways of mitogen-activated protein kinase and phosphatidylinositol-3 kinase. Amplifications of *EGFR* are more common in squamous-cell carcinoma, whereas a kinase domain mutation is more common in adenocarcinoma. The latter occurs more frequently in women, persons of Han decent, and nonsmokers who develop lung cancer. This kinase is critical because it is the target of the RTK inhibitors gefitinib and erlotinib, which have been effective in treatment of this type of lung cancer. Also a member of the EGFR family, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog (avian) (ERBB2 aka HER2) protein lacks a ligand-binding domain and therefore cannot bind growth factors. However, it does bind tightly to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signaling pathways that stimulate cell proliferation. Mutations in this gene are associated with adenocarcinoma.

KRAS is a guanine nucleotide (GDP/GTP)-binding protein that acts as a self-inactivating signal transducer. Normal KRAS can be transiently activated by signals from cell surface receptors that promote the exchange of bound GDP for GTP. The transient nature of this response is ensured by the intrinsic ability of KRAS to hydrolyze GTP, and thereby to switch itself off. Single point mutations produce KRAS proteins with amino acid substitutions that have reduced GTPase activity. Mutant KRAS proteins are thereby locked in a constitutively activated GTP-bound state. KRAS mutations are more common among or are associated with adenocarcinomas among smokers and activated NNK and BaP for KRAS bulky adducts. Mutation or amplification of *TP53*, *EGFR*, *ERBB2*, or *KRAS* thus provides a proliferative advantage to tumor cells.

Other RTKs mutated in lung cancer include Met proto-oncogene (hepatocyte growth factor receptor) (MET) and anaplastic lymphoma RTK (ALK). The ligand for MET is HGF, which despite its name is a growth factor in the lung and important in repair (Ware and Matthay, 2002). ALK belongs to the insulin receptor superfamily. A chromosomal rearrangement of the *ALK* gene that leads to fusion with echinoderm microtubule-associated protein like 4 (*EML4*) can be found in about 7% of adenocarcinomas. Other fusion proteins can be generated with the ALK protein. A specific RTK inhibitor of MET and ALK, Crizotinib, has been effective against NSCLC cell lines that express oncogenic ALK fusion proteins and has been useful in an initial clinical trial for lung cancers of this type (Rodig and Shapiro, 2010).

Serine/threonine kinase 11 (STK11 aka liver kinase B1 homolog or LKB1) regulates cell polarity and other cellular functions. Normally, STK11 protein activates AMP-activated protein kinase (AMPK), which leads to growth suppression. However, mutant STK11 proteins lack the ability to phosphorylate AMPK and to interact with TP53, which diminishes transcription of target genes. Mutations in *STK11* are associated with smoking and are common in adenocarcinoma and squamous-cell carcinoma in persons of European decent, whereas it is uncommon in adenocarcinoma in persons of Hun decent. *STK11* mutations often are present in tumors with *KRAS* mutations, but are not found in tumors with *EGFR* mutations.

Other mutations common in lung cancer occur in NK2 homeobox 1 (NKX2-1 aka thyroid transcription factor 1: TITF1), and phosphoinositide-3-kinase, catalytic, α polypeptide (PIK3CA). Cancer stem cells (CSCs) are a subset of cancer cells with the ability to initiate cancer when injected into mice (Martelli et al., 2011). CSCs are thought to be responsible for tumor onset, self-renewal/maintenance, mutation accumulation, and metastasis. The existence of CSCs could explain the high frequency of neoplasia relapse and resistance to therapies. Critical for alveolar type II cell development, NKX2-1 may have a role in modulating stem cell functions and *NKX2-1* amplification (with increased copy number) is common in adenocarcinoma. PIK3CA is the catalytic subunit of PI-3-kinase (PI3K), which is a member of the v-akt murine thymoma viral oncogene homolog 1 (AKT1) and mechanistic target of rapamycin (serine/threonine kinase) (MTOR) survival pathway. The PI3K–AKT1–MTOR pathway is downstream of EGFR and

mutation in this pathway protects against apoptosis in tumor cells. Pharmaceuticals that inhibit PI3K/AKT1/MTOR can induce apoptosis in tumors and is a new target for eliminating CSCs.

Another common alteration is increased v-myc myelocytomatosis viral oncogene homolog (avian) (MYC aka c-Myc). MYC is amplified at a higher frequency in SCLC cell lines than tumors, suggesting that MYC is more related to tumor progression and a relatively late event in lung cancer development. A transcription factor, MYC may contribute to the production of multiple growth factors that can promote and inhibit the proliferation via paracrine and autocrine loops via specific receptors.

In toxicological testing, lung tumors or cancers in laboratory animals do not always mimic those in humans. Most lung tumors in mice are peripherally located adenomas and adenocarcinomas, even after tobacco or NNK exposure. Rarely do mice or rats develop bronchial squamous-cell carcinoma found in humans that smoke. Rats on occasion develop lung tumors that are characterized by an epithelium surrounding a space filled with keratin. The mass may compress the adjacent lung parenchyma and occasionally invade it. These lesions are classified by some pathologists as bona fide tumors, whereas other pathologists characterize this type of lesion as a cyst filled with keratin. Classification of such a lesion is important because these lesions often are found in long-term tests in animals that have been exposed to chemicals that are not considered carcinogens, such as carbon black, titanium dioxide, and certain human-made fibers (ILSI, 2000). In other animal tests, a large particle number or mass is instilled into the trachea as a single exposure, which is used to mimic a daily or a lifetime exposure burden. These instillations may not be evenly distributed in the lung and histological examination often demonstrates focal accumulations of a large number of particles almost never observed in humans even after a lifetime of exposure. A unifying hypothesis postulates that because clearance mechanisms in the deep lung depend predominantly on phagocytosis and migration of pulmonary alveolar macrophages, such large doses overwhelm clearance mechanisms and elicit irresolvable inflammation (called particle overload) (Morrow, 1992). As a consequence, lung burdens of these dusts persist for months or years, and may evoke unique mechanisms of disease pathogenesis not applicable to human experience with inhaled chemicals or particles. The issue of whether particle overload is useful in defining a toxicity threshold in such experiments remains unresolved.

Pulmonary Fibrosis

The pathological hallmark of pulmonary fibrosis is increased focal staining of collagen fibers in the alveolar interstitium. Excess lung collagen is usually observed not only in the alveolar interstitium but also throughout the centriacinar region, including the alveolar ducts and respiratory bronchioles (Fig. 15-8). The foci can coalesce to form large masses of noncompliant lung. These foci can contain the inducing material including asbestos, silica, or synthetic vitreous fibers aka man-made mineral fiber (MMMF). The pleural surface of the lung may also become fibrotic and together with parenchymal stiffening prevent full lung inflation. Ultimately the lung is unable to inflate or deflate properly and thus is restricted. Restrictive lung disease can be detected by a decrease in the predicted FVC, with or without a change in the FEV1.

The pathogenesis of pulmonary fibrosis involves epithelial cell injury and macrophage activation produced by a wide range of toxic insults (Wynn, 2011). For example, macrophages can be activated by phagocytosis of crystalline silica or crocidolite asbestos, which activate inflammasome receptor-mediated TNF and IL1B formation. Epithelial cells and macrophages also release chemokines that recruit and activate other inflammatory cells including neutrophils and T cells. These cells combine to produce excessive TGFB1, TNF, IL1B, IL13, and IL17. Of these, TNF and TGFB1 are major mediators in pulmonary fibrosis because fibrosis can be diminished experimentally by inhibition of TNF (Piguet et al., 1990) or TGFB1 (Sheppard, 2004; Gharaee-Kermani et al., 2009) activity. Importantly, TGFB1 and other TGFB family members (TGFB2 and TGFB3) are secreted in inactive complexes with a latency-associated peptide (LAP), a protein derived from the N-terminal of the region of the gene product. LAP is a ligand for the integrins, heterodimeric proteins consisting of α and β subunits that bind matrix molecules or cellular counterreceptors. In the lung, integrin α v β 6 (through cell–cell contact) and integrin α v β 8 (through a proteolytic process) can activate extracellular latent TGFB1 and TGFB3.

Repeated injury and the inability to resolve macrophage activation lead to areas of focal hypoxia. The consequence of the combined TGFB1/3, cytokine release, and hypoxia leads to epithelial-to-mesenchymal transition in which lung epithelial cells transdifferentiate into fibroblast-like cells (Chapman, 2011). These cells contribute to a larger population of myofibroblasts that arise from local mesenchymal cells, and bone marrow–derived fibrocytes. The migration, proliferation, and activation of myofibroblasts contribute to excessive extracellular matrix deposition that has altered collagen cross-linking. A feature of fibrogenesis is the oxidation of lysines in the extracellular matrix (ECM), mainly in collagen, to the aldehyde, allysine by lysyl-oxidase (LOX) enzymes. Allysine undergoes a series of condensation reactions with other amino acids on neighboring collagen molecules to form irreversible cross-links that stabilize the ECM. The consequence of this process is disorderly repair and substained fibrogenesis, which leads to progressive stiffness of the fibrotic lung.

AGENTS KNOWN TO PRODUCE RESPIRATORY DISEASE IN HUMANS

Currently, the American Chemical Society (ACS) lists over 50 million chemicals with a unique Chemical Abstracts Service (CAS) Registry Number and over 280,000 are listed as inventoried/regulated chemicals by CHEMLIST, but only ~7900 compounds are listed as commonly used by industry (ACS, 2011). Many of these compounds represent hazards to the respiratory tract. For example, the American Conference of Governmental Industrial Hygienists (ACGIH) lists over 700 TLVs for atmospheric exposures (ACGIH, 2011). TLVs refer to occupational airborne concentrations of substances "that nearly all workers may be repeatedly exposed day after day without adverse health effects." These values and other exposure limits have been developed because prevention of exposure is one of the most effective approaches to prevent lung injury and disease. Nonetheless, given the large morbidity and mortality associated with current acute and chronic lung disease, a great need exists to develop additional preventative and therapeutic strategies based on the knowledge of the cellular and molecular events that determine lung injury and repair. Table 15-7 lists a portion of the respiratory toxicants that can produce acute and chronic lung injury in humans. In the following sections, a few examples of our current understanding of lung injury at the mechanistic level are discussed, with emphasis on agents directly responsible for human lung disease.

Inhalation Hazards

Acrolein As an α–β-unsaturated aldehyde, acrolein (2-propenal) is volatile at room temperature and is highly irritating to the upper and lower respiratory tract (Bein and Leikauf, 2011). Acrolein can

Table 15-7

Agents That Produce Lung Injury and Disease

TOXICANT	DISEASE	EXPOSURE	ACUTE EFFECTS	CHRONIC EFFECTS
Acrolein	Acute lung injury, chronic obstructive pulmonary disease	Biomass or hot oil cooking, firefighters, environmental tobacco smoke, biocide water treatment	Cough, shortness of breath, extreme oronasal irritation, pulmonary edema, airway hyperreactivity	Chronic obstructive pulmonary disease, possibly asthma or lung cancer
Aluminum abrasives	Shaver's disease, corundum smelter's lung, bauxite lung	Abrasives manufacturing, smelting	Alveolar edema	Interstitial fibrosis, emphysema
Aluminum dust	Aluminosis	Aluminum, firework, ceramic, paint, electrical good, and abrasive manufacturing	Cough, shortness of breath	Interstitial fibrosis
Ammonia		Farming, refrigeration operations, ammonia, fertilizer, chemical, and explosive manufacturing	Oronasal and bronchial irritation, pulmonary edema	Acute lung injury, chronic bronchitis
Arsenic		Pesticide, pigment, glass, and alloy manufacturing	Bronchitis	Laryngitis, bronchitis, and lung cancer
Asbestos	Asbestosis	Mining, construction, shipbuilding, brake repair, vermiculite contaminant		Fibrosis, pleural calcification, lung cancer, mesothelioma
Aspergillus (Mold)	Framer's lung, compost lung, malt worker's lung, aspergillosis	Working with moldy hay, compost, or barley	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Avian protein	Bird fancier's lung	Bird handling and farming with exposure to bird droppings	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Beryllium	Berylliosis	Mining, alloy, and ceramic manufacturing, milling beryllium	Pulmonary edema, pneumonia	Interstitial granulomatosis, progressive dyspnea, cor pulmonarle, fibrosis, and lung cancer
Cadmium		Welding, smelting, and electrical equipment, battery, alloy, and pigment manufacturing	Cough, pneumonia	Emphysema, cor pulmonale
Carbides of tungsten, titanium, or tantalum	Hard metal disease	Metal cutting and manufacturing	Bronchial epithelial hyper- and metaplasia	Peribronchial and perivascular fibrosis
Chlorine		Paper, plastics, chlorinated product manufacturing	Cough, hemoptysis, dyspnea, bronchitis, pneumonia	
Chromium (VI)		Chromium compound, paint, pigment, chromite ore reduction manufacturing	Oronasal and bronchial irritation	Fibrosis, lung cancer
Coal dust	Coal worker's pneumoconiosis	Coal mining		Fibrosis with emphysema
Cotton dust	Byssinosis	Textile manufacturing	Chest tightness, wheezing, dyspnea	Restrictive lung disease, chronic bronchitis
Diacetyl	Bronchiolitis obliterans ("Popcorn lung")	Manufacture of microwave popcorn, cookies, potato chip flavoring, and coffee roasting	Chest tightness, wheezing, dyspnea	Restrictive lung diseases with occlusion of bronchioles
Hydrogen fluoride		Chemical, photograph film, solvent and plastic manufacturing	Airway irritation, hemorrhagic pulmonary edema	

Table 15-7

Agents That Produce Lung Injury and Disease

TOXICANT	DISEASE	EXPOSURE	ACUTE EFFECTS	CHRONIC EFFECTS
Iron oxides	Siderotic lung disease, silver finisher's lung, hematite miner's lung, arc welder's lung	Welding, steel and jewelry manufacturing, foundry work, hematite mining,	Cough	Silver finisher's lung with subpleural and perivascular macrophage aggregates; hematite miner's lung with diffuse fibrosis-like pneumoconiosis; arc welder's lung with bronchitis
Isocyanates		Auto painting, and plastic and chemical manufacturing	Airway irritation, cough, dyspnea	Asthma
Kaolin	Kaolinosis	Pottery making		Fibrosis
Manganese	Manganese pneumonia	Chemical and metal manufacturing	Acute pneumonia (often fatal)	Recurrent pneumonia
Nickel		Nickel mining, smelting, electroplating, battery manufacturing, fossil fuel combustion	Delayed pulmonary edema, skin allergy	Acute lung injury, chronic bronchitis, non-small cell lung cancer, nasal cancer
Nitrogen oxides	Silo-filler's diseases	Silo filling, welding, explosive manufacturing	Immediate or delayed pulmonary edema	Bronchiolitis obliterans, emphysema in experimental animals
Nontuberculous mycobacteria.	Metalworking fluid hypersensitivity	Working with metal cutting fluid containing water and contaminated with mycobacteria	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Organic (sugar cane) dust (possibly contaminated with thermophilic *actinomycete*)	Bagassosis	Sugar cane and molasses manufacturing (bagasse is the fibrosis residue from sugar extraction)	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Ozone		Welding, photocopying, bleaching flour, water treatment, deodorizing	Substernal pain, exacerbation of asthma, bronchitis, pulmonary edema	Fibrosis (including airways)
Perchloroethylene (tetrachloroethene)		Dry cleaning, metal degreasing, grain fumigation	Edema	Hepatic and lung cancer
Phosgene		Plastic, pesticide, and chemical manufacturing	Severe pulmonary edema	Bronchitis and fibrosis
Silica	Silicosis, pneumoconiosis	Mining, stone cutting, sand blasting, farming, quarry mining, tunneling	Acute silicosis (inflammation)	Fibrosis, silicotuberculosis
Sulfur dioxide		Chemical manufacturing, refrigeration, bleaching, fumigation	Bronchoconstriction, cough, chest tightness	Chronic bronchitis
Talc	Talcosis	Mining, rubber manufacturing, cosmetics	Cough	Fibrosis
Thermophilic *actinomycete*	Farmer's lung, mushroom worker's lung, penguin humidifier lung	Farming (hay or grain degradation)	Bronchoconstriction, cough, chest tightness	Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
Tin		Mining, tin processing		Widespread mottling in chest x-ray often without clinical impairment
Vanadium		Metal cutting and manufacturing, specialty steel manufacturing	Airway irritation and mucus production	Chronic bronchitis

be formed by heating cooking oils and fats above 300°C (e.g., wok cooking), and thus its name refers to the pungent "acrid" (from Latin stem: acer meaning sharp or sour) smell that is produced from "oleum" (Latin meaning "oil"). Acrolein also can be formed in cigarette smoke, environmental tobacco smoke, domestic cooking with biomass fuels (WHO, 2006), smoke from fires, and automotive and diesel exhaust. Additional human exposure can result from acrolein use as a biocide or chemical feedstock.

Acrolein contains a reactive carbonyl group and an electrophilic α-carbon and thus is highly reactive (often forming cross-links) with biological macromolecules. Irritant stimuli, of which acrolein is one of the most potent, activate respiratory sensory nerve endings, especially TRPA1 (Bautista et al., 2006; Andre et al., 2008). More plentiful in cigarette smoke than PAHs, acrolein can adduct tumor suppressor p53 (TP53) DNA (Tang et al., 2011). Acrolein is also generated endogenously at sites of lung injury, and excessive breath levels (sufficient to activate metalloproteinases and increase mucin transcripts) (Deshmukh et al., 2009) have been detected in persons with COPD or asthma (Deshmukh et al., 2008). Because of its high reactivity, acrolein alters gene regulation, increases inflammation, decreases mucociliary transport, and diminishes alveolar-capillary barrier integrity (Jang et al., 2011). In laboratory animals, high acrolein exposures have led to acute lung injury and pulmonary edema (Leikauf et al., 2011) similar to that produced by smoke inhalation, whereas lower concentrations have produced bronchial hyperreactivity (Leikauf et al., 1989), excessive mucus production (Deshmukh et al., 2005), and alveolar enlargement (Borchers et al., 2007). Susceptibility to acrolein exposure is associated with differential regulation of cell surface receptor, transcription factor, and ubiquitin–proteasome genes (Fabisiak et al., 2011; Leikauf et al., 2011). Thus, acrolein contributes to the morbidity and mortality associated with acute lung injury and COPD, and possibly asthma and lung cancer. Although irritant-induced sensory responses can serve as warning signs and promote avoidance behavior or protective measures, acrolein exposure remains a global health problem because its release is closely linked to basic human needs (e.g., cooking), emergencies (e.g., firefighting), or personal habits (e.g., smoking). In addition, acrolein is present in vapor generated by e-cigarettes (Sleiman et al., 2016). Of note, these systems are designed to create small diameter aerosols that increase the deposition of particles and gasses in the distal airways and alveolus.

Asbestos Asbestos refers to a group of silicate minerals in fiber form. The most commonly mined and commercially used asbestos fibers include the serpentine chrysotile asbestos and the amphiboles crocidolite, anthophyllite, amosite, actinolite, and tremolite asbestos. Exposure to asbestos fibers occurs in mining operations and in the construction and shipbuilding industries, where asbestos was at one time widely used for its insulating and fireproofing properties. Concern about asbestos in older buildings has led to the removal of asbestos-based insulating material; abatement workers may now represent an additional population at risk.

Asbestos causes three forms of lung disease: asbestosis, lung cancer, and malignant mesothelioma (Mossman et al., 2011). Asbestosis is a form of pulmonary fibrosis with characteristically diffuse collagen foci and the presence of asbestos fibers, either free or coated with a proteinaceous material (asbestos bodies). Alveolar macrophage clearance is critical to the prevention of asbestosis and depends on fiber length, biopersistance, and dose (Churg, 2003; Churg et al., 2003). Long fibers cannot be fully engulfed by a macrophage and cause "frustrated phagocytosis." High fiber concentrations (dose) can produce particle overload, thereby augmenting inflammation, and lead to failure of the macrophages to clear particles. Asbestosis can be detected by characteristic nodular localized lesions of macrophages and asbestos fibers. Serpentine asbestos fibers tend to be cleared from the lung more rapidly and to be more "soluble" in tissue and thus are less biopersistant than amphibole fibers.

Disintegration refers to a number of processes, including the subdivision of the fibers into shorter segments; partial dissolution of components of the matrix, creating a more porous fiber of relatively unchanged external size; or surface etching of the fibers, creating a change in the external dimensions of the fibers and/or complete dissolution. For synthetic vitreous fibers (SVF or MMMF), fiber breakup is virtually determined by length. The breakdown into smaller diameter fibrils that is characteristic of asbestos fibers is seldom seen. For SVF, the relative importance of breakage into length segments, partial dissolution, and surface etching to the clearance of fibers depends upon the size and composition of the fiber. Asbestosis can produce characteristic nodular localized lesions of macrophages and asbestos fibers. Lung cancer develops in workers in the asbestos mining industry and smoking of cigarettes greatly enhances the risk. Malignant mesothelioma is a rare form of cancer that develops mainly in the pleural mesothelium, the protective lining that covers the lungs, diaphragm, and interior of the chest wall. Unlike lung cancer, mesothelioma is not associated with smoking history.

Observations in humans sometimes differ from those in laboratory animals. In animal experiments, chrysotile produces mesothelioma much more readily than do the amphibole fibers. In humans, amphibole fibers are implicated more often even when the predominant exposure is to chrysotile asbestos. Chrysotile breaks down much more readily than do the amphiboles. One possibility is that in laboratory animals, the chrysotile fibers, even if broken down, are retained longer relative to the life span of the animal than they are in humans, thus explaining the higher rate of mesothelioma developed.

Health hazards associated with asbestos exposure depend on fiber shape, length, and surface properties (Lippmann, 1994; Oberdörster et al., 2005; Donaldson et al., 2010). Chrysolite asbestos are curved fibers and tend to be less toxic than crocidolite fibers that are long and thin, straight fibers. Fibers 2 μm in length may produce asbestosis; mesothelioma is associated with fibers ≥5-μm long, and lung cancer with fibers ≥10 μm. Fiber diameter is another critical feature. Fibers with diameters larger than ~3 μm do not readily penetrate into the peripheral lung. Only fibers ≥0.15 μm in diameter are likely to produce asbestosis or lung cancer. Mesothelioma, however, is more often associated with fiber diameter <0.5 μm because thinner fibers may be translocated from their site of deposition via the lymphatics to other organs, including the pleural surface (Oberdörster et al., 2005). The surface properties of asbestos fibers also contribute to toxicity (Upadhyay and Kamp, 2003; Mossman et al., 2011). Crocidolite contains more iron (including ferrous iron [Fe^{2+}] that may participate in Fenton chemistry to generate reactive oxygen species) than chrysolite. The interaction of iron on the surface of asbestos fibers with oxygen may lead to the production of hydrogen peroxide and the highly reactive hydroxyl radical—events that have been associated with asbestos toxicity (Upadhyay and Kamp, 2003). The protection afforded by SOD or free radical scavengers in asbestos-related cell injury in vitro supports a role of reactive oxygen species and concomitant oxidized lipids as a mechanism of asbestos toxicity.

Once asbestos fibers have been deposited in the lung, they may become phagocytized by alveolar macrophages (Churg, 2003; Churg et al., 2003). Short fibers are completely ingested and

subsequently removed via the mucociliary escalator. Longer fibers are incompletely ingested, and the macrophages become unable to leave the alveoli. Activated by the fibers, macrophages release mediators such as cytokine, chemokines, and growth factors, which in turn attract immunocompetent cells or stimulate collagen production. Asbestos-related lung disease thus may be mediated through the triggering of epithelial injury and macrophage activation, and inflammatory events or through the production of changes that eventually lead to the initiation (DNA damage caused by reactive molecular species) or promotion (increased rate of cell turnover in the lung) of the carcinogenic process (Mossman et al., 2011).

Coal Dust Coal worker's pneumoconiosis (CWP aka Black Lung Disease) results from the accumulation of coal dust in the lungs and can be simple coal worker's pneumoconiosis (SCWP) and complicated coal worker's pneumoconiosis (CCWP) also called progressive massive fibrosis (PMF) (Wade et al., 2011). CWP is accompanied by anthracosis, which is the asymptomatic accumulation of carbon without a consequent cellular reaction. In varying degrees, anthracosis is common even among most urban dwellers and in tobacco smokers, but is more severe in CWP. Normally, inhaled coal dust reaches the terminal bronchioles, and the carbon is engulfed by alveolar and interstitial macrophages. Phagocytosed coal particles are transported by macrophages up the mucociliary elevator and are expelled in the mucus or through the lymphatic system. However, when clearance mechanisms are overwhelmed, the dust-laden macrophages accumulate in the alveoli and may trigger an immune response.

Histological hallmarks of CWP are areas of focal deposition of coal dust and pigment-laden macrophages, which are known as coal macules. When exposure is extended over a significant amount of time to dust particles 2 to 5 μm in diameter, activated fibroblasts secrete reticulin, which entraps the macrophages. If the dust-laden macrophages lyse, the fibroblastic response is augmented and more collagen is laid down in the area. The lymphatic tree is contained in the pulmonary interstitium, along with arterial and venous vessels. Macrophages can migrate into the lymphatic vessels. Arterioles can become surrounded by enlarging collagen deposits, and blood flow impaired from the resultant interstitial fibrosis. In PMF, this involves the accumulation of necrotic macrophages, fibroblasts, reticulin, and collagen along the vascular tree. As the blood vessels become compromised, a progressive ischemic necrosis ensues. The lesion enlarges and appears as focal macules. The focal macules can extend, and join other macules in the vicinity, forming discrete areas of interstitial fibrosis. This process can be exacerbated by tuberculosis or rheumatoid factor, which accelerates the rate of progression of focal ischemic necrosis and fibrosis. Coal mining often involves simultaneous silicon dioxide exposure due to silica-containing rock grinding. Silica is cytotoxic and produces macrophage lysis, which stimulates the fibroblasts to add more collagen to the network and enlarge the areas of pulmonary fibrosis. This growing collagen network causes distention of the respiratory bronchioles, forming focal areas of emphysema. Coal dust exposure, cigarette smoking, age, and race are significant and additive predictors of the severity of emphysema among coal miners (Kuempel, et al., 2009).

Diacetyl A diketone flavoring agent, diacetyl is commonly used to give a buttery taste to foods. Industrial exposure to diacetyl has been associated with "popcorn lung," which is a form of bronchiolitis obliterans (Kreiss et al., 2002). This condition most commonly has been reported in microwave popcorn production, but it has occurred elsewhere. In the manufacture of microwave popcorn, cumulative diacetyl exposure >0.8 ppm-year was associated with increased risk of airflow obstruction (Lockey et al., 2009). The latency period between time of first exposure and onset of symptoms is variable but is usually less than 10 years and may be as short as a few months. Additional occupational diacetyl exposures that have led to airflow obstruction occurred in cookie production (Cavalcanti et al., 2012), potato chip flavoring manufacturing (Hendrick, 2008), and coffee roasting (Bailey et al., 2015). While cases of fixed airways obstruction from flavored e-cigarettes have yet to be reported, concern has been raised about the growth in marketing and use of electronic cigarettes (Sleiman et al., 2016; Syamlal et al., 2016). Diacetyl ($C_4H_6O_2$) and the related acetyl propionyl ($C_5H_6O_2$) have been found in a large proportion of sweet-flavored electric cigarette liquids, with many of them exposing users to higher than safety levels (Farsalinos et al., 2015; Hubbs et al., 2015). Of note, diacetyl-induced pathologies somewhat consistent with bronchiolitis obliterans can be observed in rats (Palmer et al., 2011), but perhaps not in mice (Morgan et al., 2008).

The mechanism of how diacetyl produces an effect localized to the bronchioles is yet to be determined. A direct effect on the respiratory epithelium with the disorganized fibrotic repair appears most likely as the underlying mechanism. Typical chest high-resolution computerized tomographic (HRCT) scan findings include mosaic attenuation and patchy air trapping that is accentuated on expiration, bronchial wall thickening, cylindrical bronchiectasis, and upper lobe sub-pleural centrilobular nodularity, sometimes with a tree-in-bud pattern that suggests an active inflammatory process (Van Rooy et al., 2007).

Naphthalene Naphthalene occurs in cigarette smoke, tars, petroleum and is a precursor in the chemical synthesis of tanning agents, phthalic acid anhydride, carbaryl, and 2-naphthol. In laboratory animals, inhaled or parenterally administered naphthalene produces extensive necrosis in the bronchiolar epithelium of the mouse but much less necrosis in the airways of rats, hamsters, or monkeys (Buckpitt et al., 2002). The primary target in the surface epithelium is the bronchiolar secretoglobin cells. These cells were thought to be very important because they contain a stem cell subpopulation. They were once thought to be capable of cellular maintenance of the epithelium in bronchi, bronchioles, and alveolar regions. However, more recent evidence indicates that these cells are only involved in the long-term maintenance and repair of airways but not alveolar epithelium (Rawlins et al., 2009). In mice, naphthalene is metabolized to naphthalene oxide primarily through CYP2F enzymes, especially CYP2F2 (Shultz et al., 1999; Baldwin et al., 2005). The toxic metabolite thus can be used to ablate bronchiolar secretoglobin cells (Reynolds et al., 2000).

In rats and other species, including monkeys, metabolism of naphthalene is less stereospecific and the rates of formation of the epoxide are much slower than in mice (Buckpitt et al., 2002). Naphthalene epoxides may subsequently be conjugated with glutathione and form adducts that are eliminated as mercapturic acids. The epoxides can undergo rearrangement to 1-naphthol with subsequent metabolism to quinones, which are potentially toxic compounds. Naphthalene metabolites bind covalently to cellular proteins that are important in normal cellular homeostasis and protein folding, and this may be related to the mechanism of toxicity by this chemical. Interestingly, in both mice and rhesus monkeys the total amount of adducted protein is similar (Lin et al., 2006).

Silica Inhaled particles of silicon dioxide (silica) cause a characteristic human lung disease—silicosis. Crystalline silica is a major

component of the earth's crust; silicon is only second to oxygen as the most common element. Mineral forms of silicon exist primarily as crystalline SiO_2 with a central silicon atom forming a tetrahedron with four shared oxygen atoms. The three principal crystalline isomeric forms are quartz, tridymite, and cristobalite. The tetrahedral structure is biopersistent and linked to fibrogenic potential. Stishovite, a rare crystalline variant without the tetrahedral conformation, is biologically inert. Amorphous forms of silica (e.g., kieselguhr and vitreous silica) also have low fibrogenic potential. The ubiquitous presence of silica has made it an occupational hazard ever since tools were cut from stone, and silicosis remains a significant industrial hazard throughout the world in occupations such as mining and quarrying, sandblasting, and foundry work.

In addition to its structure, particle size, concentration, and surface properties affect the pathogenicity of silica both in vivo and in vitro. In humans, the most fibrogenic particle size appears to be about 1 μm (range 0.5 to 3 μm). In animal experiments (rats, hamsters), the comparable values appear to be 1 to 2 μm (range 0.5 to 5 μm). In laboratory animals, the concentration of silica dust is directly related to the intensity and rapidity of the histological reaction in the lung. When compared with stored silica or coated silica, freshly fractured silica particles produce more free radicals from their surface, increasing the respiratory burst when phagocytized, and more pulmonary inflammation (Vallyathan et al., 1995).

Silicosis may be acute or chronic with distinct pathological consequences. Acute silicosis occurs only in subjects exposed to a very high levels of silica (most often quartz or sand), small enough to be respirable (usually <5 μm) over a relatively short period, generally a few months or years. These patients have worsening dyspnea, fever, cough, and weight loss that can rapidly progress to respiratory failure, usually ending in death within two years. No known therapeutic strategy controls the relentless course of acute silicosis.

Chronic silicosis has a long latency period, usually >10 years and can be divided into simple and complicated silicosis. Even after radiographic changes, simple silicosis may be asymptomatic (i.e., no dyspnea) with little change in pulmonary function. The x-ray presents fibrotic nodules, generally in the apical portion of lung. The hilar lymph nodes have peripheral calcifications known as eggshell calcifications. Simple silicosis may progress into complicated silicosis, which is defined as the presence of conglomerate nodules larger than 1 cm in diameter. These nodules usually occur in the upper and mid-lung zones. In advanced stages, the nodules may be surrounded by emphysematous bullae. Chronic silicosis is associated with an increased incidence of tuberculosis.

The pathophysiological basis of pulmonary fibrosis in chronic silicosis is probably better understood than is the etiology of any other form of pulmonary fibrosis (Huaux, 2007). The role of pulmonary alveolar macrophages in the ingestion of silica is an initiating event. Macrophages phagocytose silica particles into phagosomes that fuse with endosomes during the internalization process (Costantini et al., 2011). Similar to the fate of microorganisms, macrophages experience phagosomal destabilization (Hornung et al., 2008) and activate pathways including the NALP3 inflammasome (Cassel et al., 2008; Dostert et al., 2008), pattern-recognition receptors, and antiviral pathways to release inflammatory cytokines (Giordano et al., 2010). In contrast to microorganisms, silica particles cannot be degraded and macrophages undergo cell death, releasing these particles that are engulfed by other macrophages, thus perpetuating the process of phagocytosis and cell death (Huaux, 2007). This leads to elevated mediator release (TGFB1, TNF, etc.) (Di Giuseppe et al., 2009), epithelial-mesenchymal transition (Chapman, 2011), and initiates and maintains myofibroblast collagen (Munger and Sheppard, 2011).

Blood-Borne Agents That Cause Pulmonary Toxicity in Humans

A number of compounds administered systematically can enter the lung through the pulmonary circulation and cause lung injury and disease. For example, intraparenteral naphthalene has marked lung toxicity in the mouse, probably due to an enrichment of an activating enzyme (CYP2F) in a specific cell in the lung epithelium (Buckpitt et al., 2002). Another example is that the ingestion of arsenic has been associated with lung cancer (Putila and Guo, 2011). Below are three examples that have had toxicological significance in clinical settings.

1,3 Bis (2-Chloroethyl)-1-Nitrosourea (BCNU) A chemotherapeutic drug that has pulmonary fibrosis as a complication is carmustine (BCNU). BCNU exerts its antitumor properties by reacting with cellular macromolecules and forms inter- and intra-strand cross-links with DNA. In humans, a dose-related pulmonary toxicity is often noticed first by a decrease in diffusion capacity, which can develop into fatal pulmonary fibrosis (Weiss et al., 1981). The mechanism of action is not entirely clear (Wu et al., 2001). It is possible that BCNU inhibits pulmonary glutathione disulfide reductase, an event that may lead to a disturbed GSH/GSSG state in pulmonary cells. Eventually, this state leaves the cell unable to cope with oxidant stress. High concentrations of oxygen in the inspired air may enhance the pulmonary toxicity of BCNU and also that of the other anticancer drugs known to affect lung tissue: bleomycin and cyclophosphamide.

Bleomycin Bleomycin is a cancer chemotherapeutic drug with a major complication—pulmonary fibrosis that can be fatal (Jules-Elysee and White, 1990). Bleomycin produces a sequence of injury and necrosis to capillary endothelial cells and alveolar type I cells. This, in turn, leads to edema formation and hemorrhage, delayed (after 1 to 2 weeks) proliferation, and apoptosis of alveolar type II cells. Eventually the alveolar walls are thickened by fibrotic changes. In many tissues, the cytosolic enzyme bleomycin hydrolase inactivates bleomycin (Schwartz et al., 1999). In lung and skin, two target organs for bleomycin toxicity, the activity of this enzyme is low compared with that in other organs. Bleomycin stimulates the production of collagen in the lung. Prior to increased collagen biosynthesis, steady-state levels of mRNA coding for fibronectin and procollagens are increased, subsequent to a bleomycin-mediated release of cytokines such as TGFB1 and TNF (Ortiz et al., 1999). In mice, bleomycin-induced TGFB1 is critical because mice deficient in its receptor TGFBR2 in alveolar epithelial cells have attenuated fibrosis and increased survival following bleomycin (Degryse et al., 2011). Bleomycin also can combine with Fe(II) to create reactive oxygen species that can lead to DNA single- and double-strand breaks. Animal models of bleomycin-induced pulmonary fibrosis have been used to study the efficacy of promising antifibrotic drugs (Giri, 2003) and stem cell therapy (Ortiz et al., 2003).

Cyclophosphamide A number of chemotherapeutic drugs can produce lung damage and pulmonary toxicity in patients treated with these drugs (Meadors et al., 2006). Cyclophosphamide is widely used as an anticancer and immunosuppressive drug. The undesirable side effects include hemorrhagic cystitis and pulmonary fibrosis (Fraiser et al., 1991). Cyclophosphamide is metabolized by cytochrome P450 to two highly reactive metabolites: acrolein and phosphoramide mustard. In the lung, cooxidation with the PGH synthase system, which has high activity in the lung, is a possibility. Although the exact mechanism of action for causing lung damage has not been established, studies with isolated lung microsomes have shown that cyclophosphamide and its metabolite acrolein initiate lipid peroxidation (Patel and Block, 1985).

EVALUATION OF TOXICANT-INDUCED LUNG DAMAGE

Humans Studies

Although the lung is susceptible to multiple toxic injuries, it is also amenable to a number of tests that allow evaluation of proper functioning (Utell and Frampton, 2000; Rennard and Spurzen, 2006; Huang and Ghio, 2009). Commonly used tests include measurement of FEV1, FVC, and airway resistance. Additional tests evaluate the maximal flow rates and different lung volumes, diffusion capacity, oxygen, and carbon dioxide content of the arterial and venous blood, distribution of ventilation, and lung and chest wall compliance. Many pulmonary function tests (e.g., FEV1 and FVC) require active collaboration by the subject examined. This noninvasive test is easy to administer to adults (ATS, 1991) and children (Beydon et al., 2007), and does not require sophisticated equipment or a hospital setting. The subject is asked first to inhale deeply and then to exhale the air as quickly as possible. The test is repeated typically three times and the result of the best effort is recorded. The test is often used in epidemiological studies or controlled clinical studies designed to assess the potential adverse effects of air pollutants. A reduction in FEV1 is usually indicative of impaired ventilation such as that found in restrictive (increased lung stiffness) or obstructive (obstructed airflow) lung disease.

To accomplish proper oxygenation of venous blood and elimination of CO_2, the gases have to diffuse across the air–blood barrier (Macintyre et al., 2005). The structural properties that control gas exchange include the lung gas volume, the path length for diffusion in the gas phase, the thickness and area of the alveolar capillary membrane, and the volume of blood in capillaries supplying ventilated alveoli. The functional properties that control gas exchange include the uniformity of ventilation and perfusion with respect to each other, the diffusion characteristics of the alveolar membrane, the binding properties of hemoglobin (Hb) in the alveolar capillaries, and the gas tensions in blood in the pulmonary vascular bed that exchanges gas with the alveoli. Gas exchange may be hindered by the accumulation of fluids or cellular elements in the alveoli (edema, pneumonic infiltrates), thickening of the alveolar wall (fibrosis), insufficient ventilation of the alveolar region (emphysema), ventilation–perfusion mismatching, or insufficient presence of oxygen transport elements (reduced alveolar blood volume or reduced amount of Hb in the blood). Gas exchange can be evaluated by measuring the arterial partial pressure of both oxygen and CO_2. In general, blood gas analysis is a comparatively insensitive assay for disturbed ventilation because of the organisms' buffering and reserve capacities, but may be a useful tool in clinical medicine. Measurement of diffusion capacity with CO, a gas that binds with 250 times higher affinity to Hb than does oxygen, is more sensitive. The test is easy to perform in humans and laboratory animals and is widely used in clinical studies.

Proper lung function in humans can be evaluated with several additional techniques. Computed tomography provides detailed roentgenographic information of airways and lung parenchyma and has been useful in screening high-risk patients for lung cancer (National Lung Screening Trial, 2011). Increased concentrations of nitric oxide are often found in exhaled air when inflammatory processes have led to induction of iNOS (Barnes et al., 2010). As an experimental method, exhaled breath or induced sputum analysis may also be useful in assessing other inflammatory indicators in exhaled breath condensate such as oxidative stress markers (e.g., hydrogen peroxide and isoprostanes), nitric oxide derivatives (e.g., nitrate and nitrates), arachidonic acid metabolites (e.g., prostanoids, leukotrienes, and epoxides), adenosine, and cytokines (Popov, 2011). Fiberoptic bronchoscopy has become one of the most valuable tools for the detection of toxic lung injury (Reynolds, 2011). The procedure allows direct visual inspection of the major lobar and segmental airways; the depth of penetration is limited by the external diameter of the bronchoscope, usually 5 mm. Bronchoscopy also allows the introduction and retrieval of saline solutions into the lung and subsequent analysis for cellular and molecular constituents (bronchoalveolar lavage). Excision of small tissue samples (biopsies) during bronchoscopy is an additional diagnostic tool, most helpful in the evaluation and staging of precancerous and cancerous lesions.

Animals Studies

The toxicology of inhaled materials has been and continues to be extensively studied in experimental animals (Mauderly, 1996). In such studies, selection of animals with a respiratory system similar to that of humans is particularly desirable (Phalen et al., 2008). The respiratory system of monkeys most closely resembles that of humans. However, the availability and cost of these animals and the necessity for special facilities for housing monkeys and performing long-term exposures, along with ethical considerations, including the confinement of primates in small exposure chambers for prolonged periods, severely limits the use of primates. Rats and mice are widely used, although fundamental differences in respiratory anatomy (e.g., lack of respiratory bronchioles) and function (rats and mice are obligate nose breathers) can complicate the extrapolation of effects to humans. Experimental studies with guinea pigs and rabbits provided the first conclusive evidence that sulfuric acid and SO_2 may damage human lungs (Amdur, 1989). The following techniques are used to study the effects of inhaled toxicants in animals.

Inhalation Exposure Systems In inhalation studies, animals are kept within a chamber that is ventilated with a defined test atmosphere (Phalen, 1976; Wong, 2007). Generation of such an atmosphere is comparatively easy for gases that are available in high purity in a compressed tank, for example, SO_2, O_2, or NO_2. Metering and dilution produce appropriate concentrations for exposure. Final concentrations within the chamber can be monitored continuously with direct reading detectors (e.g., UV for ozone or infrared for CO_2) that are calibrated. Exposure should be within 5% of the targeted concentration. Alternatively, wet chemical analysis procedures are applied after sampled gases from the chambers are bubbled through traps. Such sampling yields a time-weighted average for the concentration. More challenging is the generation of particles or complex mixtures (e.g., tobacco smoke, diesel, and gasoline exhaust or residual oil fly ash), particularly because of the possibility of interactions between individual mixture constituents and the possibility of formation of artifacts (Pauluhn, 2005).

Exposure chambers must allow for the rapid attainment of the desired concentrations of toxicants, maintenance of desired toxicant levels homogeneously throughout the chamber, adequate capacity for experimental animals, and minimal accumulation of undesired products associated with animal occupancy (usually ammonia, dander, heat, and carbon dioxide). As a general rule, the total body volume of the animals should not exceed 5% of the chamber volume. Nose-only exposure chambers avoid some of these problems (Phalen et al., 1984; Leong et al., 1998). However, nose-only exposure systems create different problems, including a great deal of stress on the animals due to confinement during exposure, and the very labor-intensive handling that is required for exposures by this route. The proper selection of exposure techniques for a given

chemical is ultimately a decision that must be made on a case-by-case basis. Finally, concern for the environment and the safety of facility personnel suggest prudence in how chambers are exhausted.

Intratracheal and Intranasal Instillation Alternative approaches are intratracheal (Driscoll et al., 2000; Rayamajhi et al., 2011) and intranasal (Szarka et al., 1997) instillation of particles in mice or rats. These approaches are useful when the quantity of material is limited. Intratracheal instillation can be done using a gavage syringe or micro-sprayer (Hasegawa-Baba et al., 2014; Lawrenz et al., 2014). In some cases, particles can be suspended in surfactant material that diminishes aggregation and improves dispersion. Because rodents are obligatory nasal breathers, intranasal exposure is performed by placing material in one nostril with a micropipette while occluding the opposing nostril. Upon inhalation, the material can be delivered to the respiratory tract. However, these approaches have a major limitation in that the dose rate does not correspond to that by inhalation. In addition, these delivery methods produce focal areas of a large quantity of particles to a relative small surface area. Thus, the high focal dose can trigger local response due to the dose intensity that may not be triggered following inhalation.

The delivered dose rate of particles to the lung is a determinant for the acute inflammatory neutrophilic response due to dust overloading (Morrow, 1988). Overload is typified by a progressive reduction of particle clearance from the distal lung, reflecting a breakdown in alveolar macrophage-mediated dust removal as a result of the loss of macrophage mobility. Inhalation or instillation of a very large amount (quantities of respirable dust far in excess of physiological loads) of even inert particles, so-called nuisance dust, into the distal lung can result in activation of alveolar macrophage, neutrophil recruitment, and lung injury. This is particularly true when the dose rate is not considered. The same dose delivered over days by inhalation as opposed to sudden instillation leads to a low response, conceivably reflecting the low release rate of phagocytosis-related inflammatory mediators (e.g., chemotactic factors) from alveolar macrophages (Oberdörster et al., 1997).

Pulmonary Function Tests in Experimental Animals Conducting pulmonary function tests in experimental animals poses distinct challenges, especially in small rodents (Reinhard et al., 2005). Experimental animals cannot be made to maximally inhale or exhale at the investigator's will. For example, FEV1 can be obtained in experimental animals only under anesthesia. Expiration is forced by applying external pressure to the thorax or negative pressure to the airways (Vinegar et al., 1979). Alternatively, analysis of pressure–volume curves is a comparatively easy test to perform in animals, not requiring a specialized apparatus (Alarie et al., 1961; Sinnett et al., 1981). The test provides some indication of lung compliance. Compliance (volume/pressure) is calculated as the slope of the volume–pressure curve. The volume–pressure curve involves deflating the lung and then inflating the lung with incremental volumes and recording the pressure, and can be obtained from lungs filled with air and/or physiological saline. The lung is then deflated with incremental volumes and and the pressure is again recorded. Compliance provides an indication of the intrinsic elastic properties of the lung parenchyma and, when measured in vivo, the thoracic cage. In emphysema, compliance of the lung increases because elastic recoil is decreased. When the inflation and deflation are performed with air, the curves will not match and this hysteresis is an indication of surfactant function. The use of saline is sensitive to structural changes in lung parenchyma, as the effects of surfactant are eliminated in a saline-filled lung.

Another pulmonary function test is the analysis of airway resistance, which can be measured in laboratory animals by restrained plethysmography (Amdur and Mead, 1958), unrestrained video-assisted plethysmography (Bates et al., 2008), or unrestrained acoustic plethysmography (Reynolds et al., 2008). An increase in airway resistance is a measure of bronchoconstriction inasmuch as the airway smooth muscle contraction or airway mucosal edema narrows the airway lumen and obstructs airflow. Airway resistance also can be measured after a challenge of incremental doses of inhaled methacholine (or histamine) (Bates and Irvin, 2003) or infused acetylcholine (Swiecichowski et al., 1993). This challenge provides a measure of airway hyperreactivity, a distinguishing feature of asthma in humans. In asthma, resting airway resistance is typically normal but when challenged, airway resistance will increase at a lower threshold dose of methacholine. Analysis of breathing pattern can also be used and may differentiate between upper airway and lower airway irritants (Alarie et al., 1988; Bates et al., 2004). In rodents, upper airway ("sensory") irritants produce a breathing pattern of decreased respiratory frequency with increased tidal volume, whereas lower airway ("pulmonary") irritants produce a breathing pattern of increased respiratory frequency and decreased minute volume (i.e., the total volume of air breathed in 1 minute).

Morphological Techniques The pathology of acute and chronic injury may be examined by gross inspection and under the microscope and should include the nasal passages, larynx, major bronchi, and the lung parenchyma.

Regional distribution of lesions in nasal passages can be assessed after fixation and decalcification. Various regions of the nasal passages can then be examined by obtaining cross sections at multiple levels. Proper fixation of the lung is done by vascular perfusion with fixative through the pulmonary artery or by instillation of fixative through the trachea. Perfusion fixation does not dislodge material (lining fluid, deposited particles) or cells in the lumen of the airways or the alveoli from their original position. Fixation by intratracheal instillation can be done under controlled pressure (usually 30-cm H_2O in rodents), which preserves alveolar architecture. This permits semiquantitative or quantitative measurements to be made. The choice of fixative depends on how the lung will be further analyzed. Formalin-based fixatives are satisfactory for routine histopathology, whereas the use of more sophisticated techniques such as electron microscopy, immunohistochemistry, and in situ hybridization require careful selection of the fixative.

Paraffin sections of respiratory tract tissue are suitable for routine histopathological analysis of gross pathological changes, for example, inflammation, fibrosis, or lung tumors. The tissue can be stained with (a) hematoxylin and eosin stain for routine assessment (Llewellyn, 2009), (b) periodic acid-Schiff's Alcian blue stain for glycoproteins (mucus cells) (Jeffery et al., 1982), or (c) Masson trichrome stain for collagen (Goldner, 1938). Plastic or Epon sections 1-μm thick are required for proper identification of additional cell types or recognition of cytoplasmic changes as in damaged BSCs. Other structural alterations, such as degenerative changes or necrosis of type I epithelial cells or capillary endothelial cells, usually are detected by transmission electron microscopy (TEM). TEM is essential for an unequivocal identification of cells in the alveolar interstitium and is used mainly in morphometric analysis of the lung. Scanning electron microscopy allows visualization of the surface of interior lung structures, reveals alterations in the tissue surface, and detects rearrangement of the overall cell population. Confocal microscopy, consisting of a laser microscope coupled to a computer, allows examination of thick sections and discovery of specific cell types deep within the tissue labeled with fluorescent markers; it is an ideal tool for three-dimensional reconstruction of normal and damaged lung.

Morphometry, the quantitative description of structure, refers to a quantitative analysis of tissue (Gehr et al., 1993). Measurements

made in two dimensions on photographs taken under the microscope allow one to measure areas, the thickness of a structure, and numerical density. Using the appropriate formula, values such as the volume occupied by a specific cell population in the entire lung parenchyma can be calculated (Hyde et al., 2007).

Additional tools for the study of toxic lung injury include immunohistochemistry, in situ hybridization, and analysis of cell kinetics. Transcriptome (Mohr et al., 2002), proteome (Miller et al., 2008), and metabolome (Fabisiak et al., 2011) profiling are additional value tools to assess the lung in health and disease. Antibodies to a variety of enzymes, mediators, and other proteins are available. It is possible to identify cell types that carry certain enzymes and their anatomic locations. In situ hybridization allows one to visualize anatomic sites where a specific gene product is expressed, for example, collagen production in a fibrotic lung. Two useful sites to obtain lung-specific expression include the Human Protein Atlas (Uhlen et al., 2010) and GenePaint (Visel et al., 2004). Ascribing a given metabolic capability to a specific cell type requires evaluation of gene expression and/or protein production in specific cells in situ. The normal adult lung is an organ for which under normal circumstances very few cells appear to die and to be replaced. When damaged by a toxic insult, the lung parenchyma is capable of repairing itself in an efficient manner. Type I cell damage is followed by proliferation of type II epithelial cells, which eventually transform into new type I cells (Adamson and Bowden, 1974; Fujino et al., 2011); in the airways, the BSCs proliferate and divide following injury to ciliated cells (Delgado et al., 2011; Rock and Hogan, 2011). Quantitative data can be obtained by either injection of the DNA precursors (tritiated thymide, bromodeoxyuridine) or visualization of proliferating cell nuclear antigen (PCNA). Flow cytometry is valuable in the study of cell populations prepared from the lung.

Pulmonary Lavage and Pulmonary Edema Pulmonary edema and/or pulmonary inflammation are early events in acute and chronic lung injury. The fluid lining the pulmonary epithelium can be recovered by bronchoalveolar lavage. Analysis of the lavage fluid is a useful tool to detect respiratory tract toxicity (Henderson, 2005; Reynolds, 2011). Influx of neutrophils or other leukocytes such as lymphocytes or eosinophils into the lavage fluid is the most sensitive sign of inflammation. Measurements of lung injury include total protein and/or albumin. Additional measurements of secretory products of macrophages and epithelial cells include fibronectin, chemokines, and other cytokines (e.g., TNF or IL1B). Reduced glutathione levels may be an indicator of oxidative stress. Lactate dehydrogenase activity (and its substituent isoenzymes), *N*-acetylglucosaminidase, acid or alkaline phosphatase, other lysosomal hydrolases, and sialic acid add additional information. In addition pulmonary edema can be assessed by determining lung wet:dry ratio or injection of Evans blue dye albumin (Patterson et al., 1992; Li et al., 2010).

In Vitro Studies

Isolated Perfused Lung In vitro systems with materials originally obtained from either human tissues or experimental animals are particularly suited for the study of mechanisms that cause lung injury (Aufderheide, 2005; Bakand and Hayes, 2010). The methods include isolated perfused lung, microdissection/organotypic tissue culture systems, and cell type–specific cell culture. The isolated perfused lung method is applicable to lungs from many laboratory species (e.g., mouse, rat, guinea pig, or rabbit) (Mehendale et al., 1981). The lung is perfused with blood or a blood substitute through the pulmonary arterial bed. At the same time, the lung is actively (through rhythmic inflation–deflation cycles with positive pressure) or passively (by creating negative pressure with an artificial thorax in which the lung is suspended) ventilated. Toxic chemicals can be introduced into the perfusate or the inspired air (Rhoades, 1984). Repeated sampling of the perfusate allows one to determine the rate of metabolism of drugs and the metabolic activity of the lung.

Airway Microdissection and Organotypic Tissue Culture Systems Many inhalants act in specific regions of the respiratory tract. For example, the terminal bronchioles, a region especially rich in the highly metabolically competent BSCs, is often a target of gases with low water solubility. Microdissection of the nasal passage (Fanucchi et al., 1999) and airways (Plopper et al., 1991) consists of stripping away surrounding tissue or parenchyma while maintaining the airway structure and exposing the epithelium. Microdissected airways can be studied in culture for up to one week, can be used to study site-specific gene expression, morphological changes in toxicant injury and repair, or can be used for biochemical analyses including enzyme activity measurements and determination of antioxidant concentrations (such as glutathione). This facilitates study of the metabolically active BSCs found in the airways (Plopper et al., 2001). Tissue culture systems have been developed in which epithelial cells maintain their polarity, differentiation, and normal function similar to what is observed in vivo. Epithelial cell surfaces are exposed to air (or a gas phase containing an airborne toxic chemical), while the basal portion is bathed by a tissue culture medium (Widdicombe and Welsh, 1980).

Lung Cell Culture Many lung-specific cell types have been isolated and can be maintained as cell culture. Human (Reynolds, 2011) and animal (Kobzik et al., 1988) alveolar or interstitial macrophages can be obtained from lavage or lung tissue. Their function can be examined in vitro with or without exposure to appropriate toxic stimuli (Brain, 1986; Brain et al., 1994). Type II alveolar epithelial cells can be isolated and primary cell cultures maintained in culture for short periods (Dobbs et al., 1997; Wang et al., 2007). Direct isolation of type I epithelial cells has also been successful (Williams, 2003).

Maintenance of the epithelial cells at the air–liquid interface is important to maintain polarity and differentiation. Epithelial cells may be seeded on top of a suitable supporting material (e.g., collagen or nitrocellulose membranes) with mesenchymal cells seeded on the other side to observe epithelial cell–fibroblast interactions. Systems are available for the isolation and culture of human bronchial epithelial cells (Lechner et al., 1982; Lechner and LaVeck, 1985) and BSCs (Belinsky et al., 1995). These cells can be differentiated into ciliated and mucin-producing cells by growth in air–liquid interface systems (Whitcutt et al., 1988; de Jong et al., 1994). Serous and mucus cells can be obtained from submucosal glands and maintained in primary cell culture (Finkbeiner et al., 2010). Lung fibroblasts are easily grown (Baglole et al., 2005) and have been studied in coculture with epithelial cells (Delgado et al., 2011). Primary cell cultures have been transformed (Gruenert et al., 1988) and/or immortalized (Reddel et al., 1988; Vaughan et al., 2006) and can be passaged and maintained for long periods in the laboratory. Cell lines established from lung tumors have been used extensively by investigators and have yielded many novel insights into lung cancer (Gazdar et al., 2010).

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REFERENCES

Abbinante-Nissen JM, Simpson LG, Leikauf GD. Neutrophil elastase increases secretory leukocyte protease inhibitor transcript levels in airway epithelial cells. *Am J Physiol*. 1993;265:L286–L292.

Adams FA. On air, water, and places. In: *The Genuine Works of Hippocrates*. Vol 1. London: Sydenham Society; 1849:179–222.

Adamson IY, Bowden DH. The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. *Lab Invest*. 1974;30:35–42.

Aguilar PR, Michelson AP, Isakow W. Obliterative bronchiolitis. *Transplantation*. 2016;100:272–283.

Aharinejad S, Taghavi S, Klepetko W, Abraham D. Prediction of lung-transplant rejection by hepatocyte growth factor. *Lancet*. 2004;363:1503–1508.

Aitken J. On dust, fogs, and clouds. *Nature*. 1880;23:195–197.

Aitken J. The remarkable sunsets. *Proc Roy Soc Edinb*. 1884;12:448–450.

Alarie Y, Schaper M, Nielsen GD, Abraham MH. Structure-activity relationships of volatile organic chemicals as sensory irritants. *Arch Toxicol*. 1988;72:125–140.

Alarie Y. Sensory irritation by airborne chemicals. *CRC Crit Rev Toxicol*. 1973;2:299–363.

Alarie Y, Stone H, Latour R, Robillard E, Dautrebande L. New studies on aerosols. XV. Influence of constricting and dilating microaerosols on "pressure-volume" curves of isolated atelectactic rats' lung. *Arch Int Pharmacodyn Ther*. 1961;133:470–480.

Albert RE, Arnett LC. Clearance of radioactive dust from the human lung. *AMA Arch Ind Health*. 1955;12:99–106.

Albert RE, Sellakumar AR, Laskin S, et al. Gaseous formaldehyde and hydrogen chloride induction of nasal cancer in the rat. *J Natl Cancer Inst*. 1982;68:597–603.

Alexis NE, Lay JC, Zeman KL, et al. In vivo particle uptake by airway macrophages in healthy volunteers. *Am J Respir Cell Mol Biol*. 2006;34:305–313.

Ali M, Lillehoj EP, Park Y, Kyo Y, Kim KC. Analysis of the proteome of human airway epithelial secretions. *Proteome Sci*. 2011;9:4.

Ali MS, Pearson JP. Upper airway mucin gene expression: a review. *Laryngoscope*. 2007;117:932–938.

Allavena P, Chieppa M, Bianchi G, et al. Engagement of the mannose receptor by tumoral mucins activates an immune suppressive phenotype in human tumor-associated macrophages. *Clin Dev Immunol*. 2010;2010:547179.

Allen-Gipson DS, Blackburn MR, Schneider DJ, et al. Adenosine activation of A2B receptor(s) is essential for stimulated epithelial ciliary motility and clearance. *Am J Physiol Lung Cell Mol Physiol*. 2011;301:L171–L180.

Amdur MO. Sulfuric acid: the animals tried to tell us. 1989 Herbert Stokinger Lecture. *Appl Ind Hyg*. 1989;4:189–197.

Amdur MO, Mead J. Mechanics of respiration in unanesthetized guinea pigs. *Am J Physiol*. 1958;192:364–368.

Amdur MO, Schulz RZ, Drinker P. Toxicity of sulfuric acid mist to guinea pigs. *AMA Arch Ind Hyg Occup Med*. 1952;5:318–329.

American Chemical Society Chemical Abstract Service. CAS Registry Numbers. Available at: http://www.cas.org/expertise/cascontent/registry/regsys.html. Accessed October 1, 2011.

American Conference of Governmental Industrial Hygienists. *Documentation of the Threshold Limit Values and Biological Exposure Indices*. 7th ed. ACGIH Publication No. 0100Doc, Cincinnati, OH; 2011.

American Thoracic Society. Lung function testing: selection of reference values and interpretative strategies. American Thoracic Society. *Am Rev Respir Dis*. 1991;1144:1202–1218.

Andre E, Campi B, Materazzi S, et al. Cigarette smoke-induced neurogenic inflammation is mediated by alpha, beta-unsaturated aldehydes and the TRPA1 receptor in rodents. *J Clin Invest*. 2008;118:2574–2582.

Anttila S, Raunio H, Hakkola J. Cytochrome P450-mediated pulmonary metabolism of carcinogens: regulation and cross-talk in lung carcinogenesis. *Am J Respir Cell Mol Biol*. 2011;44:583–590.

Arredouani MS, Palecanda A, Koziel H, et al. MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages. *J Immunol*. 2005;175:6058–6064.

Asgharian B, Price OT, Schroeter JD, et al. Derivation of mass transfer coefficients for transient uptake and tissue disposition of soluble and reactive vapors in lung airways. *Ann Biomed Eng*. 2011;39:1788–1804.

Aufderheide M. Direct exposure methods for testing native atmospheres. *Exp Toxicol Pathol*. 2005;57(suppl 1):213–226.

Baglole CJ, Reddy SY, Pollock SJ, et al. Isolation and phenotypic characterization of lung fibroblasts. *Methods Mol Med*. 2005;117:115–127.

Bailey RL, Cox-Ganser JM, Duling MG, et al. Respiratory morbidity in a coffee processing workplace with sentinel obliterative bronchiolitis cases. *Am J Ind Med*. 2015;58:1235–1245.

Bakand S, Hayes A. Troubleshooting methods for toxicity testing of airborne chemicals in vitro. *J Pharmacol Toxicol Methods*. 2010;61:76–85.

Baldwin RM, Schultz MA, Buckpitt AR. Bioactivation of the pulmonary toxicants naphthalene and 1-nitronaphthalene by rat CYP2F4. *J Pharmacol Exp Ther*. 2005;312:857–865.

Barker AF, Bergeron A, Rom WN, Hertz MI. Obliterative bronchiolitis. *N Engl J Med*. 2014;370:1820–1828.

Barnes PJ, Dweik RA, Gelb AF, et al. Exhaled nitric oxide in pulmonary diseases: a comprehensive review. *Chest*. 2010;138:682–692.

Bates J, Irvin C, Brusasco V, et al. The use and misuse of Penh in animal models of lung disease. *Am J Respir Cell Mol Biol*. 2004;31:373–374.

Bates JH, Irvin CG. Measuring lung function in mice: the phenotyping uncertainty principle. *J Appl Physiol*. 2003;94:1297–1306.

Bates JH, Thompson-Figueroa J, Lundblad LK, Irvin CG. Unrestrained video-assisted plethysmography: a noninvasive method for assessment of lung mechanical function in small animals. *J Appl Physiol*. 2008;104:253–261.

Baum A, Sachidanandam R, García-Sastre A. Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. *Proc Natl Acad Sci U S A*. 2010;107:16303–16308.

Bautista DM, Jordt SE, Nikai T, et al. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell*. 2006;124:1269–1282.

Beckman JS. Understanding peroxynitrite biochemistry and its potential for treating human diseases. *Arch Biochem Biophys*. 2009;484:114–116.

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A*. 1990;87:1620–1624.

Beeson WL, Abbey DE, Knutsen SF. Long-term concentrations of ambient air pollutants and incident lung cancer in California adults: results from the ASHMOG study. Adventist Health Study on Smog. *Environ Health Perspect*. 1998;106:813–822.

Bein K, Leikauf GD. Acrolein—a pulmonary hazard. *Mol Nutr Food Sci*. 2011;55:1342–1360.

Belinsky SA, Lechner JF, Johnson NF. An improved method for the isolation of type II and Clara cells from mice. *In Vitro Cell Dev Biol Anim*. 1995;31:361–366.

Bellomo R. The cytokine network in the critically ill. *Anaesth Intensive Care*. 1992;20:288–302.

Belperio JA, Burdick MD, Keane MP, et al. The role of the CC chemokine, RANTES, in acute lung allograft rejection. *J Immunol*. 2000;165:461–472.

Belperio JA, Keane MP, Burdick MD, et al. Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome. *J Clin Invest*. 2001;108:547–556.

Belperio JA, Keane MP, Burdick MD, et al. Role of CXCL9/CXCR3 chemokine biology during pathogenesis of acute lung allograft rejection. *J Immunol*. 2003;171:4844-4852.

Belperio JA, Keane MP, Burdick MD, et al. Role of CXCR2/CXCR2 ligands in vascular remodeling during bronchiolitis obliterans syndrome. *J Clin Invest*. 2005;115:1150–1162.

Berrington WR, Iyer R, Wells RD, et al. NOD1 and NOD2 regulation of pulmonary innate immunity to *Legionella pneumophila*. *Eur J Immunol*. 2010;40:3519–3527.

Bessac BF, Jordt SE. Breathtaking TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology (Bethesda)*. 2008;23:360–370.

Bessac BF, Sivula M, von Hehn CA, et al. Transient receptor potential ankyrin 1 antagonists block the noxious effects of toxic industrial isocyanates and tear gases. *FASEB J*. 2009;23:1102–1114.

Beydon N, Davis SD, Lombardi E, et al. An official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children. *Am J Respir Crit Care Med*. 2007;175:1304–1345.

Bingham E, Pfitzer EA, Barkley W, Radford EP. Alveolar macrophages: reduced number in rats after prolonged inhalation of lead sesquioxide. *Science*. 1968;162:1297–1299.

Bingle L, Bingle CD. Distribution of human PLUNC/BPI fold-containing (BPIF) proteins. *Biochem Soc Trans*. 2011;39:1023–1027.

Bisetti AA. Bernardino Ramazzini and occupational lung medicine. *Ann N Y Acad Sci*. 1988;534:1029–1037.

Blanc PD. Occupational and environmental medicine: the historical perspective (Chapter 2). In: Rosenstock L, Cullen MR, Brodlin CA, Redlich CA, eds. *Textbook of Clinical Occupational and Environmental Medicine*. 2nd ed. Philadelphia, PA: Elsevier Saunders; 2005:17–27.

Bond JA, Harkema JR, Russell VI. Regional distribution of xenobiotic metabolizing enzymes in respiratory airways of dogs. *Drug Metab Dispos*. 1988;16:116–124.

Borchers MT, Wesselkamper SC, Curull V, et al. Sustained CTL activation by murine pulmonary epithelial cells promotes the development of COPD-like disease. *J Clin Invest*. 2009;119:636–649.

Borchers MT, Wesselkamper SC, Harris NL, et al. CD8+ T cells contribute to macrophage accumulation and airspace enlargement following repeated irritant exposure. *Exp Mol Pathol*. 2007;83:301–310.

Bowden DH. Macrophages, dust and pulmonary diseases. *Exp Lung Res*. 1987;12:89–107.

Bowdish DM, Gordon S. Conserved domains of the class A scavenger receptors: evolution and function. *Immunol Rev*. 2009;227:19–31.

Brain JD. The effects of increased particles on the number of alveolar macrophages. *Inhaled Part*. 1970;1:209–225.

Brain JD. Toxicological aspects of alterations of pulmonary macrophage function. *Annu Rev Pharmacol Toxicol*. 1986;26:547–565.

Brain JD, Godleski J, Kreyling W. In vivo evaluation of chemical biopersistence of nonfibrous inorganic particles. *Environ Health Perspect*. 1994;102(suppl 5):119–125.

British Medical Journal. Fog fatality in London. *Br Med J*. 1880;1:254.

Brooks SM, Weiss MA, Bernstein IL. Reactive airways dysfunction syndrome (RADS). Persistent asthma syndrome after high level irritant exposures. *Chest*. 1985;88:376–384.

Buch SC, Diergaarde B, Nukui T, et al. Genetic variability in DNA repair and cell cycle control pathway genes and risk of smoking-related lung cancer. *Mol Carcinog*. 2012;51(suppl 1):E11–E20.

Buckpitt A, Boland B, Isbell M, et al. Naphthalene-induced respiratory tract toxicity: metabolic mechanisms of toxicity. *Drug Metab Rev*. 2002;34:791–820.

Buckpitt AR, Cruikshank MK. Biochemical function of the respiratory tract: metabolism of xenobiotics. In: Sipes IG, McQueen CA, Gandolfi JA, eds. *Comprehensive Toxicology, Vol 8, Toxicology of the Respiratory System*. Oxford: Elsevier Science; 1997:159–186.

Bushey RT, Chen G, Blevins-Primeau AS, et al. Characterization of UDP-glucuronosyltransferase 2A1 (UGT2A1) variants and their potential role in tobacco carcinogenesis. *Pharmacogenet Genomics*. 2011;21:55–65.

Casarett LJ. Some physical and physiological factors controlling the fate of inhaled substances. II. Retention. *Health Phys*. 1960;2:379–386.

Cassel SL, Eisenbarth SC, Iyer SS, et al. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A*. 2008;105:9035–9040.

Cavalcanti Zdo R, Albuquerque Filho AP, Pereira CA, Coletta EN. Coletta Bronchiolitis associated with exposure to artificial butter flavoring in workers at a cookie factory in Brazil. *J Bras Pneumol*. 2012;38:395–399.

Chandrashekar J, Hoon MA, Ryba NJ, Zuker CS. The receptors and cells for mammalian taste. *Nature*. 2006;444:288–294.

Chapman HA. Epithelial-mesenchymal interactions in pulmonary fibrosis. *Annu Rev Physiol*. 2011;73:413–435.

Chapman HA Jr, Stone OL, Vavrin Z. Degradation of fibrin and elastin by intact human alveolar macrophages in vitro. Characterization of a plasminogen activator and its role in matrix degradation. *J Clin Invest*. 1984;73;806–815.

Charpin JM, Stern M, Grenet D, Israël-Biet D. Insulinlike growth factor-1 in lung transplants with obliterative bronchiolitis. *Am J Respir Crit Care Med*. 2000;161:1991–1998.

Chen G, Korfhagen TR, Xu Y, et al. SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production. *J Clin Invest*. 2009;119:2914–2924.

Chen HY, Yu SL, Chen CH, et al. A five-gene signature and clinical outcome in non-small-cell lung cancer. *N Engl J Med*. 2007;356:11–20.

Chen JH, Stoltz DA, Karp PH, et al. Loss of anion transport without increased sodium absorption characterizes newborn porcine cystic fibrosis airway epithelia. *Cell*. 2010;143:911–923.

Chung KF, Pavord ID. Prevalence, pathogenesis, and causes of chronic cough. *Lancet*. 2008;371:1364–1374.

Churg A. Interactions of exogenous or evoked agents and particles: the role of reactive oxygen species. *Free Radic Biol Med*. 2003;34:1230–1235.

Churg A, Wang RD, Xie C, Wright JL. Alpha-1-Antitrypsin ameliorates cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med*. 2003;168:199–207.

Coffin DL, Gardner DE, Holzman RS, Wolock FJ. Influence of ozone on pulmonary cells. *Arch Environ Health*. 1968;16:633–636.

Costantini LM, Gilberti RM, Knecht DA. The phagocytosis and toxicity of amorphous silica. *PLoS One*. 2011;6:e14647.

Cutz E, Yeger H, Pan J. Pulmonary neuroendocrine cell system in pediatric lung disease-recent advances. *Pediatr Dev Pathol*. 2007;10:419–435.

Dalhamn T, Rodin J. Mucous flow and ciliary activity in the trachea of rats exposed to pulmonary irritant gas. *Br J Ind Med*. 1956;13:110–113.

D'Armiento J, Dalal SS, Okada Y, Berg RA, Chada K. Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. *Cell*. 1992;7:955–961.

Dautrebande L, Highman B, Alford WC. Studies on aerosols; reduction of dust deposition in lungs of rabbits by aqueous aerosols. *J Ind Hyg Toxicol*. 1948;30:103–107.

Davies CN. Dust particles and aqueous aerosols: a critical review. *J Ind Hyg Toxicol*. 1949;31:169.

De Flora S, Bennicelli C, D'Agostini F, Izzotti A, Camoirano A. Cytosolic activation of aromatic and heterocyclic amines. Inhibition by dicoumarol and enhancement in viral hepatitis B. *Environ Health Perspect*. 1994;102(suppl 6):69–74.

de Jong PM, van Sterkenburg MA, Hesseling SC, et al. Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface. *Am J Respir Cell Mol Biol*. 1994;10:271–277.

Degryse AL, Tanjore H, Xu XC, et al. TGFβ signaling in lung epithelium regulates bleomycin-induced alveolar injury and fibroblast recruitment. *Am J Physiol Lung Cell Mol Physiol*. 2011;300:L887–L897.

Delgado O, Kaisani AA, Spinola M, et al. Multipotent capacity of immortalized human bronchial epithelial cells. *PLoS One*. 2011;6:e22023.

DeLucia AJ, Hoque PM, Mustafa MG, Cross CE. Ozone interaction with rodent lung: effect on sulfhydryls and sulfhydryl-containing enzyme activities. *J Lab Clin Med*. 1972;80:559–566.

DeMaria S, Ngai J. The cell biology of smell. *J Cell Biol*. 2010;191:443–452.

Denissenko MF, Pao A, Tang M, Pfeifer GP. Referential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. *Science*. 1996;274:430–432.

Deshmukh HS, Case LM, Wesselkamper SC, et al. Metalloproteinases mediate mucin 5AC expression by epidermal growth factor receptor activation. *Am J Respir Crit Care Med*. 2005;171:305–314.

Deshmukh HS, McLachlan A, Atkinson JJ, et al. Matrix metalloproteinase-14 mediates a phenotypic shift in the airways to increase mucin production. *Am J Respir Crit Care Med*. 2009;180:834–845.

Deshmukh HS, Shaver C, Case LM, et al. Acrolein-activated matrix metalloproteinase 9 contributes to persistent mucin production. *Am J Respir Cell Mol Biol*. 2008;38:446–454.

DiGiovine B, Lynch JP 3rd, Martinez FJ, et al. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. *J Immunol* 1996;157:4194–4202.

Di Giuseppe M, Gambelli F, Hoyle GW, et al. Systemic inhibition of NF-kappaB activation protects from silicosis. *PLoS One*. 2009;4:e5689.

Ding X, Kaminsky LS. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol*. 2003;43:149–173.

Dobbs LG, Pian MS, Maglio M, Dumars S, Allen L. Maintenance of the differentiated type II cell phenotype by culture with an apical air surface. *Am J Physiol*. 1997;273:L347–L354.

Donaldson K, Murphy FA, Duffin R, Poland CA. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol*. 2010;7:5.

Dostert C, Pétrilli V, Van Bruggen R, et al. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 2008;320:674–677.

Drew RT, Eisenbud M. The pulmonary dose from 220Rn received by indigenous rodents of the Morro Do Ferro, Brazil. *Radiat Res*. 1970;42:270–281.

Drinker P, Thomson RM, Finn JL. Quantitative measurements of the inhalation, retention and exhalation of dusts and fumes by man. I. Concentrations of 50 to 450 mg per cubic meter. *J Indust Hyg Toxicol*. 1928;10:13.

Driscoll KE, Costa DL, Hatch G, et al. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol Sci*. 2000;55:24–35.

El-Gamel A, Sim E, Hasleton P, et al. Transforming growth factor beta (TGF-beta) and obliterative bronchiolitis following pulmonary transplantation. *J Heart Lung Transplant*. 1999;18:828–837.

Elinav E, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. *Immunity*. 2011;34:665–679.

Evans MJ, Van Winkle LS, Fanucchi MV, Plopper CG. Cellular and molecular characteristics of basal cells in airway epithelium. *Exp Lung Res*. 2001;27:401–415.

Evelyn J. *Fumifugium, or the Inconvenience of the Aer and Smoak of London Dissipated. Together with Some Remedies Humbly Proposed by JE Esq, to his Sacred Majesty and to the Parliament Now Assembled*. London: His Majesty's Command; 1661. (Reprinted by the National Society for Clean Air in 1961. Dorset Press, Dorchester. 1961.)

Fabisiak JP, Medvedovic M, Alexander DC, et al. Integrative metabolome and transcriptome profiling reveals discordant energetic stress between mouse strains with differential sensitivity to acrolein-induced acute lung injury. *Mol Nutr Food Res*. 2011;55:1423–1434.

Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med*. 2010;363:2233–2247.

Fanucchi MV, Plopper CG, Harkema JR, Hotchkiss JA. In vitro culture of microdissected rat nasal airway tissues. *Am J Respir Cell Mol Biol*. 1999;20:1274–1285.

Farsalinos KE, Kistler KA, Gillman G, Voudris V. Evaluation of electronic cigarette liquids and aerosol for the presence of selected inhalation toxins. *Nicotine Tob Res*. 2015;17:168-174.

Ferin J, Urbánková G, Vlcková A. Influence of tobacco smoke on the elimination of particles from the lungs. *Nature*. 1965;206:515–516.

Feron VJ, Arts JH, Kuper CF, Slootweg PJ, Woutersen RA. Health risks associated with inhaled nasal toxicants. *Crit Rev Toxicol*. 2001;31:313–347.

Ferrer-Sueta G, Radi R. Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS Chem Biol*. 2009;4:161–177.

Finkbeiner WE, Zlock LT, Mehdi I, Widdicombe JH. Cultures of human tracheal gland cells of mucous or serous phenotype. *In Vitro Cell Dev Biol Anim*. 2010;46:450–456.

Fleischer J, Breer H, Strotmann J. Mammalian olfactory receptors. *Front Cell Neurosci*. 2009;3:9.

Fletcher C, Peto R. The natural history of chronic airflow obstruction. *Br Med J*. 1977;1:1645–1648.

Fraiser LH, Kanekal S, Kehrer JP. Cyclophosphamide toxicity. Characterising and avoiding the problem. *Drugs*. 1991;42:781–795.

Frank R. The effects of inhaled pollutants on nasal and pulmonary flow-resistance. *Ann Otol Rhinol Laryngol*. 1970;79:540–546.

Freeman FA, Gutierrez H, Rubbo H. Nitric oxide: a central regulatory species in pulmonary oxidant reactions. *Am J Physiol Lung Cell Mol Physiol*. 1995;268:L697–L698.

Freeman G, Haydon GB. Emphysema after low-level exposure to NO2. *Arch Environ Health*. 1964;8:125–128.

Friberg L. Proteinuria and kidney injury among workmen exposed to cadmium and nickel dust; preliminary report. *J Ind Hyg Toxicol*. 1948;30:32–36.

Fujino N, Kubo H, Suzuki T, et al. Isolation of alveolar epithelial type II progenitor cells from adult human lungs. *Lab Invest*. 2011;91:363–378.

Ganz T, Gabayan V, Liao HI, et al. Increased inflammation in lysozyme M-deficient mice in response to Micrococcus luteus and its peptidoglycan. *Blood*. 2003;101:2388-2392.

Gardner DE, Holzman RS, Coffin DL. Effects of nitrogen dioxide on pulmonary cell population. *J Bacteriol*. 1969;98:1041–1043.

Gazdar AF, Girard L, Lockwood WW, Lam WL, Minna JD. Lung cancer cell lines as tools for biomedical discovery and research. *J Natl Cancer Inst*. 2010;102:1310–1321.

Gazi U, Martinez-Pomares L. Influence of the mannose receptor in host immune responses. *Immunobiology*. 2009;214:554–561.

Gehr P, Geiser M, Stone KC, Crapo JD. Morphometric analysis of the gas exchange region of the lung. In: Gardner DE, Crapo JD, McClellan RO, eds. *Toxicology of the Lung*. 2nd ed. New York: Raven Press; 1993:111–154.

Geiser M. Update on macrophage clearance of inhaled micro- and nanoparticles. *J Aerosol Med Pulm Drug Deliv*. 2010;23:207–217.

Geppetti P, Materazzi S, Nicoletti P. The transient receptor potential vanilloid 1: role in airway inflammation and disease. *Eur J Pharmacol*. 2006;533:207–214.

Ghanei M, Mokhtari M, Mohammad MM, Aslani J. Bronchiolitis obliterans following exposure to sulfur mustard: chest high resolution computed tomography. *Eur J Radiol*. 2004;52:164–169.

Ghantous H, Dencker L, Gabrielsson J, Danielsson BR, Bergman K. Accumulation and turnover of metabolites of toluene and xylene in nasal mucosa and olfactory bulb in the mouse. *Pharmacol Toxicol*. 1990;66:87–92.

Gharaee-Kermani M, Hu B, Phan SH, Gyetko MR. Recent advances in molecular targets and treatment of idiopathic pulmonary fibrosis: focus on TGFbeta signaling and the myofibroblast. *Curr Med Chem*. 2009;16:1400–1417.

Gibby EM, Cohen GM. Conjugation of 1-naphthol by human bronchus and bronchoscopy samples. *Biochem Pharmacol*. 1984;33:739–743.

Gilad Y, Wiebe V, Przeworski M, Lancet D, Pä ä bo S. Loss of olfactory receptor genes coincides with the acquisition of full trichromatic vision in primates. *PLoS Biol*. 2004;2:E5

Giordano G, Van den Brúle S, Re SL, et al. Type I interferon signaling contributes to chronic inflammation in a murine model of silicosis. *Toxicol Sci*. 2010;116(2):682–692.

Giri SN. Novel pharmacological approaches to manage interstitial lung fibrosis in the twenty-first century. *Annu Rev Pharmacol Toxicol*. 2003;43:73–95.

Gloede E, Cichocki JA, Baldino JB, Morris JB. A validated hybrid computational fluid dynamics-physiologically based pharmacokinetic model for respiratory tract vapor absorption in the human and rat and its application to inhalation dosimetry of diacetyl. *Toxicol Sci*. 2011;123:231–246.

Goldner J. A modification of the masson trichrome technique for routine laboratory purposes. *Am J Pathol*. 1938;14:237–243.

Goldstein BD, Balchum OJ. Effect of ozone on lipid peroxidation in the red blood cell. *Proc Soc Exp Biol Med*. 1967;126:356–358.

Goldstein E, Green GM, Seamans C. The effect of silicosis on the antibacterial defense mechanisms of the murine lung. *J Infect Dis*. 1969;120:210–216.

Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity*. 2010;32:593–604.

Green GM, Carolin D. The depressant effect of cigarette smoke on the in vitro antibacterial activity of alveolar macrophages. *N Engl J Med*. 1967;276:421–427.

Gross P, Brown JH, Hatch TF. Experimental endogenous lipoid pneumonia. *Am J Pathol*. 1952;28:211–221.

Gross P, Pfitzer EA, Tolker E, Babyak MA, Kaschak M. Experimental emphysema: its production with papain in normal and silicotic rats. *Arch Environ Health*. 1965;11:50–58.

Gruenert DC, Basbaum CB, Welsh MJ, Li M, Finkbeiner WE, Nadel JA. Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proc Natl Acad Sci U S A*. 1988;85:5951–5955.

Gwinn MR, Vallyathan V. Respiratory burst: role in signal transduction in alveolar macrophages. *J Toxicol Environ Health B Crit Rev*. 2006;9:27–39.

Haberzettl P, Schins RP, Hohr D, Wilhelmi V, Borm PJ, Albrecht C. Impact of the FcgammaII-receptor on quartz uptake and inflammatory response by alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol*. 2008;294:L1137–L1148.

Haldane JS. Preface. *Respiration*. New Haven, CT: Yale University Press; 1922:viii.

Haldane JS. The action of carbonic oxide on man. *J Physiol*. 1895;18: 430–462.

Haldane JS. The causes of death in colliery explosions. *Trans Fed Inst Mining Eng*. 1896;11:502–513.

Hall H, Leaderer B, Cain W, Fidler A. Personal risk factors associated with mucosal symptom prevalence in office workers. *Indoor Air*. 1993;3:206–209.

Harkema JR, Carey SA, Wagner JG. The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. *Toxicol Pathol*. 2006;34:252–269.

Hasegawa-Baba Y, Kubota H, Takata A, Miyagawa M. Intratracheal instillation methods and the distribution of administered material in the lung of the rat. *J Toxicol Pathol*. 2014;27:197–204.

Hatch T. Progress in industrial sanitation: with special reference to the control of industrial dust. *Am J Public Health Nations Health*. 1937;27:671–679.

Hatch T, Hemeon WC. Influence of particle size in dust exposure. *J Ind Hyg Toxicol*. 1948;30:172–180.

Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science*. 1997;277:2002–2004.

He D, Frost AR, Falany CN. Identification and immunohistochemical localization of sulfotransferase 2B1b (SULT2B1b) in human lung. *Biochim Biophys Acta*. 2005;1724:119–126.

Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst*. 1999;91:1194–1210.

Henderson MC, Siddens LK, Morré JT, Krueger SK, Williams DE. Metabolism of the anti-tuberculosis drug ethionamide by mouse and human FMO1, FMO2 and FMO3 and mouse and human lung microsomes. *Toxicol Appl Pharmacol*. 2008;233:420–427.

Henderson RF. Use of bronchoalveolar lavage to detect respiratory tract toxicity of inhaled material. *Exp Toxicol Pathol*. 2005;57(suppl 1):155–159.

Henderson Y, Haggard HW. *Noxious Gases*. 2nd ed. New York: Reinhold Pub Corp; 1943.

Hendrick DJ. "Popcorn worker's lung" in Britain in a man making potato crisp flavouring. *Thorax*. 2008;63: 267–268.

Herbst RS, Heymach JV, Lippman SM. Molecular origins of cancer: lung cancer. *N Engl J Med*. 2008;359:1367–1380.

Hertz MI, Henke CA, Nakhleh RE, et al. Obliterative bronchiolitis after lung transplantation: a fibroproliferative disorder associated with platelet-derived growth factor. *Proc Natl Acad Sci U S A*. 1992;89:10385–10389.

Hodge HC, Leach LJ, Scott JK Spiegl CJ, Steinhardt H. Acute inhalation toxicity of lithium hydride. *AMA Arch Ind Health*. 1956;14:468–470.

Hodgson M. Indoor environmental exposures and symptoms. *Environ Health Perspect*. 2002;110(suppl 4):663–667.

Hodson D. Victor Grignard (1871–1935). *Chem Br*. 1987;23:141–142.

Hoffman FL. Mortality from respiratory diseases in dusty trades (inorganic dusts). *Bulletin of the United States Bureau of Labor Statistics*. Washington: United States Bureau of Labor Statistics; 1918.

Holland CG. *Diseases of the Lungs from Mechanical Causes*. London: John Churchill; 1843.

Homer RJ, Zhu Z, Cohn L, et al. Differential expression of chitinases identify subsets of murine airway epithelial cells in allergic inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2006;291:L502–L511.

Hornung V, Bauernfeind F, Halle A, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol*. 2008;9:847–856.

Houghton AM, Quintero PA, Perkins DL, et al. Elastin fragments drive disease progression in a murine model of emphysema. *J Clin Invest*. 2006;116:753–759.

Hu G, Shi Z, Hu J, Zou G, Peng G, Ran P. Association between polymorphisms of microsomal epoxide hydrolase and COPD: results from meta-analyses. *Respirology*. 2008;13:837–850.

Huang YC, Ghio AJ. Controlled human exposures to ambient pollutant particles in susceptible populations. *Environ Health*. 2009;8:33.

Huaux F. New developments in the understanding of immunology in silicosis. *Curr Opin Allergy Clin Immunol*. 2007;7:168–173.

Hubbs AF, Cummings KJ, McKernan LT, Dankovic DA, Park RM, Kreiss K. Comment on Farsalinos et al., "Evaluation of Electronic Cigarette Liquids and Aerosol for the Presence of Selected Inhalation Toxins." *Nicotine Tob Res*. 2015;17:1288–1289.

Hukkanen J, Pelkonen O, Hakkola J, Raunio H. Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. *CRC Crit Rev Toxicol*. 2002;32:391–411.

Hyde DM, Tyler NK, Plopper CG. Morphometry of the respiratory tract: avoiding the sampling, size, orientation, and reference traps. *Toxicol Pathol*. 2007;35:41–48.

Ichinohe T, Pang IK, Iwasaki A. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol*. 2010;11: 404–410.

Iles KE, Forman HJ. Macrophage signaling and respiratory burst. *Immunol Res*. 2002;26:95–105.

ILSI Risk Science Institute Workshop. The relevance of the rat lung response to particle overload for human risk assessment. *Inhal Toxicol*. 2000;12:1–148.

International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans. In: *Beryllium, Cadmium, Mercury and Exposures in the Glass Manufacturing Industry*. Vol 58. Lyon, France: IARC; 1993.

International Agency for Research on Cancer. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. In: *Tobacco Smoke and Involuntary Smoking*. Vol 83. Lyon, France: IARC; 2004.

International Commission on Radiological Protection. *Human Respiratory Tract Model for Radiological Protection*. ICRP Publication 66; Ann. ICRP 24 (Nos. 1–3). Oxford, UK: Elsevier; 1994.

Ishikawa E, Ishikawa T, Morita YS, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J Exp Med*. 2009;206:2879–2888.

Jang AS, Concel VJ, Bein K, et al. Endothelial dysfunction and claudin 5 regulation during acrolein-induced lung injury. *Am J Respir Cell Mol Biol*. 2011;44:483–490.

Janoff A, Sloan B, Weinbaum G, et al. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am Rev Respir Dis*. 1977;115:461–478.

Jarvis J, Seed MJ, Elton R, Sawyer L, Agius R. Relationship between chemical structure and the occupational asthma hazard of low molecular weight organic compounds. *Occup Environ Med*. 2005;62:243–250.

Jeffery PK, Ayers M, Rogers D. The mechanisms and control of bronchial mucous cell hyperplasia. *Adv Exp Med Biol*. 1982;144:399–409.

John W, Wall SM, Ondo JL, Winklmayr W. Modes in the size distributions of atmospheric inorganic aerosol. *Atmos Environ*. 1990;24A:2349–2359.

Joseph P, Jaiswal AK. NAD(P)H:quinone oxidoreductase1 (DT diaphorase) specifically prevents the formation of benzo[a]pyrenequinone-DNA adducts generated by cytochrome P4501A1 and P450 reductase. *Proc Natl Acad Sci U S A*. 1994;91:8413–8417.

Jourenkova-Mironova N, Wikman H, Bouchardy C, et al. Role of glutathione S-transferase GSTM1, GSTM3, GSTP1 and GSTT1 genotypes in modulating susceptibility to smoking-related lung cancer. *Pharmacogenetics*. 1998;8:495–502.

Juarez E, Nuñez C, Sada E, et al. Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. *Respir Res*. 2010;11:2.

Jules-Elysee K, White DA. Bleomycin-induced pulmonary toxicity. *Clin Chest Med*. 1990;11:1–20.

Kadara H, Behrens C, Yuan P, et al. A five-gene and corresponding protein signature for stage-I lung adenocarcinoma prognosis. *Clin Cancer Res*. 2011;17:1490–1501.

Kao RC, Wehner NG, Skubitz KM, Gray BH, Hoidal JR. Proteinase 3. A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters. *J Clin Invest*. 1988;82:1963–1973.

Kato M, Staub NC. Response of small pulmonary arteries to unilobar hypoxia and hypercapnia. *Circ Res*. 1966;19:426–440.

Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373–384.

Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011;34:637–650.

Kern DG. Outbreak of the reactive airways dysfunction syndrome after a spill of glacial acetic acid. *Am Rev Respir Dis*. 1991;144:1058–1064.

Kesimer M, Kirkham S, Pickles RJ, et al. Tracheobronchial air-liquid interface cell culture: a model for innate mucosal defense of the upper airways? *Am J Physiol Lung Cell Mol Physiol*. 2009;296:L92–L100.

Khateri S, Ghanei M, Keshavarz S, Soroush M, Haines D. Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard agent. *J Occup Environ Med*. 2003;45:1136–1143.

King MS, Eisenberg R, Newman JH, et al. Constrictive bronchiolitis in soldiers returning from Iraq and Afghanistan. *N Engl J Med*. 2011;365:222–230.

Kivity S, Fireman E, Lerman Y. Late asthmatic response to inhaled glacial acetic acid. *Thorax.* 1994;49:727–728.

Kiyohara C, Yoshimasu K, Takayama K, Nakanishi Y. NQO1, MPO, and the risk of lung cancer: a HuGE review. *Genet Med*. 2005;7:463–478.

Kiyohara C, Yoshimasu K, Takayama K, Nakanishi Y. EPHX1 polymorphisms and the risk of lung cancer: a HuGE review. *Epidemiology*. 2006;17:89–99.

Kobzik L, Godleski JJ, Barry BE, Brain JD. Isolation and antigenic identification of hamster lung interstitial macrophages. *Am Rev Respir Dis*. 1988;138:908–914.

Kollarik M, Undem BJ. Sensory transduction in cough-associated nerves. *Respir Physiol Neurobiol*. 2006;152:243–254.

Kosaka H, Uozumi M, Tyuma I. The interaction between nitrogen oxides and hemoglobin and endothelium-derived relaxing factor. *Free Radic Biol Med*. 1989;7:653–658.

Kreiss K, Gomaa A, Kullman G, Fedan K, Simoes EJ, Enright PL. Clinical bronchiolitis obliterans in workers at a microwave-popcorn plant. *N Engl J Med*. 2002;347:330–338.

Kuempel ED, Wheeler MW, Smith RJ, Vallyathan V, Green FH. Contributions of dust exposure and cigarette smoking to emphysema severity in coal miners in the United States. *Am J Respir Crit Care Med*. 2009;180:257–264.

Kuhn M. Molecular physiology of membrane guanylyl cyclase receptors. *Physiol Rev*. 2016;96:751–804.

Landahl HD, Black S. Penetration of air-borne particulates through the human nose. *Indust Hyg Toxicol*. 1947;29:269–277.

Lanosa MJ, Willis DN, Jordt S, Morris JB. Role of metabolic activation and the TRPA1 receptor in the sensor irritation response to styrene and naphthalene. *Toxicol Sci*. 2010;115:589–595.

Laskin S, Kuschner M, Nelson N, Altshuler B, Harley JH, Daniels M. Carcinoma of the lung in rats exposed by the beta-radiation of intrabronchial ruthenium pellets. I. Dose-response relationships. *J Natl Cancer Inst*. 1963;31:219–231.

Laurell CB, Eriksson S. The electrophoretic alpha-1-globulin pattern of serum in alpha-1-antitrypsin deficiency. *Scand J Clin Invest*. 1963;15:132–140.

Lawrenz MB, Fodah RA, Gutierrez MG, Warawa J. Intubation-mediated intratracheal (IMIT) instillation: a noninvasive, lung-specific delivery system. *J Vis Exp*. 2014;93:e52261.

Lazard D, Zupko K, Poria Y, et al. Odorant signal termination by olfactory UDP glucuronosyl transferase. *Nature*. 1991;349:790–793.

Lechner JF, Haugen A, McClendon IA, Pettis EW. Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. *In Vitro*. 1982;18:633–642.

Lechner JF, LaVeck MA. A serum-free method for culturing normal bronchial epithelial cells at clonal density. *Meth Cell Sci*. 1985;9:43–48.

Lee J, Nordestgaard BG, Dahl M. EPHX1 polymorphisms, COPD and asthma in 47,000 individuals and in meta-analysis. *Eur Respir J*. 2011;37:18–25.

Leikauf GD. Hazardous air pollutants and asthma. *Environ Health Perspect*. 2002;110(suppl 4):505–526.

Leikauf GD, Borchers MT, Prows DR, Simpson LG. Mucin apoprotein expression in COPD. *Chest*. 2002;121:166S–182S.

Leikauf GD, Concel VJ, Liu P, et al. Haplotype association mapping of acute lung injury in mice implicates activin a receptor, type 1. *Am J Respir Crit Care Med*. 2011;183:1499–1509.

Leikauf GD, Leming LM, O'Donnell JR, Doupnik CA. Bronchial responsiveness and inflammation in guinea pigs exposed to acrolein. *J Appl Physiol*. 1989;66:171–178.

Leong BK, Coombs JK, Sabaitis CP, Rop DA, Aaron CS. Quantitative morphometric analysis of pulmonary deposition of aerosol particles inhaled via intratracheal nebulization, intratracheal instillation or nose-only inhalation in rats. *J Appl Toxicol*. 1998;18:149-60.

Levin SW, Butler JD, Schumacher UK, Wightman PD, Mukherjee AB. Uteroglobin inhibits phospholipase A2 activity. *Life Sci*. 1986;38:1813–1819.

Li H, Su X, Yan X, et al. Toll-like receptor 4-myeloid differentiation factor 88 signaling contributes to ventilator-induced lung injury in mice. *Anesthesiology*. 2010;113:619–629.

Liman ER. Changing senses: chemosensory signaling and primate evolution. *Adv Exp Med Biol*. 2012;739:206-217

Lin CY, Boland BC, Lee YJ, et al. Identification of proteins adducted by reactive metabolites of naphthalene and 1-nitronaphthalene in dissected airways of rhesus macaques. *Proteomics*. 2006;6:972–982.

Ling G, Gu J, Genter MB, Zhuo X, Ding X. Regulation of cytochrome P450 gene expression in the olfactory mucosa. *Chem Biol Interact*. 2004;147:247–258.

Lippmann M. Deposition and retention of inhaled fibres: effects on incidence of lung cancer and mesothelioma. *Occup Environ Med*. 1994;51:793–798.

Lippmann M, Albert RE. Use of monodisperse aerosols for studies on respiratory tract deposition and clearance. *J Air Pollut Control Assoc*. 1968;18:672–674.

Lippmann M, Leikauf GD. Introduction and background (Chapter 1). In: Lippmann M, ed. *Environmental Toxicants Human Exposures and Their Health Effects*. New York: Wiley; 2009:3–18.

Lippmann M, Yeates DB, Albert RE. Deposition, retention, and clearance of inhaled particles. *Br J Ind Med*. 1980;37:337–362.

Llewellyn BD. Nuclear staining with alum hematoxylin. *Biotech Histochem*. 2009;84:159–177.

Lockey JE, Hilbert TJ, Levin LP, et al. Airway obstruction related to diacetyl exposure at microwave popcorn production facilities. *Eur Respir J*. 2009;34:63–71.

Lo-Guidice JM, Allorge D, Cauffiez C, et al. Genetic polymorphism of the human cytochrome P450 CYP4B1: evidence for a non-functional allelic variant. *Pharmacogenetics*. 2002;12:367–374.

Loo YM, Gale M Jr. Immune signaling by RIG-I-like receptors. *Immunity*. 2011;34:680–692.

Lorenzo IM, Liedtke W, Sanderson MJ, Valverde MA. TRPV4 channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelial cells. *Proc Natl Acad Sci U S A*. 2008;105:12611–12616.

Macintyre N, Crapo RO, Viegi G, et al. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. *Eur Respir J*. 2005;26:720–735.

Malo JL, Chan-Yeung M. Agents causing occupational asthma. *J Allergy Clin Immunol*. 2009;123:545–550.

Maris NA, Dessing MC, de Vos AF, et al. Toll-like receptor mRNA levels in alveolar macrophages after inhalation of endotoxin. *Eur Respir J*. 2006;28:622–626.

Martelli AM, Evangelisti C, Follo MY, et al. Targeting the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in cancer stem cells. *Curr Med Chem*. 2011;18(18):2715–2126.

Mauderly JL. Usefulness of animal models for predicting human responses to long-term inhalation of particles. *Chest*. 1996;109(3 suppl):65S–68S.

Meadors M, Floyd J, Perry MC. Pulmonary toxicity of chemotherapy. *Semin Oncol*. 2006;33:98–105.

Mehendale HM, Angevine LS, Ohmiya Y. The isolated perfused lung—a critical evaluation. *Toxicology*. 1981;21:1–36.

Mercer RR, Crapo JD. Architecture of the acinus. In: Parent RA, ed. *Treatise on Pulmonary Toxicology: Comparative Biology of the Normal Lung*. Boca Raton, FL: CRC Press; 1991:109–120.

Meyer M, Piiper J. Nitric oxide (NO), a new test gas for study of alveolar-capillary diffusion. *Eur Respir J*. 1989;2:494–496.

Miller I, Eberini I, Gianazza E. Proteomics of lung physiopathology. *Proteomics*. 2008;8:5053–5073.

Minelli C, Wei I, Sagoo G, Jarvis D, Shaheen S, Burney P. Interactive effects of antioxidant genes and air pollution on respiratory function and airway disease: a HuGE Review. *Am J Epidemiol*. 2011;173:603–620.

Miravitlles M. Cough and sputum production as risk factors for poor outcomes in patients with COPD. *Respir Med*. 2011;105:1118–1128.

Mohr S, Leikauf GD, Keith G, Rihn BH. Microarrays as cancer keys: an array of possibilities. *J Clin Oncol*. 2002;20:3165–3175.

Morgan DL, Flake GP, Kirby PJ, Palmer SM. Respiratory toxicity of diacetyl in C57BL/6 mice. *Toxicol Sci*. 2008;103:169–180.

Morris JB. Uptake of styrene in the upper respiratory tract of the CD mouse and Sprague-Dawley rat. *Toxicol Sci*. 2000;54:222–228.

Morris JB. Overview of upper respiratory tract vapor uptake studies. *Inhal Toxicol*. 2001;13:335–345.

Morris JB, Buckpitt AR. Upper respiratory tract uptake of naphthalene. *Toxicol Sci*. 2009;111:383–391.

Morris JB, Symanowicz PT, Olsen JE, et al. Immediate sensory nerve-mediated respiratory responses to irritants in healthy and allergic airway-diseased mice. *J Appl Physiol*. 2003;94:1563–1571.

Morrow PE. Possible mechanisms to explain dust overloading of the lungs. *Fundam Appl Toxicol*. 1988;10:369–384.

Morrow PE. Dust overloading in the lungs: update and appraisal. *Toxicol Appl Phamacol*. 1992;113:1–12.

Morrow PE, Mehrhof E, Casarett LJ, Morken DA. An experimental study of aerosol deposition in human subjects. *AMA Arch Ind Health*. 1958;18:292–298.

Mossman BT, Lippmann M, Hesterberg TW, Kelsey KT, Barchowsky A, Bonner JC. Pulmonary endpoints (lung carcinomas and asbestosis) following inhalation exposure to asbestos. *Toxicol Environ Health B Crit Rev*. 2011;14:76–121.

Mudd JB, Leavitt R, Ongun A, McManus TT. Reaction of ozone with amino acids and proteins. *Atmos Environ*. 1969;3:669–682.

Mukherjee AB, Kundu GC, Mantile-Selvaggi G, et al. Uteroglobin: a novel cytokine? *Cell Mol Life Sci*. 1999;55:771–787.

Munger JS, Sheppard D. Cross talk among TGF-β signaling pathways, integrins, and the extracellular matrix. *Cold Spring Harb Perspect Biol*. 2011;3:a005017.

Murphy SD, Klingshirn DA, Ulrich DE Respiratory response of guinea pigs during acrolein inhalation and its modification by drugs. *J Pharmacol Exp Ther*. 1963;141:79–83.

Murray PJ, Allen JE, Biswas SK, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. 2014;41:14–20.

Nadel JA, Comroe JH Jr. Acute effects of inhalation of cigarette smoke on airway conductance. *J Appl Physiol*. 1961;16:713–716.

National Lung Screening Trial Research Team; Aberle DR, Adams AM, Berg CD, et al. Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med*. 2011;365:395–409.

Nebert DW, Dalton TP. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nat Rev Cancer*. 2006;6:947–960.

Nei M, Niimura Y, Nozawa M. The evolution of animal chemosensory receptor gene repertoires: roles of chance and necessity. *Nat Rev Genet*. 2008;9:951-963.

Nelson N. Some toxicologic aspects of atmospheric pollution. *Am J Public Health Nations Health*. 1955;145:1289–1301.

Nielsen GD, Wolkoff P, Alarie Y. Sensory irritation: risk assessment approaches. *Regul Toxicol Pharmacol*. 2007;48:6–18.

Niimura Y, Nei M. Evolutionary dynamics of olfactory and other chemosensory receptor genes in vertebrates. *J Hum Genet*. 2006;51:505–517.

Oberdörster G, Cox C, Gelein R. Intratracheal instillation versus intratracheal-inhalation of tracer particles for measuring lung clearance function. *Exp Lung Res*. 1997;23:17–34.

Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect*. 2005;113:823–839.

Oliver T. Dust as a cause of occupational disease (Chapter 17). In: Oliver T, ed. *Dangerous Trades. The Historical, Social, Legal Aspects of Industrial Occupations as Affecting Health, by a Number of Medical Experts*. London: John Murray; 1902.

Ortiz LA, Gambelli F, McBride C, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A*. 2003;100:8407–8411.

Ortiz LA, Lasky J, Lungarella G, et al. Upregulation of the p75 but not the p55 TNF-alpha receptor mRNA after silica and bleomycin exposure and protection from lung injury in double receptor knockout mice. *Am J Respir Cell Mol Biol*. 1999;20:825–833.

Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity*. 2011;34(5):651–664.

Ouadrhiri Y, Pilette C, Monteiro RC, Vaerman JP, Sibille Y. Effect of IgA on respiratory burst and cytokine release by human alveolar macrophages: role of ERK1/2 mitogen-activated protein kinases and NF-kappaB. *Am J Respir Cell Mol Biol*. 2002;26:315–332.

Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*. 2007;87:315–424.

Palm PE, McNernery JM, Hatch T. Respiratory dust retention in small animals; a comparison with man. *AMA Arch Ind Health*. 1956;13:355–365.

Palma AS, Feizi T, Zhang Y, et al. Ligands for the beta-glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. *J Biol Chem*. 2006;281:5771–5779.

Palmer SM, Flake GP, Kelly FL, et al. Severe airway epithelial injury, aberrant repair and bronchiolitis obliterans develops after diacetyl instillation in rats. *PLoS One*. 2011;6:e17644.

Palmes ED, Nelson N, Laskin S, Kuschner M. Inhalation toxicity of cobalt hydrocarbonyl. *Am Ind Hyg Assoc J*. 1959;20:453–468.

Pandis S. Atmospheric aerosol processes (Chapter 3). In: McMurry PH, Shepard M, Vickery JS, eds. *Particulate Matter Science for Policy Makers: A NARSTO Assessment*. Cambridge, UK: Cambridge University Press; 2004:103–107.

Parkinson OT, Liggitt HD, Rettie AE, Kelly EJ. Generation and characterization of a Cyp4b1 null mouse and the role of CYP4B1 in the activation and toxicity of Ipomeanol. *Toxicol Sci*. 2013;134:243–250.

Patel AC, Brett TJ, Holtzman MJ. The role of CLCA proteins in inflammatory airway disease. *Annu Rev Physiol*. 2009;71:425–449.

Patel JM, Block ER. Cyclophosphamide-induced depression of the antioxidant defense mechanisms of the lung. *Exp Lung Res*. 1985;8:153–165.

Patterson CE, Rhoades RA, Garcia JG. Evans blue dye as a marker of albumin clearance in cultured endothelial monolayer and isolated lung. *J Appl Physiol*. 1992;72:865–873.

Patty FA. Industrial hygiene and its relation to medicine. *Ind Med Surg*. 1949;18:225–228.

Pauluhn J. Overview of inhalation exposure techniques: strengths and weaknesses. *Exp Toxicol Pathol*. 2005;57(suppl 1):111–128.

Pauwels R, Rabe K. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet*. 2004;364:613–620.

Phalen RF. Inhalation exposure of animals. *Environ Health Perspect*. 1976;16:17–24.

Phalen RF, Mannix RC, Drew RT. Inhalation exposure methodology. *Environ Health Perspect*. 1984;56:23–34.

Phalen RF, Oldham MJ, Wolff RK. The relevance of animal models for aerosol studies. *J Aerosol Med Pulm Drug Deliv*. 2008;21:113–124.

Piguet PF, Collart MA, Grau GE, Sappino AP, Vassalli P. Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. *Nature*. 1990;344:245–247.

Pinkerton KE, Gehr P, Crapo JD. Architecture and cellular composition of the air-blood barrier. In: Parent RA, ed. *Treatise on Pulmonary Toxicology: Comparative Biology of the Normal Lung*. Boca Raton, FL: CRC Press; 1991:121–128.

Plopper CG, Chang AM, Pang A, Buckpitt AR. Use of microdissected airways to define metabolism and cytotoxicity in murine bronchiolar epithelium. *Exp Lung Res*. 1991;17:197–212.

Plopper CG, Van Winkle LS, Fanucchi MV, et al. Early events in naphthalene-induced acute Clara cell toxicity: II. Comparison of glutathione depletion and histopathology by airway location. *Am J Resp Cell Mol Biol*. 2001;24:272–281.

Plopper CG, Weir AJ, Nishio SJ, et al. Elevated susceptibility to 4-ipomeanol cytotoxicity in immature Clara cells of neonatal rabbits. *J Pharmacol Exp Ther*. 1994;269:867–880.

Pope CA 3rd, Burnett RT, Thun MJ, et al. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. *JAMA*. 2002;287:1132–1141.

Pope CA 3rd, Burnett RT, Turner MC, et al. Lung cancer and cardiovascular disease mortality associated with ambient air pollution and cigarette smoke: shape of the exposure-response relationships. *Environ Health Perspect*. 2011;119:1616–1621.

Popov TA. Human exhaled breath analysis. *Ann Allergy Asthma Immunol*. 2011;106:451–456.

Pryor WA. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ Health Perspect*. 1997;105(suppl 4):875–882.

Putila JJ, Guo NL. Association of arsenic exposure with lung cancer incidence rates in the United States. *PLoS One*. 2011;6:e25886.

Raabe OG. Some important considerations in use of power functions to describe clearance data. *Health Phys*. 1967;13:293–295.

Rabe KF, Hurd S, Anzueto A, et al. Global initiative for chronic obstructive lung disease. global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med*. 2007;176:532–555.

Rawlins EL, Okubo T, Xue Y, et al. The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem Cell*. 2009;4:525–534.

Rayamajhi M, Redente EF, Condon TV, Gonzalez-Juarrero M, Riches DW, Lenz LL. Non-surgical intratracheal instillation of mice with analysis of lungs and lung draining lymph nodes by flow cytometry. *J Vis Exp*. 2011;51: pii: 2702.

Reddel RR, Ke Y, Gerwin BI, et al. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res*. 1988;48:1904–1909.

Reid L. Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis. *Thorax*. 1960;15:132–141.

Reinhard C, Meyer B, Fuchs H, et al. Genomewide linkage analysis identifies novel genetic Loci for lung function in mice. *Am J Respir Crit Care Med*. 2005;171:880–888.

Rennard SI, Spurzen JR. Methods for evaluating the lung in human subjects. In: Gardner DE, ed. *Toxicology of the Lung*. 4th ed. Boca Raton, FL: CRC Press, Taylor & Francis; 2006:1–28.

Reynolds HY. Bronchoalveolar lavage and other methods to define the human respiratory tract milieu in health and disease. *Lung*. 2011;189:87–99.

Reynolds JS, Johnson VJ, Frazer DG. Unrestrained acoustic plethysmograph for measuring specific airway resistance in mice. *J Appl Physiol*. 2008;105:711–717.

Reynolds SD, Hong KU, Giangreco A, et al. Conditional Clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. *Am J Physiol Lung Cell Mol Physiol*. 2000;278:L1256-1263.

Rhoades RA. Isolated perfused lung preparation for studying altered gaseous environments. *Environ Health Perspect*. 1984;56:43–50.

Robson WD, Irwin DA, King EJ. Experimental silicosis: quartz, sericite and irritating gases. *Can Med Assoc J*. 1934;31:237–245.

Rock JR, Hogan BL. Epithelial progenitor cells in lung development, maintenance, repair, and disease. *Annu Rev Cell Dev Biol*. 2011;27:4.1–4.20.

Rodig SJ, Shapiro GI. Crizotinib, a small-molecule dual inhibitor of the c-Met and ALK receptor tyrosine kinases. *Curr Opin Investig Drugs*. 2010;11:1477–1490.

Rogers DF. Mucoactive agents for airway mucus hypersecretory diseases. *Respir Care*. 2007;52:1176–1193.

Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev*. 2006;86:245–278.

Rowe VK, McCollister DD, Spencer HC, Adams EM, Irish DD. Vapor toxicity of tetrachloroethylene for laboratory animals and human subjects. *AMA Arch Ind Hyg Occup Med*. 1952;5:566–579.

Russell R. *London Fogs*. London: Edward Stanford; 1880.

Rylander R. Environmental air pollutants and lung defense to airborne bacteria. *Aspen Emphysema Conf*. 1968;11:297–304.

Salam MT, Lin PC, Avol EL, Gauderman WJ, Gilliland FD. Microsomal epoxide hydrolase, glutathione S-transferase P1, traffic and childhood asthma. *Thorax*. 2007;62:1050–1057.

Salathe M. Regulation of mammalian ciliary beating. *Annu Rev Physiol*. 2007;69:401–422.

Sallenave JM. Secretory leukocyte protease inhibitor and elafin/trappin-2: versatile mucosal antimicrobials and regulators of immunity. *Am J Respir Cell Mol Biol*. 2010;42:635–643.

Salvi SS, Barnes PJ. Chronic obstructive pulmonary disease in non-smokers. *Lancet*. 2009;374:733–743.

Sanderson MJ, Sleigh MA. Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. *J Cell Sci*. 1981;47:331–347.

Sato M, Shames DS, Gazdar AF, Minna JD. A translational view of the molecular pathogenesis of lung cancer. *J Thorac Oncol*. 2007;2:327–343.

Schattgen SA, Fitzgerald KA. The PYHIN protein family as mediators of host defenses. *Immunol Rev*. 2011;243:109–118.

Schmid A, Salathe M. Ciliary beat co-ordination by calcium. *Biol Cell*. 2011;103:159–169.

Schwartz DR, Homanics GE, Hoyt DG, Klein E, Abernethy J, Lazo JS. The neutral cysteine protease bleomycin hydrolase is essential for epidermal integrity and bleomycin resistance. *Proc Natl Acad Sci U S A*. 1999;96:4680–4685.

Schweigert MK, Mackenzie DP, Sarlo K. Occupational asthma and allergy associated with the use of enzymes in the detergent industry–a review of the epidemiology, toxicology and methods of prevention. *Clin Exp Allergy*. 2000;30:1511–1518.

Selikoff IJ, Churg J, Hammond EC. Relation between exposure to asbestosis and mesothelioma. *N Engl J Med*. 1965;272:560–565.

Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg GI, Welgus HG. Human 92- and 72-kilodalton type IV collagenases are elastases. *J Biol Chem*. 1991;266:7870–7875.

Shah AS, Ben-Shahar Y, Moninger TO, Kline JN, Welsh MJ. Motile cilia of human airway epithelia are chemosensory. *Science*. 2009;325:1131–1134.

Shakeri MS, Dick FD, Ayres JG. Which agents cause reactive airways dysfunction syndrome (RADS)? A systematic review. *Occup Med (Lond)*. 2008;58:205–211.

Shapiro SD, Goldstein NM, Houghton AM, et al. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am J Pathol*. 2003;163:2329–2335.

Shapiro SD, Ingenito EP. The pathogenesis of chronic obstructive pulmonary disease: advances in the past 100 years. *Am J Respir Cell Mol Biol*. 2005;32:367–372.

Shehin-Johnson SE, Williams DE, Larsen-Su S, Stresser DM, Hines RN. Tissue specific expression of flavin-containing monooxygenase (FMO) forms 1 and 2 in the rabbit. *J Pharmacol Exp Ther*. 1995;272:1293–1299.

Sheppard D. Roles of alphav integrins in vascular biology and pulmonary pathology. *Curr Opin Cell Biol*. 2004;16:552–557.

Sheppard D, Wong WS, Uehara CF, Nadel JA, Boushey HA. Lower threshold and greater bronchomotor responsiveness of asthmatic subjects to sulfur dioxide. *Am Rev Respir Dis*. 1980;122:873–878.

Shi X, Zhou S, Wang Z, Zhou Z, Wang Z. CYP1A1 and GSTM1 polymorphisms and lung cancer risk in Chinese populations: a meta-analysis. *Lung Cancer*. 2008;59:155–163.

Shultz MA, Choudary PV, Buckpitt AR. Role of murine cytochrome P-450 2F2 in metabolic activation of naphthalene and metabolism of other xenobiotics. *J Exp Ther*. 1999;290:281–288.

Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin*. 2011;61:212–236.

Silverman L, Lee RC. A low-resistance valve and indicating flowmeter for respiratory measurements. *Science*. 1946;103:537–539.

Silverman L, Whittenberger JL. Blood changes due to ammonia inhalation? *Science*. 1949;109:121–122.

Sinnett EE, Jackson AC, Leith DE, Butler JP. Fast integrated flow plethysmograph for small mammals. *J Appl Physiol*. 1981;50:1104–1110.

Sleiman M, Logue JM, Montesinos VN, et al. Emissions from electronic cigarettes: key parameters affecting the release of harmful chemicals. *Environ Sci Technol*. 2016;50:9644–9651.

Slocum SL, Kensler TW. Nrf2: control of sensitivity to carcinogens. *Arch Toxicol*. 2011;85:273–284.

Smith BJ, Curtis JF, Eling TE. Bioactivation of xenobiotics by prostaglandin H synthase. *Chem Biol Interact*. 1991;79:245–264.

Smyth HF Jr. Solving the problem of the toxicity of new chemicals in industry. *W V Med J*. 1946;42:177.

Snider GL, Kleinerman J, Thurlbeck WM, Bengali ZH. The definition emphysema: report of a National Heart, Lung, and Blood Institute workshop. *Am Rev Respir Dis*. 1985;132:182–185.

Squadrito GL, Pryor WA. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radic Biol Med*. 1998;25:392–403.

Sterk PJ, Bel EH. Bronchial hyperresponsiveness: the need for a distinction between hypersensitivity and excessive airway narrowing. *Eur Respir J*. 1989;2:267–274.

Stokinger HE. Air pollution and the particle size-toxicity problem. *Nucleonics*. 1949;5:50.

Stokinger HE, Sprague GF 3rd, Hall Rh, Ashenburg JJ, Scott JK, Steadman LT. Acute inhalation toxicity of beryllium: four definitive studies of beryllium sulfate at exposure concentrations of 100, 50, 10, and 1 mg per cubic meter. *Arch Ind Hyg Occup Med*. 1950;1:379–397.

Stripp BR, Lund J, Mango GW, et al. Clara cell secretory protein: a determinant of PCB bioaccumulation in mammals. *Am J Physiol*. 1996;271:L656-64.

Sun S, Schiller JH, Gazadar AF. Lung cancer in never smokers—a different disease. *Nat Rev Cancer*. 2007;7:778–790.

Sutterwala FS, Ogura Y, Flavell RA. The inflammasome in pathogen recognition and inflammation. *J Leukoc Biol*. 2007;82:259–264.

Swiecichowski AL, Long KJ, Miller ML, Leikauf GD. Formaldehyde-induced airway hyperreactivity in vivo and ex vivo in guinea pigs. *Environ Res*. 1993;61:185–199.

Syamlal G, Jamal A, King BA, Mazurek JM. Electronic cigarette use among working adults—United States, 2014. *MMWR Morb Mortal Wkly Rep*. 2016;65:557–561.

Szarka RJ, Wang N, Gordon L, Nation PN, Smith RH. A murine model of pulmonary damage induced by lipopolysaccharide via intranasal instillation. *J Immunol Methods*. 1997;202:49–57.

Tamura HO, Harada Y, Miyawaki A, Mikoshiba K, Matsui M. Molecular cloning and expression of a cDNA encoding an olfactory-specific mouse phenol sulphotransferase. *Biochem J*. 1998;331:953–958.

Tang MS, Wang HT, Hu Y, et al. Acrolein induced DNA damage, mutagenicity and effect on DNA repair. *Mol Nutr Food Res*. 2011;55:1291–1300.

Tegtmeyer PJ, Craighead JE. Infection of adult mouse macrophages in vitro with cytomegalovirus. *Proc Soc Exp Biol Med*. 1968;129:690–694.

Ter-Minassian M, Asomaning K, Zhao Y, et al. Genetic variability in the metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). *Int J Cancer*. 2011;130:1338–1346.

Thackrah C. *The Effects of Arts, Trades, and Professions, and of Civic States and Habits of Living, on Health and Longevity*. 2nd ed. London: Longman; 1832.

Thorton-Manning JR, Dahl AR. Metabolic capacity of nasal tissue interspecies comparisons of xenobiotic-metabolizing enzymes. *Mutat Res*. 1997;380:43–59.

Touhara K, Vosshall LB. Sensing odorants and pheromones with chemosensory receptors. *Annu Rev Physiol*. 2009;71:307–332.

Travassos LH, Carneiro LA, Girardin SE, et al. Nod1 participates in the innate immune response to *Pseudomonas aeruginosa*. *J Biol Chem*. 2005;280:36714–36718.

Travassos LH, Girardin SE, Philpott DJ, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep*. 2004;5:1000–1006.

Tyler WS, Pearse AG. Oxidative enzymes of the interalveolar spetum of the rat. *Thorax*. 1965;20:149–152.

Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol*. 2010;28:1248–1250.

Upadhyay D, Kamp DW. Asbestos-induced pulmonary toxicity: role of DNA damage and apoptosis. *Exp Biol Med*. 2003;228:650–659.

Utell MJ, Frampton MW. Toxicologic methods: controlled human exposures. *Environ Health Perspect*. 2000;108(suppl 4):605–613.

Vallyathan V, Castranova V, Pack D, et al. Freshly fractured quartz inhalation leads to enhanced lung injury and inflammation. Potential role of free radicals. *Am J Respir Crit Care Med*. 1995;152:1003–1009.

Van Lommel A. Pulmonary neuroendocrine cells (PNEC) and neuroendocrine bodies (NEB): chemoreceptors and regulators of lung development. *Paediatr Respir Res*. 2001;2:171–176.

van Lookeren Campagne M, Wiesman C, Brown EJ. Macrophage complement receptors and pathogen clearance. *Cell Microbiol*. 2007;9:2095–2102.

Van Rooy FG, Rooyackers JM, Prokop M, et al. Heederik Bronchiolitis obliterans syndrome in chemical workers producing diacetyl for food flavorings. *Am J Respir Crit Care Med*. 2007;176:498–504.

Vaughan MB, Ramirez RD, Wright WE, Minna JD, Shay JW. A three-dimensional model of differentiation of immortalized human bronchial epithelial cells. *Differentiation*. 2006;74:141–148.

Vijayaraghavan R, Schapper M, Thompson R, Stock MF, Alarie Y. Characteristic modification of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract. *Arch Toxicol*. 1993;67:478–490.

Vinegar A, Sinnett EE, Leith DE. Dynamic mechanisms determine functional residual capacity in mice, *Mus musculus. J Appl Physiol*. 1979;46: 867–871.

Visel A, Thaller C, Eichele G. GenePaint.org: an atlas of gene expression patterns in the mouse embryo. *Nucleic Acids Res*. 2004;32(database issue):D552–D556.

Wade WA, Petsonk EL, Young B, Mogri I. Severe occupational pneumoconiosis among West Virginian coal miners: one hundred thirty-eight cases of progressive massive fibrosis compensated between 2000 and 2009. *Chest*. 2011;139:1458–1462.

Wang J, Edeen K, Manzer R, et al. Differentiated human alveolar epithelial cells and reversibility of their phenotype in vitro. *Am J Respir Cell Mol Biol*. 2007;36:661–668.

Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med*. 2000;342:1334–1339.

Ware LB, Matthay MA. Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair. *Am J Physiol Lung Cell Mol Physiol*. 2002;282:L924–L940.

Warren DL, Brown D Jr., Buckpitt AR. Evidence for cytochrome P-450 mediated metabolism in the bronchiolar damage by naphthalene. *Chem Biol Interact*. 1982;40: 287–303.

Watson TM, Reynolds SD, Mango GW, Boe IM, Lund J, Stripp BR. Altered lung gene expression in CCSP-null mice suggests immunoregulatory roles for Clara cells. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L1523–L1530.

Weibel ER. Is the lung built reasonably? The 1983 J. Burns Anderson lecture. *Am Rev Respir Dis*. 1983;128:752–760.

Weigt SS, DerHovanessian A, Wallace WD, Lynch JP 3rd, Belperio JA. Bronchiolitis obliterans syndrome: the Achilles′ heel of lung transplantation. *Semin Respir Crit Care Med*. 2013;34:336–351.

Weiss RB, Poster DS, Penta JS. The nitrosoureas and pulmonary toxicity. *Cancer Treat Rev*. 1981;8:111–124.

Wesselkamper SC, Case LM, Henning LN, et al. Gene expression changes during the development of acute lung injury: role of transforming growth factor beta. *Am J Respir Crit Care Med*. 2005;172:1399–1411.

West JA, Chichester CH, Buckpitt AR, et al. Heterogeneity of Clara cell glutathione: a possible basis for differences in cellular responses to pulmonary cytotoxicants. *Am J Respir Cell Mol Biol*. 2000;23:27–36.

Whetstine JR, Yueh MF, McCarver DG, et al. Ethnic differences in human flavin-containing monooxygenase 2 (FMO2) polymorphisms: detection of expressed protein in African-Americans. *Toxicol Appl Pharmacol*. 2000;168:216–224.

Whitby-Logan GK, Weech M, Walters E. Zonal expression and activity of glutathione S-transferase enzymes in the mouse olfactory mucosa. *Brain Res*. 2004;995:151–157.

Whitcutt MJ, Adler KB, Wu R. A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. *In Vitro Cell Dev Biol*. 1988;24:420–428.

Whitsett JA, Wert SE, Weaver TE. Alveolar surfactant homeostasis and the pathogenesis of pulmonary disease. *Annu Rev Med*. 2010;61:105–119.

Widdicombe JH, Welsh MJ. Ion transport by dog tracheal epithelium. *Fed Proc*. 1980;39:3062–3066.

Williams MC. Alveolar type I cells: molecular phenotype and development. *Annu Rev Physiol*. 2003;65:669–695.

Winkelmann A, Noack T. The Clara cell: a "Third Reich eponym"? *Eur Respir J*. 2010;36:722–727.

Wistuba II, Gazdar AF. Characteristic genetic alterations in lung cancer. In: Driscoll B, ed. *Lung Cancer Volume 1: Molecular Pathology Methods and Reviews*. Totowa, NJ: Humana Press; 2003:3–28.

Witschi H. Fritz Haber: December 9, 1868-January 29, 1934. *Toxicology*. 2000;149:3–15.

Witschi HP, Aldridge WN. Uptake, distribution and binding of beryllium to organelles of the rat liver cell. *Biochem J*. 1968;106:811–820.

Wong BA. Inhalation exposure systems: design, methods and operation. *Toxicol Pathol*. 2007;35:3–14.

Woolcock AJ, Salome CM, Yan K. The shape of the dose-response curve to histamine in asthmatic and normal subjects. *Am Rev Respir Dis*. 1984;130:71–75.

World Health Organization. *Fuel for Life: Household Energy and Health*. Geneva, Switzerland: WHO Press; 2006.

Wu M, Kelley MR, Hansen WK, Martin WJ. Reduction of BCNU toxicity to lung cells by high-level expression of O(6)-methylguanine-DNA methyltransferase. *Am J Physiol Lung Cell Mol Physiol*. 2001;280:L755–L761.

Wynn TA. Integrating mechanisms of pulmonary fibrosis. *J Exp Med*. 2011;208:1339–1350.

Xiang M, Fan J. Pattern recognition receptor-dependent mechanisms of acute lung injury. *Mol Med*. 2010;16:69–82.

Xun WW, Brennan P, Tjonneland A, et al. Single-nucleotide polymorphisms (5p15.33, 15q25.1, 6p22.1, 6q27, and 7p15.3) and lung cancer survival in the European Prospective Investigation into Cancer and Nutrition (EPIC). *Mutagenesis*. 2011;26:657–666.

Yasuda S, Yasuda T, Liu MY, et al. Sulfation of chlorotyrosine and nitrotyrosine by human lung endothelial and epithelial cells: role of the human SULT1A3. *Toxicol Appl Pharmacol*. 2011;251:104–109.

Yoshida K, Kasama K. Biotransformation of nitric oxide. *Environ Health Perspect*. 1987;73:201–206.

Young JM, Waters H, Dong C, Fülle HJ, Liman ER. Degeneration of the olfactory guanylyl cyclase D gene during primate evolution. *PLoS One*. 2007;2:e884.

Young WA, Shaw DB, Bates DV. Effects of low concentration of ozone on pulmonary function in man. *J Appl Physiol*. 1964;19:765–768.

Yuan JM, Koh WP, Murphy SE, et al. Urinary levels of tobacco-specific nitrosaminemetabolites in relation to lung cancer development in two prospective cohorts of cigarette smokers. *Cancer Res*. 2009;69:2990–2995.

Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med*. 2002;33:337–349.

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chapter 16

Toxic Responses of the Nervous System

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Acknowledgments

References

OVERVIEW OF THE NERVOUS SYSTEM

Neurotoxicants and neurotoxins have been extensively studied because of their toxic effects on humans and their utility in the study of the nervous system (NS). Many insights into the organization and function of the NS are based on observations derived from the action of neurotoxicants. The binding of exogenous compounds to membranes has been the basis for the definition of specific receptors within the brain; an understanding of the roles of different cell types in the function of the NS has stemmed from the selectivity of certain toxicants in injuring specific cell types while sparing others, and important differences in basic metabolic requirements of different subpopulations of neurons have been inferred from the effects of neurotoxicants.

It is estimated that millions of people worldwide are exposed to known neurotoxicants each year, contributing to a dramatic increase over time of neurological diseases and associated deaths (Pritchard and Rosenorn-Lanng, 2015). An even larger potential problem stems from the incomplete information on many compounds that may have neurotoxic effects. The extent to which neurological disability may be related to chronic low-level chemical exposures is currently unknown; moreover, we do not understand the overall impact of environmental contaminants on brain function.

In order to study neurotoxicological consequences of chemical exposures, one must understand the structure, function, and development of the NS. These features can be quite complex, with differential anatomy, physiology, and cell types specific for location and function. Several general aspects modulate the NS response to chemicals, including (1) the privileged status of the NS with the maintenance of a biochemical barrier between the brain and the blood, (2) the importance of the high-energy requirements of the brain, (3) the spatial extensions of the NS as long cellular processes and the requirements of cells with such a complex geometry, (4) the maintenance of an environment rich in lipids, (5) the transmission of information across extracellular space at the synapse, (6) the distances over which electrical impulses must be transmitted, coordinated, and integrated, and (7) development and regenerative patterns of the NS. Each of these features of the NS carries with it specialized metabolic/physiological requirements and unique vulnerabilities to toxic compounds.

Blood–Brain Barrier

The NS is protected from the adverse effects of many potential toxicants by a functional and anatomic barrier. In 1885, Ehrlich noticed that certain dyes did not distribute into the brain and spinal cord, whereas other tissues became stained. Conversely, when injected into the brain, the dye did not appear in the periphery. This observation pointed to the existence of an interface between the blood and the brain, or a "blood–brain barrier." Most of the brain, spinal cord, retina, and peripheral NS (PNS) maintain this barrier with the blood, with selectivity similar to the interface between cells and the extracellular space. The principal basis of the blood–brain barrier is thought to be specialized endothelial cells in the brain's microvasculature, aided, at least in part, by interactions with glia (Kniesel and Wolburg, 2000). In addition to this interface with blood, the brain, spinal cord, and peripheral nerves are also completely covered with a continuous lining of specialized cells that limits the entry of molecules from adjacent tissue. In the brain and spinal cord, this is the meningeal surface; in peripheral nerves, each fascicle of nerve is surrounded by perineurial cells.

Among the unique properties of NS endothelial cells is the presence of tight junctions between cells (Rubin and Staddon, 1999; Kniesel and Wolburg, 2000). Thus, molecules must pass through membranes of endothelial cells, rather than between them, as they do in other tissues. The blood–brain barrier also contains transporters, such as the multidrug-resistant protein, which transport some xenobiotics that have diffused into endothelial cells and back into the blood. If not actively transported into the brain, the penetration of toxicants is largely related to their lipid solubility and to their ability to pass through the plasma membranes of the cells forming the barrier (Pardridge, 1999; Stewart, 2000). There are, however, important exceptions to this general rule. In the mature NS, the spinal and autonomic ganglia as well as a small number of other sites within the brain, called circumventricular organs, do not contain specialized endothelial tight junctions and are not protected by blood–tissue barriers. Rather, a somewhat less tight barrier is provided by several layers of overlapping astrocytic foot processes. Indeed, it is this cellular anatomical arrangement that allows the endocrine-regulating components of the circumventricular organs to sense changes in blood hormone levels and respond accordingly. This discontinuity of the barrier allows entry of some chemicals, for example, the anticancer drug doxorubicin, into the sensory ganglia. This is the basis for the selective neurotoxicity of this compound to ganglionic neurons (Spencer, 2000). The blood–brain barrier is incompletely developed at birth and even less so in premature infants. This predisposes the premature infant to brain injury by toxicants, such as unconjugated bilirubin or hexachlorophene, that later in life are excluded from the NS (Lucey et al., 1964; Mullick, 1973).

Energy Requirements

Neurons and cardiac myocytes share the property of conduction of electrical impulses, and their critical dependence on aerobic respiration is due to the high metabolic demand associated with the maintenance and repetitive reinstitution of ion gradients. Neuronal cells are also postmitotic, making energy homeostasis critical to normal functioning of the NS. Membrane depolarizations and repolarizations occur with such frequency that these cells must be able to produce large quantities of high-energy phosphates even in a resting state. That the energy requirements of the brain are related to membrane depolarizations is supported by the fact that hyperactivity, as in epileptic foci, increases the energy requirements by as much as fivefold (Plum and Posner, 1985). The dependence on a continual source of energy, in the absence of energy reserves, places the neuron in a vulnerable position. To meet these high-energy requirements, the brain utilizes aerobic glycolysis and, therefore, is extremely sensitive to even brief interruptions in the supply of oxygen or glucose.

Exposure to toxicants that inhibit aerobic respiration (e.g., cyanide) or to conditions that produce hypoxia (e.g., carbon monoxide, or CO, poisoning) leads to the early signs of dysfunction in the myocardium and neurons. Damage to the NS under these conditions is a combination of direct toxic effects on neurons and secondary damage from systemic hypoxia or ischemia. For example, acute CO poisoning damages those structures in the central nervous system (CNS) that are most vulnerable to hypoxia: the neurons in specific regions of the basal ganglia and hippocampus, certain layers of the cerebral cortex, and the cerebellar Purkinje cells. Experiments utilizing several different laboratory animal species have shown that systemic hypotension is the best predictor of these lesions following CO poisoning; however, CO poisoning also may produce white matter damage, and this leukoencephalopathy may result from a primary effect of CO in the CNS (Penny, 1990). As with CO, survivors of cyanide poisoning may develop lesions in the CNS that are characteristic of systemic hypoxic or ischemic injury, and experiments in rats and monkeys have led to the conclusion that global hypoperfusion, rather than direct histotoxicity, is the major cause of CNS damage (Auer and Benveniste, 1997).

3-Nitropropionic acid (3-NP), a naturally occurring mycotoxin, is an irreversible inhibitor of succinate dehydrogenase that results in adenosine triphosphate (ATP) depletion in cerebral cortical explants and is associated with motor disorders in livestock and humans that have ingested contaminated food (Ludolph et al., 1991, 1992). Some investigators removed the complication of systemic toxicity by directly injecting 3-NP into specific regions of the brain. They have observed neuron degeneration mediated in part by excitotoxic mechanisms (Brouillet et al., 1993a). These examples demonstrate the exquisite sensitivity of neurons to energy depletion and also underscore the complex relationships between direct neurotoxicity and the effects of systemic toxicity on the NS.

Axonal Transport

In the NS, impulses are conducted over great distances at rapid speed. These impulses provide information about the environment to the organism in a coordinated manner that allows an organized response to be carried out at a specific site. However, the intricate organization of such a complex network places an unparalleled demand on the cells of the NS. Single cells, rather than being spherical and a few micrometers in diameter, are elongated and may extend over a meter in length.

The anatomy of such a complex cellular network creates features of metabolism and cellular geometry that are peculiar to the NS. The two immediate demands placed on the neuron are the maintenance of a larger cellular volume and the transport of intracellular materials over great distances. The length of neurons may exceed 200,000 times the dimensions of most other cells. For example, the cell body of a lower motor neuron is located in the spinal cord and the axon extends to the site of innervation of a muscle at a distant location. In spite of the smaller diameter of the axon, the length of the axon translates to an axonal volume that is hundreds of times greater than that of the cell body itself (Schwartz, 1991). The cellular machinery that provides protein synthesis to maintain this volume is readily visible in large neurons through the light microscope as the Nissl substance, which is formed by clusters of ribosomal complexes for the synthesis of proteins (Parent, 1996). In fact, neurons are the only cell type with such a Nissl substance, reflecting the unusual demand for protein synthesis. Axonal microtubules are

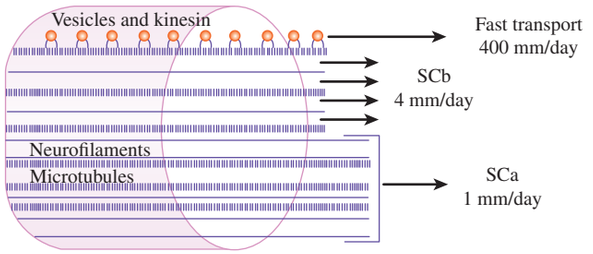


Figure 16-1. *Axonal transport.* Fast axonal transport is depicted as spherical vesicles moving along microtubules with intervening microtubule-associated motors. The slow component A (SCa) represents the movement of the cytoskeleton, composed of neurofilaments and microtubules. Slow component B (SCb) moves at a faster rate than SCa and includes soluble proteins, which are apparently moving between the more slowly moving cytoskeleton.

essential for transport of materials to and from the synapse, and the stability of microtubules is maintained by the binding of the microtubule-associated protein tau (Cleveland et al., 1977).

In addition to the increased burden of protein synthesis, the neuron is dependent on the ability to distribute materials over the distances encompassed by its processes. Protein synthesis occurs in the cell body, and the products are then transported to the appropriate site through the process of axonal transport. The assembly of the cytoskeleton at tremendous distances from their site of synthesis in the cell body represents a formidable challenge (Nixon, 1998). Through studies of the movement of radiolabeled amino acid precursors, several major components of axonal transport are known (Grafstein, 1995). The fastest component, known as fast axonal transport, carries a large number of proteins, many of which are associated with vesicles (Grafstein, 1995). This ATP-dependent process reaches a rate of 400 mm/day (Fig. 16-1) and is dependent on microtubule-associated ATPase activity and motor proteins. Kinesin and dynein are the prototypes of a class of microtubule-associated motors that provide both the mechanochemical force in the form of a microtubule-associated ATPase and the interface between microtubules as the track and vesicles as the cargo. Vesicles are transported rapidly in an anterograde direction by kinesin and in a retrograde direction by dynein (Schnapp and Reese, 1989). In the axon, multiple waves of transport can be detected in the fast component of axonal transport (Mulugeta et al., 2000).

The transport of some organelles, including mitochondria, constitutes an intermediate component of axonal transport, moving at approximately 50 mm/day (Grafstein, 1995). As with the fast component, the function is apparently the continuous replacement of organelles within the axon.

The slowest component of axonal transport represents the movement of the cytoskeleton itself (Fig. 16-1). The cytoskeleton is composed of structural elements, including microtubules formed by the association of tubulin subunits and neurofilaments formed by the association of three neurofilament protein subunits. Dynamic exchange of subunits of the filamentous structure has now been observed with high-resolution microscopy of living cells, indicating that stationary filamentous structures exchange subunits that move rapidly once dissociated (Wang et al., 2000).

Neurofilaments and microtubules move at a rate of approximately 1 mm/day and make up the majority of slow component a (SCa), which is the slowest-moving component of axonal transport (Hoffman and Lasek, 1975). Subunit structures appear to migrate and reassemble in a process that is dependent on nucleoside triphosphates, kinases, and phosphatases (Nixon, 1998; Koehnle and Brown, 1999). Moving at only a slightly more rapid rate of 2 to 4 mm/day in an anterograde direction is slow component b (SCb) (Grafstein, 1995). Included in SCb are several structural proteins, such as the component of microfilaments (actin) and several microfilament-associated proteins (M2 protein and fodrin), as well as clathrin and many soluble proteins.

This continual transport of proteins from the cell body through the various components of axonal transport is the mechanism through which the neuron provides the distal axon with its complement of functional and structural proteins. Some vesicles are also moving in a retrograde direction and provide the cell body with information concerning the status of the distal axon. The evidence for such a dynamic interchange of materials and information stems from not only the biochemical detection of these components of axonal transport but also the observations of the effects of terminating this interchange by severing the axon from its cell body.

The result of transection of an axon is that the distal axon is destined to degenerate, a process known as axonal degeneration, which is unique to the NS. The cell body of the neuron responds to the transection of the axon as well and undergoes a process of chromatolysis. Notably, defects in components of the retrograde transport pathway have been linked to neurodegenerative diseases demonstrating that disruption of the dynein–dynactin motor complex that drives retrograde transport causes motor neuron loss and muscle denervation in a transgenic mouse model (LaMonte et al., 2002). Studies in mice with either point mutations or a small deletion of the dynein heavy chain corroborate the hypothesis that neurons are preferentially susceptible to defects in dynein function, establishing that mutations in the dynein cofactor dynactin cause neurodegenerative sequelae (Perlson et al., 2010).

Axonal Degeneration

Current concepts of axonal degeneration were initially derived from nerve transections reported by Augustus Waller over a hundred years ago. Accordingly, the sequence of events that occurs in the distal stump of an axon following transection is referred to as Wallerian degeneration. Because the axonal degeneration associated with chemicals and some disease states is thought to occur through a similar sequence of events, it is often referred to as Wallerian-like axonal degeneration.

Following axotomy, there is degeneration of the distal nerve stump, followed by generation of a microenvironment supportive of regeneration and involving the distal axon, ensheathing glial cells and the blood nerve barrier. Initially there is a period during which the distal stump survives and maintains relatively normal structural, transport, and conduction properties. The duration of survival is proportional to the length of the axonal stump (Chaudry and Cornblath, 1992), and this relationship appears to be maintained across species.

Wallerian degeneration was long thought to be a passive process that proceeded inexorably after separating the axon from the trophic support provided by the cell body. This thinking was changed by the discovery of the *Wld*^{S} mouse, in which the rate of axonal degeneration triggered by a variety of insults is retarded by about 10-fold (Beirowski et al., 2005). We now know from several lines of evidence that Wallerian degeneration is an active process mediated by the axon itself, and that it is possible to slow or even halt its progression. Moreover, although axonal degeneration can be initiated by many different means, including physical, genetic, or toxic, the mechanisms of degeneration converge into common regulated pathways that are potentially subject to pharmacological intervention (Coleman, 2005; Stys, 2005).

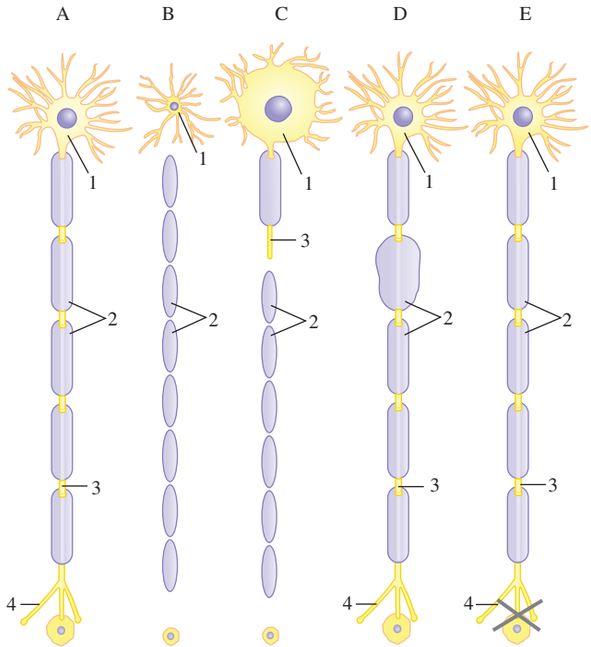


Figure 16-2. *Patterns of neurotoxic injury.* (**A**) Normal neuron showing (1) cell body and dendrites, (2) myelinating cells, encircling the (3) axon, and (4) synapse. (**B**) A neuronopathy resulting from the death of the entire neuron. Astrocytes often proliferate in response to the neuronal loss, creating both neuronal loss and gliosis. (**C**) An axonopathy occurs when the axon is the primary site of injury, the axon degenerates, and the surviving neuron shows only chromatolysis with margination of its Nissl substance and nucleus to the cell periphery. (**D**) Myelinopathy resulting from disruption of myelin or selective injury to the myelinating cells. To prevent cross talk between adjacent axons, myelinating cells divide and cover the denuded axon rapidly; however, the process of remyelination is much less effective in the CNS than in the PNS. (**E**) Some forms of toxicity are due to interruption of the process of neurotransmission, either through blocking excitation or by excessive stimulation, rather than actual cell death.

The dynamic relationships between the neuronal cell body and its axon are important in understanding the basic pathological responses to some axonal and neuronal injuries caused by neurotoxicants (Fig. 16-2). When the neuronal cell body has been lethally injured, it degenerates—in a process called neuronopathy. This is characterized by the loss of the cell body and all of its processes, with no potential for regeneration. However, when the injury is at the level of the axon, the axon may degenerate while the neuronal cell body continues to survive, a condition known as an axonopathy. In this setting, there is a potential for regeneration and recovery from the toxic injury as the axonal stump sprouts and regenerates.

Terminating the period of survival is an active proteolysis that digests the axolemma and axoplasm, leaving only a myelin sheath surrounding a swollen degenerated axon. Digestion of the axon appears to be an all-or-none event effected through endogenous proteases (Schlafer and Zimmerman, 1984) that are activated through increased levels of intracellular free Ca^{2+} (George et al., 1995). Although it is established that degeneration of the most terminal portion of the axon occurs first, whether degeneration of the remainder of the stump occurs from proximal to distal, distal to proximal, or simultaneously along its entire length remains a matter of debate. The active proteolysis phase occurs so rapidly in mammals that it has been difficult to define a spatial distribution.

In the PNS, Schwann cells respond to loss of axons by decreasing synthesis of myelin lipids, downregulating genes encoding myelin proteins, and dedifferentiating to a premyelinating mitotic Schwann cell phenotype (Stoll and Muller, 1999). They may also become phagocytic, giving rise to "myelin ovoids." The proliferating Schwann cells align along the original basal lamina, which creates a tubular structure referred to as a band of Bungner. In addition to providing physical guidance for regenerating axons, these tubes provide trophic support from nerve growth factor, brain-derived nerve growth factor, insulin-like growth factor, and corresponding receptors produced by the associated Schwann cells. Resident macrophages distributed along the endothelium within the endoneurium and the denervated Schwann cells assist in clearing myelin debris, but the recruitment of hematogenous macrophages accounts for the removal of the majority of myelin. Infiltrating macrophages express complement receptor 3, and the presence of complement 3 on the surface of degenerating myelin sheaths facilitates opsonization. Another essential role of recruited circulating macrophages is the secretion of interleukin-1, which is responsible for stimulating production of nerve growth factor by Schwann cells. In contrast to the proteolysis of the axon, processing of myelin breakdown products proceeds in an established proximal-to-distal progression.

Investigations have shown that degeneration of the distal axonal stump after transection is an active, synchronized process that can be delayed experimentally through decreasing temperature, preventing the entry of extracellular Ca^{2+}, or inhibiting calpain II–mediated proteolysis (George et al., 1995). Accompanying events in glial cells and macrophages direct and facilitate the sprouting neurite originating from the surviving proximal axon that also undergoes changes in protein expression resembling a less differentiated state. The facilitation of regeneration in the PNS by Schwann cells distinguishes it from the CNS, in which oligodendrocytes secrete inhibitory factors that impede neurite outgrowth. Eventually, though, even in the PNS, if axonal contact is not restored, Schwann cell numbers will decrease, bands of Bungner will disappear, and increased fibroblast collagen production will render regeneration increasingly unlikely.

Thus, a critical difference exists in the significance of axonal degeneration in the CNS compared with that in the PNS: peripheral axons can regenerate, whereas central axons cannot. In the PNS, glial cells and macrophages create an environment supportive of axonal regeneration, and Schwann cells transplanted to the CNS maintain this ability. In the CNS, release of inhibitory factors from damaged myelin and astrocyte scarring actually interfere with regeneration (Qiu et al., 2000). Interestingly, when this glial interference is removed through transplantation of CNS neurons to the PNS, the neurons are capable of extending neurites. There appears, however, to be more than just glial interference to account for the lack of CNS regeneration. The observation that embryonic neurons can overcome glial interference when placed into the adult NS is consistent with the development of an intrinsic sensitivity to inhibitory factors during maturation. Therefore, the inability of the CNS to regenerate appears to be due to both unfavorable environmental glial factors and properties of the mature neuron. The clinical relevance of the disparity between the CNS and PNS is that partial recovery—or, in mild cases, complete recovery—can occur after axonal degeneration in the PNS, whereas the same event is irreversible in the CNS.

Myelin Formation and Maintenance

Myelin is formed in the CNS by oligodendrocytes and in the PNS by Schwann cells. Both of these cell types form concentric layers of lipid-rich myelin by the progressive wrapping of their cytoplasmic processes around the axon in successive loops (Fig. 16-3).

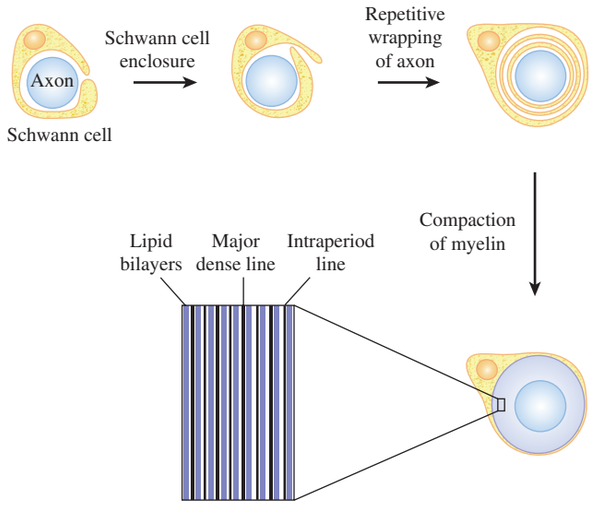


Figure 16-3. *Process of myelination.* Myelination begins when a myelinating cell encircles an axon, either Schwann cells in the peripheral nervous system or oligodendrocytes in the CNS. Simple enclosure of the axon persists in unmyelinated axons. Myelin formation proceeds by a progressive wrapping of multiple layers of the myelinating cell around the axon, with extrusion of the cytoplasm and extracellular space to bring the lipid bilayers into close proximity. The intracellular space is compressed to form the major dense line of myelin, and the extracellular space is compressed to form the intraperiod line.

Ultimately, these cells exclude cytoplasm from the inner surface of their membranes to form the major dense line of myelin (Monuki and Lemke, 1995; Parent, 1996; Quarles et al., 1997). In a similar process, the extracellular space is reduced on the extracellular surface of the bilayers, and the lipid membranes stack together, separated only by a proteinaceous intraperiod line existing between successive layers.

The formation and maintenance of myelin requires metabolic and structural proteins that are unique to the NS. Myelin basic protein, an integral protein of CNS myelin, is closely associated with the intracellular space (at the major dense line of myelin) (Monuki and Lemke, 1995; Quarles et al., 1997), and an analogous protein, P1, is located in the PNS. On the extracellular surface of the lipid bilayers is the CNS proteolipid protein at the intraperiod line of myelin. Mutation of this protein in several species, including humans, or overexpression of the wild-type gene in transgenic mice results in disorders in which myelin of the CNS does not form normally (Pham-Dinh et al., 1991; Readhead et al., 1994).

There are a variety of hereditary disorders where myelin is either poorly formed from the outset or inadequately maintained after its formation. In addition to mutation of proteolipid protein, there are a variety of inherited abnormalities of myelin proteins and myelin-specific lipid catabolism. These genetic defects have provided some insight into the special processes required to maintain the lipid-rich environment of myelin. It is now known that the maintenance of myelin is dependent on a number of membrane-associated proteins and on metabolism of specific lipids present in myelin bilayers. Some toxic compounds interfere with this complex process of the maintenance of myelin and result in the toxic "myelinopathies" (Fig. 16-2). The loss of myelin with the preservation of axons is referred to as primary demyelination, whereas degeneration of myelin accompanied by removal of the myelin sheath resulting from axonal loss is known as secondary demyelination (Wisniewski and Bloom, 1975).

Neurotransmission

Intercellular communication is achieved in the NS through the synapse. Neurotransmitter released from one axon to another neuron act as the first messenger. Binding of the transmitter to the postsynaptic receptor is followed by modulation of an ion channel or activation of a second messenger system, leading to changes in the responding cell. In the case of neuromuscular transmission, acetylcholine crosses the synaptic cleft to bind the cholinergic receptor of the myocyte and leads to muscle contraction. Chemically induced dysfunction of neurotransmission may occur in the absence of altered cellular structures; rather, the neurotoxicity expresses itself in terms of altered conduction and propagation of nerve impulses and changes in functions such as behavior, performance, and conditioning.

Chemicals acting on neurotransmission may interrupt the transmission of impulses, block or accentuate transsynaptic communication, block reuptake of neurotransmitters or precursors, or interfere with second-messenger systems. The structural similarity of many compounds with similar actions has led to the recognition of specific categories of drugs and toxicants. For example, some drugs mimic the process of neurotransmission of the sympathetic NS and are termed sympathomimetic compounds. As the targets of these drugs are located throughout the body, the responses are not localized; however, the responses are stereotyped in that each member of a class tends to have similar biological effects. At times, altered neurotransmission is beneficial to an individual, for example, by stabilizing a disease-induced imbalance, and it is this aspect of biological response that is studied in the field of neuropharmacology. However, excessive or inappropriate exposure to compounds that alter neurotransmission may result in responses considered as neurotoxic. The therapeutic index is a measure of the margin between the desirable (therapeutic) and toxic effects of a chemical.

In terms of toxicity, many side effects of neurological drugs may be viewed as short-term interactions that are reversible with time or that may be counteracted by the use of appropriate antagonists. However, some of the toxicity associated with long-term exposures may be irreversible. For example, phenothiazines, which have been used to treat chronic schizophrenia for long periods of time, may lead to the condition of tardive dyskinesia, in which the patient is left with a permanent disability that includes prominent facial grimaces (DeVeaugh-Geiss, 1982). Excessive stimulation of neurotransmitter systems may also have long-term consequences; for example, excitatory systems (e.g., glutamate) produce excitotoxicity manifest as CNS diseases and nerve cell death (Wang and Qin, 2010).

Development of the Nervous System

The NS begins development during gestation (first month in humans, day 7 in mice, day 9.5 in rats) and continues through adolescence (Bayer et al., 1993; Rice and Barone, 2000). Proliferation, migration, differentiation, synaptogenesis, apoptosis, and myelination are the basic processes that underlie development of the NS, and these occur in a tightly choreographed sequence that depends on the region, cell type, and neurotrophic signals. Both neuronal and glial precursors replicate in a discrete zone near the inner surface of the neural tube. The proliferation and migration of these cells occur in waves that are specific for brain regions, but in general, the brain develops in a caudal to rostral direction (with cerebellar development being a notable exception). During differentiation (phenotype expression) and synaptogenesis (formation of functional synaptic connections), the circuitry of the NS is established. Chemicals such as nerve growth factors, adhesive molecules, and neurotransmitters serve as morphogenic signals; neurotransmitter developmental

signals are separate from their synaptic transmission function (Lauder, 1993). Selected cells are also removed during ontogeny via apoptosis (programmed cell death), which results in the appropriate cell types in the correct regions. The glial supportive cells develop last, and myelination is protracted. The period of rapid proliferation of glial cells is known as the brain growth spurt, during which time it is particularly vulnerable to insult (Dobbing and Smart, 1974; Dobbing and Sands, 1979). Although the order of neurogenesis is conserved across species, the rate of maturation and timing of specific events relative to birth vary; consequently, consideration of these data impact extrapolation of developmental consequences from laboratory animals to humans (Dobbing and Sands, 1973; Bayer et al., 1993; Clancy et al., 2007).

The immature NS is especially vulnerable to certain chemicals (Rodier, 1995; Bondy and Campbell, 2005), and there are several factors that make the developing NS uniquely susceptible. Cell sensitivity differs with the developmental stage, leading to critical windows of vulnerability (Bayer et al., 1993; Adams et al., 2000; Selevan et al., 2000). Chemicals that alter the timing and formation of neural connections could result in permanent malformations, the consequences of which may be quite unlike the chemical's effects in the adult NS (Barone et al., 2000). Furthermore, while synaptogenesis can continue throughout life, proliferation cannot; therefore, the CNS is unique in that damaged neural cells are not readily replaced. Finally, there are physiological and kinetic differences in the developing organism that may profoundly influence its sensitivity, including the slow formation of the blood–brain barrier and lack of key metabolic enzymes to protect the brain and eliminate toxicants (Bearer, 1995; Makri et al., 2004).

Although the developing NS is often more sensitive than the mature NS to insult (depending on the stage of development), the high rate of proliferation and regeneration in the developing NS may also lead to greater recovery or plasticity (an ability of one portion of the NS to assume the function of an injured area), which could attenuate some injuries (Goldberger and Murray, 1985). An example is the faster recovery from organophosphorus (OP) pesticide-induced acetylcholinesterase (AChE) inhibition observed in younger laboratory rats, which is probably due to the rapid formation of the esterase enzyme and replacement of the phosphorylated form (Moser and Padilla, 1998). Some developmental changes may appear transient due to this plasticity and compensation, but underlying changes in NS development could become manifest with aging or some form of challenge (e.g., Barone et al., 1995, 2000; Rice, 1996; Bondy and Campbell, 2005).

In evaluating developmental neurotoxicity, chemical exposure or treatment may occur during critical windows of susceptibility or may cover the entire developmental process (i.e., during gestation, lactation, and adolescence). In general, injurious exposures early in gestation impact development of major brain regions, whereas later exposures alter biochemical, morphological, or functional features of the neural systems. Functional, neurochemical, morphometric, or neuroanatomical endpoints are often used to assess the impact of developmental exposures; multiple measures are often needed to assess the wide array of potential outcomes. The ontogeny of specific behaviors, reflexes, and motor functions has been established for laboratory rats and mice, and compared to human developmental patterns (e.g., Fox, 1965; Altman and Sudarshan, 1975; Wood et al., 2003). Markers of synaptic proteins, assays of synaptic enzymes, or challenges with pharmacologically specific chemicals are but a few methods by which to test synaptic function. Although frank neuropathology is not as common with developmental neurotoxicants, measurements of layer widths in synaptic zones may reflect physical malformations.

FUNCTIONAL MANIFESTATIONS OF NEUROTOXICITY

Although knowledge of a toxicant's complete biochemical or molecular mechanism(s) is the ultimate goal of neurotoxicology, a full understanding of the toxicity also requires knowledge of the functional outcomes of those changes. Being the final output or manifestation of the NS, function includes motor, sensory, autonomic, and cognitive capabilities. The strength of functional assessments has been exploited by many investigators and regulatory agencies to assess the neurological effects of chemicals. Tilson (1993) proposed two distinct tiers of functional testing of neurotoxicants: a first tier in which observational batteries or motor activity tests may be used to identify the presence of a neurotoxic substance, and a second tier that involves more refined tests to allow better characterization of the effects.

An overall assessment of function may be described using a series, or battery, of tests. These tests typically evaluate a variety of neurological functions, and are sometimes used to screen for potential neurotoxicity in regulatory and safety pharmacology testing (Tilson and Moser, 1992; Moser, 2000). Specific behavioral methods include observational assessments (functional observational batteries [FOBs] and Irwin screens), tests of motor activity, and expanded clinical observations. These tests have the advantage over biochemical and pathological measures in that they permit evaluation of a single animal over time to determine the onset, progression, duration, and reversibility of a neurotoxic injury. Comparisons of defined protocols of FOBs with limited numbers of compounds (Moser et al., 1997a, 1997b) suggest that these methods can identify neurotoxic compounds reliably.

Some functional tests are more specific than observations and motor activity, and may be used to more fully characterize neurotoxic effects. Many of these functions have a clinical or behavioral correlate in humans, thus improving extrapolation of the outcomes. Electrophysiological tests provide sensory-specific information on nerve conduction velocity and neuromotor integrity, and have been used to complement behavioral evaluations (Dyer, 1985; Mattsson et al., 1989). Measures of sensory function tap specific neuronal pathways that govern stimuli-dependent reflexes. For example, the acoustic startle response is a sensory-evoked motor reflex with a defined neuronal pathway (Davis et al., 1982). Treatment effects could indicate sensory, motor, or muscle fiber alterations with little or no central involvement. Autonomic function includes evaluations of cardiovascular status and cholinergic/adrenergic balance.

Deficits in cognitive function, especially in the context of developmental toxicity, represent an endpoint of great public concern and rhetoric. Behavioral toxicologists have incorporated methodologies from behavioral pharmacology and psychology to develop a variety of tests of learning and memory for laboratory animals. These procedures include spatial navigation of mazes, associations with shock, conditioned responses, and appetite-motivated operant responses. In most cases, deficits in human cognitive function may be detected in laboratory animals as well, although the affected cognitive domain may vary. For example, in humans, exposure to lead in early childhood is capable of lowering IQ and altering behavioral control. Studies in rats exposed to lead have reported deficits in spatial learning, sustained attention, activity levels, and other behaviors (e.g., Nihei et al., 2000; Moreira et al., 2001; Morgan et al., 2001). Detailed assessments such as these provide valuable insights into the damage caused by neurotoxicants. Ultimately, neurotoxicants identified by behavioral and physiological methods are evaluated at a cellular and molecular level to provide an understanding of the events in the NS that cause the neurological dysfunction.

MECHANISMS OF NEUROTOXICITY

Efforts to understand the mechanism of action of individual neurotoxic compounds have begun with the identification of the cellular or subcellular target. In the NS, this has most often been one of four targets: the neuron cell body, the axon, the myelinating cell, or the neurotransmitter system. As a result, neurotoxic compounds that cause neuronopathies, axonopathies, myelinopathies, or neurotransmitter-associated toxicity may be identified (Fig. 16-2). This is the classification system that is utilized here to organize the discussion of neurotoxic compounds and their mechanisms of action.

Neuronopathies

Certain toxicants are specific for neurons, or sometimes a particular group of neurons, resulting in their injury or, when intoxication is severe enough, their death. The loss of a neuron is irreversible and includes degeneration of all of its cytoplasmic extensions, dendrites and axons, and of the myelin ensheathing the axon (Fig. 16-2). Although the neuron is similar to other cell types in many respects, some features of the neuron are unique, placing it at risk for the action of cellular toxicants. Some of the unique features of the neuron include a high metabolic rate, a long cellular process that is supported by the cell body, and an excitable membrane that is rapidly depolarized and repolarized. Because many neurotoxic compounds act at the site of the cell body, when massive loss of axons and myelin is discovered in the PNS or CNS, the first question is whether the neuronal cell bodies themselves have been destroyed.

Although a large number of compounds are known to produce toxic neuronopathies (Table 16-1), all of these toxicants share certain features. Each toxic condition is the result of a cellular toxicant that has a predilection for neurons, most likely due to one of the neuron's particular vulnerabilities. The initial injury to neurons is followed by apoptosis or necrosis, leading to permanent loss of the neuron. These chemicals tend to be diffuse in their action, although they may show some selectivity in the degree of injury of different neuronal subpopulations or at times an exquisite selectivity for such a subpopulation. The expression of these cellular events is often a diffuse encephalopathy, with global dysfunctions; however, the symptomatology reflects the location of the injury to the brain; consequently, neurotoxicants that are selective in their action and affect only a subpopulation of neurons may lead to interruption of only a particular functionality.

Doxorubicin Doxorubicin (Adriamycin), a quinone-containing anthracycline antibiotic, is one of the most effective antimitotics in cancer chemotherapy. Unfortunately, clinical application of doxorubicin is greatly limited by its acute and chronic cardiotoxicity. In addition to its cardiac toxicity, which limits the quantity of doxorubicin that can be given to cancer patients, doxorubicin also injures neurons in the PNS, specifically those of the dorsal root ganglia and autonomic ganglia (Spencer, 2000). This selective vulnerability of peripheral ganglion cells is particularly dramatic in experimental animals.

Doxorubicin's antineoplastic properties derive from its ability to intercalate into grooves of DNA, interfering with transcription. Other important mechanisms of action of doxorubicin include its interaction with topoisomerase II, which forms a DNA-cleavable complex (Chuang and Chuang, 1979; Cheng et al., 1992) and generation of reactive oxygen species (ROS) by enzymatic electron reduction of doxorubicin by a variety of oxidases, reductases, and dehydrogenases (Kappus, 1987; Gutierrez, 2000).

The neurotoxicity of doxorubicin is quite limited in its extent, despite the fact that all neurons are dependent on the ability to transcribe DNA. The particular vulnerability of sensory and autonomic neurons appears to reflect the lack of protection of these neurons by a blood–tissue barrier within ganglia. If the blood–brain barrier is temporarily opened by the use of mannitol, the toxicity of doxorubicin is expressed in a much more diffuse manner, with injury of neurons in the cortex and subcortical nuclei of the brain (Spencer, 2000).

Methyl Mercury The neuronal toxicity of organomercurial compounds, such as methyl mercury (MeHg), was tragically revealed in large numbers of poisonings in Japan and Iraq. The residents of Minamata Bay in Japan, whose diet was largely composed of fish from the bay, were exposed to massive amounts of MeHg when mercury-laden industrial effluent was rerouted into the bay (Kurland et al., 1960; Takeuchi et al., 1962). MeHg injured even more people in Iraq, with more than 400 deaths and 6000 people hospitalized. In this epidemic, as well as in several smaller ones, the effects occurred after the consumption of grain that had been dusted with MeHg as an inexpensive pesticide (Bakir et al., 1973). Typically, environmental exposure to mercury occurs via the food chain due to accumulation of MeHg in fish. Monitoring in the United States indicates that all 50 states have fish consumption advisories covering about 40% of the nation's rivers, lakes, and streams, and 100% of the Great Lakes and connecting waters (U.S. EPA, 2013).

The clinical picture of MeHg poisoning varies with both the severity of exposure and the age of the individual at the time of exposure. In adults, the most dramatic sites of injury are the neurons of the visual cortex and the small internal granular cell neurons of the cerebellar cortex, whose massive degeneration results in blindness and marked ataxia. In children, developmental disabilities, retardation, and cognitive deficits occur. Such age-related differences are seen also in other mammals, although the specific areas damaged may differ. It has been suggested that these differences are caused by an immature blood–brain barrier causing a more generalized distribution of mercury in the developing brain. Recent studies in rats show that the neurons that are most sensitive to the toxic effects of MeHg are those that reside in the dorsal root ganglia, perhaps again reflecting the vulnerability of neurons not shielded by blood–tissue barriers (Schionning et al., 1998).

The mechanism of MeHg toxicity has been the subject of intense investigation. However, it remains unknown whether the ultimate toxicant is MeHg or the liberated mercuric ion. Although Hg^{2+} is known to bind strongly to sulfhydryl groups, it is not clear that MeHg results in cell death through sulfhydryl binding. A variety of aberrations in cellular function have been noted, including impaired glycolysis, nucleic acid biosynthesis, aerobic respiration, protein synthesis (Cheung and Verity, 1985), and neurotransmitter release (Atchison and Hare, 1994). In addition, there is evidence for enhanced oxidative injury (LeBel et al., 1992; Shanker et al., 2002) and altered calcium homeostasis (Marty and Atchison, 1997). Exposure to MeHg leads to widespread neuronal injury and subsequently to a diffuse encephalopathy. However, there is relative selectivity of the toxicant for some groups of neurons over others. The distribution of neuronal injury does not appear to be related to the tissue distribution of either MeHg or ionic mercury but rather to particular vulnerabilities of these neurons. Susceptibility of different brain regions or cell types to MeHg (Clarkson, 1997) may also be dependent on factors such as the intracellular reduced glutathione (GSH) concentration and the ability to increase glycolytic flux in the face of mitochondrial damage. These observations are consistent with morphological observations in which astrocytes that accumulate MeHg appear normal, whereas neurons that are found in

Table 16-1

Compounds Associated with Neuronal Injury (Neuronopathies)

NEUROTOXICANT	NEUROLOGICAL FINDINGS	CELLULAR BASIS OF NEUROTOXICITY	REFERENCES
Aluminum	Dementia, encephalopathy (humans), learning deficits	Spongiosis cortex, neurofibrillary aggregates, degenerative changes in cortex	Chang and Dyer (1995), Graham and Lantos (1997), Spencer and Schaumburg (2000)
6-Amino-nicotinamide	Not reported in humans, hind limb paralysis (experimental animals)	Spongy (vacuolar) degeneration in spinal cord, brainstem, cerebellum; axonal degeneration of the peripheral nervous system (PNS)	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Arsenic	Encephalopathy (acute), peripheral neuropathy (chronic)	Brain swelling and hemorrhage (acute), axonal degeneration in PNS (chronic)	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Azide	Insufficient data (humans); convulsions, ataxia (primates)	Neuronal loss in cerebellum and cortex	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Bismuth	Emotional disturbances, encephalopathy, myoclonus	Neuronal loss, basal ganglia and Purkinje cells of cerebellum	Spencer and Schaumburg (2000)
Carbon monoxide	Encephalopathy, delayed parkinsonism/dystonia	Neuronal loss in cortex, necrosis of globus pallidus, focal demyelination; blocks oxygen binding site of hemoglobin and iron-binding sites of brain	Spencer and Schaumburg (2000)
Carbon tetrachloride	Encephalopathy (secondary to liver failure)	Enlarged astrocytes in striatum, globus pallidus	Spencer and Schaumburg (2000)
Chloramphenicol	Optic neuritis, peripheral neuropathy	Neuronal loss (retina), axonal degeneration (PNS)	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Cyanide	Coma, convulsions, rapid death; delayed parkinsonism/dystonia	Neuronal degeneration, cerebellum and globus pallidus; focal demyelination; blocks cytochrome oxidase/adenosine triphosphate production	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Doxorubicin	Insufficient data (humans), progressive ataxia (experimental animals)	Degeneration of dorsal root ganglion cells, axonal degeneration (PNS)	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Ethanol	Mental retardation, hearing deficits (prenatal exposure)	Microcephaly, cerebral malformations	Graham and Lantos (1997)
Lead	Encephalopathy (acute), learning deficits (children), neuropathy with demyelination (rats)	Brain swelling, hemorrhages (acute), axonal loss in PNS (humans)	Chang and Dyer (1995), Graham and Lantos (1997), Spencer and Schaumburg (2000)
Manganese	Emotional disturbances, parkinsonism/dystonia	Degeneration of striatum, globus pallidus	Chang and Dyer (1995), Graham and Lantos (1997)
Mercury, inorganic	Emotional disturbances, tremor, fatigue	Insufficient data in humans (may affect spinal tracts; cerebellum)	Chang and Dyer (1995), Graham and Lantos (1997), Spencer and Schaumburg (2000)
Methanol	Headache, visual loss or blindness, coma (severe)	Necrosis of putamen, degeneration of retinal ganglion cells	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Methylazoxymethanol acetate (MAM)	Microcephaly, retarded development (rats)	Developmental abnormalities of fetal brain (rats)	Abou-Donia (1993)
Methyl bromide	Visual and speech impairment, peripheral neuropathy	Insufficient data	Spencer and Schaumburg (2000)
Methyl mercury (organic mercury)	Ataxia, constriction of visual fields, paresthesias (adult), psychomotor retardation (fetal exposure)	Neuronal degeneration, visual cortex, cerebellum, ganglia Spongy disruption, cortex, cerebellum	Chang and Dyer (1995), Graham and Lantos (1997), Spencer and Schaumburg (2000) Graham and Lantos (1997), Spencer and Schaumburg (2000)

(*continued*)

Table 16-1

(Continued)

NEUROTOXICANT	NEUROLOGICAL FINDINGS	CELLULAR BASIS OF NEUROTOXICITY	REFERENCES
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	Parkinsonism, dystonia (acute exposure) Early-onset parkinsonism (late effect of acute exposure)	Neuronal degeneration in substantia nigra Neuronal degeneration in substantia nigra	Graham and Lantos (1997), Spencer and Schaumburg (2000) Graham and Lantos (1997), Spencer and Schaumburg (2000)
3-Nitropropionic acid	Seizures, delayed dystonia/grimacing	Necrosis in basal ganglia	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Phenytoin (diphenyl-hydantoin)	Nystagmus, ataxia, dizziness	Degeneration of Purkinje cells (cerebellum)	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Quinine	Constriction of visual fields	Vacuolization of retinal ganglion cells	Spencer and Schaumburg (2000)
Streptomycin (aminoglycosides)	Hearing loss	Degeneration of inner ear (organ of Corti)	Spencer and Schaumburg (2000)
Thallium	Emotional disturbances, ataxia, peripheral neuropathy	Brain swelling (acute), axonal degeneration in PNS	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Trimethyltin	Tremors, hyperexcitability (experimental animals)	Loss of hippocampal neurons, amygdala pyriform cortex	Graham and Lantos (1997)

their proximity and are void of MeHg undergo cell death (Garman et al., 1975). It seems likely that MeHg toxicity is mediated by numerous reactions and that no single critical target will be identified. As these toxic events occur, the injured neurons eventually die.

Trimethyltin Organotins are used industrially as plasticizers, antifungal agents, or pesticides. Intoxication with trimethyltin has been associated with a potentially irreversible limbic-cerebellar syndrome in humans and similar behavioral changes in primates (Reuhl et al., 1985; Besser et al., 1987). Trimethyltin gains access to the NS where, by an undefined mechanism, it leads to diffuse neuronal injury. Trimethyltin triggers selective apoptosis in specific subregions of the mammalian CNS and specific subsets of immune system cells (Balaban et al., 1988; Patanow et al., 1997). The hippocampus is particularly vulnerable to this process. Following acute intoxication, the cells of the fascia dentata degenerate; with chronic intoxication, the cells of the corpus ammonis are lost. Ganglion cells and hair cells of the cochlea are similarly sensitive (Liu and Fechter, 1996). Several hypotheses are suggested for the mechanism of trimethyltin neurotoxicity, including energy deprivation and excitotoxic damage. Evidence to date suggests that organotins, such as trimethyltin, interact with the CXC region of stannin, and that trimethyltin treatment significantly alters its expression (Toggas et al., 1993). Stannin is located on human chromosome 16p13, and has a syntenic relationship to the murine chromosomal homolog (Dejneka et al., 1998). Stannin is a highly conserved, 88 amino acid membrane-bound protein localized, in part, to the mitochondria and other vesicular organelles; this protein interacts with other regulatory proteins to alter growth and apoptosis. Trimethyltin exposure results in tumor necrosis factor-α (TNF-α) production which, in turn, induces stannin gene expression.

Axonopathies

The neurotoxic disorders termed axonopathies are those in which the primary site of toxicity is the axon itself. The axon degenerates, and with it the myelin surrounding that axon; however, the neuron cell body remains intact (Fig. 16-2). John Cavanagh coined the term dying-back neuropathy as a synonym for distal axonopathy (Cavanagh, 1964). The concept of "dying back" postulated that the focus of toxicity was the neuronal cell body itself and that the distal axon degenerated progressively from the synapse, back toward the cell body with increasing injury. It now appears that, in the best studied axonopathies, a different pathogenetic sequence occurs; the toxicant results in a "chemical transection" of the axon at some point along its length, and the axon distal to the transection, biologically separated from its cell body, degenerates in a Wallerian fashion.

Because longer axons have more targets for toxic damage than shorter axons, one would predict that longer axons would be more affected in toxic axonopathies. Indeed, such is the case. The involvement of long axons of the CNS, such as ascending sensory axons in the posterior columns or descending motor axons, along with long sensory and motor axons of the PNS, prompted Spencer and Schaumburg (1976) to suggest that the toxic axonopathies in which the distal axon was most vulnerable be called central peripheral distal axonopathies, which, though cumbersome, accurately depicts the pathological loci.

The number of axonal toxicants is large and increasing in number (Table 16-2); however, they may be viewed as a group, all of which result in the pathological loss of distal axons with the survival of the cell body. Because the axonopathies pathologically resemble the actual physical transection of the axon, axonal transport appears to be a likely target in many of the toxic axonopathies. Furthermore, as these axons degenerate, the result is most often the clinical condition of peripheral neuropathy, in which sensations and motor strength are first impaired in the most distal extent of the axonal processes, the feet and hands. Hence, functional impairment is said to follow a "glove and stocking" distribution. With time and continued injury, the deficit progresses to involve more proximal areas of the body and the long axons of the spinal cord. The potential for regeneration is great when the insult is limited to peripheral nerves and may be virtually complete in axonopathies in which the initiating event can be determined and removed.

Table 16-2

Compounds Associated with Axonal Injury (Axonopathies)

NEUROTOXICANT	NEUROLOGICAL FINDINGS	BASIS OF NEUROTOXICITY	REFERENCES
Acrylamide	Peripheral neuropathy (often sensory)	Axonal degeneration, axon terminal affected in earliest stages	Graham and Lantos (1997), Spencer and Schaumburg (2000)
p-Bromophenylacetyl urea	Peripheral neuropathy	Axonal degeneration in the peripheral nervous system (PNS) and central nervous system (CNS)	Spencer and Schaumburg (2000)
Carbon disulfide	Psychosis (acute), peripheral neuropathy (chronic)	Axonal degeneration, early stages include neurofilamentous swelling	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Chlordecone (Kepone)	Tremors, in coordination (experimental animals)	Insufficient data (humans), axonal swelling and degeneration	Spencer and Schaumburg (2000)
Chloroquine	Peripheral neuropathy, weakness	Axonal degeneration, inclusions in dorsal root ganglion cells; also vacuolar myopathy	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Clioquinol	Encephalopathy (acute), subacute myelooptic neuropathy (subacute)	Axonal degeneration, spinal cord, PNS, optic tracts	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Colchicine	Peripheral neuropathy	Axonal degeneration, neuronal perikaryal filamentous aggregates; vacuolar myopathy	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Dapsone	Peripheral neuropathy, predominantly motor	Axonal degeneration (both myelinated and unmyelinated axons)	Graham and Lantos (1997)
Dichlorophenoxyacetate	Peripheral neuropathy (delayed)	Insufficient data	Spencer and Schaumburg (2000)
Dimethylaminopropionitrile	Peripheral neuropathy, urinary retention	Axonal degeneration (both myelinated and unmyelinated axons)	Spencer and Schaumburg (2000)
Ethylene oxide	Peripheral neuropathy	Axonal degeneration	Graham and Lantos (1997)
Glutethimide	Peripheral neuropathy (predominantly sensory)	Insufficient data	Spencer and Schaumburg (2000)
Gold	Peripheral neuropathy (may have psychiatric problems)	Axonal degeneration, some segmental demyelination	Graham and Lantos (1997), Spencer and Schaumburg (2000)
n-Hexane	Peripheral neuropathy, severe cases have spasticity	Axonal degeneration, early neurofilamentous swelling, PNS, spinal cord	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Hydralazine	Peripheral neuropathy	Insufficient data	Spencer and Schaumburg (2000)
β,β′-Iminodipropionitrile	No data in humans, excitatory movement disorder (rats)	Proximal axonal swellings, degeneration of olfactory epithelial cells, vestibular hair cells	Graham and Lantos (1997)
Isoniazid	Peripheral neuropathy (sensory), ataxia (high doses)	Axonal degeneration	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Lithium	Lethargy, tremor, ataxia (reversible)	Insufficient data	Spencer and Schaumburg (2000)
Methyl *n*-butyl ketone	Peripheral neuropathy	Axonal degeneration, early neurofilamentous swelling, PNS, spinal cord	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Metronidazole	Sensory peripheral neuropathy, ataxia, seizures	Axonal degeneration, mostly affecting myelinated fibers; lesions of cerebellar nuclei	Graham and Lantos (1997), Spencer and Schaumburg (2000)

(*continued*)

Table 16-2

(Continued)

NEUROTOXICANT	NEUROLOGICAL FINDINGS	BASIS OF NEUROTOXICITY	REFERENCES
Misonidazole	Peripheral neuropathy	Axonal degeneration	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Nitrofurantoin	Peripheral neuropathy	Axonal degeneration	Spencer and Schaumburg (2000)
Organophosphorus compounds (neuropathy target esterase inhibitors)	Abdominal pain (acute), peripheral neuropathy	Axonal degeneration	Chang and Dyer (1995), Graham and Lantos (1997), Spencer and Schaumburg (2000)
Paclitaxel (taxoids)	Delayed peripheral neuropathy (motor), spasticity	Axonal degeneration (delayed after single exposure), PNS, spinal cord	Abou-Donia (1993), Chang and Dyer (1995), Graham and Lantos (1997), Spencer and Schaumburg (2000)
Platinum (cisplatin)	Peripheral neuropathy	Axonal degeneration, microtubule accumulation in early stages	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Pyridinethione (pyrithione)	Movement disorders (tremor, choreoathetosis)	Axonal degeneration (variable)	Chang and Dyer (1995), Spencer and Schaumburg (2000)
Vincristine (vinca alkaloids)	Cranial (most often trigeminal) neuropathy Peripheral neuropathy, variable autonomic symptoms	Insufficient data Axonal degeneration (PNS), neurofibrillary changes (spinal cord, intrathecal route)	Spencer and Schaumburg (2000) Graham and Lantos (1997), Spencer and Schaumburg (2000)

Hexane and Gamma-Diketones It was first noted in the late 1960s that humans with chronic high exposures to *n*-hexane, a simple alkane, in a work setting develop a progressive sensorimotor distal axonopathy (Yamamura, 1969). Intentional inhalation of materials containing *n*-hexane is also common, and produces the same neurotoxic effects. An identical axonopathy was also produced by methyl *n*-butyl ketone (2-hexanone), leading to the discovery of the mechanism by which these two compounds are similarly metabolized. The carbon chain undergoes ω-1 oxidation, resulting in 2,5-hexanedione (HD), a γ-diketone. This metabolite is ultimately the toxic species produced from *n*-hexane and 2-hexanone. Other γ-diketones or precursors also produced the same axonopathy, whereas α- or β-diketones are not axonopathic to the NS (Krasavage et al., 1980).

γ-Diketones, including HD, react with amino groups on all proteins, forming pyrrole adducts (Fig. 16-4). This is an important step in the development of axonopathy, as evidenced by the inability of 3,3-dimethyl-2,5-hexanedione, a γ-diketone that is unable to form pyrrole adducts, to cause this type of neurotoxicity (Sayre et al., 1986). After forming, these pyrroles are oxidized and cross-linking occurs between neurofilament subunits. It is noteworthy that 3-acetyl-2,5-hexanedione cannot form cross-links and does not produce axonopathy, suggesting that pyrrole oxidation and cross-linking are necessary steps in the development of γ-diketone axonopathy (St. Clair et al., 1988). Neurofilaments accumulate in the distal axon, usually just proximal to a node of Ranvier, and form massive axonal swellings leading to retraction of myelin from the nodes (Graham et al., 1982). In addition to swelling, axonal atrophy is a pathological feature of γ-diketone neurotoxicity.

Axonal atrophy was previously thought to occur secondary to swelling; however, more recent investigations have suggested that it may be the more relevant pathophysiological feature. In one study, rats dosed at a lower rate (100 to 250 mg/kg/day) developed axonal swelling and atrophy, and rats given a higher dose (400 mg/kg/day) failed to consistently develop swellings, while atrophy and behavioral alterations were nearly universal. These data suggest that axonal atrophy is the pathological change that leads to nerve dysfunction and behavioral changes (Lehning et al., 2000). The mechanism responsible for axonal atrophy is still unknown; however, a depletion of tubulin subunits has been reported, and is a likely contributor to the overall neuropathological picture (LoPachin et al., 2005).

The dimethyl analog of HD, 3,4-dimethyl-2,5-hexanedione (DMHD), produces a similar neuropathy. However, due to the methyl groups, DMHD forms the cyclic adduct much more rapidly than HD, forming pyrrole adducts that oxidize and lead to cross-linking faster than HD. The faster rate of adduct formation results in DMHD producing proximal axonal swellings similar to those seen with β,β′-iminodipropionitrile (IDPN) (Anthony et al., 1983).

Carbon Disulfide The most significant exposures of humans to carbon disulfide (CS_2) have occurred in the vulcan rubber and viscose rayon industries. Manic psychoses were observed in the former setting and were correlated with very high levels of exposure (Seppaleinen and Haltia, 1980). In recent decades, interest in the human health effects has been focused on the NS and the cardiovascular system, where injury has been documented in workers exposed to much higher levels than those that are allowed today.

What is clearly established is the capacity of CS_2 to cause a distal axonopathy that is identical pathologically to that caused by *n*-hexane. There is growing evidence that covalent cross-linking of neurofilaments also underlies CS_2 neuropathy through a series of reactions that parallel the sequence of events in *n*-hexane neuropathy. Although *n*-hexane requires metabolism to 2,5-hexanedione, CS_2 is itself the ultimate toxicant, reacting with protein amino groups to form dithiocarbamate adducts (Lam and DiStefano, 1986). The dithiocarbamate adducts of lysyl amino groups undergo decomposition to isothiocyanate adducts, electrophiles that then react with protein

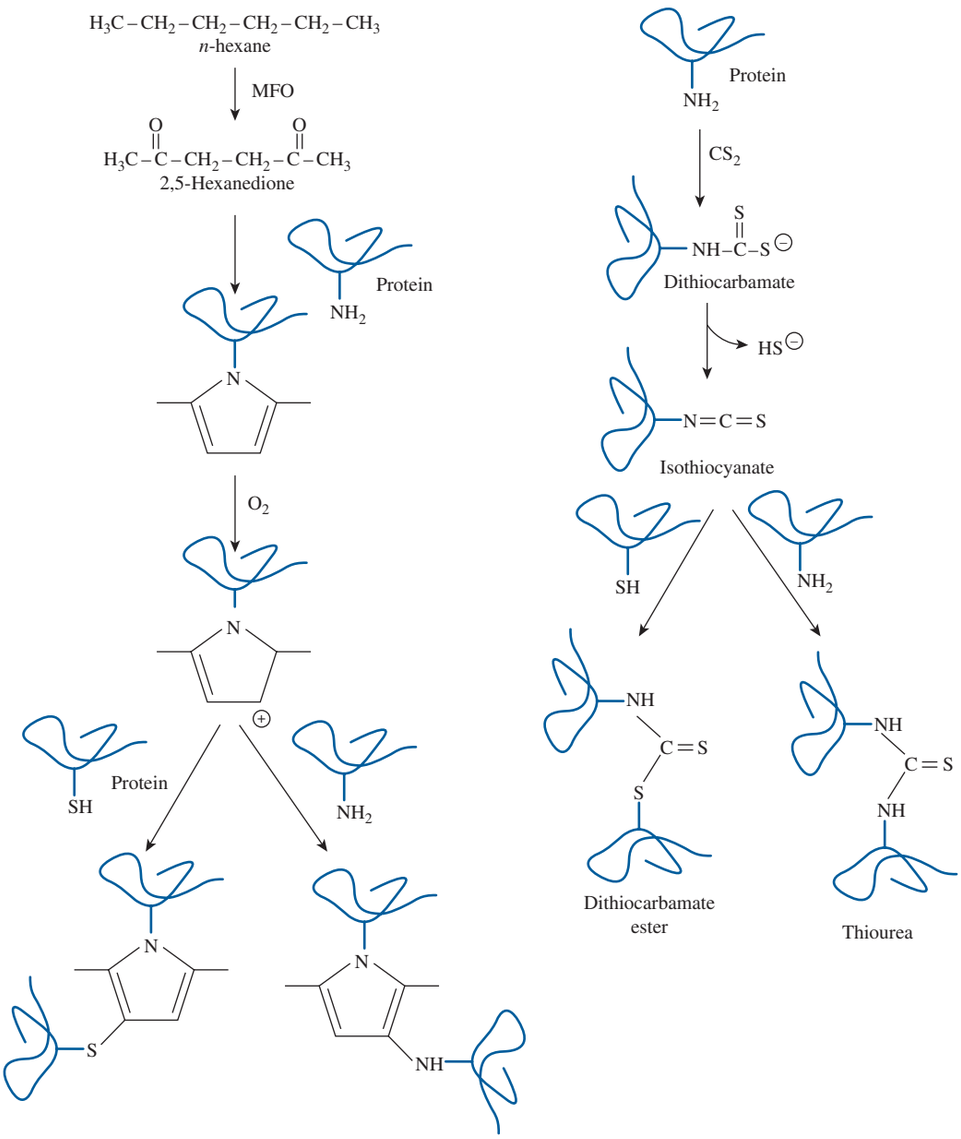


Figure 16-4. *Molecular mechanisms of protein cross-linking in the neurofilamentous neuropathies.* Both 2,5-hexanedione, produced from hexane via ω -1 oxidation function of mixed function oxidase (MFO), and CS_2 are capable of cross-linking proteins. Pyrrole formation from 2,5-hexanedione is followed by oxidation and reaction with adjacent protein nucleophiles. Dithiocarbamate formation from CS_2 is followed by formation of the protein-bound isothiocyanate and subsequent reaction with adjacent protein nucleophiles.

nucleophiles to yield covalent cross-linking (Fig. 16-4). The reaction of the isothiocyanate adducts with cysteinyl sulfhydryls to form *N*,*S*-dialkyldithiocarbamate ester cross-links is reversible, whereas the reaction with protein amino functions forms thiourea cross-links irreversibly. Over time, the thiourea cross-links predominate and are most likely the most biologically significant (Amarnath et al., 1991; Valentine et al., 1992, 1995; Graham et al., 1995).

As with *n*-hexane neuropathy, it has been postulated that the stability and long transport distance of the neurofilament determine that this protein is the toxicologically relevant target in chronic CS_2 intoxication. Nonetheless, proteins throughout the organism are derivatized and cross-linked as well. Cross-linking has been identified in erythrocyte-associated proteins including spectrin and globin as well as in the putative neurotoxic target neurofilament subunits (Valentine et al., 1993, 1997). Analysis of cross-linking in erythrocyte proteins has verified that cross-linking occurs through thiourea bridges that accumulate with continuing exposure (Erve et al., 1998a, 1998b). Neurofilament cross-linking involves all three subunits and also demonstrates a cumulative dose–response and temporal relationship consistent with it being a contributing event in the development of the axonal neurofilamentous swellings. The correlation of protein cross-linking in erythrocyte proteins and axonal proteins together with the ability to detect covalent modifications on peripheral proteins at subneurotoxic levels and at preneurotoxic time points suggests that modifications on peripheral proteins can be used as biomarkers of effect for CS_2 exposure. These biomarkers, together with morphological changes, have been used to establish CS_2 as the ultimate neurotoxic species in the peripheral neuropathy produced by oral administration of *N*,*N*-diethyldithiocarbamate (Johnson et al., 1998).

The clinical effects of exposure to CS_2 in the chronic setting are very similar to those of *n*-hexane exposure, with the development of sensory and motor symptoms occurring initially in a stocking-and-glove distribution. In addition to this chronic axonopathy, CS_2 can lead to aberrations in mood and indications of diffuse encephalopathic disease. Some of these signs and symptoms are transient at first and subsequently become more long lasting, a feature that is common in vascular insufficiency in the NS. This fact, in combination with the knowledge that CS_2 may accelerate the process of atherosclerosis, suggests that some of the effects of CS_2 on the CNS are vascular in origin.

β,β′-Iminodipropionitrile β,β′-Iminodipropionitrile (IDPN) is a synthetic, bifunctional nitrile that causes a "waltzing syndrome" in rats and other mammals, although human exposure has never been documented. Features of this waltzing syndrome include excitement, circling, head twitching, and overalertness, and are observed after a single large intraperitoneal injection to a rat (1.5 to 2.0 g/kg) (Chou and Hartmann, 1964). Although the cause of this behavior has not been conclusively determined, it has been suggested that degeneration of vestibular sensory hair cells is responsible (Llorens et al., 1993).

Other pathological changes also follow administration of IDPN, most notably in large caliber axons in spinal cord and peripheral nerves, the primary targets of neurotoxicity. The accumulation of neurofilaments in the proximal axon occurs, leading to swelling without degeneration in most animals (Gold, 2000). Quails deficient in neurofilaments demonstrate no swellings when administered IDPN, suggesting that the toxicity is due to a selective effect on neurofilaments (Mitsuishi et al., 1993). These neurofilament swellings are similar to those observed in CS_2 or γ-diketones toxicity. Repeated exposure to IDPN leads to demyelination and onion bulb formation, and eventually can produce distal axonal atrophy due to a reduction in anterograde neurofilament transport to the distal axon (Clark et al., 1980).

This impairment of axonal transport results from the disruption of the association between microtubules and neurofilaments by IDPN, causing neurofilament accumulation (Griffin et al., 1983). This leads to complete disturbance of the cytoskeleton of the axon. Although unclear, the mechanism responsible for this interference is hypothesized to result from the direct alteration of neurofilament proteins by IDPN, possibly by changing their chemical properties and causing aggregation (Anderson et al., 1991).

Acrylamide Acrylamide is a man-made vinyl monomer used widely in water purification, paper manufacturing, mining, and waterproofing. It is also used extensively in biochemical laboratories, and is present in smaller amounts in many foods prepared at high temperatures. Although it can be dangerous if not handled carefully, most toxic events in humans have been observed as peripheral neuropathies in factory workers exposed to high doses (Garland and Patterson, 1967; Kesson et al., 1977; Collins et al., 1989; Myers and Macun, 1991).

Early studies of acrylamide neuropathy revealed a distal axonopathy characterized by multiple axonal swellings (Spencer and Schaumburg, 1976). Repeated dosing results in a more proximal axonopathy, in a "dying back" process. The first changes are seen in Pacinian corpuscles (an encapsulated ending of a sensory nerve that acts as a receptor for pressure and vibration), followed by muscle spindles (sensory receptors within the body of a muscle that primarily detect changes in the length of this muscle) and the nerve terminal. These changes are caused by accumulations of neurofilaments at the nerve terminal. Paranodal swellings (swelling in the areas of the Nodes of Ranvier, also known as myelin sheath gaps, where discontinuities exist in the insulating myelin sheaths of myelinated axons) develop, leading to the retraction of myelin (Schaumburg et al., 1974; Spencer and Schaumburg, 1974). A decrease in the number of synaptic vesicles and mitochondria at the nerve terminal is also characteristic, probably due to inhibition of retrograde and anterograde axonal transport (DeGrandchamp et al., 1990; Padilla et al., 1993; Harris et al., 1994). It has also been observed that nerve terminal degeneration occurs prior to development of axonopathy, suggesting that this degeneration is the primary lesion (LoPachin et al., 2002).

Many early studies investigating acrylamide neurotoxicity noted nerve terminal degeneration, but for three decades the distal axonopathy was believed to be the lesion responsible for neurological signs and symptoms (e.g., ataxia and numbness in extremities). However, in more recent studies, neurological symptoms and nerve terminal degeneration were similarly observed in both short-term high-dose and long-term low-dose animals in the rat PNS, while axonal degeneration occurred only in low-dose studies subsequent to neurological alteration (LoPachin et al., 2002).

Organophosphorus Compounds Organophosphorus (OP) compounds (see Chap. 22, "Toxic Effects of Pesticides") are used not only as insecticides and chemical warfare agents, but also as chemical intermediates, flame retardants, fuel additives, hydraulic fluids, lubricants, pharmaceuticals, and plasticizers. The OP insecticides and nerve agents are designed to inhibit AChE, thereby causing accumulation of acetylcholine in cholinergic synapses resulting in cholinergic toxicity and death (Thompson and Richardson, 2004; Richardson, 2010). However, apart from the insecticides, nerve agents, and some of the pharmaceuticals, OP compounds produced for other applications often have little or no anti-AChE activity (Richardson and Makhaeva, 2014).

Some OP compounds, such as tri-*o*-cresyl phosphate (TOCP), are neuropathic and can cause a severe sensorimotor central peripheral distal axonopathy called OP compound–induced delayed neurotoxicity (OPIDN) without inducing cholinergic poisoning. This condition is also referred to as a delayed neuropathy or delayed polyneuropathy (OPIDP) (Lotti and Moretto, 2005). However, neuropathy usually connotes peripheral nerve disease, whereas OPIDN also involves degeneration of ascending and descending spinal cord tracts (Richardson and Makhaeva, 2014).

An OPIDN epidemic of massive proportions occurred during Prohibition in the United States, when Jamaica Ginger extract (Ginger Jake), a popular source of alcohol, was adulterated with TOCP. Another outbreak occurred in Morocco where olive oil was contaminated with TOCP. Human cases have also occurred after exposure to certain formerly used OP insecticides, such as EPN (*O*-ethyl-*O*-4-nitrophenyl phenylphosphonothionate) and leptophos [*O*-(4-bromo-2,5-dichlorophenyl)-*O*-methyl phenylphosphonothionate] (Lotti and Moretto, 2005).

Many OP compounds are lipophilic and readily enter the NS. If the parent compound or a metabolite has suitable reactivity, they can phosphorylate neural target proteins, such as various serine hydrolases (Casida and Quistad, 2005). When the principal target is AChE, cholinergic toxicity can ensue, either because of suprathreshold levels of inhibition or inhibition plus aging (Fig. 16-5). A substantial level of AChE inhibition on its own is sufficient to produce cholinergic toxicity and death. When aging of inhibited AChE also occurs (net loss of a ligand from the phosphorus of the OP-enzyme conjugate, leaving a negatively charged phosphoryl moiety attached to the active site), the qualitative nature of the toxicity does not change. Instead, the inhibited AChE becomes intractable to reactivation, rendering therapy with oximes, such

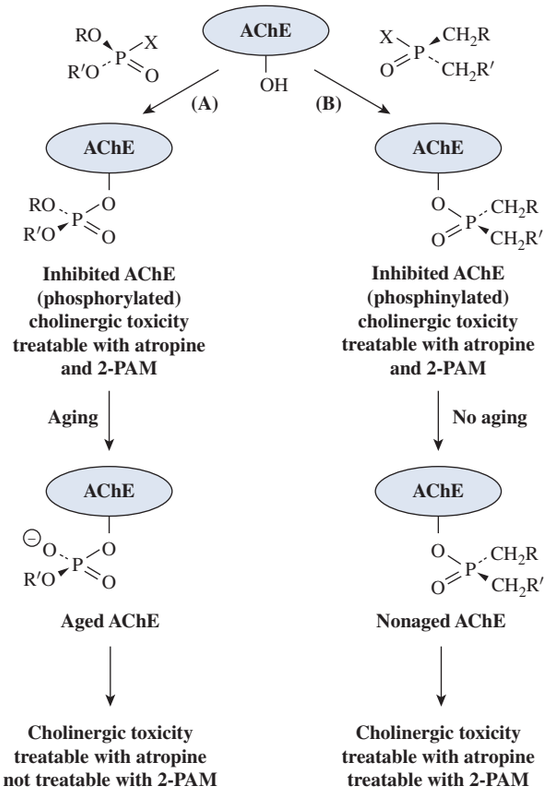


Figure 16-5. *AChE and cholinergic toxicity.* (**A**) Inhibition by an ageable OP compound produces cholinergic toxicity, treatable with atropine and 2-PAM. Aging does not alter the type of toxicity, but it obviates 2-PAM therapy. (**B**) Inhibition by a nonageable phosphinate produces cholinergic toxicity, treatable with atropine and 2-PAM. R, R′, substituted or unsubstituted alkyl or aryl groups; X, primary leaving group displaced by the AChE active site serine.

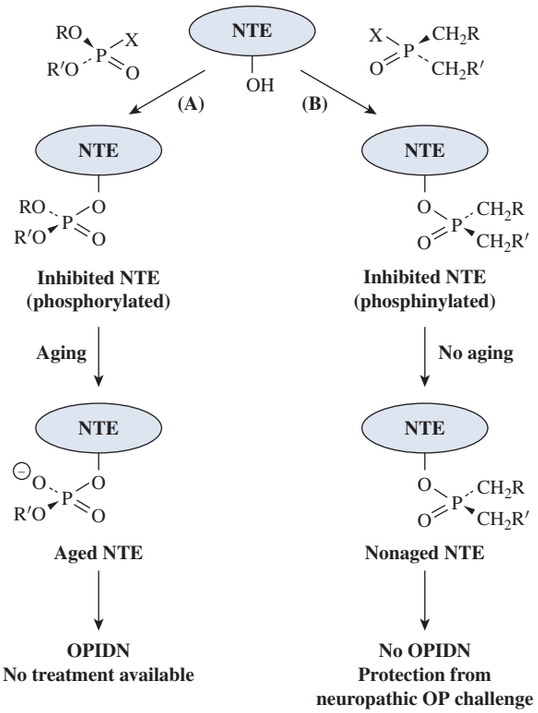


Figure 16-6. *NTE and OPIDN.* (**A**) Inhibition by an ageable OP compound; rapid aging yields a negatively charged phosphoryl conjugate resulting in OPIDN. (**B**) Inhibition by a nonageable phosphinate does not produce OPIDN but provides protection against neuropathic (ageable) NTE inhibitors. R, R′, substituted or unsubstituted alkyl or aryl groups; X, primary leaving group displaced by the NTE active site serine.

as 2-pralidoxime methiodide (2-PAM) ineffective (Thompson and Richardson, 2004; Richardson, 2010) (Fig. 16-5).

When the principal target is neuropathy target esterase (neurotoxic esterase [NTE], also known as patatin-like phospholipase domain containing 6 [PNPLA6]), OPIDN can result only if both suprathreshold (more than 70%) inhibition occurs *and* the inhibited enzyme undergoes aging. Thus, in the case of NTE and OPIDN, inhibition alone is insufficient to precipitate toxicity. It appears that the biochemical lesion is not simply a blockade of the active site. Instead, axonopathy is triggered by specific chemical modification of the NTE protein (Fig. 16-6). Neuropathic (aging) inhibitors of NTE include compounds from the phosphate, phosphonate, and phosphoramidate classes of OP compounds (Richardson, 1992; Kropp et al., 2004; Wijeyesakere and Richardson, 2010; Richardson et al., 2013) (Fig. 16-7).

Certain NTE inhibitors—including members of the phosphinate, carbamate, and sulfonylfluoride classes—do not age and do not cause OPIDN (Fig. 16-7). However, pretreatment with a nonaging NTE inhibitor prevents OPIDN from occurring after a challenge dose of a neuropathic (aging) NTE inhibitor. It has been proposed that these nonaging compounds protect against OPIDN by blocking the active site of NTE thus preventing inhibition and aging by a subsequent dose of an neuropathic (aging) inhibitor (Richardson and Makhaeva, 2014) (Fig. 16-6).

In contrast, when protective NTE inhibitors are administered following exposure to a near-threshold subclinical dose of a neuropathic OP compound, OPIDN is fully expressed (Pope et al., 1993). Because the initial treatment involves a compound that can produce OPIDN on its own and the disease is likely to be incipient rather than absent, this effect should be called potentiation; however, some authors refer to the phenomenon as promotion (Lotti, 2002). Although the potentiating chemicals inhibit NTE, this enzyme is not thought to be the target of potentiation. The level of NTE inhibition produced by the potentiator is not related to the level of potentiation observed, and these potentiators appear to exacerbate axonopathies from other causes as well, such as trauma and 2,5-hexanedione exposure. These results have been interpreted to indicate that potentiation enhances progression of the axonopathic process, inhibits repair, or both (Randall et al., 1997; Lotti, 2002).

Axonal degeneration does not commence immediately after acute exposure to a neuropathic OP compound but is delayed for at least 8 days between the acute high-dose exposure and clinical signs of axonopathy. Some effective regeneration of axons occurs in the PNS, for example, excitatory inputs to skeletal muscle from lower motor neurons in the spinal cord. In contrast, axonal degeneration is progressive and persistent in long tracts of the spinal cord, for example, inhibitory pathways from upper motor neurons in the motor cortex to lower motor neurons in the spinal cord anterior horn. Accordingly, the clinical picture of OPIDN changes from flaccid to spastic paralysis during a course of months to years (Lotti and Moretto, 2005; Richardson and Makhaeva, 2014).

Fortunately, studies of the initiation steps of OPIDN and structure–activity relationships of neuropathic OP compounds have led to

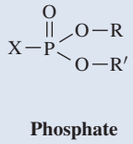
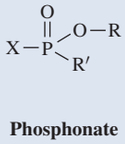
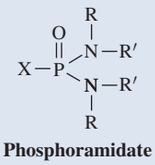
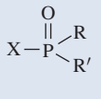
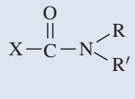
(A) **Neuropathic (ageable)**			
	Phosphate	**Phosphonate**	**Phosphoramidate**
(B) **Nonneuropathic (nonageable)** **pretreatment: protection** **posttreatment: potentiation**			
	Phosphinate	**Sulfonate**	**Carbamate**

Figure 16-7. *NTE inhibitors.* (**A**) Neuropathic (ageable). (**B**) Nonneuropathic (nonageable). R, R′, substituted or unsubstituted alkyl or aryl groups; X, primary leaving group displaced by the NTE active site serine.

highly accurate prediction of the neuropathic potential of these chemicals. Consequently, human cases of OPIDN are now rare and usually arise from intentional ingestion of massive doses of OP insecticides in suicide attempts. Nevertheless, the fact remains that OPIDN is a debilitating and incurable condition. Moreover, although the mechanism linking aged NTE to axonopathy is unknown, mutations affecting the catalytic domain of NTE produce motor neuron disease (Hein et al., 2010; Rainier et al., 2011). Furthermore, NTE is now known to have phospholipase B activity, hydrolyzing phosphatidylcholine at both the sn-2 and sn-1 positions to glycerophosphocholine and a fatty acid. Because conditional knockout of nervous system NTE produces lesions similar to those seen in OPIDN, aging inhibitors of NTE are likely disrupting phospholipid homeostasis in neurons (Glynn, 2013). Accordingly, research continues in order to enhance mechanistic understanding that could be applied to improving biosensors and biomarkers of exposure, high-throughput testing of new compounds, prophylaxis and treatment for OPIDN, and understanding of motor neuron diseases (Richardson, 2005; Wijeyesakere and Richardson, 2010; Richardson et al., 2015).

Recently, concerns about the neurotoxicity of TOCP have resurfaced in the guise of aerotoxic syndrome, a constellation of neurobehavioral symptoms attributed to breathing aircraft cabin air containing a variety of contaminants including TOCP from jet engine lubricants. Binding of the cresyl saligenin phosphate (CBDP) toxic metabolite of TOCP to NTE and other proteins has been detected, but the mechanism of aerotoxic syndrome remains to be elucidated (Schopfer et al., 2014; Hausherr et al., 2017).

The foregoing discussion has been limited to organic compounds of pentacovalent phosphorus, which are by far the most common and best studied of the OP compounds. However, organic compounds of trivalent phosphorus, such as triphenylphosphine (TPPn) and triphenylphosphite (TPPi), have relatively widespread use, particularly as antioxidants, chemical intermediates, and polymer enhancers. Both TPPn and TPPi produce axonal degeneration in the CNS and PNS, but the spatial–temporal distributions of lesions are different from that of classical OPIDN produced by organic pentacovalent phosphorous compounds and the pathogenic mechanisms are unknown (Abou-Donia, 1992). In particular, it appears that the mechanism of initiation of axonopathy by TTPn is independent of NTE inhibition and aging (Davis et al., 1999), and this relationship is unclear for TPPi (Padilla et al., 1987; Richardson et al., 2015).

Pyridinethione This compound is a chelating agent that is usually encountered as the zinc complex. Two molecules of pyridinethione are complexed with zinc to form bis[1-hydroxy-2(1*H*)-pyridine-thionato] zinc, commonly known as zinc pyridinethione or zinc pyrithione (ZPT) (Bond et al., 2002; Lewis et al., 2005).

ZPT is a biocide that has antibacterial and antifungal properties. It is the active ingredient in shampoos and other preparations for the treatment of seborrheic dermatitis and dandruff. ZPT is also used as an antifouling agent for ship paints, drywall, and tarps, and as an antibacterial agent for incorporation into cleaning sponges. Thus, the intended uses of ZPT can lead to human exposures through direct dermal contact and potential exposure to biota through leaching into marine and freshwater environments (Pierard-Franchimont et al., 2002; Grunnet and Dahllof, 2005).

Because the compound is directly applied to the human scalp in antidandruff shampoos, the finding that ZPT produced limb weakness and peripheral neuropathy in rodents after oral administration raised concern about potential neurotoxicity in humans (Sahenk and Mendell, 1979). Rats, rabbits, and guinea pigs all develop a distal axonopathy when exposed to ZPT in the diet. Fortunately, however, dermal absorption of ZPT is minimal (Klaassen, 1976), and there have been no reports of neurological findings in humans attributable to occupational or consumer ZPT exposures (Sahenk and Mendell, 2000).

Although the zinc ion appears to be an important component of the therapeutic action of ZPT, only the pyridinethione moiety is absorbed following ingestion (Klaassen, 1976), with the majority of zinc eliminated in the feces. In addition, oral sodium pyridinethione is also neurotoxic, indicating that the pyridinethione moiety is responsible for the neurotoxicity. Pyridinethione chelates zinc, copper, and other metal ions and, once oxidized to the disulfide, may lead to the formation of protein–pyridinethione mixed disulfides. However, which of these properties, if any, is responsible for the molecular mechanism of its neurotoxicity remains unknown (Sahenk and Mendell, 2000).

Although these molecular issues remain to be resolved, pyridinethione appears to interfere with the fast axonal transport systems. Although the fast anterograde system is less affected, pyridinethione impairs the turnaround of rapidly transported vesicles and slows their retrograde transport (Sahenk and Mendell, 1980). This aberration of the fast axonal transport systems is the most likely physiological basis of the accumulation of tubulovesicular structures in the distal axon. As these materials accumulate in one region of the axon, they distend the axonal diameter, resulting in axonal swellings filled with membranous profiles. As in many other distal axonopathies, the axon degenerates in its more distal regions beyond

the accumulated structures. The earliest signs are diminished grip strength and electrophysiological changes of the axon terminal, with normal conduction along the proximal axon in the early stages of exposure (Ross and Lawhorn, 1990).

Microtubule-Associated Neurotoxicity A number of plant alkaloids alter the assembly and depolymerization of microtubules in nerve axons, causing neurotoxicity. The oldest known of these are colchicine and the vinca alkaloids, which bind to tubulin and cause depolymerization of microtubules. Colchicine is an alkaloid pharmaceutical used in the treatment of gout, familial Mediterranean fever, and other disorders. A common side effect of treatment in patients with abnormal renal function is a peripheral axonal neuropathy. Although this neuropathy is generally mild, it is often accompanied by a disabling myopathy that can lead to the inability to walk (Riggs et al., 1986).

A number of vinca alkaloids, including vincristine and vinblastine—both chemotherapeutic agents—produce a peripheral axonopathy very similar to that induced by colchicine. Vincristine is commonly used to treat leukemias and lymphomas, and has greater potential for adverse toxic effects than vinblastine. The chemical binds to tubulin subunits and prevents the polymerization into microtubules (Prakash and Timasheff, 1992). Nearly all evidence of vincristine-induced neuropathy has been observed in humans. Most treated patients develop neurotoxicity to some extent, beginning with parasthesias of the fingers. General weakness and clumsiness is common, but this improves quickly with removal of treatment. Parasthesias may persist, however, and some distal sensory loss may be permanent (Schaumburg, 2000).

More recently paclitaxel (Taxol), another plant alkaloid, has become a popular chemotherapeutic drug used to treat a variety of neoplasms. However, side effects include a predominantly sensory neuropathy, beginning in the hands and feet (Sahenk et al., 1994). Like colchicine and the vinca alkaloids, paclitaxel binds to tubulin; however, instead of leading to depolymerization, it promotes the formation of microtubules. Once formed, these microtubules remain stabilized by paclitaxel even in conditions that normally lead to dissociation of tubulin subunits, including cold temperatures or the presence of calcium (Schiff and Horwitz, 1981). When paclitaxel is injected directly into the sciatic nerve of rats, microtubules aggregate along the axon, causing demyelination, axonal degeneration, and impairment of regeneration (Lipton et al., 1989; Mielke et al., 2006).

The pathologies of the axon induced by these drugs are different. Although colchicine leads to atrophy of the axon and a decrease in the number of microtubules, paclitaxel causes their aggregation to form a matrix that may inhibit fast axonal transport, which has been demonstrated with both colchicine and paclitaxel. A change in the number of microtubules has been observed in some reports and absent from others (Roytta et al., 1984; Nakata and Yorifuji, 1999). Although the mechanisms may differ slightly, both exposures result in a peripheral neuropathy that must be taken into account in medical treatments.

Myelinopathies

Myelin provides electrical insulation of neuronal processes, and its absence leads to a slowing of and/or aberrant conduction of impulses between adjacent processes, so-called ephaptic transmission. Toxicants exist that result in the separation of the myelin lamellae, termed intramyelinic edema, and in the selective loss of myelin, termed demyelination. Intramyelinic edema may be caused by alterations in the transcript levels of myelin basic protein-mRNA (Veronesi et al., 1991), and early in its evolution this pathology is reversible. However, the initial stages may progress to demyelination, with loss of myelin from the axon. Demyelination may also result from direct toxicity to the myelinating cell. Remyelination in the CNS occurs to only a limited extent after demyelination. However, Schwann cells in the PNS are capable of remyelinating the axon after a demyelinating injury. Interestingly, remyelination after segmental demyelination in peripheral nerve involves multiple Schwann cells and results, therefore, in internodal lengths (the distances between adjacent nodes of Ranvier) that are much shorter than normal and a permanent record of the demyelinating event.

The compounds in Table 16-3 all lead to a myelinopathy. Some of these compounds have created problems in humans, and many have been used as tools to explore the process of myelination of the NS and the process of remyelination following toxic disruption of myelin. In general, the functional consequences of demyelination depend on the extent of the demyelination and whether it is localized within the CNS or the PNS or is more diffuse in its distribution. Those toxic myelinopathies in which the disruption of myelin is diffuse generate a global neurological deficit, whereas those that are limited to the PNS produce the symptoms of peripheral neuropathy.

Hexachlorophene Hexachlorophene, or methylene 2,2′-methylene-bis(3,4,6-trichlorophenol), resulted in human neurotoxicity when newborn infants, particularly premature infants, were bathed with the compound to avoid staphylococcal skin infections (Mullick, 1973). Following skin absorption of this hydrophobic compound, hexachlorophene enters the NS and results in intramyelinic edema, splitting the intraperiod line of myelin in both the CNS and the PNS. The intramyelinic edema leads to the formation of vacuoles, creating a "spongiosis" of the brain (Purves et al., 1991). Experimental studies with erythrocyte membranes show that hexachlorophene binds tightly to cell membranes, resulting in the loss of ion gradients across the membrane (Flores and Buhler, 1974). This loss of the ability to exclude ions from between the layers of myelin leads to water and ion entry, which separates the myelin layers as "edema." Another, perhaps related, effect is the uncoupling of mitochondrial oxidative phosphorylation by hexachlorophene (Cammer and Moore, 1972) because this process is dependent on a proton gradient. Intramyelinic edema is reversible in the early stages, but with increasing exposure, hexachlorophene causes segmental demyelination. Swelling of the brain causes increased intracranial pressure, which may be fatal. With high-dose exposure, axonal degeneration is seen, along with degeneration of photoreceptors in the retina. It has been postulated that the pressure from severe intramyelinic edema may also injure the axon, leading to axonal degeneration; endoneurial pressure measurements support this idea (Myers et al., 1982). The toxicity of hexachlorophene expresses itself functionally in diffuse ways that reflect the disseminated process of myelin injury. Humans exposed acutely to hexachlorophene may have generalized weakness, confusion, and seizures. Progression may occur, to include coma and death.

Tellurium This metal has been shown to improve the machinability of copper and stainless steel. Tellurium addition to lead decreases the corrosive action of sulfuric acid on lead and improves its strength and hardness. It is also used as a basic ingredient in blasting caps and for chill control in cast iron. Although human exposures have not been reported, neurotoxicity of tellurium has been demonstrated in animals. Young rats exposed to tellurium in their diet develop a severe peripheral neuropathy. Within the first 2 days of dietary exposure, the synthesis of myelin lipids in Schwann cells displays striking changes (Harry et al., 1989). These include decreased synthesis of cholesterol and cerebrosides (lipids richly represented in myelin), and downregulated myelin protein mRNA

Table 16-3

Compounds Associated with Injury of Myelin (Myelinopathies)

NEUROTOXICANT	NEUROLOGICAL FINDINGS	BASIS OF NEUROTOXICITY	REFERENCES
Acetylethyltetramethyl tetralin (AETT)	Not reported in humans; hyperexcitability, tremors (rats)	Intramyelinic edema, pigment accumulation in neurons	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Amiodarone	Peripheral neuropathy	Axonal degeneration and demyelination, lipid-laden lysosomes in Schwann cells	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Cuprizone	Not reported in humans, encephalopathy (experimental animals)	Status spongiosis of white matter, intramyelinic edema (early stages); gliosis (late)	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Disulfiram	Peripheral neuropathy, predominantly sensory	Axonal degeneration, swellings in distal axons	Graham and Lantos (1997)
Ethidium bromide	Insufficient data (humans)	Intramyelinic edema, status spongiosis of white matter	Spencer and Schaumburg (2000)
Hexachlorophene	Irritability, confusion, seizures	Brain swelling, intramyelinic edema in CNS and PNS, late axonal degeneration	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Lysolecithin	Effects only on direct injection into PNS or CNS (experimental animals)	Selective demyelination	Graham and Lantos (1997)
Perhexilene	Peripheral neuropathy	Demyelinating neuropathy, membrane-bound inclusions in Schwann cells	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Tellurium	Hydrocephalus, hind-limb paralysis (experimental animals)	Demyelinating neuropathy, lipofuscinosis (experimental animals)	Graham and Lantos (1997)
Triethyltin	Headache, photophobia, vomiting, paraplegia (irreversible)	Brain swelling (acute) with intramyelinic edema, spongiosis of white matter	Chang and Dyer (1995), Graham and Lantos (1997), Spencer and Schaumburg (2000)

(Morell et al., 1994). On the contrary, the synthesis of phosphatidylcholine, a more ubiquitous membrane lipid, is unaffected. The synthesis of free fatty acids and cholesterol esters increases to some degree, and there is a marked elevation of squalene, a precursor of cholesterol. These biochemical findings demonstrate a variety of lipid abnormalities, and the simultaneous increase in squalene and decrease in cholesterol suggests that tellurium or one of its derivatives may interfere with the normal conversion of squalene to cholesterol. Squalene epoxidase, a microsomal monooxygenase that utilizes NAPDH cytochrome P450 reductase, has been strongly implicated as the target of tellurium, because its inhibition by tellurium as well as certain other organotellurium compounds shows a correlation between the potency of enzyme inhibition and demyelination in vivo (Goodrum, 1998).

In conjunction with these biochemical changes, lipids accumulate in Schwann cells within intracytoplasmic vacuoles; shortly afterward, these Schwann cells lose their ability to maintain myelin. Axons and the myelin of the CNS are impervious to the effects of tellurium. However, individual Schwann cells in the PNS disassemble their concentric layers of myelin membranes, depriving the adjacent intact axon of its electrically insulated status. Not all Schwann cells are equally affected by the process; rather, those Schwann cells that encompass the greatest distances appear to be the most affected. These cells are associated with the largest-diameter axons, encompass the longest intervals of myelination, and provide the thickest layers of myelin. Thus, it appears that the most vulnerable cells are those with the largest volume of myelin to support (Bouldin et al., 1988).

As the process of remyelination begins, several cells cooperate to reproduce the myelin layers that were previously formed by a single Schwann cell. Perhaps this diminished demand placed upon an individual cell is the reason that remyelination occurs even in the presence of continued exposure to tellurium (Bouldin et al., 1988). The expression of the neurological impairment is also short in duration, reflecting the transient cellular and biochemical events. The animals initially develop severe weakness in the hind limbs but then recover their strength after 2 weeks on the tellurium-laden diet.

Lead Lead exposure in animals results in a peripheral neuropathy with prominent segmental demyelination, a process that bears a strong resemblance to tellurium toxicity (Dyck et al., 1977). However, the neurotoxicity of lead is much more variable in humans than in rats, and there are also a variety of manifestations of lead toxicity in other organ systems.

The neurotoxicity of lead has been appreciated for centuries. In current times, adults are exposed to lead in occupational settings through lead smelting processes and soldering and in domestic settings through old paint, lead-containing pipes or solder, or through the consumption of "moonshine" contaminated with this metal. In addition, some areas contain higher levels of environmental lead,

resulting in higher blood levels in the inhabitants. Children, especially those below 5 years of age, have higher blood levels of lead than adults in the same environment, due to the mouthing of objects and the consumption of substances other than food. In addition, children absorb lead more readily, and the very young do not have the protection of the blood–brain barrier. Lead exposures in children include lead-based paint or contaminated water, soil, or food. Despite efforts to limit such exposures, many children still have blood lead levels above those which should trigger public health actions (CDC, 2017). In young children, acute massive exposures to lead result in severe cerebral edema, perhaps from damage to endothelial cells. Children seem to be more susceptible to this lead encephalopathy than adults (Johnston and Goldstein, 1998); however, adults may also develop an acute encephalopathy in the setting of massive lead exposure.

Chronic lead intoxication in adults results in peripheral neuropathy, often accompanied by manifestations outside the NS, such as gastritis, colicky abdominal pain, anemia, and the prominent deposition of lead in particular anatomic sites, creating lead lines in the gums and in the epiphyses of long bones in children (though rare today in developing countries). The effects of lead on the peripheral nerve of humans (lead neuropathy) are not entirely understood. Electrophysiological studies have demonstrated a slowing of nerve conduction. Although this observation is consistent with the segmental demyelination that develops in experimental animals, pathological studies in humans with lead neuropathy typically have demonstrated an axonopathy. Another finding in humans is the predominant involvement of motor axons, creating one of the few clinical situations in which patients present with predominantly motor symptoms. The basis for the effect on the brain (lead encephalopathy) is also unclear, although an effect on the membrane structure of myelin and myelin membrane fluidity has been shown (Dabrowski-Bouta et al., 1999). The etiologies associated with the pathogenesis of peripheral neuropathy are, like that of central neuropathy, rather speculative. One hypothesis postulates that the effects of lead on the blood–nerve barrier are similar to those on the blood–brain barrier, providing a unified mechanism for lead's effects on both the PNS and CNS. Another theory on the mechanisms associated with lead-induced effects on the PNS suggests a toxic effect on Schwann cells, and the ensuing demyelination of nerves (Powell et al., 1982).

Although the manifestations of acute and chronic exposures to lead have been long established, the effects of low-level exposures on infants and children have become realized. Initial reports noted a relationship between mild elevations of blood lead in children and school performance; more recently, correlations between elevated lead levels in decidual teeth and performance on tests of verbal abilities, attention, and behavior (nonadaptive) have been demonstrated (Needleman and Gatsonis, 1990; Needleman, 1994). Although there is a clear association between lead level and intellectual performance, there has been some discussion as to whether lead is causal. Children with higher blood levels tend to share certain other environmental factors, such as socioeconomic status and parental educational level. However, in spite of these complex social factors, it appears that lead exposure has an adverse effect on the intellectual abilities of children (Needleman, 1994; CDC, 2017), although a threshold for these effects has not yet been determined.

Astrocytes

Rather than being the passive glue described by Virchow and other pathologists, astrocytes are now known to perform and regulate a wide range of physiological functions in the CNS. Perturbations in the function of these important cells are frequently reflected in abnormal neuronal physiology, even in the absence of altered nerve cell morphology. Indeed, the astrocyte appears to be a primary means of defense in the CNS following exposure to neurotoxicants, as a spatial buffering system for osmotically active ions and as a depot for the sequestration and metabolic processing of endogenous molecules and xenobiotics. Although in its relative infancy, investigations into the role of astrocytes in normal/abnormal function of the NS will be crucial to a better understanding of neurotoxicity and its pathological sequelae.

Ammonia Hepatic encephalopathy (HE) and congenital and acquired hyperammonemia lead to excessive brain ammonia (ammonium, NH_4^+) accumulation. The condition results from liver failure. The effects of ammonia on the CNS vary with its concentration. At high CNS concentrations ammonia produces seizures, resulting from its depolarizing action on cell membranes, whereas, at lower concentrations, ammonia produces stupor and coma, consistent with its hyperpolarizing effects. Ammonia intoxication is associated with astrocytic swelling and morphological changes, yielding the so-called Alzheimer type II astrocytes, which precede any other morphological change (Mossakowski et al., 1970). The exclusive site for brain detoxification of glutamate to glutamine occurs within the astrocytes. This process requires ATP-dependent amidation of glutamate to glutamine, and it is mediated by the astrocyte-specific enzyme, glutamine synthetase (GS), and catalyzed by ammonia. Increased intracellular ammonia concentrations have also been implicated in the inhibition of neuronal glutamate precursor synthesis, resulting in diminished glutamatergic neurotransmission, changes in neurotransmitter uptake (glutamate), and changes in receptor-mediated metabolic responses of astrocytes to neuronal signals (Albrecht, 1996).

Nitrochemicals The therapeutic potential of organic nitrates has been recognized for more than a century and began with the use of nitroglycerine for the management of acute anginal episodes. The resulting peripheral vasodilation and reduction in blood pressure, while useful in treating cardiovascular disease, have recently been shown to be only one of the pharmacological properties of this class of chemical. The mitochondrion features prominently as a target for nitrochemicals; however, the causal relationship between mitochondrial dysfunction and initiation of the neurotoxic state remains to be established for many of the chemicals.

The dinitrobenzenes are important synthetic intermediates in the industrial production of dyes, plastics, and explosives. The neurotoxic compound, 1,3-dinitrobenzene (DNB), produces gliovascular lesions that specifically target astrocytes in the periaqueductal gray matter of the brainstem and deep cerebellar roof nuclei (Philbert et al., 1987). Although the molecular basis for the remarkable sensitivity of this cell population is unclear, it has been proposed that bioactivation of DNB by NADPH-dependent cytochrome *c* reductase (Romero et al., 1991; Hu et al., 1997) and subsequent induction of oxidative stress underlie its toxicity (Ray et al., 1992, 1994; Romero et al., 1995; Hu et al., 1999). Brainstem nuclei with high glucose requirements, such as the vestibular and deep cerebellar roof nuclei, are affected more severely than forebrain and mesencephalic structures that have similar or higher requirements for glucose and oxygen (Bagley et al., 1989; Mastrogiacomo et al., 1993; Calingasan et al., 1994). The molecular basis of the susceptibility of brainstem astrocytes is unknown but growing evidence suggests that differences in mitochondrial respiratory capacity, cellular antioxidant levels, and the expression of proteins that regulate the mitochondrial permeability transition pore (mtPTP) all contribute to the observed regional and cellular differences in susceptibility. In vitro studies indicate that DNB is a potent inducer of the mtPTP (for reviews of the mtPTP see Lemasters et al. [1998] and

Crompton [1999]) in cultured C6 glioma cells (Tjalkens et al., 2000). Mitochondrial inner membrane permeabilization in an in vitro model of DNB exposure is dependent on generation of ROS, in agreement with the reported capacity of DNB to deplete reduced pyridine nucleotides and GSH due to redox-cycling (Romero et al., 1995).

Metronidazole, a 5-nitroimidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], is an antimicrobial, antiprotozoal drug that is commonly used for the treatment of a wide variety of infections. Prolonged treatment with metronidazole is associated with a peripheral neuropathy characterized by paraesthesias, dysesthesias, headaches, glossitis, urticaria, and pruritus in addition to other somatosensory disorders. Long-term administration of metronidazole produces an irreversible sensorimotor deficit in the lower extremities of humans (Kapoor et al., 1999). Metronidazole is readily reduced to the highly reactive and toxic hydroxylamine intermediate and binds to cellular macromolecules including proteins and DNA (Coxon and Pallis, 1976). Use of higher intravenous doses of metronidazole for an extended period results in the expression of epileptiform seizures, hallucination, and attendant encephalopathy. The distribution of lesions is similar to preceding descriptions for DNB with the exception that both neurons and glia appear to be equally susceptible to the effects of metronidazole (Schentag, 1982). The mechanism of toxicity is well linked to the fact that metronidazole and its reduced metabolites bear close structural resemblance to the antineuritic nutrient, thiamine. Thiamine triphosphate (vitamin B1) is an essential coenzyme in the mitochondrial metabolism of α-ketoglutarate, pyruvate, and also modulates the activity of sodium channels. Given the similarity in the lesions produced by metronidazole and pyrithiamine, a common antimetabolite mode of action has been proposed as the primary mechanism of neurotoxicity (Evans et al., 1975; Watanabe and Kanabe, 1978; Kapoor et al., 1999).

Methionine Sulfoximine Methionine sulfoximine (MSO) is used as an ancillary material in upstream biomanufacturing applications and processes. However, it is best recognized for its biological properties where it can be used as an irreversible inhibitor of the astrocyte-specific enzyme, glutamine synthetase (GS) (Albrecht and Norenberg, 1990). Ingestion of large amounts of MSO leads to neuronal cell loss in the hippocampal fascia dentata and pyramidal cell layer, in the short association fibers and lower layers of the cerebral cortex, and in cerebellar Purkinje cells. MSO also leads to large increases of glycogen levels (Folbergrova, 1973), primarily within astrocytic cell bodies (Phelps, 1975), as well as swollen and damaged astrocytic mitochondria (Hevor, 1994). Although it is generally accepted that MSO inhibits GS, it remains unclear whether this inhibition represents the primary mechanism of MSO neurotoxicity. The relationship between inhibition of GS by MSO and seizure generation is also not well understood but is believed to be associated with inhibition of glutamate and gamma-aminobutyric acid (GABA) in seizure generation, because glutamine provides the precursor for these neurotransmitters. Rothstein and Tabakoff (1986) have demonstrated that the calcium-dependent, potassium-stimulated release of glutamate and aspartate is inhibited in striatal tissue after intracerebroventricular injection of MSO, and that their release correlates over time with the inhibition of GS.

Fluoroacetate and Fluorocitrate The Krebs cycle inhibitor fluorocitrate (FC) and its precursor fluoroacetate (FA) are preferentially taken up by glia. FA occurs naturally in a number of plants, and is available commercially as a rodenticide (Compound 1080). It is prevalent in the South African plant *Dichapetalum cymosum*, commonly referred to as the Gifblaar plant. Exposure to FA may also occur via exposure to the anticancer drug 5-fluorouracil (Okeda et al., 1990). Ingestion of large amounts of FA results in tonic convulsions. Animals consuming FA commonly seize within minutes, and those surviving these episodes frequently die later on due to respiratory arrest or heart failure. The actions of FC and FA have been attributed both to the disruption of carbon flux through the Krebs cycle and to impairment of ATP production (Swanson and Graham, 1994). FA can be metabolized to fluoroacetyl CoA, followed by condensation with oxaloacetate to form FC by citrate synthase. A second hypothesis implies that FA toxicity is associated with the inhibition of a bidirectional citrate carrier in mitochondrial membranes, which leads to elevated intramitochondrial citrate and could affect citrate-dependent ATP synthesis (Kirsten et al., 1978). Finally, it has been suggested that elevated citrate, secondary to inhibition of aconitase, is associated with the cytotoxicity of these compounds. FA selectively lowers the level of glutamine and inhibits glutamine formation in the brain, not by depleting glial cells of ATP, but by causing a rerouting of 2-oxoglutarate from glutamine synthesis into the tricarboxylic acid (TCA, or Krebs) cycle during inhibition of aconitase (Hassel et al., 1994). After the inhibition of aconitase, citrate accumulates, whereas the levels of isocitrate and α-ketoglutarate decrease. The reversible enzyme glutamate dehydrogenase begins to work in the opposite direction feeding more α-ketoglutarate into the TCA cycle (Martin and Waniewski, 1996).

Neurotransmission-Associated Neurotoxicity

A wide variety of naturally occurring toxins, as well as synthetic chemicals, alter specific mechanisms of intercellular communication. Some chemicals that have neurotransmitter-associated toxicity are listed in Table 16-4.

Although neurotransmitter-associated actions may be well understood for some agents, the specificity of the mechanisms should not be assumed. OP and carbamate pesticides produce their insecticidal actions by inhibiting AChE, the catalytic enzyme that ends the postsynaptic action of acetylcholine. The resultant cholinergic overstimulation produces signs of acute toxicity ranging from flu-like symptoms to gastrointestinal distress, ataxia, twitching, convulsions, coma, and death. These effects are not as well-correlated with AChE inhibition as might be expected for all such pesticides, leading to suggestions of additional mechanisms of action that have since been verified in animal and in vitro studies. These include direct actions on pre- and postsynaptic cholinergic receptors and altered reuptake of choline; such actions serve to modulate the downstream impact of cholinergic overstimulation (reviewed in Pope [1999]). Thus, multiple neurotransmitter targets may be more common than was once expected.

Nicotine Widely available in tobacco products and in certain pesticides, nicotine has diverse pharmacological actions and may be the source of considerable toxicity. These toxic effects range from acute poisoning to more chronic effects. Nicotine exerts its effects by binding to a subset of cholinergic receptors, the nicotinic receptors. These receptors are located in ganglia, at the neuromuscular junction, and within the CNS, where the psychoactive and addictive properties most likely reside. Smoking and "pharmacological" doses of nicotine accelerate heart rate, elevate blood pressure, and constrict blood vessels within the skin. Because the majority of these effects may be prevented by the administration of α- and β-adrenergic blockade, these consequences may be viewed as the result of stimulation of the ganglionic sympathetic NS (Benowitz, 1986). At the same time, nicotine leads to a sensation of "relaxation" and is associated with alterations of electroencephalographic (EEG) recordings in humans. These effects are probably related to

Table 16-4

Compounds Associated with Neurotransmitter-Associated Toxicity

NEUROTOXICANT	NEUROLOGICAL FINDINGS	BASIS OF NEUROTOXICITY	REFERENCES
Amphetamine and methamphetamine	Tremor, restlessness (acute); cerebral infarction and hemorrhage; neuropsychiatric disturbances	Bilateral infarcts of globus pallidus, abnormalities in dopaminergic, serotonergic, and cholinergic systems; acts at adrenergic receptors	Hardman et al. (1996), Spencer and Schaumburg (2000)
Atropine	Restlessness, irritability, hallucinations	Block cholinergic receptors (anticholinergic)	Hardman et al. (1996), Spencer and Schaumburg (2000)
Cocaine	Increased risk of stroke and cerebral atrophy (chronic users); increased risk of sudden cardiac death; movement and psychiatric abnormalities, especially during withdrawal Decreased head circumference (fetal exposure)	Infarcts and hemorrhages; alteration in striatal dopamine neurotransmission Structural malformations in newborns	Hardman et al. (1996), Spencer and Schaumburg (2000)
Domoic acid	Headache, memory loss, hemiparesis, disorientation, seizures	Neuronal loss, hippocampus and amygdala, layers 5 and 6 of neocortex Kainate-like pattern of excitotoxicity	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Kainate	Insufficient data in humans; seizures in animals (selective lesioning compound in neuroscience)	Degeneration of neurons in hippocampus, olfactory cortex, amygdala, thalamus Binds α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptors	Graham and Lantos (1997)
β-*N*-methylamino-L-alanine (BMAA)	Weakness, movement disorder (monkeys)	Degenerative changes in motor neurons (monkeys) Excitotoxic probably via *N*-methyl-D-aspartate receptors	Graham and Lantos (1997), Spencer and Schaumburg (2000)
Muscarine (mushrooms)	Nausea, vomiting, headache	Binds muscarinic receptors (cholinergic)	Hardman et al. (1996)
Nicotine	Nausea, vomiting, convulsions	Binds nicotinic receptors (cholinergic), low-dose stimulation, high-dose blocking	Hardman et al. (1996), Spencer and Schaumburg (2000)
β-*N*-oxalylamino-L-alanine (BOAA)	Seizures	Excitotoxic probably via AMPA class of glutamate receptors	Graham and Lantos (1997), Spencer and Schaumburg (2000)

the binding of nicotine with nicotinic receptors within the CNS, and the EEG changes may be blocked with mecamylamine, a nicotinic antagonist.

Acute overdose of nicotine has occurred in children who accidentally ingest tobacco products, in tobacco workers exposed to wet tobacco leaves (Gehlbach et al., 1974), and in workers exposed to nicotine-containing pesticides. In each of these settings, the rapid rise in circulating levels of nicotine leads to excessive stimulation of nicotinic receptors, a process that is followed rapidly by ganglionic paralysis. Initial nausea, rapid heart rate, and perspiration are followed shortly by marked slowing of heart rate with a fall in blood pressure. Somnolence and confusion may occur, followed by coma; if death results, it is often the result of paralysis of the muscles of lung ventilation.

Such acute poisoning with nicotine fortunately is uncommon. Exposure to lower levels for longer duration, in contrast, is very common, and the health effects of this exposure are of considerable epidemiological concern. In humans, however, it has been difficult to separate the effects of nicotine from those of other components of cigarette smoke. The complications of smoking include cardiovascular disease, cancers (especially malignancies of the lung and upper airway), chronic pulmonary disease, and attention deficit disorders in children of women who smoke during pregnancy. Nicotine may be a factor in some of these problems. For example, an increased propensity for platelets to aggregate is seen in smokers, and this platelet abnormality correlates with the level of nicotine. Nicotine also places an increased burden on the heart through its acceleration of heart rate and blood pressure, suggesting that nicotine may play a

role in the onset of myocardial ischemia (Benowitz, 1986). In addition, nicotine also inhibits apoptosis and may play a direct role in tumor promotion and tobacco-related cancers (Wright et al., 1993).

Cocaine and Amphetamines Cocaine and amphetamine stimulants are illegal, though still widely used. The number of adults using these drugs in the United States was approximately 9 million in 1972. This number grew to nearly 33 million in 1982, and in the 2001 National Household Survey on Drug Abuse it was reported that just over 10% of those surveyed had ever used cocaine, while approximately 2.6% claimed to have used it in the last 12 months (Fishburne et al., 1983; U.S. Department of Health and Human Services, 2001). Cocaine use is abundant in urban settings. It is estimated that from 10% to 45% of pregnant women take cocaine (Volpe, 1992), and metabolites can be detected in up to 6% of newborns in suburban hospitals (Schutzman et al., 1991).

Cocaine blocks the reuptake of dopamine (DA), norepinephrine, and serotonin at nerve terminals in the CNS, and causes release of DA from storage vesicles. The primary event responsible for the addictive properties and euphoric feeling when intoxicated is a block of the DA reuptake transporter (DAT) (Giros et al., 1996). This leads to enhanced DAergic transmission, and can result in a variety of symptoms in the user. Many individuals report a euphoric feeling and increased self-confidence, in addition to racing thoughts and a feeling of pressure. In other users, a period of paranoid psychosis ensues. The mechanism of altered neurotransmission has been linked to the DA D1 receptor, as mice lacking this receptor fail to exhibit many of the same characteristic behaviors as wild-type mice (Xu et al., 2000).

Cocaine abuse also places individuals at risk for cerebrovascular defects. Habitual users exhibit a greater degree of cerebral atrophy, compared by CT scan, and are more at risk of stroke and intracranial hemorrhage (Berliner, 2000). Cerebrovascular resistance has also been found to be higher in cocaine abusers than controls using Doppler sonography (Herning et al., 1999). In chronic cocaine users, neurodegenerative disorders have been observed, similar to those observed with amphetamine use.

Amphetamines affect catecholamine neurotransmission in the CNS and have the potential to damage monoaminergic cells directly. Amphetamines, including methylenedioxymethamphetamine (MDMA, or "ecstasy"), have become popular with young adults in recent decades due to the belief that it is a "safe" drug, and its ability to increase energy and sensation in adults. However, they also exert serious side effects. Similar to cocaine, the most pronounced effect of amphetamines is on the DAergic neurons, but they can also damage 5-HT axons and axon terminals (McCann and Ricaurte, 2004). The result is a distal axotomy of DA and 5-HT neurons.

The exact mechanism of amphetamine neurotoxicity is still unknown, but several clues have emerged recently. It seems that oxidative stress plays a key role in toxicity. Following amphetamine-triggered DA release from the neuron, the DA is oxidized to produce free radicals (Lotharius and O'Malley, 2001). Chronic use can affect superoxide dismutase (SOD) and catalase balance in rodents (Frey et al., 2006), and amphetamine neurotoxicity is attenuated by antioxidants (DeVito and Wagner, 1989). It also induces hyperthermia when given at ambient temperatures, and it has been shown that increasing environmental temperature increases the associated neurotoxicity (Miller and O'Callaghan, 2003).

Because drug use and HIV infection have been linked, the effect of cocaine and amphetamine use on toxicity induced by HIV-1 proteins Tat and gp120 has also been investigated. HIV-associated dementia (HAD) is a neurological disorder afflicting many AIDS patients. Exposure to cocaine and amphetamines in AIDS patients results in a synergistic neurotoxicity, which is attenuated by β-estradiol (Turchan et al., 2001). Oxidative stress has been implicated in this mechanism as well. When a normally nontoxic dose of cocaine is administered, Tat-induced oxidative stress is enhanced (Aksenov et al., 2006).

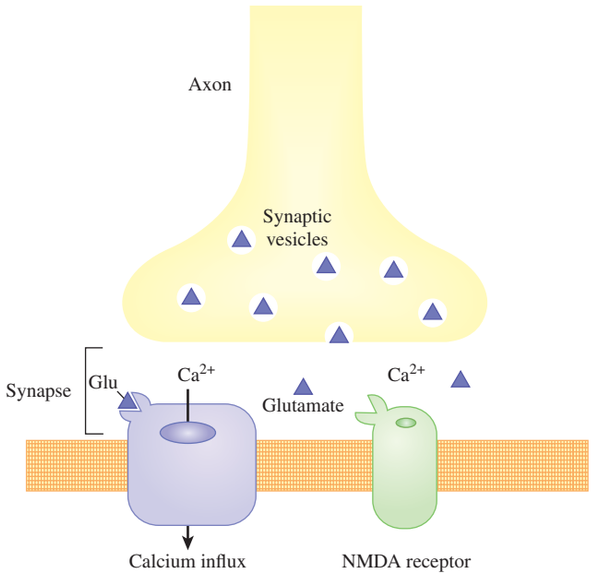


Figure 16-8. *Excitatory synapse.* Synaptic vesicles are transported to the axonal terminus, and released across the synaptic cleft to bind to the postsynaptic receptors. Glutamate, as an excitatory neurotransmitter, binds to its receptor and opens a calcium channel, leading to the excitation of the postsynaptic cell.

Excitatory Amino Acids Glutamate and certain other acidic amino acids are excitatory neurotransmitters. The discovery that these excitatory amino acids can be neurotoxic at concentrations that can be achieved in the brain has generated a great amount of interest in these "excitotoxins." In vitro systems have established that the toxicity of glutamate can be blocked by certain glutamate antagonists (Rothman and Olney, 1986), and the concept has emerged that the toxicity of excitatory amino acids may be related to such divergent conditions as hypoxia, epilepsy, and neurodegenerative diseases (Meldrum, 1987; Choi, 1988; Beal, 1992, 1995, 1998; Lipton and Rosenberg, 1994).

Glutamate is the main excitatory neurotransmitter of the brain, and its effects are mediated by several subtypes of receptors (Fig.16-8) called excitatory amino acid receptors (EAARs) (Hollmann and Heinemann, 1994; Lipton and Rosenberg, 1994; Schoepfer et al., 1994). The two major subtypes of glutamate receptors are those that are ligand-gated directly to ion channels (ionotropic) and those that are coupled with G proteins (metabotropic). Ionotropic receptors may be further subdivided by their specificity for binding kainate, quisqualate, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and *N*-methyl-D-aspartate (NMDA). The entry of glutamate into the CNS is regulated at the blood–brain barrier and, following an injection of a large dose of glutamate in infant rodents, glutamate exerts its effects in the area of the brain in which the blood–brain barrier is least developed, the circumventricular organ. Within this site of limited access, glutamate injures neurons, apparently by opening glutamate-dependent ion channels, ultimately leading to neuronal swelling and neuronal cell death (Olney, 1978; Coyle, 1987). The toxicity affects the dendrites and neuronal cell

bodies but seems to spare axons. The only known related human condition is the "Chinese restaurant syndrome," in which consumption of large amounts of monosodium glutamate as a seasoning may lead to a burning sensation in the face, neck, and chest in sensitive individuals.

The cyclic glutamate analog kainate was initially isolated in Japan from seaweed as the active component of an herbal treatment of ascariasis. Kainate is extremely potent as an excitotoxin, being 100-fold more toxic than glutamate and is selective at a molecular level for the kainate receptor (Coyle, 1987). Like glutamate, kainate selectively injures dendrites and neurons and shows no substantial effect on glia or axons. As a result, this compound has found use in neurobiology as a tool. Injected into a region of the brain, kainate can destroy the neurons of that area without disrupting the fibers that pass through the same region. Neurobiologists, with the help of this neurotoxic scalpel, are able to study the role of neurons in a particular area independent of the axonal injuries that occur when similar lesioning experiments are performed by mechanical cutting.

Development of permanent neurological deficits in individuals accidentally exposed to high doses of an EAAR agonist has underscored the potential importance of excitatory amino acids in disease (Perl et al., 1990; Teitelbaum et al., 1990). A total of 107 individuals in the Maritime Provinces of Canada were exposed to domoic acid, an analog of glutamate, and suffered an acute illness that most commonly presented as gastrointestinal disturbance, severe headache, and short-term memory loss. A subset of the more severely afflicted patients was subsequently shown to have chronic memory deficits and motor neuropathy. Neuropathological investigation of patients who died within 4 months of intoxication showed neurodegeneration that was most prominent in the hippocampus and amygdala, but also affected regions of the thalamus and cerebral cortex.

The expanding field of the excitotoxic amino acids embodies many of the same attributes that characterize the entire discipline of neurotoxicology. Neurotoxicology is generally viewed as the study of compounds that are deleterious to the NS, and the effects of glutamate and kainate may be viewed as examples of this type of injurious outcome. Exposure to these excitotoxic amino acids leads to neuronal injury and—when of sufficient degree—may kill neurons. Many of these neurotoxic compounds have become tools for neurobiologists who seek to explore the anatomy and function of the NS. Kainate, through its selective action on neuronal cell bodies, has provided a greater understanding of the functions of cells within a specific region of the brain, while previous lesioning techniques addressed only regional functions.

Toxicant-Based Models of Neurodegenerative Disease

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) Because of an error on the part of a so-called designer chemist, people who injected themselves with a meperidine derivative that was intended to serve as a substitute for heroin also received a contaminant, MPTP (Langston et al., 1983) (Fig. 16-9). Over hours to days, dozens of these patients developed the signs and symptoms of irreversible Parkinson disease (PD), some becoming immobile with rigidity (Langston and Irwin, 1986). Autopsy studies demonstrated marked degeneration of DAergic neurons in the substantia nigra (SN), with degeneration continuing many years after exposure (Langston et al., 1999). The discovery of the relationship between MPTP intoxication and parkinsonism provided researchers with a new model for studying the pathogenic mechanism of PD, and prompted a number of epidemiological and experimental investigations to reveal environmental and occupational exposures that might be associated with the disease (Dauer and Przedborski, 2003).

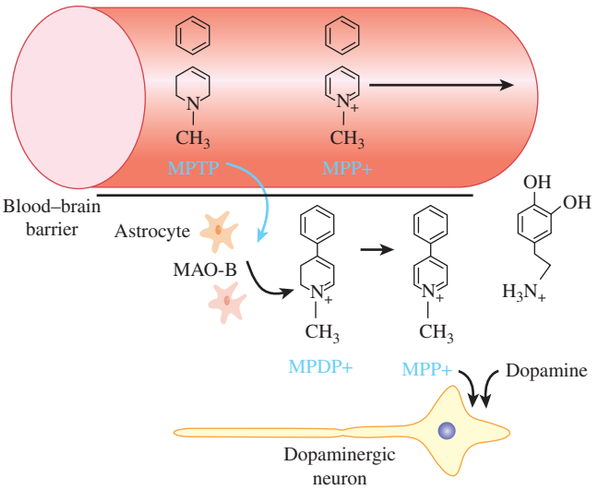


Figure 16-9. *MPTP toxicity.* MPP^+, either formed elsewhere in the body following exposure to MPTP or injected directly into the blood, is unable to cross the blood–brain barrier. In contrast, MPTP gains access and is oxidized in situ to $MPDP^+$ and MPP^+. The same transport system that carries dopamine into the dopaminergic neurons also transports the cytotoxic MPP^+.

It was initially surprising to find not only that MPTP is neurotoxic, but also that it is a substrate for the B isozyme of monoamine oxidase (MAO-B) (Gerlach et al., 1991). MPTP, an uncharged species at physiological pH, crosses the blood–brain barrier (BBB) and diffuses into cells, including astrocytes. The MAO-B of astrocytes catalyzes a two-electron oxidation to yield $MPDP^+$, the corresponding dihydropyridinium ion. A further two-electron oxidation yields the pyridinium ion MPP^+ (Fig. 16-9). MPP^+ enters DAergic neurons of the SN via the DA uptake system, resulting in injury or death of the neuron. Noradrenergic neurons of the locus ceruleus are also vulnerable to repeated exposures of MPTP (Langston and Irwin, 1986), although they are less affected by single exposures than the DAergic neurons are. Once inside neurons, MPP^+ acts as a general mitochondrial toxicant, blocking respiration at complex I (Di Monte and Langston, 2000). MPP^+ may also lead to the production of ROS and the release of DA from vesicles due to the higher pH environment of the cytosol, where the neurotransmitter undergoes autoxidation (Lotharius and O'Malley, 2000).

Consistent with the role of MAO-B in the bioactivation of MPTP to MPP^+, inhibitors of this enzyme, such as L-(–)-deprenyl (selegiline) protect against MPTP neurotoxicity. However, the protection afforded by deprenyl does not appear to arise from its inhibition of MAO-B alone, but also upon other properties, including its ability to act as an antioxidant and free radical scavenger (Ebadi et al., 2002; Mandel et al., 2003; Muralikrishnan et al., 2003; Magyar and Szende, 2004). Thus, mice deficient in Cu,Zn-SOD or GSH peroxidase show increased vulnerability to MPTP neurotoxicity (Zhang et al., 2000), whereas overexpression of Mn-SOD attenuates the toxicity (Klivenyi et al., 1998).

It should be noted that the general toxicity of the proximate neurotoxicant, MPP^+, is considerable when it is administered to animals, although systemic exposure to MPP^+ does not result in neurotoxicity because it does not cross the BBB. Moreover, compared to primates, rats are relatively resistant to DAergic neurotoxicity from systemic administration of the parent compound, MPTP,

even when the compound is injected directly in the cerebral circulation via the carotid artery. This resistance appears to be conferred, at least in part, by a high level of biotransformation of MPTP by rat endothelial cells to MPP^+ and other polar metabolites, such as MPTP-N-oxide, that are retained in endothelial cells and do not readily traverse the BBB (Scriba and Borchardt, 1989; Riachi et al., 1990; Mushiroda et al., 2001).

Individuals who were exposed to concentrations of MPTP insufficient to result in immediate parkinsonism have developed early signs of the disease years later (Calne et al., 1985). This observation presents the possibility that the onset of a neurotoxic problem may follow toxic exposure by many years (Landigan et al., 2005). It does not seem likely that an early sublethal injury to DAergic neurons later becomes lethal. Rather, smaller exposures to MPTP may cause a decrement in the population of DAergic neurons within the SN. Such a loss would most likely be silent because the symptoms of PD do not develop until approximately 80% to 90% of the SN neurons are lost. These individuals with a diminished number of neurons may be more vulnerable to further loss of DAergic neurons. The neurological picture of PD develops at an earlier age than in unexposed individuals, as a further loss of catecholaminergic neurons occurs during the process of aging.

Manganese Manganese (Mn) is an essential metal in both humans and animals. Although Mn is present in almost all diets, animals maintain stable tissue Mn levels by tightly regulating absorption and excretory processes. As an essential trace metal that is found in all tissues, Mn is required for normal metabolism of amino acids, proteins, lipids, and carbohydrates. Mn acts as a cofactor for a variety of enzymes, such as manganese metalloenzymes and Mn-dependent enzyme families. Mn metalloenzymes include arginase, glutamine synthetase (GS), phosphoenolpyruvate decarboxylase, and manganese-superoxide dismutase (Mn-SOD). Mn-dependent enzymes include oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Therefore, Mn is necessary for the function of many organ systems. In rare Mn deficiencies, clinical manifestations can include seizures, impaired growth, skeletal abnormalities, and impaired reproductive function (Critchfield et al., 1993; Wedler, 1993).

At the other end of the spectrum, it is well established that excessive exposure to Mn causes neurotoxicity (McMillan, 1999; Aschner et al., 2005). Mn accumulates in the basal ganglia and targets DAergic neurons and perhaps other neurotransmitter systems (Peres et al., 2016). The most common commercial sources of Mn have included the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT), steel factories, welding, mining plants, food, water, and Mn-containing pesticides. Occupational exposure to toxic levels of Mn in industrial workers results in psychological and neurological disturbances, including delusions, hallucinations, depression, disturbed equilibrium, compulsive or violent behavior, weakness, and apathy, followed by extrapyramidal motor system defects such as tremors, muscle rigidity, ataxia, bradykinesia, and dystonia. Although Mn toxicity has been under investigation for many years, the underlying primary molecular mechanisms of its neurotoxicity remain to be elucidated. Very few potential biomarkers have been established with no early detection markers available.

There are considerable similarities in symptoms of Mn neurotoxicity, referred to as manganism, and idiopathic Parkinson disease, but there are some differences as well (Guilarte and Gonzales, 2015). Mn toxicity causes a loss of DA neurons in the SN, and as in PD, oxidative stress appears to play a significant role in the disorder (Oestreicher et al., 1994; Kienzl et al., 1995; Montgomery, 1995). The brain area most susceptible to Mn injury is also highly sensitive to oxidative stress. Many metabolically active cell types, particularly tonically active motor neurons in the SN, require high levels of ATP for optimal function and survival. Mn accumulates in the SN, globus pallidus (GP), and striatum and interferes with ATP synthesis, analogous to effects seen with mitochondrial inhibitors or ischemia.

DAergic neurodegeneration associated with both PD and Mn exposure shares multiple common mechanisms, namely mitochondrial dysfunction, aberrant signal transduction, oxidative stress, protein aggregation, and the activation of cell death pathways. The accumulation of Mn in the striatum causes damage to the SN, reduction in tyrosine hydroxylase (TH) activity, and loss of DA neurons (Parenti et al., 1988; Tomas-Camardiel et al., 2002). Intracellular Mn is sequestered by mitochondria through the Ca^{2+} uniporter (Gavin et al., 1999). Intrastriatal injections of Mn result in excitotoxic brain injury similar to that caused by mitochondrial poisons, such as aminooxyacetic acid and 1-methyl-4-phenyl-pyridinium (Brouillet et al., 1993b). The specificity for Mn accumulation in GP and striatum likely correlates with Mn transporter distribution and the metabolic activity of these basal ganglia nuclei.

Guamanian Cycad–Induced Parkinsonism/Amyotrophic Lateral Sclerosis Syndrome An unusual prevalence of "hereditary paralysis" among the native Guam Chamorros was first reported in the early 1900s. This led to the formation of the National Institute of Neurological and Communicative Disorders and Stroke Research Center on Guam in 1939 (Rodgers-Johnson et al., 1986). In the mid-1950s the incidence of amyotrophic lateral sclerosis (ALS) and parkinsonism–dementia complex (PDC) was up to 100 times higher on Guam than anywhere else in the world (Hirano et al., 1961a). Although Guam ALS is clinically indistinguishable from ALS that occurs elsewhere in the world, Guam PDC is a distinct neurodegenerative disorder where Parkinsonism and dementia may occur simultaneously in affected Chamorros. The main clinical features included mental deterioration, parkinsonism, and evidence of motor neuron involvement. Macroscopic neuropathological features are the presence of cortical atrophy and depigmentation of the substantia nigra (SN), and microscopic evaluations revealed widespread ganglion cell degeneration and neurofibrillary tangles throughout the CNS (Hirano et al., 1961a, 1961b).

Early investigations of this Guamanian neurodegenerative complex suggested that the disorder might be related to an environmental factor, perhaps direct or indirect consumption of seeds of *Cycas circinalis* (Kurland, 1963). Cycads contain several toxins, including cycasin and β-*N*-methylamino-l-alanine (BMAA) (Schneider et al., 2002). Cycasin, which is metabolized to methylazoxymethanol (MAM), is known to be carcinogenic and to cause hepatotoxicity (Sieber et al., 1980). BMAA is a nonprotein amino acid that functions as a glutamate excitotoxin (Rakonczay et al., 1991). The toxicity of BMAA is similar to that of glutamate in vitro and can be blocked by certain EAAR antagonists (Nunn et al., 1987). Furthermore, BMAA-induced neurodegeneration may occur by an excitotoxic mechanism involving the mitochondrial permeability transition pore (mtPTP). It has been shown that BMAA increases the intracellular calcium concentration through the formation of a β-carbamate intermediate (Brownson et al., 2002). This intermediate can act as a glutamate agonist and induce glutamate excitotoxicity. Studies have also demonstrated that an increase in intracellular calcium (Dubinsky and Levi, 1998) and glutamate excitotoxicity are able to induce mtPTP (Schinder et al., 1996). In 1987, Spencer and colleagues reported that BMAA, when fed to primates in high concentrations (more than 100 mg/kg), produced a syndrome that closely resembled the neurological disorder observed on Guam (Nunn et al., 1987; Spencer et al., 1987).

Environmental Factors Relevant to Neurodegenerative Diseases

The relationship between MPTP intoxication and parkinsonism stimulated investigations into the role that environmental and occupational exposures may play in the pathogenesis of PD as well as AD, these being the most common neurodegenerative diseases. Families with early-onset PD demonstrate autosomal dominant inheritance, and studies have identified specific genes involved in these familial forms of PD (e.g., those encoding α-synuclein, parkin, ubiquitin C-terminal hydrolase L1, DJ-1, phosphatase and tensin homolog (PTEN)–induced putative kinase 1, and leucine-rich repeat kinase 2) (Kurth and Kurth, 1993; Agundez et al., 1995; Polymerpoulos et al., 1997). Nevertheless, twin studies indicate that environmental exposures play a more significant role than genetics in the vast majority of PD patients, particularly those with late-onset disease (Kuopio et al., 1999; Tanner et al., 1999). Findings such as these provide a rational basis for research concerning gene–environment interactions in the etiology of sporadic PD (Benmoyal-Segal and Soreq, 2006; Gosal et al., 2006).

Epidemiological studies implicate environmental and occupational exposure to pesticides, metals, and solvents as risk factors for PD (Gorell et al., 1997, 1998, 1999; Wirdefeldt et al., 2011; Pezzoli and Cereda, 2013; Goldman, 2014; Nandipati and Litvan, 2016). Studies of agricultural workers and communities have associated higher PD incidence with exposure to specific pesticides such as organochlorines, paraquat, rotenone, and OP compounds (Pezzoli and Cereda, 2013). Some studies point to strong correlations between occupational or environmental Mn exposure and an increased risk of PD as well as lower cognitive function (Gorell et al., 1999; Bowler et al., 2015). Meta-analyses of these studies have recently attempted to define better associations, but while most studies are supportive of environmental impacts on PD incidence, there are variations in study quality, diagnoses, exposure measurements, as well as additional risk and modifying factors.

Converging evidence in PD research suggests that mitochondrial damage and oxidative stress are the primary causes of DAergic neuronal death (Abou-Slieman et al., 2005; Mandel et al., 2005; Hauser and Hastings, 2013). Experimental and clinical studies show that mitochondrial dysfunction, such as complex I inhibition, produces excess superoxide that overwhelms the antioxidant capacity of the DAergic neurons and leads to their death. The centrality of mitochondria in these hypotheses arises primarily from findings that mitochondrial poisons, complex I inhibitors such as MPP^+ and rotenone, can induce a Parkinson-like syndrome in humans, nonhuman primates, and rodents. Moreover, neuropathological studies reveal a decrease of about 30% in complex I function in deceased PD patients, as compared with age-matched controls (Adam-Vizi, 2005). Oxidative damage also plays a significant role in DAergic neuronal cell death induced by intracranial injection of 6-OHDA, another experimental model of PD (Cannon et al., 2006). Reactive oxidative species, including H_2O_2, superoxide ions, and hydroxyl radicals, lead to lipid peroxidation, protein denaturation, and a decrease in reduced GSH: all hallmark features of postmortem PD (Jenner, 2003). Although much remains to be done, knowing that most cases of PD arise from environmental exposures that promote mitochondrial dysfunction and oxidative damage provides us with promising avenues for prevention and treatment of this debilitating disease.

In contrast, epidemiological studies have also found apparent protective effects of cigarette smoking or coffee consumption on the development of PD (Lai et al., 2002; Logroscino, 2005), and experimental studies indicate that nicotine and caffeine are protective in animal models of PD (Ross and Petrovitch, 2001; Quik, 2004; Quik et al., 2006). It is interesting that positron emission tomography (PET) studies of smokers show a marked reduction in brain MAO-B activity, similar to that produced by administration of the MAO-B inhibitor, L-deprenyl (Fowler et al., 1996). Other MAO-B inhibitors are being developed as anti-PD drugs (Mandel et al., 2005), and MAO-B inhibitors have been isolated from tobacco smoke (Khalil et al., 2006). However, it appears that MAO-B inhibition is not essential for the neuroprotective activity of these chemicals; instead, their effectiveness stems from their overall ability to preserve mitochondrial integrity and function.

The complex neurodegenerative disease in the indigenous population of Guam and surrounding islands that shares features of ALS, PD, and Alzheimer's disease (AD) were first described by Hirano and colleagues in 1961 (Hirano et al., 1961a, 1961b). Over the years, studies of this population shifted focus from a genetic to an environmental causative agent. Through a case registry established in 1958, entire pedigrees were developed using Chamorros from the same village as controls. Although several investigators have reported a high degree of familial occurrence, no definitive inheritance pattern has been established, lending support for an environmental influence involving cycad seeds (Plato et al., 2002, 2003). Traditionally, native Chamorros prepare food from cycads by washing the seeds several times before grinding them into flour (Kisby et al., 1992); however, this washing process is sufficient to remove most of the cycad toxins (Duncan et al., 1990). In 2002, Cox and Sacks suggested that the Chamorro population ingested the cycad toxins indirectly by eating flying foxes, *Pteropus mariannus*, whose diets included cycad seeds. This study demonstrated that the sharp decrease in the flying fox population was followed by a sharp decrease in the incidence of ALS (Schneider et al., 2002). The consumption of the flying foxes by the Chamorros was included in both social and ceremonial settings. It was suggested that accumulation of the neurotoxin, BMAA, in the flying fox may result in concentrations that are sufficient to cause behavioral and neuropathological changes in primates similar to those observed in the Guam ALS–PDC (Banack and Cox, 2003; Rivadeneyra-Domínguez and Rodríguez-Landa, 2014). Further assessments of the ALS–PD–dementia complex worldwide has further implicated exposures to cycad seeds in various forms (Spencer et al., 2015).

Although fewer in number compared to PD studies, some epidemiological studies of AD have shown higher risks associated with aluminum in drinking water and pesticide exposure, as well as other environmental and health factors (Santibáñez et al., 2007; Rondeau et al., 2009; Campdelacreu 2014; Yegambaram et al., 2015). An epidemic of dialysis-related dementia with some pathological resemblance to AD appears to have been related to aluminum in the dialysate, and its removal has prevented further instances of dialysis dementia. However, there is no definitive evidence that aluminum is in any way related to sporadic Alzheimer disease in the general population (Letzel et al., 2000; Chin-Chan et al., 2015).

Developmentally Neurotoxic Chemicals

Replication, migration, differentiation, apoptosis, myelination, and synapse formation are basic processes that occur in specific spatial and temporal patterns and underlie development of the NS. There are a variety of insults known to disrupt NS development, the outcomes of which may be very different depending on the time of exposure, including exposures to certain metals, solvents, antimetabolites, persistent organic pollutants, pesticides, pharmaceuticals, and ionizing radiation. Multiple mechanisms of action may be present, producing a wide array of effects in the offspring. The impact

on the developing NS may be very different, and often cannot be predicted, from effects observed in adults. A number of neurodevelopmental disorders have been, at least partially, attributed to exposures to neurotoxicological chemicals during the fetal, infant, or childhood periods. Although the number of chemicals shown to be developmentally neurotoxic in human populations is relatively small, the number of suspected toxicants from laboratory animal and other studies may be in the hundreds and growing (Grandjean and Landrigan, 2006, 2014; Miodovnik, 2011; Bellinger, 2013).

Ethanol exposure during pregnancy can result in abnormalities in the fetus, including abnormal neuronal migration and facial development, and diffuse abnormalities in the development of neuronal processes, especially the dendritic spines (Stoltenburg-Didinger and Spohr, 1983). Although the exposure may be of little consequence to the mother, it can be devastating to the fetus. There is an effect on NMDA glutamate receptors and excessive activation of GABA receptors, with induction of apoptosis throughout the brain (Ikonomidou et al., 2000). The clinical result of fetal alcohol exposure is often mental retardation, with malformations of the brain and delayed myelination of white matter (Riikonen et al., 1999). Although there remains a great deal of uncertainty concerning the molecular basis of this developmental aberration, it occurs in a variety of experimental animals, and it appears that acetaldehyde, a product of ethanol catabolism, can produce migration defects in developing animals similar to those that occur in fetal alcohol syndrome (O'Shea and Kaufman, 1979).

Some developmental neurotoxicants have been revealed by human studies or tragic poisoning occurrences. The contamination of fish in Minamata Bay, Japan, with MeHg led to developmental disabilities, including cerebral palsy, mental retardation, and seizures, in many children at birth. Children exposed to MeHg in utero show widespread neuronal loss, disruption of cellular migration, profound mental retardation, and paralysis (Reuhl and Chang, 1979; Costa et al., 2004). Studies using primates exposed in utero also have demonstrated abnormal social development (Burbacher et al., 1990). The earlier the exposure, the more generalized the damage that is observed. As with MeHg, ethanol and lead are known to produce frank neuropathology in highly exposed populations.

In recent years the concept has emerged that extremely low levels of exposure to some substances in "asymptomatic" children may have an effect on their behavioral and cognitive development. This has led to the suspicion that the number of chemically induced neurological disorders is larger than generally assumed. For example, the association between lead exposure and brain dysfunction has received experimental support in animal models and has prompted screening for lead in children (Benjamin and Platt, 1999). There is no proven safe lower limit for lead, and recent studies have associated lower IQ scores with blood lead levels less than 2 to 10 μg/dL (Winneke et al., 1994; Canfield et al., 2003; Gilbert and Weiss, 2006; Bellinger, 2008). Similarly, the debate regarding "safe" level of drinking ethanol during pregnancy is ongoing, with recent reports of no threshold for subtle cognitive effects (Sampson et al., 2000). Analyses of epidemiological data have suggested that the incidence of low-level MeHg-induced neurotoxicity has been greatly underestimated (Grandjean and Herz, 2011).

There is considerable evidence that chronic exposure to nicotine has effects on the developing fetus (reviewed in Slikker et al. [2005]). Along with decreased birth weights, attention-deficit disorders are more common in children whose mothers smoke cigarettes during pregnancy, and nicotine has been shown to lead to analogous neurobehavioral abnormalities in animals exposed prenatally to nicotine (Lichensteiger et al., 1988). Nicotinic receptors are expressed early in the development of the nervous system (NS), beginning in the developing brainstem and later expressed in the diencephalon. The role of these nicotinic receptors during development is unclear; however, it appears that prenatal exposure to nicotine alters the development of nicotinic receptors in the CNS (Van de Kamp and Collins, 1994)—changes that may be related to subsequent attention and cognitive disorders in animals and children.

Cocaine use during pregnancy is a major concern, especially in urban areas, where use can lead to a variety of acute and chronic adverse events in offspring. Cocaine is able to cross the placental barrier and the fetal blood–brain barrier, and causes reduced blood flow in the uterus. In severe events when large doses are consumed by the mother, the fetus may develop hypoxia, leading to a higher rate of birth defects (Woods et al., 1987). Maternal cocaine use is associated with low birth weight and behavioral defects, including a decreased awareness of the surroundings and altered stress and pain sensitivity (Chasnoff et al., 1985; Huber et al., 2001).

Several epidemiological studies have reported deficits in neurodevelopment and psychological performance in children exposed to polychlorinated biphenyls (PCBs) and/or dioxins (Seegal, 1996). Research in animals has shown that these persistent pollutants produce endocrine disruptions, cognitive deficits, and changes in activity levels in exposed offspring; however, the specific outcomes depend on the congener or mixture tested as well as the timing of exposure. Changes in estrogen or thyroid hormone, neurotransmitter function, and second messenger systems have been proposed as cellular bases for PCB toxicity (Seegal, 1996; Tilson and Kodavanti, 1998). Recent studies with another persistent class of hydrocarbons, polybrominated diphenyl ethers (PBDEs, used as fire retardants), have shown similarities in altering thyroid hormone metabolism and cholinergic function, and it has thus been proposed that this chemical class would also be developmentally neurotoxic (Branchi et al., 2003; Costa and Giordano, 2007). Thyroid hormone is critical to NS development, and animal studies have suggested that the developing brain may be vulnerable to environmental thyrotoxicants of all sorts (Porterfield, 1994, 2000). Finally, it has been suggested that even reversible changes in neurotransmission, such as those produced by nicotine or cholinesterase inhibitors, may alter specific growth processes and produce long-lasting deficits (Slotkin, 2004).

Chemicals That Induce Depression of Nervous System Function

The CNS maintains balance via interplay between inhibitory and excitatory influences. With general depressant chemicals, initial suppression of inhibitory systems at low doses produces excitation, such as intoxication observed with ethanol. Thus, acutely, CNS depressants produce a continuum of effects from excitation to sedation, motor impairment, coma, and ultimately death by depression of respiratory centers. Therapeutic indications of drugs causing CNS depression include anxiety, insomnia, epilepsy, and muscle relaxation (Evans and Balster, 1991).

Generalized depression of CNS function is produced by a variety of volatile solvents. These solvents include several chemical classes—aliphatic and aromatic hydrocarbons, halogenated hydrocarbons, ketones, esters, alcohols, and ethers—that are small, lipophilic molecules. They are found in a wide variety of products including nail polish removers, paints and paint thinners, industrial solvents, dry-cleaning solvents, and anesthetic gases. Human exposures range from chronic low levels in the general environment or occupational settings to high acute or chronic levels arising from household uses or solvent abuse (Dick, 2006).

There are several theories as to the mechanism of this generalized depression, but none of them are fully explanatory. Solvent

potency correlates well with the olive oil:water or octanol:water partition coefficients, leading to the once-popular Meyer–Overton hypothesis that CNS depressants exert their actions through nonspecific disruption of the lipid portions of cell membranes (e.g., Janoff et al., 1981). Anesthesia could occur as a consequence of membrane expansion or perturbations of mitochondrial calcium transport. More recent research has implicated interactions with ligand-gated ion channels as well as voltage-gated calcium channels. Specific receptors regulating these channels include gamma-aminobutyric acid type A ($GABA_A$), NMDA, and glycine receptors. These actions relate the effects of solvents to those of pharmaceutical agents such as barbiturates and benzodiazepines. Although these targets have been demonstrated mostly for ethanol (Davies, 2003), recent in vitro studies have extended this generality to other volatile solvents (e.g., Cruz et al., 2000).

A syndrome known as solvent-induced chronic toxic encephalopathy has been described for some populations with long-term and/or high-level exposure. Somewhat vague presenting symptoms include irritability, fatigue, and impaired memory or concentration, leading to the need for widely accepted diagnostic criteria (Van der Hoek et al., 2000). The absence of corroborating animal studies has prevented studies of molecular changes that may underlie these long-term effects and, indeed, has raised doubt as to the existence of such a syndrome (Ridgway et al., 2003). Specific solvents also produce other neurotoxicological actions, such as peripheral neuropathy, which are described elsewhere in this text.

IN VITRO AND OTHER ALTERNATIVE APPROACHES TO NEUROTOXICOLOGY

High-Throughput Analysis

In its report entitled "Toxicity Testing in the 21st Century: A Vision and a Strategy," the National Research Council (NRC, 2007) called for innovative experimental in vitro approaches for the assessment and characterization of chemical toxicity, including initiating molecular events. The long-term vision of the 2007 NRC report is to use high-throughput cell-based assays and quantitative structure–activity relationships (QSARs) to predict adverse outcomes. This will require the development of high-throughput approaches that can screen thousands of chemicals for presumed injurious events using time- and cost-effective models, in addition to determining the predictive effectiveness of these methods using complementary approaches. The replacement of standard in vivo neurotoxicity assessments with QSAR and high-throughput in vitro methods will require a long-term research effort (Smith, 2009; Bal-Price et al., 2010). In the meantime, we cannot rely on standard in vivo approaches that have been used successfully in the past to assess the backlog of thousands of untested chemicals.

The use of tiered testing schemes has been proposed, where the first tiers rely on high-throughput methods that test for chemical actions on critical biological receptors that initiate pathways of changes that lead to adverse outcomes, in order to identify chemicals for future testing. Second-tier tests could involve the use of alternative species, such as small fish or invertebrates, that will allow more moderate throughput, but in an intact or developing NS. Chemicals identified as having neurotoxic properties could then be tested in intact mammalian models as necessary. The mammalian models would be modified based on existing data; for example, if lower-tier data suggest effects on myelin formation, then mammalian models appropriate to test myelin-related endpoints (e.g., histopathology) should be used.

In order to develop these new testing strategies in the field of neurotoxicology, new approaches for screening and characterizing the neurotoxic potential of chemicals must be established, driven by basic research of how xenobiotics interfere with basic neurobiological processes. Critical to this process are complementary approaches that will allow assessment of the impact of chemical-induced alterations on crucial processes in intact multicellular organisms. The extraordinary conservation of both genomic/epigenomic elements and differentiation processes between mammals and nonmammalians, which has been revealed during the last two decades, makes more feasible the use of these alternative models. Finally, the results of these methods should inform and refine how, when, or if the classical mammalian model approaches are used. Each method of analysis will contribute unique information to the overall picture of neurotoxicity and inform the process and interpretation of the other avenues of investigation.

Cell Culture and Model Organism Approaches

Cell-based assays can provide critical new information regarding toxicant effects on intracellular signaling and cell lifecycle processes. Nonmammalian whole animal models can provide important understanding of effects on intercellular and system-wide signaling in an anatomically and temporally intact biological system. Emerging high-throughput and complementary models can help direct the best use of the classic mammalian models (e.g., thyroid or estrogenic effects). The integrated and tiered use of the spectrum of neurotoxicity approaches thus provides a rational approach for decreasing the vast uncertain risk posed by thousands of chemicals with no available hazard information.

Evaluation of cytotoxicity, particularly in a heterogeneous system such as the brain, is difficult in the intact animal because numerous factors (neural, hormonal, and hemodynamic) are not under experimental control. Simplified models, such as tissue culture, have been therefore indispensable as tools for understanding of basic physiology and molecular mechanisms that govern neurotoxic responses. Neural cultures offer numerous advantages over in vivo techniques. Cell morphology, protein synthesis and release, energy metabolism, receptor interaction, neurotransmitter uptake and release, as well as electrolyte and nonelectrolyte uptake and release can be directly studied. Dispersion of cells in culture permits access to clean membrane surfaces for electrophysiological studies utilizing patch clamping. Furthermore, direct effects of chemicals on a relatively homogeneous population allows for the study of specific aspects of the growth and differentiation of cells. The culture model also makes it possible to study regional specialization, and it can be extended to study cellular interactions by co-culturing various cell types. There are, however, limitations of the culture systems that should be considered. For example, cells can undergo varying degrees of differentiation, lose heterogeneous cell–cell interactions, and hence lose autocrine and paracrine signaling processes that modulate form and function of the cell. In addition, a number of different, sometimes competing, processes can influence the ability of a toxicant to damage specific cells. The reductionist approach where one removes many cell types and barriers to focus on a single cell type can facilitate diffusion or even active transport of a given toxic compound or its metabolite, limiting or enhancing toxicity. The ability of a cell to repair or replace damaged organelles or enzymes can also be critical in determining cell survival. This effect may also be dependent on neighboring cells and physical barriers, which may altogether be absent in a culture system.

Emerging alternative test species, such as *Caenorhabditis elegans*, Zebrafish (*Danio rerio*), and *Drosophila melanogaster*, are making it possible to assess the effects of small molecules rapidly, inexpensively, and on a miniaturized scale. Such model systems

provide an approach to study toxicity in an intact organism where cell–cell interactions and complex metabolic milieus influence and modify xenobiotic-induced neurotoxic potential, not possible in in vitro systems (Peterson et al., 2008). These and other species provide powerful model organisms for dissecting the components of neurodevelopment and degeneration. Moreover, these organisms are readily amenable to genetic manipulation. Embryonic stages of many of these species are optically transparent, allowing for easy real-time examination of the neuronal morphology and direct viewing of protein expression patterns. The genomic sequencing of *C. elegans*, *D. rerio*, and *D. melanogaster* has been completed, which allows for performance of whole animal PCR and provides a high-density polymorphism map of related strains. High-throughput approaches include genome-wide screening for molecular targets or mediators of toxicity and rapid, high-content chemical screens to detect potential toxicants. Genome-wide screening is important for studying any toxicant with a poorly understood mechanism of action. This screening has been accomplished using RNA interference (RNAi), DNA microarrays, and gene expression analysis.

Stem Cell–Based Approaches

Advances in human and mammalian stem cell technology has increasingly enabled in vitro study of neurotoxic properties of chemicals spanning effects from early development through maturity. The discovery that brain lipid binding protein (BLBP) expressing radial glial cells in the CNS give rise to the majority of neurons as well as astrocytes in the developing CNS sets the stage for the study of neuronal stem cell models to recapitulate brain ontogeny in vitro (Anthony et al., 2004). Further, these radial glial cells remain postnatally as adult neural stem cells (NSCs) in the subventricular zone (SVZ), one of two major niches for adult neurogenesis (Doetsch et al., 1999). The subgranular zone (SGZ) is the second major niche of adult neural stem cells (Doetsch et al., 1999; Fuentealba et al., 2012). Neural stem cells thus represent a reservoir of cells that persist into adult animals that can be utilized to generate differentiating and mature neurons and glial cells in vitro for toxicology studies, especially neurodevelopmental toxicants (Breier et al., 2008, 2010; Radio and Mundy, 2008). Such neural progenitor cells (NPCs) or neural precursor cells have been derived from both animals and humans and applied to developmental and adult neuronal toxicity testing (Breier et al., 2008; Buzanska et al., 2009; Choi et al., 2010; Fritsche et al., 2011; Stern et al., 2013) in the neurons, astrocytes, and/or oligodendrocytes derived from such stem cells. Further, three-dimensional "neurosphere" suspension cultures have enabled modeling of developmental processes such as proliferation, neuronal migration, and localized differentiation and cell death that occur in normal development to be examined in the context of toxicant exposures (Moors et al., 2009). Comparison studies of rodent and human NPC-based neurosphere models further suggests that species-specific differences in vulnerability to neurotoxicants can be observed (Baumann et al., 2016). For example, polybrominated diphenyl ethers (PBDEs), a class of flame retardants, exhibit differences in oligodendrocyte differentiation between mouse and human NPCs via distinct mechanisms (Dach et al., 2017).

In 2009, the Nobel Prize-winning work of Shinya Yamanaka and colleagues established the basis of converting adult human fibroblasts into pluripotent stem cells, termed "induced pluripotent stem cells (iPSCs)" by driving expression of a small subset of embryonic stem cell–associated genes and placing the cells into culturing conditions conducive to human stem cell maintenance (Takahashi et al., 2007). This paradigm-shifting technology has enabled the generation of pluripotent stem cells that can be directed down any of the three embryonic germ layers, including neuronal cell types, for which clinical and exposure histories of the subjects the cells are derived from may be known and used to inform neurotoxicology studies (Kumar et al., 2012; Schwartz et al., 2015). Human iPSC-based model systems have exhibited remarkable utility in evaluating vulnerability, for example, of dopamine-containing neurons to PD-relevant neurotoxicants including manganese, copper, 1-methyl-4-phenylpyridinium (MPP^+), paraquat, and maneb (Zeng et al., 2006; Aboud et al., 2012; Ryan et al., 2013; Aboud et al., 2015; Neely et al., 2017). As the human iPSC model systems are primary cells, rather than immortalized cell lines as many NPC lines may be, their ability to model neurodevelopmental features and neuronal biology is likely to be more similar to the *in vivo* condition. Thus, basic properties relevant to toxicant sensitivity such as developmental changes in the transport and processing of environmental factors may be explored. One such example is the use of developing human dopamine neurons to evaluate developmental changes in the net transport/accumulation of manganese in this neural lineage that is known to be functionally vulnerable to elevated exposure (Kumar et al., 2014). Basic properties associated with neurodevelopment may also be studied using human iPSC modeling allowing for high-throughput assessment of neurotoxic mechanisms in a human system. One such example is the use of iPSC-derived neurons to screen a library of 80 chemicals from the National Toxicology Program (NTP) known or suspected to induce neurodevelopmental toxicity for the specific effect of altering neurite outgrowth (Ryan et al., 2016). Such analysis can permit the prioritization of compounds for further developmental and adult neurotoxicity testing by in vivo and other approaches thereby speeding up efforts to identify neurotoxicants for public health research and minimize or reduce animal testing. Thus, stem cell models of neuronal differentiation have proved to be valuable model systems for the exploration of mechanisms of neurotoxicity and identification of neurotoxicants.

Systems and Computational Approaches

Despite the caveats and limitations of the "Tox 21" approach (NRC, 2007), emerging advances in analytical techniques, imaging, sensing, and massively parallel computational capabilities will permit new insights into the function and chemically induced dysfunction of the brain, spinal cord, and PNS. Systems approaches that scale from the molecular level to the whole body are currently under development and may soon revolutionize the science of neurotoxicology (Johnson and Richardson, 2017).

Nevertheless, the rudiments of the discipline of neurotoxicology remain inviolable. That is to say, considerations of dose, pharmacokinetics and dynamics, absorption, distribution, metabolism, and excretion must not be lost in the estimation of hazard and risk from exposure to neurotoxic chemicals. Fortunately, computational approaches to these considerations are already enjoying considerable success and are improving alongside advances in computing technology (Tao et al., 2015; Alqahtani, 2017).

EPIGENETICS IN NEUROTOXICOLOGY

Epigenetics can be defined broadly as transmission of heritable changes in gene expression that are not the result of changes in DNA sequence. Epigenetic changes are central to the idea that early life exposures to toxicants may lead to disease and dysfunction later in life is rapidly becoming a focus in toxicology (Heindel, 2007). This concept, termed "Fetal Basis of Adult Disease" (FEBAD), has roots in the developmental origins of health and disease (DOHaD) hypothesis popularized by Barker et al. (1989). Although much of

the research involving developmental origins of disease has been focused on epigenetic changes elicited by in utero nutritional deficiencies and their effects on cardiovascular health, metabolic syndrome, obesity, and reproductive disorders (reviewed by Barker [2007]), there are data demonstrating that this concept may also apply to neurological disorders, such as schizophrenia (Susser and Lin, 1992). An important example of this concept is the finding that children born during the Dutch Famine (1944 to 1945) have increased risk of schizophrenia (Brown and Susser, 2008) and that these children also have decreased DNA methylation in the imprinted IGF2 gene (Heijmans et al., 2008).

Based on these findings, interest has turned to epigenetic alterations as contributing factors in neuropsychiatric and neurodevelopmental disorders (Abdolmaleky et al., 2004). The first indication that epigenetic alterations were involved in neurodevelopmental disorders came with the discovery that Rett syndrome was the result of cross-linked inactivation of the methyl CpG binding protein (MeCP2) (Amir et al., 1999). MeCP2 mediates transcriptional repression through binding to CpG islands in the promoter regions of genes and through associations with histone deacetylases (HDACs) and histone methyltransferases (Fuks et al., 2003). DNA methylation is a key player in regulation of gene expression along with histone deacetylation, chromatin modification, noncoding RNAs, and RNA interference. Working in concert, these mechanisms regulate the appropriate expression of genes and protein in a time- and tissue-specific manner. Thus, alterations of the epigenetic machinery can have a powerful impact on the proper expression of genes, and potentially lead to significant alterations in development that result in disease and dysfunction.

In toxicology, research on epigenetic alterations as a result of toxicant exposure have focused primarily on the areas of carcinogenesis and reproductive function. Studies in these areas have demonstrated that exposure to environmental toxicants, such as heavy metals (Senut et al., 2012), bisphenol A (Dolinoy et al., 2007), methoxychlor, and vinclozolin (Anway et al., 2005; Zama and Uzumcu, 2009) alter DNA methylation and/or histone modifications to produce epigenetic changes associated with various toxicities later in life. From a neurological standpoint, pioneering studies by Meaney (Weaver et al., 2004) and Sweatt (Day et al., 2013) demonstrate the ability of epigenetic mechanisms to produce both long- and short-term effects on neurological function and behavior. Similarly, emerging evidence has identified epigenetic alterations as contributors to neurodegenerative diseases, including AD and PD (Labbé et al., 2016; Maloney and Lahiri, 2016), both of which may be influenced by exposure to environmental neurotoxicants. As such, there is increasing recognition for the potential of xenobiotic-induced neurotoxicity to occur through epigenetic mechanisms.

To date, the vast majority of research on epigenetic mechanisms in neurotoxicology has focused on exposure to drugs of abuse or heavy metals, with some reports on effects of endocrine disruptors and pesticides. Studies with various abused substances, including alcohol, cocaine, nicotine, and methamphetamine, have demonstrated that exposure to these substances results in a variety of alterations in DNA methylation machinery as well as histone modifications that contribute to specific gene expression changes in areas of the brain involved in addiction (Cadet, 2016). Chronic administration of L-methionine blocked sensitization to the locomotor-activating effects of cocaine that was associated with increased DNA methyltransferase (DNMT) levels and blocked cocaine-induced *c-fos* mRNA induction. This was associated with reduced methylation of the *c*-fos promotor (Wright et al., 2015). In another study, cocaine exposure was demonstrated to increase *c*-fos mRNA levels through enhanced H4 acetylation at the *c*-Fos promoter and to increase *Bdnf* mRNA through increased H3 acetylation in the BDNF promoter (Kumar et al., 2005). The functional importance of these changes was confirmed by studies demonstrating that histone deacetylase (HDAC) inhibitors could decrease cocaine self-administration (Romieu et al., 2008) and that administration of a DNA methyltransferase inhibitor (RG108) abolished cue-induced cocaine seeking (Massart et al., 2015).

With regard to heavy metals, Eid and Zawia (2016) reported that developmental exposure to Pb resulted in changes in expression of DNMTs and HDACs that were associated with alterations of proteins involved in neurodegeneration, including amyloid precursor protein. Similarly, recent work from Schneider et al. (2016) reported widespread changes in epigenetic machinery in the brain following developmental Pb exposure that are linked with behavioral dysfunction in these animals. These included changes in H3K9/14Ac and H3K9Me3 levels that were observed in an age, region, and sex-specific manner. Mercury exposure has long been known to result in a variety of neurotoxicities, but only recently has attention focused on the potential role of epigenetic changes as a contributing mechanism (Basu et al., 2014). Prenatal mercury exposure was associated with decreased DNA methylation in cord blood that was linked to decreased methylation in the paraoxonase gene promoter (Cardenas et al., 2017). Likewise, mercury exposure was linked to hypomethylation of labile regions of the genome, including the SEPP1 promoter (Goodrich et al., 2013).

Pesticides are another large functional class of chemicals for which epigenetic mechanisms are starting to be revealed as potential mediators of neurotoxicity. Exposure to the herbicide atrazine resulted in changes in copy number and expression of miRNAs associated with neurodevelopment in zebrafish (Wirbisky et al., 2016; Wirbisky and Freeman, 2017). Skinner et al. (2008) demonstrated that embryonic exposure to the fungicide vinclozolin promoted epigenetic reprogramming that was associated with transgenerational transmission of changes to the transcriptomes of the hippocampus and amygdala that are involved in axon guidance and long-term potentiation. Song et al. (2011) demonstrated that paraquat exposure induced histone acetylation in cultured cells, which was associated with increased caspase-3 activity. Inclusion of a histone acetylase inhibitor resulted in amelioration of this effect. Although the full impact of epigenetic alterations as a mechanism of neurotoxicity is in an infant stage, it is clear that research in this area will likely play a major role in helping to understand differences in neurotoxic impact based on life stage and sex, while contributing to the understanding of life-long or adult-emergent neurotoxicity resulting from developmental exposures.

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REFERENCES

Abdolmaleky HM, Smith CL, Faraone SV, et al. Methylomics in psychiatry: modulation of gene–environment interactions may be through DNA methylation. *Am J Med Genet B Neuropsychiatr Genet*. 2004;127B(1):51–59.

Aboud AA, Tidball AM, Kumar KK, et al. Genetic risk for Parkinson's disease correlates with alterations in neuronal manganese sensitivity between two human subjects. *Neurotoxicology*. 2012;33(6):1443–1449.

Aboud AA, Tidball AM, Kumar KK, et al. PARK2 patient neuroprogenitors show increased mitochondrial sensitivity to copper. *Neurobiol Dis*. 2015;73:204–212.

Abou-Donia MB. Triphenyl phosphite: a type II organophosphorus compound–induced delayed neurotoxic agent. In: Chambers JE, Levi PE, eds. *Organophosphates: Chemistry, Fate, and Effects*. San Diego, CA: Academic Press; 1992:327–351.

Abou-Donia MB, ed. *Neurotoxicology*. Boca Raton, FL: CRC Press; 1993.

Abou-Slieman PM, Mugit MM, Wood NW. Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nat Rev Neurosci*. 2005;7:207–219.

Adams J, Barone S Jr, LaMantia A, et al. Workshop to identify critical windows of exposure for children's health: neurobehavioral work group summary. *Environ Health Perspect*. 2000;108(suppl 3):535–544.

Adam-Vizi V. Production of reactive oxygen species in brain mitochondria: contribution by electron-transport chain and non-electron transport chain sources. *Antioxid Redox Signal*. 2005;7:1140–1149.

Agundez JA, Jimenex JF, Luengo A, et al. Association between the oxidative polymorphism and early onset of Parkinson's disease. *Clin Pharmacol Ther*. 1995;57:291–298.

Aksenov MY, Aksenov MV, Nath A, et al. Cocaine-mediated enhancement of Tat toxicity in rat hippocampal cell cultures: the role of oxidative stress and D1 dopamine receptor. *Neurotoxicology*. 2006;27:217–228.

Albrecht J. Astrocytes and ammonia neurotoxicity. In: Aschner M, Kimelberg HK, eds. *The Role of Glia in Neurotoxicity*. Boca Raton, FL: CRC Press; 1996:137–153.

Albrecht J, Norenberg MD. L-Methionine-DL-sulfoximine induces massive efflux of glutamine from cortical astrocytes in primary culture. *Eur J Pharmacol*. 1990;182:587–599.

Alqahtani S. In silico ADME-Tox modeling: progress and prospects. *Expert Opin Drug Metab Toxicol*. 2017;13:1147–1158.

Altman J, Sudarshan K. Postnatal development of locomotion in the laboratory rat. *Anim Behav*. 1975;23:896–920.

Amarnath V, Anthony DC, Valentine WM, et al. The molecular mechanism of the carbon disulfide mediated crosslinking of proteins. *Chem Res Toxicol*. 1991;4:148–150.

Amir RE, Van den Veyver IB, Wan M, et al. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet*. 1999;23(2):185–188.

Anderson JP, Carroll Z, Smulowitz M, et al. A possible mechanism of action of the neurotoxic agent iminodipropionitrile (IDPN): a selective aggregation of the medium and heavy neurofilament polypeptides (NF-M and NF-H). *Brain Res*. 1991;547:353–357.

Anthony DC, Boekelheide K, Graham DG. The effect of 3,4-dimethyl substitution on the neurotoxicity of 2,5-hexanedione: I. Accelerated clinical neuropathy is accompanied by more proximal axonal swellings. *Toxicol Appl Pharmacol*. 1983;71:362–371.

Anthony TE, Klein C, Fishell G, Heintz N. Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron*. 2004;41(6):881–890.

Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*. 2005;308(5727):1466–1469.

Aschner M, Erikson KM, Dorman DC. Manganese dosimetry: species differences and implications for neurotoxicity. *Crit Rev Toxicol*. 2005;35:1–32.

Atchison WD, Hare MF. Mechanisms of methylmercury-induced neurotoxicity. *FASEB J*. 1994;8:622–629.

Auer RN, Benveniste H. Hypoxia and related conditions. In: Graham DI, Lantos PL, eds. *Greenfield's Neuropathology*. 6th ed. New York: Arnold; 1997:263–314.

Bagley PR, Tucker SP, Nolan C, et al. Anatomical mapping of glucose transporter protein and pyruvate dehydrogenase in rat brain: an immunogold study. *Brain Res*. 1989;499:214–224.

Bakir F, Damluji SF, Amin-Zaki L, et al. Methylmercury poisoning in Iraq. *Science*. 1973;181:230–241.

Bal-Price AK, Hogberg HT, Buzanska L, Coecke S. Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered. *Neurotoxicol Teratol*. 2010;32:36–41.

Balaban CD, O'Callaghan JP, Billingsley ML. Trimethyltin-induced neuronal damage in the rat brain: comparative studies using silver degeneration stains, immunocytochemistry and immunoassay for neuronotypic and gliotypic proteins. *Neuroscience*. 1988;26:337–361.

Banack SA, Cox PA. Biomagnification of cycad neurotoxins in flying foxes: implications for ALS-PDC in Guam. *Neurology*. 2003;61:387–389.

Barker DJ. The origins of the developmental origins theory. *J Intern Med*. 2007;261(5):412–417.

Barker DJ, Osmond C, Law CM. The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. *J Epidemiol Community Health*. 1989;43(3):237–240.

Barone S Jr, Das KP, Lassiter TL, White LD. Vulnerable processes of nervous system development: a review of markers and methods. *Neurotoxicology*. 2000;21:15–36.

Barone S Jr, Stanton ME, Mundy WR. Neurotoxic effects of triethyltin (TET) exposure are exacerbated with aging. *Neurobiol Aging*. 1995;16:723–735.

Basu N, Goodrich JM, Head J. Ecogenetics of mercury: from genetic polymorphisms and epigenetics to risk assessment and decision-making. *Environ Toxicol Chem*. 2014;33(6):1248–1258.

Baumann J, Gassmann K, Masjosthusmann S, et al. Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events. *Arch Toxicol*. 2016;90(6):1415–1427.

Bayer SA, Altman J, Russo RJ, et al. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *Neurotoxicology*. 1993;14:83–144.

Beal MF. Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann Neurol*. 1992;31:119–130.

Beal MF. Aging, energy, and oxidative stress in neurodegenerative diseases. *Ann Neurol*. 1995;38:357–366.

Beal MF. Excitotoxicity and nitric oxide in Parkinson's disease pathogenesis. *Ann Neurol.* 1998;44:S110–S114.

Bearer CF. How are children different from adults? *Environ Health Perspect*. 1995;103(suppl 6):7–12.

Beirowski B, Adalbert R, Wagner D, et al. The progressive nature of Wallerian degeneration in wild-type and slow Wallerian degeneration (WldS) nerves. *BMC Neurosci*. 2005;6:6.

Bellinger DC. Very low lead exposures and children's neurodevelopment. *Curr Opin Pediatr*. 2008;20:172–177.

Bellinger DC. Prenatal exposures to environmental chemicals and children's neurodevelopment: an update. *Saf Health Work*. 2013;4:1–11.

Benjamin JT, Platt C. Is universal screening for lead in children indicated? An analysis of lead results in Augusta, Georgia in 1997. *J Med Assoc Georgia*. 1999;88:24–26.

Benmoyal-Segal L, Soreq H. Gene-environment interactions in sporadic Parkinson's disease. *J Neurochem*. 2006;97:1740–1755.

Benowitz NL. Clinical pharmacology of nicotine. *Annu Rev Med*. 1986;37:21–32.

Berliner R. Cocaine. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. New York: Oxford University Press; 2000:408–413.

Besser R, Kramer G, Thumler R, et al. Acute trimethyltin limbic-cerebellar syndrome. *Neurology*. 1987;37:945–950.

Bond AD, Benevellit F, Jones W. Modification of the solid-state structure of bis(1-hydroxy-2(1H)-pyridine-S^2,O)zinc(II): synthesis and characterization of a molecular solid solution incorporating 3-hydroxy-4-methyl-2-(3H)-thiazolethione. *J Mater Chem*. 2002;12:324–332.

Bondy SC, Campbell A. Developmental neurotoxicology. *J Neurosci Res*. 2005;81:605–612.

Bouldin TW, Samsa G, Earnhardt TS, et al. Schwann cell vulnerability to demyelination is associated with internodal length in tellurium neuropathy. *J Neuropathol Exp Neurol*. 1988;47:41–47.

Bowler RM, Kornblith ES, Gocheva VV, Colledge MA, Bollweg G, Kim Y, et al. Environmental exposure to manganese in air: associations with cognitive functions. *Neurotoxicology*. 2015;49:139–148.

Branchi I, Capone F, Alleva E, et al. Polybrominated diphenyl ethers: neurobehavioral effects following developmental exposure. *Neurotoxicology*. 2003;24:449–462.

Breier JM, Gassmann K, Kayser R, et al. Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: state of the science. *Neurotoxicol Teratol*. 2010;32(1):4–15.

Breier JM, Radio NM, Mundy WR, Shafer TJ. Development of a high-throughput screening assay for chemical effects on proliferation and viability of immortalized human neural progenitor cells. *Toxicol Sci*. 2008;105(1):119–133.

Brouillet E, Jenkins BG, Hyman BT, et al. Age-dependent vulnerability of the striatum to the mitochondrial toxin 3-nitropropionic acid. *J Neurochem*. 1993a;60:356–359.

Brouillet EP, Shinobu L, McGarvey U, et al. Manganese injection into the rat striatum produces excitotoxic lesions by impairing energy metabolism. *Exp Neurol*. 1993b;120:89–94.

Brown AS, Susser ES. Prenatal nutritional deficiency and risk of adult schizophrenia. *Schizophr Bull*. 2008;34(6):1054–1063.

Brownson DM, Mabry TJ, Leslie SW. The cycad neurotoxic amino acid, beta-*N*-methylamino-L-alanine (BMAA), elevates intracellular calcium levels in dissociated rat brain cells. *J Ethnopharmacol*. 2002;82:159–167.

Burbacher TM, Sackett GP, Mottet NK. Methylmercury effects on the social behavior of *Macaca fascicularis*. *Neurotoxicol Teratol*. 1990;12:65–71.

Buzanska L, Sypecka J, Nerini-Molteni S, et al. A human stem cell–Based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system. *Stem Cells*. 2009;27(10):2591–2601.

Cadet JL. Epigenetics of stress, addiction, and resilience: therapeutic implications. *Mol Neurobiol*. 2016;53(1):545–560.

Calingasan NY, Baker H, Sheu KF, et al. Selective enrichment of cholinergic neurons with the alpha-ketoglutarate dehydrogenase complex in rat brain. *Neurosci Lett*. 1994;168:209–212.

Calne DB, Langston JW, Martin WR, et al. Positron emission tomography after MPTP: observations relating to the cause of Parkinson's disease. *Nature*. 1985;317:246–248.

Cammer W, Moore CL. The effect of hexachlorophene on the respiration of brain and liver mitochondria. *Biochem Biophys Res Commun*. 1972;46:1887–1894.

Campdelacreu J. Parkinson's disease and Alzheimer disease: environmental risk factors. *Neurologia*. 2014;29:541–549.

Canfield RL, Henderson CR Jr, Cory-Slechta DA, et al. Intellectual impairment in children with blood lead concentrations below 10 microg per deciliter. *N Engl J Med*. 2003;348:1517–1526.

Cannon JR, Keep RF, Schallert T, et al. Protease-activated receptor-1 mediates protection elicited by thrombin preconditioning in a rat 6-hydroxydopamine model of Parkinson's disease. *Brain Res*. 2006;1116:177–186.

Cardenas A, Rifas-Shiman SL, Agha G, et al. Persistent DNA methylation changes associated with prenatal mercury exposure and cognitive performance during childhood. *Sci Rep*. 2017;7(1):288.

Casida JE, Quistad GB. Serine hydrolase targets of organophosphorus toxicants. *Chem Biol Interact*. 2005;157–158:277–283.

Cavanagh JB. The significance of the "dying-back" process in experimental and human neurological disease. *Int Nat Rev Exp Pathol*. 1964;7:219–267.

CDC (Centers for Disease Control and Prevention). Available at: www.dcd.gov/nceh/lead. 2017. Accessed February 2017.

Chang LW, Dyer RS. *Handbook of Neurotoxicology*. New York: M. Dekker; 1995.

Chasnoff IJ, Burns WJ, Schnoll SH, et al. Cocaine use in pregnancy. *N Engl J Med*. 1985;313:666–669.

Chaudry V, Cornblath DR. Wallerian degeneration in human nerves: serial electrophysiological studies. *Muscle Nerve*. 1992;15:687–693.

Cheng KC, Cahill DS, Kasai H, et al. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions. *J Biol Chem*. 1992;267:166–172.

Cheung MK, Verity MA. Experimental methyl mercury neurotoxicity: locus of mercurial inhibition of brain protein synthesis in vivo and in vitro. *J Neurochem*. 1985;44:1799–1808.

Chin-Chan M, Navarro-Yepes J, Quintanilla-Vega B. Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases. *Front Cell Neurosci*. 2015;9:124.

Choi DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron*. 1988;1:623–634.

Choi YS, Lee MC, Kim HS, et al. Neurotoxicity screening in a multipotent neural stem cell line established from the mouse brain. *J Korean Med Sci*. 2010;25(3):440–448.

Chou S-M, Hartmann HA. Axonal lesions and waltzing syndrome after IDPN administration in rats: with a concept "axostasis." *Acta Neuropathol*. 1964;3:428–450.

Chuang RY, Chuang LF. Inhibition of chicken myeloblastosis RNA polymerase II activity by adriamycin. *Biochemistry*. 1979;18:2069–2073.

Clancy B, Finlay BL, Darlington RB, Anand KJS. Extrapolation brain development from experimental species to humans. *Neurotoxicology*. 2007;28:931–937.

Clark AW, Griffin JW, Price DL. The axonal pathology in chronic IDPN intoxication. *J Neuropathol Exp Neurol*. 1980;39:42–55.

Clarkson TW. The toxicology of mercury. *Crit Rev Clin Lab Sci*. 1997;34:369–403.

Cleveland DW, Hwo SY, Kirschner MW. Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J Mol Biol*. 1977;116:227–247.

Coleman M. Axon degeneration mechanisms: commonality amid diversity. *Nat Rev*. 2005;6:889–898.

Collins JJ, Swaen GMH, Marsh GM, et al. Mortality patterns among workers exposed to acrylamide. *J Occup Med*. 1989;31:614–617.

Costa LG, Aschner M, Vitalone A, et al. Developmental neuropathology of environmental agents. *Ann Rev Pharmacol Toxicol*. 2004;44:87–110.

Costa LG, Giordano G. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology*. 2007;28:1047–1067.

Cox PA, Sacks OW. Cycad neurotoxins, consumption of flying foxes, and ALS-PDC disease in Guam. *Neurology*. 2002;58:956–959.

Coxon A, Pallis CA. Metronidazole neuropathy. *J Neurol Neurosurg Psychiatr*. 1976;36:403–405.

Coyle JT. Kainic acid: insights into excitatory mechanisms causing selective neuronal degeneration. In: Bock G, O'Connor M, eds. *Selective Neuronal Death*. New York: Wiley; 1987:186–203.

Critchfield JW, Carl FG, Keen CL. Anticonvulsant-induced changes in tissue manganese, zinc, copper, and iron concentrations in Wistar rats. *Metabolism*. 1993;42:907–910.

Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J*. 1999;341:233–249.

Cruz SL, Balster RL, Woodward JJ. Effects of volatile solvents on recombinant *N*-methyl-D-aspartate receptors expressed in *Xenopus oocytes*. *Br J Pharmacol*. 2000;131:1303–1308.

Dabrowski-Bouta G, Sulkowski G, Bartosz G, et al. Chronic lead intoxication affects the myelin membrane status in the central nervous system of adult rats. *J Mol Neurosci*. 1999;13:127–139.

Dach K, Bendt F, Huebenthal U, et al. BDE-99 impairs differentiation of human and mouse NPCs into the oligodendroglial lineage by species-specific modes of action. *Sci Rep*. 2017;7:44861.

Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron*. 2003;39:889–909.

Davies M. The role of GABAA receptors in mediating the effects of alcohol in the central nervous system. *J Psychiatry Neurosci*. 2003;28:263–274.

Davis M, Gendelman DS, Tischler MD, et al. A primary acoustic startle circuit: lesion and stimulation studies. *J Neurosci*. 1982;2:791–805.

Davis SL, Tanaka D Jr, Aulerich RJ, et al. Organophosphorus-induced neurotoxicity in the absence of neuropathy target esterase inhibition: the effects of triphenyl phosphine in the European ferret. *Toxicol Sci*. 1999;49:78–85.

Day JJ, Childs D, Guzman-Karlsson MC, et al. DNA methylation regulates associative reward learning. *Nat Neurosci*. 2013;16(10):1445–1452.

DeGrandchamp RL, Reuhl KR, Lowndes HE. Synaptic terminal degeneration and remodeling at the rat neuromuscular junction resulting from a single exposure to acrylamide. *Toxicol Appl Pharmacol*. 1990;105:422–433.

Dejneka NS, Polavarapu R, Deng X, et al. Chromosomal localization and characterization of the stannin (Snn) gene. *Mamm Genome*. 1998;9:556–564.

DeVeaugh-Geiss J, ed. Tardive dyskinesia: phenomenology, pathophysiology, and pharmacology. In: *Tardive Dyskinesia and Related Involuntary Movement Disorders*. Boston, MA: John Wright PSG; 1982:1–18.

DeVito MJ, Wagner GC. Methamphetamine-induced neuronal damage: a possible role for free radicals. *Neuropharmacology*. 1989;28:1145–1150.

Dick FD. Solvent neurotoxicity. *Occup Environ Med*. 2006;63:221–226.

Di Monte DA, and Langston JW. MPTP and analogs In: Spencer PS, Schaumburg HH, eds. Experimental and Clinical Neurotoxicology. New York: Oxford University Press; 2000:812–818.

Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Human Dev*. 1979;3:79–83.

Dobbing J, Sands J. Quantitative growth and development of human brain. *Arch Dis Child*. 1973;48:757–767.

Dobbing J, Smart JL. Vulnerability of developing brain and behaviour. *Br Med Bull*. 1974;30:164–168.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*. 1999;97(6):703–716.

Dolinoy DC, Huang D, Jirtle RL. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci U S A*. 2007;104(32):13056–13061.

Dubinsky JM, Levi Y. Calcium-induced activation of the mitochondrial permeability transition in hippocampal neurons. *J Neurosci Res*. 1998;53:728–741.

Duncan MW, Steele JC, Kopin IJ, et al. 2-Amino-3-(methylamino)-propanoic acid (BMAA) in cycad flour: an unlikely cause of amyotrophic lateral sclerosis and parkinsonism-dementia of Guam. *Neurology*. 1990;40:767–772.

Dyck PJ, O'Brien PC, Ohnishi A. Lead neuropathy: 2. Random distribution of segmental demyelination among "old internodes" of myelinated fibers. *J Neuropathol Exp Neurol*. 1977;36:570–575.

Dyer RS. The use of sensory evoked potentials in toxicology. *Fundam Appl Toxicol*. 1985;5:24–40.

Ebadi M, Sharma S, Shavali S, et al. Neuroprotective actions of selegiline. *J Neurosci Res*. 2002;67:285–289.

Eid A, Zawia N. Consequences of lead exposure, and it's emerging role as an epigenetic modifier in the aging brain. *Neurotoxicology*. 2016;56:254–261.

Erve JCL, Amarnath V, Graham D, et al. Carbon disulfide and *N,N*-diethyldithiocarbamate generate thiourea cross-links on erythrocyte spectrin. *Chem Res Toxicol*. 1998a;11:544–549.

Erve JCL, Amarnath V, Sills RC, et al. Characterization of a valine-lysine thiourea cross-link on rat globin produced by carbon disulfide or *N,N*-diethyldithiocarbamate in vivo. *Chem Res Toxicol*. 1998b;11:1128–1136.

Evans EB, Balster RL. CNS depressant effects of volatile organic solvents. *Neurosci Biobehav Rev*. 1991;15:233–241.

Evans WC, Evans IA, Humphreys DJ, et al. Induction of thiamine deficiency in sheep with lesions similar to those of cerebro-cortical necrosis. *J Comp Pathol*. 1975;85:253–267.

Fishburne PM, Abelson HI, Cisin I. *National Household Survey on Drug Abuse: National Institute of Drug and Alcohol Abuse Capsules, 1982*. Washington, DC: Department of Health and Human Services; 1983.

Flores G, Buhler DR. Hemolytic properties of hexachlorophene and related chlorinated biphenols. *Biochem Pharmacol*. 1974;23:1835–1843.

Folbergrova J. Glycogen and glycogen phosphorylase in the cerebral cortex of mice under the influence of methionine sulfoximine. *J Neurochem*. 1973;20:547–557.

Fowler JS, Volkow ND, Wang GJ, et al. Inhibition of monoamine oxidase B in the brains of smokers. *Nature*. 1996;379:733–736.

Fox WM. Reflex-ontogeny and behavioural development of the mouse. *Anim Behav*. 1965;13:234–241.

Frey BN, Valvassori SS, Reus GZ, et al. Changes in antioxidant defense enzymes after D-amphetamine exposure: implications as an animal model of mania. *Neurochem Res*. 2006;31:699–703.

Fritsche E, Gassmann K, Schreiber T. Neurospheres as a model for developmental neurotoxicity testing. *Methods Mol Biol*. 2011;758:99–114.

Fuentealba LC, Obernier K, Alvarez-Buylla A. Adult neural stem cells bridge their niche. *Cell Stem Cell*. 2012;10(6):698–708.

Fuks F, Hurd PJ, Wolf D, et al. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem*. 2003;278(6):4035–4040.

Garland TO, Patterson MWH. Six cases of acrylamide poisoning. *Br Med J*. 1967;4:134–138.

Garman RH, Weiss B, Evans HL. Alkylmercurial encephalopathy in the monkey: a histopathologic and autoradiographic study. *Acta Neuropathol (Berlin)*. 1975;32:61–74.

Gavin CE, Gunter KK, Gunter TE. Manganese and calcium transport in mitochondria: implications for manganese toxicity. *Neurotoxicology*. 1999;20:445–453.

Gehlbach SH, Williams WA, Perry LD, et al. Green-tobacco sickness: an illness of tobacco harvesters. *JAMA*. 1974;229:1880–1883.

George EB, Glass JD, Griffin JW. Axotomy-induced axonal degeneration is mediated by calcium influx through ion-specific channels. *J Neurosci*. 1995;15:6445–6452.

Gerlach M, Riederer P, Przuntek H, et al. MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. *Eur J Pharmacol*. 1991;12:273–286.

Gilbert SG, Weiss B. A rationale for lowering the blood lead action level from 10 to 2 microg/dL. *Neurotoxicology*. 2006;27:693–701.

Giros B, Jaber SR, Wightman RM, et al. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature*. 1996;379:606–612.

Glynn P. Neuronal phospholipid deacylation is essential for axonal and synaptic integrity. *Biochim Biophys Acta*. 2013;1831:633–641.

Gold BG. β,β′-Iminodipropionitrile. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. New York: Oxford University Press; 2000:678–679.

Goldberger ME, Murray M. Recovery of function and anatomical plasticity after damage to the adult and neonatal spinal cord. In: Cotman CW, ed. *Synaptic Plasticity*. New York: Guilford Press; 1985:77–110.

Goldman SM. Environmental toxins and Parkinson's disease. *Ann Rev Pharmacol Toxicol*. 2014;54:141–164.

Goodrich JM, Basu N, Franzblau A, Dolinoy DC. Mercury biomarkers and DNA methylation among Michigan dental professionals. *Environ Mol Mutagen*. 2013;54(3):195–203.

Goodrum JF. Role of organotellurium species in tellurium neuropathy. *Neurochem Res*. 1998;23:1313–1319.

Gorell JM, Johnson CC, Rybicki BA, et al. Occupational exposures to metals as risk factors for Parkinson's disease. *Neurology*. 1997;48:650–658.

Gorell JM, Johnson CC, Rybicki BA, et al. The risk of Parkinson's disease with exposure to pesticides, farming, well water and rural living. *Neurology*. 1998;59:1346–1350.

Gorell JM, Johnson CC, Rybicki BA, et al. Occupational exposure to manganese copper, lead, iron, mercury and zinc and the risk of Parkinson's disease. *Neurotoxicology*. 1999;20:239–247.

Gosal D, Ross OA, Toft M. Parkinson's disease: the genetics of a heterogeneous disorder. *Eur J Neurol*. 2006;13:616–627.

Grafstein B. Axonal transport: function and mechanisms. In: Waxman SG, Kocsis JD, Stys PK, eds. *The Axon: Structure, Function, and Pathophysiology*. New York: Oxford University Press; 1995:185–199.

Graham DG, Amarnath V, Valentine WM, et al. Pathogenetic studies of hexane and carbon disulfide neurotoxicity. *CRC Crit Rev Toxicol*. 1995;25:91–112.

Graham DG, Anthony DC, Boekelheide K, et al. Studies of the molecular pathogenesis of hexane neuropathy. II. Evidence that pyrrole derivatization of lysyl residues leads to protein crosslinking. *Toxicol Appl Pharmacol*. 1982;64:415–422.

Graham DI, Lantos PL, eds. *Greenfield's Neuropathology*. 6th ed. New York: Arnold; 1997.

Grandjean P, Herz KT. Methylmercury and brain development: imprecision and underestimation of developmental neurotoxicity in humans. *Mt Sinai J Med*. 2011;78:107–118.

Grandjean P, Landrigan PJ. Developmental neurotoxicity of industrial chemicals. *Lancet*. 2006;368:2167–2178.

Grandjean P, Landrigan PJ. Neurobehavioural effects of developmental toxicity. *Lancet Neurol*. 2014;13:330–338.

Griffin JW, Fahnestock KE, Price DL, et al. Microtubule-neurofilament segregation produced by β,β′-iminodipropionitrile: evidence for the association of fast axonal transport with microtubules. *J Neurosci*. 1983;3:557–566.

Grunnet KS, Dahllof I. Environmental fate of the antifouling compound zinc pyrithione in seawater. *Environ Toxicol Chem*. 2005;24:3001–3006.

Guilarte TR, Gonzales KK. Manganese-induced parkinsonism is not idiopathic Parkinson's disease: environmental and genetic evidence. *Tox Sci*. 2015;146:204-212

Gutierrez PL. The role of NAD(P)H oxidoreductase (DT-diaphorase) in the bioactivation of quinone-containing antitumor agents: a review. *Free Radic Biol Med*. 2000;29:263–275.

Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, eds. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. 9th ed. New York: McGraw-Hill; 1996.

Harris CH, Gulati AK, Friedman MA, et al. Toxic neurofilamentous axonopathies and fast axonal transport: V. Reduced bidirectional vesicle transport in cultured neurons by acrylamide and glycidamide. *J Toxicol Environ Health*. 1994;42:343–456.

Harry GJ, Goodrum JF, Bouldin TW, et al. Tellurium-induced neuropathy: metabolic alterations associated with demyelination and remyelination in rat sciatic nerve. *J Neurochem*. 1989;52:938–945.

Hassel B, Sonnewald U, Unsgard G, et al. NMR spectroscopy of cultured astrocytes: effects of glutamine and the gliotoxin fluorocitrate. *J Neurochem*. 1994;62:2187–2194.

Hauser DN, Hastings TG. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. *Neurobiol Dis*. 2013;51:35–42.

Hausherr V, Schöbel N, Liebing J, et al. Assessment of neurotoxic effects of tri-cresyl phosphates (TCPs) and cresyl saligenin phosphate (CBDP) using a combination of in vitro techniques. *Neurotoxicology*. 2017;59:210-221.

Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A*. 2008;105(44):17046–17049.

Hein ND, Stuckey JA, Rainier SR, Fink JK, Richardson RJ. Constructs of human neuropathy target esterase catalytic domain containing mutations related to motor neuron disease have altered enzymatic properties. *Toxicol Lett*. 2010;196:67–73.

Heindel JJ. Role of exposure to environmental chemicals in the developmental basis of disease and dysfunction. *Reprod Toxicol*. 2007;23(3):257–259.

Herning R, King D, Better W, et al. Neurovascular deficits in cocaine abusers. *Neuropsychopharmacology*. 1999;21:110–118.

Hevor TK. Some aspects of carbohydrate metabolism in the brain. *Biochimie*. 1994;76:111–120.

Hirano A, Kurland LT, Krooth RS, et al. Parkinsonism–dementia complex, an endemic disease on the island of Guam. I. Clinical features. *Brain*. 1961a;84:642–661.

Hirano A, Malamud N, Kurland LT. Parkinsonism–dementia complex, an endemic disease on the island of Guam. II. Pathological features. *Brain*. 1961b;84:662–679.

Hoffman PN, Lasek RJ. The slow component of axonal transport: identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J Cell Biol*. 1975;66:351–366.

Hollmann M, Heinemann S. Cloned glutamate receptors. *Ann Rev Neurosci*. 1994;17:31–108.

Hu HL, Bennett N, Holton JL, et al. Glutathione depletion increases brain susceptibility to *m*-dinitrobenzene neurotoxicity. *NeuroToxicology*. 1999;20:83–90.

Hu HL, Bennett N, Lamb JH, et al. Capacity of rat brain to metabolize *m*-dinitrobenzene: an in vitro study. *NeuroToxicology*. 1997;18:363–370.

Huber JD, Darling SF, Park K, et al. The role of NMDA receptors in neonatal cocaine-induced neurotoxicity. *Pharmacol Biochem Behav*. 2001;69:451–459.

Ikonomidou C, Bittigau P, Ishimaru MJ, et al. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science*. 2000;287:1056–1060.

Janoff AS, Pringle MJ, Miller KW. Correlation of general anesthetic potency with solubility in membranes. *Biochim Biophys Acta*. 1981;649:125–128.

Jenner P. Oxidative stress in Parkinson's disease. *Ann Neurol*. 2003;53(suppl 3):S26–S36.

Johnson DE, Richardson RJ. eds. *Computational Systems Pharmacology and Toxicology.* Cambridge, UK: Royal Society of Chemistry; 2017:332.

Johnson DJ, Graham DG, Amarnath V, et al. Release of carbon disulfide is a contributing mechanism in the axonopathy produced by *N,N*-diethyldithiocarbamate. *Toxicol Appl Pharmacol*. 1998;148:288–296.

Johnston MV, Goldstein GW. Selective vulnerability of the developing brain to lead. *Curr Opin Neurol*. 1998;11:689–693.

Kapoor K, Chandra M, Nag D, et al. Evaluation of metronidazole toxicity: a prospective study. *Int J Clin Pharm Res*. 1999;19:83–88.

Kappus H. Oxidative stress in chemical toxicity. *Arch Toxicol*. 1987;60:144–149.

Kesson CM, Baird AW, Lawson DH. Acrylamide poisoning. *Postgrad Med J*. 1977;53:16–17.

Khalil AA, Davies B, Castagnoli N Jr. Isolation and characterization of a monoamine oxidase B selective inhibitor from tobacco smoke. *Bioorg Med Chem*. 2006;14:3392–3398.

Kienzl E, Puchinger L, Jellinger K, et al. The role of transition metals in the pathogenesis of Parkinson's disease. *J Neurol Sci*. 1995;134:69–78.

Kirsten E, Sharma ML, Kun E. Molecular toxicology of (–)erythro-fluorocitrate: selective inhibition of citrate transport I mitochondria and the binding of fluorocitrate to mitochondrial proteins. *Mol Pharmacol*. 1978;14:172–184.

Kisby GE, Ellison M, Spencer PS. Content of the neurotoxins cycasin (methylazoxymethanol beta-D-glucoside) and BMAA (beta-*N*-methylamino-L-alanine) in cycad flour prepared by Guam Chamorros. *Neurology*. 1992;42:1336–1340.

Klaassen CD. Absorption, distribution, and excretion of zinc pyridinethione in rabbits. *Toxicol Appl Pharmacol*. 1976;35:581–587.

Klivenyi P, Clair D, Wermer M, et al. Manganese superoxide dismutase overexpression attenuates MPTP toxicity. *Neurobiol Dis*. 1998;5:253–258.

Kniesel U, Wolburg H. Tight junctions of the blood-brain barrier. *Cell Mol Neurobiol*. 2000;20:57–76.

Koehnle TJ, Brown A. Slow axonal transport of neurofilament protein in cultured neurons. *J Cell Biol*. 1999;144:447–458.

Krasavage WJ, O'Donoghue JL, DiVincenzo GD, et al. The relative neurotoxicity of MnBk, *n*-hexane, and their metabolites. *Toxicol Appl Pharmacol*. 1980;52:433–441.

Kropp TJ, Glynn P, Richardson RJ. The mipafox-inhibited catalytic domain of human neuropathy target esterase ages by reversible proton loss. *Biochemistry*. 2004;43:3716–3722.

Kumar A, Choi KH, Renthal W, et al. Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron*. 2005;48(2):303–314.

Kumar KK, Aboud AA, Bowman AB. The potential of induced pluripotent stem cells as a translational model for neurotoxicological risk. *Neurotoxicology*. 2012;33(3):518–529. PMC3358591.

Kumar KK, Lowe EW, Aboud AA, et al. Cellular manganese content is developmentally regulated in human dopaminergic neurons. *Sci Rep*. 2014;4:6801.

Kuopio AM, Marttila RJ, Helenius H, et al. Changing epidemiology of Parkinson's disease in southwestern Finland. *Neurology*. 1999;52:302–308.

Kurland LT. Epidemiological investigations of neurological disorders in the Mariana islands. In: Pemberton J, ed. *Epidemiology Reports on Research and Teaching*. New York: Oxford University Press; 1963:219–223.

Kurland LT, Faro SN, Siedler J. Minamata disease. *World Neurol*. 1960;1:370–395.

Kurth MC, Kurth JH. Variant cytochrome P450 CYP2D6 allelic frequencies in Parkinson's disease. *Am J Med Genet*. 1993;48:166–168.

Labbé C, Lorenzo-Betancor O, Ross OA. Epigenetic regulation in Parkinson's disease. *Acta Neuropathol*. 2016;132(4):515–530.

Lai BC, Marion SA, Teschke K, et al. Occupational and environmental risk factors for Parkinson's disease. *Parkinsonism Relat Disord*. 2002;8:297–309.

Lam G-W, DiStefano V. Characterization of carbon disulfide binding in blood and to other biological substances. *Toxicol Appl Pharmacol*. 1986;86:235–242.

LaMonte BH, Wallace KE, Holloway BA, et al. Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. *Neuron*. 2002;34:715–727.

Landigan PJ, Sonawane B, Butler RN, et al. Early environmental origins of neurodegenerative disease in later life. *Environ Health Perspect*. 2005;113:1230–1233.

Langston JW, Ballard P, Tetrud JW, et al. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*. 1983;219:979–980.

Langston JW, Forno LS, Tetrud J, et al. Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. *Ann Neurol*. 1999;46:598–605.

Langston JW, Irwin I. MPTP: Current concepts and controversies. *Clin Neuropharmacol*. 1986;9:485–507.

Lauder JM. Neurotransmitters as growth regulatory signals: role of receptors and second messengers. *Trends Neurosci*. 1993;16:233–240.

LeBel CP, Ali SF, Bondy SC. Deferoxamine inhibits methyl mercury–induced increases in reactive oxygen species formation in rat brain. *Toxicol Appl Pharmacol*. 1992;112:161–165.

Lehning EJ, Jortner BS, Fox JH, et al. γ-Diketone peripheral neuropathy. I. Quantitative morphometric analyses of axonal atrophy and swelling. *Toxicol Appl Pharmacol*. 2000;165:127–140.

Lemasters JJ, Nieminen AL, Qian T, et al. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta*. 1998;366:177–196.

Letzel S, Lang CJ, Schaller KH, et al. Longitudinal study of neurotoxicity with occupational exposure to aluminum dust. *Neurology*. 2000;54:997–1000.

Lewis JA, Tran BL, Puerta DT, et al. Synthesis, structure and spectroscopy of new thiopyrone and hydroxypyridine transition–metal complexes. *Dalton Trans*. 2005;7:2588–2596.

Lichensteiger W, Ribary U, Schlumpf M, et al. Prenatal adverse effects of nicotine on the developing brain. In: Boer GJ, Feenstra MGP, Mirmiran M, et al., eds. *Progress in Brain Research*. Vol 73. Amsterdam: Elsevier; 1988:137–157.

Lipton RB, Apfel SC, Dutcher JP, et al. Taxol produces a predominantly sensory neuropathy. *Neurology*. 1989;39:368–373.

Lipton SA, Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med*. 1994;330:613–622.

Liu Y, Fechter LD. Comparison of the effects of trimethyltin on the intracellular calcium levels in spiral ganglion cells and outer hair cells. *Acta Otolaryngol*. 1996;116:417–421.

Llorens J, Dememes D, Sans A. The behavioral syndrome caused by 3,3′-iminodipropionitrile and related nitriles in the rat is associated with degeneration of the vestibular sensory hair cells. *Toxicol Appl Pharmacol*. 1993;123:199–210.

Logroscino G. The role of early life environmental risk factors in Parkinson disease: what is the evidence? *Environ Health Perspect*. 2005;113:1234–1238.

LoPachin RM, Jortner BS, Reid ML, et al. γ-Diketone central neuropathy: quantitative analyses of cytoskeletal components in myelinated axons of the rat rubrospinal tract. *Neurotoxicology*. 2005;26:1021–1030.

LoPachin RM, Ross JF, Reid ML, et al. Neurological evaluation of toxic axonopathies in rats: acrylamide and 2,5-hexanedione. *Neurotoxicology*. 2002;23:95–110.

Lotharius J, O'Malley KL. The parkinsonian-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. *J Biol Chem*. 2000;275:38581–38588.

Lotharius J, O'Malley KL. Role of mitochondrial dysfunction and dopamine-dependent oxidative stress in amphetamine-induced toxicity. *Ann Neurol*. 2001;49:79–89.

Lotti M. Promotion of organophosphate-induced delayed polyneuropathy by certain esterase inhibitors. *Toxicology*. 2002;181–182:245–248.

Lotti M, Moretto A. Organophosphate-induced delayed polyneuropathy. *Toxicol Rev*. 2005;24:37–49.

Lucey JF, Hibbard E, Behrman RE, et al. Kernicterus in asphyxiated newborn rhesus monkeys. *Exp Neurol*. 1964;9:43–58.

Ludolph AC, He F, Spencer PS, et al. 3-Nitropropionic acid: exogenous animal neurotoxin and possible human striatal toxin. *Can J Neurol Sci*. 1991;18:492–498.

Ludolph AC, Seelig M, Ludolf A, et al. 3-Nitropropionic acid decreases cellular energy levels and causes neuronal degeneration in cortical explants. *Neurodegeneration*. 1992;1:155–161.

Magyar K, Szende B. (–)-Deprenyl, a selective MAO-B inhibitor, with apoptotic and anti-apoptotic properties. *Neurotoxicology*. 2004;25:233–242.

Makri A, Goveia M, Balbus J, et al. Children's susceptibility to chemicals: a review by developmental stage. *J Toxicol Environ Health Part B*. 2004;7:417–435.

Maloney B, Lahiri DK. Epigenetics of dementia: understanding the disease as a transformation rather than a state. *Lancet Neurol*. 2016;15(7):760–774.

Mandel S, Grunblatt E, Riederer P, et al. Neuroprotective strategies in Parkinson's disease: an update on progress. *CNS Drugs*. 2003;17:729–762.

Mandel S, Weinreb O, Amit T, et al. Mechanism of neuroprotective action of the anti-Parkinson drug rasagiline and its derivatives. *Brain Res Brain Res Rev*. 2005;48:379–387.

Martin DL, Waniewski RA. Precursor synthesis and neurotransmitter uptake by astrocytes as targets of neurotoxicants. In: Aschner M, Kimelberg HK, eds. *The Role of Glia in Neurotoxicity*. Boca Raton, FL: CRC Press; 1996:335–357.

Marty MS, Atchison WD. Pathways mediating Ca^{2+} entry in rat cerebellar granule cells following in vitro exposure to methyl mercury. *Toxicol Appl Pharmacol*. 1997;147:319–330.

Massart R, Barnea R, Dikshtein Y, et al. Role of DNA methylation in the nucleus accumbens in incubation of cocaine craving. *J Neurosci*. 2015;35(21):8042–8058.

Mastrogiacomo F, Bergeron C, Kish SJ. Brain alpha-ketoglutarate dehydrogenase complex activity in Alzheimer's disease. *J Neurochem*. 1993;61:2007–2014.

Mattsson JL, Albee RR, Eisenbrandt DL. Neurological approach to neurotoxicological examination in laboratory animals. *Int J Toxicol*. 1989;8:271–286.

McCann UD, Ricaurte GA. Amphetamine neurotoxicity: accomplishments and remaining challenges. *Neurosci Behav Rev*. 2004;27:821–826.

McMillan DE. A brief history of the neurobehavioral toxicity of manganese: some unanswered questions. *Neurotoxicology*. 1999;20:499–507.

Meldrum B. Excitatory amino acid antagonists as potential therapeutic agents. In: Jenner P, ed. *Neurotoxins and Their Pharmacological Implications*. New York: Raven Press; 1987:33–53.

Mielke S, Sparreboom A, Mross K. Peripheral neuropathy: a persisting challenge in paclitaxel-based regimes. *Eur J Cancer*. 2006;42:24–30.

Miller DB, O'Callaghan JP. Elevated environmental temperature and methamphetamine neurotoxicity. *Environ Res*. 2003;92:48–53.

Miodovnik A. Environmental neurotoxicants and developing brain. *Mt Sinai J Med*. 2011;78:58–77.

Mitsuishi K, Takahashi A, Mizutani M, et al. Beta, beta′-iminodipropionitrile toxicity in normal and congenitally neurofilament-deficient Japanese quails. *Acta Neuropathol*. 1993;86:578–581.

Montgomery EB Jr. Heavy metals and the etiology of Parkinson's disease and other movement disorders. *Toxicology*. 1995;97:3–9.

Monuki ES, Lemke G. Molecular biology of myelination. In: Waxman SG, Kocsis JD, Stys PK, eds. *The Axon: Structure, Function, and Pathophysiology*. New York: Oxford University Press; 1995:144–163.

Moors M, Rockel TD, Abel J, et al. Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. *Environ Health Perspect*. 2009;117(7):1131–1138.

Moreira EG, Vassilieff I, Vassilieff VS. Developmental lead exposure: behavioral alterations in the short and long term. *Neurotoxicol Teratol*. 2001;23:489–495.

Morell P, Toews AD, Wagner M, et al. Gene expression during tellurium-induced primary demyelination. *Neurotoxicology*. 1994;15:171–180.

Morgan RE, Garavan H, Smith EG, et al. Early lead exposure produces lasting changes in sustained attention, response initiation, and reactivity to errors. *Neurotoxicol Teratol*. 2001;23:519–531.

Moser VC. Observational batteries in neurotoxicity testing. *Int J Toxicol*. 2000;19:407–411.

Moser VC, Becking GC, Cuomo V, et al. The IPCS collaborative study on neurobehavioral screening methods. III. Results of proficiency studies. *Neurotoxicology*. 1997a;18:939–946.

Moser VC, Becking GC, Cuomo V, et al. The IPCS collaborative study on neurobehavioral screening methods: V. Results of chemical testing. *Neurotoxicology*. 1997b;18:969–1055.

Moser VC, Padilla S. Age- and gender-related differences in the time-course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. *Toxicol Appl Pharmacol*. 1998;149:107–119.

Mossakowski MJ, Renkawek K, Krasnicka Z, et al. Morphology and histochemistry of Wilsonian and hepatic gliopathy in tissue culture. *Acta Neuropathol (Berlin)*. 1970;16:1–16.

Mullick FG. Hexachlorophene toxicity: human experience at the AFIP. *Pediatrics*. 1973;51:395–399.

Mulugeta S, Ciavarra RP, Maney RK, et al. Three subpopulations of fast axonally transported retinal ganglion cell proteins are differentially trafficked in the rat optic pathway. *J Neurosci Res*. 2000;59:247–258.

Muralikrishnan D, Samantaray S, Mohanakumar KP. D-Deprenyl protects nigrostriatal neurons against 1-methyl-4-1,2,3,6-tetrahydropyridine-induced dopaminergic neurotoxicity. *Synapse*. 2003;50:7–13.

Mushiroda T, Ariyoshi N, Yokoi T, et al. Accumulation of the 1-methyl-4-pyridinium ion in suncus (*Suncus murinus*) brain: implication for flavin-containing monooxygenase activity in brain microvessels. *Chem Res Toxicol*. 2001;14:228–232.

Myers JE, Macun I. Acrylamide neuropathy in a South African factory: an epidemiologic investigation. *Am J Indust Med*. 1991;19:487–493.

Myers RR, Mizisin AP, Powell HC, et al. Reduced nerve blood flow in hexachlorophene neuropathy: relationship to elevated endoneurial pressure. *J Neuropathol Exp Neurol*. 1982;41:391–399.

Nakata T, Yorifuji H. Morphological evidence of the inhibitory effect of taxol on the fast axonal transport. *Neurosci Res*. 1999;35:113–122.

Nandipati S, Litvan I. Environmental exposures and Parkinson's disease. *Int J Environ Res Public Health*. 2016;13(9). pii: E881.

National Research Council (NRC). *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Washington, DC: National Academies Press; 2007.

Needleman HL. Childhood lead poisoning. *Curr Opin Neurol*. 1994;7:187–190.

Needleman HL, Gatsonis CA. Low-level lead exposure and the IQ of children: a meta-analysis of modern studies. *JAMA*. 1990;263:673–678.

Neely MD, Davison CA, Bowman AB. From the Cover: Manganese and Rotenone-Induced Oxidative Stress Signatures Differ in iPSC-Derived Human Dopamine Neurons. *Toxicol Sci*. 2017 Oct 1;159(2):366–379.

Nihei MK, Desmond NL, McGlothan JL, et al. *N*-Methyl-D-aspartate receptor subunit changes are associated with lead-induced deficits of long-term potentiation and spatial learning. *Neuroscience*. 2000;99:233–242.

Nixon RA. Dynamic behavior and organization of cytoskeleton proteins in neurons: reconciling old and new findings. *Bioessays*. 1998;20:798–807.

Nunn PB, Seelig M, Zagoren JC, et al. Stereospecific acute neurotoxicity of "uncommon" plant amino acids linked to human motor system diseases. *Brain Res*. 1987;410:375–379.

Oestreicher E, Sengstock GJ, Riederer P, et al. Degeneration of nigrostriatal dopaminergic neurons increases iron within the substantia nigra: a histochemical and neurochemical study. *Brain Res*. 1994;660:8–18.

Okeda R, Shibutani M, Matsuo T, et al. Experimental neurotoxicity of 5-fluorouracil and its derivatives is due to poisoning by the monofluorinated organic metabolites, monofluoroacetic acid and alpha-fluoro-beta-alanine. *Acta Neuropathol (Berlin)*. 1990;81:66–73.

Olney JW. Neurotoxicity of excitatory amino acids. In: McGeer EG, Olney JW, McGeer PL, eds. *Kainic Acid as a Tool in Neurobiology*. New York: Raven Press; 1978:95–122.

O'Shea KS, Kaufman MH. The teratogenic effect of acetaldehyde: implications for the study of fetal alcohol syndrome. *J Anat*. 1979;128:65–76.

Padilla S, Atkinson MB, Breuer AC. Direct measurement of fast axonal organelle transport in the sciatic nerve of rats treated with acrylamide. *J Toxicol Environ Health*. 1993;39:429–445.

Padilla S, Grizzle TB, Lyerly D. Triphenyl phosphite: in vivo and in vitro inhibition of rat neurotoxic esterase. *Toxicol Appl Pharmcol*. 1987;87:249–256.

Pardridge WM. Blood–brain barrier biology and methodology. *J Neurovirol*. 1999;5:556–569.

Parent A. *Carpenter's Human Neuroanatomy*. 9th ed. Baltimore, MD: Williams & Wilkins; 1996:131–198.

Parenti M, Rusconi L, Cappabianca V, et al. Role of dopamine in manganese neurotoxicity. *Brain Res*. 1988;473:236–240.

Patanow CM, Day JR, Billingsley ML. Alterations in hippocampal expression of SNAP-25, GAP-43, stannin and glial fibrillary acidic protein following mechanical and trimethyltin-induced injury in the rat. *Neuroscience*. 1997;76:187–202.

Penny DG. Acute carbon monoxide poisoning: animal models: a review. *Toxicology*. 1990;62:123–160.

Peres TV, Schettinger MR, Chen P, et al. Manganese-induced neurotoxicity: a review of its behavioral consequences and neuroprotective strategies. *BMC Pharmacol Toxicol*. 2016;17(1):57.

Perl TM, Bedard L, Kosatsky T, et al. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N Engl J Med*. 1990;322:1775–1780.

Perlson E, Maday S, Fu MM, Moughamian AJ, Holzbaur EL. Retrograde axonal transport: pathways to cell death? *Trends Neurosci*. 2010;33:335–344.

Peterson RT, Nass R, Boyd WA, Freedman JH, Dong K, Narahashi T. Use of non-mammalian alternative models for neurotoxicological study. *Neurotoxicology*. 2008;29:546–555.

Pezzoli G, Cereda E. Exposure to pesticides or solvents and risk of Parkinson disease. *Neurology*. 2013;80:2035–2041.

Pham-Dinh D, Popot JL, Boespflug-Tanguy O, et al. Pelizaeus–Merzbacher disease: a valine to phenylalanine point mutation in a putative extracellular loop of myelin proteolipid. *Proc Natl Acad Sci U S A*. 1991;88:7562–7566.

Phelps CH. An ultrastructural study of methionine sulphoximine-induced glycogen accumulation in astrocytes of the mouse cerebral cortex. *J Neurocytol*. 1975;4:479–490.

Philbert MA, Nolan CC, Cremer JE, et al. 1,3-Dinitrobenzene-induced encephalopathy in rats. *Neuropathol Appl Neurobiol*. 1987;13:371–389.

Pierard-Franchimont C, Goffin V, Decroix J, et al. A multicenter randomized trial of ketoconazole 2% and zinc pyrithione 1% shampoos in severe dandruff and seborrheic dermatitis. *Skin Pharmacol Appl Skin Physiol*. 2002;15:434–441.

Plato CC, Galasko D, Garruto R, et al. ALS and PDC of Guam: forty-year follow-up. *Neurology*. 2002;58:765–773.

Plato CC, Garruto RM, Galasko D, et al. Amyotrophic lateral sclerosis and parkinsonism–dementia complex of Guam: changing incidence rates during the past 60 years. *Am J Epidemiol*. 2003;157:149–157.

Plum F, Posner JB. Neurobiologic essentials. In: Smith LH Jr, Thier SO, eds. *Pathophysiology: The Biological Principles of Disease*. Philadelphia. PA: Saunders; 1985:1009–1036.

Polymerpoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science*. 1997;276:2045–2047.

Pope CN. Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J Toxicol Environ Health B*. 1999;2:161–181.

Pope CN, Tanaka D Jr, Padilla S. The role of neurotoxic esterase (NTE) in the prevention and potentiation of organophosphorus-induced delayed neurotoxicity (OPIDN). *Chem Biol Interact*. 1993;87:395–406.

Porterfield SP. Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system. *Environ Health Perspect*. 1994;102(suppl 2):125–130.

Porterfield SP. Thyroidal dysfunction and environmental chemicals—potential impact on brain development. *Environ Health Perspect*. 2000;108(suppl 3):433–438.

Powell HC, Myers RR, Lampert PW. Changes in Schwann cells and vessels in lead neuropathy. *Am J Pathol*. 1982;109:193–205.

Prakash V, Timasheff SN. Aging of tubulin at neutral pH: the destabilizing effect of Vinca alkaloids. *Arch Biochem Biophys*. 1992;295:137–145.

Pritchard C, Rosenorn-Lanng E. Neurological deaths of American adults (55–74) and the over 75's by sex compared with 20 Western countries 1989–2010: cause for concern. *Surg Neurol Int*. 2015;6:123–134.

Purves DC, Garrod IJ, Dayan AD. A comparison of spongiosis induced in the brain by hexachlorophene, cuprizone, and triethyl tin in the Sprague–Dawley rat. *Hum Exp Toxicol*. 1991;10:439–444.

Qiu J, Cai D, Filbin MT. Glial inhibition of nerve regeneration in mature mammalian CNS. *Glia*. 2000;29:166–174.

Quarles RH, Farrer RG, Yim SH. Structure and function of myelin, an extended and biochemically modified cell surface membrane. In: Juurlink BHJ, Devon RM, Doucette JR, et al., eds. *Cell Biology and Pathology of Myelin: Evolving Biological Concepts and Therapeutic Approaches*. New York: Plenum Press; 1997:1–12.

Quik M. Smoking, nicotine and Parkinson's disease. *Trends Neurosci*. 2004;27:561–568.

Quik M, Parameswaran N, McCallum SE, et al. Chronic oral nicotine treatment protects against striatal degeneration in MPTP-treated primates. *J Neurochem*. 2006;98:1866–1875.

Radio NM, Mundy WR. Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. *NeuroToxicology*. 2008;29(3):361–376.

Rainier S, Albers JW, Dyck PJ, et al. Motor neuron disease due to neuropathy target esterase gene mutation: clinical features of the index families. *Muscle Nerve*. 2011;43:19–25.

Rakonczay Z, Matsuoka Y, Giacobini E. Effects of L-beta-*N*-methylamino-L-alanine (L-BMAA) on the cortical cholinergic and glutamatergic systems of the rat. *J Neurosci Res*. 1991;29:121–126.

Randall JC, Yano BL, Richardson RJ. Potentiation of organophosphorus compound–induced delayed neurotoxicity (OPIDN) in the central and peripheral nervous system of the adult hen: distribution of axonal lesions. *J Toxicol Environ Health*. 1997;51:571–590.

Ray DE, Abbott NJ, Chan MW, et al. Increased oxidative metabolism and oxidative stress in m-dinitrobenzene neurotoxicity. *Biochem Soc Trans*. 1994;22:407S.

Ray DE, Brown AW, Cavanagh JB, et al. Functional/metabolic modulation of the brain stem lesions caused by 1,3-dinitrobenzene in the rat. *Neurotoxicology*. 1992;13:379–388.

Readhead C, Schneider A, Griffiths I, et al. Premature arrest of myelin formation in transgenic mice with increased proteolipid protein gene dosage. *Neuron*. 1994;12:583–595.

Reuhl KR, Chang LW. Effects of methylmercury on the development of the nervous system: a review. *Neurotoxicology*. 1979;1:21–55.

Reuhl KR, Gilbert SG, Mackenzie BA, et al. Acute trimethyltin intoxication in the monkey *(Macaca fascicularis)*. *Toxicol Appl Pharmacol*. 1985;79:436–452.

Riachi NJ, Dietrich WD, Harik SI. Effects of internal carotid administration of MPTP on rat brain and blood–brain barrier. *Brain Res*. 1990;533:6–14.

Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect*. 2000;108(suppl 3):511–533.

Rice DC. Evidence for delayed neurotoxicity produced by methylmercury. *Neurotoxicology*. 1996;17:583–596.

Richardson RJ. Interactions of organophosphorus compounds with neurotoxic esterase. In: Chambers JE, Levi PE, eds. *Organophosphates: Chemistry, Fate, and Effects*. San Diego, CA: Academic Press; 1992:299–323.

Richardson RJ. Organophosphate poisoning, delayed neurotoxicity. In: Wexler P, ed. *Encyclopedia of Toxicology*. 2nd ed. Oxford: Elsevier; 2005:302–306.

Richardson RJ. Anticholinesterase insecticides. In: McQueen C. ed. *Comprehensive Toxicology*. Vol 13. 2nd ed. Oxford: Academic Press; 2010:433–444.

Richardson RJ, Hein ND, Wijeyesakere SJ, Fink JK, Makhaeva GF. Neuropathy target esterase (NTE): overview and future. *Chem Biol Interact*. 2013;203:238–244.

Richardson RJ, Makhaeva GF. Organophosphorus compounds. In: Wexler P, ed. *Encyclopedia of Toxicology*. Vol 3. 3rd ed. Elsevier; 2014:714–719.

Richardson RJ, Worden RM, Wijeyesakere SJ, et al. Neuropathy target esterase as a biomarker and biosensor of delayed neuropathic agents. In: Gupta RC, ed. *Handbook of Toxicology of Chemical Warfare Agents*. 2nd ed. Amsterdam: Academic Press; 2015:935–952.

Ridgway P, Nixon TE, Leach JP. Occupational exposure to organic solvents and long-term nervous system damage detectable by brain imaging, neurophysiology or histopathology. *Food Chem Toxicol*. 2003;41:153–187.

Riggs JE, Schochet SS, Gutmann L, et al. Chronic human colchicine neuropathy and myopathy. *Arch Neurol*. 1986;43:521–523.

Riikonen R, Salonen I, Partanen K, et al. Brain perfusion SPECT and MRI in foetal alcohol syndrome. *Dev Med Child Neurol*. 1999;41:652–659.

Rivadeneyra-Domínguez E, Rodríguez-Landa JF. Cycads and their association with certain neurodegenerative diseases. *Neurologia*. 2014;29:517–522.

Rodgers-Johnson P, Garruto RM, Yanagihara R, et al. Amyotrophic lateral sclerosis and parkinsonism-dementia on Guam: a 30-year evaluation of clinical and neuropathologic trends. *Neurology*. 1986;36:7–13.

Rodier PM. Developing brain as a target of toxicity. *Environ Health Perspect*. 1995;103(suppl 6):73–76.

Romero I, Brown AW, Cavanagh JB, et al. Vascular factors in the neurotoxic damage caused by 1,3-dinitrobenzene in the rat. *Neuropathol Appl Neurobiol*. 1991;17:495–508.

Romero IA, Lister T, Richards HK, et al. Early metabolic changes during *m*-dinitrobenzene neurotoxicity and the possible role of oxidative stress. *Free Radic Biol Med*. 1995;18:311–319.

Romieu P, Host L, Gobaille S, et al. Histone deacetylase inhibitors decrease cocaine but not sucrose self-administration in rats. *J Neurosci*. 2008;28(38):9342–9348.

Rondeau V, Jacqmin-Gadda H, Commenges D, Helmer C, Dartigues JF. Aluminum and silica in drinking water and the risk of Alzheimer's disease or cognitive decline: findings from 15-year follow-up of the PAQUID cohort. *Am J Epidemiol*. 2009;169:489–496.

Ross GW, Petrovitch H. Current evidence for neuroprotective effects of nicotine and caffeine against Parkinson's disease. *Drugs Aging*. 2001;18:797–806.

Ross JF, Lawhorn GT. ZPT-related distal axonopathy: behavioral and electrophysiologic correlates in rats. *Neurotoxicol Teratol*. 1990;12:153–159.

Rothman SM, Olney JM. Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann Neurol*. 1986;19:105–111.

Rothstein JD, Tabakoff B. Regulation of neurotransmitter aspartate metabolism by glial glutamine synthetase. *J Neurochem*. 1986;46:1923–1928.

Roytta M, Horwitz SB, Raine CS. Taxol-induced neuropathy: short-term effects of local injection. *J Neurocytol*. 1984;13:685–701.

Rubin LL, Staddon JM. The cell biology of the blood–brain barrier. *Annu Rev Neurosci*. 1999;22:11–28.

Ryan KR, Sirenko O, Parham F, et al. Neurite outgrowth in human induced pluripotent stem cell-derived neurons as a high-throughput screen for developmental neurotoxicity or neurotoxicity. *Neurotoxicology*. 2016;53:271–281.

Ryan SD, Dolatabadi N, Chan S, et al. Isogenic human iPSC Parkinson's model shows nitrosative stress–induced dysfunction in MEF2-PGC1α transcription. *Cell*. 2013;155(6):1351–1364.

Sahenk Z, Barohn R, New P, et al. Taxol neuropathy: electrodiagnostic and sural nerve biopsy findings. *Arch Neurol*. 1994;51:726–729.

Sahenk Z, Mendell JR. Ultrastructural study of zinc pyrdinethione–induced peripheral neuropathy. *J Neuropathol Exp Neurol*. 1979;38:532–550.

Sahenk Z, Mendell JR. Axoplasmic transport in zinc pyridinethione neuropathy: evidence for an abnormality in distal turn-around. *Brain Res*. 1980;186:343–353.

Sahenk Z, Mendell JR. Pyrithione. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. New York: Oxford University Press; 2000:1050–1054.

Sampson PD, Streissguth AP, Bookstein FL, et al. On categorizations in analyses of alcohol teratogenesis. *Environ Health Perspect*. 2000;108(suppl 3):421–428.

Santibáñez M, Bolumar F, García AM. Occupational risk factors in AD: a review assessing the quality of published epidemiological studies. *Occup Environ Med*. 2007;64:723–732.

Sayre LM, Shearson CM, Wongmongkolrit T, et al. Structural basis of γ-diketone neurotoxicity: non-neurotoxicity of 3,3-dimethyl-2,5-hexanedione a γ-diketone incapable of pyrrole formation. *Toxicol Appl Pharmacol*. 1986;84:36–44.

Schaumburg HH, Wisniewski HM, Spencer PS. Ultrastructural studies of the dying-back process I. Peripheral nerve terminal and axon degeneration in systemic acrylamide intoxication. *J Neuropathol Exp Neurol*. 1974;33:260–284.

Schaumburg HH. Vinca alkaloids. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. New York: Oxford University Press; 2000:1232–1236.

Schentag JJ. Mental confusion in a patient treated with metronidazole—a concentration-related effect? *Pharmacotherapy*. 1982;2:384–387.

Schiff PB, Horwitz SB. Taxol assembles tubulin in the absence of exogenous guanosine 59-triphosphate or microtubule-associated proteins. *Biochemistry*. 1981;20:3242–3252.

Schinder AF, Olson EC, Spitzer NC, et al. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J Neurosci*. 1996;16:6125–6133.

Schionning JD, Larsen JO, Tandrup T, et al. Selective degeneration of dorsal root ganglia and dorsal nerve roots in methyl mercury–intoxicated rats: a stereological study. *Acta Neuropathol*. 1998;96:191–201.

Schlafer WW, Zimmerman U-JP. Calcium-activated protease and the regulation of the axonal cytoskeleton. In: Elam JS, Cancalon P, eds. *Advances in Neurochemistry*. New York: Plenum Press; 1984:261–273.

Schnapp BJ, Reese TS. Dynein is the motor for retrograde axonal transport of organelles. *Proc Natl Acad Sci U S A*. 1989;86:1548–1552.

Schneider D, Wink M, Sporer F, et al. Cycads: their evolution, toxins, herbivores and insect pollinators. *Naturwissenschaften*. 2002;89:281–294.

Schneider JS, Anderson DW, Kidd SK, Sobolewski M, Cory-Slechta DA. Sex-dependent effects of lead and prenatal stress on post-translational histone modifications in frontal cortex and hippocampus in the early postnatal brain. *Neurotoxicology*. 2016;54, 65–71.

Schoepfer R, Monyer H, Sommer B, et al. Molecular biology of glutamate receptors. *Prog Neurobiol*. 1994;42:353–357.

Schopfer M, Masson P, Lamourette P, et al. Detection of cresyl phosphate-modified butyrylcholinesterase in human plasma for chemical exposure associated with aerotoxic syndrome. *Anal Biochem*. 2014;461:17–26.

Schutzman DL, Frankenfield-Chernicoff M, Clatterbaugh HE, et al. Incidence of intrauterine cocaine exposure in a suburban setting. *Pediatrics*. 1991;88:825–827.

Schwartz JH. The cytology of neurons. In: Kandel ER, Schwartz JH, Jessell TM, eds. *Principles of Neural Science*. 3rd ed. Norwalk, CT: Appleton & Lange; 1991:37–48.

Schwartz MP, Hou Z, Propson NE, et al. Human pluripotent stem cell–derived neural constructs for predicting neural toxicity. *Proc Natl Acad Sci U S A*. 2015;112(40):12516–12521.

Scriba GK, Borchardt RT. Metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by bovine brain microvessel endothelial cells. *Brain Res*. 1989;501:175–178.

Seegal RF. Epidemiological and laboratory evidence of PCB-induced neurotoxicity. *Crit Rev Toxicol*. 1996;26:709–737.

Selevan SG, Kimmel CA, Mendola P. Identifying critical windows of exposure for children's health. *Environ Health Perspect*. 2000:108 (suppl 3):451–455.

Senut MC, Cingolani P, Sen A, et al. Epigenetics of early-life lead exposure and effects on brain development. *Epigenomics*. 2012;4(6):665–674.

Seppaleinen AM, Haltia M. Carbon disulfide. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. Baltimore, MD: Williams & Wilkins; 1980:356–373.

Shanker G, Mutkus LA, Walker S, et al. Methylmercury enhances arachidonic acid release and cytosolic phospholipase A_2 expression in primary cultures of neonatal astrocytes. *Mol Brain Res*. 2002;106:1–11.

Sieber SM, Correa P, Dalgard DW, et al. Carcinogenicity and hepatotoxicity of cycasin and its aglycone methylazoxymethanol acetate in nonhuman primates. *J Natl Cancer Inst*. 1980;65:177–189.

Skinner MK, Anway MD, Savenkova MI, Gore AC, Crews D (2008) Transgenerational Epigenetic Programming of the Brain Transcriptome and Anxiety Behavior. PLoS ONE 35(2): e3745. https://doi.org/10.1371/journal.pone.0003745.

Slikker W Jr, Xu ZA, Levin ED, et al. Mode of action: disruption of brain cell replication, second messenger, and neurotransmitter systems during development leading to cognitive dysfunction—developmental neurotoxicity of nicotine. *Crit Rev Toxicol*. 2005;35:703–711.

Slotkin TA. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol Appl Pharmacol*. 2004;15:132–151.

Smith RA. Twenty-first century challenges for in vitro neurotoxicity. *Altern Lab Anim*. 2009;37:367–375.

Song C, Kanthasamy A, Jin H, Anantharam V, Kanthasamy AG. Paraquat induces epigenetic changes by promoting histone acetylation in cell culture models of dopaminergic degeneration. *Neurotoxicology*. 2011;32(5):586-595.

Spencer PS. Doxorubicin and related anthracyclines. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. New York: Oxford University Press; 2000:529–533.

Spencer PS, Garner E, Palmer VS, Kisby GE. Environmental neurotoxins linked to a prototypical neurodegenerative disease. In: Aschner M, Costa L, eds. *Environmental Factors in Neurodevelopment and Neurodegenerative Disorders*. New York: Elsevier; 2015:212–237.

Spencer PS, Nunn PB, Hugon J, et al. Guam amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant excitant neurotoxin. *Science*. 1987;237:517–522.

Spencer PS, Schaumburg HH. Ultrastructural studies of the dying-back process III. The evolution of experimental peripheral giant axonal degeneration. *J Neuropathol Exp Neurol*. 1974;36:276.

Spencer PS, Schaumburg HH. Central-peripheral distal axonopathy: the pathology of dying-back polyneuropathies. In: Zimmerman H, ed. *Progress in Neuropathology*. Vol 3. New York: Grune & Stratton; 1976:253–295.

Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. 2nd ed. New York: Oxford University Press; 2000.

St. Clair MBG, Amarnath V, Moody MA, et al. Pyrrole oxidation and protein crosslinking are necessary steps in the development of γ-diketone neuropathy. *Chem Res Toxicol*. 1988;1:179–185.

Stern M, Gierse A, Tan S, Bicker G. Human Ntera2 cells as a predictive in vitro test system for developmental neurotoxicity. *Arch Toxicol*. 2013;88(1):127–136.

Stewart PA. Endothelial vesicles in the blood–brain barrier: are they related to permeability? *Cell Mol Neurobiol*. 2000;20:149–163.

Stoll G, Muller HW. Nerve injury, axonal degeneration and neural regeneration: basic insights. *Brain Pathol*. 1999;9:313–325.

Stoltenburg-Didinger G, Spohr HL. Fetal alcohol syndrome and mental retardation: spine distribution of pyramidal cells in prenatal alcohol exposed rat cerebral cortex. *Brain Res*. 1983;11:119–123.

Stys P. General mechanisms of axonal damage and its prevention. *J Neurol Sci*. 2005;233:3–13.

Susser ES, Lin SP. Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944–1945. *Arch Gen Psychiatry*. 1992;49(12):983–988.

Swanson RA, Graham SH. Fluorocitrate and fluoroacetate effects on astrocyte metabolism in vitro. *Brain Res*. 1994;664:94–100.

Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131(5):861–872.

Takeuchi T, Morikawa H, Matsumoto H, et al. A pathological study of Minamata disease in Japan. *Acta Neuropathol*. 1962;2:40–57.

Tanner CM, Ottman R, Goldman SM, et al. Parkinson disease in twins: an etiologic study. *JAMA*. 1999;281:341–346.

Tao I, Zhang P, Qin C, et al. Recent progresses in the exploration of machine learning methods as in-silico ADME prediction tools. *Adv Drug Deliv Rev*. 2015;86:83–100.

Teitelbaum JS, Zatorre RJ, Carpenter S, et al. Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. *N Engl J Med*. 1990;322:1781–1787.

Thompson CM, Richardson RJ. Anticholinesterase insecticides. In: Marrs TC, Ballantyne B, eds. *Pesticide Toxicology and International Regulation*. Chichester, England: Wiley; 2004:89–127.

Tilson HA. Neurobehavioral methods used in neurotoxicological research. *Toxicol Lett* 1993;68:231–240.

Tilson HA, Kodavanti PR. The neurotoxicity of polychlorinated biphenyls. *Neurotoxicology*. 1998;19:517–525.

Tilson HA, Moser VC. Comparison of screening approaches. *Neurotoxicology*. 1992;13:1–14.

Tjalkens RB, Ewing MM, Philbert MA. Differential cellular regulation of the mitochondrial permeability transition in an in vitro model of 1,3-dinitrobenzene-induced encephalopathy. *Brain Res*. 2000;874:165–177.

Toggas SM, Krady JK, Thompson TA, et al. Molecular mechanisms of selective neurotoxicants: studies on organotin compounds. *Ann N Y Acad Sci*. 1993;679:157–177.

Tomas-Camardiel M, Herrera AJ, Venero JL, et al. Differential regulation of glutamic acid decarboxylase mRNA and tyrosine hydroxylase mRNA expression in the aged manganese-treated rats. *Brain Res Mol Brain Res*. 2002;103:116–129.

Turchan J, Anderson C, Hauser KF, et al. Estrogen protects against the synergistic toxicity by HIV proteins, methamphetamine and cocaine. *BMC Neurosci*. 2001;2:3–13.

U.S. Department of Health and Human Services, Substance Abuse and Mental Health Services Administration, Office of Applied Studies. *National Household Study on Drug Abuse*. Washington, DC: USDHHS; 2001.

U.S. EPA. *2011 National Listing of Fish Advisories*. EPA-820-F-13-058; December 2013.

Valentine WM, Amarnath V, Amarnath K, et al. Carbon disulfide–mediated protein cross-linking by *N,N*-diethyldithiocarbamate. *Chem Res Toxicol*. 1995;8:96–102.

Valentine WM, Amarnath V, Graham DG, et al. Covalent cross-linking of proteins by carbon disulfide. *Chem Res Toxicol*. 1992;5:254–262.

Valentine WM, Amarnath V, Graham DG, et al. CS_2 mediated cross-linking of erythrocyte spectrin and neurofilament protein: dose response and temporal relationship to the formation of axonal swellings. *Toxicol Appl Pharmacol*. 1997;142:95–105.

Valentine WM, Graham DG, Anthony DC. Covalent cross-linking of erythrocyte spectrin in vivo. *Toxicol Appl Pharmacol*. 1993;121:71–77.

Van de Kamp JL, Collins AC. Prenatal nicotine alters nicotinic receptor development in the mouse brain. *Pharmacol Biochem Behav*. 1994;47:889–900.

Van der Hoek JA, Verberk MM, Hageman G. Criteria for solvent-induced chronic toxic encephalopathy: a systematic review. *Int Arch Occup Environ Health*. 2000;73:362–368.

Veronesi B, Jones K, Gupta S, et al. Myelin basic protein-messenger RNA (MBP-mRNA) expression during triethyltin-induced myelin edema. *Neurotoxicology*. 1991;12:265–276.

Volpe JJ. Effect of cocaine use on the fetus. *N Engl J Med*. 1992;327:399–407.

Wang L, Ho CL, Sun D, et al. Rapid movement of axonal neurofilaments interrupted by prolonged pauses. *Nat Cell Biol*. 2000;2:137–141.

Wang Y, Qin ZH. Molecular and cellular mechanisms of excitotoxic neuronal death. *Apoptosis*. 2010;15:1382–1402.

Watanabe I, Kanabe S. Early edematous lesion of pyrithiamine-induced acute thiamine deficient encephalopathy in the mouse. *J Neuropath Exp Neurol*. 1978;37:401–413.

Weaver IC, Cervoni N, Champagne FA, et al. Epigenetic programming by maternal behavior. *Nat Neurosci*. 2004;7(8):847–854.

Wedler FC. Biological significance of manganese in mammalian systems. *Prog Med Chem*. 1993;30:89–133.

Wijeyesakere SJ, Richardson RJ. Neuropathy target esterase. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. 3rd ed. San Diego, CA: Elsevier; 2010:1435–1478.

Winneke G, Altmann L, Kramer U, et al. Neurobehavioral and neurophysiological observations in six-year-old children with low lead levels in East and West Germany. *Neurotoxicology*. 1994;15:705–713.

Wirbisky SE, Freeman JL. Atrazine exposure elicits copy number alterations in the zebrafish genome. *Comp Biochem Physiol C Toxicol Pharmacol*. 2017;194:1–8.

Wirbisky SE, Weber GJ, Schlotman KE, Sepúlveda MS, Freeman JL. Embryonic atrazine exposure alters zebrafish and human miRNAs associated with angiogenesis, cancer, and neurodevelopment. *Food Chem Toxicol*. 2016;98(pt A):25–33.

Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. *Eur J Epidemiol*. 2011;26(suppl 1):S1–S58.

Wisniewski HM, Bloom BR. Primary demyelination as a nonspecific consequence of a cell-mediated immune reaction. *J Exp Med*. 1975;141:346–359.

Wood SL, Beyer BK, Cappon GD. Species comparison of postnatal CNS development: functional measures. *Birth Defects Res (Part B)*. 2003;68:391–407.

Woods JR, Plessinger MA, Clark KE. Effect of cocaine on uterine blood flow and fetal oxygenation. *JAMA*. 1987;257:957–961.

Wright KN, Hollis F, Duclot F, et al. Methyl supplementation attenuates cocaine-seeking behaviors and cocaine-induced *c*-Fos activation in a DNA methylation-dependent manner. *J Neurosci*. 2015;35(23):8948–8958.

Wright SC, Zhong J, Zheng H, et al. Nicotine inhibition of apoptosis suggests a role in tumor production. *FASEB J*. 1993;7:1045–1051.

Xu M, Guo Y, Vorhees CV, et al. Behavioral responses to cocaine and amphetamine administration in mice lacking the dopamine D1 receptor. *Brain Res*. 2000;852:198–207.

Yamamura Y. *n*-Hexane polyneuropathy. *Folia Psychiatr Neurol*. 1969;23:45–57.

Yegambaram M, Manivannan B, Beach TG, Halden RU. Role of environmental contaminants in the etiology of Alzheimer's disease: a review. *Current Alzheimer Res*. 2015;12:116–146.

Zama AM, Uzumcu M. Fetal and neonatal exposure to the endocrine disruptor methoxychlor causes epigenetic alterations in adult ovarian genes. *Endocrinology*. 2009;150(10):4681–4691.

Zeng X, Chen J, Deng X, et al. An in vitro model of human dopaminergic neurons derived from embryonic stem cells: MPP^+ toxicity and GDNF neuroprotection. *Neuropsychopharmacology*. 2006;31(12):2708–2715.

Zhang J, Graham DG, Montine TS, et al. Enhanced *N*-methyl-4-tetrahydropyridine toxicity in mice deficient in CuZn-superoxide dismutase or glutathione peroxidase. *J Neuropathol Exp Neurol*. 2000;59:53–61.

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chapter 17

Toxic Responses of the Cornea, Retina, and Central Visual System

Donald A. Fox and William K. Boyes

Introduction to Ocular and Visual System Toxicology

Exposure to the Eye and Visual System
- Ocular Pharmacodynamics and Pharmacokinetics
- Ocular Drug Delivery and Engineered Nanoparticles
- Ocular Drug Metabolism and Transport
- Light, UV Radiation, and Photoinduced Toxicity
- Central Visual System Pharmacokinetics

Evaluating Corneal, Retinal, and Central Visual Function
- Evaluation of Corneal Irritancy and Toxicity
- Ophthalmological Evaluations and the Pupillary Light Reflex
- Electrophysiological Techniques
- Behavioral and Psychophysical Techniques
- Color Vision Testing
- Visual Toxicity Screening Procedures

Target Sites and Mechanisms of Action: Cornea
- Acids
- Bases or Alkalies
- Organic Solvents
- Surfactants

Target Sites and Mechanisms of Action: Retina
- Retinotoxicity of Systemically Administered Therapeutic Drugs
  - *Cancer Chemotherapeutics*
  - *Chloroquine and Hydroxychloroquine*
  - *Digoxin and Digitoxin*
  - *Indomethacin*
  - *Erectile Dysfunction (ED) Drugs*
  - *Tamoxifen*
  - *Vigabatrin*
- Retinotoxicity of Known Neurotoxicants
  - *Inorganic Lead*
  - *Methanol*
  - *Organic Solvents*
  - *Organophosphates*

Target Sites and Mechanisms of Action: Mitochondrial Optic Neuropathies (MONs)
- Acrylamide
- Carbon Disulfide
- Cuban Epidemic of Optic Neuropathy
- Ethambutol

Target Sites and Mechanisms of Action: The Central Visual System
- Lead
- Methyl Mercury

Toxicants as Risk Factors for Adult-Onset Retinal Diseases

Acknowledgments

References

INTRODUCTION TO OCULAR AND VISUAL SYSTEM TOXICOLOGY

Environmental and occupational exposure to chemicals, toxicants, gases, and vapors as well as the off target effects of systemic and ocular therapeutic drugs may result in structural and functional alterations to the eye and central visual system (Anger and Johnson, 1985; Grant and Schuman, 1993; Otto and Fox, 1993; Fox, 1998; Santaella and Fraunfelder, 2007; Bartlett and Jaanus, 2008; Fox, 2015; Simunovic, 2016). Almost half of all neurotoxic chemicals affect some aspect of sensory function (Crofton and Sheets, 1989). The most frequently reported sensory system alterations occur in the retina and visual system (Anger and Johnson, 1985; Crofton and Sheets, 1989; Grant and Schuman, 1993; Fox, 1998, 2015). Approximately 3000 substances are toxic to the eye and visual system (Grant and Schuman, 1993). In many cases, alterations in retinal and visual function are the initial symptoms following chemical or toxicant exposure (Damstra, 1978; Hanninen et al., 1978; Baker et al., 1984; Anger and Johnson, 1985; Mergler et al., 1987; Iregren et al., 2002). This suggests that sensory systems, and in particular the retina and central visual system, may be especially vulnerable to toxic insult. Alterations in the structure and/or function of the eye or central visual system are among the criteria utilized for setting permissible occupational or environmental exposure levels for many different chemicals in the United States (http://www.cdc.gov/niosh/npg/; http://www.epa.gov/iris/index.html). New or existing drugs may have visual side effects (Grant and Schuman, 1993; Novack, 2003; Brigell et al., 2005; Santaella and Fraunfelder, 2007). Subtle alterations in visual processing of information (e.g., visual perception, visual motor) can have profound immediate, long-term, and delayed effects on the mental, social, and physical health and performance of an individual. Among the elderly, reduced visual function is a major factor contributing to decreased ability to conduct routine activities of daily living, decreased ability to live independently, and increased risk of falls, car crashes, and other hazards. Ocular and visual system impairments can lead to

Table 17-1

Ocular, Retinal, and Central Visual System Sites of Action of Selected Xenobiotics Following Systemic Exposure

XENOBIOTIC	CORNEA	LENS	RPE	OUTER RETINA: RODS AND CONES	INNER RETINA: BCS, ACS, IPCS	RGCS, OPTIC NERVE OR TRACT	LGN, VISUAL CORTEX
Acrylamide				−	−	++	++
Amiodarone	+	+				+	
Carbon disulfide				+	−	++	+
Chloroquine	+		+	+		+	
Chlorpromazine	+	+	+	+			
Corticosteroids		++				+	
Digoxin and digitoxin	+	+	+	++		+	+
Ethambutol				+		++	
Hexachlorophene				+			++
Indomethacin	+		+	+			
Isotretinoin	+						
Lead	+		+	++	+	+	+
Methanol			+	++	−	++	+
Methyl mercury, mercury			+	−	−	++	+
n-Hexane			+	+		+	
Naphthalene		+		+			
Organic solvents				+			+
Organophosphates		+		+		+	+
Sildenafil	−	−	−	+	+	+	−
Styrene				+			
Tamoxifen	+			+		+	
Vigabatrin	−	−	−	+	+	+	−

RPE, retinal pigment epithelium; BC, bipolar cell; AC, amacrine cell; IPC, interplexiform cell; RGC, retinal ganglion cell; LGN, lateral geniculate nucleus. "+" indicates that this site of action was cited in one or more case reports, review articles, clinical or animal studies. "−" indicates that this site of action showed no adverse effect as cited in one or more case reports, review articles, clinical or animal studies, productivity, and a distinct decrease in the overall quality of life.

increased occupational injuries, loss of productive work time, costs for providing medical and social services, lost productivity, and a distinct decrease in the overall quality of life (Gupta et al., 2007).

The overall goals of this chapter are to provide essential information on ocular pharmacodynamics, pharmacokinetics, and drug metabolism; review the procedures for testing visual function; and evaluate and review the structural and functional alterations in the mammalian eye and central visual system produced by environmental and workplace chemicals, toxicants, gases and vapors, and off-target effects of major therapeutic drugs. The adverse effects of these chemicals on the different compartments of the eye (i.e., cornea, lens, retina, and retinal pigment epithelium [RPE]), central visual pathway (i.e., optic nerve and optic tract), and central processing areas (i.e., lateral geniculate nucleus [LGN] and visual cortex) are addressed (Table 17-1). To further understand the potential effects of these chemicals and drugs on the eye, the distribution of phase I and phase II drug metabolizing enzymes, and xenobiotic uptake and efflux transporters in ocular tissues are presented in Tables 17-2 and 17-3, respectively. Table 17-4 provides examples of common signs, symptoms, and potential pathophysiological mechanisms of visual dysfunction associated with acute or chronic exposure to toxicants and selected drugs. Some of the symptoms are very nonspecific (e.g., "blurred vision") whereas others are more selective.

The ophthalmological evaluation of the eye and the testing of visual function are discussed in this chapter, as the results from clinical, behavioral, and electrophysiological studies form the basis of diagnosis and understanding of adverse visual system effects. Many of the chemicals discussed below initially appear to affect a single site and, by inference, mechanism of action, whereas others have several sites and corresponding mechanisms of action. However, a more in-depth examination reveals that depending upon the dose (concentration) many of these chemicals have multiple sites of action. A few examples illustrate the point. First, as described below in more detail, carbon disulfide produces optic nerve/tract degeneration and also adversely affects the neurons and vasculature of the retina, resulting in photoreceptor and retinal ganglion cell (RGC) structural and functional alterations (Raitta et al.,

Table 17-2

Distribution of Ocular Xenobiotic-Biotransforming Phase I and Phase II Enzymes

	ENZYMES	TEARS	CORNEA	IRIS/CILIARY	LENS	RETINA	CHORIOD
Phase I enzymes	Acetylcholinesterase (AChE)	+		+		+	+
	Alcohol dehydrogenase		+		−	+	+
	Aldehyde dehydrogenase		+		+	+	+
	Aldehyde reductase		+	+	+	+	+
	Aldose reductase		+		+	+	
	Carboxylesterase	+	+	+		+	+
	Catalase	−	+	+	+	+	+
	Cu/Zn superoxide dismutase	+	+	−	±	+	
	MAO-A or MAO-B	+		+		+	+
	CYP1A1 or CYP1A2	+	+	+	−	+	+
	CYP1B1		+	+	+	+	
	CYP2B1 or CYP2B2				+	+	
	CYP2C11				+		
	CYP3A1				+	+	
	CYP4A1 or CYP4B2		+	+		+	
	CYP27A1					+	
Phase II enzymes	Glutathione peroxidase	−	+	+	+	+	+
	Glutathione reductase		+		+	+	
	Glutathione-*S*-transferase		+	+	+	+	
	Sulfotransferases				+	+	
	UDP-glucuronosyl transferases				+	+	
	N-Acetyltransferase		+	+	+	+	+

"+" and "−" indicate that the enzyme was present (localized by immunohistochemistry, immunogold electron microscopy, Western blot, or gene expression) or absent, respectively, in human, monkey, or rodent tissues.

Table 17-3

Distribution of Ocular Xenobiotic Uptake and Efflux Transporters (TPs)

	XENOBIOTIC TRANSPORTER	CORNEA: EPITHELIUM	CORNEA: ENDOTHELIUM	RPE	RODS & CONES	INNER RETINA	NFL
TPs	SLC22A7 (OAT2)	+	−	+	−	+	+
	SLC22A3 (OCT3)	+	+	+	+	−	−
	ABCB1 (MDR1; P-gp)	−	−	−	−	−	−
	ABCC1 (MRP1)	−	−	−	−	−	−
	ABCC4 (MRP4)	+	−	−	−	−	−
	ABCC5 (MRP5)	−	−	−	−	−	−
	ABCG2 (BCRP)	+	−	−	−	+	+

SLC, solute carrier; OAT, organic anion transporter; ABC, ATP binding cassette; MDR1, multidrug resistance protein 1; P-gp, permeability glycoprotein; MRP, multidrug resistance-associated protein efflux pump; BCRP, breast resistance protein; RPE, retinal pigment epithelium; NFL, nerve fiber layer. "+" and "−" indicate that the enzyme was present (localized by immunohistochemistry, Western blot, or gene expression) or absent, respectively, in human tissues.

Table 17-4

Common Signs and Symptoms of Visual System Dysfunction

COMMON SIGNS AND SYMPTOMS	POSSIBLE PATHOPHYSIOLOGICAL BASIS	EXAMPLES OF CHEMICALS PRODUCING THIS EFFECT
Cornea		
Pigment deposits in corneal epithelium (verticillate keratopathy)	Intralysosomal accumulation of lipids	Amphiphilic medications such as amiodarone, chloroquine, clofazimine, phenothiazines, suramin
Lens		
Cataracts anterior cortical (AC), posterior subcapsular (PSC)	Chemical deposition, photochemical oxidation	Long-term systemic use of phenothiazine (AC), corticosteroids (PSC)
Pupil		
Pupil constriction (miosis)	Anticholinesterases	Organophosphate and carbamate insecticides, nerve gas agents such as sarin, soman, and tabun
Pupil dilation (mydriasis) and photophobia	Cholinergic antagonists	Atropine or belladonna
	Adrenergic agonists	Amphetamine, cocaine, phenylephrine
Ocular motility		
Diplopia (double vision), nystagmus	Oculomotor impairment, damage or dysfunction in vestibular/oculomotor reflex pathways	Acute alcohol intoxication, barbiturate toxicity, acute solvent exposure
Retinal pigment epithelium		
Loss of central vision (central scotoma)	Degeneration of retinal pigment epithelium and underlying photoreceptors	Carbon disulfide, chloroquine
Retina		
Poor night (scotopic) vision and impaired dark adaptation	Damage to and apoptosis of rod photoreceptors,	Lead, methyl mercury, vigabatrin
	Acetylcholinesterase inhibitors	Organophosphate and carbamate insecticides, nerve gas agents
Altered color perception, central scotoma	Inhibition of cone photoreceptor sodium-pumps	Digitalis/digitoxin
Altered color perception	Inhibition of cone photoreceptor cGMP-phosphodiesterase	Sildenafil and tadalafil
Impaired color discrimination (blue/yellow)	Damage to cone photoreceptors and inner retina	Chronic exposure to styrene and organic solvents, trimethadione, chronic high dose antibiotics
Impaired color discrimination (red/green)	Acquired damage to cone photoreceptors, neural retina and/or afferent visual pathway	Higher level chronic exposure to organic solvents, carbon disulfide or hexane, chronic carbon monoxide, chronic alcoholism, ethambutol
Loss of peripheral vision (tunnel vision, peripheral scotoma, visual field constriction)	Degeneration of peripheral retina and nerve fiber layer	Methyl mercury, vigabatrin
Reduced contrast sensitivity and visual acuity	Degeneration of the retinal ganglion cells and optic tract, microaneurysms and retinal vasculopathy	Acrylamide, carbon disulfide
Optic nerve and optic tract		
Reduced contrast sensitivity and visual acuity	Optic neuritis and/or degeneration of the optic tract, generally affecting mitochondrial ATP production	Higher level chronic exposure to organic solvents such as carbon disulfide or hexane, ethambutol, ethylene glycol, isoniazid, linezolid and chloramphenicol, methanol, vigabatrin
Monocular and/or binocular visual loss	Nonarteritic anterior ischemic optic neuropathy	Amiodarone, sildenafil, and tadalafil
Lateral geniculate and visual cortex		
Central scotoma	Degeneration of calcarine fissure of visual cortex	Methyl mercury
Visuomotor deficits and reduced contrast sensitivity	Visual and motor cortex dysfunction	Lead, chronic exposure to carbon disulfide, hexane and other solvents, toluene

Inclusion in this table indicates that this drug, chemical, or toxicant was cited in one or more case reports, review articles, clinical or animal studies. The pathophysiological causes and chemicals listed are provided as examples and are not exhaustive.

1974, 1981; Palacz et al., 1980; Seppalainen and Haltia, 1980; De Rouck et al., 1986; Eskin et al., 1988; Merigan et al., 1988; Fox, 2015). Second, gestational and postnatal exposure to inorganic lead affects rod photoreceptors in developing and adult mammals, resulting in rod-mediated (scotopic) vision deficits; however, structural and functional deficits at the level of the RGCs, visual cortex, and oculomotor system also are observed (Otto and Fox, 1993; Fox et al., 1997, 2011; Fox, 1998, 2015; Rothenberg et al., 2002; He et al., 2003; Giddabasappa et al., 2011; Chaney et al., 2016). Although both gestational and postnatal lead exposure produce scotopic flash electroretinographic (ERG) deficits, the amplitude changes are in opposite directions and their underlying mechanisms are distinctly different. Finally, some environmental and occupational neurotoxicants (e.g., acrylamide or lead) have been utilized for in vivo and in vitro animal models to examine the pathogenesis of different retinal, neuronal, or axonal diseases; the basic functions of the retinocortical pathways; and/or the molecular mechanisms of apoptosis (Fox and Sillman, 1979; Vidyasagar, 1981; Lynch et al., 1992; He et al., 2000, 2003).

To write this chapter, information was synthesized and condensed from several classic resources on different aspects of ocular, retinal, and visual system anatomy, biochemistry, cell and molecular biology, histology, pharmacology, physiology, and toxicology (Dayhaw-Barker et al., 1986; Grant and Schuman, 1993; Wässle, 2004; Banh et al., 2005; Albert et al., 2008; Bartlett and Jaanus, 2008; Simunovic, 2010, 2016; Wu, 2010; Levin et al., 2011). The interested reader should consult these sources for more detail than is provided below.

EXPOSURE TO THE EYE AND VISUAL SYSTEM

Ocular Pharmacodynamics and Pharmacokinetics

Toxic chemicals and systemic drugs can affect all parts of the eye (Fig. 17-1; Table 17-1). Several factors determine whether a chemical reaches a particular ocular site of action, including the physiochemical properties of the chemical, concentration and duration of exposure/treatment, route of exposure, and the movement of the chemical into and across the different ocular barriers and compartments. The cornea and external adnexa of the eye, including the conjunctiva (the delicate membranes covering the inner surface of the eyelids and the exposed surface of the sclera) and eyelids are often exposed directly to chemicals (i.e., acids, bases, solvents), gases, particles, and drugs. The first site of action is the tear film: a four-layered structure with both hydrophobic and hydrophilic properties. The outermost tear film layer is a thin (0.1 μm) hydrophobic layer that is secreted by the meibomian (sebaceous) glands. This superficial lipid layer helps stabilize the tear film by retarding evaporation and protects the underlying thicker (7 μm) aqueous layer that is produced by the lacrimal glands. The third layer, which has both hydrophobic and hydrophilic properties, is the very thin (0.02 to 0.05 μm) mucoid layer. It is secreted by the goblet cells of the conjunctiva and acts as an interface between the hydrophilic layer of the tears and the hydrophobic layer of the corneal epithelial cells. The fourth layer, the glycocalyx layer, consists of a meshwork of membrane-bound mucins, which are organic hydrophilic molecules that anchor the mucin to the ocular surface. The aqueous layer is the largest portion of the tear film, and therefore water-soluble chemical compounds more readily mix with the tears and gain access to the cornea.

The cornea, an avascular tissue, is the external barrier to the internal ocular structures. Once a chemical interacts with the tear film and subsequently contacts the cornea and conjunctiva, the majority of what is absorbed locally enters the anterior segment by passing across the cornea. In contrast, a greater systemic absorption and higher blood concentration occurs through contact with the vascularized conjunctiva (Edelhauser, 2006; Fig. 17-2). The human cornea, which is approximately 500-μm thick, has several distinct layers, or barriers, through which a chemical must pass in order to reach the anterior chamber (see discussion on cornea). The first is the corneal epithelium: a stratified squamous, non-keratinized, and multicellular hydrophobic layer. These cells have a relatively low ionic conductance through apical cell membranes, and due to the tight junctions (desmosomes), they have a high-resistance paracellular pathway. The primary barrier to chemical penetration of the cornea is the set of tight junctions at the superficial layer of the corneal epithelial cells. Thus, the permeability of the corneal epithelium as a whole is low and only lipid-soluble chemicals readily pass through this layer. Bowman's membrane separates the epithelium from the stroma. The corneal stroma makes up 90% of the corneal thickness and is composed of water, collagen, and glycosaminoglycans. It contains approximately 200 lamellae, each about 1.5- to 2.0-μm thick. Due to the composition and structure of the stroma, hydrophilic chemicals easily dissolve in this thick layer, which can also act as a reservoir for these chemicals. The inner edge of the corneal stroma is bounded by a thin, limiting basement membrane, called Descemet membrane, which is secreted by the corneal endothelium. The innermost layer of the cornea, the corneal endothelium, is composed of a single layer of large-diameter hexagonal cells connected by terminal bars and surrounded by lipid membranes. The endothelial cells have a relatively low ionic conductance through apical cell surface and a high-resistance paracellular pathway. Although, the permeability of the corneal endothelial cells to ionized chemicals is relatively low, it is still 100 to 200 times more permeable than the corneal epithelium. The sodium-potassium pump is located on the basolateral membrane while the energy-dependent sodium-bicarbonate transporter is located on the apical membrane (Edelhauser, 2006).

There are two separate arterial systems in the human eye (Flammer and Mozaffarieh, 2008; Nickla and Wallman, 2010): (1) uveal arteries, which supply the vascular beds of the iris, ciliary body, and choroid, and (2) retinal arteries, which supply the inner retina. All ocular arteries are derived from the ophthalmic artery, which is a branch of the internal carotid artery. The ophthalmic artery branches into the central retinal artery, which enters the eye with the optic nerve and further branches into four major vessels serving each of the retinal quadrants. The two posterior ciliary arteries (long and short) do not enter with the optic nerve. In the anterior segment of the eye, there is a blood–aqueous barrier that has relatively tight junctions between the endothelial cells of the iris capillaries and nonpigmented cells of the ciliary epithelium. The major function of the ciliary epithelium is the production of aqueous humor from the plasma filtrate present in the stroma of the ciliary processes.

The retina of humans, monkeys, pigs, dogs, rats, and mice has a dual circulatory supply: choroidal and retinal. The outer or distal retina, which consists of the RPE, rod, and cone photoreceptor outer segments (ROS, COS) and inner segments (RIS, CIS), and the photoreceptor outer nuclear layer (ONL), are avascular. These areas of the retina are supplied by the choriocapillaris: a dense, one-layered network of fenestrated vessels formed by the short posterior ciliary arteries and located next to the RPE. Consistent with their known structure, these capillaries have loose endothelial junctions and

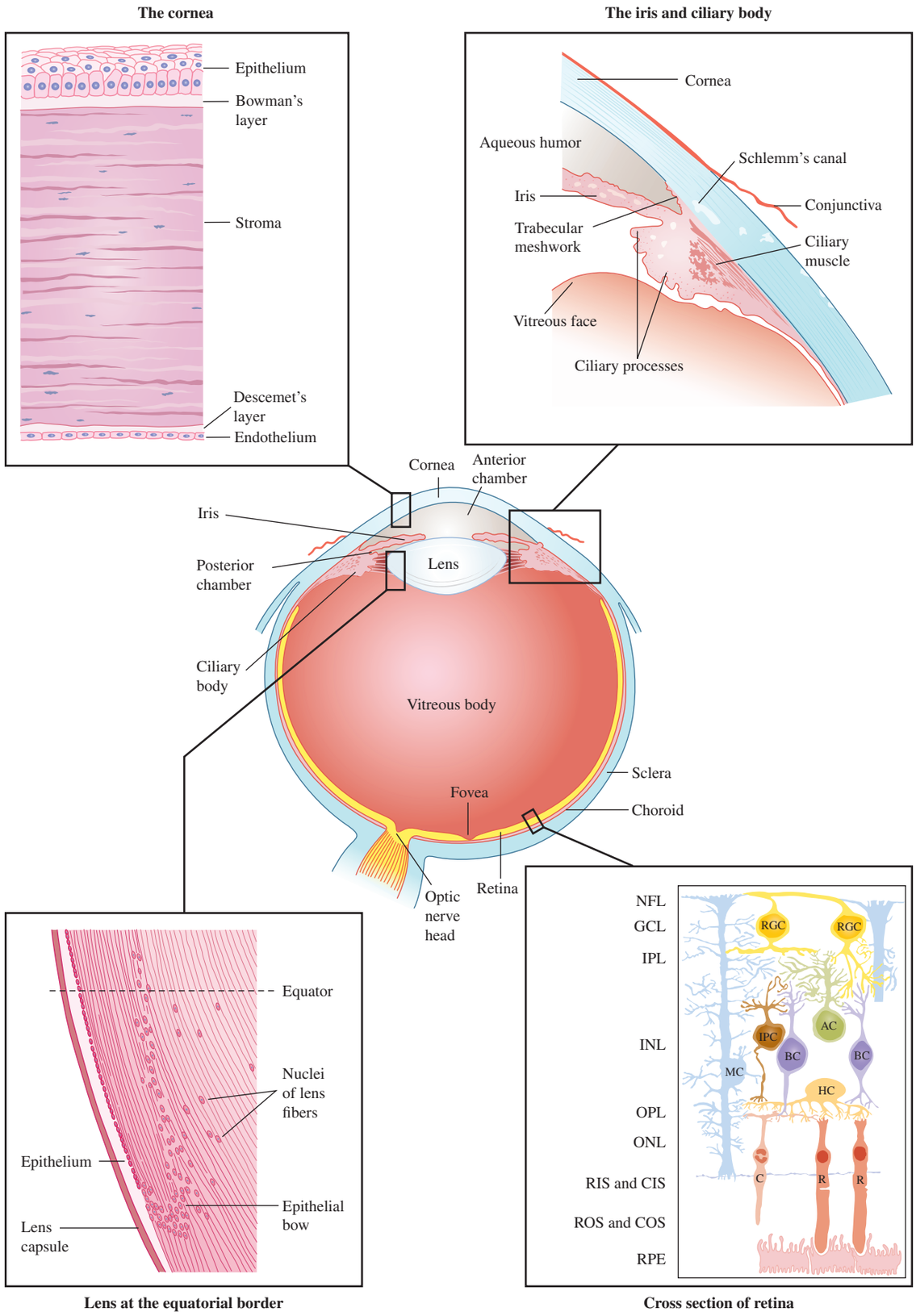

Figure 17-1. *Diagrammatic horizontal cross section of the eye, with medium-power enlargement of details for the cornea, iris and ciliary body, lens, and retina.* The morphological features, their role in ocular pharmacodynamics, pharmacokinetics, drug metabolism, and the adverse effects of drugs and chemicals on these sites are discussed in the text.

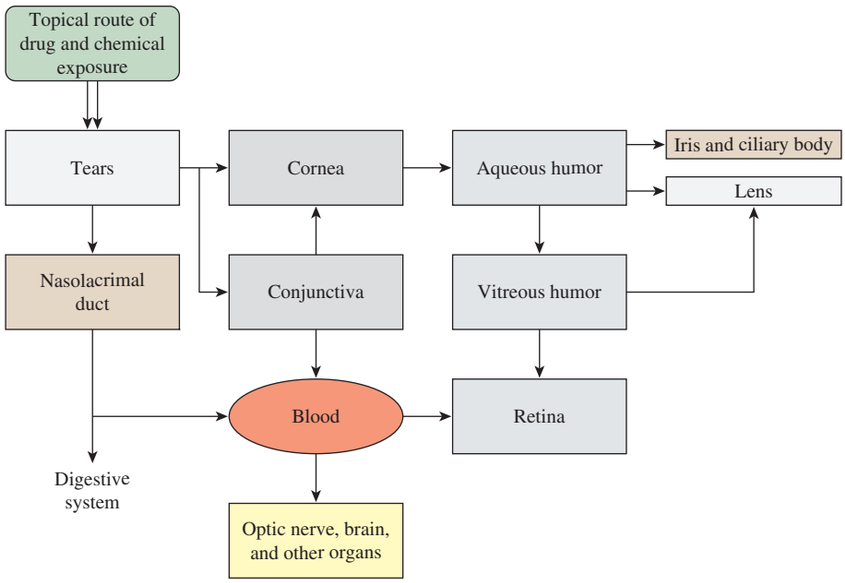

Figure 17-2. *Ocular absorption and distribution of drugs and chemicals following the topical route of exposure.* The details for movement of drugs and chemicals between compartments of the eye and subsequently to the optic nerve, brain, and other organs are discussed in the text.

abundant fenestrae; they are highly permeable to large proteins. The retinal blood vessels are distributed within the inner or proximal portion of the retina, which consists of the outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). The endothelial cells of capillaries of the retinal vessels have tight junctions similar to those that form the blood–brain barrier in the cerebral capillaries. These capillaries form the blood–retinal barrier, and under normal physiological conditions, they are largely impermeable to chemicals such as glucose and amino acids. However, at the level of the optic disc, the blood–retinal barrier lacks these tight-junction types of capillaries and thus hydrophilic molecules can enter the optic nerve head by diffusion from the extravascular space (Flammer and Mozaffarieh, 2008; Nickla and Wallman, 2010) and cause selective damage at this site of action.

Following systemic exposure to drugs and chemicals by the oral, inhalation, dermal, or parenteral route, these compounds are distributed to all parts of the eye by the blood in the uveal blood vessels and retinal vessels (Fig. 17-3). Most of these drugs and chemicals equilibrate rapidly with the extravascular space of the choroid where they are separated from the retina and vitreous body by the retinal pigment epithelium (RPE) and endothelial cells of the retinal capillaries, respectively. Hydrophilic molecules with molecular weights less than 200 to 300 Da can cross the ciliary epithelium and iris capillaries and enter the aqueous humor (Sears, 1984). Thus, the corneal endothelium, the cells responsible for maintaining normal hydration and transparency of the corneal stroma, could be exposed to chemical compounds by the aqueous humor and limbal capillaries. Similarly, the anterior surface of the lens can also be exposed as a result of its contact with the aqueous humor. The most likely retinal target sites following systemic drug and chemical exposure appear to be the RPE and photoreceptors in the distal retina because the endothelial cells of the choriocapillaris are permeable to proteins smaller than 50 to 70 kDa. However, the cells of the RPE are joined on their basolateral surface by tight junctions, zonula occludens, which limit the passive penetration of large molecules into the neural retina (Strauss, 2005; Mecklenburg and Schraemeyer, 2007).

The presence of intraocular melanin plays a special role in ocular toxicology. First, it is found in several different locations in the eye: pigmented cells of the iris, ciliary body, RPE, and uveal tract. Second, it has a high binding affinity for polycyclic aromatic hydrocarbons, electrophiles, calcium, and toxic heavy metals such as aluminum, iron, lead, and mercury (Meier-Ruge, 1972; Potts and Au, 1976; Dräger, 1985; Ulshafer et al., 1990; Eichenbaum and Zheng, 2000). Although this initially may play a protective role, it can also result in the excessive accumulation, long-term storage, and slow release of numerous drugs and chemicals from melanin. For example, atropine binds more avidly to pigmented irides and thus its duration of action is prolonged (Bartlett and Jaanus, 2008). In addition, lead accumulates in the human retina such that its concentration is 5 to 750 times higher than that in other ocular tissues (Eichenbaum and Zheng, 2000).

Ocular Drug Delivery and Engineered Nanoparticles

Ocular drug delivery and targeting create significant challenges and obstacles for most drugs that must enter the eye and reach their site of action. The main ocular target sites of importance for disease treatment and neuroprotection are the anterior segment, retinal neurons (i.e., photoreceptors, retinal ganglion cells: RGCs), retinal vasculature, and RPE. As noted above, there are numerous superficial barriers, blood–retina barriers, transporters, and depot sites that restrict bioavailability, decrease therapeutic efficacy, and increase side effects. Two new approaches involve the development of nanoscale suspensions for topical drug delivery and intravitreal injections of different types/categories of nanoparticles as drug carriers. The goal of nanoscale formulations is to enhance penetration from the cornea, deliver a wide variety of drugs and molecules, and increase the concentration and contact time of drugs with these tissues (Diebold and Calonge, 2010; Bisht et al., 2017). A wide variety of new intravitreal nanoparticles as drug carriers have been developed including chitosan-coated, compacted DNA, drug-coated, lipid-based, liposome, magnetic, unimolecular

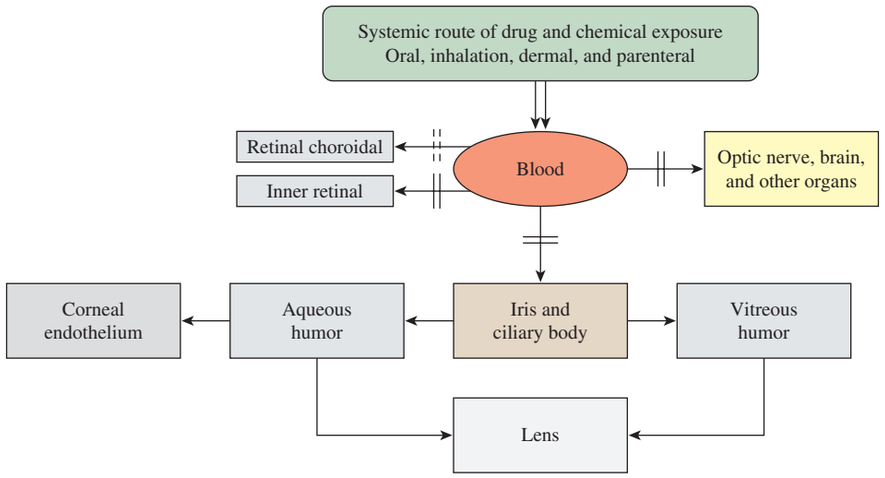

Figure 17-3. *Distribution of drugs and chemicals in the anterior and posterior segments of the eye, optic nerve, brain, and other organs following the systemic route of exposure.* The details for movement of drugs and chemicals between compartments of the eye are discussed in the text. The solid and dotted double lines represent the different blood–tissue barriers present in the anterior segment of the eye, retina, optic nerve, and brain. The solid double lines represent tight endothelial junctions, whereas the dotted double lines represent loose endothelial junctions.

micelles, etc. (Diebold and Calonge, 2010; Nagpal et al., 2010; Seyfoddin et al., 2010; Adijanto and Naash, 2015; Battaglia et al., 2016; Bisht et al., 2017; Giannaccini et al., 2017; Zhao et al., 2017). These nanoparticles target various retinal cells (e.g., photoreceptors, RGCs) and the retinal vasculature. Magnetic nanoparticles can target drug delivery to the choroidal layer (Giannaccini et al., 2017). All of these vehicles for topical and intravitreal ocular drug delivery need to have low toxicity to ocular tissues (Prow, 2010; Jo et al., 2011; Penha et al., 2011). In addition, among the thousands of materials being developed and incorporated into hundreds of consumer products, little attention has focused on the potential of engineered nanomaterials for ocular and neurotoxicity (Boyes et al., 2012; Migliore et al., 2015) other than in vitro assessments of potential phototoxicity (vide infra). In addition, nanomaterials are readily transformed in environmental or biological media, making it difficult to know the final active form of nano-enabled ocular drugs, or engineered nanomaterials developed for other purposes that might inadvertently come into contact with ocular tissues (Boyes et al., 2017).

Ocular Drug Metabolism and Transport

Metabolism and excretion of xenobiotics occurs in all compartments of the eye by well-known phase I and II xenobiotic-biotransforming enzymes, and the less well-studied drug transporters in ocular tissues. Drug metabolizing enzymes (see Chap. 6) such as acetylcholinesterase, carboxylesterase (also known as pseudocholinesterase), alcohol and aldehyde dehydrogenase, aldehyde and aldose reductase, catalase, monoamine oxidase A and/or B, and Cu^{2+}/Zn^{2+} superoxide dismutase as well as several types of proteases are present in the tears, iris–ciliary body, choroid, and retina of many different species (Shanthaverrappa and Bourne, 1964; Waltman and Sears, 1964; Bausher, 1976; Atalla et al., 1988; Crouch et al., 1991; Watkins and Wirthwein, 1991; Gondhowiardjo and van Haeringen, 1993; Downes and Holmes, 1995; Gaudet et al., 1995; Behndig et al., 1998; King et al., 1999; Nakamura et al., 2005; Choudhary et al., 2006; Fox et al., 2011; Table 17-2).

Cytochrome P450 (CYP) is a superfamily of hemoproteins involved with xenobiotic and endogenous metabolism. There are age-, gender-, and ocular region-specific differences in CYP expression in developing and adult human and rodent eyes (Nakamura et al., 2005; Choudhary et al., 2006; Doshi et al., 2006; Lee et al., 2006; Nakano et al., 2014). Mutations or alterations in CYP expression (e.g., CYP1B1) during development can produce ocular diseases and defects such as primary congenital glaucoma and corneal opacity (Choudhary et al., 2006; Nakano et al., 2014). Numerous other CYP family members are located in the eye. For example, CYP1A1 and CYP1A2 are found in all bovine and mouse ocular tissues except the lens and can be induced by 3-methylcholanthrene and β-naphthoflavone (Shichi and Nebert, 1982; Zhao and Shichi, 1995). Moreover, CYP4A1 in mouse and CYP4B1 in rabbit are present in corneal epithelium and can be induced by phenobarbital and the peroxisome proliferator clofibrate (Shichi, 1996; Zhao et al., 1996; Mastyugin et al., 1999). This corneal epithelial CYP monooxygenase metabolizes arachidonic acid to two of its major metabolites: 12(*R*)-HETE [12(*R*)-hydroxy-5,8,10,14-eicosatrienoic acid] and 12(*R*)-HETrE [12(*R*)-hydroxy-5,8,14-eicosatrienoic acid] (Asakura et al., 1994; Schwartzman, 1997; Mastyugin et al., 1999). In the corneal epithelium, 12(*R*)-HETE is a potent inhibitor of Na^+,K^+-ATPase, whereas 12(*R*)-HETrE is a potent angiogenic and chemotactic factor (Schwartzman, 1997).

The phase II conjugating enzymes found in bovine, rabbit, and rat ocular tissues include UDP glucuronosyltransferase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase, and *N*-acetyltransferase (Awasthi et al., 1980; Shichi and Nebert, 1982; Penn et al., 1987; Watkins et al., 1991; Srivastava et al., 1996; Singh and Shichi, 1998; Nakamura et al., 2005; see Table 17-2). Although the activity of these enzymes varies between species and ocular tissues, the whole lens appears to have low biotransformational activity. Metabolically, the lens is a heterogeneous tissue, with glutathione-*S*-transferase activity found in the lens epithelium and not in the lens cortex or nucleus (Srivastava et al., 1996). Overall, these findings suggest that ocular tissues that contact the external environment have a blood supply possessing both CYPs and phase II conjugating enzymes, especially those enzymes related to glutathione conjugation. The presence and need for a competent glutathione conjugation system is clearly understandable in ocular tissues that directly interact with UV radiation, light, and xenobiotics. Further work is needed to determine the presence and activity of other CYP family members in ocular tissue, the factors (i.e., age,

gender, tissue-specific, xenobiotics) that regulate their expression, and their endogenous and exogenous substrates.

Drug uptake and excretion (efflux) transport in ocular tissues has received increasing attention (Dahlin et al., 2013; Nakano et al., 2014). Gene and protein expression studies have identified several ATP binding cassette (ABC) families of transporters, solute carrier (SLC) transporters, and organic-anion and cation transporters (OATs; OCTs): especially in the cornea, retina, and blood–retinal barriers (Dahlin et al., 2013; Nakano et al., 2014). Their cellular and subcellular roles in normal homeostatic function, chemical/drug/toxicant exposures, diseases, and pathophysiological responses in the retina and other tissues are active and important areas of investigation (see Table 17-3).

Light, UV Radiation, and Photoinduced Toxicity

The most important oxidizing agents are visible light and UV radiation, particularly UV-A (320 to 400 nm) and UV-B (290 to 320 nm). Light- and UV-induced photo-oxidation can generate reactive oxygen species that produce oxidative damage over time. Higher-energy UV-C (100 to 290 nm) is even more damaging. Fortunately, at sea level the atmosphere filters out virtually all UV-C and all but a small fraction of UV-B derived from solar radiance (AMA, 1989). The cornea absorbs about 45% of radiation below 280 nm, but only about 12% between 320 and 400 nm. The lens absorbs much of the light between 300 and 400 nm and transmits 400 nm and above to the retina (Banh et al., 2005). Absorption of light energy in the lens can trigger a variety of photoreactions, including the generation of fluorophores and pigments that lead to the yellow–brown coloration of the lens. Sufficient exposure to infrared radiation, as occurs to glassblowers, or microwave radiation can produce cataracts through direct heating of the ocular tissues.

Drugs, diagnostic dyes, dietary supplements, nanoparticles, and other chemicals can serve as mediators of photo-induced toxicity in the cornea, lens, or retina (Dayhaw-Barker et al., 1986; Roberts, 2001, 2002; Glickman, 2002; Siu et al., 2008; Zhao et al., 2010; Wielgus and Roberts, 2012). This occurs when the chemical structure allows absorption of light energy in the UV or visible spectrum and the subsequent generation of activated intermediates, free radicals and reactive oxygen species. Chemical structures likely to participate in such phototoxic mechanisms have tricyclic, heterocyclic, or porphyrin ring structures because, with light, they produce stable triplet reactive molecules leading to free radicals and reactive oxygen species. The propensity of chemicals to cause phototoxic reactions can be predicted using photophysical and in vitro procedures (Roberts, 2001; Glickman, 2002; Wielgus and Roberts, 2012). Recently, high efficiency light–emitting diode (LED) lighting, rich in the more phototoxic blue wavelengths of the spectrum, has come under scrutiny for potential contributions to retinal phototoxicity. This includes both LEDs used for interior lighting (Krigel et al., 2016) and also for frequent users of smart phones (Lin et al., 2017).

The phototoxic properties of chemicals are being exploited for photodynamic therapies where photoactive chemicals are delivered to pathological tissues such as cancerous tumors or inappropriate angiogenic vessels in age-related macular degeneration. Wavelength-specific light is introduced to the tissue causing the photoactive chemical to activate thereby initiating a free-radical cascade that kills the pathological tissues. These chemicals also are being developed to utilize long wavelengths near the red/infrared end of the spectrum where the irradiation penetrates deeper into the tissue. A high-throughput screening assay can rapidly screen candidate chemicals for phototoxicity (Butler et al., 2010). However, photoactive chemicals that are not intentionally introduced for photodynamic therapy also can generate phototoxic reactions. Examples, which exhibit in vitro ocular phototoxicity, are fullerene or carbon allotropes (Roberts et al., 2008), the antibiotic ciprofloxacin (Zhao et al., 2010), and titanium dioxide nanomaterials (Sanders et al., 2012).

Central Visual System Pharmacokinetics

The penetration of chemicals into visual areas of the central nervous system (CNS) is governed, like other parts of the CNS, by the blood–brain barrier (Fig. 17-3). The blood–brain barrier is formed through a combination of tight junctions in brain capillary endothelial cells and foot processes of astrocytic glial cells that surround the brain capillaries. Together these structures serve to limit the penetration of blood-borne compounds into the brain and in some cases actively exclude compounds from brain tissue. The concept of an absolute barrier is not correct, however, because the blood–brain barrier is differentially permeable to compounds depending on their size, charge, and lipophilicity. Compounds that are large, highly charged, or otherwise not very lipid soluble tend to be excluded from the brain, whereas smaller, uncharged, and lipid-soluble compounds more readily penetrate into the brain tissue. In some cases, toxic compounds may be actively transported into the brain by mimicking the natural substrates of active transport systems. A few areas of the brain lack a blood–brain barrier, consequently some blood-borne compounds can penetrate into these regions.

EVALUATING CORNEAL, RETINAL, AND CENTRAL VISUAL FUNCTION

Testing for potential toxic effects of compounds on the cornea, retina, and central visual system can be divided into tests of corneal toxicity and tests of retinal/visual function. Such a categorization might include tests of corneal irritancy/toxicity, ophthalmological evaluations, electrophysiological studies of retinal/visual system function, behavioral and/or psychophysical evaluations of visual thresholds and aspects of perception, color vision, and visual screening for toxicity.

Evaluation of Corneal Irritancy and Toxicity

Topical injury to the eye, especially the cornea, can result from contact with household and workplace products and chemicals. Accidents involving common household products produce over 100,000 eye injuries per year (McGwin et al., 2006; Peate, 2007). Regulatory agencies require ocular irritancy testing to determine whether chemicals and/or products can produce eye damage as well as the type and degree of damage. Based on this information, chemicals are classified as ocular hazard and appropriately labeled. Since 1944, the standard procedures for evaluating ocular irritation were based on the Draize method (Draize et al., 1944). Over this time, the Draize test with some additions and revisions formed the basis of safety evaluations in data submitted to several government regulatory bodies including the European Economic Community and several federal agencies within the United States. Traditionally, albino rabbits were the subjects evaluated in the Draize test, although the Environmental Protection Agency (EPA) protocol allows different test species to be used if sufficient justification is provided. The standard procedure involves instillation of 0.1 mL of a liquid or 100 mg of a solid into the conjunctival sac of

one eye and then gently holding the eye closed for 1 second. The untreated eye serves as a control. Both eyes are evaluated at 1, 24, 48, and 72 hours after treatment. If there is evidence of damage in the treated eye at 72 hours, the examination time may be extended. The cornea, iris, and conjunctiva are evaluated and scored according to a weighted scale. The cornea is scored for both the degree of opacity and the area of involvement, with each measure having a potential range from 0 (none) to 4 (most severe). The iris receives a single score (0 to 2) for irritation, including degree of swelling, congestion, and degree of reaction to light. The conjunctiva is scored for the redness (0 to 3), chemosis (swelling 0 to 4), and discharge (0 to 3). The individual scores are then multiplied by a weighting factor: five for the cornea, two for the iris, and five for the conjunctiva. The results are summed for a maximum total score of 110. Photographic examples of lesions receiving each score are provided in Datson and Freeberg (1991). In this scale, the cornea accounts for 80 (73%) of the total possible points, in accordance with the severity associated with corneal injury.

The Draize test has been criticized due to its high interlaboratory variability, subjective nature of scoring, poor predictive value for human irritants, and most significantly for causing undue pain and distress to the tested animals. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), a committee representing 14 agencies of the U.S. Federal Government, has evaluated alternative testing procedures to assess the potential for chemicals to cause ocular corrosivity and irritation. ICCVAM currently lists seven such alternative testing procedures that have been accepted by U.S. Regulatory Agencies, adopted by Organization for Economic Co-operation and Development (OECD) and endorsed by the European Union (ICCVAM, 2017); see the full report at https://ntp.niehs.nih.gov/iccvam/docs/ocutox_docs/oteval/otevalrpt.pdf.

Ophthalmological Evaluations and the Pupillary Light Reflex

There are many ophthalmological procedures for evaluating the health of the eye. The procedures available range from routine clinical screening evaluations to sophisticated imaging techniques for targeted purposes: the latter are beyond the scope of this chapter. A clinical evaluation of the eye examines the adnexa, and the anterior and posterior structures in the eye. Examination of the adnexa includes evaluating the eyelids, lacrimal apparatus, and palpebral (covering the eyelid) and bulbar (covering the eye) conjunctiva. The anterior structures or anterior segment include the cornea, iris, lens, and anterior chamber. The posterior structures include the retina, retinal vasculature, choroid, optic nerve, sclera, and retinal pigment epithelium (RPE). These and other ocular examinations are described in several publications (Harroff, 1991; Peiffer et al., 2000; Albert et al., 2008).

An ophthalmological examination may also involve a standard or automated (i.e., infrared pupillography) examination of the pupillary light reflex (PLR). The direct PLR involves shining a bright light into the eye and observing the reflexive pupil constriction in the same eye. The consensual PLR is observed in the eye not stimulated. Both the direct and consensual PLRs are dependent on function of a reflex arc involving cells in the retina, which travel through the optic nerve, optic chiasm, and optic tract to project to neurons in the pretectal area. Pretectal neurons project to ipsilateral (for the direct reflex) and contralateral (for the consensual reflex) parasympathetic neurons of the midbrain accessory oculomotor (Edinger–Westphal) nucleus. Preganglionic neurons from the Edinger–Westphal nucleus project through the oculomotor nerve to the ciliary ganglion. Postganglionic neurons from the ciliary ganglion then innervate the smooth muscle fibers of the iridal pupillary sphincter. Rod and cone photoreceptors, and the intrinsically photosensitive retinal ganglion cells (ipRGCs) differentially contribute to the PLR (McDougal and Gamlin, 2010). The absence of a PLR is indicative of damage somewhere in the reflex pathway, and differential impairment of the direct or consensual reflexes can indicate the location of the lesion (Kawasaki, 1999; Muppidi et al., 2013). Drugs (e.g., stimulants, sedatives, depressants) can change the speed and degree of the PLR (Hou et al., 2006; Lindauer et al., 2008; Monticelli et al., 2010). The presence of a PLR, however, is not synonymous with normal visual function. PLRs can be maintained even with significant damage to the retina or optic nerve. In addition, lesions in visual areas outside of the reflex pathway, such as in the visual cortex, may also leave the reflex function intact. Nevertheless, examining the pupillary reflex can be useful, such as in demonstrating effects of mercury vapor exposure on the autonomic nervous system (Milioni et al., 2017).

Electrophysiological Techniques

Many electrophysiological or neurophysiological procedures are available for non-invasively and objectively testing visual function in a toxicological context. In a simple sense, most of these procedures involve stimulating the eyes with visual stimuli and electrically recording potentials generated by visually responsive cells or neurons. Different techniques and stimuli are used to selectively study the function of different/specific retinal or visual cortical neurons. In drug and toxicology studies that assess retinal and visual function, the most commonly used procedures are the electroretinograms (ERGs: flash, pattern or PERG, multifocal or mfERG) and visual-evoked potentials (VEPs). These procedures have numerous variations of stimulation and recording protocols depending on the specific aspects of visual system being evaluated.

ERGs are typically elicited with a brief flash of light and recorded from an electrode placed in contact with the cornea. A typical ERG waveform (Fox and Farber, 1988; Rosolen et al., 2005; Heckenlively and Arden, 2006) includes an initial negative-going waveform, called the a-wave, that reflects the activation of photoreceptors, and a following positive b-wave that reflects the activity of retinal ON-bipolar cells (Abd-El-Barr et al., 2009). In addition, a series of oscillatory potentials can be observed overriding the b-wave, of which the neural generators are somewhat uncertain, but they presumably reflect various stages of intraretinal signal processing. A standard set of ERG procedures has been recommended for screening assessments of human clinical patients (Heckenlively and Arden, 2006). These procedures include the recording of (1) a response reflective of only rod photoreceptor function in the dark-adapted eye, (2) the maximal response in the dark-adapted eye, (3) a response developed by cone photoreceptors, (4) oscillatory potentials, and (5) the response to rapidly flickered light. These recommendations were used to create a protocol for screening the retinal function of dogs in toxicological studies (Jones et al., 1994). For testing retinal function beyond a screening level evaluation, ERG amplitude and latency versus log stimulus intensity functions are very useful (Fox and Farber, 1988; Rosolen et al., 2005; Heckenlively and Arden, 2006). Although flash-evoked ERGs do not reflect the function of the retinal ganglion cell (RGC) layer, PERGs elicited with pattern-reversal stimuli reflect the activation of RGCs (Heckenlively and Arden, 2006). The mfERG enables simultaneous stimulation of multiple retinal areas and recording of each response independently. This significantly improves the usefulness of the ERG as it provides a topographic measure of retinal activity (Heckenlively and Arden, 2006; Dettoraki and Moschos, 2016). In

addition, mfERGs may be a more sensitive indicator of early retinal dysfunction than visual acuity, color vision, visual field analysis, and/or dark adaptation. Furthermore, they can be correlated with optical coherence tomography (OCT) results and thereby increase the localization of the retinal dysfunction.

Visual evoked potentials (VEPs) are elicited with stimuli similar to those used to evoke ERGs. However, VEPs are recorded from electrodes overlying the visual (striate) cortex. Consequently, VEPs reflect the activity of the retinogeniculostriate pathway and the activity of cells in the visual cortex. Flash-elicited VEPs have been used in a number of studies of potentially neurotoxic compounds in laboratory animals (Fox et al., 1977; Herr and Boyes, 1995). Pattern-elicited VEPs are more widely used in human clinical evaluations because of their diagnostic value. Long–Evans pigmented rats, but not albino rats, produce usable pattern VEPs (Boyes and Dyer, 1983; Boyes et al., 2016). While recording pattern VEPs and conducting psychophysical studies with pigmented Long–Evans hooded rats, Fox (1984) found that the spatial frequency functions yielded almost the same visual acuity values (1.4 cycles per degree) as the psychophysically determined spatial resolution limit values (1.8 cycles per degree). These values are in good agreement with those obtained by others using single-cell electrophysiological and behavioral techniques (Birch and Jacobs, 1979). Moreover, pattern and flash-elicited VEPs have exhibited differential sensitivity to some neurotoxic chemicals (Boyes and Dyer, 1984; Fox, 1984). The US EPA published guidelines for conducting VEP testing in a toxicological context, along with analogous sensory-evoked potential procedures for evaluating auditory and somatosensory function (EPA, 1998a).

Behavioral and Psychophysical Techniques

Behavioral and psychophysical testing procedures typically vary the parameters of the visual stimulus and then determine whether the subject can discriminate or perceive the stimulus (Woodhouse and Barlow, 1982; Maurissen, 1995). Many facets of visual function in humans and laboratory animals are studied using these procedures. Often, the goal is to resolve the spatial or temporal limits of visual discrimination; however, most visual scenes and targets in our daily life involve discrimination of objects with low to middle spatial and temporal frequencies (Woodhouse and Barlow, 1982). Contrast sensitivity functions are used to assess these parameters. In addition, as discussed below, other visual parameters also have been investigated using psychophysical procedures.

Contrast sensitivity refers to the ability to resolve small differences in luminance contrast, such as the difference between subtle shades of gray. Contrast sensitivity should be measured for a series of visual patterns that differ in pattern size. Typically, such patterns are a series of sine-wave gratings (striped patterns where the luminance changes across the pattern in a sinusoidal manner) where the spatial frequency of the pattern (i.e., the width of the bars measured in cycles/degree) varies in octave steps. The patterns vary in contrast across the image (i.e., the difference between brightness and darkness of the bars). The resulting data, when plotted on log/log coordinates, forms a contrast sensitivity function that is representative of the ability to detect visual patterns over the range of visible pattern sizes. Contrast sensitivity functions are dependent primarily on the neural as opposed to the optical properties of the visual system. The contrast sensitivity functions generally form an inverted U-shaped profile with highest sensitivity to contrast at intermediate spatial frequencies. Sensitivity to high spatial frequencies is equivalent to a measure of visual acuity, whereas sensitivity to mid-range spatial frequencies is important for facial recognition (Ginsburg, 2003). The peak of the function as well as the limits of resolution on both the spatial frequency and contrast axes varies across species (Birch and Jacobs, 1979; Fox, 1984).

Some visual parameters that have been investigated include (1) the absolute luminance threshold, which is the threshold value for detecting an illuminated target by a dark-adapted subject in a dark-adapted environment; (2) visual acuity, which is the spatial resolution of the visual system: approximately 50 cycles per degree in humans (Woodhouse and Barlow, 1982) and 1.1 to 1.8 cycles per degree in albino and hooded rats (Birch and Jacobs, 1979; Dean, 1981; Fox, 1984); (3) color and spectral discriminations (Simunovic, 2010, 2016); (4) critical flicker fusion frequency, which is the threshold value for detecting a flickering light at different luminance intensities; and (5) the peak spatial and temporal contrast sensitivity functions at different luminance levels (Woodhouse and Barlow, 1982). Most of these tests are dependent upon the quality of the ocular optics and the ability to obtain a sharply focused visual image on the retina. Thresholds for detecting luminance, contrast, flicker, and color are primarily dependent on retinal and central mechanisms of neural function, although optical impairments (e.g., cataracts) interfere with these functions. The assessment of visual acuity and contrast sensitivity has been recommended for field studies of humans potentially exposed to neurotoxic substances (Agency for Toxic Substances and Disease Registry [ATSDR], 1992).

Color Vision Testing

Color vision deficits are either inherited or acquired. Hereditary red–green color deficits occur in about 8% of males (X-linked) whereas only about 0.5% of females show similar congenital deficits (Simunovic, 2010, 2016). Inherited color deficiencies generally occur in two common forms: protanopia, a red–green confusion caused by abnormality or absence of the long-wavelength (red) sensitive cones (L-type cones); and deuteranopia caused by abnormality or absence of the middle-wavelength sensitive (green) cones (M-type cones). Deuteranopes demonstrate a concomitant confusion of red–green and blue–yellow due to the lack of M-type cones. Congenital loss of short-wavelength cones, resulting in a blue–yellow confusion (tritanopia, or type III), is extremely rare. Most acquired color vision deficits, such as those caused by drug and chemical exposure, begin with a reduced ability to perform blue–yellow discriminations (Bartlett and Jaanus, 2008; Simunovic, 2010, 2016). With increased or prolonged low-level exposure, the color confusion can progress to the red–green axis as well. Because of the rarity of inherited tritanopia, it is generally assumed that blue–yellow deficits, when observed, are acquired deficits. Bilateral lesions in the area V4 of visual cortex can also lead to color blindness (prosopagnosia).

Color vision has been evaluated in multiple occupationally and environmentally exposed populations (Iregren et al., 2002). Color vision may be evaluated using several different testing procedures. Commonly used procedures in human toxicological evaluations include the Ishihara color plates and chip arrangement tests such as the Farnsworth-Munson 100 Hue (FM-100) test and the simplified 15-chip tests using either the saturated hues of the Farnsworth D-15 or the desaturated hues of the Lanthony Desaturated Panel D-15 (Iregren et al., 2002; Simunovic, 2016). The Ishihara plates involve a series of colored spots arranged in patterns that take advantage of perceived difference in shades resulting from congenital protan or deutan anomalies. Normal observers perceive different sets of embedded numbers than those with color vision deficits. The Farnsworth-Munson procedure involves arrangement of 85 chips in

order of progressively changing color. The relative chromatic value of successive chips induces those with color perception deficits to abnormally arrange the chips. The pattern is indicative of the nature of the color perception anomaly. The FM-100 is considered more diagnostically reliable but takes considerably longer to administer than the similar but more efficient Farnsworth and Lanthony tests. The desaturated hues of the Lanthony D-15 are designed to better identify subtle acquired color vision deficits. For this reason, and because it requires only a few minutes to administer, the Lanthony D-15 was recommended as the procedure for color vision screening of potentially occupationally and environmentally exposed populations (ATSDR, 1992). A critical review of this procedure, its test–retest reliability, and its use in toxicological applications should be consulted prior to its use (Geller and Hudnell, 1997; Good et al., 2005).

Visual Toxicity Screening Procedures

As noted above, there is a clear need for retinal and visual function testing as a component of the toxicological evaluation of environmental and occupational chemicals. The extent and magnitude of potential retinal and central visual system alterations in humans and experimental animals exposed to most drugs and toxicants is not known because this has not been systematically evaluated. The EPA/OECD screening batteries for neurotoxicological evaluation of laboratory animals only include minimal evaluations of visual function: a functional observational battery (FOB), an automated measurement of motor activity and neuropathology (EPA, 1998b). Only the FOB evaluates visual function and the extent of these measures is very limited. The entire assessment of visual function includes observing the animal's response to an approaching object such as a pencil and observing the pupil's response to a light (vide supra). These procedures are limited as they do not explore responsiveness over a range of stimulus features such as luminance, color, contrast, spatial frequency, and/or temporal frequency. In addition, they do not evaluate rod or cone sensory thresholds or maximal responses, nor do they isolate potential motor or integrative contributions to task performance. Despite these shortcomings, the FOB has been successful at detecting visual deficits produced by exposure to 3,3-iminodipropionitrile (Moser and Boyes, 1993) or carbon disulfide (Moser et al., 1998), which illustrates the importance of conducting at least this level of visual function screening in routine testing programs. Some studies include ophthalmological and/or ocular pathological evaluations. Comprehensive visual toxicity studies should include ophthalmological, histological, and pathological evaluation of ocular tissues as well as assessments of simple and complex visual function.

Another potentially limiting problem in product safety testing is the routine use of albino animals, whose capacity for visual function is limited at best (vide supra). The toxicity of many polycyclic aromatic compounds is mediated through interactions with melanin (Potts, 1964; Meier-Ruge, 1972), which is absent in the eyes of albino strains. In addition, light-related ocular lesions, including cataracts and retinal degeneration, are often observed in control albino rats and mice used in 2-year product-safety evaluations as the retinas of these animals are particularly susceptible to light-induced damage (Rapp et al., 1990). For example, one study showed that the proportion of Fisher 344 rats in the control group with photoreceptor lesions ranged from less than 10% of rats housed on the bottom row of the cage racks to over 55% of rats housed on the top row, where the luminance was greater (Rao, 1991). Even under reduced light levels, the incidence of these effects was as high as 15%. If albino animals are used as the test subjects, it is important to control the overall level of cage illumination to less than 100 lux and also to periodically rotate the animals among the rows of the cage racks. Even then, it is difficult to interpret pathological changes in albino rats and mice exposed to test compounds against such high rates of background retinal lesions.

Sensory dysfunction can confound other measures of neurotoxicity. Many behavioral and observational evaluations of neurotoxicity involve presentation of sensory stimuli to human or animal subjects followed by the observation or measurement of a behavioral or motor response. In many cases, the inferences drawn from such measures are stated in terms of the cognitive abilities of the test subject, such as whether learning or memory have been compromised as a function of exposure to the test compound. If the subject was unable to clearly and precisely perceive the test stimuli, which are often complex patterns or contain color, task performance may be affected independently of any effect on cognition. Controlling for visual deficits may alter the interpretation of performance or cognitive tasks (Anger et al., 1994; Hudnell et al., 1996; Walkowiak et al., 1998; Cestnick and Coltheart, 1999).

TARGET SITES AND MECHANISMS OF ACTION: CORNEA

The cornea is the anterior covering of the eye and as such must provide three essential functions. First, it must provide a clear refractive surface. The air-to-fluid/tissue interface at the cornea is the principal refractive surface of the eye, providing approximately 48 diopters of refraction. The curvature of the cornea must be correct for the visual image to be focused at the retina. Second, the cornea provides tensile strength to maintain the appropriate shape of the globe. Third, the cornea protects the eye from external factors, including potentially toxic chemicals. The anatomy is reviewed in the discussion of the section "Ocular Pharmacodynamics and Pharmacokinetics" above.

The cornea is transparent to wavelengths of light ranging between 310 nm (UV) and 2500 nm (IR) in wavelengths. Exposure to UV light below this range can damage the cornea. It is most sensitive to wavelengths of approximately 270 nm. Excessive UV exposure leads to photokeratitis and corneal pathology, the classic example being welder's arc burns.

The corneal epithelial cells can be damaged by topical or systemic exposure to drugs and chemicals (Table 17-1). Direct chemical exposure to the eye requires emergency medical attention. Acid and alkali chemicals that come into contact with the cornea can be extremely destructive. Products at pH extremes ≤ 2.5 or ≥ 11.5 are considered as extreme ocular irritants. They can cause severe ocular damage and permanent loss of vision. Damage that extends to the corneal endothelium is associated with poor repair and recovery. The most important therapy is immediate and adequate irrigation with large amounts of water or saline, whichever is most readily available. The extent of damage to the eye and the ability to achieve a full recovery are dependent upon the nature of the chemical, the concentration and duration of exposure, and the speed and magnitude of the initial irrigation. The American Academy of Ophthalmology classifies chemical injury to the cornea into four grades of severity as well as treatment for each: see online *EyeNet Magazine* (https://www.aao.org/eyenet/article/treating-acute-chemical-injuries-of-cornea).

Several drugs cause keratopathies. A list of the drugs, their symptoms, adverse reactions, and reversibility upon cessation of exposure have been described (Diamante and Fraunfelder, 1998; Raizman et al., 2017). A common finding is vortex dystrophy caused by cationic amphiphilic chemicals that easily cross cell membranes

and produce intracellular phospholipid accumulation. Other drugs and chemicals cause corneal deposits. There are precious few studies on environmental or occupational corneal injury. An analysis, of approximately 600 agricultural and industrial chemicals (raw materials, intermediates, formulation components, and sales products), evaluated using the Draize procedure, reported that over half of the materials tested caused no (18% to 31%) or minimal (42% to 51%) irritation. Depending on the chemical category, 9% to 17% of compounds were graded as slightly irritant, whereas 1% to 6% were graded as strong or extreme irritants (Kobel and Gfeller, 1985).

Acids

Strong acids with a pH≤2.5 can be highly injurious. Among the most significant acidic chemicals in terms of the tendency to cause clinical ocular damage are hydrofluoric acid, sulfurous acid, sulfuric acid, chromic acid, hydrochloric acid, nitric acid, and acetic acid (McCulley, 1998). Injuries may be mild if contact is with weak acids or with dilute solutions of strong acids. Compounds with a pH between 2.5 and 7.0 produce pain or stinging; but with only brief contact, they will cause no lasting damage (Grant and Schuman, 1993). Following mild burns, the corneal epithelium may become turbid as the corneal stroma swells. Mild burns are typically followed by rapid regeneration of the corneal epithelium and full recovery. In more severe burns, the epithelium of the cornea and conjunctiva become opaque and necrotic and may disintegrate over the course of a few days. In severe burns, there may be no sensation of pain because the corneal nerve endings are destroyed (Grant and Schuman, 1993).

Acid chemical burns of the cornea occur through hydrogen ion-induced denaturing and coagulation of proteins. As epithelial cell proteins coagulate, glycosaminoglycans precipitate and stromal collagen fibers shrink. These events cause the cornea to become cloudy. The protein coagulation and shrinkage of the collagen is protective in that it forms a barrier and reduces further penetration of the acid. The collagen shrinkage, however, contracts the eye and can lead to a dangerous acute increase in intraocular pressure. The pH of the acid is not the only determinant of the severity of injury; however, as equimolar solutions of several chemicals adjusted to the same pH of 2 produce a wide range of outcomes. Both the hydrogen ion and anionic portions of the acid molecules contribute to protein coagulation and precipitation. The tissue proteins also tend to act as buffers (Grant and Schuman, 1993).

Bases or Alkalies

Compounds with a basic pH are potentially even more damaging to the eye than are strong acids. Among the compounds of clinical significance in terms of frequency and severity of injuries are ammonia or ammonium hydroxide, sodium hydroxide (lye), potassium hydroxide (caustic potash), calcium hydroxide (lime), and magnesium hydroxide (McCulley, 1998). One of the reasons that caustic chemicals are so dangerous is their ability to rapidly penetrate the ocular tissues. This is particularly true for ammonia, which has been measured in the aqueous humor just seconds after application to the cornea. The toxicity of these substances is a function of their pH, being more toxic with increasing pH values. As with acid burns, the concentration of the solution and the duration of contact with the eye are important determinants of the eventual clinical outcome. Rapid and extensive irrigation after exposure and removal of particles, if present, is the immediate therapy of choice (Grant and Schuman, 1993).

A feature of caustic burns that differentiates them from acid burns is that the former has two phases of injury. First, there is an acute phase from exposure up to 1 week. Depending on the extent of injury, direct damage from exposure is observed in the cornea, adnexa, and possibly in the iris, ciliary body, and lens. The presence of strong hydroxide ions causes rapid necrosis of the corneal epithelium and, if sufficient amounts are present, penetration through and/or destruction of the successive corneal layers. Strong alkali substances attack membrane lipids, causing necrosis and enhancing penetration of the substance to deeper tissue layers. The cations also react with the carboxyl groups of glycosaminoglycans and collagen, the latter reaction leading to hydration of the collagen matrix and corneal swelling. The cornea may appear clouded or become opaque immediately after exposure as a result of stromal edema and changes to, or precipitation of, proteoglycans. The denaturing of the collagen and loss of protective covering of the glycosaminoglycans is thought to make the collagen fibrils more susceptible to subsequent enzymatic degradation. Intraocular pressure may increase as a result of initial hydration of the collagen fibrils and later through the blockage of aqueous humor outflow. Conversely, if the alkali burn extends to involve the ciliary body, the intraocular pressure may decrease due to reduced formation of aqueous humor. The acute phase of damage is typically followed by a second phase of corneal repair. The repair process may involve corneal neovascularization along with regeneration of the corneal epithelium. Approximately, 2 to 3 weeks after alkali burns, however, damaging ulceration of the corneal stroma often occurs. The formation of these lesions is related to the inflammatory infiltration of polymorphonuclear leukocytes and fibroblasts and the release of degratory proteolytic enzymes. Clinically, anti-inflammatory therapy limits ulcerative damage. Stromal ulceration usually stops when the corneal epithelium is restored (Grant and Schuman, 1993).

Organic Solvents

When organic solvents are splashed into the eye, the result is typically a painful immediate reaction. As in the case of acids and bases, exposure of the eye to solvents should be treated rapidly with abundant water irrigation. Highly lipophilic solvents can damage the corneal epithelium and produce swelling of the corneal stroma. Most organic solvents do not have a strongly acid or basic pH and therefore cause minimal chemical burns to the cornea. In most cases, the corneal epithelium will be repaired over the course of a few days and there will be no residual damage. Exposure to solvent vapors may produce small transparent vacuoles in the corneal epithelium, which may be asymptomatic or associated with moderate irritation and tearing (Grant and Schuman, 1993).

Surfactants

These compounds have water-soluble (hydrophilic) properties at one end of the molecule and lipophilic properties at the other end that help to dissolve fatty substances in water and also serve to reduce water surface tension. The widespread use of these chemicals in soaps, shampoos, detergents, cosmetics, and similar consumer products leads to abundant opportunities for exposure to ocular tissues. Many of these chemicals may be irritating or injurious to the eye. The hydrophilic portion of these compounds may be anionic, cationic, or neutral. In general, the cationic substances tend to be stronger irritants and more injurious than the other types, and anionic compounds more so than neutral ones (Grant and Schuman, 1993). Because these compounds are by design soluble in both aqueous and lipid media, they readily penetrate the sandwiched aqueous and lipid barriers of the cornea (see discussion of the section "Ocular Pharmacodynamics and Pharmacokinetics" above). This property has implications in drug delivery; for example, low

concentrations of the preservative benzalkonium chloride to ophthalmic solutions enhance ocular penetration of topically applied medications (Bartlett and Jaanus, 2008), but also add to their corneal toxicity (Liang et al., 2011).

TARGET SITES AND MECHANISMS OF ACTION: RETINA

The adult mammalian retina is a highly differentiated tissue containing eight distinct layers plus the RPE, 11 major types of neurons, and a Müller glial cell (Fig. 17-1). The eight layers of the neural retina, which originate from the cells of the inner layer of the embryonic optic cup, are the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), rod and cone photoreceptor inner segment layer (RIS; CIS), and the rod and cone photoreceptor outer segment layer (ROS; COS). The retinal pigment epithelium (RPE), which originates from the cells of the outer layer of the embryonic optic cup, is a single layer of cuboidal epithelial cells that lies on Bruch membrane adjacent to the vascular choroid. Between the RPE and photoreceptor outer segments lies the subretinal space, which is similar to the brain ventricles. The 11 major types of neurons are the rod and cone photoreceptors, (depolarizing) ON-rod and ON-cone bipolar cells, (hyperpolarizing) OFF-cone bipolar cells, horizontal cells, numerous subtypes of amacrine cells, an interplexiform cell, and different types of retinal ganglions cells (RGCs): ON-RGCs, OFF-RGCs, and ipRGCs. The Müller glial cell (MGC) is the only glial cell in the retina. The somas of the MGCs are in the INL. The end feet of the MGCs in the proximal or inner retina along with a basal lamina form the internal limiting membrane of the retina, which is similar to the pial surface of the brain. In the distal retina, the MGC end feet join with the photoreceptors and zonula adherens to form the external limiting membrane, which is located between the ONL and RIS/CIS. The interested reader is referred to the excellent references in the introduction section as well as to numerous outstanding Web sites devoted exclusively to the retina (http://webvision.med.utah.edu; http://cvs.anu.edu.au; http://retina.anatomy.upenn.edu) for basic information on the anatomic, biochemical, cell and molecular biological, and physiological aspects of retinal structure and function.

The mammalian retina is highly vulnerable to toxicant-induced structural and/or functional damage due to (1) the presence of a highly fenestrated choriocapillaris that supplies the distal or outer retina as well as a portion of the inner retina; (2) the very high rate of oxidative mitochondrial metabolism, especially in photoreceptors (Ahmed et al., 1993; Medrano and Fox, 1995; Braun et al., 1995; Winkler, 1995; Shulman and Fox, 1996; Perkins et al., 2012; Rueda et al., 2016); (3) high daily turnover of rod and cone outer segments (LaVail, 1976); (4) high susceptibility of the rod and cones to degeneration due to inherited retinal dystrophies as well as associated syndromes and metabolic disorders (van Soest et al., 1999; Rueda et al., 2016); (5) presence of specialized ribbon synapses and synaptic contact sites (Wässle, 2004; tom Dieck and Brandstätter, 2006; Johnson et al., 2007); (6) presence of numerous neurotransmitter and neuromodulatory systems, including extensive glutamatergic, GABAergic, and glycinergic systems (Kalloniatis and Tomisich, 1999; Wässle, 2004); (7) presence of numerous and highly specialized gap junctions used in the information signaling process (Bloomfield and Völgyi, 2009); (8) presence of melanin in the choroid, RPE, uvea, and iris (Meier-Ruge, 1972; Potts and Au, 1976; Karlsson and Lindquist, 2016); (9) a very high choroidal blood flow rate, as high as 10 times that of the gray matter of the brain (Nickla and Wallman, 2010); and (10) the additive or synergistic toxic action of certain chemicals with light (Dayhaw-Barker et al., 1986; Backstrom et al., 1993; Roberts, 2001, 2002; Glickman, 2002; Siu et al., 2008; Zhao et al., 2010; Wielgus and Roberts, 2012). The retina is the only part of the central nervous system also exposed to light.

The retina is also an excellent model system for studying the effects of chemicals on the developing and mature CNS. Its structure–function relationships are well established. The histogenic steps of development of the neurons and glial components are well characterized. The development of the CNS and most retinal cells occurs early during gestation in humans (Hendrickson, 1992; Hendrickson and Drucker, 1992) and continues for an additional 7 to 14 days postnatally in the rat (Dobbing and Sands, 1979; Raedler and Sievers, 1975). Therefore, toxicological effects in the pigmented rodent retina have relevance for chemical exposure during the early gestation period in humans as well as during early postnatal development. The retina contains a wide diversity of synaptic transmitters and second messengers whose developmental patterns are well described. Moreover, the rodent retina is easily accessible, it has most of the same anatomical and functional features found in the developing and mature human retina, and the rat rod pathway is similar to that in other mammals (Finlay and Sengelaub, 1989; Chun et al., 1993). Finally, rat rods have similar dimensions, photochemistry, and photocurrents as human and monkey rods (Baylor et al., 1984; Schnapf et al., 1988). These general and specific features underscore the relevance and applicability of using the pigmented rodent retina to investigate the effects of chemicals on this target site as well as a model to investigate the neurotoxic effects of chemicals during development.

Each retinal layer, which has its own metabolic profile (Rueda et al., 2016), can undergo specific as well as general toxic effects. These alterations and deficits include, but are not limited to visual field deficits, scotopic vision deficits such as night blindness and increases in the threshold for dark adaptation, cone-mediated (photopic) deficits such as decreased color perception, decreased visual acuity, macular and general retina edema, retinal hemorrhages and vasoconstriction, and pigmentary changes. The list of drugs and other chemicals that cause retinal alterations is extensive, as evidenced by an examination of Table 17-1, Grant's *Toxicology of the Eye* (Grant and Schuman, 1993), Bartlett and Jaanus (2008) discussion of the adverse retinal effects of therapeutic systemic drugs, and a review by Wolfensberger (1998) that addresses the adverse effects of drugs on the RPE. The overall aim of this section is to discuss in detail several chemicals, solvents, and drugs: (1) that are used as drugs or that are environmentally relevant neurotoxicants; (2) whose behavioral, physiological, and/or pathological effects on the retina are known; and (3) whose retinal site(s) and/or mechanism of action are well characterized.

The chemical- and drug-induced alterations in retinal structure and function are grouped into two major categories. The first category focuses on retinotoxicity of systemically administered therapeutic drugs. Six major drugs are discussed in detail: chloroquine/hydroxychloroquine, digoxin/digitoxin, indomethacin, erectile dysfunction drugs (i.e., sildenafil, tadalafil, and vardenafil), tamoxifen, and vigabatrin. The second category focuses on well-known neurotoxicants that produce retinotoxicity: inorganic lead, methanol, selected organic solvents, and organophosphates. See Chaps. 16 and 23 for information on the effects of lead on the brain and other target organs and Chap. 24 for additional information on methanol and the organic solvents discussed below.

Retinotoxicity of Systemically Administered Therapeutic Drugs

Cancer Chemotherapeutics Ocular toxicity is a common side effect of cancer chemotherapy (Imperia et al., 1989; Schmid et al., 2006). Symptoms include blurred vision, diplopia, decreased color vision, decreased visual acuity, optic/retrobulbar neuritis, transient cortical blindness, and demyelination of the optic nerves (Imperia et al., 1989; Schmid et al., 2006). The retina, due to its high metabolic activity and choroidal circulation (vide infra), appears to be particularly vulnerable to numerous cytotoxic drugs such as the alkylating chemicals cisplatin, carboplatin, and carmustine; the antimetabolites cytosine arabinoside, 5-fluorouracil, and methotrexate; and the mitotic inhibitors such as docetaxel. The ocular toxicity of various drugs is dependent upon the dose, duration of dosage, and route of administration. However, if not detected at an early stage of toxicity, the ocular complications are often irreversible even after chemotherapy is discontinued (Imperia et al., 1989; Schmid et al., 2006). One strategy to avoid such retinal complications is to conduct prospective ophthalmological examinations as well as scotopic and photopic ERG testing prior to the onset and during chemotherapy. The ocular side effects of tamoxifen, an estrogen antagonist used in oncology, are discussed below.

Chloroquine and Hydroxychloroquine Two of the most extensively studied retinotoxic drugs are chloroquine (Aralen) and hydroxychloroquine (Plaquenil). The first case of chloroquine-induced retinopathy was reported more than 40 years ago (Bartlett and Jaanus, 2008). These 4-aminoquinoline derivatives are used as antimalarial and anti-inflammatory drugs. The low-dose therapy used for malaria is essentially free from toxic side effects; however, the chronic, high-dose therapy used for rheumatoid arthritis, and discoid and systemic lupus erythematosus (initially 400 to 600 mg/day for 4 to 12 weeks and then 200 to 400 mg/day; Ellsworth et al., 1999) can cause irreversible loss of retinal function. Chloroquine, its major metabolite desethylchloroquine, and hydroxychloroquine have high affinity for melanin, which results in very high concentrations of these drugs accumulating in the choroid and retinal pigment epithelium (RPE), ciliary body, and iris during and following drug administration (Rosenthal et al., 1978). Prolonged exposure of the retina to these drugs, especially chloroquine, may lead to an irreversible retinopathy, whose early symptoms produce the pathognomonic "bull's-eye retina" visualized as a dark, central pigmented area involving the macula, surrounded by a pale ring of depigmentation, which is surrounded by another ring of pigmentation. In humans and monkeys, long-term chloroquine administration results in sequential degeneration of the retinal ganglion cells (RGCs), photoreceptors, and RPE and the eventual migration of RPE pigment into the outer nuclear and plexiform layers, ONL and OPL, respectively (Ramsey and Fine, 1972; Rosenthal et al., 1978). Chloroquine binding to melanin was not the pathological mechanism of action, but rather phospholipidosis in the retinal synapses of both albino and pigmented rats produced the retinal degeneration (Lezmi et al., 2013). Approximately, 20% to 30% of patients who received high doses of chloroquine exhibited some type of retinal abnormality, whereas 5% to 10% showed severe changes in retinal function (Burns, 1966; Shearer and Dubois, 1967; Sassaman et al., 1970; Krill et al., 1971). Hydroxychloroquine has fewer side effects and less ocular toxicity. Doses less than 400 mg/day appear to produce little or no retinopathy even after prolonged therapy (Johnson and Vine, 1987).

Digoxin and Digitoxin The cardiac glycosides digoxin and digitoxin are digitalis derivatives used in the treatment of congestive heart disease and in certain cardiac arrhythmias. As part of the extract of the plant foxglove, digitalis was recommended for heart failure (dropsy) over 200 years ago. Digitalis-induced visual system abnormalities such as decreased vision, flickering areas of vision loss (scotomas), and altered color vision were documented during that time (Withering, 1785). Approximately, 20% to 60% of patients with cardiac glycoside serum levels in the therapeutic range and 50% to 80% of the patients with cardiac glycoside serum levels in the toxic range complain of visual system disturbances within 2 weeks after the onset of therapy (Robertson et al., 1966; Haustein et al., 1982; Duncker et al., 1994). Digoxin produces more toxicity than digitoxin due to its greater volume of distribution and plasma protein binding (Haustein and Schmidt, 1988). The most frequent visual complaints are color vision impairments and hazy or snowy vision, although complaints of flickering light, colored spots surrounded by bright halos, blurred vision, and glare sensitivity also are reported. The color vision disturbances have been confirmed with the Farnsworth-Munsell 100 Hue Test (Haustein et al., 1982; Haustein and Schmidt, 1988; Duncker and Krastel, 1990). Clinical examinations show that these patients have decreased visual acuity and central scotomas, but no funduscopic changes. ERG analysis revealed reduced rod and cone amplitudes, increased rod and cone implicit times, and elevated rod and cone thresholds (Robertson et al., 1966; Alken and Belz, 1984; Duncker and Krastel, 1990; Madreperla et al., 1994). Taken together, these ophthalmological, behavioral, and electrophysiological findings demonstrate that the photoreceptors are a primary target site of the cardiac glycosides digoxin and digitoxin.

The above results suggest that cone photoreceptors are more susceptible to the effects of cardiac glycosides than rod photoreceptors. Electrophysiological experiments by Madreperla et al. (1994) showed that cones were ~50 times more sensitive to digoxin and were impaired to a greater degree at the same digoxin concentration than the rods. Following short-duration saturating light flashes, rods appear to recover faster and more completely than cones. However, neither recovers to their dark-adapted baseline response level. These findings correlate with the slow recovery of the electroretinogram (ERG) seen in patients following termination of digoxin exposure (Duncker and Krastel, 1990; Madreperla et al., 1994) and is most likely due to the high affinity and slow off-rate of digoxin binding to the cardiac glycoside site located on the extracellular side of the catalytic α-subunit of the sodium-potassium ATPase enzyme (Sweadner, 1989).

Digitalis glycosides, like ouabain, are potent inhibitors of retinal Na^+,K^+-ATPase (Winkler and Riley, 1977; Fox et al., 1991b; Shulman and Fox, 1996). Digoxin-binding studies show that the retina has the highest number of Na^+,K^+-ATPase sites of any ocular tissue, even higher than those of brain (Lissner et al., 1971). There are three different isoforms of the α subunit of Na^+,K^+-ATPase (i.e., α1, α2, and α3), and they differ significantly in their sensitivity to cardiac glycoside inhibition (Sweadner, 1989). In the rat retina, the α1-low and α3-high ouabain affinity isoforms of the enzyme account for ≥97% of the Na^+,K^+-ATPase mRNA. The α3 isoform is localized to rat photoreceptors, horizontal cells, and bipolar cells. Photoreceptors predominantly express the α3 mRNA (approximately 85%), a small amount of α1 mRNA (approximately 15%), and almost no detectable α2 mRNA. Electron microscopic immunocytochemistry studies reveal that the α3 isoform is localized exclusively to the plasma membrane of the rat photoreceptor inner segments (McGrail and Sweadner, 1989; Schneider et al., 1991). The α3 isozyme accounts for most of the rod Na^+,K^+-ATPase activity (Shulman and Fox, 1996). The rat rod photoreceptor Na^+,K^+-ATPase specific activity is approximately threefold higher than

whole retinal (Fox et al., 1991b; Shulman and Fox, 1996) or whole brain values (Marks and Seeds, 1978). This also is reflected in the two- to threefold greater ouabain-sensitive oxygen consumption in the dark-adapted outer retina relative to the whole or inner retina, respectively (Medrano and Fox, 1995; Shulman and Fox, 1996). Interestingly, the endogenously made 12(*R*)-HETE (vide supra) is a very potent inhibitor of Na^+,K^+-ATPase (Schwartzman, 1997).

Indomethacin Indomethacin is a potent nonsteroidal anti-inflammatory drug with analgesic and antipyretic properties that is frequently used for the management of arthritis, gout, and musculoskeletal discomfort. It inhibits prostaglandin synthesis by inhibiting cyclooxygenase. The first cases of indomethacin-induced retinopathy were reported approximately 30 years ago (Bartlett and Jaanus, 2008). Chronic administration of 50 to 200 mg/day of indomethacin for 1 to 2 years has been reported to produce corneal opacities, discrete pigment scattering of the retinal pigment epithelium (RPE) perifoveally, paramacular depigmentation, decreases in visual acuity, altered visual fields, increases in the threshold for dark adaptation, blue–yellow color deficits, and decreases in ERG and electro-oculogram (EOG) amplitudes (Burns, 1966, 1968; Henkes et al., 1972; Koliopoulos and Palimeris, 1972; Peponis et al., 2010). Decreases in the ERG a- and b-wave amplitudes, with larger changes observed under scotopic dark-adapted than light-adapted conditions, have been reported. Upon cessation of drug treatment, the ERG waveforms and color vision changes return to near normal, although the pigmentary changes are irreversible (Burns, 1968; Henkes et al., 1972). The mechanism of retinotoxicity is unknown; however, it appears likely that the RPE is a primary target site.

Erectile Dysfunction (ED) Drugs The three major drugs for ED (sildenafil, tadalafil, and vardenafil) are cGMP-specific phosphodiesterase (PDE) type 5 inhibitors (Corbin et al., 2002; Moschos and Nitoda, 2016). The protypical ED drug sildenafil citrate (Viagra) is only a 10-fold weaker inhibitor of cGMP PDE type 6, which is present in rod and cone photoreceptors (Corbin et al., 2002; Zhang et al., 2005). Tadalafil and vardenafil are much more selective for PDE5 than PDE6 and thus have fewer retinal side effects (Moschos and Nitoda, 2016). Following sildenafil usage, transient visual symptoms such as a blue tinge to vision, increased brightness of lights and blurry vision as well as alterations in scotopic and photopic ERGs have been reported (Laties and Zrenner, 2002; Jagle et al., 2004). Sildenafil also has been associated with the occurrence of nonarteritic anterior ischemic optic neuropathy (NAION) in at-risk patients (i.e., those with small cup-to-disc ratios and/or arteriosclerotic risk profiles) within minutes to hours after the ingestion of the drug (Fraunfelder et al., 2006; Moschos and Nitoda, 2016). Available data suggest that the risk of occurrence of NAION in patients taking sildenafil or tadalafil may not be significantly different from the general population (Fraunfelder et al., 2006; Gorkin et al., 2006; Laties, 2009).

Tamoxifen Tamoxifen (Nolvadex, Tamoplex), a triphenylethylene derivative, is a nonsteroidal antiestrogenic drug that competes with estrogen for its receptor sites. It is a highly effective antitumor drug used for the treatment of metastatic breast carcinoma in postmenopausal women. Tamoxifen-induced retinopathy following chronic high-dose therapy (180 to 240 mg/day for approximately 2 years) was first reported over 35 years ago (Kaiser-Kupfer et al., 1981). At this dose, there is widespread axonal degeneration in the macular and perimacular area, as evidenced by the presence of different sized yellow–white refractile opacities in the inner plexiform layer (IPL) and nerve fiber layer (NFL) observed during fundus examination. Macular edema may or may not be present. Clinical symptoms include a permanent decrease in visual acuity and abnormal visual fields, as the axonal degeneration is irreversible (Ah-Song and Sasco, 1997; Bartlett and Jaanus, 2008). Several prospective studies, with sample sizes ranging from 63 to 303 women with breast cancer, have shown that chronic low-dose tamoxifen (20 mg/day) can result in a small but significant increase in the incidence ($\leq$10%) of corneal lesion or keratopathy (Pavlidis et al., 1992; Gorin et al., 1998; Lazzaroni et al., 1998; Noureddin et al., 1999). In addition, these studies showed that retinopathy is much less frequently observed than with high-dose therapy and, except for a few reports of altered color vision and decreased visual acuity, there were no significant alterations in visual function. Following cessation of low-dose tamoxifen therapy, most of the keratopathy and retinal alterations except the corneal opacities and retinopathy were reversible (Pavlidis et al., 1992; Gorin et al., 1998; Noureddin et al., 1999).

Vigabatrin Vigabatrin is an irreversible inhibitor of GABA-transaminase that is used to treat refractory complex partial seizures and infantile spasms (Tolman and Faulkner, 2009). After 20 years of use in Europe, it was approved in 2009 by the FDA for use in the United States despite its risk of retinopathy characterized by irreversible bilateral, concentric peripheral visual constriction, and decreased retinal nerve fiber layer (NFL) thickness (Maguire et al., 2010; Clayton et al., 2011; Plant and Sergott, 2011). Clinical and meta-analysis studies revealed that the prevalence of the asymptomatic visual field loss was ~50% (Maguire et al., 2010; Clayton et al., 2011; Plant and Sergott, 2011). Onset of the visual field loss was variable as was observed after as little as 6 weeks of exposure, but generally required a couple of years (Maguire et al., 2010; Clayton et al., 2011; Plant and Sergott, 2011). In addition, rod and cone ERGs, mfERGs, and ERG flicker responses are altered, indicating that retinal damage also occurs (Daneshvar et al., 1999; Wang et al., 2008; Dettoraki and Moschos, 2016). Recent dose–response studies in mice revealed disorganization of the photoreceptors and the outer plexiform layer (OPL), the first synaptic layer, following 1 month of vigabatrin exposure (Wang et al., 2008). The drug is recommended for epileptic children and adults only when there are no alternative choices.

Retinotoxicity of Known Neurotoxicants

Inorganic Lead Inorganic lead is probably the oldest known and most studied environmental toxicant. For almost 100 years, it has been known that overt lead poisoning (mean blood lead [BPb] $\geq$80 μg/dL) in humans produces visual system pathology and overt visual symptoms (reviewed in: Otto and Fox, 1993; Fox, 1998, 2015). Clinical manifestations include amblyopia, blindness, optic neuritis or atrophy, peripheral and central scotomas, paralysis of eye muscles, and decreased visual function. Moderate- to high-level lead exposure produces scotopic and temporal visual system deficits in occupationally exposed factory workers and developmentally lead-exposed monkeys and rodents (Fox and Farber, 1988; Otto and Fox, 1993; Fox et al., 1997; Kohler et al., 1997; Fox, 1998, 2015; Rice, 1998; Perkins et al., 2012; Giddabasappa et al., 2011). A prospective epidemiological study in 7- to 10-year-old children revealed that low-level gestational lead exposure produces long-lasting scotopic supernormal flash ERG deficits (Rothenberg et al., 2002), which was confirmed in a case report (Nagpal and Brodie, 2009). Similar results were found in a rodent model of low-level gestational lead exposure (Fox et al., 2008). Subsequently it was determined that gestational lead

exposure increased retinal progenitor cell proliferation, and rod photoreceptor and bipolar cell neurogenesis (Giddabasappa et al., 2011), consistent with the supernormal ERGs (Fox et al., 2008), as well as delayed rod and bipolar cell differentiation (Chaney et al., 2016). Retinal and visual deficits can adversely affect learning and memory as well as experimental procedures used to assess these cognitive parameters (Anger et al., 1994; Hudnell et al., 1996; Walkowiak et al., 1998; Cestnick and Coltheart, 1999).

Studies in Occupationally Exposed Lead Workers Clinical and electrophysiological studies in lead-exposed factory workers assessed the site of action and extent of injury. Several cases of retrobulbar optic neuritis and optic nerve atrophy were observed following chronic moderate-level or acute high-level lead exposure (Baghdassarian, 1968; Karai et al., 1982; Fox, 2015). Most of these cases presented with fundus lesions, peripheral or paracentral scotomas whereas the most severe cases also had a central scotoma. Generally, the scotomas were not observed until approximately 5 years of continuous lead exposure. Interestingly, the earliest observable scotomas were not detected under standard photopic viewing conditions but became evident only under scotopic or mesopic (rod- and cone-mediated) viewing conditions. These ophthalmological findings correlate directly with the ERG data observed in similarly exposed lead workers. No alterations in the critical flicker fusion threshold (i.e., temporal resolution) were observed when the test was conducted under photopic conditions or when using red lights. However, consistent decreases in temporal resolution were observed when the test was conducted under scotopic conditions or when green lights were used (reviewed in: Fox, 2015). Moreover, in occupationally lead-exposed workers with or without visual acuity deficits or no observable alterations following ophthalmological examination, the sensitivity and amplitude of the a-wave and/or b-wave of the dark-adapted ERG were decreased (Scholl and Zrenner, 2000). In other lead-exposed workers, one funduscopic study noted the presence of a grayish lead pigmentary deposit in the area peripheral to the optic disc margins (Sonkin, 1963).

A recent quantitative retinal structural assessment compared three groups of young (mean 27 to 32 years) lead-exposed battery workers (exposed ~4 years) with mean BPb of 16.9, 29.3, 46.2 μg/dL lead to an age-matched control group of office workers with a mean BPb of 2.85 μg/dL lead. All subjects had full ophthalmological exams and were corrected for best visual acuity. Spectral density (SD)-OCT imaging technology was used to determine retinal nerve fiber layer (NFL) thickness, macular thickness, and choroidal thickness. Retinal NFL thickness exhibited concentration-dependent decreases compared to controls, although the two lower groups were not significantly different from each other. Macular thickness and choroidal thickness exhibited concentration-dependent decreases for each lead-exposed group compared to controls (Ekinci et al., 2014).

In addition to the retinal deficits, oculomotor deficits occur in chronically lead-exposed workers who have no observable ophthalmological abnormalities. Results from three independent studies, including a follow-up, show that the mean accuracy of rapid intermittent (saccadic) eye movements is lower in lead-exposed workers and the number of overshoots is increased (reviewed in: Fox, 2015). In addition, these studies also revealed that the saccade maximum velocity was decreased. Another study found abnormal smooth pursuit eye movements in lead-exposed workers (reviewed in: Fox, 2015). Although the site and mechanism of action underlying these alterations are unknown, they most likely result from CNS-mediated deficits.

In summary, these results suggest that occupational lead exposure produces concentration- and time-dependent alterations in the retina such that higher levels of lead directly and adversely affect both the retina and optic nerve, whereas lower levels of lead appear to primarily affect the rod photoreceptors and their pathway. Interestingly, these latter clinical findings showing preferential lead-induced rod-selective deficits in sensitivity and temporal resolution are observed in both in vivo and in vitro animal studies (vide infra). Furthermore, these retinal and oculomotor alterations were, in most cases, correlated with the blood lead concentrations and occurred in the absence of observable ophthalmological changes, CNS symptoms, and abnormal performance test scores. Thus, these measures of temporal visual function may be among the most sensitive for the early detection of the neurotoxic effects of inorganic lead.

In Vivo and In Vitro Animal Studies with Lead Lead exposure to adult animals and postnatally developing animals produces retinal, optic nerve, and visual cortical damage and functional deficits. The degree and extent of these alterations depends upon the dose, age, and duration of lead exposure (Otto and Fox, 1993; Fox et al., 1997; Perkins et al., 2012; Fox, 2015). Retinas from rhesus monkeys and mice exposed prenatally and/or postnatally to lead had decreased tyrosine hydroxylase immunoreactivity (TH-IR) in the large dopaminergic amacrine cells and a complete loss of TH-IR in a small subset of amacrine cells (Kohler et al., 1997; Fox et al., 2011). These results suggest that lead exposure produces a decrease in TH synthesis, a finding consistent with other studies (Lasley and Lane, 1988; Jadhav and Ramesh, 1997), and/or a loss of a subset of TH-IR amacrine cells, a finding consistent with in vitro work (Scortegagna and Hanbauer, 1997). In contrast to these studies, 6 weeks of moderate-level lead exposure to adult rats (Fox et al., 1997) or 3 weeks of lead exposure to neonatal rats or mice from birth to weaning produced rod and bipolar cell apoptotic cell death, as well as persistent rod and cone synaptic pathophysiology (Fox and Chu, 1988; Fox et al., 1997; Perkins et al., 2012). Brief (15 minutes) exposure of isolated adult rat retinas to nM to μM Pb^{2+}, concentrations regarded as pathophysiologically relevant (Al-Modhefer et al., 1991), produce rod-selective apoptosis (He et al., 2000, 2003). These results are consistent with numerous studies showing that elevated levels of rod photoreceptor Ca^{2+} and/or Pb^{2+} play a key role in the process of apoptotic rod cell death in patients with retinitis pigmentosa and cancer-associated retinopathy (Thirkill et al., 1987; van Soest et al., 1999), mice with retinal degeneration (*rd*) (Chang et al., 1993; Fox et al., 1999), rats injected with antirecoverin monoclonal antibodies (Adamus et al., 1998), rats with hypoxic-ischemic injury (Crosson et al., 1990), or light-induced damage (Edward et al., 1991).

In vivo and in vitro data suggest that Pb^{2+} produces a dose (concentration)-dependent inhibition of rod cGMP PDE, which elevates rod cGMP (Fox and Farber, 1988; Fox et al., 1991a, 1997; Srivastava et al., 1995a, 1995b) and the rod Ca^{2+} concentration (Fox and Katz, 1992; Medrano and Fox, 1994; He et al., 2000, 2003). Detailed kinetic analysis revealed that pM Pb^{2+} competitively and directly inhibits rod cGMP PDE relative to mM Mg^{2+} (Srivastava et al., 1995a, 1995b). In addition, nM Pb^{2+} can elevate the rod Ca^{2+} (and Pb^{2+}) concentration via its competitive inhibition of retinal Na^+,K^+-ATPase relative to MgATP (Fox et al., 1991b). Once inside the rod, both Ca^{2+} and Pb^{2+} enter the mitochondria via the Ca^{2+} uniporter and induce mitochondrial depolarization, swelling, and cytochrome *c* release (He et al., 2000, 2003). The effects of Ca^{2+} and Pb^{2+} were additive and blocked by the mitochondrial permeability transition pore inhibitor cyclosporin A. Following cytochrome *c* release, caspase-9 and caspase-3 are sequentially and selectively

activated. These results are consistent with numerous studies from different tissues demonstrating that Pb^{2+} is preferentially associated with mitochondria and particularly with the inner membrane and matrix fractions (Barltrop et al., 1971; Bull, 1980; Pounds, 1984). Taken together, the results suggest that Ca^{2+} and Pb^{2+} bind to the internal divalent metal binding site of the mitochondrial permeability transition pore (Szabo et al., 1992) and subsequently open it, which initiates the cytochrome *c*-caspase cascade of apoptosis in rods (He et al., 2000, 2003).

In vitro extracellular and intracellular electrophysiological recordings in isolated whole retinas or photoreceptors are consistent with postnatal lead exposure studies. That is, nM to μM Pb^{2+} selectively depress the amplitude and absolute sensitivity of the rod but not cone photoreceptor potential (Fox and Sillman, 1979; Otto and Fox, 1993). These electrophysiological results are similar to the ERG alterations observed in occupationally lead-exposed workers and in adult rats exposed to low and moderate levels of lead only during development (Otto and Fox, 1993; Fox, 1998, 2015). In addition, these postnatally lead-exposed rats exhibit rod-mediated increases in dark and light adaptation time, decreases in critical flicker fusion frequency (i.e., temporal resolution), decreases in relative sensitivity, and increases in a- and b-wave latencies (Fox and Farber, 1988; Fox and Rubinstein, 1989; Fox et al., 1991a; Fox and Katz, 1992) and decreases in the temporal response properties of both sustained (X-type) and transient (Y-type) retinal ganglion cells (RGCs), such as decreased optimal temporal frequency and temporal resolution (Ruan et al., 1994). By extension, these results suggest that there is a common underlying biochemical mechanism responsible for these rod-mediated deficits: the Pb^{2+}-induced inhibition of cGMP PDE (vide supra). If rods and blue-sensitive cones in humans exhibit the same sensitivity to a lead-induced inhibition of cGMP-PDE as they do to the drug-induced inhibition of cGMP-PDE (Zrenner and Gouras, 1979; Zrenner et al., 1982; Fox and Farber, 1988), it was predicted that blue-cone (short wavelength or S-cones) color vision deficits as well as scotopic deficits may be found in adults and children exposed to lead (Fox and Farber, 1988). S-cone deficits were observed in an occupationally lead-exposed worker (Scholl and Zrenner, 2000).

Methanol Methanol is a low-molecular-weight (32 Da), colorless, and volatile liquid that is widely used as an industrial solvent; a chemical intermediate; a fuel source for picnic stoves, racing cars, and soldering torches; an antifreeze agent; and an octane booster for gasoline. The basic toxicology and references were reviewed (Eells, 1992). Briefly, methanol is readily and rapidly absorbed from all routes of exposure (i.e., dermal, inhalation, and oral), easily crosses all membranes, and thus is uniformly distributed to organs and tissues in direct relation to their water content. Following different routes of exposures, the highest concentrations of methanol are found in the blood, aqueous, and vitreous humors, and bile as well as the brain, kidneys, lungs, and spleen. In the liver, methanol is oxidized sequentially to formaldehyde by alcohol dehydrogenase in human and nonhuman primates or by catalase in rodents and then to formic acid. It is excreted as formic acid in the urine or oxidized further to carbon dioxide and then excreted by the lungs. Formic acid is the toxic metabolite that mediates the metabolic acidosis as well as the retinal and optic nerve toxicity observed in humans, monkeys, and rats with a decreased capacity for folate metabolism (Murray et al., 1991; Eells, 1992; Lee et al., 1994; Garner et al., 1995a, 1995b; Eells et al., 1996; Seme et al., 1999).

Human and nonhuman primates are highly sensitive to methanol-induced neurotoxicity due to their limited capacity to oxidize formic acid. The toxicity occurs in several stages. First there is a mild CNS depression, followed by an asymptomatic 12- to 24-hour latent period, then by a syndrome consisting of formic acidemia, uncompensated metabolic acidosis, ocular and visual toxicity, coma, and possibly death (Eells, 1992). The treatment of methanol poisoning involves both combating acidosis and preventing methanol oxidation, but it is not discussed further herein (see Chaps. 24 and 33).

Administration of methanol to rats with a decreased capacity for folate metabolism resulted in toxic blood formate concentrations of 8 to 16 mM (Murray et al., 1991; Lee et al., 1994; Garner et al., 1995a, 1995b; Eells et al., 1996; Seme et al., 1999). Permanent visual damage occurred in humans and monkeys when the blood formate levels exceeded 7 mM (Eells, 1992). Acute methanol poisoning in humans, monkeys, and experimental rats resulted in profound and permanent structural alterations in the retina and optic nerve and visual impairments ranging from blurred vision to decreased visual acuity and light sensitivity to blindness. Ophthalmological studies of exposed humans and monkeys reveal varying degrees of edema of the papillomacular bundle and optic nerve head (Benton and Calhoun, 1952; Potts, 1955; Baumbach et al., 1977; Hayreh et al., 1980). Histopathological and ultrastructural investigations in methanol-exposed monkeys and folate-modified rats showed retinal edema, swollen and degenerated photoreceptors, degenerated retinal ganglion cells (RGCs), swollen retinal pigment epithelial cells, axonal (optic nerve) swelling, and mitochondrial swelling and disintegration in each of these cells, but especially in the photoreceptors and optic nerve (Baumbach et al., 1977; Hayreh et al., 1980; Murray et al., 1991; Seme et al., 1999). Considering the differences in species, methanol exposures, time course of analysis, and procedures utilized, the overall data for the acute effects of methanol on the flash electroretinogram (ERG) are remarkably consistent. Following methanol exposure, the ERG b-wave amplitude in humans, monkeys, and folate-modified rats starts to decrease significantly when the blood formate concentration exceeded 7 mM (Potts, 1955; Ruedeman, 1961; Ingemansson, 1983; Murray et al., 1991; Lee et al., 1994). These ERG b-wave alterations, as well as flicker-evoked ERG alterations (Seme et al., 1999), occur at lower formate concentrations than those associated with structural changes in the retina and optic nerve, as discussed above. Decreases in the a-wave amplitude are delayed, relative to the b-wave and occur when blood formate concentrations further increase (Ruedeman, 1961; Ingemansson, 1983; Murray et al., 1991; Eells et al., 1996). In addition, it has been shown that intraretinal metabolism of methanol is necessary for the formate-mediated alterations in the ERG (Garner et al., 1995a), although intravenous infusion of formate in monkeys does induce optic nerve edema (Martin-Amat et al., 1978). Finally, in the folate-modified rats, it appears that photoreceptors that respond to a 15-Hz flicker/510-nm wavelength mesopic–photopic stimulus are more sensitive to methanol than the ultraviolet-sensitive (UV) cones (Seme et al., 1999). The data from the ERG b-wave methanol studies suggest that the initial effect of formate is directly on the ON-type rod bipolar cells, Müller glial cells (MGCs), and/or synaptic transmission between the photoreceptors and bipolar cells. A well-designed series of pharmacological, ERG, and potassium-induced Müller cell depolarization studies using several controls and folate-modified rats revealed a direct toxic effect of formate on MGC function (Garner et al., 1995a, 1995b). These studies also provided evidence that formate does not directly affect depolarizing rod bipolar cells or synaptic transmission between the photoreceptors and bipolar cells. Formate also appears to directly and adversely affect the photoreceptors as evidenced by the markedly decreased ERG a-wave and flicker response data (Ruedeman, 1961; Ingemansson, 1983; Murray et al., 1991; Eells et al., 1996; Seme et al., 1999).

Several findings suggest that the mechanism of methanol poisoning involves a disruption in oxidative energy metabolism. First, the whole retinal ATP concentration is decreased in folate-deficient rats 48 hours following methanol exposure, the time point when the b-wave was lost (Garner et al., 1995b). Second, both formate (10 to 200 mM) and formaldehyde (0.5 to 5 mM) inhibited oxygen consumption in isolated ox retina, and formaldehyde was considerably more potent (Kini and Cooper, 1962). Third, similar concentrations of formaldehyde inhibited oxidative phosphorylation of isolated ox retinal mitochondria, with greater effects observed using FAD-linked than NADH-linked substrates (Kini and Cooper, 1962). Unfortunately, the effects of formate were not examined. Fourth, and consistent with the above results, formate inhibits succinate-cytochrome *c* reductase and cytochrome oxidase activity (K_i = 5 to 30 mM), but not NADH-cytochrome *c* reductase activity in isolated beef heart mitochondria and/or submitochondrial particles (Nicholls, 1976). Fifth, ultrastructural studies reveal swollen mitochondria in rat photoreceptor inner segment and optic nerve 48 to 72 hours after nitrous oxide/methanol exposure (Murray et al., 1991; Seme et al., 1999). To date, there are no such studies conducted on the MGCs. Taken together, these results suggest formate is a mitochondrial poison that inhibits oxidative phosphorylation of photoreceptors, MGCs, and optic nerve.

Organic Solvents

n-Hexane, Perchloroethylene, Styrene, Toluene, Trichloroethylene, Xylene, and Mixtures The neurotoxicity of organic solvents is well established (see Chaps. 16 and 24). In addition, exposures to organic solvents and other volatile hydrocarbons are associated with deficits in color vision, contrast sensitivity, and visual-motor performance (Fox, 2015). Similar deficits have been reported after occupational, residential, and recreational exposures: the latter among inhalant drug abusers. However, there is a paucity of mechanistic studies on the adverse effects of organic solvents on the retina and visual system.

Loss of color vision (acquired dyschromatopsia) and contrast sensitivity have been reported in workers exposed to organic solvents and related compounds such as alcohols, *n*-hexane, toluene, trichloroethylene, styrene, xylene, and solvent mixtures (Mergler et al., 1987, 1988, 1991; Iregren et al., 2002; Paramei et al., 2004; Benignus et al., 2005; Attarchi et al., 2010). Workers in microelectronic plants, print shops, and paint manufacturing facilities, and painters who were exposed to concentrations of solvents that exceeded the threshold limit values, had acquired dyschromatopsia as assessed by the Lanthony D-15 desaturated color arrangement panel (Mergler et al., 1987, 1988, 1991; Iregren et al., 2002). These workers had no observable clinical abnormalities as assessed by biomicroscopy, funduscopy, and peripheral visual field tests. The color vision losses were characterized initially as an increase in blue–yellow confusion errors, although more severe red–green deficits were reported with extended duration or higher concentrations of exposure. As a general rule, acquired blue–yellow losses may result from lens opacification or outer retinal alterations, whereas red–green losses are traditionally associated with retrobulbar, or central visual pathway alterations (Simunovic, 2010, 2016). In addition, many of the occupationally exposed workers also exhibited lower contrast sensitivity at intermediate spatial frequencies, which likely reflects alterations in neural function (Mergler et al., 1991; Broadwell et al., 1995). The data from Mergler et al. (1987, 1988, 1991) appear to show sex differences in these adverse visual effects. A study of female workers, where the Lanthony D-15 desaturated test was used to assess color vision, showed a trend toward increased prevalence of color vision impairment following exposure to low to moderate concentrations of toluene (Zavalic et al., 1996). The neurotoxic compounds, *n*-hexane, its metabolite 2,5-hexanedione and carbon disulfide have been associated with macular changes (Raitta et al., 1974, 1978, 1981) and rod and cone degeneration (Backstrom and Collins, 1992; Backstrom et al., 1993). Photoreceptor damage and retinal pathology have not been reported for other hydrocarbon solvents. Clearly more detailed, well-designed, and well-executed studies are needed to determine the (1) specific solvents that cause alterations in color vision, (2) vulnerability of spatial and temporal contrast sensitivity, (3) dose (concentration)–response relations between exposures and effects, (4) possible sex differences, (5) potential reversibility of deficits if exposure is terminated, and (6) pathophysiological and toxicological basis for these changes. Prospective occupational studies would be particularly helpful given the difficulties in obtaining appropriately matched control populations in cross-sectional study designs.

Perchloroethylene (Tetrachloroethylene; PERC) Long-term low-level occupational and environmental PERC exposure produces adverse retinal and central visual system effects. Dry cleaners exposed to low-level PERC for 8 to 10 years had blue–yellow deficits (Cavalleri et al., 1994). A 2-year follow-up study showed that these deficits increased in workers that were exposed to higher concentrations of PERC for 2 more years, yet remained the same when the concentration decreased (Cavalleri et al., 1994; Gobba et al., 1998). A population-based retrospective cohort study found an association between prenatal and early childhood PERC-contaminated drinking water and blue–yellow color and spatial contrast sensitivity deficits at high spatial frequencies in 30-year-old adults, without retinal disease, exposed during development (Getz et al., 2012). Structural MRI studies found no alterations in white or gray matter volumes or white matter hypointensities (Janulewicz et al., 2013), suggesting that the observed color vision and contrast sensitivity alterations were due to retinal changes (i.e., photoreceptors and parvocellular or P-type retinal ganglion cells). At first glance, the spatial contrast sensitivity and color vision deficits appear similar to those from children prenatally exposed to TCE (Till et al., 2005). However, the children with prenatal TCE exposure had reductions in contrast sensitivity in the low and intermediate frequency range and abnormal red–green, but not blue–yellow, vision (Till et al., 2005).

Deficits in visual function such as contrast sensitivity have been observed in residents of neighborhoods containing dry cleaners using PERC (Altmann et al., 1995) and in residents of apartment buildings with co-located dry cleaners (Schreiber et al., 2002). For both of these residential studies, the atmospheric concentrations of PERC were below those typical of occupational settings. Laboratory experiments with human subjects exposed to PERC for 4 hours for 4 days revealed increased peak latency delays in the N75, P100, and N150 of the visual evoked potential (VEP) as well as decreases in contrast sensitivity at low and intermediate spatial frequencies (Altmann et al., 1990). Acute PERC inhalation exposure also reduced pattern VEP amplitudes in rats (Boyes et al., 2009). Recently, a study by the New York State Department of Health reported mild, but statistically significant, deficits of contrast sensitivity in children living in apartment buildings with co-located PERC dry cleaners in comparison to residents of control buildings without dry cleaners (Storm et al., 2011). The above results reveal that PERC is toxic to both the developing and adult visual system.

Styrene Six independent studies report that workers exposed to mean atmospheric concentrations of styrene ranging from 20 to 70 ppm exhibit concentration-dependent alterations in color vision (Gobba et al., 1991; Fallas et al., 1992; Chia et al., 1994;

Campagna et al., 1995; Eguchi et al., 1995; Iregren et al., 2005). A combined data analysis from two of the above studies (Gobba et al., 1991; Campagna et al., 1995) suggests that the threshold for color visual impairments is ≤4 ppm styrene (Campagna et al., 1996). This was well below the threshold limit value-time-weighted average (TLV–TWA) value for any country: range 20 to 50 ppm. The similarity of the styrene-induced blue–yellow color vision deficits observed by five independent groups demonstrates the reproducibility of these color vision deficits. In addition, two meta-analysis studies of solvent exposure and color vision are consistent with these results. Paramei et al. (2004) evaluated the color confusion index (CCI) scores from 15 studies of workers exposed to toluene, styrene, or mixed solvents. They concluded that while 13 of the original 15 studies reported CCI values indicative of impaired performance in the solvent-exposed group, the large variations among the effect sizes across studies obscured an overall statistically significant association between exposure and CCI values for all the substances except styrene, which was significantly associated with impaired color discrimination. Benignus et al. (2005) focused on only reports of styrene exposure from the six independent studies and also observed a statistically significant relationship between cumulative styrene exposure and increased CCI scores. The slope of the function observed suggested that the magnitude of decrement in CCI scores for a worker exposed to 20 ppm styrene for eight years would be equivalent to an additional 1.7 years of aging (Benignus et al., 2005). The reversibility of these impairments has not been thoroughly studied, although in one study no recovery was found after a 1-month period of no exposure (Gobba et al., 1991). Rats exposed to styrene for 13 weeks showed lower retinal dopamine content and fewer tyrosine hydroxylase immunoreactive retinal amacrine cells (Vettori et al., 2000). In summary, there is a concordance of evidence that styrene exposure is associated with retinal toxicity in experimental animals and color vision deficits in occupationally exposed workers.

Toluene Toluene is one of the most widely used substances in industry and commerce. It is used as a solvent, degreaser, constituent of products such as paints and glues, and is a substantial component of gasoline and other fuels. Among the neurotoxic consequences of occupational toluene exposure are impaired color vision (Cavalleri et al., 2000; Campagna et al., 2001) and reduced visual contrast sensitivity (Donoghue et al., 1995). Acute toluene exposure impairs oculomotor function (Niklasson et al., 1995) and reduces pattern VEP amplitudes in proportion to estimated concentration of toluene in the brain (Boyes et al., 2007). Moreover, toluene has a relatively low irritancy and a high euphoric potential making it a favored selection among inhalant drug abusers; many are young and expose themselves repeatedly to very high toluene concentrations. Abuse of toluene is associated with poor visual acuity, altered or unrecordable pattern VEPs, optic neuropathy, abnormal MRI signals in visual cortex, decreased perfusion of thalamus and cerebral cortex, and visual hallucinations (Kamran and Bakshi, 1998; Ryu et al., 1998; Kiyokawa et al., 2005). Recent ERG and pattern VEP experimental studies in pigmented rats reveals that 13 weeks, but not 4 weeks, of 1000 ppm toluene exposure decreased the peak b-wave amplitude, but did not alter the VEP, up to 1 year following exposure (Boyes et al., 2016). Based on histology and immunohistochemistry results, the authors suggested that inner retinal neurons were selectively affected (Boyes et al., 2016).

Organophosphates The neurotoxicity of organophosphates is well established (see Chap. 16); however, the link between organophosphate exposure and retinotoxicity is unresolved. Clinical studies conducted in Japan report on ocular toxicity from laboratory animals exposed to organophosphates, and reports to the EPA by pesticide manufacturers suggest that various organophosphates produce retinotoxicity and chronic ocular damage (Ishikawa, 1973; Dementi, 1994). However, many of the early clinical reports were poorly designed and remain unconfirmed. The evidence for organophosphate-induced retinal toxicity is strongest for fenthion (dimethyl 3-methyl-4-methylthiophenyl phosphorothionate) (Imai et al., 1983; Misra et al., 1985; Boyes et al., 1994; Tandon et al., 1994). Two epidemiological studies of licensed pesticide applicators and their spouses did not find a statistically increased risk of retinal degeneration from use of organophosphate insecticides as a class, but risks were increased for some individual organophosphate chemicals (Kamel et al., 2000; Kirrane et al., 2005). Interestingly, both studies identified an increased risk of retinal degeneration in individuals exposed to fungicides. There were also reports that Japanese children exposed to organophosphates had a high incidence of myopia (Ko et al., 1988). Experimentally, the visual control of ocular growth, which is cholinergically mediated, was impaired in the eyes of chicks exposed to the organophosphate insecticide chlorpyrifos (Geller et al., 1998). Embryonic chick retinal cells did not develop normally when exposed to diazinon in vitro (Paraoanu et al., 2006). Currently, the mechanisms of ocular toxicity, sites of action, and whether the effects are restricted to some specific organophosphates such as fenthion or are more general to the chemical class are unknown. The use of organophosphate insecticides has been restricted (but not eliminated) in North America and Europe in recent years, but continues in much of the developing world. Thus, it is important to resolve the potential risks of ocular toxicity for this class of chemicals.

TARGET SITES AND MECHANISMS OF ACTION: MITOCHONDRIAL OPTIC NEUROPATHIES (MONS)

The optic nerve consists primarily of retinal ganglion cell (RGC) axons carrying visual information from the retina to several distinct anatomic destinations in the CNS. Both myelinated and nonmyelinated axons are present and grouped into bundles of axons that maintain a topographic distribution with respect to the site of origin in the retina. Optic nerve axons have a high rate of oxygen consumption and the unmyelinated prelaminar portion of the optic nerve has a higher number of mitochondria and sodium-gated ion channels than the myelinated postlaminar region (Bristow et al., 2002). The fibers of the papillomacular bundle (PMB), a group of RGCs that carry the information from central retina to the optic nerve, are the most vulnerable to acquired and genetic damage as they have small diameter long unmyelinated fibers in the retina (Wang and Sadun, 2013). Mitochondrial Optic Neuropathies (MON) include the genetic MONs (e.g., Leber's hereditary optic neuropathy and autosomal dominant optic atrophy), nutritional MONs (e.g., decreased folic acid and B12), and drug-induced MONs (Wang and Sadun, 2013). The clinical features of MONs include relatively symmetric visual impairment, loss of high spatial frequency contrast sensitivity (decreased visual acuity), early and profound loss of color vision (often red), central visual field deficits with spared peripheral vision, (delayed) temporal pallor of the optic discs and normal pupillary light reflex (Altiparmak, 2013; Wang and Sadun, 2013). Numerous tools are needed to properly diagnose the early changes (e.g., visual field, color vision, and contrast sensitivity testing; VEP P100 amplitude; OCT).

At the optic chiasm, the fibers split so that, in humans and other primates, about 50% of those fibers originating from the

temporal retina continue in the optic tract toward the ipsilateral side of the brain, while 50% of those fibers originating in the nasal half of the retina cross the midline and project to the contralateral side of the brain (Neveu and Jeffery, 2007). In pigmented rodents, about 90% of the fibers cross the midline, whereas in albino rodents this is almost 100% (Neveu and Jeffery, 2007). Fibers from the optic nerve terminate in the dorsal LGN, the superior colliculus, and pretectal areas. Information passing through the LGN to the visual cortex gives rise to conscious visual perception. Information traveling to the superior colliculus is used to generate eye movements. Pathways leading to the pretectal areas subserve the pupil response. The lateral geniculate nucleus (LGN) of primates contains six histological layers that are alternately innervated by cells from the contralateral and ipsilateral eyes. The cells projecting to and from the ventral two layers of the LGN have large cell bodies, and consequently, this pathway is referred to as the magnocellular system. RGCs projecting to the magnocellular layers of the LGN are referred to as either M-type or Pα cells. Magnocellular neurons are sensitive to fast moving stimuli and to low levels of luminance contrast, but are insensitive to differences in color. The cells from the magnocellular pathway are involved in motion perception. On the dorsal side of the LGN, the cells are smaller and form the parvocellular pathway. RGCs projecting to the parvocellular layers of the LGN are referred to as P-type or Pβ cells. Parvocellular neurons are sensitive to color and to fine detailed patterns, have slower conduction velocities, and are involved in perception of color and form (Kaplan and Benardete, 2001). Compared to magnocellular cells, parvocellular cells have greater spatial, but lower temporal resolution.

Acrylamide

Acrylamide monomer is used in a variety of industrial and laboratory applications, where it serves as the basis for the production of polyacrylamide gels and other polyacrylamide products. Exposure to acrylamide produces a distal axonopathy in large-diameter axons of the peripheral nerves and spinal cord (Spencer and Schaumburg, 1974a, 1974b). Visual effects of acrylamide exposure occur at dose levels sufficient to cause substantial peripheral neuropathy, but the selective nature of the visual deficits and associated neuropathology is very instructive. Although the large-diameter and long axons are most vulnerable to acrylamide in the peripheral nerve and spinal cord, this is not the case in the optic tract. The middle diameter axons of the P_{β}-type retinal ganglion cells (RGCs) that project to the parvocellular layers of the LGN of New- and Old-World primates degenerate after prolonged treatment with acrylamide (Eskin and Merigan, 1986; Lynch et al., 1989). The larger-diameter P_{α}-type RGCs that project to the magnocellular layers of the LGN are apparently spared. Visual function testing in these primates, without a functional parvocellular system, revealed selective perceptual deficits in detecting visual stimuli with high spatial–frequencies (i.e., fine visual patterns) and low temporal–frequencies (i.e., slowly modulating sine waves) (Merigan and Eskin, 1986). However, the monkeys' perception of larger visual patterns, modulated at higher temporal rates, was not impaired. These experiments helped elucidate the functional differentiation of primate parvocellular and magnocellular visual systems. Why the axons of the optic nerve and tract show a different size-based pattern of vulnerability than do axons of the peripheral nerve and spinal cord is not understood. Norwegian tunnel workers examined 2 to 10 years after using acrylamide and *n*-methylolacrylamide, for grouting operations, had altered color discrimination, light sensitivity, and VEP latencies (Goffeng et al., 2008a, 2008b). This indicates the sensitivity of and persistent adverse effects on the human visual system to occupational acrylamide exposure. Many of the histopathological, clinical, and behavioral effects of acrylamide are consistent with MONs. Although the mechanism of action of acrylamide is unknown, several reports indicate that altered mitochondrial and energy metabolism underlie the pathophysiology (Sabri, 1994; Chen et al., 2013; Liu et al., 2015).

Carbon Disulfide

Carbon disulfide (CS_2) is used in industry to manufacture viscose rayon, carbon tetrachloride, and cellophane. The neurotoxicity of CS_2 is well known and involves damage to the peripheral and CNSs (see Chap. 16) as well as profound effects on vision (Beauchamp et al., 1983). The peripheral neuropathy results from a distal axonal degeneration of the large-caliber and long axons of the peripheral nerves and spinal cord, probably through the reactions with the sulfhydryl groups of axonal neurofilament proteins, yielding covalent cross linkages that lead to filamentous tangles and axonal swellings (Graham and Valentine, 2000). Workers chronically exposed to CS_2 experience loss of visual function accompanied by observable lesions in the retinal vasculature. The visual function and structural deficits reported in viscose rayon workers include central scotoma, depressed visual sensitivity in the peripheral visual field, optic atrophy, pupillary disturbances, blurred vision, and disorders of color perception. A workplace study of 123 Belgian viscose rayon workers found statistical associations between a weighted cumulative CS_2 exposure score and deficits in color vision measured using the Farnsworth-Munsell 100-HUE test, as well as excess microaneurysms observed ophthalmoscopically and by fundus photographs (Vanhoorne et al., 1996). This association was not observed in the 42 workers who were never exposed to levels above the TLV value of 31 mg/m^3. The coexistence of retinal microaneurysms with functional loss led to the presumption that the visual deficits were a secondary consequence of vascular disease and perhaps of retinal hemorrhages.

This association was addressed in CS_2-exposed macaque monkeys used in visual psychophysical, fluorescein angiography, and fundus photography studies as well as postmortem neuropathological evaluations (Merigan et al., 1988; Eskin et al., 1988). They observed markedly decreased contrast sensitivity functions, decreased visual acuity, and degeneration of the retinal ganglion cells (RGCs), all of which occurred in the absence of retinal microaneurysms or hemorrhages. There was little evidence of effects on the other retinal neurons. These findings indicate that the retinal and optic nerve pathology produced by CS_2 are likely a direct neuropathological action and not the indirect result of vasculopathy. Interestingly, and importantly, after cessation of exposure, the visual acuity measures recovered temporarily in two of the CS_2-treated monkeys; however, the contrast sensitivity measures did not recover. This demonstrates the independence of these two measures and the utility and importance of independent evaluations of contrast sensitivity and visual acuity. In these studies, many of the histopathological, clinical, and behavioral effects of CS_2 were consistent with MONs. Although the mechanism of action of CS_2 is unknown, several reports indicate that altered mitochondrial and energy metabolism underlie the pathophysiology (Tarkowski and Sobczak, 1971; Tarkowski et al., 1980; Guo et al., 2014; Pan et al., 2016).

Cuban Epidemic of Optic Neuropathy

During 1992 and 1993, an epidemic occurred in Cuba in which over 50,000 people suffered from optic neuropathy, sensory and

autonomic peripheral neuropathy, high-frequency neural hearing loss, and myelopathy. This is the largest epidemic of neurological disease in the 20th century (Roman, 1998). The affected individuals were characterized as having bilateral low visual acuity, impaired color perception, impaired visual contrast sensitivity, central scotoma, optic disc pallor, and, in particular, loss of nerve fibers from the papillomacular bundle (Sadun et al., 1994a; Hedges et al., 1997). Individuals with neurological findings demonstrated stocking-glove sensory deficits, leg cramps, sensory ataxia, altered reflexes, and complaints of memory loss (Mojon et al., 1997). Various authors noticed similarities between the Cuban cases and nutritional or alcohol–tobacco amblyopia, B12 deficiency, Leber's hereditary optic neuropathy, and Strachan disease (Sadun et al., 1994b; Mojon et al., 1997). The optic neuropathy resembled methanol poisoning (Roman, 1998; Sadun, 1998). The outbreak of the epidemic was linked to nutritional deficiencies (Hedges et al., 1997; Mojon et al., 1997; Roman, 1998). Exposure to toxicants could not be documented in most of the people identified in the epidemic, suggesting nutritional deficit as the principal cause. However, coexposure to low levels of mitochondrial toxicants or other factors may have pushed individuals over a threshold for causing nerve damage. Regardless, many of the clinical and behavioral effects observed in these affected individuals were consistent with MON.

Ethambutol

The dextro isomer of ethambutol is widely used as an antimycobacterial drug for the treatment of tuberculosis. Of the over 9 million new cases of tuberculosis worldwide, over half of the patients take ethambutol and 100,000 develop optic neuropathy (Wang and Sadun, 2013). It is well known that ethambutol produces dose-related alterations in the visual system, such as blue–yellow and red–green dyschromatopsias, decreased contrast sensitivity, reduced visual acuity, and visual field loss. The earliest visual symptoms appear to be a decrease in contrast sensitivity and color vision, although impaired red–green color vision is the most frequently observed and reported complaint. However, the loss of contrast sensitivity may explain why some patients with normal visual acuity and color perception still complain of visual disturbance. These visual system alterations can occur with a few weeks of doses equal to or greater than 20 mg/kg body weight; however, they usually become manifest after several months of treatment (Koliopoulos and Palimeris, 1972; Polak et al., 1985; Salmon et al., 1987). The symptoms are primarily associated with one of two forms of optic neuropathy. The most common form, seen in almost all cases, involves the central optic nerve fibers or papillomacular bundle (PMB) and typically results in a central or paracentral scotoma in the visual field and is associated with impaired red–green color vision and decreased visual acuity, whereas the second form involves the peripheral optic nerve fibers and typically results in a peripheral scotoma and visual field loss (Lessell, 1998; Bartlett and Jaanus, 2008). The most common clinical and behavioral effects observed in patients taking ethambutol are pathognomonic for a drug-induced MON.

In experimental animals, ethambutol causes retinal ganglion cell (RGC) and optic nerve degeneration and possibly amacrine and bipolar cell alterations (van Dijk and Spekreijse, 1983; Grant and Schuman, 1993; Sjoerdsma et al., 1999). Although the mechanism responsible for producing the RGC and optic nerve degeneration is unknown, recent in vivo studies in rats and in vitro rat RGC cell culture experiments suggest that ethambutol causes RGC death secondary to glutamate-induced excitotoxicity (Heng et al., 1999). Pharmacological studies, using the in vivo and in vitro models, show that although ethambutol is not a direct NMDA-receptor agonist, it makes RGCs more sensitive to endogenous levels of glutamate. Using the fluorescent Ca^{2+} dyes calcium green 1-AM and rhod-2, Heng et al. (1999) showed that following application of ethambutol in the presence, but not absence, of glutamate to isolated RGCs, there was a decrease in cytosolic Ca^{2+} and a subsequent increase in mitochondrial Ca^{2+}. Interestingly, the increase in mitochondrial Ca^{2+} resulted in an increase in the mitochondrial membrane potential as measured by the mitochondrial membrane potential sensitive dye JC-1. The authors (Heng et al., 1999) postulate that this latter phenomenon occurs as a result of an ethambutol-mediated chelation of Zn^{2+} from the mitochondrial ATPase inhibitor protein IF1 (Rouslin et al., 1993) that subsequently results in the inhibition of mitochondrial ATP synthesis and elevation of mitochondrial membrane potential.

TARGET SITES AND MECHANISMS OF ACTION: THE CENTRAL VISUAL SYSTEM

Many areas of the cerebral cortex are involved in the perception of visual information. The primary visual cortex (V1) (i.e., Brodmann area 17 or striate cortex) receives the primary projections of visual information from the lateral geniculate nucleus (LGN) and also from the superior colliculus. Neurons from the LGN project to the visual cortex maintaining a topographic representation of the receptive field origin in the retina. The receptive fields in the left and right sides of area 17 reflect the contralateral visual world and representations of the upper and lower regions of the visual field are separated below and above, respectively, the calcarine fissure. Cells in the posterior aspects of the calcarine fissure have receptive fields located in the central part of the retina. Cortical cells progressively deeper in the calcarine fissure have retinal receptive fields that are located more and more peripherally in the retina. The central part of the fovea has tightly packed photoreceptors for resolution of fine detailed images. The cortical representation of the central fovea is proportionately larger than the peripheral retina, which accommodates a proportionately larger need for neural image processing. The magnocellular and parvocellular pathways project differently to the histologically defined layers of primary striate visual cortex and then to extrastriate visual areas. The receptive fields of neurons in the visual cortex are more complex than the circular center-surround arrangement found in the retina and LGN. Cortical cells respond better to lines of a particular orientation than to simple spots. The receptive fields of cortical cells are thought to represent computational summaries of a number of simpler input signals. As the visual information proceeds from area V1 to extrastriate visual cortical areas, the representation of the visual world reflected in the receptive fields of individual neurons becomes progressively more complex (Kaplan and Benardete, 2001).

Lead

In addition to the well-documented retinal effects of lead (vide supra), lead exposure during adulthood or perinatal development produces structural, biochemical, and functional deficits in the visual cortex of humans, nonhuman primates, and rats (Fox et al., 1977; Costa and Fox, 1983; Otto et al., 1985; Lilienthal et al., 1988; Reuhl et al., 1989; Murata et al., 1993; Otto and Fox, 1993; Winneke et al., 1994; Altmann et al., 1998; Ethier et al., 2012). Quantitative morphometric studies in monkeys exposed to either high levels of lead from birth or infancy to 6 years of age revealed a decrease in visual cortex (areas V1 and V2), cell volume density, and a decrease in the number of initial arborizations among

pyramidal neurons (Reuhl et al., 1989). The former results may be due to an absolute decrease in total cell numbers, possibly resulting from lead-induced apoptosis as observed in the retina (Fox et al., 1997; He et al., 2000, 2003). This may also account for the decreased density of cholinergic muscarinic receptors found in the visual cortex of adult rats following moderate level developmental lead exposure (Costa and Fox, 1983). The morphometric results on neuronal branching are reminiscent of earlier findings in the neocortex of rats following high level developmental lead exposure (Petit and LeBoutillier, 1979), and recent findings in the somatosensory cortex of rats following low or moderate level developmental lead exposure (Wilson et al., 2000). These alterations could contribute to the decreases in contrast sensitivity (Fox, 1984; Rice, 1998), flash and pattern-reversal evoked potentials (Fox et al., 1977; Otto et al., 1985; Lilienthal et al., 1988; Murata et al., 1993; Winneke et al., 1994; Altmann et al., 1998), and visual function (Hansen et al., 1989; Muñoz et al., 1993; Ethier et al., 2012) in lead-exposed laboratory animals or humans.

Methyl Mercury

Methyl mercury became notorious in two episodes of mass poisoning (see Chaps. 16 and 23). In the 1950s, industrial discharges of mercury into Minamata Bay in Japan became biomethylated to form methyl mercury, which then accumulated in the food chain and reached toxic concentrations in the fish and shellfish consumed in the surrounding communities. Hundreds of people were poisoned, showing a combination of sensory, motor, and cognitive deficits. A more widespread episode of methyl mercury poisoning affected thousands of Iraqi citizens who mistakenly ground wheat grain into flour that had been treated with methyl mercury as a fungicide and that was intended for planting and not for direct human consumption.

Visual deficits are a prominent feature of methyl mercury intoxication in adult humans, along with several other neurological manifestations such as difficulties with somatosensory sensation, gait, memory, and cognition. Methyl mercury poisoned individuals experienced a striking and progressive constriction of the visual field (peripheral scotoma) as patients became progressively less able to see objects in the visual periphery (Iwata, 1977). The narrowing of the visual field gives impression of looking through a long tunnel, hence the term tunnel vision. Visual field constrictions also have been observed in methyl mercury-poisoned monkeys (Merigan, 1979). On autopsy of some of the Minamata patients, focal neurological degeneration was observed in several brain regions including motor cortex, cerebellum, and calcarine fissure of visual cortex (Takeuchi and Eto, 1977). The histopathological feature was a destruction of the cortical neural and glial cells, with sparing of the subcortical white matter, optic radiations, and lateral geniculate nucleus (LGN). Monkeys and dogs that were treated experimentally with methyl mercury showed greater damage in the calcarine fissure, associated with higher regional concentrations of protein-bound mercury, than in other brain regions (Yoshino et al., 1966; Berlin et al., 1975). In the Minamata patients, there was a regional distribution of damage observed within striate cortex, such that the most extensive damage occurred deep in the calcarine fissure and was progressively less in the more posterior portions. Thus, the damage was most severe in the regions of primary visual cortex that subserved the peripheral visual field, with relative sparing of the cortical areas representing the central vision. This regional distribution of damage corresponded with the progressive loss of peripheral vision while central vision was relatively preserved. Methyl mercury-poisoned individuals also experienced poor night vision (i.e., scotopic vision deficits), also attributable to peripheral visual field losses. Similar changes were observed in adult monkeys exposed to methyl mercury (Berlin et al., 1975). Mercury also accumulates in the retina of animals exposed to methyl mercury (DuVal et al., 1987). Acute exposure of isolated retinas to mercury or methyl mercury produces rod-selective electrophysiological and morphological alterations (Fox and Sillman, 1979; Braekevelt, 1982), whereas subacute dosing with methyl mercury alters the photopic ERG prior to the scotopic ERG (Goto et al., 2001). The neurological damage in adult cases of Minamata disease was focally localized in the calcarine cortex and other areas but was more globally distributed throughout the brain in those developmentally exposed.

The levels of methyl mercury exposure experienced by people in Minamata Bay were undoubtedly high. Studies of visual function in nonhuman primates exposed to methyl mercury during perinatal development demonstrate a decrease in visual contrast sensitivity, visual acuity, and temporal flicker resolution at dose levels lower than those associated with constriction of the visual fields (Rice and Gilbert, 1990; Rice, 1996). Monkeys exposed to methyl mercury from birth onward or in utero plus postnatally exhibited spatial vision deficits under both high and low luminance conditions, although the deficits were greater under scotopic illumination (Rice, 1998). The effects on temporal vision were different. That is, monkeys exposed from birth displayed superior low-luminance temporal vision, whereas high-luminance temporal vision was not impaired. In contrast, monkeys exposed to methyl mercury in utero plus postnatally exhibited deficits in low-frequency high-luminance temporal vision, whereas low-luminance temporal vision was superior to that of control monkeys (Rice, 1998). These data indicate that the spatial and temporal vision deficits produced by developmental exposure to methyl mercury are different from those produced during adulthood. These findings are reminiscent of the opposite results in the retinal ERGs and histology following gestational lead exposure (Giddabasappa et al., 2011; Chaney et al., 2016) compared to postnatal lead exposure (Fox et al., 1997; He et al., 2003).

TOXICANTS AS RISK FACTORS FOR ADULT-ONSET RETINAL DISEASES

The interaction of toxicant exposure with underlying genetics is an area of increased concern and study by the WHO and NIH (WHO, 2004; NIEHS, 2012). Several recent studies suggest that occupational exposure to neurotoxicants may increase the risk for a number of age-related retinal diseases. Increased lead accumulation, as measured by hair lead levels, was positively associated with increased primary open-angle glaucoma in females ~60 years old, but not in males that were ~50 years old (Yuki et al., 2009). Moreover, this association was significant in females with low, but not high tension, glaucoma (Yuki et al., 2009). Elevated neural retinal lead was also positively associated with an increased incidence of age-related macular degeneration (AMD) in 50 donor eyes compared to 72 control eyes (Erie et al., 2009a). Reduced levels of retinal pigment epithelium (RPE) copper and zinc were found in donor retinas with defined AMD relative to controls (Erie et al., 2009b). Lead accumulates in the retina and RPE of non-occupationally exposed humans (Eichenbaum and Zheng, 2000; Erie et al., 2005), avidly binds to melanin like copper and zinc (Ulshafer et al., 1990), and can displace copper and zinc from several proteins (Kirberger and Yang, 2008). Thus, lead may increase the risk of age-related macular degeneration (AMD) via divalent cation displacement. Alternatively, lead-induced increases in oxidative stress and/or inflammatory cytokine production (Stohs and Bagchi,

1995) are potential mechanisms contributing to AMD. Consistent with these findings, adult rats exposed to lead had increased blood–retinal permeability, which is a risk factor for AMD and diabetic retinopathy (Shen et al., 2016). Three different studies suggest that occupational exposure to organic solvents could trigger Leber's Hereditary Optic Neuropathy: a maternally inherited loss of central vision due to one of three mitochondrial DNA (mtDNA) point mutations (Johns et al., 1992; Rufa et al., 2005; Carelli et al., 2007).

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REFERENCES

Abd-El-Barr MM, Pennesi ME, Saszik SM, et al. Genetic dissection of rod and cone pathways in the dark-adapted mouse retina. *J Neurophysiol*. 2009;102:1945–1955.

Adamus G, Machnicki M, Elerding H, et al. Antibodies to recoverin induce apoptosis of photoreceptor and bipolar cells in vivo. *J Autoimmun*. 1998;11:523–533.

Adijanto J, Naash MI. Nanoparticle-based technologies for retinal gene therapy. *Eur J Pharm Biopharm*. 2015;95(Pt B):353–367.

Agency for Toxic Substances and Disease Registry. *Neurobehavioral Test Batteries for Use in Environmental Health Field Studies*. Atlanta, GA: U.S. Department of Health, Public Health Service; 1992.

Ahmed J, Braun RD, Dunn R Jr, Linsenmeier RA. Oxygen distribution in the macaque retina. *Invest Ophthalmol Vis Sci*. 1993;34:516–521.

Ah-Song R, Sasco AJ. Tamoxifen and ocular toxicity. *Cancer Detect Prev*. 1997;21:522–531.

Albert D, Miller JW, Azar DT, Blodi BA. *Albert & Jakobiec's Principles & Practice of Ophthalmology*. 3rd ed. New York: Saunders; 2008.

Alken RG, Belz GG. A comparative dose-effect study with cardiac glycosides assessing cardiac and extracardiac responses in normal subjects. *J Cardiovasc Pharmacol*. 1984;6:634–640.

Altiparmak UE. Toxic optic neuropathies. *Curr Opin Ophthalmol*. 2013;24:534–539.

Altmann L, Bottger A, Wiegand H. Neurophysiological and psychophysical measurements reveal effects of acute low-level organic solvent exposure in humans. *Int Arch Occup Environ Health*. 1990;62:493–499.

Altmann L, Florian Neuhann H, Kramer U, Witten J, Jermann E. Neurobehavioral and neurophysiological outcomes of chronic low-level tetrachloroethene exposure measured in neighborhoods of dry cleaning shops. *Environ Res*. 1995;69:83–89.

Altmann L, Sveinsson K, Kramer U, et al. Visual functions in 6-year-old children in relation to lead and mercury levels. *Neurotoxicol Teratol*. 1998;20:9–17.

Al-Modhefer AJA, Bradbury MWB, Simons TJB. Observations on the chemical nature of lead in human blood serum. *Clin Sci*. 1991;81:823–829.

American Medical Association. Harmful effects of ultraviolet radiation. Council on Scientific Affairs. *JAMA*. 1989;262:380–384.

Anger WK, Johnson BL. Chemicals affecting behavior. In: O'Donoghue JL, ed. *Neurotoxicity of Industrial and Commercial Chemicals*. Boca Raton, FL: CRC Press; 1985:51–148.

Anger WK, Letz R, Chrislip DW, et al. Neurobehavioral test methods for environmental health studies of adults. *Neurotoxicol Teratol*. 1994;16:489–497.

Asakura T, Matsuda M, Matsuda S, Shichi H. Synthesis of 12(R)- and 12(S)-hydroxyeicosatetraenoic acid by porcine ocular tissues. *J Ocul Pharmacol*. 1994;10:525–535.

Atalla LR, Sevanian A, Rao NA. Immunohistochemical localization of glutathione peroxidase in ocular tissue. *Curr Eye Res*. 1988;7:1023–1027.

Attarchi MS, Labbafinejad Y, Mohammadi S. Occupational exposure to different levels of mixed organic solvents and colour vision impairment. *Neurotoxicol Teratol*. 2010;32:558–562.

Awasthi YC, Saneto RP, Srivastava SK. Purification and properties of bovine lens glutathione *S*-transferase. *Exp Eye Res*. 1980;30:29–39.

Backstrom B, Collins VP. The effects of 2,5-hexanedione on rods and cones of the retina of albino rats. *NeuroToxicology*. 1992;13:199–202.

Backstrom B, Nylen P, Hagman M, et al. Effect of exposure to 2,5-hexanediol in light or darkness on the retina of albino and pigmented rats. I. Morphology. *Arch Toxicol*. 1993;67:277–283.

Baghdassarian SA. Optic neuropathy due to lead poisoning. *Arch Ophthalmol*. 1968;80:721–723.

Baker EL, Feldman RG, White RA, et al. Occupational lead neurotoxicity: a behavioral and electrophysiological evaluation. *Br J Ind Med*. 1984;41:352–361.

Banh A, Bantseev V, Choh V, Moran KL, Sivak JG. The lens as a focusing device and its response to stress. *Prog Retin Eye Res*. 2005;25:189–206.

Barltrop D, Barrett AJ, Dingle JT. Subcellular distribution of lead in the rat. *J Lab Clin Med*. 1971;77:705–712.

Bartlett JD, Jaanus SD. *Clinical Ocular Pharmacology*. 5th ed. Boston, MA: Butterworth-Heinemann; 2008.

Battaglia L, Serpe L, Foglietta F, et al. Application of lipid nanoparticles to ocular drug delivery. *Expert Opin Drug Deliv*. 2016;13:1743–1757.

Baumbach GL, Cancilla PA, Martin-Amat G, et al. Methyl alcohol poisoning: IV. Alterations of the morphological findings of the retina and optic nerve. *Arch Ophthalmol*. 1977;95:1859–1865.

Bausher LP. Identification of A and B forms of monoamine oxidase in the iris-ciliary body, superior cervical ganglion, and pineal gland of albino rabbits. *Invest Ophthalmol*. 1976;15:529–537.

Baylor DA, Nunn BJ, Schnapf JL. The photocurrent, noise and spectral sensitivity of rods of the monkey *Macaca fascicularis*. *J Physiol*. 1984;357:575–607.

Beauchamp RO Jr, Bus JS, Popp JA, et al. A critical review of the literature on carbon disulfide toxicity. *CRC Crit Rev Toxicol*. 1983;11:168–277.

Behndig A, Svensson B, Marklund SL, Karlsson K. Superoxide dismutase isoenzymes in the human eye. *Invest Ophthalmol Vis Sci*. 1998;39:471–475.

Benignus VA, Geller AM, Boyes WK, Bushnell PJ. Human neurobehavioral effects of long-term exposure to styrene: a meta-analysis. *Environ Health Perspect*. 2005;113:532–538.

Benton CD, Calhoun FP. The ocular effects of methyl alcohol poisoning: report of a catastrophe involving three hundred and twenty persons. *Trans Am Acad Ophthalmol*. 1952;56:875–883.

Berlin M, Grant CA, Hellberg J, et al. Neurotoxicity of methyl mercury in squirrel monkeys. Cerebral cortical pathology, interference with scotopic vision, and changes in operant behavior. *Arch Environ Health*. 1975;30:340–348.

Birch D, Jacobs GH. Spatial contrast sensitivity in albino and pigmented rats. *Vision Res*. 1979;19:933–937.

Bisht R, Mandal A, Jaiswal JK, Rupenthal ID. Nanocarrier mediated retinal drug delivery: overcoming ocular barriers to treat posterior eye diseases. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*. 2017 Apr 20. [Epub ahead of print].

Bloomfield SA, Völgyi B. The diverse roles and regulation of neuronal gap junctions in the retina. *Nat Rev Neurosci*. 2009;10:496–506.

Boyes WK, Bercegeay M, Degn L, et al. Toluene inhalation exposure for 13 weeks causes persistent changes in electroretinograms of Long-Evans rats. Toluene inhalation exposure for 13 weeks causes persistent changes in electroretinograms of Long-Evans rats. *Neurotoxicology*. 2016;53:257–270.

Boyes WK, Bercegeay M, Krantz QT, et al. Acute toluene exposure and rat visual function in proportion to momentary brain concentration. *Toxicol Sci*. 2007;99:572–581.

Boyes WK, Bercegeay M, Oshiro WM, et al. Acute perchloroethylene exposure alters rat visual-evoked potentials in relation to brain concentrations. *Toxicol Sci*. 2009;108:159–172.

Boyes WK, Chen R, Chen C, Yokel RA. The neurotoxic potential of engineered nanomaterials. *NeuroToxicology*. 2012;33:902–910.
Boyes WK, Dyer RS. Pattern reversal visual evoked potentials in awake rats. *Brain Res Bull*. 1983;10:817–823.
Boyes WK, Dyer RS. Chlordimeform produces profound but transient changes in visual evoked potentials of hooded rats. *Exp Neurol*. 1984;86:434–447.
Boyes WK, Tandon P, Barone S Jr, Padilla S. Effects of organophosphates on the visual system of rats. *J Appl Toxicol*. 1994;14:135–143.
Boyes WK, Thornton BL, Al-Abed SR, et al. Comprehensive framework for evaluating the environmental health and safety implications of engineered nanomaterials. *Crit Rev Toxicol.* 2017;47:767–810.
Braekevelt CR. Morphological changes in rat retinal photoreceptors with acute methyl mercury intoxication. In: Hollyfield JG, ed. *The Structure of the Eye*. New York: Elsevier; 1982:123–131.
Braun RD, Linsenmeier RA, Goldstick TK. Oxygen consumption in the inner and outer retina of the cat. *Invest Ophthalmol Vis Sci*. 1995;36:542–554.
Brigell M, Dong CJ, Rosolen S, Tzekov R. An overview of drug development with special emphasis on the role of visual electrophysiological testing. *Doc Ophthalmol*. 2005;110:3–13.
Bristow EA, Griffiths PG, Andrews RM, et al. The distribution of mitochondrial activity in relation to optic nerve structure. *Arch Ophthalmol*. 2002;120:791-796.
Broadwell DK, Darcey DJ, Hudnell HK, et al. Worksite clinical and neurobehavioral assessment of solvent-exposed workers. *Am J Indust Med*. 1995;27:677–698.
Bull RJ. Lead and energy metabolism. In: Singhal RL, Thomas JA, eds. *Lead Toxicity*. Baltimore, MD: Urban and Schwarzenberg; 1980:119–168.
Burns CA. Ocular effects of indomethacin. Slit lamp and electroretinographic: ERG study. *Invest Ophthalmol*. 1966;5:325–331.
Burns CA. Indomethacin, reduced retinal sensitivity and corneal deposits. *Am J Ophthalmol*. 1968;66:825–835.
Butler MC, Itotia PN, Sullivan JM. A high-throughput biophotonics instrument to screen for novel ocular photosensitizing therapeutic agents. *Invest Ophthalmol Vis Sci*. 2010;51:2705–2720.
Campagna D, Gobba F, Mergler D, et al. Color vision loss among styrene-exposed workers: neurotoxicological threshold assessment. *NeuroToxicology*. 1996;17:367–373.
Campagna D, Mergler D, Huel G, et al. Visual dysfunction among styrene-exposed workers. *Scand J Work Environ Health*. 1995;21:382–390.
Campagna D, Stengel B, Mergler D, et al. Color vision and occupational toluene exposure. *Neurotoxicol Teratol*. 2001;23:473–480.
Carelli V, Franceschini F, Venturi S, et al. Grand rounds: could occupational exposure to n-hexane and other solvents precipitate visual failure in Leber hereditary optic neuropathy? *Environ Health Perspect*. 2007;115:113–115.
Cavalleri A, Gobba F, Nicali E, Fiocchi V. Dose-related color vision impairment in toluene-exposed workers. *Arch Environ Health*. 2000;55:399–404.
Cavalleri A, Gobba F, Paltrinieri M, et al. Perchloroethylene exposure can induce colour vision loss. *Neurosci Lett*. 1994;179:162–166.
Cavalleri A, Gobba F, Nicali E, Fiocchi V. Dose-related color vision impairment in toluene-exposed workers. *Arch Environ Health*. 2000;55:399–404.
Cestnick L, Coltheart M. The relationship between language-processing and visual-processing deficits in developmental dyslexia. *Cognition*. 1999;71:231–255.
Chang GQ, Hao Y, Wong F. Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. *Neuron*. 1993;11:595–605.
Chaney SY, Mukherjee S, Giddabasappa A, et al. Increased proliferation of late-born retinal progenitor cells by gestational lead exposure delays rod and bipolar cell differentiation. *Mol Vis*. 2016;22:1468–1489.
Chen JH, Yang CH, Wang YS, et al. Acrylamide-induced mitochondria collapse and apoptosis in human astrocytoma cells. *Food Chem Toxicol*. 2013;51:446–452.
Chia SE, Jeyaratnam J, Ong CN, et al. Impairment of color vision among workers exposed to low concentrations of styrene. *Am J Ind Med*. 1994;26:481–488.
Chun MH, Han SH, Chung JW, Wässle H. Electron microscopic analysis of the rod pathway of the rat retina. *J Comp Neurol*. 1993;332:421–432.
Choudhary D, Jansson I, Sarfarazi M, Schenkman JB. Physiological significance and expression of P450s in the developing eye. *Drug Metab Rev*. 2006;38:337–52.
Clayton LM, Dévilé M, Punte T, et al. Retinal nerve fiber layer thickness in vigabatrin-exposed patients. *Ann Neurol*. 2011;69:845–854.
Corbin JD, Francis SH, Webb DJ. Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology*. 2002;60:4–11.
Costa LG, Fox DA. A selective decrease in cholinergic muscarinic receptors in the visual cortex of adult rats following developmental lead exposure. *Brain Res*. 1983;276:259–266.
Crofton KM, Sheets LP. Evaluation of sensory system function using reflex modification of the startle response. *J Am Coll Toxicol*. 1989;8:199–211.
Crosson CE, Willis JA, Potter DE. Effect of the calcium antagonist, nifedipine, on ischemic retinal dysfunction. *J Ocul Pharmacol*. 1990;6:293–299.
Crouch RK, Goletz P, Snyder A, Coles WH. Antioxidant enzymes in human tears. *J Ocul Pharmacol*. 1991;7:253–258.
Dahlin A, Geier E, Stocker SL, et al. Gene expression profiling of transporters in the solute carrier and ATP-binding cassette superfamilies in human eye substructures. *Mol Pharm*. 2013;10:650–663.
Damstra T. Environmental chemicals and nervous system dysfunction. *Yale J Biol Med*. 1978;51:457–468.
Daneshvar H, Racette L, Coupland SG, et al. Symptomatic and asymptomatic visual loss in patients taking vigabatrin. *Ophthalmology*. 1999;106:1792–1798.
Datson GP, Freeberg FE. Ocular irritation testing. In: Hobson DW, ed. *Dermal and Ocular Toxicity: Fundamentals and Methods*. Boca Raton, FL: CRC Press; 1991:509–539.
Dayhaw-Barker P, Forbes D, Fox DA, et al. Drug photoxicity and visual health. In: Waxler M, Hitchins VM, eds. *Optical Radiation and Visual Health*. Boca Raton, FL: CRC Press; 1986:147–175.
Dean P. Visual pathways and acuity hooded rats. *Behav Brain Res*. 1981;3:239–271.
Dementi B. Ocular effects of organophosphates: a historical perspective of Saku disease. *J Appl Toxicol*. 1994;14:119–129.
De Rouck A, De Laey JJ, Van Hoorne M, et al. Chronic carbon disulfide poisoning: a 4-year follow-up study of the ophthalmological signs. *Int Ophthalmol*. 1986;9:17–27.
Dettoraki M, Moschos MM. The role of multifocal electroretinography in the assessment of drug-induced retinopathy: A review of the literature. *Ophthalmic Res*. 2016;56:169-177.
Diamante GG, Fraunfelder FT. Adverse effects of therapeutic agents on cornea and conjunctiva. In: Leibowitz HM, Waring GO, eds. *Corneal Disorders, Clinical Diagnosis and Management*. 2nd ed. Philadelphia, PA: Saunders; 1998:736–769.
Diebold Y, Calonge M. Applications of nanoparticles in ophthalmology. *Prog Retin Eye Res*. 2010;29:596–609.
Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev*. 1979;3:79–91.
Donoghue AM, Dryson EW, Wynn-Williams G. Contrast sensitivity in organic-solvent-induced chronic toxic encephalopathy. *J Occup Environ Med*. 1995;37:1357–1363.
Doshi M, Marcus C, Bejjani BA, Edward DP. Immunolocalization of CYP1B1 in normal, human, fetal and adult eyes. *Exp Eye Res*. 2006;82:24–32.
Downes JE, Holmes RS. Purification and properties of murine corneal alcohol dehydrogenase. Evidence for class IV ADH properties. *Adv Exp Med Biol*. 1995;372:349–354.
Dräger UC. Calcium binding in pigmented and albino eyes. *Proc Natl Acad Sci U S A*. 1985;82:6716–6720.
Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther*. 1944;82:377–389.
Duncker G, Krastel H. Ocular digitalis effects in normal subjects. *Lens Eye Tox Res*. 1990;7:281–303.
Duncker GI, Kisters G, Grille W. Prospective, randomized, placebo-controlled, double-blind testing of colour vision and electroretinogram at therapeutic and subtherapeutic digitoxin serum levels. *Ophthalmologica*. 1994;208:259–261.

DuVal G, Grubb BR, Bentley PJ. Mercury accumulation in the eye following administration of methyl mercury. *Exp Eye Res*. 1987;44:161–164.

Edelhauser HF. The balance between corneal transparency and edema: the Proctor Lecture. *Invest Ophthalmol Vis Sci*. 2006;47:1754–1767.

Edward DP, Lam TT, Shahinfar S, et al. Amelioration of light-induced retinal degeneration by a calcium overload blocker: flunarizine. *Arch Ophthalmol*. 1991;109:554–562.

Eells JT. Methanol. In: Thurman RG, Kauffman FC, eds. *Browning's Toxicity and Metabolism of Industrial Solvents: Alcohols and Esters*. Vol 3. Amsterdam: Elsevier; 1992:3–20.

Eells JT, Salzman MM, Lewandowski MF, Murray TG. Formate-induced alterations in retinal function in methanol-intoxicated rats. *Toxicol Appl Pharmacol*. 1996;140:58–69.

Eguchi T, Kishi R, Harabuchi I, et al. Impaired colour discrimination among workers exposed to styrene: relevance of a urinary metabolite. *Occup Environ Med*. 1995;52:534–538.

Eichenbaum JW, Zheng W. Distribution of lead and transthyretin in human eyes. *Clin Toxicol*. 2000;38:371–381.

Ekinci M, Ceylan E, Cağatay HH, et al. Occupational exposure to lead decreases macular, choroidal, and retinal nerve fiber layer thickness in industrial battery workers. *Curr Eye Res*. 2014;39:853–858.

Ellsworth AJ, Witt DM, Dugdale DC, Oliver LM. *Mosby's 1999-2000 Medical Drug Reference*. St Louis, MO: Mosby; 1999:184–185.

Erie JC, Butz JA, Good JA, et al. Heavy metal concentrations in human eyes. *Am J Ophthalmol*. 2005;139:888–893.

Erie JC, Good JA, Butz JA. Excess lead in the neural retina in age-related macular degeneration. *Am J Ophthalmol*. 2009a;148:890–894.

Erie JC, Good JA, Butz JA, et al. Reduced zinc and copper in the retinal pigment epithelium and choroid in age-related macular degeneration. *Am J Ophthalmol*. 2009b;147:276–282.

Eskin TA, Merigan WH. Selective acrylamide-induced degeneration of color opponent ganglion cells in macaques. *Brain Res*. 1986;378:379–384.

Eskin TA, Merigan WH, Wood RW. Carbon disulfide effects on the visual system: II. Retinogeniculate degeneration. *Invest Ophthalmol Vis Sci*. 1988;29:519–527.

Ethier AA, Muckle G, Bastien C, et al. Effects of environmental contaminant exposure on visual brain development: a prospective electrophysiological study in school-aged children. *NeuroToxicology*. 2012;33:1075–1085.

Fallas C, Fallas J, Maslard P, Dally S. Subclinical impairment of colour vision among workers exposed to styrene. *Br J Ind Med*. 1992;49:679–682.

Finlay BL, Sengelaub DR. *Development of the Vertebrate Retina*. New York: Plenum Press; 1989.

Flammer J, Mozaffarieh M. Autoregulation, a balancing act between supply and demand. *Can J Ophthalmol*. 2008;43:317–321.

Fox DA. Psychophysically and electrophysiologically determined spatial vision deficits in lead-exposed rats have a cholinergic component. In: Narahashi T, ed. *Cellular and Molecular Basis of Neurotoxicity*. New York: Raven Press; 1984:123–140.

Fox DA. Sensory system alterations following occupational exposure to chemicals. In: Manzo L, Costa LG, eds. *Occupational Neurotoxicology*. Boca Raton, FL: CRC Press; 1998:169–184.

Fox DA. Retinal and visual system: occupational and environmental toxicology. *Handb Clin Neurol*. 2015;131:325–340.

Fox DA, Campbell ML, Blocker YS. Functional alterations and apoptotic cell death in the retina following developmental or adult lead exposure. *NeuroToxicology*. 1997;18:645–665.

Fox DA, Chu LWF. Rods are selectively altered by lead: II. Ultrastructure and quantitative histology. *Exp Eye Res*. 1988;46:613–625.

Fox DA, Farber DB. Rods are selectively altered by lead: I. Electrophysiology and biochemistry. *Exp Eye Res*. 1988;46:579–611.

Fox DA, Hamilton RW, Johnson JE, et al. Gestational lead exposure selectively decreases retinal dopamine amacrine cells and dopamine content in adult mice. *Toxicol Appl Pharmacol Toxicol*. 2011;256:258–267.

Fox DA, Kala SV, Hamilton WR, Johnson JE, O'Callaghan JP. Low-level human equivalent gestational lead exposure produces supernormal scotopic electroretinograms, increased retinal neurogenesis and decreased dopamine utilization in rats. *Environ Health Perspect*. 2008;116:618–625.

Fox DA, Katz LM. Developmental lead exposure selectively alters the scotopic ERG component of dark and light adaptation and increases rod calcium content. *Vision Res*. 1992;32:249–252.

Fox DA, Katz LM, Farber DB. Low-level developmental lead exposure decreases the sensitivity, amplitude and temporal resolution of rods. *NeuroToxicology*. 1991a;12:641–654.

Fox DA, Lewkowski JP, Cooper GP. Acute and chronic effects of neonatal lead exposure on the development of the visual evoked response in rats. *Toxicol Appl Pharmacol*. 1977;40:449–461.

Fox DA, Poblenz AT, He L. Calcium overload triggers rod photoreceptor apoptotic cell death in chemical-induced and inherited retinal degenerations. *Ann N Y Acad Sci*. 1999;893:282–286.

Fox DA, Rubinstein SD. Age-related changes in retinal sensitivity, rhodopsin content and rod outer-segment length in hooded rats following low level lead exposure during development. *Exp Eye Res*. 1989;48: 237–249.

Fox DA, Rubinstein SD, Hsu P. Developmental lead exposure inhibits adult rat retinal, but not kidney, Na^+,K^+-ATPase. *Toxicol Appl Pharmacol*. 1991b;109:482–493.

Fox DA, Sillman AJ. Heavy metals affect rod, but not cone photoreceptors. *Science*. 1979;206:78–80.

Fraunfelder FW, Pomeranz HD, Egan RA. Nonarteritic anterior ischemic optic neuropathy and sildenafil. *Arch Ophthalmol*. 2006;124:733–734.

Garner CD, Lee EW, Louis-Ferdinand RT. Muller cell involvement in methanol-induced retinal toxicity. *Toxicol Appl Pharmacol*. 1995b;130:101–107.

Garner CD, Lee EW, Terzo TS, Louis-Ferdinand RT. Role of retinal metabolism in methanol-induced retinal toxicity. *J Toxicol Environ Health*. 1995a;44:43–56.

Gaudet SJ, Razavi P, Caiafa GJ, Chader GJ. Identification of arylamine *N* acetyltransferase activity in the bovine lens. *Curr Eye Res*. 1995;14:873–877.

Geller AM, Abdel-Rahman AA, Peiffer RL, et al. The organophosphate pesticide chlorpyrifos affects form deprivation myopia. *Invest Ophthalmol Vis Sci*. 1998;39:1290–1294.

Geller AM, Hudnell HK. Critical issues in the use and analysis of the Lanthony desaturate color vision test. *Neurotoxicol Teratol*. 1997;19:455–465.

Getz KD, Janulewicz PA, Rowe S, et al. Prenatal and early childhood exposure to tetrachloroethylene and adult vision. *Environ Health Perspect*. 2012;120:1327–1332.

Giannaccini M, Pedicini L, De Matienzo G, et al. Magnetic nanoparticles: a strategy to target the choroidal layer in the posterior segment of the eye. *Sci Rep*. 2017;7:43092.

Giddabasappa A, Hamilton WR, Chaney S, et al. Gestational lead exposure selectively increases the proliferation and number of late-born rod photoreceptors and bipolar cells. *Environ Health Perspect*. 2011;119:71–77.

Ginsburg AP. Contrast sensitivity and functional vision. *Int Ophthalmol Clin*. 2003;43:5–15.

Glickman RD. Phototoxicity to the retina: mechanisms of damage. *Int J Toxicol*. 2002;21:473–490.

Gobba F, Galassi C, Imbriani M, et al. Acquired dyschromatopsia among styrene-exposed workers. *J Occup Med*. 1991;33:761–765.

Gobba F, Righi E, Fantuzzi G, et al. Two-year evolution of perchloroethylene-induced color- vision loss. *Arch Environ Health*. 1998;53:196–198.

Goffeng LO, Heier MS, Kjuus H, et al. Nerve conduction, visual evoked responses and electroretinography in tunnel workers previously exposed to acrylamide and N-methylolacrylamide containing grouting agents. *Neurotoxicol Teratol*. 2008a;30:186–194.

Goffeng LO, Kjuus H, Heier MS, Alvestrand M, Ulvestad B, Skaug V. Colour vision and light sensitivity in tunnel workers previously exposed to acrylamide and N-methylolacrylamide containing grouting agents. *NeuroToxicology*. 2008b;29:31–39.

Gondhowiardjo TD, van Haeringen NJ. Corneal aldehyde dehydrogenase, glutathione reductase, and glutathione-*S*-transferase in pathologic corneas. *Cornea*. 1993;12:310–314.

Good GW, Schepler A, Nichols JJ. The reliability of the Lanthony Desaturated D-15 test. *Optom Vis Sci*. 2005;82:1054–1059.

Gorin MB, Day R, Costantino JP, et al. Long-term tamoxifen citrate use and potential ocular toxicity. *Am J Ophthalmol*. 1998;125:493–501.

Gorkin L, Hvidsten K, Sobel RE, Siegel R. Sildenafil citrate use and the incidence of nonarteritic anterior ischemic optic neuropathy. *Int J Clin Pract*. 2006;60:500–503.

Goto Y, Shigematsu J, Tobimatsu S, et al. Different vulnerability of rat retinal cells to methylmercury exposure. *Curr Eye Res*. 2001;23:171–178.

Graham DG, Valentine WM. Carbon disulfide. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. 2nd ed. New York: Oxford University Press; 2000:315–317.

Grant WM, Schuman JS. *Toxicology of the Eye*. 4th ed. Springfield, IL: Charles C Thomas; 1993.

Guo Y, Wang W, Dong Y, et al. Carbon disulfide induces rat testicular injury via mitochondrial apoptotic pathway. *Chemosphere*. 2014;108:367–375.

Gupta OP, Brown GC, Brown MM. Age-related macular degeneration: the costs to society and the patient. *Curr Opin Ophthalmol*. 2007;18:201–205.

Hanninen H, Nurminen M, Tolonen M, Martelin T. Psychological tests as indicators of excessive exposure to carbon disulfide. *Scand J Work Environ*. 1978;19:163–174.

Hansen ON, Trillingsgaard A, Beese I, et al. A neuropsychological study of children with elevated dentine lead level: assessment of the effect of lead in different socioeconomic groups. *Neurotoxicol Teratol*. 1989;11:205–213.

Harroff HH. Pathological processes of the eye related to chemical exposure. In: Hobson DW, ed. *Dermal and Ocular Toxicity: Fundamentals and Methods*. Boca Raton, FL: CRC Press; 1991:493–508.

Haustein KO, Oltmanns G, Rietbrock N, Alken RG. Differences in color vision impairment caused by digoxin, digitoxin, or pengitoxin. *J Cardiovasc Pharmacol*. 1982;4:536–541.

Haustein KO, Schmidt C. Differences in color discrimination between three cardioactive glycosides. *Int J Clin Pharmacol Ther Toxicol*. 1988;26:517–520.

Hayreh MM, Hayreh SS, Baumbach GL, et al. Ocular toxicity of methanol: an experimental study. In: Merigan WH, Weiss B, eds. *Neurotoxicity of the Visual System*. New York: Raven Press; 1980:33–53.

He L, Perkins GA, Poblenz AT, et al. Bcl-xL overexpression blocks bax-mediated mitochondrial contact site formation and apoptosis in rod photoreceptors of lead-exposed mice. *Proc Natl Acad Sci U S A*. 2003;100:1022–1027.

He L, Poblenz AT, Medrano CJ, Fox DA. Lead and calcium produce photoreceptor cell apoptosis by opening the mitochondrial permeability transition pore. *J Biol Chem*. 2000;275:12175–12184.

Heckenlively JR, Arden G. *Principles and Practice of Clinical Electrophysiology of Vision*. Cambridge: MIT Press; 2006.

Hedges TR III, Hirano M, Tucker K, Caballero B. Epidemic optic and peripheral neuropathy in Cuba: a unique geopolitical public health problem. *Surv Ophthalmol*. 1997;41:341–353.

Hendrickson A. A morphological comparison of foveal development in man and monkey. *Eye*. 1992;6:136–144.

Hendrickson A, Drucker D. The development of parafoveal and mid-peripheral human retina. *Behav Brain Res*. 1992;49:21–31.

Heng JE, Vorwerk CK, Lessell E, et al. Ethambutol is toxic to retinal ganglion cells via an excitotoxic pathway. *Invest Ophthalmol Vis Sci*. 1999;40:190–196.

Henkes HE, van Lith GHM, Canta LR. Indomethacin retinopathy. *Am J Ophthalmol*. 1972;73:846–856.

Herr DW, Boyes WK. Electrophysiological analysis of complex brain systems: sensory evoked potentials and their generators. In: Chang LW, Slikker W, eds. *Neurotoxicology: Approaches and Methods*. New York: Academic Press; 1995:205–221.

Hou RH, Scaife J, Freeman C, et al. Relationship between sedation and pupillary function: comparison of diazepam and diphenhydramine. Relationship between sedation and pupillary function: comparison of diazepam and diphenhydramine. *Br J Clin Pharmacol*. 2006;61:752–760.

Hudnell HK, Otto DA, House DE. The influence of vision on computerized neurobehavioral test scores: a proposal for improving test protocols. *Neurotoxicol Teratol.* 1996;18:391–400.

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). https://ntp.niehs.nih.gov/pubhealth/evalatm/iccvam/acceptance-of-alternative-methods/index.html#Ocular-Corrosivity-and-Irritation. Accessed 26 May, 2017.

Imai H, Miyata M, Uga S, Ishikawa S. Retinal degeneration in rats exposed to an organophosphate pesticide (fenthion). *Environ Res*. 1983;30:453–465.

Ingemansson SO. Studies on the effect of 4-methylpyrazole on retinal activity in the methanol poisoned monkey by recording the electroretinogram. *Acta Ophthalmol*. 1983;(suppl 158):1–24.

Imperia PS, Lazarus HM, Lass JH. Ocular complications of systemic cancer chemotherapy. *Surv Ophthalmol*. 1989;34:209–230.

Iregren A, Andersson M, Nylén P. Color vision and occupational chemical exposures: I. An overview of tests and effects. *NeuroToxicology*. 2002;23:719–733.

Iregren A, Johnson A-C, Nylén P. Low-level styrene exposure and color vision in Swedish styrene workers. *Environ Toxicol Pharmacol*. 2005;19:511–516.

Ishikawa S. Chronic optica-neuropathy due to environmental exposure of organophosphate pesticides (Saku disease): clinical and experimental study. *Nippon Ganka Gakkai Zasshi*. 1973;77:1835–1886.

Iwata K. Neuro-ophthalmological findings and a follow-up study in the Agano area, Niigata Pref. In: Tsubaki T, Irukayama K, eds. *Minamata Disease: Methylmercury poisoning in Minamata and Niigata, Tokyo*. Tokyo: Kodansha; 1977:166–185.

Jadhav AL, Ramesh GT. Pb-induced alterations in tyrosine hydroxylase activity in rat brain. *Mol Cell Biochem*. 1997;175:137–141.

Jagle H, Jagle C, Serey L, et al. Visual short-term effects of Viagra: double-blind study in healthy young subjects. *Am J Ophthalmol*. 2004;137:842–849.

Janulewicz PA, Killiany RJ, White RF, et al. Structural Magnetic Resonance Imaging in an adult cohort following prenatal and early postnatal exposure to tetrachloroethylene (PCE)-contaminated drinking water. *Neurotoxicol Teratol*. 2013;38:13-20.

Jo DH, Lee TG, Kim JH. Nanotechnology and nanotoxicology in retinopathy. *Int J Mol Sci*. 2011;12:8288–8301.

Johns DR, Smith KH, Miller NR. Leber's hereditary optic neuropathy. Clinical manifestations of the 3460 mutation. *Arch Ophthalmol*. 1992;110:1577–1581.

Johnson JE Jr, Perkins GA, Giddabasappa A, et al. Spatiotemporal regulation of ATP and Ca^{2+} dynamics in vertebrate rod and cone ribbon synapses. *Mol Vis*. 2007;13:887–919.

Johnson MW, Vine AK. Hydroxychloroquine therapy in massive total doses without retinal toxicity. *Am J Ophthalmol*. 1987;104:139–144.

Jones RD, Brenneke CJ, Hoss HE, Loney ML. An electroretinogram protocol for toxicological screening in the canine model. *Toxicol Lett*. 1994;70:223–234.

Kaiser-Kupfer MI, Kupfer C, Rodriques MM. Tamoxifen retinopathy. A clinocopathologic report. *Ophthalmology*. 1981;88:89–93.

Kalloniatis M, Tomisich G. Amino acid neurochemistry of the vertebrate retina. *Prog Retinal Eye Res*. 1999;18:811–866.

Kamel F, Boyes WK, Gladen BC, et al. Retinal degeneration in licensed pesticide applicators. *Am J Ind Med*. 2000;37:618–628.

Kamran S, Bakshi R. MRI in chronic toluene abuse: low signal in the cerebral cortex on T2-weighted images. *Neuroradiology*. 1998;40: 519–521.

Kaplan E, Benardete E. The dynamics of primate retinal ganglion cells. *Prog Brain Res*. 2001;134:17–34.

Karai I, Horiguchi SH, Nishikawa N. Optic atrophy with visual field defect in workers occupationally exposed to lead for 30 years. *J Toxicol Clin Toxicol*. 1982;19:409–418.

Karlsson O, Lindquist NG. Melanin and neuromelanin binding of drugs and chemicals: toxicological implications. *Arch Toxicol*. 2016;90:1883–1891.

Kawasaki A. Physiology, assessment, and disorders of the pupil. *Curr Opin Ophthalmol*. 1999;10:394–400.

King G, Hirst L, Holmes R. Human corneal and lens aldehyde dehydrogenases. Localization and function(s) of ocular ALDH1 and ALDH3 isozymes. *Adv Exp Med Biol*. 1999;463:189–198.

Kini MM, Cooper JR. Biochemistry of methanol poisoning. 4. The effect of methanol and its metabolites on retinal metabolism. *Biochem J*. 1962;82:164–172.

Kirberger M, Yang JJ. Structural differences between Pb^{2+}- and Ca^{2+}-binding sites in proteins: implications with respect to toxicity. *J Inorg Biochem*. 2008;102:1901–1909.

Kirrane EF, Hoppin JA, Kamel F, et al. Retinal degeneration and other eye disorders in wives of farmer pesticide applicators enrolled in the Agricultural Health Study. *Am J Epidemiol*. 2005;161:1020–1029.

Kiyokawa M, Mizota A, Takasoh M, Adachi-Usami E. Pattern visual evoked cortical potentials in patients with toxic optic neuropathy caused by toluene abuse. *Jpn J Ophthalmol*. 2005;43:438–442.

Kobel W, Gfeller W. Distribution of eye irritation scores of industrial chemicals. *Food Chem Toxicol*. 1985;23:311–312.

Kohler K, Lilienthal H, Guenther E, et al. Persistent decrease of the dopamine-synthesizing enzyme tyrosine hydroxylase in the rhesus monkey retina after chronic lead exposure. *NeuroToxicology*. 1997;18: 623–632.

Koliopoulos J, Palimeris G. On acquired colour vision disturbances during treatment with ethambutol and indomethacin. *Mod Probl Ophthalmol*. 1972;11:178–184.

Ko LS, Shum JT, Chen YL, Wang YS. Pesticides and myopia, a working hypothesis. *Acta Ophthalmol Suppl*. 1988;185:145–146.

Krigel A, Berdugo M, Picard E, et al. Light-induced retinal damage using different light sources, protocols and rat strains reveals LED phototoxicity. *Neuroscience*. 2016;339:296–307.

Krill AE, Potts AM, Johanson CE. Chloroquine retinopathy. Investigation of discrepancy between dark adaptation and electroretinographic findings in advanced stages. *Am J Ophthalmol*. 1971;71:530–543.

Lasley SM, Lane JD. Diminished regulation of mesolimbic dopaminergic activity in rat after chronic inorganic lead exposure. *Toxicol Appl Pharmacol*. 1988;95:474–483.

Laties A. Vision disorders and phosphodiesterase 5 inhibitors: a review of the evidence to date. *Druf Saf*. 2009;32:1–18.

Laties A, Zrenner E. Viagra (sildenafil citrate) and ophthalmology. *Prog Retin Eye Res*. 2002;21:485–506.

LaVail MM. Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. *Science*. 1976;194:1071–1074.

Lazzaroni F, Scorolli L, Pizzoleo CF, et al. Tamoxifen retinopathy: does it really exist? *Graefes Arch Clin Exp Ophthalmol*. 1998;236:669–673.

Lee AY, Chung SS. Involvement of aldose reductase in naphthalene cataract. *Invest Ophthalmol Vis Sci*. 1998;39:193–197.

Lee EW, Garner CD, Terzo TS. A rat model manifesting methanol-induced visual dysfunction suitable for both acute and long-term exposure studies. *Toxicol Appl Pharmacol*. 1994;128:199–206.

Lee JW, Fuda H, Javitt NB, Strott CA, Rodriguez IR. Expression and localization of sterol 27-hydroxylase (CYP27A1) in monkey retina. *Exp Eye Res*. 2006;83:465–469.

Leske MC, Chylack LT Jr, Wu SY. The lens opacities case-control study. Risk factors for cataract. *Arch Ophthalmol*. 1991;109:244–251.

Lessell S. Toxic and deficiency optic neuropathies. In: Miller NR, Newman NJ, eds. *Walsh and Hoyt's Clinical Neuro-Ophthalmology*. 5th ed. Baltimore, MD: Williams & Wilkins; 1998.

Levin LA, Nilsson SFE, Ver Hoeve J, et al. *Adler's Physiology of the Eye*. 10th ed. New York: Elsevier Press; 2011.

Lezmi S, Rokh N, Saint-Macary G, et al. Chloroquine causes similar electroretinogram modifications, neuronal phospholipidosis and marked impairment of synaptic vesicle transport in albino and pigmented rats. *Toxicology*. 2013;308:50–59.

Liang H, Pauly A, Riancho L, Baudouin C, Brignole-Baudouin F. Toxicological evaluation of preservative-containing and preservative-free topical prostaglandin analogues on a three-dimensional-reconstituted corneal epithelium system. *Br J Ophthalmol*. 2011;95: 869–875.

Lilienthal H, Lenaerts C, Winneke G, Hennekes R. Alteration of the visual evoked potential and the electroretinogram in lead-treated monkeys. *Neurotoxicol Teratol*. 1988;10:417–422.

Lin CH, Wu MR, Li CH, et al. Editor's Highlight: Periodic exposure to smartphone-mimic low-luminance blue light induces retina damage through Bcl-2/BAX-dependent apoptosis. *Toxicol Sci*. 2017;157:196–210.

Lindauer A, Siepmann T, Oertel R, et al. Pharmacokinetic/pharmacodynamic modelling of venlafaxine: pupillary light reflex as a test system for noradrenergic effects. *Clin Pharmacokinet*. 2008;47:721–731.

Lissner W, Greenlee JE, Cameron JD, Goren SB. Localization of tritiated digoxin in the rat eye. *Am J Ophthalmol*. 1971;72:608–614.

Liu Z, Song G, Zou C, et al. Acrylamide induces mitochondrial dysfunction and apoptosis in BV-2 microglial cells. *Free Radic Biol Med*. 2015;84:42–53.

Lynch JJ III, Eskin TA, Merigan WH. Selective degeneration of the parvocellular-projecting retinal ganglion cells in a New World monkey, *Saimiri sciureus*. *Brain Res*. 1989;499:325–332.

Lynch JJ III, Silveira LC, Perry VH, Merigan WH. Visual effects of damage to P ganglion cells in macaques. *Vis Neurosci*. 1992;8:575–583.

Madreperla SA, Johnson MA, Nakatani K. Electrophysiological and electroretinographic evidence for phototreceptor dysfunction as a toxic effect of digoxin. *Arch Ophthalmol*. 1994;112:807–812.

Maguire MJ, Hemming K, Wild JM, et al. Prevalence of visual field loss following exposure to vigabatrin therapy: a systematic review. *Epilepsia*. 2010;51:2423–2431.

Marks MJ, Seeds NW. A heterogeneous ouabain-ATPase interaction in mouse brain. *Life Sci*. 1978;23:2735–2744.

Martin-Amat G, McMartin KE, Hayreh SS, et al. Methanol poisoning: ocular toxicity produced by formate. *Toxicol Appl Pharmacol*. 1978;45:201–208.

Mastyugin V, Aversa E, Bonazzi A, et al. Hypoxia-induced production of 12-hydroxyeicosanoids in the corneal epithelium: involvement of a cytochrome P-4504B1 isoform. *J Pharmacol Exp Ther*. 1999;289:1611–1619.

Maurissen JPJ. Neurobehavioral methods for the evaluation of sensory functions. In: Chang LW, Slikker W, eds. *Neurotoxicology: Approaches and Methods*. New York: Academic Press; 1995:239–264.

McCulley JP. Chemical injuries of the eye. In: Leibowitz HM, Waring GO. eds. *Corneal Disorders, Clinical Diagnosis and Management*. 2nd ed. Philadelphia, PA: Saunders; 1998:770–790.

McDougal DH, Gamlin PD. The influence of intrinsically-photosensitive retinal ganglion cells on the spectral sensitivity and response dynamics of the human pupillary light reflex. *Vision Res*. 2010;50:72–87.

McGrail KM, Sweadner KJ. Complex expression patterns for Na^+, K^+-ATPase isoforms in retina and optic nerve. *Eur J Neurosci*. 1989;2:170–176.

McGwin G Jr, Hall TA, Seale J, Xie A, Owsley C. Consumer product-related eye injury in the United States, 1998-2002. *J Safety Res*. 2006;37:501–506.

Mecklenburg L, Schraemeyer U. An overview on the toxic morphological changes in the retinal pigment epithelium after systemic compound administration. *Toxicol Pathol*. 2007;35:252–267.

Medrano CJ, Fox DA. Substrate-dependent effects of calcium on rat retinal mitochondria respiration: physiological and toxicological studies. *Toxicol Appl Pharmacol*. 1994;125:309–321.

Medrano CJ, Fox DA. Oxygen consumption in rat outer and inner retina: light- and pharmacologically induced inhibition. *Exp Eye Res*. 1995;61:273–284.

Meier-Ruge W. Drug-induced retinopathy. *CRC Toxicol*. 1972;1:325–360.

Mergler D, Belanger S, Grosbois S, Vachon N. Chromal focus of acquired chromatic discrimination loss and solvent exposure among printshop workers. *Toxicology*. 1988;49:341–348.

Mergler D, Blain L, Lagace JP. Solvent related colour vision loss: an indicator of damage? *Int Arch Occup Environ Health*. 1987;59:313–321.

Mergler D, Huel G, Bowler R, et al. Visual dysfunction among former microelectronics assembly workers. *Arch Environ Health*. 1991;46:326–334.

Merigan WH. Effects of toxicants on visual systems. *Neurobehav Toxicol*. 1979;1(suppl 1):15–22.

Merigan WH, Eskin TA. Spatio-temporal vision of macaques with severe loss of P beta retinal ganglion cells. *Vision Res*. 1986;26:1751–1761.

Merigan WH, Wood RW, Zehl D, Eskin TA. Carbon disulfide effects on the visual system: I. Visual thresholds and ophthalmoscopy. *Invest Ophthalmol Vis Sci*. 1988;29:512–518.

Migliore L, Uboldi C, Di Bucchianico S, Coppedè F. Nanomaterials and neurodegeneration. *Environ Mol Mutagen*. 2015;56:149–170.

Milioni ALV, Nagy BV, Moura ALA, et al. Neurotoxic impact of mercury on the central nervous system evaluated by neuropsychological tests and on the autonomic nervous system evaluated by dynamic pupillometry. *NeuroToxicology*. 2017;59:263–269.

Misra UK, Nag D, Misra NK, et al. Some observations on the macula of pesticide workers. *Hum Toxicol*. 1985;4:135–145.

Mojon DS, Kaufmann P, Odel JG, et al. Clinical course of a cohort in the Cuban epidemic optic and peripheral neuropathy. *Neurology*. 1997;48:19–22.

Monticelli F, Preiss U, Hitzl W, Keller T. Pupil function as an indicator of being under the influence of central nervous system-acting substances from a traffic-medicine perspective: Part II. *Med Sci Law*. 2010;56:19–25.

Moschos MM, Nitoda E. Pathophysiology of visual disorders induced by phosphodiesterase inhibitors in the treatment of erectile dysfunction. *Drug Des Devel Ther*. 2016;8:3407–3413.

Moser VC, Boyes WK. Prolonged neurobehavioral and visual effects of short-term exposure to 3,3-iminodipropionitrile (IDPN) in rats. *Fund Appl Toxicol*. 1993;21:277–290.

Moser VC, Phillips PM, Morgan DL, Sills RC. Carbon disulfide neurotoxicity in rats: VII. Behavioral evaluations using a functional observational battery. *NeuroToxicology*. 1998;19:147–158.

Muñoz H, Romiew I, Palazuelos E, et al. Blood lead level and neurobehavioral development among children living in Mexico City. *Arch Environ Health*. 1993;48:132–139.

Muppidi S, Adams-Huet B, Tajzoy E, et al. Dynamic pupillometry as an autonomic testing tool. *Clin Auton Res*. 2013;23:297–303.

Murata K, Araki S, Yokoyama K, et al. Assessment of central, peripheral, and autonomic nervous system functions in lead workers: neuroelectrophysiological studies. *Environ Res*. 1993;61:323–336.

Murray TG, Burton TC, Rajani C, et al. Methanol poisoning. A rodent model with structural and functional evidence for retinal involvement. *Arch Ophthalmol*. 1991;109:1012–1016.

Nagpal AG, Brodie SE. Supranormal electroretinogram in a 10-year-old girl with lead toxicity. *Doc Ophthalmol*. 2009;118:163–166.

Nagpal K, Singh SK, Mishra DN. Chitosan nanoparticles: a promising system in novel drug delivery. *Chem Pharm Bull*. 2010;58:1423–1430.

Nakamura K, Fujiki T, Tamura HO. Age, gender and region-specific differences in drug metabolising enzymes in rat ocular tissues. *Exp Eye Res*. 2005;81:710–715.

Nakano M, Lockhart CM, Kelly EJ, Rettie AE. Ocular cytochrome P450s and transporters: roles in disease and endobiotic and xenobiotic disposition. *Drug Metab Rev*. 2014;46:247–260.

Neveu MM, Jeffery G. Chiasm formation in man is fundamentally different from that in the mouse. *Eye (Lond)*. 2007;21:1264–1270.

Nicholls P. The effect of formate on cytochrome aa3 and on electron transport in the intact respiratory chain. *Biochim Biophys Acta*. 1976;430:13–29.

Nickla DL, Wallman J. The multifunctional choroid. *Prog Retin Eye Res*. 2010;29:144–168.

National Institute of Environmental Health (NIEHS). *Advancing Science, Improving Health: A Plan for Environmental Health Research. Strategic Plan 2012–2017*. National Institute of Environmental Health Sciences, NIH Pub. No. 12-7935, 2012.

Niklasson M, Stengard K, Tham R. Are the effects of toluene on the vestibulo and opto-ocular motor system inhibited by the action of GABAB antagonist CGP 35348? *Neurotoxicol Teratol*. 1995;17:351–357.

Noureddin BN, Seoud M, Bashshur Z, et al. Ocular toxicity in low-dose tamoxifen: a prospective study. *Eye*. 1999;13:729–733.

Novack GD. Emerging drugs for ophthalmic diseases. *Expert Opin Emerg Drugs*. 2003;8:251–266.

Otto DA, Fox DA. Auditory and visual dysfunction following lead exposure. *NeuroToxicology*. 1993;14:191–208.

Otto DA, Robinson G, Baumann S, et al. 5-Year follow-up study of children with low-to-moderate lead absorption: electrophysiological evaluation. *Environ Res*. 1985;38:168–186.

Palacz O, Szymanska K, Czepita D. Studies on the ERG in subjects with chronic exposure to carbon disulfide: I. Assessment of the condition of the visual system taking into account ERG findings depending on the duration of exposure to CS2. *Klin Oczna*. 1980;82:65–68.

Pan X, Yan D, Wang D, et al. Mitochondrion-mediated apoptosis induced by acrylamide is regulated by a balance between Nrf2 antioxidant and MAPK signaling pathways in PC12 cells. *Mol Neurobiol*. 2017;54:4781–4794.

Paramei GV, Meyer-Baron M, Seeber A. Impairments of colour vision induced by organic solvents: a meta-analysis study. *NeuroToxicology*. 2004;25:803–816.

Paraoanu LE, Mocko JB, Becker-Roeck M, et al. Exposure to diazinon alters in vitro retinogenesis: retinospheroid morphology, development of chicken retinal cell types, and gene expression. *Toxicol Sci*. 2006;89:314–324.

Pavlidis NA, Petris C, Briassoulis E, et al. Clear evidence that long-term, low-dose tamoxifen treatment can induce ocular toxicity. A prospective study of 63 patients. *Cancer*. 1992;69:2961–2964.

Peate WF. Work-related eye injuries and illnesses. *Am Fam Physician*. 2007;75:1017–1022.

Peiffer RL, McCary B, Bee W, et al. Contemporary methods in ocular toxicology. *Toxicol Methods*. 2000;10:17–39.

Penha FM, Rodrigues EB, Furlani BA, et al. Toxicological considerations for intravitreal drugs. *Expert Opin Drug Metab Toxicol*. 2011;7:1021–1034.

Penn JS, Naash MI, Anderson RE. Effect of light history on retinal antioxidants and light damage susceptibility in the rat. *Exp Eye Res*. 1987;44:779–788.

Peponis V, Kyttaris VC, Chalkiadakis SE, et al. Ocular side effects of antirheumatic medications: what a rheumatologist should know. *Lupus*. 2010;19:675–682.

Perkins GA, Scott R, Perez A, et al. Bcl-xL-mediated remodeling of rod and cone synaptic mitochondria after postnatal lead exposure: electron microscopy, tomography and oxygen consumption. *Mol Vis*. 2012;18:3029–3048.

Petit TL, LeBoutillier JC. Effects of lead exposure during development on neocortical dendritic and synaptic structure. *Exp Neurol*. 1979;64:482–492.

Plant GT, Sergott RC. Understanding and interpreting vision safety issues with vigabatrin therapy. *Acta Neurol Scand Suppl*. 2011;192:57–71.

Polak BC, Leys M, van Lith GH. Blue-yellow colour vision changes as early symptoms of ethambutol oculotoxicity. *Ophthalmologica*. 1985;191:223–226.

Potts AM. The visual toxicity of methanol: VI. The clinical aspects of experimental methanol poisoning treated with base. *Am J Ophthalmol*. 1955;39:76–82.

Potts AM. The reaction of uveal pigment in vitro with polycyclic compounds. *Invest Ophthalmol*. 1964;3:405–416.

Potts AM, Au PC. The affinity of melanin for inorganic ions. *Exp Eye Res*. 1976;22:487–491.

Pounds JG. Effect of lead intoxication on calcium homeostasis and calcium-mediated cell function: a review. *NeuroToxicology*. 1984;5:295–332.

Prow TW. Toxicity of nanomaterials to the eye. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*. 2010;2:317–333.

Raedler A, Sievers J. The development of the visual system of the albino rat. *Adv Anat Embryol Cell Biol*. 1975;50:7–87.

Raitta C, Seppalainen AN, Huuskonen MS. *n*-hexane maculopathy in industrial workers. *Von Graefes Arch Klin Exp Ophthalmol*. 1978;209:99–110.

Raitta C, Teir H, Tolonen M, Nurminen M, Helpio E, Malmstrom S. Impaired color discrimination among viscose rayon workers exposed to carbon disulfide. *J Occup Med*. 1981;23:189–192.

Raitta C, Tolonen M, Nurminen M. Microcirculation of ocular fundus in viscose rayon workers exposed to carbon disulfide. *Arch Klin Exp Ophthalmol*. 1974;191:151–164.

Raizman MB, Hamrah P, Holland EJ, et al. Drug-induced corneal epithelial changes. *Surv Ophthalmol*. 2017;62:286–301.

Ramsey MS, Fine BS. Chloroquine toxicity in the human eye. Histopathologic observations by electron microscopy. *Am J Ophthalmol*. 1972;73:229–235.

Rao GN. Light intensity-associated eye lesions of Fisher 344 rats in long-term studies. *Toxicol Pathol*. 1991;19:148–155.

Rapp LM, Tolman BL, Koutz CA, Thum LA. Predisposing factors to light-induced photoreceptor cell damage: retinal changes in maturing rats. *Exp Eye Res*. 1990;51:177–184.

Reuhl KR, Rice DC, Gilbert SG, Mallett J. Effects of chronic developmental lead exposure on monkey neuroanatomy: visual system. *Toxicol Appl Pharmacol*. 1989;99:501–509.

Rice DC. Sensory and cognitive effects of developmental methylmercury exposure in monkeys, and a comparison to effects in rodents. *NeuroToxicology*. 1996;17:139–154.

Rice DC. Effects of lifetime lead exposure on spatial and temporal visual function in monkeys. *NeuroToxicology*. 1998;19:893–902.

Rice DC, Gilbert SG. Effects of developmental exposure to methylmercury on spatial and temporal visual function in monkeys. *Toxicol Appl Pharmacol*. 1990;102:151–163.

Roberts JE. Ocular phototoxicity. *J Photochem Photobiol B*. 2001;64:136–143.

Roberts JE. Screening for ocular phototoxicity. *Int J Toxicol*. 2002;21:491–500.

Roberts JE, Wielgus AR, Boyes WK, Andley U, Chignell CF. Phototoxicity and cytotoxicity of fullerol in human lens epithelial cells. *Toxicol Appl Pharmacol*. 2008;228:49–58.

Robertson DM, Hollenhorst RW, Callahan JA. Ocular manifestations of digitalis toxicity. *Arch Ophthalmol*. 1966;76:640–645.

Roman G. Tropical myeloneuropathies revisited. *Curr Opin Neurol*. 1998;11:539–544.

Rosenthal AR, Kolb H, Bergsma D, et al. Chloroquine retinopathy in the Rhesus monkey. *Invest Ophthalmol Vis Sci*. 1978;17:1158–1175.

Rosolen SG, Rigaudiere F, Le Gargasson JF, Brigell MG. Recommendations for a toxicological screening ERG procedure in laboratory animals. *Doc Ophthalmol*. 2005;110:57–66.

Rothenberg SJ, Schnaas L, Salgado-Valladares M, et al. Increased ERG a-and b-wave amplitudes in 7-10 year old children resulting from prenatal lead exposure. *Invest Ophthalmol Vis Sci*. 2002;43:2036–2044.

Rouslin W, Broge CW, Chernyak BV. Effects of Zn^{2+} on the activity and binding of the mitochondrial ATPase inhibitor protein, IF1. *J Bioenerg Biomembr*. 1993;25:297–306.

Ruan DY, Tang LX, Zhao C, Guo YJ. Effects of low-level lead on retinal ganglion sustained and transient cells in developing rats. *Neurotoxicol Teratol*. 1994;16:47–53.

Rueda EM, Johnson JE Jr, Giddabasappa A, et al. The cellular and compartmental profile of mouse retinal glycolysis, tricarboxylic acid cycle, oxidative phosphorylation, and ~P transferring kinases. *Mol Vis*. 2016;22:847–885.

Ruedeman AD. The electroretinogram in chronic methyl alcohol poisoning in human beings. *Trans Am Ophthalmol Soc*. 1961;59:480–529.

Rufa A, Dotti MT, Cardaioli E, et al. Leber hereditary optic neuropathy in 2 of 4 siblings with 11778 mtDNA mutation: clinical variability or effect of toxic environmental exposure? *Eur Neurol*. 2005;53:32–34.

Ryu YH, Lee JD, Yoon PH, Jeon P, Kim DI, Shin DW. Cerebral perfusion impairment in a patient with toluene abuse. *J Nucl Med*. 1998;39:632–633.

Sabri MI. Attenuation of glyceraldehyde-3-phosphate dehydrogenase activity and ATP levels in rat brain synaptosomes by acrylamide. *Neurochem Res*. 1994;19:1439–1444.

Sadun AA. Acquired mitochondrial impairment as a cause of optic nerve disease. *Trans Am Ophthalmol Soc*. 1998;96:881–923.

Sadun AA, Martone JF, Muci-Mendoza R, et al. Epidemic optic neuropathy in Cuba. Eye findings. *Arch Ophthalmol*. 1994a;112:691–699.

Sadun AA, Martone JF, Reyes L, et al. Optic and peripheral neuropathy in Cuba. *JAMA*. 1994b;271:663–664.

Salmon JF, Carmichael TR, Welsh NH. Use of contrast sensitivity measurement in the detection of subclinical ethambutol toxic optic neuropathy. *Br J Ophthalmol*. 1987;71:192–196.

Sanders K, Degn LL, Mundy WR, et al. In vitro phototoxicity and hazard identification of nano-scale titanium dioxide. *Toxicol App Pharmacol*. 2012;258:226–236.

Santaella RM, Fraunfelder FW. Ocular adverse effects associated with systemic medications: recognition and management. *Drugs*. 2007;67:75–93.

Sassaman FW, Cassidy JT, Alpern M, Maaseidvaag F. Electroretinography in patients with connective tissue diseases treated with hydroxychloroquine. *Am J Ophthalmol*. 1970;70:515–523.

Schmid KE, Kornek GV, Scheithauer W, Binder S. Update on ocular complications of systemic cancer chemotherapy. *Surv Ophthalmol*. 2006;51:19–40.

Schnapf JL, Kraft TW, Nunn BJ, Baylor DA. Spectral sensitivity of primate photoreceptors. *Vis Neurosci*. 1988;1:255–261.

Schneider BG, Shygan AW, Levinson R. Co-localization and polarized distribution of Na, K-ATPase: α3 and β2 subunits in photoreceptor cells. *J Histochem Cytochem*. 1991;39:507–517.

Scholl HPN, Zrenner E. Electrophysiology in the investigation of acquired retinal disorders. *Surv Ophthalmol*. 2000;45:29–47.

Schreiber JS, Hudnell HK, Geller AM, et al. Apartment residents' and day care workers' exposures to tetrachloroethylene and deficits in visual contrast sensitivity. *Environ Health Perspect*. 2002;110:655–664.

Schwartzman ML. Cytochrome P450 and arachidonic acid metabolism in the corneal epithelium. In: Green K, Edelhauser HF, Hackett RB, et al., eds. *Advances in Ocular Toxicology*. New York: Plenum Press; 1997:3–20.

Scortegagna M, Hanbauer I. The effect of lead exposure and serum deprivation on mesencephalic primary cultures. *NeuroToxicology*. 1997;18:331–340.

Sears ML. *Pharmacology of the Eye*. Berlin: Springer-Verlag; 1984.

Seme MT, Summerfelt P, Henry MM, et al. Formate-induced inhibition of photoreceptor function in methanol intoxication. *J Pharmacol Exp Ther*. 1999;289:361–370.

Seppalainen AM, Haltia M. Carbon disulfide. In: Spencer PS, Schaumberg HH, eds. *Experimental and Clinical Neurotoxicology*. Baltimore, MD: William & Wilkins; 1980:356–373.

Seyfoddin A, Shaw J, Al-Kassas R. Solid lipid nanoparticles for ocular drug delivery. *Drug Delivery*. 2010;17:467–489.

Shanthaverrappa TR, Bourne GH. Monoamine oxidase distribution in the rabbit eye. *J Histochem Cytochem*. 1964;12:281–287.

Shearer RV, Dubois EL. Ocular changes induced by long-term hydroxychloroquine (Plaqenil) therapy. *Am J Ophthalmol*. 1967;64:245–252.

Shen XF, Huang P, Fox DA, et al. Adult lead exposure increases blood–retinal permeability: A risk factor for retinal vascular disease. *NeuroToxicology*. 2016;57:145–152.

Shichi H. Immunocytochemical study of cytochrome P4504A induction in mouse eye. *Exp Eye Res*. 1996;63:747–751.

Shichi H, Nebert DW. Genetic differences in drug metabolism associated with ocular toxicity. *Environ Health Perspect*. 1982;44:107–117.

Shulman LM, Fox DA. Dopamine inhibits mammalian photoreceptor Na^+,K^+-ATPase activity via a selective effect on the α3 isozyme. *Proc Natl Acad Sci U S A*. 1996;93:8034–8039.

Singh AK, Shichi H. A novel glutathione peroxidase in bovine eye. Sequence analysis, mRNA level, and translation. *J Biol Chem*. 1998;273:26171–26178.

Simunovic MP. Colour vision deficiency. *Eye (Lond)*. 2010;24:747–755.

Simunovic MP. Acquired color vision deficiency. *Surv Ophthalmol*. 2016;61:132–155.

Siu TL, Morley JW, Coroneo MT. Toxicology of the retina: advances in understanding the defence mechanisms and pathogenesis of drug- and light-induced retinopathy. *Clin Exp Ophthalmol*. 2008;36:176–185.

Sjoerdsma T, Kamermans M, Spekreijse H. Effect of the tuberculostaticum ethambutol and stimulus intensity on chromatic discrimination in man. *Vision Res*. 1999;39:2955–2962.

Sonkin N. Stippling of the retina: a new physical sign in the early diagnosis of lead poisoning. *N Engl J Med*. 1963;269:779–780.

Spencer PS, Schaumburg HH. A review of acrylamide neurotoxicity: Part I. Properties, uses and human exposure. *Can J Neurol Sci*. 1974a;1:145–150.

Spencer PS, Schaumburg HH. A review of acrylamide neurotoxicity: Part II. Experimental animal neuorotoxicity and pathologic mechanisms. *Can J Neurol Sci*. 1974b;1:152–169.

Srivastava D, Fox DA, Hurwitz RL. Effects of magnesium on cyclic GMP hydrolysis by the bovine retinal rod cyclic GMP phosphodiesterase. *Biochem J*. 1995a;308:653–658.

Srivastava D, Hurwitz RL, Fox DA. Lead- and calcium-mediated inhibition of bovine rod cGMP phosphodiesterase: interactions with magnesium. *Toxicol Appl Pharmacol*. 1995b;134:43–52.

Srivastava SK, Singhal SS, Awasthi S, et al. A glutathione *S*-transferases isozyme (bGST 5.8) involved in the metabolism of 4-hydroxy-2-transnonenal is localized in bovine lens epithelium. *Exp Eye Res*. 1996;63:329–337.

Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med*. 1995;18:321–336.

Storm JE, Mazor KA, Aldous KM, Blount BC, Brodie SE, Serle JB. Visual contrast sensitivity in children exposed to tetrachloroethylene. *Arch Environ Occup Health*. 2011;66:166–177.

Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev*. 2005;85:845–881.

Sweadner KJ. Isozymes of Na^+/K^+-ATPase. *Biochem Biophys Acta*. 1989;989:185–220.

Szabo I, Bernardi P, Zoratti M. Modulation of the mitochondrial megachannel by divalent cations and protons. *J Biol Chem*. 1992;267:2940–2946.

Takeuchi T, Eto K. Pathology and pathogenesis of Minamata disease. In: Tsubaki T, Irukayama K, eds. *Minamata Disease: Methylmercury Poisoning in Minamata and Niigata, Japan*. Tokyo; Kodansha; Amsterdam: Elsevier; 1977;103–141.

Tandon P, Padilla S, Barone S Jr, et al. Fenthion produces a persistent decrease in muscarinic receptor function in the adult rat retina. *Toxicol Appl Pharmacol*. 1994;125:271–280.

Tarkowski S, Kołakowski J, Górny R, Opacka J. The content of high-energy phosphates and ultrastructure of mitochondria in the brain of rats exposed to carbon disulphide. *Toxicol Lett*. 1980;5:207–212.

Tarkowski S, Sobczak H. Oxidation and phosphorylation processes in brain mitochondria of rats exposed to carbon disulphide. *J Neurochem*. 1971;18:177–82.

Taylor A, Nowell T. Oxidative stress and antioxidant function in relation to risk for cataract. *Adv Pharmacol*. 1997;38:515–536.

Thirkill CE, Roth AM, Keltner JL. Cancer-associated retinopathy. *Arch Ophthalmol*. 1987;105:372–375.

Till C, Westall CA, Koren G, et al. Vision abnormalities in young children exposed prenatally to organic solvents. *NeuroToxicology*. 2005;26:599–613.

Tolman JA, Faulkner MA. Vigabatrin: a comprehensive review of drug properties including clinical updates following recent FDA approval. *Expert Opin Pharmacother*. 2009;10:3077–3089.

tom Dieck S, Brandstätter JH. Ribbon synapses of the retina. *Cell Tiss Res*. 2006;326:339–346.

Ulshafer RJ, Allen CB, Rubin ML. Distributions of elements in the human retinal pigment epithelium. *Arch Ophthalmol*. 1990;108:113–117.

United States Environmental Protection Agency. *Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-98-242. Health Effects Test Guideline*. OPPTS 870.6855. *Neurophysiology: Sensory Evoked Potentials*. August 1998a. Available at: http:/www.epa.gov/docs/OPPTS Harmonized/870 Health Effects Test Guidelines/Series/.

United States Environmental Protection Agency. *Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-98-238. Health Effects Test Guideline*. OPPTS 870.6200. *Neurotoxicity Screening Battery*. August 1998b. Available at: http:/www.epa.gov/docs/OPPTS Harmonized/870 Health Effects Test Guidelines/Series/.

van Dijk BW, Spekreijse H. Ethambutol changes the color coding of carp retinal ganglion cells reversibly. *Invest Ophthalmol Vis Sci*. 1983;24:128–133.

Vanhoorne M, De Rouck A, Bacquer D. Epidemiological study of the systemic ophthalmological effects of carbon disulfide. *Arch Environ Health*. 1996;51:181–188.

van Soest S, Westerveld A, de Jong PT, et al. Retinitis pigmentosa: defined from a molecular point of view. *Surv Ophthalmol*. 1999;43:321–334.

Vettori MV, Corradi D, Coccini T, et al. Styrene-induced changes in amacrine retinal cells: an experimental study in the rat. *NeuroToxicology*. 2000;21:607–614.

Vidyasagar TR. Optic nerve components may not be equally susceptible to damage by acrylamide. *Brain Res*. 1981;224:452–455.

Walkowiak J, Altmann L, Kramer U, et al. Cognitive and sensorimotor functions in 6-year-old children in relation to lead and mercury levels: adjustment for intelligence and contrast sensitivity in computerized testing. *Neurotoxicol Teratol*. 1998;20:511–521.

Waltman S, Sears ML. Catechol-*O*-methyltransferase and monoamine oxidase activity in the ocular tissues of albino rabbits. *Invest Ophthalmol*. 1964;3:601–605.

Wang MY, Sadun AA. Drug-related mitochondrial optic neuropathies. *J Neuroophthalmol*. 2013;33:172–178.

Wang QP, Jammoul F, Duboc A, et al. Treatment of epilepsy: the GABA-transaminase inhibitor, vigabatrin, induces neuronal plasticity in the mouse retina. *Eur J Neurosci*. 2008;27:2177–2187.

Wässle H. Parallel processing in the mammalian retina. *Nat Rev Neurosci*. 2004;5:747–757.

Watkins JB III, Wirthwein DP, Sanders RA. Comparative study of Phase II biotransformation in rabbit ocular tissues. *Drug Metab Dispos*. 1991;19:708–713.

World Health Organization (WHO). Selected Occupational Risk Factors, Chapter 21. In: Ezzati M, Lopez AD, Rodgers A, et al., eds. *Comparative Quantification of Health Risks: Global and Regional Burden of Diseases Attributable to Selected Major Risk Factors*. Vol 1. Geneva, Switzerland: World Health Organization; 2004:1651–1802.

Wielgus AR, Roberts JE. Retinal photodamage by endogenous and xenobiotic agents. *Photochem Photobiol*. 2012;88:1320–1345.

Wilson MA, Johnston MV, Goldstein GW, Blue ME. Neonatal lead exposure impairs development of rodent barrel field cortex. *Proc Natl Acad Sci U S A*. 2000;97:5540–5545.

Winkler BS. A quantitative assessment of glucose metabolism in the rat retina. In: Christen Y, Doly M, Droy-Lefaix MT, eds. *Vision and Adaptation, Les Seminaires Ophtamologiques d'IPSEN*. Vol 6. Paris: Elsevier; 1995:78–96.

Winkler BS, Riley MV. Na^+,K^+ and HCO_3-ATPase activity in retina: dependence on calcium and sodium. *Invest Ophthalmol Vis Sci*. 1977;16:1151–1154.

Winneke G, Altmann L, Krämer U, et al. Neurobehavioral and neurophysiological observations in six year old children with low lead levels in East and West Germany. *NeuroToxicology*. 1994;15:705–713.

Withering W. *An Account of the Foxglove and Some of Its Medicinal Uses: With Practical Remarks on Dropsy and Other Diseases*. London: Broomsleigh Press; 1785.

Wolfensberger TJ. Toxicology of the retinal pigment epithelium. In: Marmor MF, Wolfensberger TJ, eds. *The Retinal Pigment Epithelium Function and Disease*. New York, NY: Oxford University Press; 1998:621–647.

Woodhouse JM, Barlow HB. Spatial and temporal resolution and analysis. In: Barlow HB, Mollon JD, eds. *The Senses*. Cambridge: Cambridge University Press; 1982:133–164.

Wu SM. Synaptic organization of the vertebrate retina: general principles and species-specific variations: the Friedenwald lecture. *Invest Ophthalmol Vis Sci*. 2010;51:1263–1274.

Yoshino Y, Mozai T, Nako K. Distribution of mercury in the brain and its subcellular units in experimental organic mercury poisonings. *J Neurochem*. 1966;13:397–406.

Yuki K, Dogru M, Imamura Y, et al. Lead accumulation as possible risk factor for primary open-angle glaucoma. *Biol Trace Elem Res*. 2009;132:1–8.

Zavalic M, Turk R, Bogadi-Sare A, Skender L. Colour vision impairment in workers exposed to low concentrations of toluene. *Arh Hig Rada Toksikol*. 1996;47:167–175.

Zhang X, Feng Q, Cote RH. Efficacy and selectivity of phosphodiesterase-targeted drugs in inhibiting photoreceptor phosphodiesterase (PDE6) in retinal photoreceptors. *Invest Ophthalmol Vis Sci*. 2005;46:3060–3066.

Zhao B, Chignell CF, Rammal M, et al. Detection and prevention of ocular phototoxicity of ciprofloxacin and other fluoroquinolone antibiotics. *Photochem Photobiol*. 2010;86:798–805.

Zhao C, Schwartzman ML, Shichi H. Immunocytochemical study of cytochrome P450 4A induction in mouse eye. *Exp Eye Res*. 1996;63:747–751.

Zhao C, Shichi H. Immunocytochemical study of cytochrome P450 (1A1/1A2) induction in murine ocular tissues. *Exp Eye Res*. 1995;60:143–152.

Zhao L, Chen G, Li J, Fu Y, et al. An intraocular drug delivery system using targeted nanocarriers attenuates retinal ganglion cell degeneration. *J Control Release*. 2017;247:153–166.

Zrenner E, Gouras P. Blue-sensitive cones of the cat produce a rod like electroretinogram. *Invest Ophthalmol Vis Sci*. 1979;18:1076–1081.

Zrenner E, Kramer W, Bittner C, et al. Rapid effects on colour vision following intravenous injection of a new, non-glycoside positive ionotropic substance (AR-L 115BS). *Doc Ophthalmol Proc Ser*. 1982;33:493–507.

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chapter 18

Toxic Responses of the Heart and Vascular System

Matthew J. Campen

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  - *Antimicrobial and Antiviral Agents*
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  - *Antihistamines*
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  - *Antineoplastic Drugs*
  - *Analgesics and Nonsteroidal Anti-inflammatory Drugs*
  - *Oral Contraceptives*
- Natural Products
  - *Bacterial Endotoxins*
  - *Homocysteine*
  - *Hydrazinobenzoic Acid*
  - *T-2 Toxin*
  - *Vitamin D*
  - *β-Amyloid*
  - *Uremic Toxins*
- Environmental Pollutants and Industrial Chemicals
  - *Carbon Monoxide*
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  - *Aromatic Hydrocarbons*

Acknowledgments

References

INTRODUCTION

Cardiovascular toxicology is concerned with the adverse effects of extrinsic and intrinsic stresses on the heart and vascular system. Extrinsic stress involves exposure to therapeutic drugs, natural products, and environmental toxicants. Notably, the heart and vessels are somewhat protected from xenobiotics due to classical defense and detoxification organs (e.g., skin, lungs, and liver), thus many extrinsic cardiotoxins are intentionally administered substances. Intrinsic stress refers to exposure to toxic metabolites derived from nontoxic compounds such as those found in food additives and supplements. The intrinsic exposures also include secondary neurohormonal disturbance such as overproduction of inflammatory cytokines derived from pressure overload of the heart and counter-regulatory responses to hypertension or uremic toxins that arise from renal failure. These toxic exposures result in alterations in biochemical pathways, defects in cellular structure and function, and pathogenesis of the affected cardiovascular system. The manifestations of toxicological response of the heart include cardiac arrhythmia, hypertrophy, and overt heart failure. The responses of the vascular system include changes in blood pressure and lesions in blood vessels in the form of atherosclerosis, hemorrhage, and edema.

This chapter is divided into two parts, separately focusing on the heart and the vascular systems. For a better understanding of the toxic manifestations of the cardiovascular system, an overview of the physiology and biochemistry of the heart and the vascular system is presented in relation to toxicological concerns. The toxicological responses of the heart and the vascular system and the mechanisms underlying these responses are the major focus of this chapter. This chapter also presents a brief discussion of chemicals that affect the heart and the vascular system.

OVERVIEW OF THE HEART

Overview of Cardiac Structural and Physiological Features

Cardiac muscle, along with nerve, skeletal muscle, and smooth muscle, is one of the excitable tissues of the body. It shares many bioelectrical properties with other excitable tissues, and also has unique features associated with cardiac structural and physiological specificities. Fig. 18-1 illustrates the basic anatomy of the heart. With regard to cardiac toxicology, this section will only review some features of cardiac physiology and structures. There are many textbooks of cardiac anatomy and physiology that provide extensive knowledge basis of cardiac physiology and structural properties, which will not be repeated in this section.

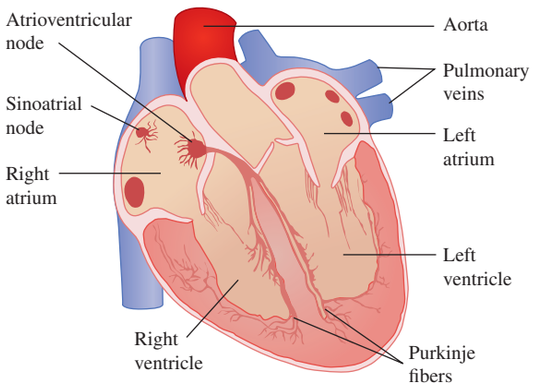

Figure 18-1. *Diagram illustrating the basic anatomy of the heart.*

Review of Cardiac Structure The primary contractile unit within the heart is the cardiac muscle cell, or cardiac myocyte. Cardiac myocytes are composed of several major structural features and organelles, as illustrated in Fig. 18-2. A primary component is the contractile element known as the myofibril. Each myofibril consists of a number of smaller filaments (the thick and thin myofilaments). The thick filaments are special assemblies of the protein myosin, while thin filaments are made up primarily of the protein actin. Cardiac myosin is a hexamer composed of one pair of myosin heavy chains (MHCs) and two pairs of myosin light chains (MLCs). Two isoforms of MHC, α and β, are expressed in cardiac muscle; the expression of these is under developmental control and may be altered by a variety of physiological, pathological, and pharmacological stimuli (Martin et al., 1996; Metzger et al., 1999). In addition, the predominant isoform expressed in normal adult cardiac tissue also depends on the species examined. Similarly, two isoforms of actin are expressed in the heart (cardiac and skeletal α-actin), and, as with MHC, actin isoform expression is influenced by developmental, physiological, pathological, and pharmacological stimuli, and the primary isoform of actin found in normal adult cardiac muscle also depends on the species examined.

Under electron microscopy, these essential structural components of myocardial contractile proteins display alternating dark bands (A bands, predominantly composed of myosin) and light bands (I bands, predominantly composed of actin). Visible in the middle of the I band is a dense vertical Z line. The area between two Z lines is called a sarcomere, the fundamental unit of muscle contraction. Although cardiac and skeletal muscle share many similarities, a major difference lies in the organization of cardiac myocytes

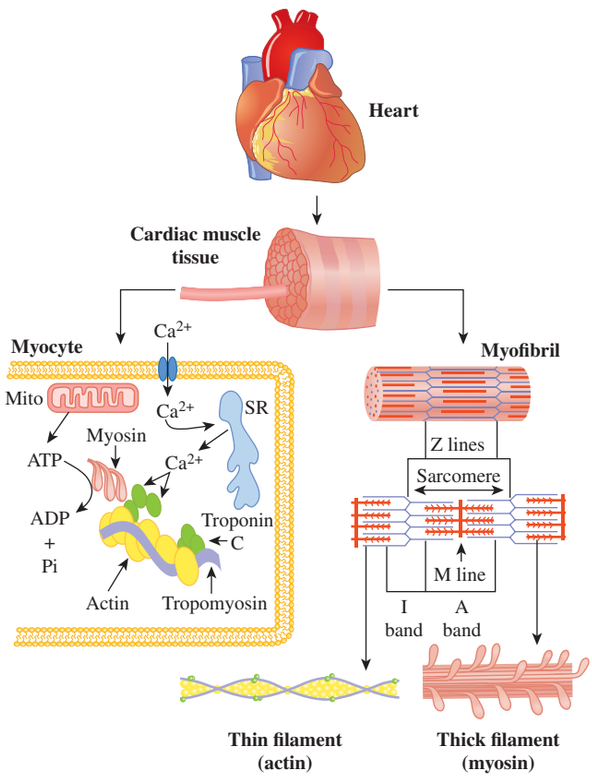

Figure 18-2. *Structural organization of cardiac muscle tissue.*

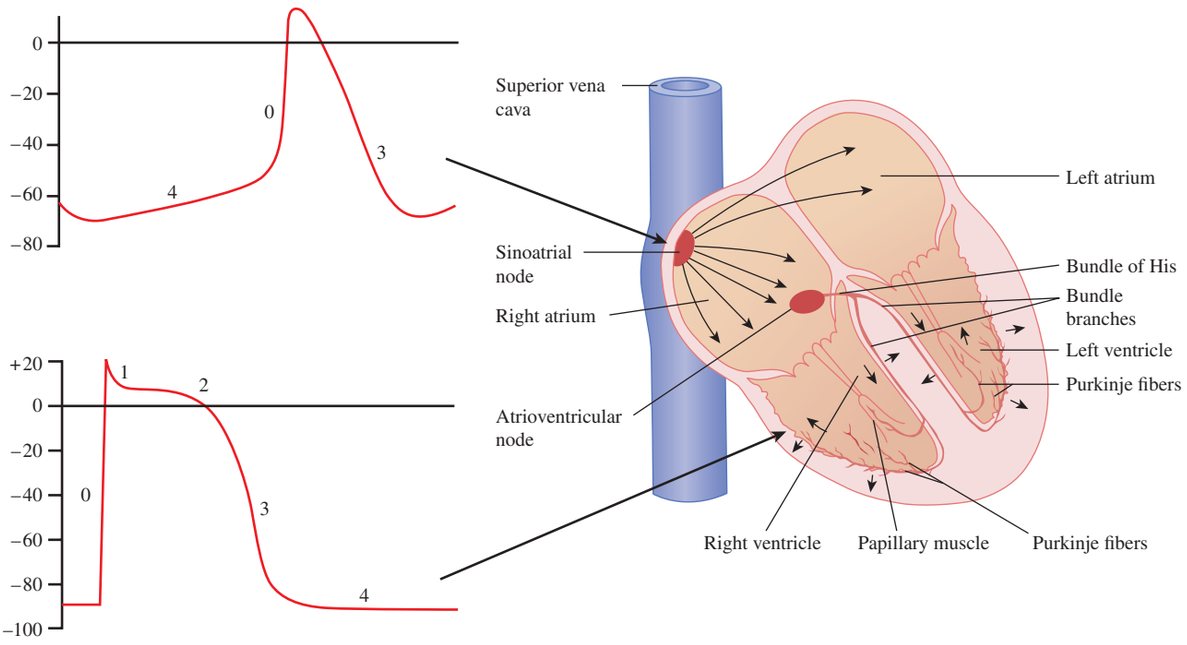

Figure 18-3. *Characteristic cardiac action potential recorded from sinoatrial node pacemaking cells (top) and Purkinje fibers (bottom) as indicated.* (From Berne and Levy [1983], with permission from Elsevier.)

into a functional syncytium where cardiac myocytes are joined end-to-end by dense structures known as intercalated disks. Within these, there are tight gap junctions that facilitate action potential propagation and intercellular communication. About 50% of each cardiac myocyte is composed of myofibrils. The rest of the intracellular space contains the remaining major components of the cell: mitochondria (33%), one or more nuclei (5%), the sarcoplasmic reticulum (SR) (2%), lysosomes (very low), glycogen granules, a Golgi network, and cytosol (12%) (Opie, 1996).

Cardiac myocytes are the largest cells in the heart and contributes to the majority of cardiac mass. However, cardiac myocytes make up only about one-quarter of all the cells in the heart. Cardiac fibroblasts, vascular cells, specialized conducting Purkinje cells, and other connective tissue cells make up the majority of cell number in the heart. Cardiac fibroblasts make up approximately 90% of these "nonmuscle" cells. Cardiac myocytes are generally considered to be terminally differentiated, although numerous innate and therapeutic pathways for myocardial regeneration via pluripotent stem cells have been recently discovered. Carbon dating experiments suggest approximately 1% to 2% of cardiomyocytes are replaced annually in healthy adults, and effect that diminishes with advanced age (Bergmann et al., 2009). The heart undergoes a significant increase in size and mass throughout growth of the organism, but the increase in heart size and mass is produced by enlargement (or hypertrophy) of preexisting cardiac myocytes (Li et al., 1996). With regard to this developmental period, cardiac hypertrophy is considered to be a normal physiological process. Under pathological conditions, hypertrophy of remaining cardiac myocytes is a hallmark of cardiac remodeling following myocardial injuries, such as myocardial infarction. Cardiac fibroblasts may continue to proliferate after birth, particularly in response to injury. Cardiac fibroblasts also contribute to cardiac remodeling following myocardial infarction and are thought to promote fibrosis and scarring of injured cardiac tissue. Thus, from a toxicological perspective, the heart is vulnerable to injury because of limited proliferative capacity of cardiac myocytes, and promotion of cardiac fibroblast proliferation and remodeling following injury.

Electrophysiology The electrophysiology of the heart is concerned with bioelectricity and its related cardiac physiological function. Bioelectricity is the result of charge generated from the movement of positively and negatively charged ions in tissues. In cardiac myocytes, three major positively charged ions make a significant contribution to the bioelectricity of the heart: calcium (Ca^{2+}), sodium (Na^{+}), and potassium (K^{+}). Each of the ions has specific channels and transporters (pumps) on the membrane of cardiac myocytes. Through the movement of these ions across the cell membrane, an action potential is generated and propagated from one cell to another, so that electric conductance is produced in the heart. The cardiomyocytes themselves can propagate a wavefront of cellular depolarization that leads to contraction, but specialized cells also exist, such as pacemaker cells in the sinoatrial nods and Purkinje fibers traveling through the ventricular septum that control the timing and organization of the conduction.

Action Potential Cardiac myocytes produce an action potential when activated. In the resting state, the resting potential of a myocyte is about –60 to –90 mV relative to the extracellular fluid potential. A sudden depolarization changes the membrane potential from negative inside to positive inside, followed by a repolarization to reset the resting potential. The process of an action potential from depolarization to the completion of repolarization is divided into five phases in cardiac Purkinje fibers, as shown in Fig. 18-3. Phase 0 represents a rapid depolarization due to the inward current of Na^{+}, which is caused by the rapid opening of voltage-gated sodium channels. Phase 1 is associated with an immediate rapid repolarization, during which the Na^{+} inward current is inactivated and a transient K^{+} outward current is activated, followed by an action potential plateau or phase 2, which is dominated by slowly

decreasing inward Ca^{2+} current and a slow activation of an outward K^+ current. Phase 3 reflects a fast K^+ outward current and inactivation of the plateau Ca^{2+} inward current, and phase 4 is the diastolic interval for the resetting of the resting potential.

Automaticity A group of specialized cells in the heart are capable of repetitively spontaneous self-excitation, which generate and distribute each impulse through the heart in a highly coordinated manner to control the normal heartbeat. These cells include the sinoatrial (SA) node pacemaking cells and Purkinje fibers in the ventricles. Other cells such as the atrial-specialized fibers under normal conditions have a much slower automaticity and are normally controlled or "entrained" by the pacemaking rhythm, but can increase firing rate and become an ectopic pacemaker under abnormal conditions. The sinus node pacemaker cells have only three distinct phases of action potential (Fig. 18-3): phase 0, rapid depolarization; phase 3, plateau and repolarization; and phase 4, slow depolarization, often referred to as *pacemaker potential*. It is the pacemaker potential that brings the membrane potential to a level near the threshold for activation of the voltage-gated calcium channels and inward Ca^{2+} current, which triggers the phase 0 rapid depolarization and makes the pacemaker cells of automaticity. In pacemaker cells, phase 0 is mediated almost entirely by increased conductance of Ca^{2+} ions. Additionally, cells of the atrioventricular (AV) node exhibit a slower automaticity, such that under normal conditions the AV pace is entrained by the SA node rhythm. The AV node slows conduction through the heart to allow proper filling of ventricles prior to contraction, and further serves as a safety net in the event of SA nodal electrophysiologic dysfunction.

Contractility Cardiac myocytes, like other muscle cells, exhibit the specific functional feature of contractility. Myocyte contraction occurs when an action potential causes the release of Ca^{2+} from the SR as well as the entry of extracellular Ca^{2+} into the cell. This action potential–triggered Ca^{2+} increase in the plasma and myocyte contraction is called *excitation–contraction coupling* (Fig. 18-4). The increase in Ca^{2+} concentrations in the cell allows Ca^{2+} to bind to troponin and tropomyosin, leading to conformational change in the contractile unit of the cardiac myocyte: thin filament. This conformational change permits interaction between the actin and myosin filaments through the crossbridges (myosin heads). Adenosine triphosphate (ATP) is hydrolyzed by ATPase present in the crossbridges to release energy for the movement of the crossbridges in a ratchet-like fashion. This action increases the overlap of the actin and myosin filaments, resulting in shortening of the sarcomeres and contraction of the myocardium. Synchronized contraction of atrial and ventricular myocytes leads to pumping of blood during the systolic phase of the cardiac cycle. After each contraction, the heart must then relax to permit filling from the atria, during a period called diastole.

Electrotonic Cell-to-Cell Coupling Myocardium as a whole has to synchronize the contraction and relaxation of individual myocytes in order to perform its pump function. This is achieved by a special structural feature of cell-to-cell interaction, electrotonic cell-to-cell coupling via the gap junction. Through the gap junction, major ionic fluxes between adjacent cardiomyocytes are spread, thus allowing electrical synchronization of contraction. Each single gap junction is composed of 12 connexin 43 (Cx43) units, assembled in two hexametric connexons (hemichannels) that are contributed, one each, by the two participating cells. The connexins interact with other proteins within the cell so that connexons are not only important for cell-to-cell coupling, but also involved in cell signaling and volume regulation. An important feature of the connexon-controlled electrotonic cell-to-cell coupling is the electrotonic current flow that attenuates the differences in action potential duration of individual cardiac myocytes. Anatomically speaking, the distribution of gap junctions at the ends of myocytes facilitates a linear directionality of electrical conduction while discouraging transmission of abnormal "ectopic" electrical conductions traveling in the transverse direction to minimize risk of arrhythmic events.

Electrocardiograph The electrophysiological features of cardiac myocytes and the electrotonic cell-to-cell coupling give rise to charge at any given locus in the heart with a magnitude and a direction. Therefore, at any given moment in the cardiac cycle, there is

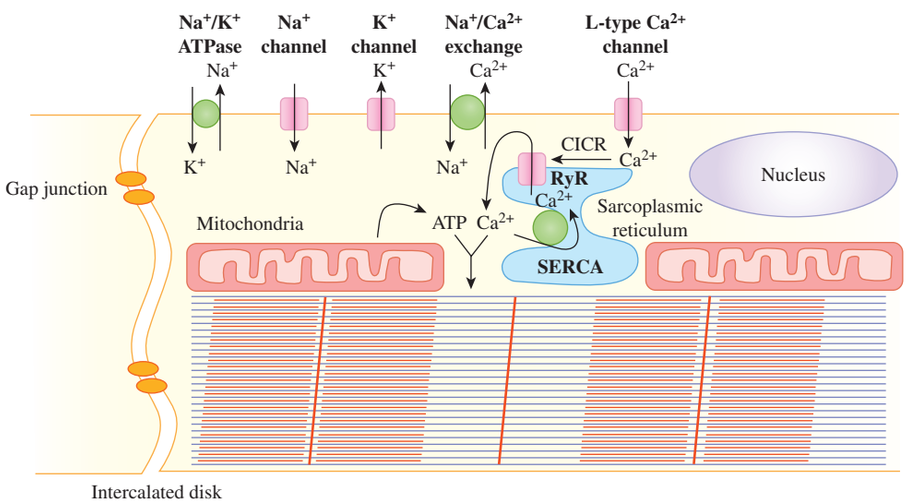

Figure 18-4. *Overview of excitation–contraction coupling in cardiac myocytes.* Upon rapid depolarization (rapid influx of Na^+ through fast channels; phase 0 of the action potential), L-type Ca^{2+} channels are opened allowing a slower but sustained influx of Ca^{2+} down the electrochemical gradient (phase 2 of the action potential). During the process of Ca^{2+}-induced Ca^{2+} release (CICR), slight elevation in intracellular-free Ca^{2+} stimulates Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR). The SR provides the majority of Ca^{2+} required for contraction. The mitochondria provide energy for contraction in the form of ATP. Repolarization of the cell occurs largely by activation of K^+ channels and efflux of K^+ (phase 3 of the action potential). For relaxation, the SR Ca^{2+} ATPase (SERCA) actively pumps Ca^{2+} back into the SR, although some Ca^{2+} may be removed by the Na^+/Ca^{2+} exchanger or by sarcolemmal Ca^{2+} pumps.

a complex pattern of electrical charges across the membranes of myriad cells in the heart. The sum of all the individual cells that exist at any given time within the heart is the *resultant cardiac vector*. The changes in the resultant cardiac vector throughout the cardiac cycle can be recorded as a *vector cardiograph*. Lead systems are used to record certain projections of the resultant cardiac vector. The potential difference between two recording electrodes represents the projection of the vector on the line between the two leads. Components of vectors projected on such lines eliminate their directions and have a sum of magnitude, being scalar quantities. Thus, a recording of the changes with time in the potential differences between two points on the surface of the skin is the so-called *scalar electrocardiograph*.

In general, the pattern of the scalar electrocardiograph consists of P, QRS, and T waves, as shown in Fig. 18-5. The PR interval is a measure of the time from the onset of atrial activation to the onset of ventricular activation. The QRS complex represents the conduction pathways through the ventricles. The ST segment is the interval during which the entire ventricular myocardium is depolarized, and lies on the isoelectric line under normal conditions. The QT interval is sometimes referred to as the period of "electrical systole" of the ventricles, and mirrors the action potential duration of the mass of ventricular myocytes. The QT interval prolongation is recognized as a major life-threatening factor of drug cardiac toxicity, which is brought about by a reduction of outward currents and/or enhanced inward currents during phase 2 and 3 of the action potential. The electrocardiogram is therefore a valuable tool for diagnosing abnormalities of cardiac physiology. Beyond the single cardiac cycle, beat-to-beat variation in the pacing of the heart can be an important reflection of autonomic nervous system activity, a phenomenon referred to as heart rate variability.

Neurohormonal Regulation Although the heartbeat is governed by the automaticity of the sinus node P cells, neurohormonal regulation of cardiac electrophysiology and contraction controls cardiac function under normal and abnormal conditions. Toxicants often exert their effects on the cardiac system through interference with the neurohormonal regulation, thus this regulatory system is of significant relevance to cardiac toxicology. There are many neurohormonal systems that have significant impact on the heart. A detailed description of the regulatory system will be provided in the following sections in association with specific discussion of cardiac functional regulation, compensatory, and maladaptive responses to toxic exposures.

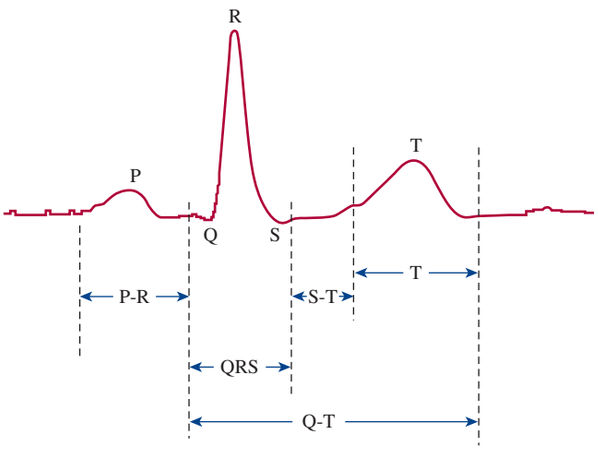

Figure 18-5. *A typical electrocardiogram (ECG) with the illustration of important deflections and intervals.*

Overview of Cardiac Energy Metabolism and Biochemistry

ATP and the Heart It is easy to understand that the need of energy for the heart is high. The chemical energy in the form of ATP is absolutely needed to support the systolic and diastolic work of the heart (Ventura-Clapier et al., 2004). In the heart, the primary ATP-utilizing reactions are catalyzed by actomyosin ATPase in the myofibril, the Ca^{2+}-ATPase in the SR, and the Na^+,K^+-ATPase in the sarcolemma (Ingwall and Weiss, 2004). ATP is also needed for molecular synthesis and degradation in the heart as in other organ systems. ATP synthesis by oxidative phosphorylation in the mitochondria is usually sufficient to support the normal needs of the heart, even when the work output of the heart increases three- to fivefold (Ingwall and Weiss, 2004). In addition, the glycolytic pathway and the tricarboxylic acid cycle also make small contributions to ATP synthesis. The concentration of ATP does not define the energetic state of the heart. The amount of ATP made and used at any given time is many times greater than the size of the measurable ATP pool (Ingwall and Weiss, 2004). Thus, cardiac myocytes contain high concentrations of mitochondria, which ensure that the ATP supply remains constant through oxidation of a variety of carbon-based fuels for ATP synthesis under different conditions. Under conditions of ischemia or impaired blood flow when oxygen is limited (e.g., myocardial infarction), ATP is rapidly consumed in the heart and reduced to adenosine, which can act as a vasodilator and have other protective effects. However, adenosine readily crosses the myocyte phospholipid membrane and leaves the cell, impeding recovery following prolonged ischemia (Jennings, 2013).

Phosphocreatine and the Heart A unique feature in energy metabolism of the heart is the use of energy reserve systems, such as phosphocreatine (PCr), to maintain a high phosphorylation potential to drive ATPase reactions under highly demanding conditions (Ingwall et al., 1985). PCr exists in the heart at twice the ATP concentration (Bittl and Ingwall, 1985). The enzyme creatine kinase (CK) transfers the phosphoryl group between ATP and PCr at a rate about 10 times faster than the rate of ATP synthesis by oxidative phosphorylation. The reaction catalyzed by CK is: PCr + ADP + H^+ ↔ Creatine + ATP. Under the conditions when ATP demand exceeds ATP supply, the use of PCr is a major pathway to maintain a constant supply of ATP. The CK reaction is also important to maintain low ADP and Pi concentrations, thereby retaining high phosphorylation potential (Saupe et al., 2000). Creatine is not made in the heart but accumulates against a large concentration gradient facilitated by a creatine transporter. In the normal heart, about two-thirds of the total creatine pool is phosphorylated through the CK reaction to form PCr (Wallimann et al., 1998; Neubauer et al., 1999).

Metabolic Pathways The continuous synthesis of ATP via mitochondrial oxidative phosphorylation is mandatory for the work of the heart (Huss and Kelly, 2005). Under normal conditions, the oxidation of fatty acids (FA) is the major pathway, providing about 65% of the total energy demand. In contrast, the oxidation of glucose provides about 30% of the total energy demand (Shipp et al., 1961; Wisneski et al., 1987). In hypertrophic and failing hearts, there is a metabolic shift from FA- to glucose-dependent energy supply. Thus, decreased FA oxidation and increased glucose utilization in association with depressed FA deposition and increased glucose uptake are observed in hypertrophic and failing hearts (van Bilsen et al., 2004). This shift enhances the glycolytic pathway, and thus increases the anaerobic metabolism. However, it remains debated whether this metabolic shift to the so-called "fetal

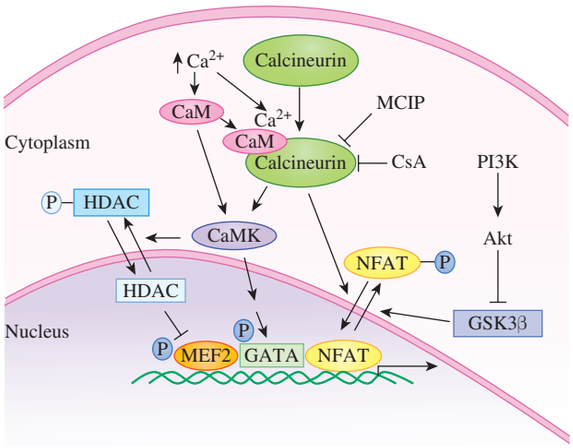

Figure 18-6. *Calcineurin signal transduction pathways in regulation of transcription factors involved in hypertrophic growth of cardiac myocytes.* Sustained increases in intracellular Ca^{2+} concentrations, along with calmodulin (CaM), activate calcineurin, which in turn causes dephosphorylation of nuclear factor of activated T cells (NFAT), enabling NFAT to translocate to the nucleus where it interacts with GATA4 to regulate gene expression. Phosphorylation of NFAT is stimulated by glycogen synthase kinase 3β (GSK3β), whose activity is inhibited by activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway. Calcineurin also regulates CaM activation of CaM-dependent kinase (CaMK), which activates GATA transcription factor. CaMK also phosphorylates histone deacetylases (HDAC), leading to HDAC translocation from nucleus to cytoplasm. Otherwise, HDAC in the nucleus inhibits myocyte-enhancer factor 2 (MEF2) transcription activity. Cyclosporine A (CsA) and modulatory calcineurin-interacting protein (MCIP) inhibit calcineurin activation.

phenotype" is adaptive or maladaptive. It is important to note that the "shift" is only partial, and even when the proportion of ATP synthesized from glucose increases many fold, aerobic metabolism still remains dominant (van Bilsen et al., 2004). With regard to the concern of cardiac toxicology, the metabolic shift is often observed with mitochondrial dysfunction. In response to toxic exposure, mitochondrial damage leads to impaired oxidative phosphorylation and a metabolic shift from aerobic to anaerobic and a reliance on glucose utilization.

Calcium, Calmodulin, and Calcineurin The role of calcium in cardiac toxic responses has been extensively investigated. As an important ion regulating cardiac cycle and contraction, direct disturbance of calcium concentrations or channel function can have profound proarrhythmic consequences. Furthermore, continually elevated levels of cytosolic calcium can also promote cardiomyocyte hypertrophy. When carefully examining the current literature, one can find that there are very few mechanistic studies that specifically probe the role of calcium in cardiac toxicity, although numerous studies have implicated intracellular Ca^{2+} as a signal for cardiac responses to environmental toxic insults (Shier and Dubourdieu, 1992; Toraason et al., 1997; Buck et al., 1999). In response to myocardial stress by environmental toxic exposures, calcium concentrations are increased in the myocardial cells (Sleight, 1996). This is consistent with the speculation that Ca^{2+} coordinates physiological responses to stresses. Notably, calcium levels become augmented in systole with increased myocyte force generation in response to increased vascular resistance; in latter stages of heart failure, diastolic dysfunction is characterized by elevated resting levels of calcium.

The role of calcium in mediating myocardial hypertrophic signals has been extensively studied (Stemmer and Klee, 1994). A sustained increase in intracellular Ca^{2+} concentrations activates calcineurin. Calcineurin is a ubiquitously expressed serine/threonine phosphatase that exists as a heterodimer, comprises a 59-kDa calmodulin (CaM)-binding catalytic A subunit and a 19-kDa Ca^{2+}-binding regulatory B subunit (Molkentin et al., 1998). Activation of calcineurin is mediated by binding of Ca^{2+} to the regulatory subunit and CaM to the catalytic subunit (Fig. 18-6). Calcineurin in turn dephosphorylates a transcription factor, nuclear factor of activated T cells (NFAT), which then translocates to the nucleus and transcribes a battery of pro-growth elements (Bers, 2008). Of toxicological relevance is that calcineurin is activated by a sustained increase in Ca^{2+} concentration and is insensitive to transient Ca^{2+} fluxes, such as in response to cardiomyocyte contraction (Stemmer and Klee, 1994).

Similarly, certain G protein–coupled receptors (e.g., for endothelin and angiotensin) and other growth factor receptors can activate phospholipase C, leading to the formation of diacylglycerol and inositol triphosphate (IP_3). IP3 activates nuclear IP3 receptors that, together with calcium release, activate calmodulin and calmodulin kinase IIδ. Within the nucleus, this activity phosphorylates histone deacetylases, which are exported from the nucleus, thereby removing an important suppressor of hypertrophic myocyte enhancer factor 2 (MEF2) transcription, described in the section "Myocyte-Enhancer Factor 2" in greater detail (Bers, 2008).

Numerous studies have demonstrated important roles for Ras, mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) signaling pathways in myocardial responses to hypertrophic stimuli (Jalili et al., 1999). All of these signal transduction pathways are associated with increase in intracellular Ca^{2+} concentrations (Ho et al., 1998). The coordinating role of calcium in cardiac hypertrophic response has been demonstrated (Stemmer and Klee, 1994) as follows. Hypertrophic stimuli, such as angiotensin II and phenylephrine, cause an elevation of intracellular Ca^{2+} that results in activation of calcineurin as well as generation of IP_3. A series of reactions occur through activated calcineurin, including dephosphorylation of NFAT and its translocation to the nucleus, where it interacts with GATA4 (Fig. 18-6). Calcineurin also acts through an NFAT-independent mechanism to regulate hypertrophic gene expression.

AMP-Activated Protein Kinase Activation of AMP-activated protein kinase (AMPK) often occurs when the myocardial metabolic phenotype shifts to the fetal form. Activation of AMPK occurs with changes in high-energy phosphate metabolism in hypertrophic and failing hearts. The increase in AMP/ATP ratio occurs when the PCr/ATP ratio decreases due to a decrease in PCr, with or without a concomitant decrease in ATP. The decrease in PCr/ATP ratio is an index of decreased energy reserve and correlates with the severity of heart failure and is of prognostic value (Neubauer et al., 1997). Activation of AMPK leads to translocation of the insulin-dependent glucose transporter (GLUT4) from intracellular stores to the sarcolemma (Russell et al., 1999). Mice overexpressing an active form of AMPK suffer from pathological cardiac glucogen accumulation (Arad et al., 2003). Furthermore, the AMPK-dependent phosphorylation of the enzyme 6-phosphofructo-2-kinase stimulates glycolysis (Marsin et al., 2000). These pathways indicate the importance of AMPK activation for the cardiac metabolic shift to the energy supply reliance on glucose metabolism.

Mitogen-Activated Protein Kinases MAPKs play a major role in cardiac response to toxic insults. A generalized diagram for MAPK signaling is presented in Fig. 18-7. The MAPKs consist of a series of successively acting kinases and three major branches are

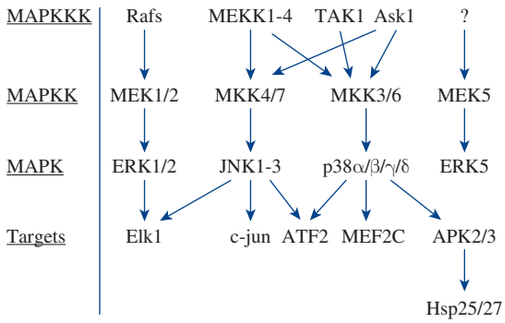

Figure 18-7. *Schematic representation of the hierarchy constituents of the MAPK signaling pathways.* MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase.

involved in the classic MAPK signaling pathway. These branches are divided based on their terminal effector kinases: the extracellular signal–regulated protein kinases (ERK), the *c*-jun NH_2-terminal kinases (JNK), and p38 MAPKs (Sugden and Clerk, 1998). Each branch of the MAPKs has a hierarchy control system beginning at the MAPK kinase kinase (MAPKKK), as shown in Fig. 18-7. Among the MAPKs, p38 MAPKs have been extensively studied in myocardial apoptosis. This subfamily consists of p38α, p38β, p38γ, and p38δ (Sugden and Clerk, 1998). Several studies have identified p38 MAPKs as an important group of signaling molecules that mediate environmental stress responses in various cell types (Tibbles and Woodgett, 1999). In noncardiac cells, p38 MAPKs have been implicated in gene expression, morphological changes, and cell death in response to endotoxin, cytokines, physical stress, and chemical insults (Tan et al., 1996; Wang and Ron, 1996). In cardiac cells, p38 MAPKs are associated with the onset of apoptosis in ischemia reperfusion (Yin et al., 1997). In particular, transfection experiments using primary cultures of neonatal rat cardiomyocytes have shown that p38α is critically involved in myocyte apoptosis (Wang et al., 1998).

Adriamycin, a cancer chemotherapeutic drug also known as doxorubicin, is a cardiac toxicant and two observations indicate that the p38 MAPK is involved in Adriamycin-induced myocyte apoptosis (Kang et al., 2000a). First, a time-course analysis revealed that p38 MAPK activation preceded the onset of apoptosis. As early as 30 minutes after Adriamycin administration, myocyte apoptosis occurs, detected by a sensitive method of fluorescein isothiocyanate (FITC) conjugation of annexin V (annexin V-FITC). However, p38 MAPK activation detected by the FITC-conjugated anti-phospho p38 antibody and confocal microscopy is observed 20 minutes after Adriamycin treatment (Kang et al., 2000a). Second, SB203580, a specific inhibitor of p38 MAPKs, inhibits Adriamycin-induced myocyte apoptosis (Kang et al., 2000a). Because SB203580 acts as a specific inhibitor of p38α and p38β, but not p38γ and p38δ, the involvement of the former specific isoforms of the p38 MAPK in the Adriamycin-induced myocyte apoptosis is implicated. p38α is involved in apoptosis of neonatal rat cardiomyocytes in primary cultures and p38β mediates hypertrophy of these cells (Wang et al., 1998).

Protein Kinase C PKC is among the most extensively studied signaling molecules in the heart. Many cardiac toxicological studies have examined the role of PKC in mediating toxic signals. Several excellent reviews on PKC in myocardial signaling pathways leading to cardiac hypertrophy and heart failure are available (Puceat and Vassort, 1996). PKC is a ubiquitously expressed serine/threonine kinase, which is activated predominantly by G_q/G_{11}-coupled receptors. The PKC family consists of 11 isoforms, which are divided into three subgroups: conventional PKCs (cPKCs) including α, β (I and II), and γ; novel PKCs (nPKCs) including ε, δ, η, ζ, and θ; and atypical PKCs (aPKCs) including ι, λ, and μ (Newton, 1995). The cPKCs are activated by Ca^{2+} and diacylglycerol (DAG), as well as phorbol esters. The nPKCs do not bind Ca^{2+}, but respond to DAG and phorbol ester stimulation. The aPKCs do not respond to Ca^{2+}, DAG, or phorbol esters. PKC has been demonstrated to participate in the regulation of transcription, the maintenance of cell growth and membrane structure, and modulation of immune responses. Disturbances in PKC signaling pathways lead to cardiac hypertrophy and heart failure, which is of toxicological significance.

Transcription Factors Transcription factors activate or deactivate myocardial gene expression, which affects the function and phenotype of the heart. Many transcription factors have been studied in myocardial tissue. Several of them are of toxicological significance as described below.

Activator Protein-1 Activator protein-1 (AP-1) is a transcription factor composed of *Jun* and *Fos* gene family members (McMahon and Monroe, 1992). The AP-1-binding site is the TRE (12-*O*-tetradecanoyl phorbol 13-acetate response element), and the binding of AP-1 to the TRE initiates transcription of the target genes (Diamond et al., 1990). Elevated levels of *c*-Jun are seen in cardiomyocytes with ischemia–reperfusion (Brand et al., 1992). In volume-overload hypertrophy, AP-1 plays an important role in the regulation of Fas and FasL activities (Wollert et al., 2000). Overstretching of myocardium induces Fas expression (Cheng et al., 1995). Subsequently, Fas-dependent signaling pathways can lead to myocardial cell apoptosis. However, there are other studies that indicate activation of AP-1 is not associated with the induction of apoptosis (Lenczowski et al., 1997). AP-1 has been implicated in transcriptional regulation of several genes associated with a hypertrophic response (Paradis et al., 1996).

Myocyte-Enhancer Factor 2 Myocyte-enhancer factor 2 (MEF2) is a transcription factor that binds to A-/T-rich DNA sequences within the promoter regions of a number of cardiac genes, including muscle CK gene, β-MHC, MLC1/3, MLC2v, skeletal α-actin, SR Ca^{2+}-ATPase, cardiac troponin T, C, and I, desmin, and dystrophin (Black and Olson, 1998). MEF2 is critically involved in the regulation of inducible gene expression during myocardial hypertrophy. The activation of MEF2 involves phosphorylation of the transcription factor by p38 MAPK or ERK5-MAPK. The ERK5-MEF2 pathway has been observed in the generation of cardiac hypertrophy. An important function of MEF2 is the convergence in the binary downstream pathway of Ca^{2+} signaling. Increased intracellular Ca^{2+} binds to and activates Ca^{2+}-binding proteins including CaM, which regulates calcineurin and Ca^{2+}/CaM-dependent protein kinase (CaMKs). Activation of either calcineurin or CaMKs induces cardiac hypertrophy. CaMKs stimulate MEF2 through phosphorylation of the transcriptional suppressor, histone deacetylases (HDACs). Nuclear CaMK is considered as a HDAC kinase whose activity is enhanced by calcineurin. Thus, MEF2 converges the stimulating signaling of both CaMKs and calcineurin leading to activation of hypertrophic gene expression (Fig. 18-6).

Nuclear Factor of Activated T Cells 3 NFAT3 is a member of a multigene family that contains four members: NFATc, NFATp, NFAT3, and NFAT4 (Rao et al., 1997). These transcription factors bind to the consensus DNA sequence GGAAAAT as monomers or dimers through a Rel homology domain (Rooney et al., 1994). Unlike the other three members that are restricted in their

expression to T cells and skeletal muscle, NFAT3 is expressed in a variety of tissues including the heart. NFAT3 plays a major role in cardiac hypertrophy (Pu et al., 2003). Hypertrophic stimuli, such as angiotensin II and phenylephrine, cause an increase in intracellular Ca^{2+} levels in myocardial cells. This elevation in turn results in activation of calcineurin. NFAT3 is localized within the cytoplasm and is dephosphorylated by the activated calcineurin. This dephosphorylation enables NFAT3 to translocate to the nucleus where it interacts with GATA4 (Fig. 18-6). NFAT3 can also activate some hypertrophic responsive genes through mechanisms independent of GATA4.

GATA GATA factors are a family of nuclear transcriptional regulatory proteins that are related structurally within a central DNA-binding domain, but are restricted in expression to distinct sets of cell types (Yamamoto et al., 1990). Currently, six different family members have been characterized in vertebrate species. They are GATA1, 2, 3, 4, 5, and 6. Each protein contains two similar repeats of a highly conserved zinc finger of the form $CXNCX_6LWRRX_7CNAC$. The C-terminal repeat constitutes a minimal DNA-binding domain sufficient for sequence-specific recognition of a "GATA" *cis*-element, usually (A/T)GATA(A/G) or a related DNA sequence, present in promoters and/or enhancers of target genes (Evans et al., 1988). It has been shown that GATA-1/2/3 regulates various aspects of hematopoiesis (Orkin, 1992), whereas GATA-4/5/6 regulates cardiogenesis (Yamamoto et al., 1990). The significance of GATA-4 in regulation of hypertrophic response in myocardial cells has been demonstrated (Evens, 1997). Cardiac hypertrophy induced by angiotensin II is mediated by an angiotensin II type$_{1\alpha}$ receptor ($AT_{1\alpha}R$). A GATA motif exists in the $AT_{1\alpha}R$ promoter. Mutations introduced to the consensus-binding site for GATA factor abolished the pressure overload response (Evens, 1997). Moreover, interactions between AP-1 and GATA-4, and between NFAT3 and GATA-4 are essential in myocardial hypertrophic responses.

CARDIAC TOXIC RESPONSES

Basic Concepts and Definitions

The ultimate functional effect of cardiac toxic manifestations is decreased cardiac output and peripheral tissue hypoperfusion, resulting from alterations in biochemical pathways, energy metabolism, cellular structural and function, electrophysiology, and contractility of the heart. The morphological and functional alterations induced by toxic exposure that reduce cardiac output are referred to as toxicological cardiomyopathy. The critical cellular event leading to toxicological cardiomyopathy is myocardial cell death, which leads to extracellular matrix (ECM) remodeling (fibrotic scarring) in the place of lost cells, along with functional changes of surviving cells in response to the reduced contractile function. The recognition of the role of apoptosis in the development of heart failure has significantly enhanced our knowledge of myocardial cell death (James, 1994; Haunstetter and Izumo, 1998; Sabbah and Sharov, 1998).

For a better understanding of cardiac toxicology, a triangle model of cardiac toxicity is presented in Fig. 18-8. In this model, complexity of the interaction between environmental stresses and the heart, and the balance between myocardial protection and deleterious dose and time effects are considered. First, it is important to recognize that chemicals can lead to heart failure without cardiac hypertrophy. Second, a chemical can lead to activation of both protective and destructive responses in the myocardium and often it is the balance of such responses, varying among individuals, that determines the outcome. Third, long-term toxicological responses often result in maladaptive hypertrophy, which can ultimately lead to heart failure and/or predispose the heart for malignant ventricular arrhythmia, leading to sudden cardiac death.

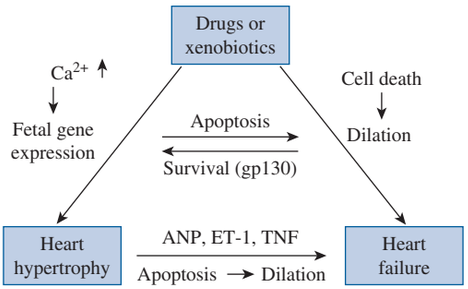

Figure 18-8. *Triangle analytical model of cardiac responses to drugs and xenobiotics.* Drugs or xenobiotics can directly cause both heart failure and heart hypertrophy. Under severe acute toxic insults, myocardial cell death becomes the predominant response leading to cardiac dilation and heart failure. In most cases, myocardial survival mechanisms can be activated so that myocardial apoptosis is inhibited. The survived cardiomyocytes often become hypertrophy through activation of calcium-mediated fetal gene expression and other hypertrophic program. If toxic insult continues, the counter-regulatory mechanisms against heart hypertrophy such as activation of cytokine-medicated pathways eventually lead to myocardial cell death through apoptosis or necrosis, dilated cardiomyopathy, and heart failure.

Acute Cardiac Toxicity Acute cardiac toxicity may arise in response to a single exposure to a high dose of cardiotoxic chemicals. It is often manifested by cardiac arrhythmia or a profound depression of pump function. However, myocardial apoptosis is also involved in acute cardiac toxicity. It is not difficult to define acute cardiac toxicity; however, it sometimes is technically difficult to quantify acute cardiac toxicity. In particular, the impact of acute cardiac toxicity on the ultimate outcome of cardiac function is not often easily recognized. For instance, a single high dose of arsenic can lead to cardiac arrhythmia and sudden cardiac death, which is easy to measure (Goldsmith and From, 1980). However, a single oral dose of the antibiotic monensin (20 mg/kg) can progressively impair cardiac function, but long-term observation (often a few months for clinical signs of heart failure) is needed to observe this chronic manifestation from an acute injection (Van Vleet et al., 1983; Litwak et al., 2005). As shown in Fig. 18-8, toxic exposure can directly lead to heart failure, which is different from an often-observed hypertrophic response, which may or may not progress to heart failure. In contrast, a single bolus injection of monocrotaline, a pyrrolizidine alkaloid found in plants of the genus *Crotalaria*, can cause right-sided cardiac hypertrophy and ultimately lead to failure in rats. Thus, acute exposures to certain toxins are capable of leading to chronic cardiac impacts, albeit from a variety of different mechanisms. An important consideration is the reversibility of effects. Acute activation of vagal tone may depress cardiac rate and output, but is likely temporary and/or reversible. However, myocardial apoptosis or fibrosis will cause more permanent dysfunction.

Chronic Cardiac Toxicity Chronic cardiac toxicity is the cardiac response to long-term exposure to chemicals, which is often manifested by cardiac hypertrophy and the transition to heart failure. About 25% of human patients with cardiomyopathy are categorized as having idiopathic cardiomyopathy. At least a portion of these patients with idiopathic cardiomyopathy are due to chemical exposure. Environmental exposure to ambient particulate matter air pollution, for example, is associated with cardiac remodeling

(Gordon and Reibman, 2000; Dockery, 2001; Van Hee et al., 2009). Recognition of chronic cardiac toxicity in the pathogenesis of cardiomyopathy is of clinical relevance, and this knowledge can be used to identify and treat patients at risk of toxicological cardiomyopathy.

Cardiac Arrhythmia Arrhythmias may arise from chronic remodeling of the heart, which alters the normal electrical pathways, or acutely from the effect of toxicants on normal cellular electrophysiology. Additionally, acute induction of arrhythmias may occur due to sudden loss of blood flow and reduced oxygen availability, such as may be induced by coronary artery vasospasm. Cardiac rhythms under physiological conditions are set by pacemaker cells, located in the sinoatrial node, that are normally capable of developing spontaneous depolarization and responsible for generating the cardiac rhythm, the so-called automatic rhythm. A cardiac rhythm that deviates from the normal automatic rhythm is called cardiac arrhythmia, often manifested in the form of tachycardia (fast heart rate) or bradycardia (slow heart rate). There are several classes of tachycardia, including sinus tachycardia, atrial tachycardia, ventricular tachycardia, and Torsades de Pointes, a life-threatening ventricular tachycardia notably arising from drug-induced prolongation of myocyte monophasic action potentials. Subclasses such as atrial fibrillation, atrial flutter, and accelerated idioventricular rhythm can be identified from standard electrocardiographic traces and provide important etiologic information about the underlying abnormality. Mechanisms for different classes of arrhythmia are discussed in the section "Arrhythmogenesis: QT Prolongation and Sudden Cardiac Death."

Cardiac Hypertrophy There are two basic forms of cardiac hypertrophy: concentric hypertrophy, which is often observed during pressure overload and is characterized by new contractile-protein units assembled in parallel resulting in a relative increase in the width of individual cardiac myocytes (De Simone, 2003). By contrast, eccentric or dilated hypertrophy is characterized by the assembly of new contractile-protein units in series resulting in a relatively greater increase in the length than in the width of individual myocytes (Kass et al., 2004). Toxicological cardiomyopathy is often manifested in the form of eccentric hypertrophy. The development of cardiac hypertrophy can be divided into three stages: developing hypertrophy, during which period the cardiac workload exceeds cardiac output; compensatory hypertrophy, in which the workload/mass ratio is normalized and normal cardiac output is maintained; decompensatory hypertrophy, in which ventricular dilation develops and cardiac output progressively declines, and overt heart failure occurs (Richey and Brown, 1998). Pathophysiologically, the early stages of heart failure entail compensatory involvement of the renal, vascular, and neurohormonal systems. When these compensatory mechanisms fail, perfusion of the body decreases below the needs of the systemic tissues and cardiac decompensation occurs. Most commonly, hypertrophy disproportionately occurs on the left side of the heart, which pumps blood to the systemic circulation, but syndromes related to pulmonary hypertension, wherein the pulmonary vascular resistance is significantly increased, can lead to right-sided hypertrophy and failure. The compound monocrotaline is a unique toxin in this regard, known for its ability to damage the pulmonary vasculature and cause right heart failure.

Heart Failure A traditional definition of heart failure is the inability of the heart to maintain cardiac output sufficient to meet the metabolic and oxygen demands of peripheral tissues. This definition has been modified to include changes in systolic and diastolic function that reflect specific alterations in ventricular function and abnormalities in a variety of subcellular processes (Piano et al., 1998). Early stages of heart failure are characterized by physiological compensatory changes to offset compromised systemic perfusion. In addition to cardiac hypertrophy, the body also increases fluid retention through activation of the renin-angiotensin system and increases overall sympathetic tone. When such pathways are no longer sufficient to increase cardiac output and perfusion owing to severe cardiac deterioration, decompensation occurs. The final stages of decompensation occur relatively rapidly and are difficult to manage pharmacologically even to extend life briefly.

Myocardial Degeneration and Regeneration

Myocardial degeneration is the ultimate response of the heart to toxic exposure, which can be measured by both morphological and functional degenerative phenotypes. However, myocardial degeneration should not necessarily be considered an irreversible toxic response. In the past, the heart has been considered incapable of regenerating, so that cardiac injury in the form of cell loss or scar tissue formation was considered permanent damage to the heart. However, evidence now indicates myocardial regeneration and recovery from cardiomyopathy are possible, although at present these remain very slow processes.

Myocardial Degenerative Responses Myocardial cell death, fibrosis (scar tissue formation), and contractile dysfunction are considered as degenerative responses, which can result in cardiac arrhythmia, hypertrophy, and heart failure. If acute cardiac toxicity does not affect the capacity of myocardial regeneration, the degenerative phenotype may be reversible. Both acute and chronic toxic stresses can lead to irreversible degeneration, depending on whether or not the cardiac repair and regeneration mechanisms are overwhelmed. Cell death is the most common phenotype of myocardial degeneration. Both apoptosis and necrosis occur in the process of myocardial cell death, which is discussed in the next section. Myocardial cell death is accompanied by hypertrophy of the remaining cardiac myocytes so that in the hypertrophic heart, the total number of cardiac myocytes is reduced but the size or volume of individual cells is increased.

During myocardial remodeling after cell death, not only is there an increase in the size of cardiac myocytes, but also cardiac fibrosis occurs. Myocardial fibrosis results from excess accumulation and modification of ECM components, which mainly consist of collagen proteins. Because ECM fibers are noncontractile, they offer resistance to both contraction and relaxation of the heart, diminishing both systolic and diastolic function. The net accumulation of ECM connective tissue results from enhanced synthesis or diminished break down of the matrix, or both. Collagens, predominately types I and III, are the major fibrous proteins in ECM and their synthesis may increase in response to toxic insults. The degradation of ECM is dependent on the activity of matrix metalloproteinases (MMPs). According to their substrate specificity, MMPs fall into five categories: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, and MMP-11), membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and metalloelastase (MMP-12). These MMPs are organ specific so that not all are present in the heart. The activities of these enzymes are altered during the processes of fibrogenesis and fibrinolysis. Under toxic stress conditions, the imbalance between fibrogenesis and fibrinolysis can lead to excess collagen accumulation and fibrosis.

The MMPs are inhibited by specific endogenous tissue inhibitor of metalloproteinases (TIMPs), which comprise a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3, and TIMP-4.

In general, during heart remodeling the concentration of TIMPs increases. Synthetic inhibitors generally contain a chelating group that binds the catalytic zinc atom at the MMP active site tightly. Common chelating groups include hydroxamates, carboxylates, thiols, and phosphinyls. Hydroxymates are particularly potent inhibitors of MMPs and other zinc-dependent enzymes, due to their bidentate chelation of the zinc atom. Other substituents of TIMPs are usually designed to interact with various binding pockets on the MMP of interest, making the inhibitor more or less specific for a given MMP.

Toxic Effect on Myocardial Regeneration The mainstay of cardiac medicine and therapy has centered on the concept that the heart is a terminally differentiated organ and that cardiac myocytes are incapable of proliferating. Thus, cell death would lead to a permanent loss of the total number of cardiac myocytes. However, this view has been challenged recently due to the identification of cardiac progenitor cells (Anversa et al., 2006). These cells are characterized and proposed to be responsible for cardiac repair because these cells can make myocytes and vascular structures. These cells possess the fundamental properties of stem cells; therefore, they are also called cardiac stem cells. They are self-renewing, clonogenic, and multipotent, as demonstrated by reconstitution of infarcted heart by intramyocardial injection of cardiac progenitor cells or the local activation of these cells by growth factors. It is important to note that toxicological studies of the cardiac progenitor cells have not been done, and it is important to determine the potential of cardiac stem cells to help recover from toxic insults. The effect of chemicals on the cardiac progenitor cells is unknown. One speculation is that when severe damage to cardiac progenitor cells occurs, the potential for recovery from severe cardiac injury would be limited.

Myocardial vascularization is required for myocardial regeneration. Many toxic insults affect the capacity of angiogenesis in the myocardium, so that cardiac ischemia occurs. The combination of cardiac ischemia and the direct toxic insults to cardiomyocytes constitute synergistic damage to the heart. During regeneration, coronary arterioles and capillary structures are formed to bridge the dead tissue (scar tissue) and supply nutrients for the survival of the regenerated cardiomyocytes. There is an orderly organization of myocytes within the myocardium and a well-defined relationship between the myocytes and the capillary network. This proportion is altered under cardiac toxic conditions; either toxicological hypertrophy or diminished capillary formation can lead to hypoperfusion of myocytes in the myocardium. Unfortunately, our understanding of the toxic effects on myocardial angiogenesis is limited.

Reversible and Irreversible Toxic Responses Cardiomyopathy was viewed not to be recoverable in the past, but there is cumulative evidence that demonstrates reversibility of cardiomyopathy. The issue related to whether or not toxicological cardiac lesions are reversible has not been explored. However, it can be speculated that there would be reversible and irreversible manifestations of the cardiac response to toxic insults. A key factor determining the reversibility of cardiac toxicity is the degree of scarification or ECM accumulation. The removal of scar tissue or fibrosis in the myocardium in the past has been considered impossible. Although there are no studies that have shown scar tissue is removable in humans, there are observations in animal models of hypertensive heart disease that myocardial fibrosis is recoverable (Weber, 2005). Toxicity leading to substantial fibrosis and/or apoptosis of cardiomyocytes would be difficult to reverse, while cardiac effects from toxicants that act as ligands acutely affecting ion channel function or autonomic nervous system tone would be readily reversible.

Myocardial Cell Death and Signaling Pathways

Apoptosis and Necrosis Toxic insults trigger a series of reactions in cardiac cells leading to measurable changes. Mild injuries can be repaired and mild disturbances of homeostasis can be balanced physiologically. However, severe injuries will lead to cell death in the modes of apoptosis and necrosis. If the cell survives the insults, structural and functional adaptations will take place.

Apoptosis was found to be involved in cardiomyopathy in 1994 (Gottlieb et al., 1994). The loss of cardiac myocytes is a fundamental component of myocardial injury, which initiates and aggravates cardiomyopathy. An important mode of myocardial cell loss is apoptosis, which has been demonstrated in heart failure patients (Olivetti et al., 1997). Myocardial apoptosis has been shown to play an important role in cardiac toxic effects induced by Adriamycin (Kang et al., 2000a; Wang et al., 2001a), an important anticancer drug whose clinical application is limited by its cardiotoxicity. Exposure of primary cultures of cardiomyocytes to cadmium also induces apoptosis (El-Sherif et al., 2000). Endogenous factors, such as the uremic toxin *p*-cresyl sulfate, which builds up in the circulation in renal failure, can also induce cardiomyocyte apoptosis (Han et al., 2015).

Many in vivo studies have shown that only a very small percentage of myocardial cell populations undergo apoptosis under pathological conditions. For example, less than 0.5% of cells appeared apoptotic in myocardial tissue under the stress of dietary copper deficiency in mice (Kang et al., 2000b). At first glance, this number seems to be too insignificant to account for myocardial pathogenesis. In a carefully designed time-course study (Kajstura et al., 1996), it was estimated that cardiomyocyte apoptosis is completed in less than 20 hours in rats. Myocytes that undergo apoptosis are lost and may not be replaced under toxicological conditions. Although the possibility of myocardial regeneration has been identified (Anversa et al., 2006), xenobiotics often cause degenerative effect through apoptosis as well as inhibitory effect on regeneration. Adriamycin-induced cardiomyopathy is a good example for the pathogenesis resulting from both degeneration and inhibition of regeneration. If apoptosis occurs at a constant rate of about 0.5% myocytes a day (Kang et al., 2000b), this will clearly overwhelm maximal regenerative processes (about 2% annually) and could easily lead to substantial functional deficits in a matter of months.

Necrosis is a term that had been widely used to describe myocardial cell death in the past. Myocardial infarction, in particular, had been considered as a consequence of necrosis (Eliot et al., 1977). It is now recognized that apoptosis contributes significantly to myocardial infarction (Yaoita et al., 2000). However, the importance of necrosis in myocardial pathogenesis cannot be underestimated. The contribution of necrosis to cardiomyopathy induced by environmental toxicants and pollutants is particularly important. A critical issue is how to distinguish apoptosis from necrosis.

Apoptosis and necrosis were originally described as two distinct forms of cell death that can be clearly distinguished (Wyllie, 1994). However, these two modes of cell death can occur simultaneously in tissues and cultured cells. The intensity and duration of insults may determine the outcome. Triggering events can be common for both types of cell death. A downstream controller, however, may direct cells toward a programmed execution of apoptosis. If the apoptotic program is aborted before this control point and the initiating stimulus is severe, cell death may occur by necrosis (Leist et al., 1997).

To distinguish apoptosis from necrosis, specific oligonucleotide probes have been developed to recognize different aspects of

DNA damage (Didenko et al., 1998), and have been successfully applied, in combination with confocal microscopy, to identify apoptotic and necrotic cell death in the heart with different pathogenic challenges.

Single-Strand DNA Breaks A monoclonal mouse anti-ssDNA antibody has been developed that is specifically reactive with ssDNA, but does not recognize dsDNA. An immunohistochemical assay for detection of ssDNA using this antibody in combination with a terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay can distinguish repairable ssDNA breaks from apoptotic DNA damage in the heart.

Apoptotic DNA Damage Apoptotic DNA damage produces end products that are fragments of double-strand DNA cleavage with three overhangs, which can be specifically identified by *Tag* polymerase-generated probe (Didenko et al., 1998). The specificity of this molecular probe to identify apoptosis has been confirmed by other methods such as dual labeling of TdT and caspase-3 (Frustaci et al., 2000). In addition, this apoptotic specific probe in combination with fluorescence labeling of different cellular components allows quantitative detection of apoptotic cells, with the possibility of identifying the origin of the apoptotic cells, such as myocytes (stained with α-sarcomeric actin), endothelial cells (stained with factor VIII), and fibroblasts (stained with vimentin) in the heart (Anversa, 2000).

Necrotic DNA Damage Necrotic DNA damage is characterized by double-strand DNA cleavage with blunt ends. That is because during necrosis, the release of lysosomal proteases degrades histones, resulting in loss of DNA protection and exposure to endonucleases and exonucleases. Endonucleases produce double-strand DNA cleavage with three overhangs, but exonucleases remove terminal nucleotides, leading to a blunt end of the damaged DNA. A probe generated by *pfu* polymerase can specifically recognize these blunt-end DNAs (Anversa, 2000). Its specific reaction with necrotic DNA has been confirmed by other methods such as the permeability of myosin antibody into necrotic cells (Guerra et al., 1999), and the disruption of the sarcolemma by vinculin staining, which can clearly define the continuity of the sarcolemmal surface (Yamashita et al., 2001).

Proportion of Apoptotic and Necrotic Cell Death in the Heart The proportion of apoptotic and necrotic cell death in the heart can be estimated by the combination of the above procedures. First, a conventional TUNEL procedure can be used to identify the total TUNEL-positive cells. Second, the procedure to define double-strand DNA breaks with blunt ends can be used to quantify the proportion of necrotic cells in the total TUNEL-positive population. Finally, the combination of the procedure to identify double-strand DNA breaks with 3′ overhangs, and the specific antibody to identify total ssDNA breaks can distinguish the proportion of apoptotic cells from those with ssDNA breaks only.

Distinguishing Apoptotic Myocytes from Nonmyocytes in the Myocardium An in situ TUNEL assay in combination with a dual immunohistochemical detection of α-sarcomeric actin has been used to distinguish apoptotic myocytes from nonmyocytes (Kang et al., 2000a). Apoptotic myocytes are dually stained by TUNEL and α-sarcomeric actin, and apoptotic nonmyocytes are stained only by TUNEL. Another procedure is immuno-gold TUNEL and electron microscopic examination of the apoptotic cells (Kang et al., 2000a). The gold standard for identification of apoptotic cells is morphological examination by electron microscopy. The immuno-gold TUNEL and electron microscopic procedure defines the cell type and morphological characteristics of apoptotic cells.

Telomeres and Telomerase Telomeres are base pair repeats of TTAGGG at the 3′ chromosomal ends that provide genomic stability in the face of oxidative stress. The number of repeats, normally hundreds to thousands, can be reduced with aging, cellular division, and oxidative stress. Numerous proteins support and maintain telomere length, but the function of these proteins may become compromised by disease or toxicity. When telomeres are truncated to a critical threshold, cells can enter a senescent phenotype that may progress to apoptosis. Telomere attrition appears most significant in extreme age (more than 80 years) and may contribute to cardiac failure late in life. Mice with artificially shortened telomeres exhibit an eccentric cardiomyopathy (Leri et al., 2003), which can be prevented by activation of the protein telomerase, which lengthens telomeres (Oh et al., 2001). Limited data exist showing that telomere structure and function can be impacted by toxicants; Adriamycin has been used often as a model of cardiac failure in rodents and insult to the telomere system has been implicated as central to this pathology (Piegari et al., 2013).

Mitochondrial Control of Cell Death The role of mitochondria in myocardial response to toxicants as well as therapeutic drugs has long been a focus of investigation. Mitochondrial control of cell death is an important topic of apoptotic research. Factors affecting mitochondrial control of cell death are presented in Fig. 18-9. These factors have the same target effect: modification of mitochondrial permeability transition (MPT).

Mitochondrial Permeability Transition MPT occurs under toxic insults (Kroemer et al., 1997). This MPT behaves like a membrane pore that allows diffusion of solutes less than 1500 Da in size. Although MPT can occur as a temporary event, it can rapidly become irreversible, with the resulting loss of mitochondrial homeostasis and high-amplitude mitochondrial swelling. Because the inner membrane has a larger surface area than the outer membrane, mitochondrial swelling can cause the rupture of the outer membrane, releasing intermembrane proteins into the cytosol (Reed et al., 1998). Among the intermembrane proteins is cytochrome *c*. Another possible mechanism that leads to mitochondrial cytochrome *c* release is the action of Bax, a proapoptotic protein of the Bcl-2 family (Adams and Cory, 1998). Overexpression of Bax under oxidative stress conditions has been observed in a number of studies in different tissues including the heart (Cook et al., 1999). It has been shown that Bax is translocated from cytosol to mitochondria and forms pores in mitochondrial outer membranes, leaving the inner membranes intact (Jurgensmeier et al., 1998). This mechanism implies that Bax-mediated cytochrome *c* release is independent of MPT (Saikumar et al., 1998).

The release of cytochrome *c* from mitochondria into the cytosol is a critical initiation step in myocardial apoptosis. Cytochrome *c* aggregates with apoptotic protease activating factor-1 (apaf-1, another factor released from mitochondria under oxidative stress), procaspase-9, and dATP, and subsequently activates caspase-9, which activates caspase-3. The apoptotic pathway involving mitochondrial cytochrome *c* release and caspase-3 activation is presented in Fig. 18-10. To determine the significance of the caspase-3-activated apoptotic pathway in the pathogenesis of toxicological cardiomyopathy, a caspase-3-specific inhibitor, Ac-DEVD-cmk, is often used. For instance, treatment of cultured cardiomyocytes isolated from neonatal mice with Ac-DEVD-cmk efficiently suppressed caspase-3 activity and reduced the number of apoptotic cells in cultures under treatment with Adriamycin (Wang et al., 2001a).

Defective Mitochondrial Oxidative Phosphorylation Defective mitochondrial oxidative phosphorylation has been extensively

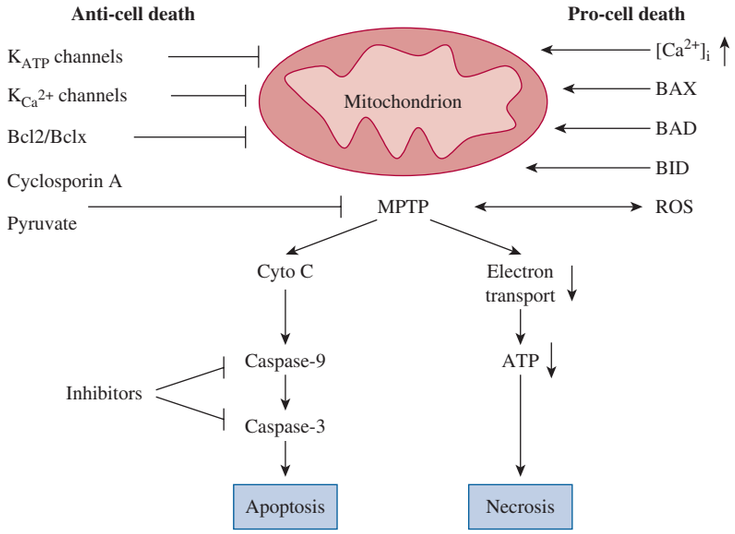

Figure 18-9. *Major factors affecting mitochondrial MPTP and myocardial cell death.* Mitochondrial cytochrome *c* release is a critical factor controlling cardiomyocyte apoptosis. Mitochondrial permeability transition pore (MPTP) opening is a determinant factor for cytochrome *c* release, as well as for electron transport collapsing leading to decreases in ATP production. The factors affecting MPTP thus are classified as pro-cell death and anti-cell death. Many other factors also affect cell death programs such as apoptosis inducing factor (AIF) released from mitochondria, but the involvement of MPTP is not evidenced.

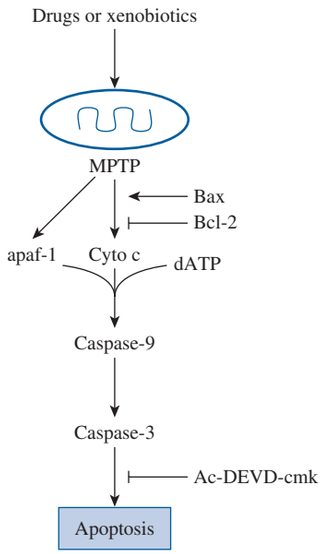

Figure 18-10. *Mitochondrial control of myocardial apoptotic pathway.* Exposure to drugs or xenobiotics can cause mitochondrial MPTP opening and cytochrome *c* release, which in association with apaf-1 and in the presence of dATP activates caspase-9. Caspase-9 consequently activates caspase-3 leading to apoptosis. Factors regulating the apoptotic pathway include those listed in Fig. 18-9, such as Bax and Bcl-2. Ac-DEVD-cml is a selective inhibitor of caspase-3.

investigated ever since the identification of mitochondrial oxidative phosphorylation. The link between defective oxidative phosphorylation and pathogenesis of cardiomyopathy has been revealed recently. The early phase of defects in oxidative phosphorylation increases mitochondrial outer membrane permeability, leading to cytochrome *c* release, thus resulting in cytochrome *c*–mediated caspase-9 activation and thereby caspase-3 activation, leading to apoptosis (Fosslien, 2001). The defective oxidative phosphorylation also leads to depletion of cellular ATP levels, resulting in necrosis (Fosslien, 2001). Fig. 18-11 presents a generalized mitochondrial oxidative phosphorylation process, including electron-transferring complexes and ATP production. Detection of mutated or otherwise defective components in oxidative phosphorylation is important for understanding the myocardial cell death by xenobiotics.

Abnormal Mitochondrial Biosynthesis Abnormal mitochondrial biosynthesis is also linked to myocardial pathogenesis. Both nuclear and mitochondrial DNA encode mitochondrial proteins. Therefore, nuclear DNA damage can lead to mutated products and abnormal mitochondrial biosynthesis. However, mitochondrial DNA encodes essential elements for mitochondrial function. Mitochondrial DNA is subjected to far more oxidative injury than nuclear DNA due to the lack of histones and high exposure to reactive oxygen species (ROS) generated by the electron transport chain. Mitochondrial DNA repair mechanisms exist, although they are limited compared to nuclear DNA repair. Due to these unique characteristics of mitochondrial DNA, cumulative mitochondrial DNA damage under oxidative stress conditions such as Adriamycin treatment leads to irreversible mitochondrial dysfunction in the heart (Zhou et al., 2001). This cumulative and relatively irreversible oxidative mitochondrial dysfunction concept has an important impact on our understanding of chronic as well as late-onset cardiomyopathy of anthracyclines. These drugs cause cardiomyopathy sometimes months to years after cessation of the drug therapy. During this time period, subtle pathological changes that may not be detectable but may continue to accumulate and lead to an overt toxic event.

Generation of Reactive Oxygen Species Generation of ROS has been described as an "unwanted" function of mitochondria. Drugs and other chemicals have been studied individually to determine how they produce ROS; however, debate continues regarding the importance of each identified pathway as well as the site of ROS generation in mitochondria. In general, it is accepted that changes in mitochondrial membrane potential are critically involved in ROS generation. There are two important potassium channels that play important roles in mitochondrial membrane permeability. The first is the mitochondrial ATP-sensitive potassium channel (Akao et al., 2001), and the second is the Ca^{2+}-activated potassium channel in

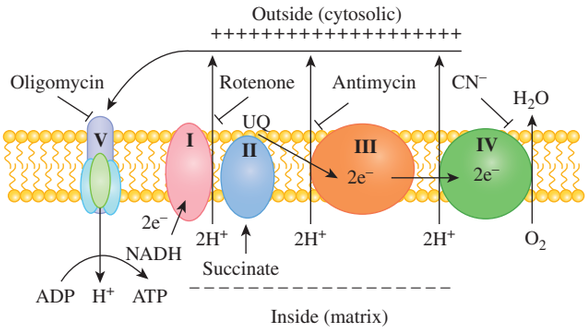

Figure 18-11. *Diagram of the electron-transferring complexes I, II, III, and IV and the ATPase (V) present in the inner mitochondrial membrane.* The respective complexes are I, NADH: ubiquinone oxidoreductase; II, succinate: ubiquinone oxidoreductase; III, ubiquinol: ferrocytochrome *c* oxidoreductase; IV, ferrocytochrome *c*: oxygen oxidoreductase; V, ATP synthesase; UQ, ubiquinone-5 or coenzyme Q10. The mitochondrial electron transport chain is coupled at three points so that the electron transfer between carriers is sufficiently exergonic to drive the transport of protons and to establish an electrochemical proton gradient upon which ATP formation depends. Various inhibitors and their sites are also noted.

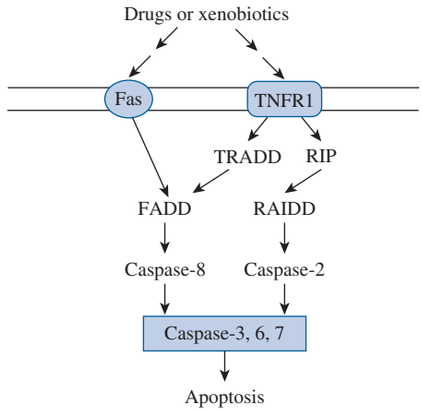

Figure 18-12. *Simplified presentation of death receptors–mediated myocardial apoptosis.* The cardiotoxicity-related death receptors include Fas and tumor necrotic factor receptor 1 (TNFR1), as shown. Other death receptors such as DR3 and DR4/5 are not included in the figure. Both Fas- and TNFR1-activated apoptotic pathways can be altered by drugs and xenobiotics directly or indirectly through changes in the production of Fas ligand (FasL) or TNF-α. Fas activation activates caspase-8 through Fas-associated death domain (FADD). TNFR1 activates TNF-R1-associated death domain (TRADD), leading to activation of FADD. TNFR1 also activates a receptor-interacting protein (RIP), which activates caspase-2 through the adaptor protein, RIP-associated ICH-1/CED-3 homologous protein with a death domain (RAIDD). Both caspase-8 and caspase-3 activate the apoptosis executor proteins, caspase-3, -6, and -7.

the cardiac inner mitochondrial membrane (Xu et al., 2002). It has been shown that diazoxide, an antihypertensive agent that dilates arteries, opens mitochondrial ATP-sensitive potassium channels and preserves mitochondrial integrity, as well as suppresses hydrogen peroxide-induced apoptosis in cardiomyocytes (Akao et al., 2001). The Ca^{2+}-activated potassium channels, in contrast, contribute to mitochondrial potassium uptake of myocytes, and opening of these channels protects the heart from infarction (Xu et al., 2002).

Death Receptors and Signaling Pathways Death receptor–mediated apoptotic signaling pathway has been a major focus of cardiotoxicity research. In this regard, cytokines that trigger the death receptor signaling pathways have been studied. Among these cytokines is tumor necrosis factor-α (TNF-α) (Kubota et al., 2001), the most studied cytokine in myocardial cell death signaling pathways. The pathway leading to TNF-α-induced myocardial apoptosis is mediated by TNF receptors TNFR1 and TNFR2. TNF-α binding of these receptors leads to activation of caspase 8, which in turn cleaves the Bcl-2 homology 3 (BH3) interacting–domain death agonist (BID), a BH3 domain-containing proapoptotic Bcl2 family member. The truncated BID is translocated from cytosol to mitochondria, inducing the clustering of mitochondria around the nuclei and release of cytochrome *c*, followed by the loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation (i.e., apoptosis). Caspase 8 also directly activates caspase-3, leading to apoptosis (Fig. 18-12). Besides TNF-α, Fas ligand is also able to induce apoptosis of cardiomyocytes through the death receptor–mediated signaling pathway (Hayakawa et al., 2002).

The death receptor–mediated apoptosis thus can be divided into mitochondrion-dependent and mitochondrion-independent signaling pathways. There is a bewildering diversity of programmed cell death paradigms related to the mitochondrion-controlled process, eventually leading to caspase-3 activation and apoptosis. Under chronic toxic insults, the relative importance of mitochondrial MPT pore opening, cytochrome *c* release, and electron transport defects needs to be critically examined in order to understand the process or mode of cell death.

There are two important questions that need to be carefully addressed. First, how important is apoptosis in the overall pathogenesis of cardiomyopathy under different conditions? It essentially is a universal observation that apoptosis is involved in the cardiotoxicity of drugs and chemicals; however, there is very limited quantitative evidence supporting its significance in cardiomyopathy. This concern is followed by the second important question: can caspase inhibitors offer long-term protection against myocardial cell death leading to prevention of cardiomyopathy? It is important to note that apoptosis and necrosis are linked phenomena sharing common triggers, such as MPT pore opening, as depicted in Fig. 18-13. For instance, some studies have shown that caspase inhibitors effectively inhibit apoptosis, but cell death occurred by necrosis instead of apoptosis (Suzuki et al., 2001). It is thus important to define the most efficient approach to blocking myocardial cell death rather than inhibiting a particular cell death program (Kang, 2001).

It is important to note that apoptosis is an energy-dependent process and that the switch in the decision between apoptosis and necrosis depends on ATP concentrations (Eguchi et al., 1997), as depicted in Fig. 18-13. In particular, conditions causing ischemia by many drug and chemical exposures to myocardial cells result in significant reduction and eventual depletion of adenine nucleotides. Loss of more than 70% of the total ATP pool present in myocardial cells results in a switch from apoptosis to necrosis (Leist et al., 1999). This extent of ATP depletion is observed in myocardial infarction.

Mitochondrial Dynamics and Autophagy

The importance of mitochondria in cardiac response to toxic insults and in the process of toxicological cardiomyopathy is related not only to the control of cell death, but also to autophagy, the tightly regulated cellular "housekeeping" process responsible for the degradation of damaged and dysfunctional cellular organelles and protein aggregates.

Autophagy occurs in all eukaryotic cells under the stress of starvation, hypoxia, and toxic insults, as well as under physiological stimulation such as hormones and developmental signals.

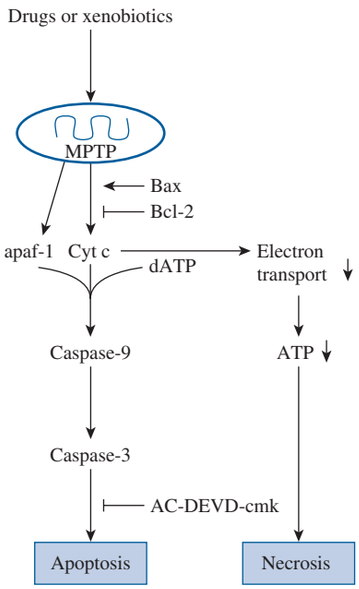

Figure 18-13. *Interchange between apoptotic and necrotic cell death pathways regulated by mitochondrial MPTP.* Mitochondrial MPTP opening leads to cytochrome *c*-mediated activation of apoptotic pathway, which is ATP dependent. However, MPTP formation also leads to electron transport collapse and reduced ATP production, which eventually leads to necrosis. Inhibition of caspase-3 also potentially switches the cell death program from apoptotic to necrotic if the exposure to drugs or xenobiotics is persistent.

Autophagy can be selective or nonselective. Selective autophagy of mitochondria is termed mitophagy, which is triggered by MPT pore opening and loss of mitochondrial membrane potential (Tolkovsky, 2009). In cardiomyocytes and other terminally differentiated cells, mitophagy is a continuous process of mitochondrial turnover, but the rate of this turnover is influenced by stresses and makes a critical contribution to myocardial pathogenesis. Nonselective autophagy has been observed in response to nutrient starvation; the degradation of cytosolic components including mitochondria via autophagy provides amino acids and lipid substrates for intermediate metabolism (Tolkovsky, 2009).

Mitochondrial Dynamics The well-known and comprehensively depicted function of mitochondria is the generation of ATP through oxidative phosphorylation. However, it has been known that mitochondria also carry out other critical functions, including regulation of programmed cell death or apoptosis, the synthesis and degradation of essential metabolites, heme and steroid synthesis, regulation of cell proliferation, maintenance of plasma membrane potential, and calcium signaling. These diverse functions demand mitochondrial biogenesis and turnover highly regulated. In cardiomyocytes and other terminally differentiated cells, this regulation is far more important for the vital function of the cell.

Mitochondria are dynamic organelles and the morphology of mitochondria undergoes interchange between two distinct arrangements: elongated interconnected mitochondrial networks and discrete fragments. The former is accomplished by mitochondrial fusion and the latter by mitochondrial fission. In the heart, mitochondrial fusion and fission constitute a major response of cardiomyocytes to stresses. For instance, sustained pressure overload promotes structural, functional, and metabolic remodeling of cardiomyocyte mitochondria to compensate for changes in energy demands and to remove damaged organelles. This process ultimately determines the fate of a cardiomyocyte: survival versus death. The changes in mitochondrial morphology are orchestrated by a group of mitochondrial fusion and fission proteins. These proteins were first identified in yeast or *Drosophila*, and the function of these proteins has been a major focus in the study of mitochondrial dynamics. But the identification of these proteins in the mammalian heart has made a significant impact on the role of mitochondrial dynamics in the pathogenesis of cardiac disease.

Mitochondrial Fusion Mitochondrial fusion is a process of an elongated interconnected mitochondrial network formation. This is a fundamental process in the life of eukaryotic cells. Mitochondrial fusion requires coordinated joining of both the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). In this process, the constituents in the matrix and intermembrane space are secured—not to be released or mixed. Therefore, the fusion machinery involves OMM and IMM fusion, and their coordination.

OMM fusion is directed by mitofusins 1 and 2 (MFN1 and MFN2), large GTPase located in the OMM. They contain two transmembrane regions in the OMM, with a short loop in the intermembrane space and the major parts of the protein facing the cytosol. In mammalian cells, early during fusion two mitochondria approach each other in a tethered docking step. The carboxy-terminal heptad repeats of MFN1 have been shown to form an intermolecular antiparallel coiled coil that may tether adjacent mitochondria before fusion (Koshiba et al., 2004). The coiled coil formation by mitofusins thus draws the membranes close together and initiates lipid bilayer mixing, and the GTPase provides biomechanical energy for outer membrane fusion. It has been shown that mutation of the GTPase domain of MFN2 results in an accumulation of tethered but unfused mitochondria (Eura et al., 2003).

IMM fusion is governed by Mgm1, a dynamin-related large GTPase that is essential for inner membrane fusion in yeast (Meeusen et al., 2006). The mammalian Mgm1 ortholog, optic atrophy 1 (OPA1) protein, is tethered to the IMM and in the intermembrane space (Olichon et al., 2002). Differential splicing of *Opa1* transcripts generates multiple variants (Song et al., 2007). A number of proteases localized in mitochondria cleave OPA1 disrupting OPA1 function and inhibiting fusion. Mutations in OPA1 selectively block inner membrane fusion. After the completion of outer membrane fusion, OPA1 is required in *trans* on both inner membranes of the fusion partners. A normal membrane potential is required for fusion of IMM. Loss of membrane potential promotes cleavage of OPA1, causing that outer membrane fusion proceeds in the absence of inner membrane fusion.

Mitochondrial Fission Mitochondrial fusion is a process of the fragmented discrete mitochondrial morphogenesis. This process is regulated by dynamin-related protein 1 (DRP1) in mammals. This protein is soluble and contains an N-terminal GTPase, a middle domain, and a C-terminal GTPase effector, which is involved in self-assembly. Cells lacking DRP1 contain highly interconnected mitochondrial networks (Smirnova et al., 1998). During fission, DRP1 is recruited from the cytoplasm to mitochondria. Mammalian fission protein 1 (FIS1) in the OMM interacts with DRP1. FIS1 overexpression promotes mitochondrial fragmentation and FIS1 depletion stimulates interconnected mitochondrial networks (James et al., 2003; Yoon et al., 2003). In yeast, a mitochondrial division protein 1 (Mdv1) has been shown to coexist with Fis1 and interact with DRP1, but its metazoan homologs have not been identified, indicating a significant difference between metazoan and yeast mitochondrial fission machineries.

The function of mitochondrial fission machinery has been extensively understood in yeast, but less comprehensively in

metazoans. In yeast, the fis1–Mdv1–Dnm1 complex is important for mitochondrial fission, but the lack of Mdv1 in metazoans makes a different mitochondrial fission machinery. It has been shown that knockdown of human FIS1 does not affect the distribution of DRP1 in mitochondria (Lee et al., 2004). A mitochondrial fission factor (MFF) has been found in mammalians, but not in yeast (Gandre-Babbe and van der Bliek, 2008). MFF is a tail-anchored protein containing heptad repeats and a C-terminal transmembrane domain that is embedded in the OMM. Depletion of MFF suppresses mitochondrial fission in mammalian cells. However, MFF and FIS1 exist in separate complexes, suggesting that they may act differently in mitochondrial fission (Gandre-Babbe and van der Bliek, 2008).

Regulation of Mitochondrial Dynamics Multiple factors affect mitochondrial fusion and fission. Cell cycle control of mitochondrial dynamics is often observed in mitotically active cells. Mitochondrial fusion is found during G_1-S phase, but mitochondrial fission is necessary in cell division. In differentiated cells such as cardiomyocytes, the cell cycle control of mitochondrial dynamics is less important. However, reactivation of fetal gene program in adult cardiomyocytes in cardiac hypertrophy and heart failure would affect mitochondrial dynamics, which has not been understood.

Oxidative and nitrosative stress often induces mitochondrial fission. Mitochondria are the major site for the production of ROS in the heart. The process not only affects mitochondrial dynamics, but also generates ROS-induced damage to proteins, lipids, and DNA. Studies have shown overexpression of MFN inhibits ROS-induced mitochondrial fission and protects cardiomyocytes from oxidative injury (Yu et al., 2011).

Ca^{2+} regulation of mitochondrial dynamics is more related to the role of mitochondria in Ca^{2+} transport and homeostasis. Activation of calcineurin (CaN) and calmodulin-dependent protein kinase 1 (CaMK1) by Ca^{2+} affects the recruitment of DRP1 and mitochondrial translocation. Inhibition of mitochondrial Ca^{2+} uptake attenuates mitochondrial fission, indicating the importance of intermitochondrial Ca^{2+} concentrations in the regulation of mitochondrial dynamics.

Metabolic regulation of mitochondrial dynamics is the fundamental process of mitochondrial biological function. Mitochondrial dynamics and energy substrate metabolism are tightly linked. Increased metabolic rates upregulate mitochondrial fusion and dense packing of cristae. Downregulation of OPA1 or MFN leads to fragmented mitochondria with reduced membrane potential and oxygen consumption. Glucose concentrations play a critical role in the regulation of mitochondrial dynamics. Changes in fusion-related proteins have been observed in animal models and patients with type 2 diabetes (Zorzano et al., 2010). High glucose levels stimulate mitochondrial fragmentation in H9c2 cardiomyoblasts and neonatal rat cardiomyocytes (Yu et al., 2008).

Toxicological Significance of Mitochondrial Dynamics Toxicological significance of mitochondrial dynamics in the heart can be observed under a diversity of exogenous and endogenous stress conditions. Mitochondrial dynamics is a physiological process as well as a feature of pathogenesis. Under physiological conditions, mitochondrial fusion and fission are essential to maintain metabolic homeostasis and the balance between energy production and consumption. Disruption of this physiological process under stress conditions or toxicological exposures leads to overwhelming of mitochondrial fusion or fission. Under these conditions, cell death and maladaptation occur, leading to toxicological pathogenesis.

Autophagy Intricately regulated degradation and turnover of subcellular components ensure normal cellular function, growth, and development. The major catabolic pathway responsible for the disposal of damaged organelles and protein aggregates is autophagy, a lysosome-dependent proteolytic pathway capable of processing cellular components, including damaged organelles and protein aggregates. During this process, damaged organelles and proteins are encircled in a double-membrane vesicle, so-called autophagosome, delivered to lysosomes. The contents in the autophagosome are hydrolyzed to yield amino acids, fatty acids, and substrates for ATP generation that can be recycled to synthesize new proteins, high-energy phosphates, and renewed cellular components. Autophagy is a conserved mechanism for cell survival under conditions of starvation and stress. Therefore, autophagy has been recognized to play an important role in maintaining cellular homeostasis, and upregulation of autophagy under stress conditions may serve as an adaptive process.

Autophagy can be subdivided into three different processes: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is often referred to as autophagy that is characterized by the sequestration of organelles and proteins within an autophagosome, as described above. Microautophagy refers to protrusion of the lysosomal membranes per se around a region of cytoplasm. Chaperone-mediated autophagy is defined as that degradation which is restricted only to those proteins with a consensus peptide sequence recognized by specific chaperone complexes. Current mechanistic understanding mostly focuses on macroautophagy or autophagy.

There are multiple proteins and interrelated signaling pathways involved in autophagy. This complex process can be divided into four distinct but consecutive steps: (1) induction of autophagy; (2) assembly of autophagosomes; (3) docking and fusion of autophagosome with lysosomal membrane; and (4) breakdown of the autophagic body.

The induction of autophagy requires the participation of three different protein complexes: autophagy-specific gene 1 (Atg1) complex, target of rapamycin (TOR) complex 1 (TORC1), and class III phosphatidylinositol-1 kinase (PI3K) complex. In this step, TORC1 acts as a sensor of cellular nutrient status. Nutrient stress leads to the partial dephosphorylation of Atg13, which in turn increases the binding affinity of Atg13 to Atg1, a pivotal precursor for subsequent autophagosome formation.

The autophagosome assembly and formation is the most complex phase of autophagy. The Atg5 complex and microtubule-associated protein 1 light chain 3A (LC3-II) work in concert to initiate elongation or expansion of the isolated membrane that will eventually form the matured autophagosome. There are several Atg genes involved in this phase and they work sequentially. It has been shown that Atg5 is essential for autophagy. Mice with a cardiac-specific disruption of *Atg-5* develop premature age-related heart failure, indicating that autophagy is an essential regulatory mechanism for normal cardiac function over the course of a life (Taneike et al., 2010).

Autophagosome docking and fusion have been studied in yeast and found to involve multiple vesicle trafficking and membrane fusion proteins. The final stage of autophagy, breakdown of autophagic body and its content, generates substances for protein synthesis, lipid turnover, and ATP production.

Cardiac Hypertrophy and Heart Failure

Adaptive and Maladaptive Responses Myocardial adaptation refers to the general process by which the ventricular myocardium changes in structure and function. This process is often referred to as "remodeling." During maturation, myocardial remodeling is a normal feature for adaptation to increased demands. However, in

response to pathological stimuli, such as exposure to environmental toxicants, myocardial remodeling is adaptive in the short term, but is maladaptive in the long term, and often results in further myocardial dysfunction. The central feature of myocardial remodeling is an increase in myocardial mass associated with a change in the shape of the ventricle (Frey and Olson, 2003).

At the cellular level, the increase in myocardial mass is reflected by cardiac myocyte hypertrophy, which is characterized by enhanced protein synthesis, heightened organization of the sarcomere, and the eventual increase in cell size. At the molecular level, the phenotype changes in cardiac myocytes are associated with reintroduction of the so-called fetal gene program, characterized by the patterns of gene expression mimicking those seen during embryonic development. These cellular and molecular changes are observed in both adaptive and maladaptive responses, thus distinguishing adaptive from maladaptive responses is difficult.

Adaptive Response There are both physiological hypertrophy and pathological hypertrophy of the heart. Physiological hypertrophy is considered an adaptive response, which is an adjustment of cardiac function for an increased demand of cardiac output. Such an adaptive hypertrophy is the increase in cardiac mass after birth and in response to exercise. A biochemical distinction of the adaptive hypertrophy is that myocardial accumulation of collagen does not accompany the hypertrophy. Functionally, the increased mass is associated with enhanced contractility and cardiac output. In response to toxicological stresses, the heart also often increases its mass, which has been viewed as an adaptive response as well. However, most recent evidence suggests that cardiac hypertrophy is a maladaptive process of the heart in response to intrinsic and extrinsic stresses.

Maladaptive Response Although toxic stress-induced hypertrophy can normalize wall tension, it is a risk factor for sudden cardiac death and has a high potential to progress to overt heart failure. A distinction between adaptive and maladaptive hypertrophy is whether the hypertrophy is necessary for the compensatory function of the heart under physiological and pathological stress conditions. Many studies using genetically manipulated mouse models, either in the form of gain-of-function or loss-of-function, have supported the hypothesis that cardiac hypertrophy is neither required nor necessarily compensatory. For instance, forced expression of a dominant-negative calcineurin mutant confers protection against hypertrophy and fibrosis after abdominal aortic construction (Zou et al., 2001). Also, the elimination of hypertrophy in animals by calcineurin suppression did not cause compromised hemodynamic changes over a period of several weeks (Hill et al., 2000). Therefore, in these experimental approaches, hypertrophic growth could be abolished in the presence of continuous pressure overload, but the compensatory response could not be compromised. An interesting observation is that an almost complete lack of cardiac hypertrophy in response to aortic banding in a transgenic mouse model was accompanied with a significant slower pace of deterioration of systolic function (Esposito et al., 2002). These observations indicate that cardiac hypertrophy in response to extrinsic and intrinsic stressors is not a compensatory response. However, cardiac hypertrophy increases the risk for malignant arrhythmia and heart failure, and thus is now viewed as a maladaptive response.

Hypertrophic Signaling Pathways Extrinsic and intrinsic stresses activate signaling transduction pathways leading to fetal gene program activation, enhanced protein synthesis of adult cardiomyocytes, and the eventual hypertrophic phenotype. The signaling pathways include several components: G protein–coupled receptors, protein kinases including MAPK, PKC, and AMPK, calcium and calcineurin, and phosphoinositide 3-kinase (PI3K)/glycogen synthase kinase 3β (GSK3β), and transcription factors. Activation of each of the components is sufficient to induce myocardial hypertrophic growth. These components also affect each other through cross talk. The diagram presented in Fig. 18-14 briefly summarizes these pathways and their interactions. Among these pathways, protein kinases, calcium/calcineurin, and transcription factors have been discussed above. A brief summary for the G protein–coupled receptors and the PI3K/GSK3 pathway is presented as follows.

G Protein–Coupled Receptors Myocardial adrenergic, angiotensin, and endothelin (ET-1) receptors belong to G protein–coupled receptors, which are coupled to three major classes of heterotrimeric GTP-binding proteins: $G_{\alpha s}$, $G_{\alpha q}/G_{\alpha 11}$, and $G_{\alpha i}$. Activation of $G_{\alpha q}$-coupled receptors is sufficient to induce myocyte hypertrophy in vitro (Adams et al., 1998). Cardiac-specific ablation of $G_{\alpha q}/G_{\alpha 11}$ in adult animals causes an almost complete lack of cardiac hypertrophy in response to aortic banding (Wettschureck et al., 2001). Overexpression of a dominant-negative mutant of $G_{\alpha q}$ in transgenic mouse hearts suppresses pressure-overload hypertrophy (Akhter et al., 1998). Cardiac overexpression of $G_{\alpha s}$, the downstream effector of β1-adenergic receptors in the heart, initially increases contractility, but eventually results in cardiac hypertrophy, fibrosis, and heart failure (Bisognano et al., 2000).

Phosphoinositide 3-Kinase/Glycogen Synthase Kinase 3β Pathway Activation of PI3K is found in both physiological and pathological hypertrophy. Insulin-like growth factor (IGF) is involved in the growth of the heart after birth (Shioi et al., 2002). Overexpression of IGF induces cardiac hypertrophy (Delaughter et al., 1999). IGF signals through PI3K to the serine/threonine kinase Akt or protein kinase B. Both PI3K and the Akt induce hypertrophic growth of adult hearts. Overexpression of constitutively active PI3K mutant in the heart leads to increased heart size in mice, and expression of dominant-negative PI3K results in a small heart (Shioi et al., 2000). Overexpression of Akt induces cardiac hypertrophy in transgenic mice without adverse effects on systolic function (Matsui et al., 2002). Akt phosphorylates GSK3β, and thus inhibits the activation of GSK3β. Otherwise, the activated GSK3β phosphorylates transcription factors of the NFAT family (Fig. 18-14). As discussed above, activation of calcineurin dephosphorylates NFAT3 in the cytoplasm, which enables NFAT3 to translocate to the nucleus where it can activate hypertrophic gene expression dependent on or independent of GATA4. Phosphorylation of NFAT3 in the nucleus by GSK3β promotes NFAT3 translocation to the cytoplasm, becoming inactive. Hypertrophic stimuli such as β-adrenergic agonist isoproterenol, ET-1, and phenylepherine all induce GSK3β phosphorylation in a PI3K-dependent fashion, indicating possible requirement of inactivation of GSK3β through phosphorylation in hypertrophic growth of the heart.

Transition from Cardiac Hypertrophy to Heart Failure Pathological hypertrophy is a risk factor for malignant arrhythmia and heart failure. The link of heart hypertrophy to malignant arrhythmia is discussed in the next section. The critical cellular event of this transition is myocardial apoptosis triggered by inflammatory cytokines, such as TNF-α, and neurohormonal factors, such as atrial natriuretic peptide (ANP), which leads to dilated cardiomyopathy and deterioration of cardiac function. Toxicological exposures may cause dilated cardiomyopathy or heart failure without an intermediate hypertrophic stage. Myocardial cell death also plays an essential role in direct cardiac dilation pathogenesis.

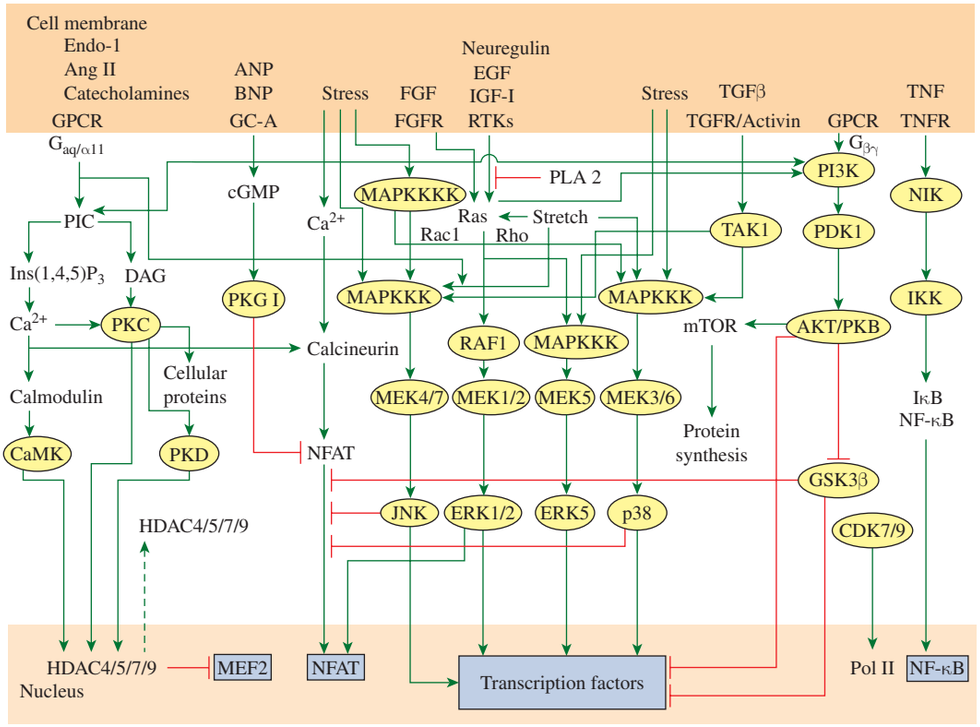

Figure 18-14. *Overview of signaling transduction pathways involved in cardiac hypertrophic growth and their cross-talk interactions.* The signaling that occurs at the sarcolemmal membrane is shown at the top and the intermediate transduction of signals by various kinases and phosphatases is shown in the middle. The nucleus is shown at the bottom. ANP, atrial natriuretic peptide; Ang II, angiotensin II; BNP, B-type natriuretic peptide; CaMK, calmodulin-dependent kinase; CDK, cyclin-dependent kinase; DAG, diacylglycerol; EGF, epidermal growth factor; Endo-1, endothelin-1; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; GC-A, guanyl cyclase-A; GPCR, G protein–coupled receptors; GSK3β, glycogen synthase kinase-3β; HDAC, histone deacetylases; IκB, inhibitor of NF-κB; IGF-I, insulin-like growth factor-I; IKK, inhibitor of NF-κB kinase; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKKKK, MAPKKK kinase; MEF, myocyte-enhancer factor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; PLA2, phospholipase A2; PLC, phospholipase C; Pol II, RNA polymerase II; RTK, receptor tyrosine kinase; TAK, TGF-β-activated kinase; TGF-β, transforming growth factor-beta; TGFR, TGF receptor; TNF-α, tumor necrosis factor-α; TNFR, TNF-α receptor. (From Heineke and Molkentin [2006].)

Fig. 18-15 illustrates the process of xenobiotic-induced transition from cardiac hypertrophy to heart failure.

Alterations of biochemical reactions in the myocardium are often seen soon after exposure to environmental toxicants. These include alterations in ionic homeostasis, such as changes in intracellular calcium concentrations, which occur in most exposures to environmental toxicants (Symanski and Gettes, 1993). Aberrant energy metabolism is another early response to environmental toxicants in the heart, resulting in decreased production and/or enhanced consumption of ATP (Abas et al., 2000). Alterations in enzymatic reactions are often described in cardiac toxic responses (Depre and Taegtmeyer, 2000). The early signaling pathways leading to myocardial toxic responses are the focus of current cardiac toxicology research (Piano, 1994). Detailed descriptions of these pathways and their role in cardiotoxicity are yet to be explored. It is likely that activation of signaling pathways is a critical response of myocardial cells to environmental toxic insults (Cheng et al., 1999a). The cross talk between signaling pathways determines the ultimate outcome of myocardial responses to chemicals.

Physiological alterations occur both as early responses to environmental toxicants and as subsequent events in the late development of cardiomyopathy. The most obvious myocardial dysfunction that occurs in the early responses to toxicants is cardiac arrhythmia (Peters et al., 2000), which often results from the changes in intracellular calcium concentrations and other biochemical alterations, leading to miscommunication between cells and misconduction of electricity (Rosen, 1995). These changes, if not accompanied by cardiomyopathy, do not involve myocardial cell death and are reversible. In contrast, the late phase of cardiac dysfunction and arrhythmia, however, often result from cardiomyopathy.

Changes in myocardial morphology take place when extensive toxic insults are imposed on the heart and/or toxic exposures persist (He et al., 1996). Cardiac hypertrophy is often observed as a consequence of long-term toxic insults. From cardiac hypertrophy to heart failure, activation of compensatory mechanisms, including the sympathetic nervous system and the renin–angiotensin system, occurs (Holtz, 1993). The compensatory response in turn activates counter-regulatory mechanisms such as upregulation of ANP expression (Francis and Chu, 1995) and increases in cytokines, such as TNF-α production. Extensive biochemical, physiological, and molecular changes result in myocardial remodeling (Swynghedauw, 1999) and remarkable cell death, ultimately leading to heart failure.

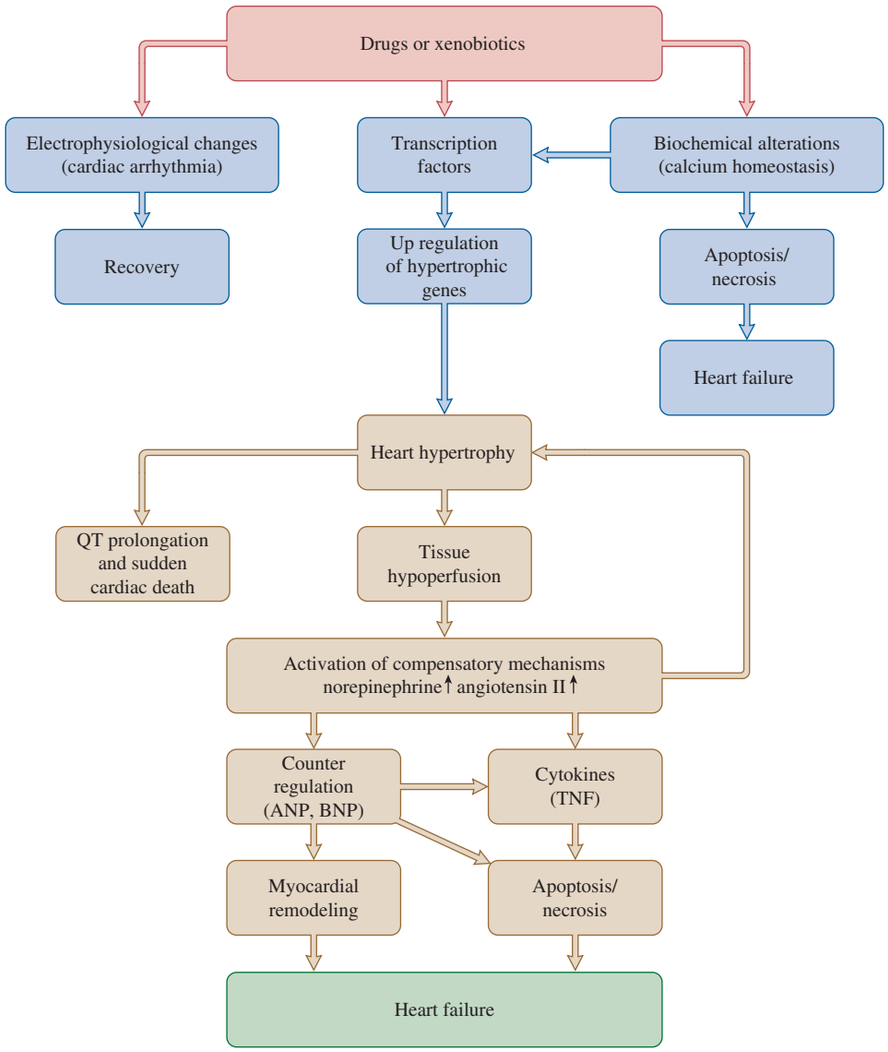

Figure 18-15. *Acute and chronic toxic exposure–induced heart failure and the transition from heart hypertrophy to heart failure.* Acute exposure to drugs or xenobiotics can cause cardiac arrhythmia, which is often observed. But if the toxic insult is so severe, myocardial apoptosis and necrosis become predominant leading to dilated cardiomyopathy and heart failure. However, the heart often survives from toxic insults through adaptive mechanisms involving upregulation of hypertrophic genes and heart hypertrophy. Heart hypertrophy increases the risk for QT prolongation and sudden cardiac death, and also activates neurohormonal regulatory mechanisms including elevation of plasma concentration of sympathetic neural transmitters and angiotensins. These compensatory mechanisms in turn activate counter-regulatory mechanisms such as ANP, BNP, and TNF-α. A long-term action of the counter-regulatory mechanisms leads to myocardial remodeling and the transition from heart hypertrophy to heart failure.

Arrhythmogenesis: QT Prolongation and Sudden Cardiac Death

The electrical system of the heart works exquisitely, conducting a heartbeat approximately every second of every day for the lifetime of an individual. When the electrical conduction pattern is compromised, however, it can lead to sudden death in an otherwise healthy individual. Numerous pathological scenarios, mostly related to remodeling or scarification, can induce arrhythmias, but many drugs and other chemicals can adversely affect the monophasic action potential of cardiomyocytes, pacemaker cells, and Purkinje fibers to induce major arrhythmias.

Cardiomyocyte Monophasic Action Potential Cardiomyocytes contract in a rhythmic pattern due to the cyclic release and subsequent reuptake of sarcoplasmic calcium that interacts with the actin–myosin contractile proteins. Calcium release is activated by cellular depolarization as part of the monophasic action potential (MAP) that is a characteristic feature of cardiomyocytes (Fig. 18-3). The cardiomyocyte MAP is typically described as having five discrete phases: 0 to 4. In cardiac action potential, phase 0 represents the rapid depolarization of myocytes caused by opening of sodium channels and the rapid influx of the positive sodium ion from the extracellular environment. Phase 1 of the cardiac action potential is recognized as a partial repolarization of the membrane due to inactivation of cardiac sodium channels, and activation of transit outward potassium channels. Phase 2 of the action potential is generated primarily by the opening of L-type calcium channels on the intracellular sarcoplasmic reticulum, which leads to Ca^{2+} ions entering the cytosol to cause contraction. This phase is sensitive to small changes in ion currents and is a

critical determinant of the duration of the action potential. Phase 3 of the cardiac action potential represents myocardial cell repolarization due to outward potassium currents. There are two critical potassium channels that terminate the plateau phase (phase 2) and initiate the final repolarization phase (phase 3): I_{Kr} and I_{Ks}. I_{Kr} is the rapidly activating delayed rectifier potassium current, and I_{Ks} is the slowly activating delayed rectifier potassium current. Phase 4 is a resting phase, dominated by resetting currents and activity of the Na^+,K^+-ATPase.

In pacemaking cells, as opposed to the larger mass of cardiomyocytes, the phase 4 period is notable for the increased slope of phase 4, due to a "leaky" pacemaker channel I_f. This leakiness causes a gradual increase in cellular potential, until the voltage achieves the appropriate window for activating voltage-gated calcium channels. Unlike cardiomyocytes, pacemaker cells initiate the phase 0 depolarization through these voltage-gated calcium channels, rather than sodium. Pacemaker cells also lack a prolonged phase 2/3 plateau, as they are not contractile cells. Most cardiomyocytes have some degree of automaticity, or the ability to self-pace, but due to the reduced role of the I_f current, the time between automatic beats is much slower than pacemaker cells. Thus, the more rapidly firing pacemaking cells *entrain* the rest of the cardiomyocytes due to the spread of depolarization signals through gap junctions.

The relationship between a cellular depolarization and the electrocardiogram (ECG) is complicated due to combined influence of both the MAP and anatomical patterns of conduction. Initial depolarization in the pacemaking cells and atria are typically observed and reflect the P-wave of the ECG. The phase 0 depolarization of the larger ventricular mass of cardiomyocytes is reflected as the QRS wave on the ECG. The repolarization phase of ventricular myocytes correlates with the T wave on the electrocardiogram. Therefore, the duration of the QT interval is related to the length of the ventricular action potentials. Notably, repolarization of atrial myocytes occurs during the QRS period and is generally too small to influence the morphology of this wave.

Cellular Abnormalities: Afterdepolarizations The normal monophasic action potential is dependent on specific timing of numerous ion channels in terms of three general biophysical states: Resting, Open, and Inactive. Ions can pass through the channel only during the Open state. Channels in the Inactive state cannot be opened until they reset to the Resting state; during the Inactive state, channels are said to be refractory, as they cannot be triggered to open. Ion channel cycles during the MAP are overlapping and well-synchronized, thus selective interference with the normal function of on channel type can have significant consequences for the cardiomyocyte. An important phenomenon related to this is called an afterdepolarization (Fig. 18-16), whereby a given channel becomes open abnormally and leads to depolarization of the cell prior to the cell being fully recovered from the previous beat (i.e., toward the completion of phase 3). As a concrete example, if a cardiomyocyte is treated with a potassium channel blocker, the duration of the MAP can be prolonged. However, because the selective potassium channel blocker does not have activity on the sodium channels that activate the initial phase 0 depolarization, these channels will change from Inactive to Open and, because the cardiomyocyte is still depolarized because of the prolonged MAP, the Open sodium channels may be triggered to open because they are dependent on voltage for activation.

In such scenarios, the cell may lose polarity, stimulate adjacent cells abnormally, or have a significant delay in recovery such that it can interfere with subsequent conductions from the normal pacemaking cells. Cells that become independent of the normal conduction pattern from the pacemaking cells can become "ectopic" pacemaking cells, and significantly disrupt cardiac electrophysiological function.

Organ-level Abnormalities: Reentry and Capture Major, life-threatening ventricular arrhythmias arise largely due to interference with the normal spread of depolarizations that arise from atrioventricular pacemaking cells. This interference leads to self-propagating waveforms that "capture" and entrain the cardiac tissue, leading to often rapidly successive depolarizations that cause tachyarrhythmias, or wholly disorganized wavefronts that cause ventricular fibrillation. Capture often arises due to regions of myocardium that poorly conducts, causing the normal wavefront to travel around this region and possibly looping backwards as a result, a phenomenon called "reentry" (Fig. 18-16). Toxicants may contribute to such life-threatening electrocardiographic disturbances (1) by chronically causing abnormal growth that alters the normal pattern of wavefront propagation; (2) by acutely disrupting the MAP, leading to afterdepolarizations and causing ectopic conductors or areas of non-conducting tissue; and (3) through effects of increasing oxygen demand or limiting oxygen delivery (for instance, via coronary artery vasospasm).

QT Prolongation Recognition of QT prolongation and its associated adverse effects on the heart has been a major focus in drug discovery and development during the last two decades. A number of drugs have been found to cause QT prolongation and Torsades de Pointes, a life-threatening ventricular arrhythmia, and thus were removed from the market or relabeled for restricted use. It has been known for a long time that quinidine causes sudden cardiac death; however, the severe and lethal side effect of QT prolongation did not draw sufficient attention until the last decade, due to the lack of knowledge and experimental approaches to obtain a comprehensive understanding of QT prolongation. Knowledge on QT prolongation has accumulated and regulatory guidelines for a battery of preclinical tests to assess QT liability of a potential drug are recommended.

A simple definition for QT prolongation is that the length of QT interval observed from a typical electrocardiogram is prolonged (Fig. 18-16). Clinically, long QT syndrome is defined when the QT interval is longer than 460 milliseconds. However, Torsades de Pointes occurs with an average increase in QT interval by approximately 200 milliseconds (a normal QT interval is about 300 milliseconds). A human study has found that Torsades de Pointes does not occur with a QT interval shorter than 500 milliseconds (Joshi et al., 2004). In general, the long QT syndrome can be divided into two classes: congenital and acquired. Congenital long QT syndrome is caused by rare genetic traits, while acquired long QT syndrome is among the major concerns for drug cardiac toxicity in pharmaceutical discovery and development.

Molecular Basis of QT Prolongation A reduction in net outward current and/or an increase in inward current are potential contributors to the prolongation of cardiac action potential, thereby QT prolongation on the electrocardiogram. Although many channels are potentially involved in the prolongation of the cardiac action potential, current studies have identified three important channels that play a critical role in the plateau phase (phase 2) of the cardiac action potential, sodium inward channels, and potassium outward channels (I_{Kr} and I_{Ks}).

Sodium Channel Dysfunction Sodium channel dysfunction in congenital long QT syndrome is related to mutations in *SCN5A* gene that encodes the α-subunits of sodium channels. Mutational analyses have found 14 distinct mutations of *SCN5A* associated with long QT syndrome (Splawski et al., 2000). It has been hypothesized

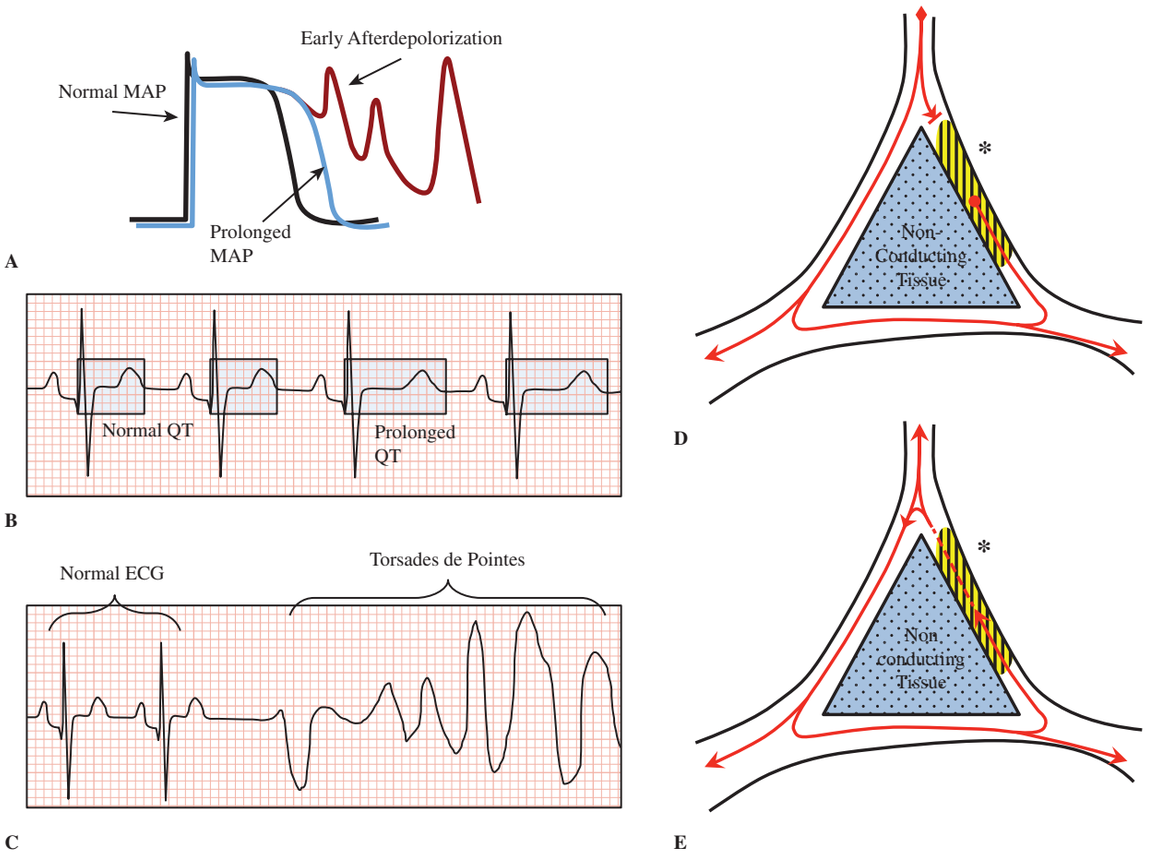

Figure 18-16. *Cellular and tissue level abnormalities central to arrhythmogenesis.* Prolongation of the cardiomyocyte monophasic action potential (**A**) may lead to reactivation of sodium or calcium channels contributing to the development of an early afterdepolarization. Prolongation of the monophasic action potentials of ventricular myocytes can lead to QT prolongation (**B**), which is a substrate for the initiation of Torsades de Pointes (**C**). Reentry and capture are demonstrated by the diagrams in **D** and **E**. Around a region of damaged nonconducting tissue, the depolarization wave spreads around the tissue to the left, but to the right the wavefront encounters refractory cardiac tissue that slows the conduction (*). As the normal wavefront wraps around the nonconducting tissue counterclockwise, it encounters this area of poorly conducting tissue again from the other side. If the wavefront is unable to pass through the poorly conducting tissue (as in **D**), the normal conductions arising from the SA node will predominate. However, if the wavefront is able to pass through it may be able to propagate a new reentrant excitation that captures the cardiac rhythm, setting the stage for a major ventricular arrhythmia.

that gain-of-function mutations in *SCN5A* would cause long QT syndrome because reopening of the sodium channels during the plateau phase of action potential, even in a small inward current, would lengthen the duration of the cardiac action potential. Sodium channel inactivation immediately following depolarization (phase 1) is important for the transition to phase 2 of the action potential. A mutation of *SCN5A* has been found to destabilize the inactivation gate (Bennett et al., 1995). Activation of these mutant sodium channels is normal and the rate of inactivation appears slightly faster than normal, but these mutant channels can reopen during the plateau phase of the action potential, leading to a prolonged plateau phase.

The I_{Kr} *potassium channels* critically affect the length of the plateau phase of the cardiac action potential. The human *ether-à-go-go*-related gene (*HERG*) is expressed primarily in the heart and encodes the α-subunit of the cardiac I_{Kr} potassium channel. There are 94 mutations of *HERG*, which have been identified to represent 45% of the total number of mutations related to long QT syndrome to date (Splawski et al., 2000). The HERG α-subunits assemble with MiRP1 β-subunits to form cardiac I_{Kr} channels. The I_{Kr} potassium channel is one of the two channels that are primarily responsible for termination of the plateau phase of the action potential. During the repolarization of the action potential, the I_{Kr} channels open, resulting in an increase in the magnitude of I_{Kr} current during the first half of phase 3 repolarization. Many *HERG* mutations occur around the membrane-spanning domains and the pore region of the channel. Most of these mutations have a loss-of-function effect and many long QT syndrome–associated mutations in *HERG* are missense mutations, which lead to a dominant-negative effect on I_{Kr} channels because the functional I_{Kr} potassium channels are composed of heteromultimers including several HERG subunits. Therefore, the loss-of-function mutations in *HERG* make a critical contribution to the long QT syndrome due to the prolonged plateau phase of cardiac potential.

The I_{Ks} *potassium channel* is the other one of the two channels primarily responsible for the termination of the plateau phase of the action potential. The I_{Ks} potassium channel is assembled from KVLQT1 α-subunits and the minK β-subunits. There are two molecular mechanisms that possibly account for reduced KVLQT1 function in the long QT syndrome (Wollnik et al., 1997). First, intragenic deletions of one *KVLQT1* allele result in synthesis of abnormal α-subunits that do not assemble with normal subunits, leading to a 50% reduction in the number of the functional channels. Second, missense mutations result in synthesis of KVLQT1 subunits with structural abnormalities, which can assemble with normal subunits. Channels formed from the mutant KVLQT1

subunits have reduced or no function. Both of these mutations result in a dominant-negative effect. Interestingly, both *KVLQT1* and *minK* are expressed in the inner ear, in which the channels function to produce a potassium-rich fluid known as endolymph that bathes the organ of Corti, the cochlear organ responsible for hearing. Individuals with Jervell and Lange–Nielsen syndrome have homozygous mutations of *KVLQT1* or minK, thus having no functional I_{Ks} channels. These individuals have severe arrhythmia susceptibility and congenital neural deafness.

Therefore, the molecular basis of the QT prolongation on the electrocardiogram is the prolongation of cardiac action potential. In this regard, the inward sodium channels and outward potassium channels play an important role in increasing the length of the plateau phase of action potentials. Congenital long QT syndrome is related to gain-of-function mutations in sodium channels and/or loss-of-function mutations in potassium channels. Acquired long QT syndrome is also related to altered function of these channels; however, many other factors that affect the phenotype of long QT syndrome and the clinical manifestations.

Torsade De Pointes and Sudden Cardiac Death The abnormalities of different channels in different regions of the heart at varying levels result in channel dysfunction with regional variability. The regional abnormalities of cardiac repolarization or conductance provide a substrate for arrhythmia. Under these conditions, arrhythmia is induced if a trigger mechanism is implanted. The trigger for arrhythmia in the long QT syndrome is thought to be spontaneous secondary depolarization that arises during or just following the plateau of the action potential. This small action potential is the so-called early afterdepolarization, which occurs preferentially in M cells and Purkinje cells due to reactivation of the L-type calcium channels and/or activation of the sodium–calcium exchange current. Once triggered, a self-propagating waveform captures the rhythm of the heart creating a high-frequency waveform on the ECG referred to as Torsades de Pointes, French for "twisting of the points" (Fig. 18-16). Torsades de Pointes can spontaneously revert to a normal rhythm, but it can also be a life-threatening arrhythmia, leading to sudden cardiac death. Drugs causing Torsades de Pointes are considered severe cardiac toxic agents. There are several drugs that were removed from the market due to their "Torsadogenic" effect. Torsades-inducing drugs are seen in a number of therapeutic areas, including antipsychotic, antiarrhythmic, and antimicrobial drugs, and even recently removed cyclooxygenase-2 (COX-2) inhibitors Vioxx and Bextra, which is discussed in the section "Cardiac Toxic Chemicals." Because of the inherent risk to otherwise healthy subjects, QT prolongation effect is now a required test by the U.S. Food and Drug Administration for early phases of drug development.

Parameters Affecting QT Prolongation and Torsadogenesis Alterations in the function of cardiac channels or "cardiac channelopathies" occur at the cellular level. However, electrotonic cell-to-cell coupling influences the dispersion of repolarization. If myocardial cells with intrinsically different duration of action potential are well coupled, electrotonic current flow attenuates the differences in action potential duration in individual cardiomyocytes. Therefore, torsadogenesis results from not only cardiomyocytes, but also other types of cells and the interaction among these cells. There are many factors that affect the clinical manifestations of QT prolongation and torsadogenesis. Genetic polymorphisms and female gender are two distinct risk factors. The mechanism of the polymorphisms and the rationale for high susceptibility of females to QT prolongation and torsadogenesis are yet to be determined.

Drugs and Environmental Toxicants Drug-induced QT prolongation is a major acquired long QT syndrome. Selective blockers of potassium channels, including the drugs so-called class III antiarrhythmics, have been developed for the treatment of various atrial arrhythmias. However, these drugs predictably produce long QT syndrome, which is sufficient to cause Torsades de Pointes in 5% to 7% of recipients. Environmental exposure to particulate air pollution in air is a risk factor for QT prolongation in elders, children, and individuals with compromised hearts.

Disturbances in Ion Homeostasis Hypokalemia in combination with torsadogenic drugs is a most recognized risk factor for QT prolongation and Torsades de Pointes. It is also shown that sodium supplementation can diminish the long QT syndrome due to the gain-of-function mutations in sodium channels. Stress-induced Ca^{2+} overload in myocardial cells increases the likelihood of arrhythmia. The electrode imbalance exerts more effect on compromised hearts.

Abnormal Gap Junction Gap junction–mediated intercellular communication is essential in the propagation of electrical impulse in the heart. The gap junction is composed of connexons, as described in the overview of cardiac structural and physiological features section. Under normal conditions, the gap junction electrotonic current flow attenuates the differences in action potential duration of myocardial cells. Toxicological exposures cause damage to connexons leading to disruption of electrotonic cell-to-cell coupling, thus the differences in the action potential duration would be dominant, particularly under the influence of torsadogenic drugs or conditions.

Myocardial Ischemic Injury Acute myocardial ischemia can cause immediate arrhythmia due to disturbance in ionic homeostasis, which is often transient. However, acute ischemia induces myocardial infarction that can lead to the block of cardiac conductance. Under the myocardial infarction, the areas separated by the scar tissue would be uncoupled, making the differences in the duration of action potential of myocardial cells in different regions apparent. The infarct heart thus is more susceptible to drug-induced QT prolongation and Torsades.

Cardiac Hypertrophy Purkinje fibers are derived from myogenic precursors during embryonic development. The normal distribution of Purkinje fibers in the myocardium is proportional to the mass of the heart. Cardiac hypertrophy resulting from the hypertrophic growth of cardiac myocytes would lead to unbalanced distribution of Purkinje fibers in the remodeling heart. The conduction of pacemaker potentials would thus be interrupted.

Myocardial Fibrosis Dilated cardiomyopathy in alcoholics often involves myocardial fibrosis, which simulates the effect of myocardial infarction on the electrical conduction in the heart and block of cardiac conductance.

Heart Failure Most individuals with failing hearts die suddenly of cardiac arrhythmias. Heart failure presents a common, acquired form of the long QT syndrome. In human heart failure, selective downregulation of two potassium channels, I_{to1} and I_{K1}, has been shown to be involved in action potential prolongation. The I_{to1} current is involved in phase 1 of the action potential and opposes the depolarization. The increase in depolarization may be adaptive in the short term because it provides more time for excitation–contraction coupling, mitigating the decrease in cardiac output. However, downregulation of potassium channels becomes maladaptive in the long term because it predisposes the individual to early afterdepolarization, inhomogeneous repolarization, and polymorphic ventricular tachycardia.

Biomarkers for Cardiovascular Toxicity

Myocardial injury can be divided into two major classes: structural and nonstructural injuries. The structural damage of the heart includes cell death and the associated histopathological changes such as myocardial infarction. Functional deficits often accompany the structural injury. Nonstructural damage represents functional deficits without apparent structural alterations. Myocardial adaptation to intrinsic and extrinsic stresses leading to myocardial structural changes such as hypertrophy should be in the category of structural damage because the progression of hypertrophy leads to heart failure in which cell death is a major determinant factor. Myocardial structural changes and functional alterations can be indirectly observed by echocardiography and electrocardiogram in combination with stress testing. The data generated from these measurements can be considered in a broad sense as biomarkers. However, in clinical practice and experimental approach, biomarkers are referred to as indexes of myocardial injury measured from blood samples. The fundamental principle of the biomarkers is that molecules that are released from the myocardium under various injury conditions are readily detectable from blood samples.

Validation of Biomarkers For a biomarker to be indicative of myocardial damage, an important question needs to be addressed is what characteristics are required for a valid biomarker. In 2000, the Expert Working Group (EWG) on biomarkers of drug-induced cardiac toxicity was established under the Advisory Committee for Pharmaceutical Sciences of the Center for Drug Evaluation and Research of the U.S. Food and Drug Administration. The report from this EWG has summarized the characteristics of ideal cardiac toxic injury biomarkers (Wallace et al., 2004). These characteristics include cardiac specificity, sensitivity, predictive value, robust, bridge preclinical to clinical, and noninvasive procedure/accessibility. These characteristics are adapted as a standard for development and validation of a biomarker of myocardial injury.

Availability of Biomarkers Currently, validated biomarkers (Table 18-1) that are included in clinical diagnostic testing guidelines are all related to myocardial structural injury. Developing biomarkers for nonstructural injury is most challenging and demands implementation of more advanced technologies such as functional genomics and proteomics. In addition, currently available biomarkers have limitations, although they are useful within the context of any given study design.

Several circulating biomarkers of vascular injury and inflammation have been extensively evaluated with regard to clinical outcomes, drug-induced vascular injury, or both. For the most part, markers of vascular inflammation have some limited predictive capacity for adverse coronary events (such as C-reactive protein [CRP]), but often lack specificity. Biomarkers may arise from acute phase responses (CRP, IL-6), endothelial cell activation (adhesion molecules), peptidase activation (MMP9), or interference with the nitric oxide synthase system (asymmetric dimethylarginine, total NO). For the study of pharmaceutical safety and xenobiotic-induced vascular injury, a battery of complementary biomarkers is typically applied.

Biomarker Applications and Limitations All the biomarkers described in Table 18-1 have been used as indices of myocardial or vascular injury in clinical practice and experimental studies. The major concern of most of the biomarkers is their specificity. Creatinine kinase isozyme MB (CK-MB) is present in small quantities in skeletal muscle and other tissues, thus elevations of CK-MB occur in some diseases involving skeletal muscle injury. Myoglobin is found in all muscle types and its concentrations vary significantly between species and even within species. B-type natriuretic peptide (BNP) has been proposed to use as a prognostic indicator of disease progression and outcome of congestive heart failure. However, the actual utility of this biomarker is untested. BNP is involved in the counter-regulation of heart hypertrophy, thus the changes in serum BNP concentrations as a function of time in the transition from cardiac hypertrophy to heart failure need to be understood comprehensively. Higher levels of BNP may not necessarily indicate more severity of the heart disease, indicating that more scrutiny to analysis is needed. CRP is a biomarker of inflammation, and its use in myocardial injury is more supplementary to other tests than having independently predictive value.

Considering all of the limitations above, more reliable biomarkers are needed. Novel markers have been explored, including extracellular endothelial vesicles (exosomes), micrRNA, or function in vitro assays using peripheral blood monocytes or platelets, or application of whole serum or plasma on cells to assess inflammatory capacity in total. However, such innovative approaches require substantial laboratory sophistication and may be unlikely to develop further without robust clinical validation. A significant advance in the development and validation of biomarkers for myocardial and vascular injury is the promising clinical experience with cardiac troponin T (cTnT), which has absolute myocardial tissue specificity and high sensitivity. cTnT is now accepted by the clinical community as the biomarker of choice for assessing myocardial damage in humans. Its preclinical value for monitoring drug cardiac toxicity and in drug development needs to be evaluated.

CARDIAC TOXIC CHEMICALS

Many substances can cause cardiac toxic responses directly or indirectly. However, only chemicals that primarily act on the heart or whose cardiac toxicity is the primary concern should be categorized as cardiac toxic chemicals. Clinically, the most recognized toxicological cardiomyopathy is found in alcoholic heart muscle disease, which is often referred to as alcoholic cardiomyopathy (ACM). In this section, ACM will be discussed as a prototype of toxicological cardiomyopathy. However, cardiomyopathy induced by therapeutic drugs is also well recognized, but it is often referred to as cardiomyopathy induced by specific chemicals, such as anthracycline cardiomyopathy. In this context, the chemicals that cause cardiac toxicity can be classified in multiple ways; however, this chapter categorizes them into (1) pharmaceutical chemicals, (2) natural products, and (3) environmental and industrial chemicals. Because general mechanisms of cardiotoxicity have been discussed above, these cardiac toxic chemicals will be briefly discussed.

Alcohol and Alcoholic Cardiomyopathy

ACM has been recognized for a long time (Klatsky, 1998), and the prevalence of ACM in selected populations ranges from 23% to 40% (McKenna et al., 1998; Fauchier et al., 2000). Alcohol is considered to be the causal chemical in up to 40% of all patients with nonischemic, dilated cardiomyopathy (Gavazzi et al., 2000). ACM is characterized by an increase in myocardial mass, dilation of the ventricles, wall thinning, ventricular dysfunction, and heart failure (Piano, 2002).

The pathogenesis of ACM is not completely understood. However, it is clear that the duration of heavy alcohol use in patients is a critical factor. Clinical data have shown that ACM typically is seen after a long term of consistent consumption of at least 80 g of alcohol per day (Regan, 1990; Lazarevic et al., 2000). In general, asymptomatic ACM patients with changes in cardiac structure and

Table 18-1

Biomarkers for Cardiovascular Toxicity, Injury, and Inflammation

CARDIAC MARKERS	BACKGROUND	USE
Creatine kinase	Elevation of specific isoform CK-MB in the serum is a specific marker of acute myocardial infarction	Routinely used clinical and preclinical myocardial injury marker
Myoglobin	Elevation of serum myoglobin is likely reflective of the extent of myocardial damage, although not specific to cardiac muscle	Readily available clinical and preclinical marker, although lack of specificity has led to reduced utilization
B-type natriuretic peptide	Cardiac neurohormone secreted by the ventricular myocardium in response to volume and pressure overload, and the release of BNP is a valuable indicator of heart failure	BNP is an important diagnostic marker as well as a drug used to alleviate congestive heart failure symptoms in cardiac decompensation. BNP has value for preclinical models of heart failure across species
Cardiac troponins	Cardiac troponin T (cTnT) and I (cTnI) are constituents of the myofilaments and expressed exclusively in cardiomyocytes. It is thus of absolute myocardial tissue specificity	"Gold Standard" for diagnosis of myocardial infarction. Value extends to preclinical safety and experimental models
VASCULAR MARKERS	**BACKGROUND**	**USE**
Acute-phase proteins (e.g., CRP, serum amyloid A, and IL-6)	CRP is a marker of systemic and vascular inflammation, which appears to predict future cardiac events in asymptomatic individuals. SAA and IL6 are similarly reflective of systemic inflammation, but not used as extensively as CRP for clinical prediction.	Clinically relevant to long-term disease, but may lack specificity/sensitivity for preclinical safety assessment.
Soluble adhesion molecules	Soluble intracellular and vascular cell adhesion molecule (ICAM-1 and VCAM-1) and E- and P-selectins are cell surface receptors on endothelial cells that can be shed following injury or inflammatory activation of peptidases	Clinical and preclinical values, although not routinely used for cardiovascular diagnostic purposes
Endothelin-1	A potent vasoconstrictive peptide, appears elevated in response to a number of toxicants	Clearly involved in certain clinical pathologies, especially pulmonary hypertension. Mostly used in preclinical experimental settings
Thrombospondin-1	May be released from a variety of tissues and is a nonspecific biomarker for a number of diseases including vascular injury, but may also be a driver of inflammation and endothelial cell activation via the CD36 receptor	Principally preclinical and experimental research values
Vascular endothelial growth factor (VEGF)	A peptide that stimulates angiogenesis, may reflect vascular injury or conditions necessitating vascular growth or healing	Used clinically most often for cancer-related outcomes, but often used for preclinical safety related to vascular injury
Cytokines (MCP-1, CINC/IL-8, KC/GRO)	Numerous cytokines are released into the circulation from atheromas and areas of vasculitis. Specific cytokines may be more pertinent than others depending on the injury or pathology of interest	Used widely clinically and experimentally, but not specific enough for diagnostic or prognostic value

function had a history of consuming more than 90 g/day of alcohol for more than 5 years (Lazarevic et al., 2000). For the symptomatic ACM, some limited data have shown that more than 10 years of excessive alcohol consumption in alcoholics produces congestive heart failure (Mathews et al., 1981).

The pathogenesis of heart failure begins after an index event such as alcohol-induced cardiac muscle injury that produces an initial decline in pumping capacity of the heart. Following this initial decline, a variety of compensatory mechanisms are activated, including the adrenergic nervous system, the renin–angiotensin system, and the cytokine system. Some of these compensatory changes have been detected in alcoholic patients (Adams and Hirst, 1986). However, with time, the sustained activation of these systems can lead to secondary end-organ damage within the ventricle by activating and accelerating the left ventricle remodeling and subsequent cardiac decompensation, resulting in the transition from asymptomatic to symptomatic heart failure (Mathews et al., 1981).

It was proposed that the metabolite acetaldehyde is responsible for some of the cardiac injury associated with ethanol consumption. The metabolic enzyme responsible for the conversion of ethanol to acetaldehyde is alcohol dehydrogenase, which is absent in cardiac myocytes. Studies have indicated that the impaired liver function of alcoholics may be sufficient to generate quantities of acetaldehyde that can reach the heart. The direct effects of acetaldehyde on the

myocardium include inhibition of protein synthesis, inhibition of Ca^{2+} sequestration by the SR, alterations in mitochondrial respiration, and disturbances in the association of actin and myosin. The exact mechanism of ACM is unresolved. It has been suggested that a combination of multiple factors is involved, including malnutrition, cigarette smoking, systemic hypertension, and beverage additives, in addition to a long-term consumption of alcohol in the ACM patients (Ahmed and Regan, 1992).

The generation of reactive oxidative metabolites from the biotransformation of ethanol has been suggested to be a major contributing factor for ACM, because these metabolites lead to lipid peroxidation of cardiac myocytes or oxidation of cytosolic and membraneous protein thiols (Ribiere et al., 1992; Kannan et al., 2004). Most experimental approaches involve alcohol-containing liquid diet feeding to rodent models of ACM for several weeks to several months. However, a key factor for the development of ACM in humans is the duration of excessive consumption of alcohol. The simulation of daily excessive amount of alcohol consumption in rodents without disturbances in food intake to produce nutritional deficiency is a constant challenge. One of the difficulties in using rodent models is that the short life span of the animals does not allow a sufficient long period of alcohol exposure to produce some of the critical pathological changes such as myocardial fibrosis observed in humans.

A recent study using a mouse model in which alcohol-induced heart hypertrophy and fibrosis were all produced may have been a breakthrough. In this mouse model, a zinc-regulatory protein, metallothionein (MT), is genetically deleted and when zinc was exogenously added to the alcohol-containing liquid diet, alcohol-induced cardiac fibrosis, but not heart hypertrophy, in the MT knockout (MTKO) mice was prevented (Wang et al., 2005). Zinc deficiency is an important feature in alcoholic patients and animal models (McClain and Su, 1983; Bogden et al., 1984). Zinc homeostasis within the cell is dependent on MT, which under physiological conditions binds seven atoms of zinc (Kagi, 1991). The role of MT in the regulation of zinc homeostasis has been revealed only recently (Maret, 2000; Kang, 2006). The most important feature of MT is that under oxidative stress conditions, it releases zinc (Maret, 2000; Kang, 2006). In the MT-KO mice, alcohol-induced myocardial pathological changes are either accelerated or severely altered relative to those in WT mice. These pathological changes, particularly the myocardial fibrosis, resemble the pathology observed in patients with ACM.

There are several interesting clues that have been provided from the MT-KO mouse studies. First, the link between the deficiency in endogenously stored zinc due to the lack of MT and the alcohol-induced myocardial fibrosis is suggested by the fact that supplementation with zinc inhibits alcoholic myocardial fibrosis. Second, the dissociation between alcohol-induced heart hypertrophy and myocardial fibrosis is suggested by the fact that supplementation with zinc only inhibits fibrosis but not heart hypertrophy. Third, possible involvement of oxidative stress in the fibrogenesis is suggested by the fact that MT functions as an antioxidant (Kang, 1999) and zinc release from MT is an essential response of MT to oxidative stress (Maret, 2000; Kang, 2006), suggesting that oxidative stress is involved in the fibrogenesis. Further studies following the same direction will provide more comprehensive insights into the pathogenesis of ACM.

Pharmaceutical Chemicals

Cardiac toxicity of pharmaceutical chemicals is a major problem in drug development and their clinical application. The pharmaceutical chemicals that cause cardiac toxic responses can be simply classified as drugs that are used to treat cardiac disease, and others that are used to treat noncardiac disease. In the category of drugs used to treat cardiac disease, cardiac toxicity is often produced by overexpression of the principal pharmaceutical effects. Although overdosing of these drugs can be a major factor for untoward effects, cardiac toxicity is often inevitable for this group of drugs. Drugs such as digitalis, quinidine, and procainamide often cause acute cardiac toxicity in the form of arrhythmia, which is reversible upon cessation of their use. Other cardiac drugs may cause cardiac toxicity by mechanisms different from that of the therapeutic action. For instance, catecholamines may cause cardiac toxicity through oxidative stress, rather than by their pharmaceutical action on the sympathetic nervous system. The other category is noncardiac drugs that produce cardiac toxicity. For instance, anthracyclines, such as Adriamycin, are effective anticancer drugs, but their ability to produce severe cardiac toxicity limits their use in cancer patients. Vioxx is a selective COX-2 inhibitor used as an anti-inflammatory drug, but it causes QT prolongation and increases the risk for sudden cardiac death.

Antiarrhythmic Agents For decades, many sodium and potassium channel blockers were administered to suppress certain arrhythmias without evidence that they actually benefited patients long-term. Results from the CAST-I and CAST-II trials demonstrated that such approaches actually increased mortality, suggesting that antiarrhythmic medications may increase risk of fatal arrhythmias, or contribute to mortality in other ways (The Cardiac Arrhythmia Suppression Trial Investigators, 1992). Thus, utilization of antiarrhythmic drugs has evolved to incorporate increased scrutiny in terms of which conditions and patients will truly benefit. Antiarrhythmic drugs have historically been classified based upon a primary mechanism of action: Na^+ channel blockers (class I), β-adrenergic blockers (class II), drugs that prolong action potential duration, especially K^+ channel blockers (class III), and Ca^{2+} channel blockers (class IV). However, this classification is artificial because most of the drugs have multiple mechanisms of action.

Class I Antiarrhythmic Agents These are primarily Na^+ channel blockers, including disopyramide, encainide, flecainide, lidocaine, mexiletine, moricizine, phenytoin, procainamide, propafenone, quinidine, and tocainide. Blockade of cardiac Na^+ channels results in reduction of conduction velocity, prolonged QRS duration, decreased automaticity, and inhibition of triggered activity from delayed afterdepolarizations or early afterdepolarizations (Roden, 1996). The primary concern of Na^+ channel blocker toxicity is that proarrhythmic effects are seen at a much higher incidence in those patients with a previous history of myocardial infarction or with acute myocardial ischemia (Nattel, 1998). The proarrhythmic effects of these drugs would also be more prevalent in patients with other cardiac complications.

Class II Antiarrhythmic Drugs These are β-adrenergic receptor–blocking drugs, including acebutolol, esmolol, propranolol, and sotalol. The catecholamines increase contractility, heart rate, and conduction through activation of β-adrenergic receptors in the heart. These effects can be explained by increased adenylyl cyclase activity, increased cyclic AMP, activation of protein kinase A, and phosphorylation and activation of L-type Ca^{2+} channels, thereby increasing intracellular Ca^{2+}, and particularly the amplitude of the Ca^{2+} transient. Therefore, antagonists of β-adrenergic receptors in the heart lead to effects that are opposite that of catecholamines, and are useful for the treatment of supraventricular tachycardia. The main adverse cardiovascular effect of β-adrenergic receptor antagonists is hypotension. These drugs may also exacerbate AV

conduction deficits (e.g., heart block) and promote arrhythmias during bradycardia.

Class III Antiarrhythmic Drugs These are primarily K^+ channel blockers. These drugs include amiodarone, bretylium, dofetilide, ibutilide, quinidine, and sotalol. Blockade of K^+ channels increases action potential duration and increases refractoriness. Prolonged action potential duration contributes to the development of early afterdepolarizations and promotion of tachycardia, especially polymorphic ventricular tachycardia (such as Torsdaes). The most noticeable adverse effect of these drugs is QT prolongation and torsadogenesis. Most of the drugs in this class also affect other ion channels and/or receptors. Amiodarone and quinidine also block Na^+ channels, whereas sotalol inhibits β-adrenergic receptors in the heart. Amiodarone prolongs action potential duration and effective refractory period of Purkinje fibers and ventricular myocytes, and the most common adverse cardiovascular effect of amiodarone is bradycardia. Amiodarone may also have cardiotoxic effects by stimulating excessive Ca^{2+} uptake, especially in the presence of procaine (Gotzsche and Pedersen, 1994).

Class IV Antiarrhythmic Drugs These are Ca^{2+} channel blockers and include diltiazem and verapamil. The dihydropyridine Ca^{2+} channel blockers are not used to treat arrhythmias because they have a greater selectivity for vascular cells; however, these drugs may also alter cardiac ion homeostasis when plasma concentrations of the drugs are elevated. The dihydropyridines interact with Ca^{2+} channels in the inactivated state of the channel, and because vascular smooth muscle resting potentials are lower than cardiac cells, the time spent in the inactivated state is relatively longer in vascular smooth muscle, thus providing some preference of dihydropyridines for the vasculature (Galan et al., 1998). Bepridil, verapamil, and diltiazem exert negative inotropic and chronotropic effects. These drugs also exert a negative chronotropic effect, thus they may produce bradycardia. In contrast, the dihydropyridine Ca^{2+} channel blockers typically induce a reflex tachycardia subsequent to peripheral vascular dilation and baroreceptor reflex leading to increased sympathetic outflow from the medulla.

Inotropic Drugs Drugs involved in this category include the cardiac glycosides, Ca^{2+} sensitizing agents, catecholamines, and other sympathomimetic drugs. As with the antiarrhythmic drugs, inotropic drugs may exert cardiotoxic effects through extensions of their pharmacological action.

Cardiac Glycosides These (digoxin and digitoxin) are inotropic drugs used for the treatment of congestive heart failure. Ouabain is a cardiac glycoside commonly used in the laboratory for electrophysiological experiments in cardiac myocytes. The mechanism of inotropic action of cardiac glycosides involves inhibition of Na^+, K^+-ATPase, elevation of intracellular Na^+, activation of Na^+/Ca^{2+} exchange, and increased availability of intracellular Ca^{2+} for contraction. Consequently, cardiotoxicity may result from Ca^{2+} overload, potentially including reduction in resting membrane potential (less negative), delayed afterdepolarizations, and premature ventricular contraction or ectopic beats. Cardiac glycosides also exhibit parasympathomimetic activity through vagal stimulation and facilitation of muscarinic transmission; however, at higher doses, sympathomimetic effects may occur as sympathetic outflow is enhanced. The principal adverse cardiac effects of cardiac glycosides include slowed AV conduction with potential block, ectopic beats, and bradycardia. During overdose, when the resting membrane potential is significantly altered and ectopic beats are prevalent, ventricular tachycardia may develop and can progress to ventricular fibrillation. A wide variety of drug interactions with digoxin have been reported, including both pharmacokinetic interactions (drugs that alter serum concentrations of digoxin) and pharmacodynamic interactions (drugs that alter the cardiac effects of digoxin).

Ca^{2+}-Sensitizing Drugs Ca^{2+}-sensitizing drugs including adibendan, levosimendan, and pimobendan are useful as inotropic drugs for the treatment of heart failure. In contrast to the main mechanism by which many other inotropic drugs act through elevating intracellular-free Ca^{2+} ($[Ca^{2+}]_i$) during the Ca^{2+} transient (i.e., increase the amplitude of the Ca^{2+} transient), these Ca^{2+}-sensitizing drugs increase Ca^{2+} sensitivity of cardiac myocytes, thereby avoiding Ca^{2+} overload (Lee and Allen, 1997). Although cardiotoxicity resulting from Ca^{2+} overload would not be expected following administration of these new drugs, some experimental data suggest that they may still exert proarrhythmic effects (Lee and Allen, 1997). The possibility that such Ca^{2+}-sensitizing drugs interfere with diastolic function (relaxation) requires further investigation but may contribute to the ventricular arrhythmias associated with these drugs. Other Ca^{2+}-sensitizing drugs include the xanthine oxidase inhibitors allopurinol and oxypurinol, which have been shown to increase contractile force but decrease Ca^{2+} transient amplitude (Perez et al., 1998).

Catecholamines and Sympathomimetics Catecholamines represent a chemical class of neurotransmitters synthesized in the adrenal medulla (epinephrine and norepinephrine) and in the sympathetic nervous system (norepinephrine). These neurotransmitters exert a wide variety of cardiovascular effects. Because of their ability to activate α- and β-adrenergic receptors, especially in the cardiovascular system, a number of synthetic catecholamines have been developed for the treatment of cardiovascular disorders and other conditions such as asthma and nasal congestion. Inotropic and chronotropic catecholamines used to treat bradycardia, cardiac decompensation following surgery, or to increase blood pressure (e.g., hypotensive shock) include epinephrine, isoproterenol, and dobutamine, and these drugs typically display nonselective activation of adrenergic receptors. More selective β_2-adrenergic receptor agonists used for bronchodilatory effects in asthma include albuterol, bitolterol, fenoterol, formoterol, metaproterenol, pirbuterol, procaterol, salmeterol, and terbutaline. High oral doses of albuterol or terbutaline or inhalation doses (i.e., enhanced delivery to the stomach instead of the lungs with subsequent systemic absorption) of these drugs may lead to nonselective activation of β_1-adrenergic receptors in the heart with subsequent tachycardia.

Sympathomimetic drugs that are more selective for α-adrenergic receptors include the nasal decongestants ephedrine, phenylephrine, phenylpropanolamine, and pseudoephedrine. As with the asthma drugs, at high doses these nasal decongestants can produce tachycardia, and a number of deaths have been reported. Of particular interest is the high concentration of ephedra alkaloids that may be present in some herbal remedies or "neutraceuticals," especially in products containing ma huang (Gurley et al., 1998). Tachycardia may occur from the consumption of large amounts of ephedra alkaloids, which may predispose the myocardium to ventricular arrhythmias.

High circulating concentrations of epinephrine (adrenaline) and norepinephrine (noradrenaline) and high doses of synthetic catecholamines, such as isoproterenol, may cause cardiac myocyte death. Many of the catecholamines and related drugs have been shown to induce cardiac myocyte hypertrophic growth in vitro. Catecholamine-induced cardiotoxicity involves pronounced pharmacological effects, including increased heart rate, enhanced myocardial oxygen demand, and an overall increase in systolic arterial blood pressure.

Other possible mechanisms for the cardiotoxicity of high concentrations of catecholamines include coronary insufficiency resulting from coronary vasospasm, decreased levels of high-energy phosphate stores caused by mitochondrial dysfunction, increased sarcolemmal permeability leading to electrolyte alterations, altered lipid metabolism resulting in the accumulation of FAs, and intracellular Ca^{2+} overload (Dhalla et al., 1992).

Central Nervous System Acting Drugs Some of central nervous system (CNS)–acting drugs have considerable effects on the cardiovascular system, including tricyclic antidepressants (TCAs), general anesthetics, some of the opioids, and antipsychotic drugs.

TCAs including amitriptyline, desipramine, doxepin, imipramine, and protriptyline have significant cardiotoxic effects, particularly in cases of overdose. The effects of TCAs on the heart include ST segment elevation, QT prolongation, supraventricular and ventricular arrhythmias (including Torsades), and sudden cardiac death. In addition, as a result of peripheral α-adrenergic blockade, TCAs cause postural hypotension—the most prevalent cardiovascular effect. Although many of these adverse effects are related to the quinidine-like actions, anticholinergic effects, and adrenergic actions of these drugs, the tricyclics also have direct actions on cardiac myocytes and Purkinje fibers, including depression of inward Na^+ and Ca^{2+} and outward K^+ currents (Pacher et al., 1998). Furthermore, the risk of TCA-induced cardiotoxicity is significantly enhanced in children and by concomitant administration of other drugs that alter ion movement or homeostasis in the heart (e.g., the Na^+ channel–blocking class I antiarrhythmic agents), or use in patients with cardiovascular disease.

Antipsychotic Drugs These include the phenothiazines (acetophenazine, chlorpromazine, fluphenazine, mesoridazine, perphenazine, thioridazine, and trifluoperazine), chlorprothixene, thiothixene, and other heterocyclic compounds (clozapine, haloperidol, loxapine, molindone, pimozide, and risperidone). As with TCAs, the most prominent adverse cardiovascular effect of antipsychotic drugs is orthostatic hypotension. However, the phenothiazines (e.g., chlorpromazine and thioridazine) may exert direct effects on the myocardium, including negative inotropic actions and quinidine-like effects (Baldessarini, 1996). Some ECG changes induced by these drugs include prolongation of the QT and PR intervals, blunting of T waves, and depression of the ST segment. Through anticholinergic actions, clozapine can produce substantial elevations in heart rate (tachycardia).

General Anesthetics General anesthetics as exemplified by enflurane, desflurane, halothane, isoflurane, methoxyflurane, and sevoflurane have adverse cardiac effects, including reduced cardiac output by 20% to 50%, depression of contractility, and production of arrhythmias (generally benign in healthy myocardium but more serious in cardiac disease). These anesthetics may sensitize the heart to the arrhythmogenic effects of endogenous epinephrine or to β-receptor agonists. Halothane has been found to block the L-type Ca^{2+} channel by interacting with dihydropyridine-binding sites, to disrupt Ca^{2+} homeostasis associated with the SR, and to modify the responsiveness of the contractile proteins to activation by Ca^{2+} (Bosnjak, 1991). Propofol is an intravenously administered general anesthetic that also decreases cardiac output and blood pressure. In addition, propofol causes a negative inotropic effect by its direct action on cardiac myocytes. Propofol has been shown to antagonize β-adrenergic receptors, inhibit L-type Ca^{2+} current, and reduce Ca^{2+} transients (Zhou et al., 1997, 1999; Guenoun et al., 2000).

Local Anesthetics In general, local anesthetics have few undesirable cardiac effects. However, when high systemic concentrations of cocaine and procainamide are attained, these chemicals may have prominent adverse effects on the heart.

Cocaine This acts as a local anesthetic agent by blocking conduction in nerve fibers through reversibly inhibiting Na^+ channels and stopping the transient rise in Na^+ conductance. In the heart, cocaine decreases the rate of depolarization and the amplitude of the action potential, slows conduction speed, and increases the effective refractory period. The other major pharmacological action of cocaine is its ability to inhibit the reuptake of norepinephrine and dopamine into sympathetic nerve terminals (sympathomimetic effect). Cocaine also, indirectly through its actions on catecholamine reuptake, stimulates β- and α-adrenergic receptors, leading to increased cyclic AMP and inositol triphosphate levels. These second messengers will, in turn, provoke a rise in cytosolic Ca^{2+}, which causes sustained action potential generation and extrasystoles. The net effect of these pharmacological actions is to elicit and maintain ventricular fibrillation. In addition, cocaine causes cardiac myocyte death and myocardial infarction, but the mechanism of action remains to be elucidated.

Other Local Anesthetic Drugs These include benzocaine, bupivacaine, etidocaine, lidocaine, mepivacaine, pramoxine, prilocaine, procaine, procainamide, proparacaine, ropivacaine, and tetracaine. Lidocaine and procainamide are also used as antiarrhythmic drugs. Extremely high doses of these drugs cause decreases in electrical excitability, conduction rate, and force of contraction likely through inhibition of cardiac Na^+ channels (Catterall and Mackie, 1996).

Anthracyclines, Tyrosine Kinase Inhibitors, and Other Antineoplastic Agents Cardiotoxicity is recognized as a serious side effect of chemotherapy for malignant cancers, especially with well-known antitumor agents such as doxorubicin, daunorubicin, imatinib, sunitinib, 5-fluorouracil, and cyclophosphamide (Havlin, 1992).

Anthracyclines These (doxorubicin [Adriamycin] and daunorubicin) are widely used antineoplastic drugs for the treatment of breast cancer, leukemias, and a variety of other solid tumors. Unfortunately, the clinical usefulness of these drugs is limited because of acute and chronic cardiotoxic effects. The acute effects mimic anaphylactic-type responses, such as tachycardia and various arrhythmias. These effects are usually manageable and most likely are due to the potent release of histamine from mast cells sometimes observed in acute dosing. In addition, large acute doses can also cause left ventricular failure. The greatest limiting factor of the anthracyclines is associated with long-term exposure, which usually results in the development of cardiomyopathies and, in severe cases, congestive heart failure (Havlin, 1992).

Two additional anthracyclines were introduced to the U.S. market in 1999, and several lipid formulations of doxorubicin (liposomal doxorubicin) have been approved in the past decade. *Valrubicin* is a semisynthetic derivative of doxorubicin approved for treatment of carcinoma in situ of the bladder. It is administered locally for bladder cancer and therefore induces only mild systemic toxicities; however, systemic absorption from the bladder may occur, but valrubicin seems to exhibit a lower propensity for cardiotoxicity than doxorubicin (Hussar, 2000). *Epirubicin* is a semisynthetic derivative of daunorubicin approved for treatment of breast cancer. Like doxorubicin, epirubicin is given systemically and may induce cardiotoxicity. However, epirubicin is more lipophilic than doxorubicin and is biotransformed by conjugative pathways in the liver, resulting in a shorter half-life and a lower incidence of cardiotoxicity than with doxorubicin (Hussar, 2000).

Several major hypotheses have been suggested to account for the onset of anthracycline-induced cardiomyopathy: (1) oxidative

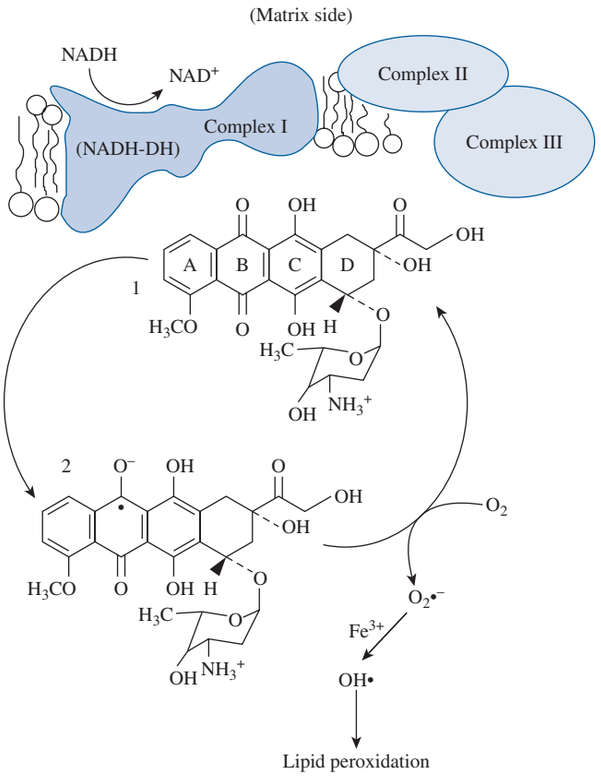

Figure 18-17. *Production of superoxide anions by oxidation–reduction cycling of doxorubicin at the level of the mitochondria.* NADH dehydrogenase (NAD-DH), which is located within complex I, has been proposed as the enzyme that catalyzes the one-electron reduction of doxorubicin (1) to a semiquinone radical (2). The semiquinone then may be reoxidized back to the parent compound by means of the reduction of molecular oxygen (O_2) to the superoxide anion ($O_2^{\bullet -}$).

stress from redox cycling or mitochondrial Ca^{2+} cycling, (2) defects in mitochondrial integrity and subsequent deterioration of myocardial energetics, (3) alterations in both SR Ca^{2+} currents and mitochondrial Ca^{2+} homeostasis, and (4) altered cardiac myocyte gene expression and induction of apoptosis. The cause-and-effect relationships of the proposed mechanisms of cardiotoxicity have not been determined, and no single theory adequately explains the exact mechanism for anthracycline-induced cardiomyopathy.

The free radical hypothesis has received the most attention in the understanding of anthrocycline-induced cardiotoxicity. The formation of ROS by doxorubicin (Fig. 18-17) has been attributed to redox cycling of the drug (Powis, 1989). Doxorubicin can undergo futile redox cycling that results in the production of oxygen free radicals; these ROS may then oxidize proteins, lipids, and nucleic acids and potentially cause DNA strand scission. The quinone-like structure of doxorubicin permits this molecule to accept an electron and form a semiquinone radical. Oxidation of the semiquinone back to the parent quinone by molecular oxygen results in the formation of superoxide radical ions that can initiate oxidative stress. The enzymatic reduction thought to be responsible for the generation of superoxide by doxorubicin has been proposed to occur between complexes I and III of the mitochondrial respiratory chain. Doxorubicin has high affinity for cardiolipin, a phospholipid found on the inner mitochondrial membrane, where NADH dehydrogenase converts the drug to a semiquinone radical (Marcillat et al., 1989). In the presence of oxygen, this radical is responsible for the generation of ROS, which then may peroxidize unsaturated membrane lipids and initiate myocardial cell injury.

Several alternate hypotheses to explain the cardiotoxicity of doxorubicin have been proposed and tested. For example, several studies have tested the hypothesis that doxorubicin induces a cycling of mitochondrial Ca^{2+} that is associated with the production of ROS and dissipation in the mitochondrial membrane potential, which in turn may result in depletion of cellular ATP (Chacon and Acosta, 1991; Solem et al., 1994). It is important to note that the observed changes in ROS accumulation, disruption of Ca^{2+} homeostasis, and mitochondrial damage are not isolated, but rather these changes occur sequentially or simultaneously. It is extremely difficult to dissect the sequences of these changes, leading to several alternate hypotheses, which may in fact occur sequentially. One of the ultimate consequences of these changes is myocardial cell death. Many studies have demonstrated that anthracycline-induced cardiotoxicity includes induction of apoptosis (Andrieu-Abadie et al., 1999; Sawyer et al., 1999; Arola et al., 2000; Kang et al., 2000a; Wang et al., 2001a).

Tyrosine Kinase Inhibitors Hematologic malignancies have proven susceptible to small molecule tyrosine kinase inhibitors, including imatinib and sunitinib. However, these agents are notable for the induction of cardiac side effects including hypertension and ventricular systolic dysfunction. Recent studies suggest that the intended action of tyrosine kinase inhibitors, specifically blocking Abelson oncogene 1 (c-Abl), is not the reason for cardiac toxicity. Rather, small molecule tyrosine kinase inhibitors may interfere with intracellular autophagy and accumulate in lysosomes (Hu et al., 2012). Not surprisingly, next-generation tyrosine kinase inhibitors appear to be more efficacious with fewer cardiac adverse events, even with treatments spanning a decade. Dasatinib and nilotinib were reported to elicit rare occurrence of pulmonary hypertension and QT prolongation, respectively, in early development, but little consistent evidence of such outcomes has been reported since approval. Nilotinib does appear to promote vascular toxicity, in terms of worsening peripheral artery disease, but this is also rare (Moslehi and Deininger, 2015).

5-Fluorouracil Clinical evidence of 5-fluorouracil cardiotoxicity ranges from mild precordial pain and ECG abnormalities (ST segment elevation, high peaked T waves, T-wave inversions, and sinus tachycardia) to severe hypotension, atrial fibrillation, and abnormalities of ventricular wall motion. The mechanism of cardiotoxicity of fluorouracil is unknown, but it may relate to impurities present in commercial products of the drug, one of which is metabolized to fluoroacetate, a compound that might participate in fluorouracil-induced cardiotoxicity.

Cyclophosphamide High doses of cyclophosphamide given to cancer or transplant patients may lead to severe hemorrhagic cardiac necrosis. The mechanism of the cardiotoxicity of this drug is not clear, but there is suggestive evidence that the toxic metabolite of cyclophosphamide, 4-hydroperoxycyclophosphamide, may alter the ion homeostasis in cardiac myocytes, resulting in increased Na^+ and Ca^{2+} content and reduced K^+ levels (Levine et al., 1993).

Antimicrobial and Antiviral Agents Cardiotoxicity associated with the clinical use of antimicrobial and antiviral drugs is often observed in overdosage and in patients with preexisting cardiovascular dysfunction.

Aminoglycosides These include amikacin, gentamicin, kanamycin, netilmicin, streptomycin, and tobramycin. Gentamicin is a representative aminoglycoside and has an inhibitory action on slow inward Ca^{2+} channels in heart muscle. Aminoglycosides inhibit the uptake or

binding of Ca^{2+} at sarcolemmal sites, thus reducing the concentration of membrane-bound Ca^{2+} available for movement into the myoplasm during depolarization of the sarcolemma. The principal mechanism of cardiodepression by gentamicin is the dislocation of Ca^{2+} from slow-channel-binding sites on the external surface of the sarcolemma, which results in a blockade of the channels (Hino et al., 1982).

Macrolides These include azithromycin, clarithromycin, dirithromycin, and erythromycin. Erythromycin is associated with QT prolongation and cardiac dysrhythmias characterized by polymorphic ventricular tachycardia (Torsades). These effects occur primarily in patients with underlying cardiac disease.

Fluoroquinolones Fluoroquinolones are a group of rapid growing antibacterial chemicals in terms of numbers of new drugs released into the market in the United States. Fluoroquinolone antibacterial drugs include ciprofloxacin, enoxacin, gatifloxacin, gemifloxacin, grepafloxacin, levofloxacin (levorotatory isomer of ofloxacin), lomefloxacin, moxifloxacin, norfloxacin, ofloxacin, sparfloxacin, and trovafloxacin (Pickerill et al., 2000). Grepafloxacin, moxifloxacin, and sparfloxacin are associated with QT prolongation in perhaps a higher incidence than macrolides. In fact, grepafloxacin was voluntarily removed from the U.S. market because of the relatively high incidence of QT prolongation and risk of Torsades de Pointes.

Tetracycline and Chloramphenicol Tetracycline and chloramphenicol have been reported to depress myocardial contractility by direct cardiac myocyte interaction or an indirect effect that lowers Ca^{2+} concentrations in the plasma or extracellular spaces. Tetracyclines are Ca^{2+} chelating agents, which explain the action of tetracyclines on myocardial contractility.

Antifungal Agents Antifungal agents, such as amphotericin B, may depress myocardial contractility by blocking activation of slow Ca^{2+} channels and inhibiting the influx of Na^{+}. Ventricular tachycardia and cardiac arrest have been reported in patients treated with amphotericin B. Flucytosine is another antifungal drug that has been associated with cardiotoxicity. In fungal cells, flucytosine is converted to 5-fluorouracil, which then exerts antifungal effects. However, flucytosine may be converted to 5-fluorouracil by gastrointestinal microflora in humans, which then may be absorbed systemically and induce cardiotoxicity as discussed above. Cardiac arrest has been reported in individuals receiving flucytosine.

Antiviral Drugs Antiviral drugs that are potentially cardiotoxic include the nucleoside analog reverse transcriptase inhibitors used for the treatment of human immunodeficiency virus (HIV) infections. Clinical studies of direct cardiotoxicity of these drugs in HIV patients are complicated by cardiomyopathy related to disease progression. The direct evidence for cardiotoxicity of zidovudine (AZT) has been obtained from a study using transgenic mice expressing replication-incompetent HIV (Lewis et al., 2000). AZT-induced cardiotoxicity in this model is related to alteration in Ca^{2+} homeostasis and/or mitochondrial toxicity. The mitochondrial toxicity of AZT has also been shown in skeletal muscle biopsy samples from AIDS patients (Dalakas et al., 1990).

Although cardiotoxicity of individual antiviral drugs is rare in clinical setting, the combination of several antiviral drugs for highly active antiretroviral therapy (HAART) has generated a major concern of cardiotoxicity (Bozkurt, 2004). HAART has dramatically improved the life expectancy of patients with HIV. The majority of the studies examining the incidence of cardiac effects demonstrated an increase in cardiac adverse event rate with HAART in the HIV-infected population. Overall, the cardiotoxicity risk appears to be greater in the HIV-infected population than in the general population, and the increased cardiac risk is associated with HAART, particularly with protease inhibitor use. However, there is general consensus that the benefits of HAART far outweigh toxicity-related risks of the treatment with HAART.

Anti-inflammatory Agents Nonsteroidal anti-inflammatory drugs (NSAIDs) include aspirin, Motrin, and Naprosyn, which are classified as nonselective NSAIDs because they are inhibitors for both COX-1 and COX-2. Inhibition of COX-1 is associated with gastrointestinal toxicity because COX-1 exerts a protective effect on the lining of the stomach. A newer class of NSAIDs has been developed including rofecoxib (Vioxx), celecoxib (Celebrex), and valdecoxib (Bextra), which are selective inhibitors of COX-2. In September 2004, Vioxx was voluntarily withdrawn from the market based upon the data from a clinical trial that showed after 18 months of use Vioxx increased the relative risk for cardiovascular events, such as heart attack and stroke (Arellano, 2005). In April 2005, Bextra was removed from the market based on the potential increased risk for serious cardiovascular adverse events and increased risk of serious skin reactions (e.g., toxic epidermal necrolysis, Stevens–Johnson syndrome, and erythema multiforme) (Talhari et al., 2005). Emerging information indicates the risk of cardiovascular events may be increased in patients receiving Celebrex (Solomon et al., 2005). The cardiovascular events induced by COX-2 inhibitors are presumably related to thrombotic events and other sequelae related to downregulation of prostacyclin production. Studies have also indicated the link of Vioxx to long QT syndrome and the increased risk for Torsades and sudden cardiac death (Fitzgerald 2004; Arellano 2005).

Antihistamines The most severe adverse effect of the second-generation histamine H_1 receptor antagonists (antihistamines) is their association with life-threatening ventricular arrhythmias and sudden cardiac death (Simons, 1994). Terfenadine and astemizole cause altered repolarization, notched inverted T waves, prominent TU waves, prolonged QT interval, first- and second-degree AV block, ventricular tachycardia or fibrillation, and Torsades de Pointes. These antihistamines produce cardiac arrhythmias by blocking the delayed rectifier K^{+} channel and prolonging action potential duration in cardiac myocytes. The prolonged action potential duration promotes early afterdepolarizations and predisposes the myocardium to ventricular arrhythmias. However, terfenadine also inhibits L-type Ca^{2+} channels in rat ventricular myocytes at concentrations near or below that required to inhibit delayed rectifier K^{+} current (Liu et al., 1997). Therefore, both inhibition of Ca^{2+} and inhibition of K^{+} current likely contribute to the cardiotoxic actions of terfenadine. As a result of cardiotoxicity, both astemizole and terfenadine have been removed from the U.S. market. However, the understanding of astemizole- and terfenadine-induced cardiotoxicity continues to be an important consideration in drug development, and other drugs have demonstrated similar clinical limitations (e.g., cisapride and fluoroquinolone antibacterial agents).

Immunosuppressants Rapamycin and tacrolimus may produce adverse cardiovascular effects, including hypertension, hypokalemia, and hypomagnesemia. Rapamycin and tacrolimus (FK506) interact with a protein that associates with ryanodine receptors (RyRs), and the protein carries the tacrolimus- or FK506-binding protein (FKBP). When rapamycin or tacrolimus binds to FKBP in cardiac myocytes, RyR becomes destabilized, resulting in Ca^{2+} leak from the SR (Marks, 1997). Tacrolimus has been shown to be associated with hypertrophic cardiomyopathy in pediatric patients, a condition that was reversed by discontinuation of tacrolimus and

administration of cyclosporin A; some of these patients developed severe heart failure (Atkison et al., 1995).

Miscellaneous Drugs Several drugs that are not included in the categories discussed earlier have significant cardiotoxic concerns and are briefly discussed below: cisapride, methylxanthines, and sildenafil.

Cisapride Cisapride is a chemical that has been used as a prokinetic drug for gastrointestinal hypomotility. However, cisapride has been removed from the U.S. market because of risk of potentially life-threatening arrhythmias (Torsades) associated with its use. Like astemizole and terfenadine, cisapride inhibits delayed rectifier K^+ current, prolongs action potential duration, prolongs the QT interval, and predisposes the heart to ventricular arrhythmias.

Methylxanthines Methylxanthines (including caffeine, theobromine, and theophylline) can be found in significant quantities in coffee, tea, chocolate, soft drinks, and other foods. Theophylline has been used for many decades for the treatment of asthma, although the mechanism of action has not been fully understood. Overdose of theophylline or rapid intravenous administration of therapeutic doses of aminophylline (theophylline complexed with ethylenediamine to increase water solubility) may produce life-threatening ventricular arrhythmias; these effects may in part be explained by direct actions of theophylline on cardiac myocyte SR or by inhibition of phosphodiesterase and elevation of cyclic AMP. The cardiac effects of methylxanthines observed in vivo (including increases in cardiac output and heart rate) may also be explained by elevated catecholamines, as theophylline has been shown to increase plasma epinephrine concentrations (Vestal et al., 1983). High concentrations of caffeine stimulate massive release of Ca^{2+} from the SR, an effect that is often utilized experimentally to determine SR function. Although it rarely occurs, caffeine-associated ventricular arrhythmias have been reported.

Sildenafil Sildenafil is a relatively specific inhibitor of phosphodiesterase-5, which is responsible for the degradation of cyclic guanosine monophosphate (cGMP; a vasodilatory second messenger). Interestingly, sildenafil was originally developed as a potential drug for treating angina; however, it was not very effective for this purpose and was subsequently developed for treatment of erectile dysfunction, where it produces vasodilation and filling of the corpus cavernosum. The primary concern regarding adverse effects of sildenafil is nonspecific inhibition of PDE3 in the heart and vasculature (Hussar, 1999). In vitro studies have revealed that sildenafil increases cyclic AMP in cardiac tissue without significant effects on cGMP (Stief et al., 2000); however, whether these effects are associated with cardiotoxicity is not known.

Natural Products

Natural products include naturally occurring catecholamines, hormones, and cytokines, as well as animal and plant toxins. Many of these products have been shown to cause cardiac toxic responses. However, it is difficult to define whether or not the cardiac toxicity results directly from the action of these products in vivo, although these products cause deleterious effects on cultured cardiomyocytes. The exposure levels of these chemicals tested in vitro in general are much higher than the concentration reached in cardiac tissue under in vivo exposure conditions. Therefore, extrapolation of in vitro data related to cardiac toxicity of natural products to in vivo conditions is challenging. However, there are some products that have clearly demonstrated cardiac toxic effects, and mechanisms of action of these products have been determined.

Catecholamines The naturally occurring sympathomimetic amines, such as epinephrine and norepinephrine, are potent and can cause deleterious effects to the heart. The synthetic catecholamine, isoproterenol, is able to cause massive necrotic changes in the myocardium and is often used as a prototype compound for the study of catecholamine cardiotoxicity, which has been discussed in the therapeutic drugs that cause cardiotoxicity.

Steroids and Related Hormones Estrogens, progestins, androgens, and adrenocortical steroids are major steroid hormones produced by mammals including humans. Myocardial tissue contains steroid receptors; therefore, the heart serves as a target organ for steroid effects. It also has been shown that cardiac tissue can synthesize steroid hormones, although the capacity for synthesis may be much lower than more classic steroid synthesizing tissues. There are two major mechanisms of action of the hormones: the first is to alter gene expression and the second is to change signaling transduction pathways.

Estrogens Estrogens are synthesized in ovaries, testes, and adrenal glands, and estrogen is an active metabolite of testosterone. Endogenous estrogens include 17β-estradiol (E_2), estrone, and estriol. Synthetic estrogens include diethylstilbestrol (nonsteroidal), equilin, esterified versions of E_2, ethinyl estradiol, mestranol, and quinestrol. In addition, many other synthetic chemicals have been shown to exert estrogenic activity, including the pesticides DDT and methoxychlor, the plasticizer bisphenol A, other industrial chemicals including polychlorinated biphenyls, and some compounds found in soybeans and tofu (e.g., phytoestrogens). Estrogens (frequently in combination with progestins) have been used for over 40 years as oral contraceptive drugs. The older versions of estrogenic oral contraceptives that contained high amounts of estrogens were associated with increased risk of coronary thrombosis and myocardial infarction; however, lower doses of estrogens have been found by numerous investigators to impart protective effects on the cardiovascular system, including antiapoptotic effects, and beneficial effects on lipid metabolism such as decreased low-density lipoproteins (LDL cholesterol) and increased high-density lipoproteins (HDL cholesterol). Estrogens alter cardiac fibroblast proliferation, but they can either increase or decrease proliferation of these cells.

Progestins Progestins are also synthesized in the ovaries, testes, and adrenal glands. Naturally occurring and synthetic progestins include desogestrel, hydroxyprogesterone, medroxyprogesterone, norethindrone, norethynodrel, norgestimate, norgestrel, and progesterone. As part of hormone replacement therapy, progestins serve an opposing role to estrogens. Unfortunately, estrogen treatment opposed with progestins may negate the cardiovascular benefits of estrogens on lipid metabolism (Kalin and Zumoff, 1990). Very little is known about the direct effects of progestins on the heart. Although progestins could exert deleterious effects on the heart, more studies are required to investigate mechanisms.

Androgens Androgens cause adverse cardiovascular effects (Rockhold, 1993; Melchert and Welder, 1995). The principal androgens are testosterone and its active metabolite dihydrotestosterone. Testosterone is synthesized in the testes, ovaries, and adrenal glands, and dihydrotestosterone mediates most androgen actions. Synthetic anabolic-androgenic steroids include the alkylated and orally available drugs danazol, fluoxymesterone, methandrostenolone, methenolone, methyltestosterone, oxandrolone, oxymetholone, and stanozolol. The nonalkylated drugs with poor oral bioavailability include androstenedione and dehydroepiandrosterone (both sold in various "nutraceutical" formulations), boldenone

(veterinary product), nandrolone (19-nortestosterone), and testosterone. Nearly all of these chemicals have received significant illicit use, particularly in extremely high doses with attempts to improve physical appearance or performance. Anabolic-androgenic steroids have been associated with alterations in lipid metabolism, including increased LDL cholesterol and decreased HDL cholesterol; therefore, these chemicals may predispose individuals to atherosclerosis (Melchert and Welder, 1995). Evidence indicating the direct cardiac toxic effect of anabolic-androgenic steroids includes alteration of Ca^{2+} fluxes in cardiac myocytes induced by testosterone (Koenig et al., 1989), hypertrophic growth of neonatal rat cardiac myocytes stimulated by testosterone and dihydrotestosterone (Marsh et al., 1998), and mitochondrial abnormalities and myofibrillar lesions induced by methandrostenolone given intramuscularly to rats (Behrendt and Boffin, 1977). In humans, high-dose anabolic-androgenic steroid use has been associated with cardiac hypertrophy and myocardial infarction. However, the mechanisms responsible for the cardiotoxic effects of anabolic-androgenic steroids remain poorly understood.

Glucocorticoids and Mineralocorticoids Glucocorticoids and mineralocorticoids are primarily synthesized in the adrenal glands. Naturally occurring glucocorticoids include corticosterone, cortisone, and hydrocortisone (cortisol), and the mineralocorticoid is aldosterone. A large number of synthetic glucocorticoids are used for treatment of various autoimmune and inflammatory diseases. These drugs include alclometasone, amcinonide, beclomethasone, betamethasone, clobetasol, desonide, desoximetasone, dexamethasone, diflorasone, fludrocortisone, flunisolide, fluocinolone, fluocinonide, fluorometholone, flurandrenolide, halcinonide, medrysone, methylprednisolone, mometasone, paramethasone, prednisolone, prednisone, and triamcinolone. Most of these drugs are primarily used topically, intranasally, or inhalationally. The primary glucocorticoids used systemically include cortisone, hydrocortisone, dexamethasone, methylprednisolone, prednisolone, and prednisone. The mineralocorticoid aldosterone is not used clinically; however, the aldosterone receptor antagonist spironolactone has been used for years to treat hypertension and is now thought to decrease morbidity and mortality associated with congestive heart failure. Both aldosterone and glucocorticoids appear to stimulate cardiac fibrosis by regulating cardiac collagen expression independently of hemodynamic alterations (Young et al., 1994; Robert et al., 1995). Furthermore, aldosterone and glucocorticoids induce hypertrophic growth and alter expression of Na^+, K^+-ATPase, Na^+/H^+ antiporter, and chloride/bicarbonate exchanger of cardiac myocytes in vitro. Clinically relevant cardiac hypertrophy has been observed in premature infants undergoing dexamethasone treatment. The mechanisms responsible for the direct effects of these chemicals remain poorly understood.

Thyroid Hormones These include thyroxine (T_4) and triiodothyronine (T_3). These hormones exert profound effects on the cardiovascular system. Hypothyroid states are associated with decreased heart rate, contractility, and cardiac output, whereas hyperthyroid states are associated with increased heart rate, contractility, cardiac output, ejection fraction, and heart mass. Patients with underlying cardiovascular disease may display arrhythmias under the treatment of thyroid hormones. Thyroid hormones also alter expression of cardiac SR Ca^{2+} handling proteins including increased expression of SR Ca^{2+} ATPase (SERCA) and decreased expression of phospholamban, an inhibitory protein of SERCA (Kaasik et al., 1997).

Cytokines More than 100 different cytokines have been found, and the cardiovascular effects of these substances can be classified as pro-inflammatory, anti-inflammatory, or cardioprotective (Pulkki, 1997). Members of the pro-inflammatory class include TNF-α; interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8); Fas ligand; and chemokines (e.g., CC chemokines such as MCP-1, macrophage chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; and RANTES, regulated on activation normally T-cell-expressed and secreted). Members of the anti-inflammatory class typically downregulate expression of pro-inflammatory cytokines and include interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13), and transforming growth factor-beta (TGF-β). The cardioprotective cytokines include cardiotrophin-1 (CT-1) and leukemia inhibitor factor, which have been shown to inhibit cardiac myocyte apoptosis from a number of different stimuli. Many of these cytokines are elevated during cardiovascular diseases such as I/R injury, myocardial infarction, and congestive heart failure.

IL-1β IL-1β is known to exert negative inotropic actions and induce apoptosis of cardiac myocytes. The effects of IL-1β on cardiac myocytes are likely mediated through induction of nitric oxide synthase (NOS) and/or increased production of nitric oxide (NO) (Arstall et al., 1999). Superoxide anion and peroxynitrite formation was associated with reduced left ventricular ejection fraction in dogs treated with microspheres containing IL-1β, suggesting the involvement of peroxynitrite in IL-1β cardiotoxicity (Cheng et al., 1999b).

TNF-α TNF-α induces apoptotic cell death in myocardium (Krown et al., 1996). The mechanisms responsible for TNF-α-induced apoptosis of cardiac myocytes are not entirely clear. TNF-α also exerts negative inotropic effects on cardiac myocytes at least potentially through increased production of sphingosine (Sugishita et al., 1999). However, it has been shown that TNF-α is essential for the cardiac protective response to stresses such as ischemic cardiac injury (Kurrelmeyer et al., 2000).

IL-6 IL-6 has been shown to induce negative inotropic effects on cardiac myocytes, possibly through induction of NOS expression and increased NO production (Sugishita et al., 1999).

IL-2 IL-2 may decrease the mechanical performance and metabolic efficiency of the heart, and these myocardial effects may be related to changes in NO synthesis and Na^+/H^+ exchange.

Interferon Interferon may result in cardiac arrhythmias, dilated cardiomyopathy, and signs of myocardial ischemia. Interferon-γ acts synergistically with IL-1β to increase NO formation in the heart, and induce Bax expression and apoptosis in cardiac myocyte cultures (Arstall et al., 1999).

Animal and Plant Toxins Animal toxins in the venom of snakes, spiders, scorpions, and marine organisms have profound effects on the cardiovascular system. There are also a number of plants—such as foxglove, oleander, and monkshood—that contain toxic constituents and have adverse effects on the cardiovascular system.

Environmental Pollutants and Industrial Chemicals

There are many chemicals classified in this category that cause cardiac toxicity. Metals and metalloids can be found both in environmental pollutants and industrial chemicals. Some heavy metals, such as cadmium, block calcium channels that affect cardiac rhythm leading to arrhythmia, others such as arsenic have high affinity for sulfhydryl groups, and interfere with sulfhydryl-containing proteins, such as receptors, regulatory proteins, and transporters. During the last decade, epidemiological

and experimental studies have identified an association between particulate matter air pollution and cardiac toxicity; however, mechanistic insights into cardiac toxicity induced by particulate matter remain elusive. In this section, a brief discussion of selected industrial agents with their prominent cardiotoxic effects and proposed mechanisms of cardiotoxicity is presented. For a more comprehensive review of industrial chemicals and their cardiotoxic potential, the reader is referred to the study of Zakhari (1992).

Airborne Particulate Matter Inhalation of air pollutants, especially particulate matter, can cause a variety of cardiac and vascular disease outcomes. A clear relationship between ambient concentration of particulate matter and cardiovascular morbidity and mortality has been documented over the past 30 years. While many of the outcomes of inhaled particles relate to vascular toxicity (discussed below), numerous studies have confirmed that particulate inhalation can lead to arrhythmias and electrocardiographic abnormalities and also negatively impact cardiac function. How inhaled particulate matter leads to cardiac effects remains incompletely understood, but it is generally thought to arise from "spillover" of intermediate reactants from the lung that have inflammatory actions on cardiovascular cells, direct translocation of particulate matter or soluble particulate constituents to the cardiac tissue, or activation of pulmonary nerves that in turn modulate autonovmic tone (Brook et al., 2010).

Solvents Industrial solvents can exert adverse effects on the heart directly or indirectly; both are related to their inherent lipophilicity. Solvents may affect cardiac physiological functions such as contraction and energy production by directly dispersing into plasma membranes. However, the effects of solvents on the heart would be more related to their actions on the neurohormonal regulation of cardiac function. Solvents may disrupt sympathetic and parasympathetic control of the heart as well as cause release of circulating hormones such as catecholamines, vasopressin, and serotonin, which in turn affects cardiac function.

Alcohols and Aldehydes On a molar basis, there is a relationship between increased carbon chain length of the alcohol and cardiotoxicity. Metabolic oxidation of alcohols yields aldehydes. Aldehydes have sympathomimetic activity as a result of their effect on releasing catecholamines. Unlike alcohols, the sympathomimetic activity of aldehydes decreases with increased chain length. The acute cardiodepressant effects of alcohols and aldehydes may be related to inhibition of intracellular Ca^{2+} transport and/or generation of oxidative stress. Chronic alcoholic cardiotoxicity is more related to a long-term consumption of alcohol, referred to as ACM, which has been discussed above. The environmental exposure to alcohols is more related to acute cardiotoxicity, including a negative dromotropic effect (reduced conductivity) and a decreased threshold for ventricular fibrillation. The common industrial alcohols include methanol (methyl alcohol or wood alcohol) and isopropyl alcohol (isopropanol). Methanol is metabolized by alcohol dehydrogenase and aldehyde dehydrogenase to formaldehyde and formic acid, and often causes reduction in heart rate. Isopropanol is metabolized to acetone, and both isopropanol and its metabolite are potent CNS depressants. Acetone is metabolized to formic acid and acetic acid, which have the potential to induce mild acidosis. Tachycardia is the most prominent clinical finding of isopropanol exposure.

Halogenated Alkanes The highly lipophilic nature of halogenated alkanes allows them to cross the blood–brain barrier readily. This action, coupled with their CNS-depressant activity, makes these compounds ideally suited for anesthetics (halothane, methoxyfluorane, and enflurane). Halogenated hydrocarbons depress heart rate, contractility, and conduction. In addition, some of these agents sensitize the heart to the arrhythmogenic effects of β-adrenergic receptor agonists such as endogenous epinephrine. Fluorocarbons (freons) have been reported to have this sensitizing effect on the myocardium. Chronic exposure to halogenated hydrocarbons may cause myocardial degenerative response.

Metals and Metalloids The most common heavy metals that have been associated with cardiotoxicity are cadmium, lead, and cobalt. These metals exhibit negative inotropic and dromotropic effects and can also produce structural changes in the heart. Chronic exposure to cadmium has been reported to cause cardiac hypertrophy. Lead has an arrhythmogenic sensitizing effect on the myocardium. In addition, lead has been reported to cause degenerative changes in the heart. Cobalt has been reported to cause cardiomyopathy. The cardiotoxic effects of heavy metals are attributed to their ability to form complexes with intracellular macromolecules and their ability to antagonize intracellular Ca^{2+}.

Other metals that have been reported to affect cardiac function are manganese, nickel, and lanthanum. Their mechanism of action appears to block Ca^{2+} channels. However, high concentrations are required to block Ca^{2+} channels (e.g., millimolar range). Barium is another metal that can affect cardiac function. Barium chloride given intravenously in high doses to laboratory animals has been reported to induce arrhythmias. This arrhythmogenic effect of barium chloride has been utilized to screen antiarrhythmic agents.

Arsenic is a metalloid, which has been shown to cause cardiotoxicity directly. Arsenic has a high affinity for sulfhydryl proteins, which are involved in multiple cellular metabolism and function. Besides environmental exposure to arsenic such as through drinking water contamination, therapeutic use of arsenic in the form of arsenic trioxide for the treatment of acute promyelocytic leukemia (APL) is another important route of exposure. Clinical reports have shown serious ventricular tachycardia at the therapeutic doses of arsenic trioxide in APL patients. Experimental studies using a dose regiment that has been shown to produce plasma concentrations of arsenic within the range of those present in arsenic-treated APL patients have shown that arsenic causes myocardial cell death through apoptosis, and results in decreased systolic and diastolic function of the heart (Li et al., 2002).

Organophosphates Intentional poisoning with organophosphate pesticides such as malathion, chlorpyrifos, and dichlorvos, remains a significant problem in many parts of the world, including Southeast Asia. While acute effects largely relate to respiratory failure, bradycardia and hypotension are important contributors to lethality. Furthermore, survivors often face long-term cardiovascular consequences, including predisposition to arrhythmia, coronary artery disease, and heart failure (Hung et al., 2015). Numerous experimental and case studies indicate electrocardiographic abnormalities arising following poisoning, including QT prolongation and slowing of conductance (AV nodal and bundle branch blockade).

OVERVIEW OF VASCULAR SYSTEM

Vascular Physiology and Structural Features

The vascular system consists of blood vessels of varying size and different cellular composition. Blood vessels can be divided into arterial, venous, and capillary systems. In addition, the lymphatic

system belongs to the vascular system, but it only carries extracellular fluid, or lymph. Although blood is the content of the vascular system and changes in the status of blood and circulation are associated with the functional alteration and toxicity of the vascular system, the blood is not included in this chapter because Chap. 11 specifically addresses the toxic responses of the blood. The main function of the vascular system is to provide oxygen and nutrients to and remove carbon dioxide and metabolic by-products from organ systems (Fig. 18-18). In addition, the vascular system is a conduit that delivers hormones and cytokines to target organs. The vascular system also has regulatory functions to manipulate organ system responses under certain toxicological conditions. For instance, endothelins produced by vascular endothelial cells alter cardiac rhythm and affect myocardial contractility. Many regulatory systems control the physiological function of the vascular system, so that the changes in the vascular system reflect either local action of chemicals or disruption of the regulatory systems, or both. The distinction between primary and secondary vascular toxic responses is challenging, and often the line between toxicity and pathology is blurred. To better understand the toxic responses of the vascular system, the following presents an overview of cell biology, physiology, and regulatory mechanism of the vascular system.

Endothelial Cells All vessels of the circulatory system are lined with a single layer of endothelial cells. These cells represent a major homeostatic organ to modulate blood flow, transport of circulating factors to end-target organs, and removal of waste products. Endothelial cells are vital to restricting activation of thrombocytes, via the production of nitric oxide (NO) and prostacyclin, both of which also mediate vasodilation to increase regional blood flow. NO is a soluble gas derived from the metabolism of arginine to citrulline and NO by the enzyme endothelial NOS (eNOS). eNOS is a dimeric protein that appears to be readily downregulated (either by regulatory phosphorylation or uncoupled electrochemically) by oxidative stress and inflammation. In parallel, healthy endothelial function can be easily perturbed by a wide range of oxidative and inflammatory insults. In many cases, such endothelial dysfunction is reversible but can acutely contribute to adverse outcomes or, if chronic, can promote vascular lesion development. In addition to NO and prostacyclin, endothelial cells also produce a number of other vasodilators, such as hydrogen sulfide, epoxyeicosatrienoic acids (EETs), and hydrogen peroxide. These factors lead to smooth muscle cell hyperpolarization and are often referred to as endothelium-derived hyperpolarizing factors (EDHFs). Endothelial cells can also create a number of vasoconstrictors, including endothelin-1 and thromboxane A2, which also act on the vascular smooth muscle.

In addition to regulating local flow and perfusion of blood, the endothelium is a central player in inflammatory responses and chronic vascular pathologies (Ross, 1999). In the earliest phases of atherosclerosis, endothelial cells respond to toxins, injury, or certain pathogens by becoming activated and expressing cell surface adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These adhesion molecules work in concert with chemokines to recruit leukocytes to regions of injury. During active inflammation, gaps between endothelial cells enlarge to facilitate extravasation of immune cells into the vessel wall. Over time, inflammatory cells build up in the vessel wall, contributing to the enlargement of atheromatous lesions.

Vascular Smooth Muscle Cells Arteries and veins are surrounded by vascular smooth muscle. The thickness of the smooth muscle layer is greater in arteries than veins, and also is lower in arterioles and venules, and absent in capillaries, except as a sphincter mechanism at the opening of capillaries to control the passage of erythrocytes. Smooth muscle controls the gauge, or diameter, of the vessels by constriction and dilation, primarily via modulation of cytosolic calcium levels. Much of the control of vascular smooth muscle contraction and relaxation arises from the endothelial cell lining. EDHFs and depolarizing factors, like endothelin-1, can alter the cytosolic levels of calcium, thereby affecting the activity of the actin-myosin contractile apparatus. Smooth muscle can also be sensitized to calcium, in a manner that makes them hypercontractile (Uehata et al., 1997). Under hypertensive, oxidative, and/or inflammatory conditions, smooth muscle cells can undergo hypertrophy and hyperplasia, leading to thickening of the vessel wall or growth of atheromatous lesions. Additionally, smooth muscle cells can differentiate from a contractile to a synthetic phenotype, which further promotes inflammation of the vascular wall.

Arterial System and Physiological Function The arterial system is composed of the aorta, major arteries, and small arterioles. The aorta and major arteries are thick-walled structures with vascular smooth muscle, elastic, and connective tissues (Fig. 18-19). Blood flow within the arterial system is initiated by contraction of the heart and begins at the ascending aorta. The ascending aorta receives all of the output of the heart with the exception of the coronary blood flow. Blood is distributed to the organ systems of the body through the major arteries that branch from the aorta. All these arteries further branch to give rise to smaller arteries and become arterioles that connect to capillaries for the delivery of oxygen and nutrients to target tissues.

Aorta and Large Arteries The aorta and the large arteries provide supporting structures and maintain the blood flow from the heart to the target organs. Therefore, these vessels function primarily as

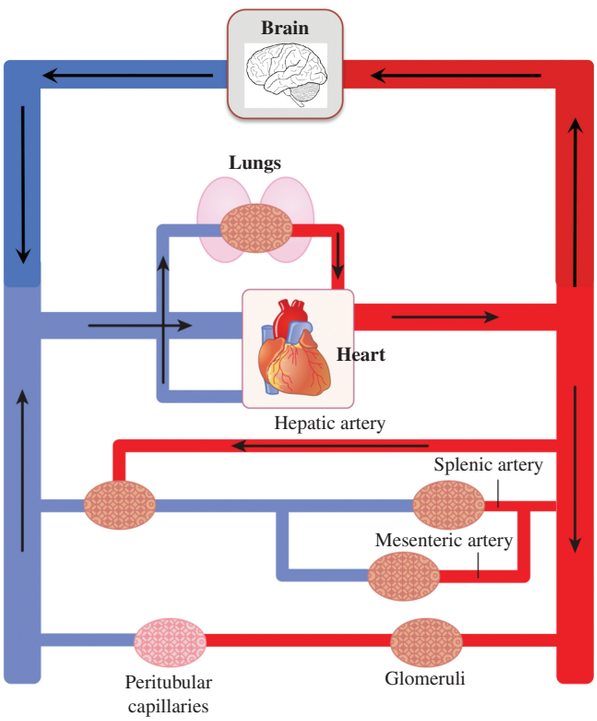

Figure 18-18. *Schematic diagram of vascular supply to selected organs.* The capillary beds represented by a meshwork connecting the arteries (right) with the veins (left); the distribution of the vasculature in several organs (liver, kidney, and lung) indicates the importance of the vascular system in toxicology.

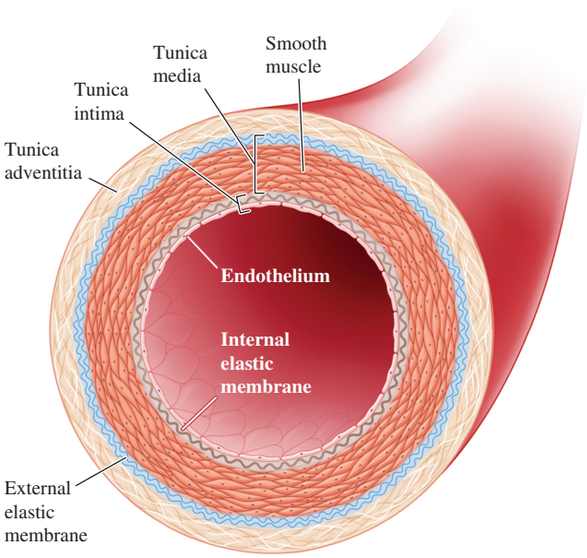

Figure 18-19. *Cross-sectional representation of the vascular wall of large and medium-size blood vessels.* The tunica intima is composed of endothelial cells, facing the vessel lumen, which rest on a thin basal lamina. The tunica media consists mainly of vascular smooth muscle cells interwoven with collagen and elastin. The tunica adventia is a layer of fibroblasts, collagen, elastin, and glycosaminoglycans.

conduits. The driving force for the blood flow is derived from the contraction of the heart and the elasticity of the aorta and large arteries under the regulation of the neurohormonal system and the resistance of the target organs. Chemicals can affect directly the arterial systems leading to alterations in blood flow or indirectly act through the regulatory system to change the blood flow.

Arterioles and Vascular Resistance The arterioles are composed of a tube of endothelial cells, surrounded by connective tissue basement membrane, a single or double layer of vascular smooth muscle cells, and a thin outer adventitial layer. The vascular smooth muscle cells are critical for the regulation of vascular resistance. The tension developed by the vascular smooth muscle cells alters the vessel diameter. Therefore, arterioles are primarily responsible for regulation of peripheral vascular resistance and blood flow. Chemicals affecting the structure and function of the vascular smooth muscle cells can thus change the physiological regulation of the vascular resistance, leading to excess dilation or restriction.

Capillaries and Microcirculation Capillaries directly connect to the distal portion of the arterioles serving as the communication site between blood and tissues, and constitute the major part of the microcirculation where nutrients, water, gases, hormones, cytokines, and waste products are exchanged between blood and tissues. In other parts of the vascular system, blood is separated from tissues by the walls of arterial and venous vessels. In contrast, capillaries are only one cell-layer thick. The passage of molecules through the capillary wall can occur both between and through the endothelial cells. Lipid-soluble molecules such as oxygen and carbon dioxide readily pass through the endothelial cell membranes. Water-soluble molecules diffuse between endothelial cells. The other pathway for water-soluble molecules is the cell membrane vesicle formation via pinocytosis (inward) and exocytosis (outward) on both the luminal and the tissue side of the capillary wall. This pathway can transfer even the largest molecules. The endothelial cells are targets of xenobiotics, whose effects can be measured by accumulation of toxic metabolites in the target organs and the pathophysiological changes in the microcirculation.

In general, there is some capillary reserve in tissues so that a particular area of a tissue can be perfused by more than one capillary, but not all of the capillaries open up at any given time. This capillary reserve serves two important functions: one is to meet the increase in metabolic demands. Under the condition that metabolic demands increase, more capillaries can open up. The other is to compensate for lesions or disruption of certain capillaries. For instance, if an occlusion occurs due to a thrombus, capillaries in close proximity can open to provide perfusion to the vulnerable area. However, in the brain, this capillary reserve appears absent so that any occlusion may result in damage.

Venous System and Physiological Function The venous system is composed of venules, veins, and vena cava. The blood flow in the venous system starts from the thin-walled venules that collect the blood from the target tissues of the body. These vessels have a relatively large surface area facilitating the reabsorption of filtered plasma from the tissue. The venules merge to veins and eventually drain into the vena cava, returning blood to the heart. Therefore, the important physiological function of the venous system is collecting blood from organ systems of the body and returning the blood to the heart. The other important function of the venous system is the capacitance of the circulatory system. This is an important protective mechanism of the circulatory system. The large veins contain vascular smooth muscle cells that can increase the blood return to the heart by constriction and increase the capacitance by dilation. If hemorrhage occurs, the circulatory system can rapidly adjust the capacitance and maintain perfusion of the tissues. Xenobiotics can exert adverse effects on the vascular smooth muscle cells so that the capacitance function of the venous system can be compromised.

Lymphatic System and Physiological Function Lymphatic vessels are endothelial tubes within tissues. The lymphatic system begins as blind-ended lymphatic end bulbs that drain into a meshwork of interconnected lymphatic vessels. This is a low-pressure system that collects excess tissue water and plasma proteins that have not been reabsorbed by the venous system. In general, in all organ systems with the exception of the CNS, more fluid is filtered than reabsorbed by the venous system. Therefore, removal of the excess fluid as well as plasma proteins that diffuse into the interstitial spaces by the lymphatic system is essential. All the lymphatics ultimately drain into the vena cava. Therefore, the physiological function of the lymphatic system is to maintain a negative interstitial pressure by removing any excess fluid and plasma proteins. Toxic insults to the lymphatic system can lead to elevated interstitial pressures and subsequent tissue edema.

Unique Vascular Beds Quite often, vascular beds are essential to target organ toxicity. While most respiratory toxicants are due to inhalation of material, in the uncommon scenario of monocrotaline toxicity, it is the pulmonary vascular bed that induces pathological consequences. Below, three other specific vascular beds are described (coronary, cerebral, and placental) as examples of how localized vascular toxicity confers harm to target organs.

Coronary Coronary arteries branch from the aorta immediately past the mitral valve. Supply of oxygen and nutrients to the heart is primarily supported by the coronary arteries. The coronary blood flow can increase about fourfold to supply additional oxygen needed by the heart muscle under demanding conditions, such as during exercise. However, even when the body is at rest, the oxygen supply through the coronary arteries to the heart is more

than the supply to an equal mass of skeletal muscle during vigorous exercise. Heart tissue extracts most of the oxygen from blood during resting conditions, and the coronary blood flow increases with the workload demands on the heart. The increase in blood flow is the major mechanism of the heart to increase energy production. Coronary vasculature disruption both anatomically and physiologically leads to reduction in cardiac blood flow and cardiac work, which is an important effect of xenobiotics. In fact, pathology of the coronary vasculature is the cause of death in about one-third of the population in industrialized societies. Recent studies indicate that particulates in the air are associated with coronary heart disease (Gordon and Reibman, 2000; Dockery, 2001).

Cerebrovasculature and the Blood–Brain Barrier The brain is uniquely protected by exceptionally tight endothelial cell gap junctions, along with a secondary barrier of pericyte cells and astrocytes. Under normal conditions, the blood–brain barrier (BBB) is permeable to water, soluble gases, and small molecule mediators, but larger circulating proteins and molecules cannot penetrate to the neural compartment. Additionally, transporter proteins such as P-glycoprotein (or multidrug resistance protein-1) act to remove larger hydrophobic molecules and prevent their uptake into the brain. Toxicants can directly or indirectly impair the BBB, permitting influx of circulating factors that initiate neuroinflammatory responses (Aragon et al., 2017).

Placenta The placenta develops during early pregnancy, in parallel with implantation of the blastocyst into the maternal endometrium, and continues to develop throughout pregnancy. The organ exists to transfer nutrients to and remove waste products from the growing fetus. As such, it is predominantly an interface of two vascular systems (maternal and fetal) and is largely populated by endothelial cells (in addition to immune cells, fibroblasts, stem cells, etc.). Limited research has been conducted on the toxicity of xenobiotics to the placental vasculature, but there is a growing appreciation that such insults may drive adverse birth outcomes.

Regulatory Mechanisms of the Vascular System

The vascular system includes conduits and microcirculation. Under physiological conditions, this system is regulated by the demands of tissue metabolism. The mechanisms controlling vascular physiology can be divided into neural, hormonal, and local controls. However, this categorization is artificial because the performance of the vascular system at any given time is the result of the integration of all three controlling mechanisms, and each of the three mechanisms affects the other two. The controlling mechanisms can be divided into remote (or systemic) and local regulation. The remote regulation includes both neural and hormonal mechanisms.

Neurohormonal Regulation Most arteries, arterioles, venules, and veins, with the exception of those of the external genitalia, receive sympathetic innervation only. Norepinephrine is the usual transmitter that binds to α_1-adrenergic receptors. These receptors are distributed to vascular smooth muscle cells, and activation of the receptors leads to contraction of the vascular smooth muscle and thus the constriction of blood vessels. There are also β_2-adrenergic receptors in vascular smooth muscle cells, to which binds the circulating epinephrine released from the adrenal medulla. Activation of the β_2-adrenergic receptors leads to vascular smooth muscle relaxation and vasodilation. Coronary and skeletal muscle arteries are highly responsive to the epinephrine-induced vasodilation. In addition, the blood vessels of skeletal muscles receive sympathetic cholinergic innervation in addition to their sympathetic adrenergic innervation, whose activation leads to vascular smooth muscle relaxation and vasodilation. The CNS regulates the activity of the autonomic nerves at several levels. Interactions between the central and autonomic nerves take place in the spinal cord, medulla, and hypothalamus. These central nervous regulations are reviewed in many physiology textbooks and not covered in this chapter.

There are many hormones that control the vascular system. Catecholamines, renin–angiotensin–aldosterone, antidiuretic hormone (ADH), and atrial natriuretic peptide (ANP) are important hormones that affect the vascular system and will be briefly reviewed.

Catecholamines Catecholamines are primarily secreted from the adrenal medulla. During the activation of the sympathetic nervous system, the adrenal medulla releases epinephrine (more than 90%) and norepinephrine (less than 10%) into the blood. As discussed above, the increase in circulating epinephrine activates the β_2-adrenergic receptors localized in the coronary and skeletal muscle arteries leading to vascular smooth muscle relaxation and vasodilation. This occurs during defense and exercise.

The Renin–Angiotensin–Aldosterone System This is critically involved in the regulation of blood pressure and volume. Renin is released from the kidney in response to reduced arterial pressure and blood volume, which catalyzes the conversion of a plasma protein angiotensinogen to angiotensin I. Angiotensin I is further converted to angiotensin II by an angiotensin-converting enzyme. Angiotensin II is a powerful arteriolar vasoconstrictor and also causes the release of aldosterone from the adrenal cortex. One of the important actions of the aldosterone is to reduce renal sodium excretion, resulting in retention of water and increase in blood volume.

ADH ADH is released from the posterior pituitary gland under the control of the hypothalamus. ADH is a vasoconstrictor but is not present in plasma in high concentrations under physiological conditions. However, ADH release is increased under the conditions of hemorrhage, decreased atrial stretch receptor firing, and increased plasma osmolarity. The effect of ADH on the vascular system is to increase the retention of water by the kidney, and thus increasing blood volume.

ANP and a related protein, B-type natriuretic peptide, are released from cardiac muscle cells when they are stimulated by stretch. ANP increases the excretion of sodium so that it decreases the blood volume. Therefore, ANP regulation of blood volume is a counter-regulatory mechanism of the renin–angiotensin–aldosterone system and ADH. BNP also causes natriuresis, as well as may cause dilation, especially of venous beds.

Local Metabolic Regulation The local regulation of the vascular system is primarily referred to as the control of microcirculation. The microcirculation is also controlled by the above neurohormonal system. This section focuses on the local metabolic regulation of microcirculation. In addition, chemicals released from endothelial cells are major local regulators of microcirculation and highly toxicologically relevant.

Oxygen is a major regulator of microcirculation. Oxygen is not stored in the end organs, and has to be replenished constantly from the blood flow. Therefore, change in metabolic rate in the end organ will require a parallel change in oxygen supply from the blood flow. The change in oxygen tension signals vascular muscles to relax or constrict. The vascular smooth muscle is not sensitive enough to respond to changes in oxygen tension except for extremely low or high oxygen tensions. However, decrease in oxygen tension along

with increases in metabolic rate causes the release of adenine nucleotides, free adenosine, and Krebs cycle intermediates. In addition, lactic acid accumulates under hypoxia conditions. All these cause vasodilation at physiological concentrations.

An important mechanism of local regulation of microcirculation is the substance released by endothelial cells, endothelium-derived relaxing factor. This substance is NO generated from arginine by NOS. The mechanism of action of the endothelium-derived relaxing factor involves the increase in cGMP and the subsequent activation of intracellular signaling pathways leading to relaxation of vascular smooth muscle cells. The endothelium-derived relaxing factor also suppresses platelet activation and reduces adhesion of leukocytes to endothelial cells.

VASCULAR SYSTEM TOXIC RESPONSES

Mechanisms of Vascular Toxicity

The effects of chemicals on the vascular system have been studied for some time, but the significance of the toxic responses of this system in affecting physiological function and pathogenesis of other organ systems is not fully understood. The potential effects of vascular toxicity on overall health and disease status are most likely underestimated. All chemicals, after absorption, contact the vascular system. Vascular endothelial cells are the immediate targets of the chemicals and are of the most frequent risk for toxic insults. These cells are the major component of the microcirculation system. Vascular smooth muscle cells are the next important targets of the chemicals.

Responses of Vascular Endothelial Cells to Toxic Insults Vascular endothelial cells play a critical role in both vascular protection from toxic insults and triggering detrimental cascade in response to toxic insults. Damage to vascular endothelial cells is a critical starting point for vascular injury. In response to toxic insults, production of NO and ROS increases in endothelial cells. Notably, NO rapidly reacts with superoxide to form peroxynitrite, which in turn can covalently modify protein tyrosine residues to form nitrotyrosine adducts. In so doing, not only is bioactive NO scavenged, but also proteins such as NOS can be rendered dysfunctional. Substances mimicking agonists activate numerous types of receptors on the endothelial cells and trigger intracellular signaling transduction, leading to activation of nuclear factor kappa-B (NF-κB) and MAPK activity. The downstream signaling transduction pathways triggered by NF-κB, MAPK, NO, and ROS then activate gene expression and regulate posttranslational modification of proteins leading to cytoprotective action against toxic insults, or the production of cytokines, chemokines, and adhesion molecules to protect the circulatory system and the affected organ systems.

Angiogenesis is an adaptive response to damages that follow toxic insults. This process helps form new blood vessels and deliver nutrients and oxygen to damaged tissue to repair the lesion. Vascular endothelial cells are both central to initiating and promoting the formation of new blood vessels and essential for blood vessel formation by forming initial tube-like structures. Xenobiotics can both promote and suppress angiogenesis, and the primary target is the vascular endothelial cell. Apoptosis is a major mechanism for cell death of the vascular endothelial cells and mechanisms and molecular signaling pathways leading to apoptosis are basically the same as described for cardiomyocytes. Until recently, it was thought that preexisting endothelial cells are the sole cellular source for angiogenesis in adults. Recent results indicate the existence of endothelial progenitor cells. The injured endothelial monolayer can be regenerated by circulating bone marrow–derived endothelial progenitor cells, which accelerates re-endothelialization and limits atherosclerotic lesion formation. These endothelial progenitor cells may be important targets of xenobiotics (O'Toole et al., 2010), although only a few studies have been conducted.

Repeated or chronic injury to endothelial cells results in an inflammatory activation, wherein endothelial cells produce chemokines and activate cytoskeletal rearrangement to permit the recruitment of leukocytes from the circulation to aid in repair (Bentzon et al., 2014). Extravasated leukocytes transform into macrophages and can accumulate in the vascular wall, causing the atherosclerotic lesion to progress. Macrophages take up fats, especially oxidized fats, and further differentiate into foam cells. The pathogenesis of atherosclerosis requires decades in humans, but endothelial activation is a crucial, ever-present component of this long-term disease. Additionally, in the setting of an existing advanced plaque, toxicants may promote a weakening of the fibrotic wall, leading to erosion (loss of endothelium) or rupture of the plaque, the result of which may be an occlusive coagulative event.

Responses of Smooth Muscle Cells to Toxic Insults The consequence of damage to vascular smooth muscle cells involves changes in the vascular tone and atherosclerosis. Receptors localized on the plasma membrane of smooth muscle cells mediate the environmental changes and change the contractility of the blood vessels. The activation of these receptors leads to signaling transduction and changes in calcium conductance. Elevation of intracellular calcium activates the contractile apparatus, leading to alteration of vascular tone. Toxic substances influence calcium homeostasis in multiple ways. Calcium homeostasis is regulated by several regulatory mechanisms as well as affects many downstream actions. Toxic targets include the calcium-binding proteins, the calcium homeostasis regulatory proteins, the calcium-activated proteins such as calcineurin, and the calcium storing and releasing process.

Proliferation and migration of medial smooth muscle cells are primarily responsible for the formation of sclerosis. Under certain circumstances, smooth muscle cells lose most of their contractility and become transformed smooth muscle cells. In some cases, this transformation is reversible. These differentiated smooth muscle cells synthesize collagen and accumulate low-density lipoproteins along with a loss in the number of myofilaments. This phenotype transformation of the smooth muscle cells occurs in atherosclerosis. Toxic substances affect the phenotype transformation of smooth muscle cells through multiple pathways. However, the growth-promoting effect is apparently a major action involved in the atherosclerotic lesion.

Oxidative Stress and Vascular Injury Many xenobiotics generate ROS during their biotransformation. Both endothelial and smooth muscle cells are capable of producing ROS and the subsequent oxidative injury (Gurtner and Burke-Wolin, 1991). The mechanism of ROS generation from xenobiotics involves enzymatic and nonenzymatic reactions. Enzymes involved in the generation of ROS in vascular cells include amine oxidase, cytochrome P450 monooxygenases, and prostaglandin synthetase. These enzymes use a diversity of substrates to produce ROS. The nonenzymatic reaction involves free iron and copper in the circulation system, which catalyze the Fenton reaction to produce ROS.

An increase in ROS in endothelial cells is related to the stimulation by angiotensin II, as well as other neurohormonal factors and cytokines. Angiotensin II binds to the AT1 receptor on the plasma membrane of endothelial cells. In response to receptor activation, cGMP production increases, leading to activation of endothelial CaM-dependent NOS and the production of NO. Stimulation of the AT1 receptor also leads to release of superoxide anion ($O_2^{\bullet -}$),

through activation of a tyrosine phosphatase pathway. The release of both NO and ($O_2^{\bullet -}$) is a major oxidative stress response of endothelial cells. The NO and ($O_2^{\bullet -}$) production and release cause damage not only to endothelial cells, but also to other cells in the vascular system.

Vascular smooth muscle cells in response to angiotensin II markedly increase the production of ($O_2^{\bullet -}$). The source of ($O_2^{\bullet -}$) in these cells likely comes from NADPH oxidase, although NADH is also indicated in the production of ($O_2^{\bullet -}$) in these cells. Xenobiotics, as discussed in the section "Vascular System Toxic Chemicals," can activate the enzyme system and lead to more extensive production of ($O_2^{\bullet -}$), or inhibit the antioxidant system including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase resulting in accumulation of ROS in the cells.

Inflammatory Lesions Inflammatory lesions of the vascular system are a common response of the vascular system. Inflammatory lesions are also called vasculitis. The pathogenesis of vasculitis has been studied both in vivo and in vitro, but the causes of many types of vasculitis are unknown. The initial injury to endothelial cells and the release of chemicals from the injured cells are responsible for the initiation of the inflammatory response, including recruitment of inflammatory cells to the injured site. Cytokines released from the activated inflammatory cells further propagate the inflammatory response leading to the eventual lesion or vasculitis.

Toxic Responses of Blood Vessels

Hypertension and Hypotension The vasculature pressure change is a major phenotype of vascular injury. Hypertension results from excessive constriction of the arterial vasculatures and/or increased resistance of the microcirculation system. However, the primary problem of sustained hypertension is an elevated vascular resistance in all organs. Once hypertension is established, it becomes a disease of the microvasculature, particularly the arteriolar microvasculature. An increased incidence of temporary or, in some cases, permanent closure of small arterioles is associated with increased resistance of the end organs. The vascular wall becomes hypertrophied, with increased smooth muscle cell growth and division arising in response to circumferential pressure as well as many neurohumoral factors that may be involved. However, the most predominant change is that all vascular smooth muscle cells are exceptionally responsive to norepinephrine. Sustained hypertension is a major complication of primary cardiovascular diseases. Complications include accelerated atherosclerosis and overload of the left ventricular muscle due to high arterial pressure. Toxic substances may directly or indirectly affect the sympathetic nervous system or alter the turnover of catecholamines in the circulation, resulting in hypertension. However, sustained hypertension by xenobiotics may involve more complicated metabolic changes in the end organs and thus changes in microcirculation also take place. For example, chemicals may enhance the renin–angiotensin system as well as renal toxicity, which may cause hypertension.

Hypotension is practically defined as the symptoms caused by low blood pressure. There are several regulatory mechanisms in the vascular system and the integration of these regulations that maintain blood flow and blood pressure within the normal range. Baroreceptors, volume receptors, chemoreceptors, and pain receptors are all involved in the integrated regulatory action to maintain adequate blood pressure. During chemical exposure, these mechanisms may be affected individually or jointly resulting in a disturbance in the integration of the regulatory mechanisms. Both transient and sustained hypotension can be produced by xenobiotics. The most common adverse effect of antihypertension drugs is hypotension. Another major cause of hypotension is hemorrhage. Alcohol overdose also causes hypotension.

Atherosclerosis The most common vascular structural injury is atherosclerosis (Ross 1999). The primary problem is the mechanical occlusion of the blood vessels such that blood flow is inadequate for the metabolic demands of the organs. Additionally, the intimal surface of atherosclerotic lesions has reduced capacity to generate antiplatelets and anticoagulant factors, providing a risk for adherent thrombi. As discussed above, activation of vascular smooth muscle cells is critically involved in atherosclerosis. Once stimulated, the vascular smooth muscle cells proliferate, differentiate into synthetic phenotype cells, and migrate to the lesion site. These synthetic cells increase the production of type I and II collagen, dermatan sulfate, proteoglycan, and stromelysins. In addition, the smooth muscle cells produce cytokines including macrophage colony-stimulating factor, TNF-α, and monocyte chemoattractant protein-1. The recruitment of inflammatory cells to the lesion site perpetuates the process of atherosclerosis.

The classic definition of atherosclerotic plaque is a combination of changes in the intima of arteries consisting of local accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue, and calcium deposits. However, this classic definition does not sufficiently describe the advanced atherosclerotic plaques that invade the media and produce bulging or enlarged arteries, cellular infiltration, and neovascularization. During the early development of atherosclerotic plaques, the lipid core has been described as the starting step of atherosclerosis. A lipid core develops and accumulates in the deep layer of the lesions before fibrous plaque formation begins. Inflammation and immune response are involved in both the early deposition of the lipid core and the late stage of fibrous accumulation. The inflammatory response includes cytokines, such as IL-6 and TNF-α. These cytokines are produced by vascular smooth muscle cells and infiltrated inflammatory cells. Many chemicals affect the oxidative metabolism of lipids in the circulatory system to trigger the formation and development of the lipid core of atherosclerosis. Toxic effects on smooth muscle cells can also initiate the formation of atherosclerotic plaques, which may be different from the lipid core–mediated atherosclerosis. In addition, macrophages and monocytes are also targets for xenobiotics and have been shown to be importantly involved in the progression of atherosclerosis.

Hemorrhage A direct mechanical injury to blood vessels causes bleeding. Chemical-induced hemorrhages are seen when damage to capillaries takes place. In addition, toxic effects on blood clotting increase the probability of hemorrhage. A classic example of chemical-induced hemorrhage is observed in snake venom poisoning. Zinc-dependent metalloproteinases are major components of snake venom and responsible for the hemorrhage. Snake venom metalloproteinases degrade various components of the basement membrane and hydrolyze endothelial cell membrane proteins, such as integrins and cadherins, involved in cell–matrix and cell–cell adhesion. These actions weaken the capillary wall and perturb the interactions between endothelial cells and the basement membrane. Thus, the transmural pressure acting on the weakened capillary wall causes distention. As a consequence, endothelial cells become very thin and eventually, the integrity of the capillary wall is lost. In addition, endothelial cells become more susceptible to blood flow–dependent shear stress, further contributing to the capillary wall disruption.

Edema The capillary exchange of fluid is bidirectional and capillaries and venules may alter the balance of hydrostatic and colloid

osmotic pressure. Filtration occurs most likely at the arteriolar end of capillaries, where filtration forces exceed absorption forces. The absorption of water occurs in the venular end of the capillary and small venules. The capillary pressure is determined by both the resistance of and the blood pressure in arterioles and venules. Xenobiotics can change the pressure gradient and cause more filtration than reabsorption of the extracellular liquid by the capillary system. In addition, more fluid is filtered than is reabsorbed by the venous system under physiological conditions. Therefore, the removal of excess fluid as well as plasma proteins that diffuse into the interstitial space is essential. This is accomplished by the lymphatic system. The lymphatics ultimately drain into the vena cava. Toxic insults to the lymphatic system can lead to elevated interstitial pressures and the subsequent tissue edema.

VASCULAR SYSTEM TOXIC CHEMICALS

Chemicals that cause vascular toxicity can also be classified into pharmaceutical chemicals, natural products, and environmental pollutants and industrial chemicals. Additionally, endogenously formed compounds can promote chronic vascular disease. Blood vessels are the target organ of these chemicals. However, some of these chemicals affect both the blood vessels and the heart. For instance, blood vessels in the heart belong to the vascular system, so that the toxicity of vascular toxic chemicals may express their toxicity in the form of cardiac toxic manifestations. Endothelial cells are major target cells of the chemicals affecting the vascular system, which are also found in the heart and make a contribution to cardiac toxicity. The same principle applies to other organ systems. Due to the distribution of vascular system in the end organs, vascular toxicity affects the organs in which the vessels are localized and is often accompanied with functional defects of the organ.

Pharmaceutical Chemicals

Vascular toxicity of pharmaceutical chemicals that are used to treat vascular disease or used to treat nonvascular disease is well known clinically. The major manifestation and mechanisms of action of selected therapeutic drugs are briefly discussed below.

Sympathomimetic Amines The sympathomimetic amines, including epinephrine, norepinephrine, dopamine, and isoproterenol, can damage the arterial vasculature by a variety of mechanisms. Large doses of norepinephrine produce toxic effects on the endothelium of the thoracic aorta of rabbits, including degenerative changes in the aortic arch in the form of increased numbers of microvilli and many focal areas of unusual endothelial cytoarchitecture. Repeated exposure to catecholamines induces atherosclerotic lesions in several animal species. Experimental data suggest that catecholamines cause the proliferative disturbances in vascular cells via α-receptors because prazosin, an α-receptor antagonist, effectively prevents the toxic response (Nakaki et al., 1990). Smooth muscle cells subjected to increased stress by diabetes, hypertension, and balloon injury are more susceptible to the effects of catecholamines. Thus, the formation of arteriosclerotic lesions in certain forms of hypertension may be initiated and/or potentiated by high levels of circulating catecholamines.

Nicotine Nicotine is an alkaloid found in various plants and mimics the actions of acetylcholine at nicotinic receptors throughout the body. At pharmacological concentrations, nicotine increases heart rate and blood pressure as a result of stimulation of sympathetic ganglia and the adrenal medulla (Marano et al., 1999). Epidemiological and experimental studies have suggested that nicotine is a causative or aggravating factor in myocardial and cerebral infarction, gangrene, and aneurysm. The effects of nicotine are related to competitive inhibition of cyclooxygenase. Recent rise in the use of electronic nicotine delivery systems, or e-cigarettes, raises many questions and concerns of potential cardiovascular toxicity of inhaled nicotine, as delivery in the absence of those combustion-related toxicants from cigarettes may lead to altered dosages or outcomes (Bhatnagar et al., 2014).

Cocaine The central actions of cocaine are to increase the circulating levels of catecholamines and cause a generalized state of vasoconstriction. Hypertension, coronary vasospasm, and cerebral strokes are common vascular complications. In pregnant women, cocaine-induced vascular changes have been associated with abortions and abruptio placentae. Studies have shown that cocaine enhances leukocyte migration across the cerebral vessel wall during inflammatory conditions. This effect is exerted through a cascade of augmented expression of inflammatory cytokines and endothelial adhesion molecules and may in fact underlie the cerebrovascular complications associated with cocaine abuse (Gan et al., 1999).

Psychotropic Drugs Trifluoperazine and chlorpromazine among the psychotropic drugs have been shown to cause intracellular cholesterol accumulation in cultured cells of the aortic intima (Iakushkin et al., 1992). Aside from the atherogenic effects, postural hypotension has been identified as the most common cardiovascular side effect of TCAs and anxiolytics such as benzodiazepine and olanzapine. Along with drugs like cocaine, nicotine, and caffeine, numerous case studies on psychotropic agents indicating acute myocardial distress (angina, infarction, heart failure) may relate to a cluster of physiological responses that misbalance oxygen supply and demand to the heart.

Antineoplastic Drugs The vasculotoxic responses elicited by antineoplastic drugs range from asymptomatic arterial lesions to thrombotic microangiopathy. Pulmonary veno-occlusive disease has been reported after the administration of various drugs, including 5-fluorouracil, doxorubicin, and mitomycin. Cyclophosphamide causes cerebrovascular and viscerovascular lesions, resulting in hemorrhages. Chronic infusions of 5-fluoro-2-deoxyuridine into the hepatic artery in dogs resulted in gastrointestinal hemorrhage and portal vein thrombosis.

Analgesics and Nonsteroidal Anti-inflammatory Drugs Aspirin can produce endothelial damage as part of a pattern of gastric erosion. Studies in rats have shown early changes in the basement membrane of endothelial cells of the capillaries and postcapillary venules, leading to obliteration of small vessels and ischemic infarcts in the large intestine. Regular use of analgesics containing phenacetin has been associated with an increased risk of hypertension and cardiovascular morbidity. NSAIDs may induce glomerular and vascular renal lesions. Certain selective cyclooxygenase 2 (COX2) inhibitors have specifically been associated with adverse vascular events, which may be due to the inhibition of prostacyclin production without the inhibition of thromboxane production typical of nonselective COX inhibitors. As such, endothelial dilation and platelet inhibition may become downregulated, enhancing the risk of occlusive events.

Oral Contraceptives Oral contraceptive steroids can produce thromboembolic disorders. Epidemiological studies have shown that oral contraceptive users have an increased risk of myocardial infarction relative to nonusers, a correlation that is markedly exacerbated by smoking, and oral contraceptive users experience an increased risk of cerebral thrombosis, hemorrhage, venous

thrombosis, and pulmonary embolism (Stolley et al., 1989). However, the mechanism by which oral contraceptives increase the risk of vascular disease is unclear, and lower doses used in contemporary formulations have reduced this concern dramatically.

Natural Products

Natural products that cause vascular toxicity include those discussed for drugs causing cardiotoxicity. In addition, many other drugs also cause vascular lesions and toxicity such as bacterial endotoxins and homocysteines, which have unique vascular toxic effects.

Bacterial Endotoxins Bacterial endotoxins are potent toxic agents to the vascular system and cause a variety of toxic effects in many vascular beds. In the liver, they cause swelling of endothelial cells and adhesion of platelets to sinusoid walls. In the lung, endotoxins produce increased vascular permeability and pulmonary hypertension. Infusion of endotoxin into experimental animals produces thickening of endothelial cells and the formation of fibrin thrombi in small veins. The terminal phase of the effects of endotoxin on the systemic vasculature results in marked hypotension. The ability of vitamin E to prevent disseminated intravascular coagulation induced by bacterial endotoxins in the rat suggests that action of these agents is somehow related to oxidative stress mechanisms.

Homocysteine Moderately elevated levels of homocysteine have been associated with atherosclerosis and venous thrombosis. Conditions including increases in the circulating homocysteine involve cardiac complications, such as hypertrophic cardiomyopathy and heart failure. Toxicity may involve oxidative injury to vascular endothelial and/or smooth muscle cells, leading to deregulation of vascular smooth muscle growth, synthesis and deposition of matrix proteins, and adverse effects on anticoagulant systems (Harpel, 1997).

Hydrazinobenzoic Acid Hydrazinobenzoic acid is a nitrogen–nitrogen bonded chemical that is present in the cultivated mushroom *Agaricus bisporus*. This hydrazine derivative causes smooth muscle cell tumors in the aorta and large arteries of mice when administered over the life span of the animals (McManus et al., 1987). These tumors have the characteristic appearance and immunocytochemical features of vascular leiomyomas and leiomyosarcomas. Smooth muscle cell lysis with vascular perforation apparently precedes malignant transformation.

T-2 Toxin Trichothecene mycotoxins, commonly classified as tetracyclic sesquiterpenes, are naturally occurring cytotoxic metabolites of *Fusarium* species. These mycotoxins, including T-2 toxin (4β,15-diacetoxy-8α-(3-methylbutyryloxy)-3α-hydroxy-12,13-epoxytrichothec-9-ene), are major contaminants of foods and animal feeds and may cause illness in animals and humans. Intravenous infusion of T-2 toxin in rats causes an initial decrease in heart rate and blood pressure, followed by tachycardia and hypertension and finally by bradycardia and hypotension (McManus et al., 1987). Acute T-2 toxin exposure causes extensive destruction of myocardial capillaries, while repeated dosing promotes thickening of large coronary arteries.

Vitamin D The toxic effects of vitamin D may be related to its structural similarity to 25-hydroxycholesterol, a potent vascular toxin. The manifestations of vitamin D hypervitaminosis include medial degeneration, calcification of the coronary arteries, and smooth muscle cell proliferation in laboratory animals.

β-Amyloid Accumulation of amyloid-β (Aβ) is a major lesion in the brain of Alzheimer's patients. Studies have shown that administration of Aβ produces extensive vascular disruption, including endothelial and smooth muscle damage, adhesion, and migration of leukocytes across arteries and venules (Thomas et al., 1997). Most importantly, the vascular actions of Aβ appear to be distinct from the neurotoxic properties of the peptide, potentially mediating its effect as a ligand for the CD36 scavenger receptor (Park et al., 2011). It appears that vascular toxicity of Aβ makes contributions to the progression of Alzheimer's dementia.

Uremic Toxins Hundreds of small molecule waste products known as uremic toxins build up in the circulation in patients with kidney disease. Among these metabolites, indoxyl sulfate, urea, cresyl sulfate, and acrolein are known to have direct toxicity to vascular endothelial cells. Indoxyl sulfate has specifically been shown to induce IL-1β inflammatory pathways and inhibit angiogenesis. Such endogenous toxins may contribute to the substantially increased risk of adverse cardiovascular outcomes in patients with chronic kidney disease.

Environmental Pollutants and Industrial Chemicals

The environmental pollutants and industrial chemicals discussed in cardiac toxicity section all have toxic effects on the vascular system. As discussed above, the cardiac effect of some of these agents and pollutants may result primarily from the vascular effect. The by-products of vascular tissue damage or the secreted substances, such as cytokines derived from vascular injury, can affect the heart either directly because of the residual of the vascular system in the heart, or indirectly through blood circulation. In this context, some of the chemicals discussed in the cardiotoxicity will not be further described. Some unique vascular toxicity will be presented.

Carbon Monoxide Carbon monoxide induces focal intimal damage and edema in laboratory animals at a concentration (180 ppm) to which humans may be exposed from environmental sources such as automobile exhaust, tobacco smoke, and fossil fuels. However, it is difficult to distinguish the direct effects of carbon monoxide from those of chemicals such as sulfur oxides, nitrogen oxides, aldehydes, and hydrocarbons on humans because most sources of carbon monoxide are complex mixtures of chemicals. Degenerative changes of myocardial arterioles have been produced experimentally in dogs forced to smoke. Similar changes have also been detected in humans who were heavy smokers and died of noncardiac causes (Wald and Howard, 1975). Tobacco smoke not only exerts a direct atherogenic effect (endothelial injury, changes in lipid profiles, and proliferation of smooth muscle cells), but also facilitates thrombosis by modulation of platelet function and vascular spasm.

Short-term exposure to carbon monoxide is associated with direct damage to vascular endothelial and smooth muscle cells. Injury to endothelial cells increases intimal permeability and allows the interaction of blood constituents with underlying components of the vascular wall. This response may account in part for the ability of carbon monoxide to induce atherosclerotic lesions in several animal species. The toxic effects of carbon monoxide have been attributed to its reversible interaction with hemoglobin. As a result of this interaction, carboxyhemoglobin decreases the oxygen-carrying capacity of blood, eventually leading to functional anemia. In addition, carbon monoxide interacts with cellular proteins such as myoglobin and cytochrome *c* oxidase and elicits a direct vasodilatory response of the coronary circulation.

Particulate Matter Recent epidemiological studies have provided a strong body of evidence that elevated levels of ambient particulate matter air pollution are associated with increased cardiovascular and respiratory morbidity and mortality. The pathways mediating such effects are complex and not fully understood. Available clinical and experimental evidence lend support to the vascular toxicity of inhaled particles, demonstrating endothelial dysfunction and promotion of atherosclerotic lesions. Importantly, such vascular lesions lead to release or secretion of cytokines and chemokines, worsening cardiac complications (Sun et al., 2005). For instance, particulate matter exposure significantly increases serum total endothelin concentrations and worsens premature ventricular complexes of the electrocardiograms that occur in the myocardial infarct rats (Kang et al., 2002). Several recent findings suggest that vascular effects of particulate inhalation may explain several seemingly noncardiovascular outcomes by impacting the vascular beds of the brain (Aragon et al., 2017) or uterus/placenta (Stapleton et al., 2015). Advances in manufacturing technology to produce and utilize nanomaterials with potentially unique toxicological properties pose additional concerns for vascular health outcomes. Particulate matter effects on the vascular system and the consequences are important health-related topics and further studies are needed to substantiate our current understanding of mechanisms for particulate matter adverse vascular effects.

Carbon Disulfide Carbon disulfide (dithiocarbonic anhydride) occurs in coal tar and crude petroleum and is commonly used in the manufacture of rayon and soil disinfectants. This chemical has been identified as an atherogenic agent in laboratory animals. The mechanism for carbon disulfide–atheroma production may involve direct injury to the endothelium coupled with hypothyroidism, because thiocarbamate (thiourea), a potent antithyroid substance, is a principal urinary metabolite of carbon disulfide. Carbon disulfide also modifies low-density lipoprotein in vitro and enhances arterial fatty deposits induced by a high-fat diet in mice (Lewis et al., 1999).

1,3-Butadiene Studies have shown that 1,3-butadiene, a chemical used in the production of styrene–butadiene, increases the incidence of cardiac hemangiosarcomas, which are tumors of endothelial origin (Miller and Boorman, 1990). Although hemangiosarcomas have also been observed in the liver, lung, and kidney, cardiac tumors are a major cause of death in animals exposed to this chemical. The toxic effects of 1,3-butadiene are dependent on its metabolic activation by cytochrome P450 to toxic epoxide metabolites. The ultimate outcomes of exposure probably are influenced by the rates of glutathione-mediated detoxification of oxidative metabolites.

Metals and Metalloids The vascular toxicity of food- and water-borne elements (selenium, chromium, copper, zinc, cadmium, lead, and mercury) as well as airborne elements (vanadium and lead) involves reactions of metals with sulfhydryl, carboxyl, or phosphate groups. Metals such as cobalt, magnesium, manganese, nickel, cadmium, and lead also interact with and block calcium channels. Intracellular calcium-binding proteins, such as CaM, are biologically relevant targets of heavy metals, including cadmium, mercury, and lead, although the contribution of this mechanism to the toxic effects of metals has been fully understood.

Cadmium Cadmium effects on the vascular system have been studied in the greatest detail. Although cadmium is not preferentially localized in blood vessels relative to other tissues, when present, cadmium is localized in the elastic lamina of large arteries, with particularly high concentrations at arterial branching points (Perry et al., 1989). A large portion of the cadmium that accumulates in the body is tightly bound to hepatic and renal MT. The low MT levels in vascular tissue may actually predispose a person to the toxic effects of cadmium (Perry et al., 1989). Long-term exposure of laboratory animals to low levels of cadmium has been associated with the development of atherosclerosis and hypertension in the absence of other toxic effects. Selenium and zinc inhibit, whereas lead potentiates the hypertensive effects of cadmium. Calcium has antagonistic effect on cadmium-induced high blood pressure. Cadmium increases sodium retention, induces vasoconstriction, increases cardiac output, and produces hyperreninemia. Any one of these mechanisms could account for the putative hypertensive effects of cadmium.

Lead Lead has been shown from epidemiological studies to be associated with essential hypertension in a large percentage of patients (Batuman et al., 1983). Elevated blood pressure has also been observed during childhood lead poisoning. The direct vasoconstrictor effect of lead may be related to the putative hypertensive response. This effect can be complemented by the ability of lead to activate the renin–angiotensin–aldosterone system. Lead also directly affects vascular endothelial and smooth muscle cells. For instance, lead inhibits the repair process in damaged endothelial cells (Fujiwara et al., 1997) and modulates spontaneous release of fibrinolytic proteins from subendothelial cells through intracellular calcium-independent pathways (Yamamoto et al., 1997). Acute lead-induced neuropathy may be due to cerebral capillary dysfunction. Inorganic lead alters arterial elasticity and causes sclerosis of renal vessels.

Mercury Mercury produces vasoconstriction of preglomerular vessels and disrupts the integrity of the blood–brain barrier. The opening of the blood–brain barrier results in extravasation of plasma protein across vascular walls into adjoining brain tissues. Mercury added to platelet-rich plasma causes a marked increase in platelet thromboxane B_2 production and platelet responsiveness to arachidonic acid.

Arsenic Arsenic poisoning causes vasodilation and capillary dilation. These actions have been associated with extravasation, transudation of plasma, and decreased intravascular volume. A severe form of arteriosclerosis, blackfoot disease, in Taiwan has been shown to be associated with high levels of arsenic in the soil and water. Blackfoot disease is an endemic peripheral vascular occlusive disease that exhibits arteriosclerosis obliterans and thromboangiitis. The ability of arsenic to induce these changes has been attributed to its effects on vascular endothelial cells. Arsenic has been reported to cause noncirrhotic portal hypertension in humans.

Aromatic Hydrocarbons Aromatic hydrocarbons, including polycyclic aromatic hydrocarbons and polychlorinated dibenzo-*p*-dioxins, are persistent toxic environmental contaminants. Aromatic hydrocarbons have been identified as vascular toxicants that can initiate and/or promote the atherogenic process in experimental animals (Ou and Ramos, 1992). The atherogenic effect is associated with cytochrome P450–mediated conversion of the parent compound to toxic metabolic intermediates, but aromatic hydrocarbons can also initiate the atherogenic process. However, studies have also shown that treatment with several polycyclic hydrocarbons increases the size but not the frequency of atherosclerotic lesions (Albert et al., 1977; Penn and Snyder, 1988), suggesting that polycyclic aromatic hydrocarbons act as promoters of the atherosclerotic process. Although additional studies are required to define the "initiating" versus "promotional" actions of polycyclic aromatic hydrocarbons, their ability to readily associate with plasma lipoproteins may play a critical role in vascular toxicity.

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REFERENCES

Abas L, Bogoyevitch MA, Guppy M. Mitochondrial ATP production is necessary for activation of the extracellular-signal-regulated kinases during ischaemia/reperfusion in rat myocyte-derived H9c2 cells. *Biochem J*. 2000;349:119–126.

Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science*. 1998;281:1322–1326.

Adams JW, Sakata Y, Davis MG, et al. Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. *Proc Natl Acad Sci U S A*. 1998;95:10140–10145.

Adams MA, Hirst M. Ethanol-induced cardiac hypertrophy: correlation between development and the excretion of adrenal catecholamines. *Pharmacol Biochem Behav*. 1986;24:33–38.

Ahmed SS, Regan TJ. Cardiotoxicity of acute and chronic ingestion of various alcohols. In: Acosta D, ed. *Cardiovascular Toxicology*. 2nd ed. New York: Raven Press; 1992:345–407.

Akao M, Ohler A, O'Rourke B, et al. Mitochondrial ATP-sensitive potassium channels inhibit apoptosis induced by oxidative stress in cardiac cells. *Circ Res*. 2001;88:1267–1275.

Akhter SA, Luttrell LM, Rockman HA, et al. Targeting the receptor–Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science*. 1998;280:574–577.

Albert RE, Vanderlaan M, Burns FJ, et al. Effect of carcinogens on chicken atherosclerosis. *Cancer Res*. 1977;37:2232–2235.

Andrieu-Abadie N, Jaffrezou JP, Hatem S, et al. L-Carnitine prevents doxorubicin-induced apoptosis of cardiac myocytes: role of inhibition of ceramide generation. *FASEB J*. 1999;13:1501–1510.

Anversa P. Myocyte death in the pathological heart. *Circ Res*. 2000;86(2):121–124.

Anversa P, Leri A, Kajstura J. Cardiac regeneration. *J Am Coll Cardiol*. 2006;47:1769–1776.

Arad M, Moskowitz IP, Patel VV, et al. Transgenic mice overexpressing mutant PRKAG2 define the cause of Wolff–Parkinson–White syndrome in glycogen storage cardiomyopathy. *Circulation*. 2003;107:2850–2856.

Aragon MJ, Topper L, Tyler CR, et al. Serum-borne bioactivity caused by pulmonary multiwalled carbon nanotubes induces neuroinflammation via blood-brain barrier impairment. *Proc Natl Acad Sci U S A*. 2017;114:E1968–E1976.

Arellano FM. The withdrawal of rofecoxib. *Pharmacoepidemiol Drug Saf*. 2005;14:213–217.

Arola OJ, Saraste A, Pulkki K, et al. Acute doxorubicin cardiotoxicity involves cardiomyocyte apoptosis. *Cancer Res*. 2000;60:1789–1792.

Arstall MA, Sawyer DB, Fukazawa R, et al. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circ Res*. 1999;85:829–840.

Atkison P, Joubert G, Barron A, et al. Hypertrophic cardiomyopathy associated with tacrolimus in paediatric transplant patients. *Lancet*. 1995;345:894–896.

Baldessarini RJ. Drugs and the treatment of psychiatric disorders. In: Hardman JG, Limbird LE, Molinoff PB, et al., eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 9th ed. New York: McGraw-Hill; 1996:399–430.

Batuman V, Landy E, Maesaka JK, et al. Contribution of lead to hypertension with renal impairment. *N Engl J Med*. 1983;309:17–21.

Behrendt H, Boffin H. Myocardial cell lesions caused by an anabolic hormone. *Cell Tissue Res*. 1977;181:423–426.

Bennett PB, Yazawa K, Makita N, et al. Molecular mechanism for an inherited cardiac arrhythmia. *Nature*. 1995;376:683–685.

Bentzon JF, Otsuka F, Virmani R, et al. Mechanisms of plaque formation and rupture. *Circ Res*. 2014;114:1852–1866.

Bergmann O, Bhardwaj RD, Bernard S, et al. Evidence for cardiomyocyte renewal in humans. *Science*. 2009;324:98–102.

Berne RM, Levy MN. *Physiology*. St. Louis, MO: Mosby; 1983.

Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol*. 2008;70:23–49.

Bhatnagar A, Whitsel LP, Ribisl KM, et al.; American Heart Association Advocacy Coordinating Committee, Council on Cardiovascular and Stroke Nursing, Council on Clinical Cardiology, and Council on Quality of Care and Outcomes Research. Electronic cigarettes: a policy statement from the American Heart Association. *Circulation*. 2014;130:1418–1436.

Bisognano JD, Weinberger HD, Bohlmeyer TJ, et al. Myocardial-directed overexpression of the human beta(1)-adrenergic receptor in transgenic mice. *J Mol Cell Cardiol*. 2000;32:817–830.

Bittl JA, Ingwall JS. Reaction rates of creatine kinase and ATP synthesis in the isolated rat heart: A 31P NMR Magnetization Transfer Study. *J Biol Chem*. 1985;260:3512–3517.

Black BL, Olson EN. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol*. 1998;14:167–196.

Bogden JD, Al Rabiai S, Gilani SH. Effect of chronic ethanol ingestion on the metabolism of copper, iron, manganese, selenium, and zinc in an animal model of alcoholic cardiomyopathy. *J Toxicol Environ Health*. 1984;14:407–417.

Bosnjak ZJ. Cardiac effects of anesthetics. In: Blank TJJ, Wheeler DM, eds. *Mechanisms of Anesthetic Action in Skeletal, Cardiac and Smooth Muscle*. New York: Plenum Press; 1991:91–96.

Bozkurt B. Cardiovascular toxicity with highly active antiretroviral therapy: review of clinical studies. *Cardiovas Toxicol*. 2004;4:243–260.

Brand T, Sharma HS, Fleischmann KE, et al. Proto-oncogene expression in porcine myocardium subjected to ischemia and reperfusion. *Circ Res*. 1992;71:1351–1360.

Brook RD, Rajagopalan S, Pope CA III, et al. Particulate matter air pollution and cardiovascular disease: an update to the scientific statement from the American Heart Association. *Circulation*. 2010;121:2331–2378.

Buck ED, Lachnit WG, Pessah IN. Mechanisms of delta-hexachlorocyclohexane toxicity: I. Relationship between altered ventricular myocyte contractility and ryanodine receptor function. *J Pharmacol Exp Ther*. 1999;289:477–485.

Catterall W, Mackie K. Local anesthetics. In: Hardman JG, Limbird LE, Molinoff PB, et al., eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 9th ed. New York: McGraw-Hill; 1996:331–347.

Chacon E, Acosta D. Mitochondrial regulation of superoxide by Ca^{2+}: an alternate mechanism for the cardiotoxicity of doxorubicin. *Toxicol Appl Pharmacol*. 1991;107:117–128.

Cheng TH, Shih NL, Chen SY, et al. Reactive oxygen species modulate endothelin-I-induced *c*-fos gene expression in cardiomyocytes. *Cardiovasc Res*. 1999a;41:654–662.

Cheng W, Li B, Kajstura J, et al. Stretch-induced programmed myocyte cell death. *J Clin Invest*. 1995;96:2247–2259.

Cheng XS, Shimokawa H, Momii H, et al. Role of superoxide anion in the pathogenesis of cytokine-induced myocardial dysfunction in dogs in vivo. *Cardiovasc Res*. 1999b;42:651–659.

Cook SA, Sugden PH, Clerk A. Regulation of Bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential. *Circ Res*. 1999;85:940–949.

Dalakas MC, Illa I, Pezeshkpour GH, et al. Mitochondrial myopathy caused by long-term zidovudine therapy. *N Engl J Med*. 1990;322:1098–1105.

De Simone G. Left ventricular geometry and hypotension in end-stage renal disease: a mechanical perspective. *J Am Soc Nephrol*. 2003;14:2421–2427.

Delaughter MC, Taffet GE, Fiorotto ML, et al. Local insulin-like growth factor I expression induces physiologic, then pathologic, cardiac hypertrophy in transgenic mice. *FASEB J*. 1999;13:1923–1929.

Depre C, Taegtmeyer H. Metabolic aspects of programmed cell survival and cell death in the heart. *Cardiovasc Res*. 2000;45:538–548.

Dhalla NS, Yates JC, Maimark B, et al. Cardiotoxicity of catecholamines and related agents. In Acosta D, ed. *Cardiovascular Toxicology*. 2nd ed. New York: Raven Press; 1992:239–282.

Diamond MI, Miner JN, Yoshinaga SK, et al. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science*. 1990;249:1266–1272.

Didenko VV, Tunstead JR, Hornsby PJ. Biotin-labeled hairpin oligonucleotides: probes to detect double-strand breaks in DNA in apoptotic cells. *Am J Pathol*. 1998;152:897–902.

Dockery DW. Epidemiologic evidence of cardiovascular effects of particulate air pollution. *Environ Health Perspect*. 2001;109:483–486.

Eguchi Y, Shimizu S, Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res*. 1997;57:1835–1840.

El-Sherif L, Wang GW, Kang YJ. Suppression of cadmium-induced apoptosis in metallothionein-overexpressing transgenic mouse cardiac myocytes. *FASEB J*. 2000;14:1193.

Eliot RS, Clayton FC, Pieper GM, et al. Influence of environmental stress on pathogenesis of sudden cardiac death. *Fed Proc*. 1977;36:1719–1724.

Esposito G, Rapacciuolo A, Naga Prasad SV, et al. Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. *Circulation*. 2002;105:85–92.

Eura Y, Ishihara N, Yokota S, Mihara K. Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. *J Biochem*. 2003;134:333–344.

Evans T, Reitman M, Felsenfeld G. An erythrocyte-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. *Proc Natl Acad Sci U S A*. 1988;85:5976–5980.

Evens T. Regulation of cardiac gene expression by GATA-4/5/6. *Trends Cardiovasc Med*. 1997;7:75–83.

Fauchier L, Babuty D, Poret P, et al. Comparison of long-term outcome of alcoholic and idiopathic dilated cardiomyopathy. *Eur Heart J*. 2000;21:306–314.

Fitzgerald GA. Coxibs and cardiovascular disease. *N Engl J Med*. 2004;351:1709–1711.

Fosslien E. Mitochondrial medicine—molecular pathology of defective oxidative phosphorylation. *Ann Clin Lab Sci*. 2001;31:25–67.

Francis GS, Chu C. Compensatory and maladaptive responses to cardiac dysfunction. *Curr Opin Cardiol*. 1995;10:260–267.

Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol*. 2003;65:45–79.

Frustaci A, Kajstura J, Chimenti C, et al. Myocardial cell death in human diabetes. *Circ Res*. 2000;87:1123–1132.

Fujiwara Y, Kaji T, Sakurai S, et al. Inhibitory effect of lead on the repair of wounded monolayers of cultured vascular endothelial cells. *Toxicology*. 1997;117:193–198.

Galan L, Talavera K, Vassort G, et al. Characteristics of Ca^{2+} channel blockade by oxodipine and elgodipine in rat cardiomyocytes. *Eur J Pharmacol*. 1998;357:93–105.

Gan X, Zhang L, Berger O, et al. Cocaine enhances brain endothelial adhesion molecules and leukocyte migration. *Clin Immunol*. 1999;91:68–76.

Gandre-Babbe S, van der Bliek AM. The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. *Mol Biol Cell*. 2008;19:2402–2412.

Gavazzi A, De Maria R, Parolini M, et al. Alcohol abuse and dilated cardiomyopathy in men. *Am J Cardiol*. 2000;85:1114–1118.

Goldsmith S, From AH. Arsenic-induced atypical ventricular tachycardia. *N Engl J Med*. 1980;303:1096–1098.

Gordon T, Reibman J. Cardiovascular toxicity of inhaled ambient particulate matter. *Toxicol Sci*. 2000;56:2–4.

Gottlieb RA, Burleson KO, Kloner RA, et al. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest*. 1994;94:1621–1628.

Gotzsche LS, Pedersen EM. Dose-dependent cardiotoxic effect of amiodarone in cardioplegic solutions correlates with loss of dihydropyridine binding sites: in vitro evidence for a potentially lethal interaction with procaine. *J Cardiovasc Pharmacol*. 1994;23:13–23.

Guenoun T, Montagne O, Laplace M, et al. Propofol-induced modifications of cardiomyocyte calcium transient and sarcoplasmic reticulum function in rats. *Anesthesiology*. 2000;92:542–549.

Guerra S, Leri A, Wang X, et al. Myocyte death in the failing human heart is gender dependent. *Circ Res*. 1999;85:856–866.

Gurley BJ, Gardner SF, White LM, et al. Ephedrine pharmacokinetics after the ingestion of nutritional supplements containing *Ephedra Sinica* (Ma Huang). *Ther Drug Monit*. 1998;20:439–445.

Gurtner GH, Burke-Wolin T. Interactions of oxidant stress and vascular reactivity. *Am J Physiol*. 1991;260:L207–L211.

Han H, Zhu J, Zhu Z, et al. *p*-Cresyl sulfate aggravates cardiac dysfunction associated with chronic kidney disease by enhancing apoptosis of cardiomyocytes. *J Am Heart Assoc*. 2015;4:e001852.

Harpel PC. Homocysteine, atherogenesis and thrombosis. *Fibrinolysis Proteolysis*. 1997;11:77–80.

Haunstetter A, Izumo S. Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circ Res*. 1998;82:1111–1129.

Havlin KA. Cardiotoxicity of anthracyclines and other antineoplastic agents. In: Acosta D, ed. *Cardiovascular Toxicology*. 2nd ed. New York: Raven Press; 1992:143–164.

Hayakawa K, Takemura G, Koda M, et al. Sensitivity to apoptosis signal, clearance rate, and ultrastructure of Fas ligand-induced apoptosis in in vivo adult cardiac cells. *Circulation*. 2002;105:3039–3045.

He SY, Matoba R, Sodesaki K, et al. Morphological and morphometric investigation of cardiac lesions after chronic administration of methamphetamine in rats. *Nippon Hoigaku Zasshi*. 1996;50:63–71.

Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006;7:589–600.

Hill JA, Karimi M, Kutschke W, et al. Cardiac hypertrophy is not a required compensatory response to short-term pressure overload. *Circulation*. 2000;101:2863–2869.

Hino N, Ochi R, Yanagisawa T. Inhibition of the slow inward current and the time-dependent outward current of mammalian ventricular muscle by gentamicin. *Pflugers Arch*. 1982;394:243–249.

Ho PD, Zechner DK, He H, et al. The Raf-MEK-ERK Cascade represents a common pathway for alteration of intracellular calcium by Ras and protein kinase C in cardiac myocytes. *J Biol Chem*. 1998;273:21730–21735.

Holtz J. Pathophysiology of heart failure and the renin–angiotensin–system. *Basic Res Cardiol*. 1993;88:183–201.

Hu W, Lu S, McAlpine I, et al. Mechanistic investigation of imatinib-induced cardiac toxicity and the involvement of c-Abl kinase. *Toxicol Sci*. 2012;129:188–199.

Hung DZ, Yang HJ, Li YF, et al. The long-term effects of organophosphates poisoning as a risk factor of CVDs: a nationwide population-based cohort study. *PLoS One*. 2015;10:e0137632.

Huss JM, Kelly DP. Mitochondrial energy metabolism in heart failure: a question of balance. *J Clin Invest*. 2005;115:547–555.

Hussar DA. New drugs of 1998. *J Am Pharm Assoc (Wash)*. 1999;39:151–206.

Hussar DA. New drugs of 1999. *J Am Pharm Assoc (Wash)*. 2000;40:181–221.

Iakushkin VV, Baldenkov GN, Tertov VV, et al. Atherogenic properties of phenothiazine drugs manifesting in cultured cells of the human aortic intima. *Kardiologiia*. 1992;32:66–68.

Ingwall JS, Kramer MF, Fifer MA, et al. The creatine kinase system in normal and diseased human myocardium. *N Engl J Med*. 1985;313:1050–1054.

Ingwall JS, Weiss RG. Is the failing heart energy starved? On using chemical energy to support cardiac function. *Circ Res*. 2004;95:135–145.

Jalili T, Takeishi Y, Walsh RA. Signal transduction during cardiac hypertrophy: the role of G alpha Q, PLC Beta I, and PKC. *Cardiovasc Res*. 1999;44:5–9.

James DI, Parone PA, Mattenberger Y, Martinou JC. hFis1, a novel component of the mammalian mitochondrial fission machinery. *J Biol Chem*. 2003;278:36373–36379.

James TN. Normal and abnormal consequences of apoptosis in the human heart. From postnatal morphogenesis to paroxysmal arrhythmias. *Circulation*. 1994;90:556–573.

Jennings RB. Historical perspective on the pathology of myocardial ischemia/reperfusion injury. *Circ Res*. 2013;113:428–438.

Joshi A, Dimino T, Vohra Y, et al. Preclinical strategies to assess QT liability and torsadogenic potential of new drugs: the role of experimental models. *J Electrocardiol*. 2004;37:7–14.

Jurgensmeier JM, Xie Z, Deveraux Q, et al. Bax directly induces release of cytochrome *c* from isolated mitochondria. *Proc Natl Acad Sci U S A*. 1998;95:4997–5002.

Kaasik A, Minajeva A, Paju K, et al. Thyroid hormones differentially affect sarcoplasmic reticulum function in rat atria and ventricles. *Mol Cell Biochem*. 1997;176:119–126.

Kagi JH. Overview of metallothionein. *Methods Enzymol*. 1991;205:401–414.

Kajstura J, Cheng W, Reiss K, et al. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest*. 1996;74:86–107.

Kalin MF, Zumoff B. Sex hormones and coronary disease: a review of the clinical studies. *Steroids*. 1990;55:330–352.

Kang YJ. The antioxidant function of metallothionein in the heart. *Proc Soc Exp Biol Med*. 1999;222:263–273.

Kang YJ. Molecular and cellular mechanisms of cardiotoxicity. *Environ Health Perspect*. 2001;109:27–34.

Kang YJ. Metallothionein redox cycle and function. *Exp Biol Med (Maywood)*. 2006;231:1459–1467.

Kang YJ, Li Y, Zhou Z, et al. Elevation of serum endothelins and cardiotoxicity induced by particulate matter (PM 2.5) in rats with acute myocardial infarction. *Cardiovasc Toxicol*. 2002;2:253–261.

Kang YJ, Zhou ZX, Wang GW, et al. Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of P38 mitogen-activated protein kinases. *J Biol Chem*. 2000a;275:13690–13698.

Kang YJ, Zhou ZX, Wu H, et al. Metallothionein inhibits myocardial apoptosis in copper-deficient mice: role of atrial natriuretic peptide. *Lab Invest*. 2000b;80:745–757.

Kannan M, Wang L, Kang YJ. Myocardial oxidative stress and toxicity induced by acute ethanol exposure in mice. *Exp Biol Med (Maywood)*. 2004;229:553–559.

Kass DA, Saavedra WF, Sabbah HN. Reverse remodeling and enhanced inotropic reserve from the cardiac support device in experimental cardiac failure. *J Card Fail*. 2004;10:S215–S219.

Klatsky AL. Alcohol and cardiovascular diseases: a historical overview. *Novartis Found Symp*. 1998;216:2–12.

Koenig H, Fan CC, Goldstone AD, et al. Polyamines mediate androgenic stimulation of calcium fluxes and membrane transport in rat heart myocytes. *Circ Res*. 1989;64:415–426.

Koshiba T, Detmer SA, Kaiser JT, Chen H, McCaffery JM, Chan DC. Structural basis of mitochondrial tethering by mitofusin complexes. *Science*. 2004;305:858–862.

Kroemer G, Zamzami N, Susin SA. Mitochondrial control of apoptosis. *Immunol Today*. 1997;18:44–51.

Krown KA, Page MT, Nguyen C, et al. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *J Clin Invest*. 1996;98:2854–2865.

Kubota T, Miyagishima M, Frye CS, et al. Overexpression of tumor necrosis factor-alpha activates both anti- and pro-apoptotic pathways in the myocardium. *J Mol Cell Cardiol*. 2001;33:1331–1344.

Kurrelmeyer KM, Michael LH, Baumgarten G, et al. Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. *Proc Natl Acad Sci U S A*. 2000;97:5456–5461.

Lazarevic AM, Nakatani S, Neskovic AN, et al. Early changes in left ventricular function in chronic asymptomatic alcoholics: relation to the duration of heavy drinking. *J Am Coll Cardiol*. 2000;35:1599–1606.

Lee JA, Allen DG. Calcium sensitisers: mechanisms of action and potential usefulness as inotropes. *Cardiovasc Res*. 1997;36:10–20.

Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Mol Biol Cell*. 2004;15:5001–5011.

Leist M, Single B, Castoldi AF, et al. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med*. 1997;185:1481–1486.

Leist M, Single B, Naumann H, et al. Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. *Exp Cell Res*. 1999;249:396–403.

Lenczowski JM, Dominguez L, Eder AM, et al. Lack of a role for jun kinase and AP-1 in Fas-induced apoptosis. *Mol Cell Biol*. 1997;17:170–181.

Leri A, Franco S, Zacheo A, et al. Ablation of telomerase and telomere loss leads to cardiac dilatation and heart failure associated with p53 upregulation. *EMBO J*. 2003;22:131–139.

Levine ES, Friedman HS, Griffith OW, et al. Cardiac cell toxicity induced by 4-hydroperoxycyclophosphamide is modulated by glutathione. *Cardiovasc Res*. 1993;27:1248–1253.

Lewis JG, Graham DG, Valentine WM, et al. Exposure of C57BL/6 mice to carbon disulfide induces early lesions of atherosclerosis and enhances arterial fatty deposits induced by a high fat diet. *Toxicol Sci*. 1999;49:124–132.

Lewis W, Grupp IL, Grupp G, et al. Cardiac dysfunction occurs in the HIV-1 transgenic mouse treated with zidovudine. *Lab Invest*. 2000;80:187–197.

Li F, Wang X, Capasso JM, et al. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J Mol Cell Cardiol*. 1996;28:1737–1746.

Li Y, Sun X, Wang L, et al. Myocardial toxicity of arsenic trioxide in a mouse model. *Cardiovasc Toxicol*. 2002;2:63–73.

Litwak KN, Mcmahan A, Lott KA, et al. Monensin toxicosis in the domestic bovine calf: a large animal model of cardiac dysfunction. *Contemp Top Lab Anim Sci*. 2005;44:45–49.

Liu S, Melchert RB, Kennedy RH. Inhibition of L-type Ca^{2+} channel current in rat ventricular myocytes by terfenadine. *Circ Res*. 1997;81:202–210.

Marano G, Ramirez A, Mori I, et al. Sympathectomy inhibits the vasoactive effects of nicotine in conscious rats. *Cardiovasc Res*. 1999;42:201–205.

Marcillat O, Zhang Y, Davies KJ. Oxidative and non-oxidative mechanisms in the inactivation of cardiac mitochondrial electron transport chain components by doxorubicin. *Biochem J*. 1989;259:181–189.

Maret W. The function of zinc metallothionein: a link between cellular zinc and redox state. *J Nutr*. 2000;130:1455S–1458S.

Marks AR. Intracellular calcium-release channels: regulators of cell life and death. *Am J Physiol*. 1997;272:H597–H605.

Marsh JD, Lehmann MH, Ritchie RH, et al. Androgen receptors mediate hypertrophy in cardiac myocytes. *Circulation*. 1998;98:256–261.

Marsin AS, Bertrand L, Rider MH, et al. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr Biol*. 2000;10:1247–1255.

Martin XJ, Wynne DG, Glennon PE, et al. Regulation of expression of contractile proteins with cardiac hypertrophy and failure. *Mol Cell Biochem*. 1996;157:181–189.

Mathews EC Jr, Gardin JM, Henry WL, et al. Echocardiographic abnormalities in chronic alcoholics with and without overt congestive heart failure. *Am J Cardiol*. 1981;47:570–578.

Matsui T, Li L, Wu JC, et al. Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart. *J Biol Chem*. 2002;277:22896–22901.

McClain CJ, Su LC. Zinc deficiency in the alcoholic: a review. *Alcohol Clin Exp Res*. 1983;7:5–10.

McKenna CJ, Codd MB, Mccann HA, et al. Alcohol consumption and idiopathic dilated cardiomyopathy: a case control study. *Am Heart J*. 1998;135:833–837.

McMahon SB, Monroe JG. Role of primary response genes in generating cellular responses to growth factors. *FASEB J*. 1992;6:2707–2715.

McManus BM, Toth B, Patil KD. Aortic rupture and aortic smooth muscle tumors in mice. Induction by *p*-hydrazinobenzoic acid hydrochloride of the cultivated mushroom *Agaricus bisporus*. *Lab Invest*. 1987;57:78–85.

Meeusen S, DeVay R, Block J, et al. Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. *Cell*. 2006;127:383–395.

Melchert RB, Welder AA. Cardiovascular effects of androgenic-anabolic steroids. *Med Sci Sports Exerc*. 1995;27:1252–1262.

Metzger JM, Wahr PA, Michele DE, et al. Effects of myosin heavy chain isoform switching on Ca^{2+}-activated tension development in single adult cardiac myocytes. *Circ Res*. 1999;84:1310–1317.

Miller RA, Boorman GA. Morphology of neoplastic lesions induced by 1,3-butadiene in B6C3F1 mice. *Environ Health Perspect*. 1990;86:37–48.

Molkentin JD, Lu JR, Antos CL, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. 1998;93:215–228.

Moslehi JJ, Deininger M. Tyrosine kinase inhibitor–associated cardiovascular toxicity in chronic myeloid leukemia. *J Clin Oncol*. 2015;33:4210–4218.

Nakaki T, Nakayama M, Yamamoto S, et al. Alpha 1-adrenergic stimulation and beta 2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol Pharmacol*. 1990;37:30–36.

Nattel S. Experimental evidence for proarrhythmic mechanisms of antiarrhythmic drugs. *Cardiovasc Res*. 1998;37:567–577.

Neubauer S, Horn M, Cramer M, et al. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. *Circulation*. 1997;96:2190–2196.

Neubauer S, Remkes H, Spindler M, et al. Downregulation of the Na(+)-creatine cotransporter in failing human myocardium and in experimental heart failure. *Circulation*. 1999;100:1847–1850.

Newton AC. Protein kinase C: structure, function, and regulation. *J Biol Chem*. 1995;270:28495–28498.

Oh H, Taffet GE, Youker KA, et al. Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. *Proc Natl Acad Sci U S A*. 2001;98:10308–10313

Olichon A, Emorine LJ, Descoins E, et al. The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. *FEBS Lett*. 2002;523:171–176.

Olivetti G, Abbi R, Quaini F, et al. Apoptosis in the failing human heart. *N Engl J Med*. 1997;336:1131–1141.

Opie L. Heart cells and organelles. In: Opie L, ed. *The Heart*. New York: Harcourt Brace Jovanovich; 1996:15–29.

Orkin SH. GATA-binding transcription factors in hematopoietic cells. *Blood*. 1992;80:575–581.

O'Toole TE, Hellmann J, Wheat L, et al. Episodic exposure to fine particulate air pollution decreases circulating levels of endothelial progenitor cells. *Circ Res*. 2010;107:200–203.

Ou X, Ramos KS. Modulation of aortic protein phosphorylation by benzo(*a*) pyrene: implications in PAH-induced atherogenesis. *J Biochem Toxicol*. 1992;7:147–154.

Pacher P, Ungvari Z, Kecskemeti V, et al. Review of cardiovascular effects of fluoxetine, a selective serotonin reuptake inhibitor, compared to tricyclic antidepressants. *Curr Med Chem*. 1998;5:381–390.

Paradis P, Maclellan WR, Belaguli NS, et al. Serum response factor mediates AP-1-dependent induction of the skeletal alpha-actin promoter in ventricular myocytes. *J Biol Chem*. 1996;271:10827–10833.

Park L, Wang G, Zhou P, et al. Scavenger receptor CD36 is essential for the cerebrovascular oxidative stress and neurovascular dysfunction induced by amyloid-beta. *Proc Natl Acad Sci U S A*. 2011;108:5063–5068.

Penn A, Snyder C. Arteriosclerotic plaque development is "promoted" by polynuclear aromatic hydrocarbons. *Carcinogenesis*. 1988;9:2185.

Perez NG, Gao WD, Marban E. Novel myofilament Ca^{2+}-sensitizing property of xanthine oxidase inhibitors. *Circ Res*. 1998;83:423–430.

Perry HM Jr, Erlanger MW, Gustafsson TO, et al. Reversal of cadmium-induced hypertension by D-myo-inositol-1,2,6-trisphosphate. *J Toxicol Environ Health*. 1989;28:151–159.

Peters A, Liu E, Verrier RL, et al. Air pollution and incidence of cardiac arrhythmia. *Epidemiology*. 2000;11:11–17.

Piano MR. Cellular and signaling mechanisms of cardiac hypertrophy. *J Cardiovasc Nurs*. 1994;8:1–26.

Piano MR. Alcoholic cardiomyopathy: incidence, clinical characteristics, and pathophysiology. *Chest*. 2002;121:1638–1650.

Piano MR, Bondmass M, Schwertz DW. The molecular and cellular pathophysiology of heart failure. *Heart Lung*. 1998;27:3–19.

Pickerill KE, Paladino JA, Schentag JJ. Comparison of the fluoroquinolones based on pharmacokinetic and pharmacodynamic parameters. *Pharmacotherapy*. 2000;20:417–428.

Piegari E, De Angelis A, Cappetta D, et al. Doxorubicin induces senescence and impairs function of human cardiac progenitor cells. *Basic Res Cardiol*. 2013;108:334-352.

Powis G. Free radical formation by antitumor quinones. *Free Radic Biol Med*. 1989;6:63–101.

Pu WT, Ma Q, Izumo S. NFAT transcription factors are critical survival factors that inhibit cardiomyocyte apoptosis during phenylephrine stimulation in vitro. *Circ Res*. 2003;92:725–731.

Puceat M, Vassort G. Signalling by protein kinase C isoforms in the heart. *Mol Cell Biochem*. 1996;157:65–72.

Pulkki KJ. Cytokines and cardiomyocyte death. *Ann Med*. 1997;29: 339–343.

Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol*. 1997;15:707–747.

Reed JC, Jurgensmeier JM, Matsuyama S. Bcl-2 family proteins and mitochondria. *Biochim Biophys Acta*. 1998;1366:127–137.

Regan TJ. Alcohol and the cardiovascular system. *JAMA*. 1990;264:377–381.

Ribiere C, Hininger I, Rouach H, et al. Effects of chronic ethanol administration on free radical defence in rat myocardium. *Biochem Pharmacol*. 1992;44:1495–1500.

Richey PA, Brown SP. Pathological versus physiological left ventricular hypertrophy: a review. *J Sports Sci*. 1998;16:129–141.

Robert V, Silvestre JS, Charlemagne D, et al. Biological determinants of aldosterone-induced cardiac fibrosis in rats. *Hypertension*. 1995;26:971–978.

Rockhold RW. Cardiovascular toxicity of anabolic steroids. *Annu Rev Pharmacol Toxicol*. 1993;33:497–520.

Roden DM. Antiarrhythmic drugs. In: Hardman JG, Limbird LE, Molinoff PB, et al., eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 9th ed. New York: McGraw-Hill; 1996:839–874.

Rooney JW, Hodge MR, Mccaffrey PG, et al. A common factor regulates both Th1- and Th2-specific cytokine gene expression. *EMBO J*. 1994;13:625–633.

Rosen MR. Cardiac arrhythmias and antiarrhythmic drugs: recent advances in our understanding of mechanism. *J Cardiovasc Electrophysiol*. 1995;6:868–879.

Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999;340:115–126.

Russell RR III, Bergeron R, Shulman GI, et al. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol*. 1999;277:H643–H649.

Sabbah HN, Sharov VG. Apoptosis in heart failure. *Prog Cardiovasc Dis*. 1998;40:549–562.

Saikumar P, Dong Z, Patel Y, et al. Role of hypoxia-induced bax translocation and cytochrome *c* release in reoxygenation injury. *Oncogene*. 1998;17:3401–3415.

Saupe KW, Spindler M, Hopkins JC, et al. Kinetic, thermodynamic, and developmental consequences of deleting creatine kinase isoenzymes from the heart. Reaction kinetics of the creatine kinase isoenzymes in the intact heart. *J Biol Chem*. 2000;275:19742–19746.

Sawyer DB, Fukazawa R, Arstall MA, et al. Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane. *Circ Res*. 1999;84:257–265.

Shier WT, Dubourdieu DJ. Sodium- and calcium-dependent steps in the mechanism of neonatal rat cardiac myocyte killing by ionophores. I. The sodium-carrying ionophore, monensin. *Toxicol Appl Pharmacol*. 1992;116:38–46.

Shioi T, Kang PM, Douglas PS, et al. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO J*. 2000;19:2537–2548.

Shioi T, McMullen JR, Kang PM, et al. Akt/protein kinase B promotes organ growth in transgenic mice. *Mol Cell Biol*. 2002;22:2799–2809.

Shipp JC, Opie LH, Challoner D. Fatty acid and glucose metabolism in the perfused heart. *Nature*. 1961;189:1018–1019.

Simons FE. The therapeutic index of newer H1-receptor antagonists. *Clin Exp Allergy*. 1994;24:707–723.

Sleight P. Calcium antagonists during and after myocardial infarction. *Drugs*. 1996;51:216–225.

Smirnova E, Shurland DL, Ryazantsev SN, van der Bliek AM. A human dynamin-related protein controls the distribution of mitochondria. *J Cell Biol*. 1998;143:351–358.

Solem LE, Henry TR, Wallace KB. Disruption of mitochondrial calcium homeostasis following chronic doxorubicin administration. *Toxicol Appl Pharmacol*. 1994;129:214–222.

Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trual for colorectal adenoma prevntion. *N Engl J Med*. 2005;352:1071–1080.

Song Z, Chen H, Fiket M, Alexander C, Chan DC. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. *J Cell Biol*. 2007;178:749–755.

Splawski I, Shen J, Timothy KW, et al. Spectrum of mutations in long-QT syndrome genes. KVLQT1, HERG, SCN5A, KCNE1, and KCNE2. *Circulation*. 2000;102:1178–1185.

Stapleton PA, Nichols CE, Yi J, et al. Microvascular and mitochondrial dysfunction in the female F1 generation after gestational TiO2 nanoparticle exposure. *Nanotoxicology*. 2015;9:941–951.

Stemmer PM, Klee CB. Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. *Biochemistry*. 1994;33:6859–6866.

Stief CG, Uckert S, Becker AJ, et al. Effects of sildenafil on camp and cGMP levels in isolated human cavernous and cardiac tissue. *Urology*. 2000;55:146–150.

Stolley PD, Strom BL, Sartwell PE. Oral contraceptives and vascular disease. *Epidemiol Rev*. 1989;11:241–243.

Sugden PH, Clerk A. "Stress-responsive" mitogen-activated protein kinases (C-Jun N-terminal kinases and P38 mitogen-activated protein kinases) in the myocardium. *Circ Res*. 1998;83:345–352.

Sugishita K, Kinugawa K, Shimizu T, et al. Cellular basis for the acute inhibitory effects of IL-6 and TNF-alpha on excitation–contraction coupling. *J Mol Cell Cardiol*. 1999;31:1457–1467.

Sun Q, Wang A, Jin X, et al. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. *JAMA*. 2005;294:3003–3010.

Suzuki K, Kostin S, Person V, et al. Time course of the apoptotic cascade and effects of caspase inhibitors in adult rat ventricular cardiomyocytes. *J Mol Cell Cardiol*. 2001;33:983–994.

Swynghedauw B. Molecular mechanisms of myocardial remodeling. *Physiol Rev*. 1999;79:215–262.

Symanski JD, Gettes LS. Drug effects on the electrocardiogram. A review of their clinical importance. *Drugs*. 1993;46:219–248.

Talhari C, lauceviciute I, Enderlein E, et al. COX-2-selective inhibitor valdecoxib induces severe allergic skin reactions. *J Allergy Clin Immunol*. 2005;115:1089–1090.

Tan Y, Rouse J, Zhang A, et al. FGF and stress regulate CREB and ATF-1 via A pathway involving P38 MAP kinase and MAPKAP kinase-2. *EMBO J*. 1996;15:4629–4642.

Taneike M, Yamaguchi O, Nakai A, et al. Inhibition of autophagy in the heart induces age-related cardiomyopathy. *Autophagy*. 2010;6:600–606.

The Cardiac Arrhythmia Suppression Trial Investigators. Effect of the antiarrhythmic agent moricizine on survival after myocardial infarction. *N Engl J Med*. 1992;327:227–233.

Thomas T, Sutton ET, Bryant MW, et al. In vivo vascular damage, leukocyte activation and inflammatory response induced by beta-amyloid. *J Submicrosc Cytol Pathol*. 1997;29:293–304.

Tibbles LA, Woodgett JR. The stress-activated protein kinase pathways. *Cell Mol Life Sci*. 1999;55:1230–1254.

Tolkovsky AM. Mitophagy. *Biochim Biophys Acta*. 2009;1793:1508–1515.

Toraason M, Wey HE, Richards DE, et al. Altered Ca^{2+} mobilization during excitation–contraction in cultured cardiac myocytes exposed to antimony. *Toxicol Appl Pharmacol*. 1997;146:104–115.

Uehata M, Ishizaki T, Satoh H, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*. 1997;389:990–994.

van Bilsen M, Smeets PJ, Gilde AJ, et al. Metabolic remodelling of the failing heart: the cardiac burn-out syndrome? *Cardiovasc Res*. 2004;61:218–226.

Van Hee VC, Adar SD, Szpiro AA, et al. Exposure to traffic and left ventricular mass and function: the Multi-Ethnic Study of Atherosclerosis. *Am J Respir Crit Care Med*. 2009;179:827–834.

Van Vleet JF, Amstutz HE, Weirich WE, et al. Clinical, clinicopathologic, and pathologic alterations in acute monensin toxicosis in cattle. *Am J Vet Res*. 1983;44:2133–2144.

Ventura-Clapier R, Garnier A, Veksler V. Energy metabolism in heart failure. *J Physiol*. 2004;555:1–13.

Vestal RE, Eiriksson CE Jr, Musser B, et al. Effect of intravenous aminophylline on plasma levels of catecholamines and related cardiovascular and metabolic responses in man. *Circulation*. 1983;67:162–171.

Wald N, Howard S.Smoking, carbon monoxide and arterial disease. *Ann Occup Hyg*. 1975;18:1–14.

Wallace KB, Hausner E, Herman E, et al. Serum troponins as biomarkers of drug-induced cardiac toxicity. *Toxicol Pathol*. 2004;32:106–121.

Wallimann T, Dolder M, Schlattner U, et al. Some new aspects of creatine kinase (CK): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. *Biofactors*. 1998;8:229–234.

Wang GW, Klein JB, Kang YJ. Metallothionein inhibits doxorubicin-induced mitochondrial cytochrome *c* release and caspase-3 activation in cardiomyocytes. *J Pharmacol Exp Ther*. 2001a;298:461–468.

Wang L, Zhou Z, Saari JT, et al. Alcohol-induced myocardial fibrosis in metallothionein-null mice: prevention by zinc supplementation. *Am J Pathol*. 2005;167:337–344.

Wang XZ, Ron D. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by P38 MAP kinase. *Science*. 1996;272:1347–1349.

Wang Y, Huang S, Sah VP, et al. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem*. 1998;273:2161–2168.

Weber KT. Are myocardial fibrosis and diastolic dysfunction reversible in hypertensive heart disease? *Congest Heart Fail*. 2005;11:322–324.

Wettschureck N, Rutten H, Zywietz A, et al. Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes. *Nat Med*. 2001;7:1236–1240.

Wisneski JA, Gertz EW, Neese RA, et al. Myocardial metabolism of free fatty acids. Studies with ^{14}C-labeled substrates in humans. *J Clin Invest*. 1987;79:359–366.

Wollert KC, Heineke J, Westermann J, et al. The cardiac Fas (APO-1/CD95) receptor/Fas ligand system: relation to diastolic wall stress in volume-overload hypertrophy in vivo and activation of the transcription factor AP-1 in cardiac myocytes. *Circulation*. 2000;101:1172–1178.

Wollnik B, Schroeder BC, Kubisch C, et al. Pathophysiological mechanisms of dominant and recessive KVLQT1 K^+ channel mutations found in inherited cardiac arrhythmias. *Hum Mol Genet*. 1997;6:1943–1949.

Wyllie AH. Death from inside out: an overview. *Philos Trans R Soc Lond B Biol Sci*. 1994;345:237–241.

Xu W, Liu Y, Wang S, et al. Cytoprotective role of Ca^{2+}-activated K^+ channels in the cardiac inner mitochondrial membrane. *Science*. 2002;298:1029–1033.

Yamamoto C, Miyamoto A, Sakamoto M, et al. Lead perturbs the regulation of spontaneous release of tissue plasminogen activator and plasminogen activator inhibitor-1 from vascular smooth muscle cells and fibroblasts in culture. *Toxicology*. 1997;117:153–161.

Yamamoto M, Ko LJ, Leonard MW, et al. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes Dev*. 1990;4:1650–1662.

Yamashita K, Kajstura J, Discher DJ, et al. Reperfusion-activated Akt kinase prevents apoptosis in transgenic mouse hearts overexpressing insulin-like growth factor-1. *Circ Res*. 2001;88:609–614.

Yaoita H, Ogawa K, Maehara K, et al. Apoptosis in relevant clinical situations: contribution of apoptosis in myocardial infarction. *Cardiovasc Res*. 2000;45:630–641.

Yin T, Sandhu G, Wolfgang CD, et al. Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney. *J Biol Chem*. 1997;272:19943–19950.

Yoon Y, Krueger EW, Oswald BJ, McNiven MA. The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Mol Cell Biol*. 2003;23:5409–5420.

Young M, Fullerton M, Dilley R, et al. Mineralocorticoids, hypertension, and cardiac fibrosis. *J Clin Invest*. 1994;93:2578–2583.

Yu T, Jhun BS, Yoon Y. High-glucose stimulation increases reactive oxygen species production through the calcium and mitogen-activated protein kinase-mediated activation of mitochondrial fission. *Antioxid Redox Signal*. 2011;14:425–437.

Yu T, Sheu SS, Robotham JL, Yoon Y. Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. *Cardiovasc Res*. 2008;79:341–351.

Zakhari S. Cardiovascular toxicology of halogenated hydrocarbons and other solvents. In: Acosta D, ed. *Cardiovascular Toxicology*. New York: Raven Press; 1992:409–454.

Zhou S, Starkov A, Froberg MK, et al. Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. *Cancer Res*. 2001;61:771–777.

Zhou W, Fontenot HJ, Liu S, et al. Modulation of cardiac calcium channels by propofol. *Anesthesiology*. 1997;86:670–675.

Zhou W, Fontenot HJ, Wang SN, et al. Propofol-induced alterations in myocardial beta-adrenoceptor binding and responsiveness. *Anesth Analg*. 1999;89:604–608.

Zorzano A, Hernández-Alvarez MI, Palacín M, Mingrone G. Alterations in the mitochondrial regulatory pathways constituted by the nuclear cofactors PGC-1alpha or PGC-1beta and mitofusin 2 in skeletal muscle in type 2 diabetes. *Biochim Biophys Acta*. 2010;1797:1028–1033.

Zou Y, Hiroi Y, Uozumi H, et al. Calcineurin plays a critical role in the development of pressure overload-induced cardiac hypertrophy. *Circulation*. 2001;104:97–101.

chapter 19

Toxic Responses of the Skin

Donald V. Belsito

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References

INTRODUCTION

As the body's first line of defense against external insult, the skin's vast surface area (1.5 to 2 m^2) is repeatedly exposed to chemical assaults and other types of injuries. As such, it may serve as a portal of entry for topical contactants. Recognizing the potential hazards of skin exposure, the National Institute of Occupational Safety and Health (NIOSH) characterizes skin disease as one of the most pervasive occupational health problems in the United States. In 2012, NIOSH ranked skin disease as the second leading work related illness based on frequency, and estimated its annual cost at greater than 1 billion dollars (NIOSH, 2017a). Although substantial reduction in the reported incidence has occurred in recent years due to better workplace environments and personal protective equipment, further improvements in prevention and management are clearly needed. Skin conditions resulting from occupational or consumer exposures not resulting in lost work are poorly recorded. Hence, their incidence is likely to be greatly underestimated.

NIOSH recently changed its cutaneous categorization to more accurately reflect the risks to workers after topical exposures (NIOSH, 2017b). Previously, inhalation exposures were thought to produce the most risk to workers, with skin exposure being only a secondary pathway. The new guidelines reflect increased understanding of several mechanisms by which skin exposure can lead to disease: systemic toxicity via skin absorption (SYS), direct effects that damage the skin (DIR), and immune-mediated responses to chemicals that contact the skin (SEN). The new notations utilize these categories to designate hazard specific skin exposures, and have incorporated standardized criteria to allow for consistency in how chemicals are designated. Determining the hazard potential of a particular chemical is based upon physicochemical properties of the substance, toxicokinetic studies, epidemiological data, in vitro or in vivo laboratory testing, and in silico computational predictions. These criteria are specified in the revised chemical designation issued by NIOSH (NIOSH, 2017b).

SKIN AS A BARRIER

A large (10% of body mass) and highly accessible organ, the skin protects the body against external insults, thus maintaining homeostasis. Its biochemical properties allow it to perform a myriad of functions beyond that of barrier. Physiologically, the skin participates directly in thermal, electrolyte, hormonal, metabolic, antimicrobial, and immune regulation. Rather than merely repelling noxious agents, the skin may react to them with a variety of defensive mechanisms preventing widespread cutaneous and/or internal injuries. If an insult is severe or sufficiently intense to overwhelm the protective function of the skin, acute or chronic injury manifests in various ways. The specific presentation depends upon a variety of intrinsic and extrinsic factors (Belsito, 1989) including body site, duration of exposure, other environmental conditions, and the physicochemical properties of the insult (Table 19-1).

Skin Histology

The skin consists of two major components: the outer epidermis and the underlying dermis, which are separated by a basement membrane (Fig. 19-1). The junction ordinarily is not flat, but rather

Table 19-1

Factors Influencing Cutaneous Responses

VARIABLE	COMMENT
Body site	
Palms/soles	Thick stratum corneum—good physical barrier Common site of contact with chemicals Occlusion with protective equipment
Intertriginous areas (axillae, groin, neck, finger webs, umbilicus, and genitalia)	Moist, occluded areas Chemical trapping Enhanced percutaneous absorption
Face	Exposed frequently Surface lipid interacts with hydrophobic substances Chemicals frequently transferred from hands
Eyelids	Poor barrier function—thin epidermis Sensitive to irritants/allergens
Postauricular area	Chemical trapping Occlusion
Scalp	Chemical trapping Hair follicles susceptible to metabolic damage
Predisposing cutaneous illnesses	
Atopic dermatitis	Increased sensitivity to irritants Impaired barrier function
Psoriasis	Impaired barrier function
Genetic factors	Predisposition to skin disorders Variation in sensitivity to irritants Susceptibility to contact sensitization
Temperature	Vasodilation—improved percutaneous absorption Increased sweating—trapping
Humidity	Increased sweating—trapping
Season	Variation in relative humidity Chapping and wind-related skin changes

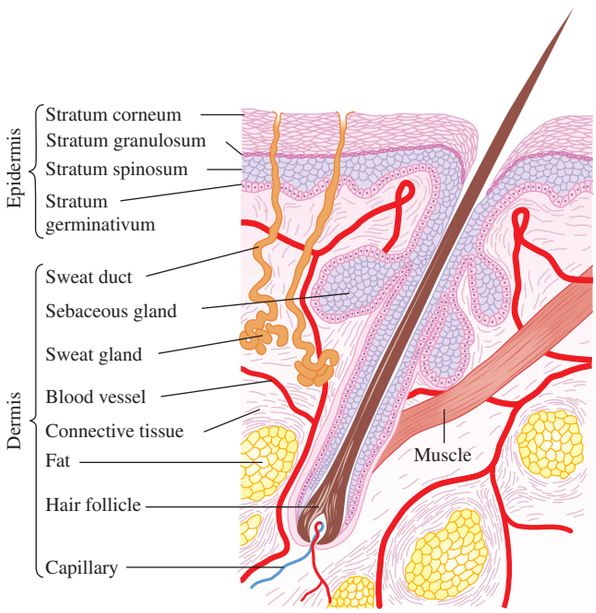


Figure 19-1. *Diagram of a cross-section of human skin.* The epidermis and pilosebaceous units are shown in pink and purple.

undulating (rete ridges). In addition, epidermal appendages (hair follicles, sebaceous glands, and eccrine glands) span the epidermis and embed into the dermis. In thickness, the dermis comprises approximately 90% of the skin, and largely has a supportive function. It has a high content of collagen and elastin secreted by fibroblasts, thus providing the skin with resilience and elasticity. Separating the dermis from underlying tissues is a layer of fat (adipocytes), whose accumulation of lipids has a cushioning and thermoregulatory action. The blood supply to the epidermis originates in the capillaries located in the rete ridges at the dermoepidermal junction. Capillaries also supply the bulbs of the hair follicles and the secretory cells of the sebaceous and eccrine glands. The ducts from the eccrine glands carry a dilute salt solution to the surface of the skin, where its evaporation provides thermoregulation. The ducts of the sebaceous gland provide a protective oil covering to the skin.

The interfollicular epidermis is stratified squamous epithelium consisting primarily of keratinocytes. These cells are tightly attached to each other by desmosomes and to the basement membrane by hemidesmosomes. Melanocytes, whose melanin granules give the skin its color, are interspersed among the basal cells and distributed in the papillae of hair follicles. Migrating through the epidermis are numerous Langerhans cells, which are dendritic, antigen presenting cells involved in the immune response of skin to foreign xenobiotics.

The cellular composition of the epidermis is dominated by the keratinocyte. Keratinocytes of the basal layer comprise the germinative component. When a basal cell divides, one of the progeny detaches from the basal lamina and migrates upwards. As cells move toward the skin surface, they undergo a programmed terminal differentiation, gradually expressing new protein markers, and accumulating keratin proteins. These keratins form insoluble intermediate filaments, which account for nearly 40% of the total cellular protein in the spinous layer of the epidermis. At the granular layer of the epidermis, keratinocytes undergo a striking morphological transformation, becoming flattened and increasing in volume nearly 40-fold. Lipid granules fuse with the plasma membrane at the granular layer/stratum corneum interface, filling the intercellular spaces of the stratum corneum with lipid, as opposed to the aqueous intercellular environment of the underlying viable epidermis. These surface lipids perform a variety of defensive functions, both in preventing diffusion of water and ions out of the body, and preventing access of toxicants and bacteria into the skin (Elias, 2005). Meanwhile, the plasma membranes of the now moribund keratinocytes become permeable, lose their reducing environment, and undergo extensive disulfide bonding of the keratin proteins. Cell organelles are degraded, while a protein envelope is synthesized immediately beneath the plasma membrane. These membrane alterations are characterized by the loss of phospholipids and the addition of sphingolipids.

This programmed terminal differentiation and cell death, beginning as keratinocytes leave the basal layer, produces the outer most layer of the skin, the stratum corneum. No longer viable, the mature cells (called corneocytes) are approximately 80% keratin in content. They are gradually shed from the surface and replaced from beneath. Epidermal turnover typically takes 28 days: 2 weeks for basal cells to reach the stratum corneum, and another 2 weeks to be shed from the surface. In psoriasis, the migration of cells to the surface is nearly 10-fold faster than normal, resulting in a stratum corneum populated by cells that are not completely mature. In instances in which the outer layer is deficient due to disease (e.g., atopic dermatitis) or physicochemical trauma, the barrier to the environment that the skin provides is suboptimal (Elias and Schmuth, 2009).

Percutaneous Absorption

During the past 50 years, the stratum corneum has been recognized as the primary barrier (Scheuplein and Blank, 1971). Diseases (e.g., atopic dermatitis) or other conditions (e.g., abrasions/wounding) in which this barrier is compromised can permit greatly increased uptake of poorly permeable substances, as does removal of the stratum corneum by tape stripping (under experimental conditions) or organic solvents such as toluene (in occupational settings). The viable layer of epidermis provides a much less protective barrier than does the stratum corneum, because hydrophilic chemicals can readily diffuse into the intercellular water, while hydrophobic chemicals can partition into the cell membranes, and each can gain access to the blood supply in the rete ridges of the dermis.

The stratum corneum is the perfect biologic membrane to prevent water loss from underlying tissues by evaporation. Its hydrophobic characteristics result from the lipid content of its intercellular spaces, approximately 15% of the total volume (Elias, 1992). The lipids, a major component being sphingolipids, have a high content of long chain ceramides, removal of which seriously compromises barrier function as measured by transepidermal water loss (TEWL). The stratum corneum is hydrated (approximately 20% water), the moisture residing in its protein content. However, the stratum corneum can take up a great deal more water upon prolonged immersion, thereby reducing the effectiveness of the barrier to hydrophilic chemicals. Indeed, occlusion of the skin with impermeable wraps, permitting the retention of perspiration underneath, is commonly employed to enhance uptake of chemicals, particularly medications, applied to the skin's surface. Although penetration from air is generally too low to be of concern, protection from skin uptake may be advisable for some compounds (e.g., organophosphates) at concentrations sufficient to require respiratory protection.

Finding the rate at which the uptake of xenobiotics through the skin occurs is important for estimating the consequences of exposure to the many agents encountered environmentally. Uptake through the skin is now incorporated into pharmacokinetic modeling to estimate potential risks from exposures. The degree of uptake depends upon exposure conditions, and is typically proportional to solute concentration (assuming it has been diluted), time, and the exposed skin surface. In addition, intrinsic factors contribute to the absorption rate of a given compound; most notably, its hydrophobicity that affects its ability to partition into the epidermal lipid and its rate of diffusion through the corneal barrier. A measure of hydrophobicity is the commonly used octanol/water partitioning ratio (K_{ow}). However, partitioning of a chemical into the skin is greatly affected by its solubility in, or adhesion to, the medium in which it is contained (including soil). Similarly, very hydrophobic compounds, once in the stratum corneum, may diffuse only very slowly into the less hydrophobic regions below. Another important factor relates to molecular weight, where there is an inverse relationship with absorption. Thus, hydrophobic agents of low molecular weight permeate the skin better than those of high-molecular-weight, or low-molecular-weight hydrophilic agents. For small molecules, hydrophobicity is the dominant factor in penetration.

Although only small amounts of chemical may penetrate the stratum corneum, those particularly potent may lead to significant systemic effects (Table 19-2). Previous findings of percutaneous *N*-nitrosamines absorption (e.g., *N*-nitrosodiethanolamine) from cosmetics raised concerns, and led to their monitoring in consumer products (Fan et al., 1977). Children and adolescents harvesting tobacco have been found to be especially susceptible to poisoning by contact with nicotine, a natural pesticide present in the leaves (McKnight and Spiller, 2005). Conditions of topical treatment of livestock for pest control must take into consideration not only the tolerance of the animals, but also the residuals in meat and milk resulting from skin penetration. High level skin exposure to chemicals considered safe at low levels can be dangerous (e.g., the nervous system toxicity and deaths of infants exposed to hexachlorophene mistakenly added to talcum powder for their diapers [Martin-Bouyer et al., 1982]). Percutaneous toxicity in neonates is of particular concern given their increased body surface area to weight, the known skin barrier defects in premature infants, and controversy surrounding at what point in the neonatal process the cutaneous barrier is fully mature.

Considerable empirical information has been collected on some compounds of particular interest (pharmaceuticals, pesticides, and pollutants) to allow for in silico quantification of penetration based upon structural/activity relationships. Thus, mathematical formulas to estimate skin penetration (P_{cw}) have been put forward using empirically derived constants (C_1, C_2, C_3) as noted below (Potts and Guy, 1992).

$$\log P_{cw} = C_1 - C_2(\mathrm{MW}) + C_3 \log K_{ow}$$

Such relations describe steady-state conditions, in which a chemical leaves the stratum corneum at the same rate it enters.

Table 19-2

Percutaneous Systemic Toxicity

Neurotoxicity	Hexachlorophene Organophosphates Lindane Acrylamide Epichlorohydrin *n*-Hexane Methyl hexane derivatives (Lucel-7) Methyl-*n*-butyl ketone Lead tetraethyl Musk tetralin (AETT)
Reproductive Toxicity	Formamides Glycol ethers Hexachlorophene Benzene Chlorinated hydrocarbons
Carcinogenicity	Chemicals found unsafe for use in cosmetics by the Cosmetic Ingredient Review due to the potential for percutaneous carcinogenicity: HC Blue No. 1 4-Methoxy-m-Phenylenediamine & salts Pyrocatechol Possibly Disperse Yellow 3: dermal absorption data needed to assess the safety because Disperse Yellow 3 has carcinogenic potential.

Because rates of transfer of various hydrophobic chemicals into the aqueous phase of the spinous layer are slow, saturation of the stratum corneum can provide a depot leading to continued penetration into the body for relatively long periods after external exposure to a given chemical has stopped. Recognition that such estimates over predict uptake from short exposures has led to modeling of limited doses (Guy, 2010a).

Diffusion through the epidermis is considerably faster at some anatomical sites than others. A list in order of increasing permeability gives the following: foot/sole, palm, forehead, and abdomen (Scheuplein and Blank, 1971). Scrotal skin has the highest permeability for most topical chemicals (Fisher, 1989). Under ordinary conditions, absorption through the epidermal appendages is usually neglected, despite the ability of chemicals to bypass the stratum corneum by this route, because the combined appendageal surface area is a relatively small fraction of the total available area for uptake. However, if loading of the stratum corneum is slow, penetration through the appendages can constitute an appreciable fraction of the total amount following short exposures. In some cases, the effects of appendageal absorption can even be dominant. For example, benzo[a] pyrene penetrates the skin of haired mice severalfold faster than that of hairless strains (Kao and Hall, 1987). The hair follicles thus serve as important routes of entry for hydrophilic agents, and could potentially be exploited for therapeutic purposes (Otberg et al., 2008).

Transdermal Drug Delivery The ability of the stratum corneum to serve as a reservoir for exogenously applied chemicals is the basis for transdermal delivery of pharmaceuticals. Application of drugs to the skin can produce systemic affects, a phenomenon observed unintentionally before the ability of the skin to serve as a delivery system was appreciated. For example, topical exposure of young girls to estrogen has led to reports of pseudoprecocious puberty, while in young males, such exposure has produced gynecomastia (Amin et al., 1998). Transfer of topically applied hormonal medication from a treated to an untreated individual has been reported to cause adverse effects (Busse and Maibach, 2011).

Eloquently designed patches are currently used to deliver an increasing number of different drugs (including estradiol, testosterone, nitroglycerin, scopolamine, etc.) for therapeutic purposes (Guy, 2010b). The advantage of the transdermal approach over oral dosing include (a) providing a steady infusion of drug over extended periods, thereby avoiding large variations in plasma concentrations; (b) preventing exposure to the acidic pH of the stomach, thus preventing biodegradation; and (c) avoiding biotransformation in the gastrointestinal tract from first pass removal by the liver. The contrast in plasma concentration kinetics between different methods of delivery is evident for chemicals that are rapidly metabolized, such as nitroglycerin, which has a half-life of minutes. A variety of chemicals, chosen to minimize irritation and allergenicity, have been incorporated into the delivery systems of pharmaceutical preparations to enhance absorption and penetration. In addition, encapsulation of drug into small vesicles of phospholipids/nonionic surfactants (micelles) and nanoparticle technologies can improve delivery through the epidermis and hair follicles (Desai et al., 2010). Finally, various microneedle systems (Prausnitz et al., 2009) have been used to deliver drugs, vaccines, and gene therapy across the skin; these systems have been critical to the delivery of insulin to brittle diabetics (continuous glucose monitoring devices).

Measurements of Penetration Traditionally, a pharmacokinetic approach to penetration has used experimental animals. To simplify determination of penetration kinetics, skin flaps have been employed and the capillary blood-flow monitored. For this purpose, pig skin was useful (Riviere and Brooks, 2005). Because penetration through rodent skin is usually faster than through human skin, the former can provide an overestimate for behavior of the latter. Therefore, human skin grafts onto athymic mice have been used to minimize species differences, since human skin survives well on athymic mice and retains its normal barrier properties. For risk assessment and pharmaceutical design, the most useful subject for experimentation is, of course, human. In pharmaceutical studies, volunteers are dosed, plasma and/or urine concentrations are quantified at suitable intervals, and amounts excreted from the body are estimated. Previously, isotopic labels were used coupled with an ultrasensitive detection by accelerator mass spectrometry (Buchholz et al., 1999). For in vitro work, there is one European Center for the Validation of Alternative Methods (ECVAM) model that has been accepted by the Organisation for Economic Co-operation and Development (OECD TG 428). In this procedure, skin static or flow-through diffusion cells consisting of rat, pig, or human skin are used under standardized in vitro conditions. This accepted OECD test guideline (OECD TG 428) is not only for cosmetic testing, but also for regulatory issues. In this model, various concentrations of the test substance are typically used to reflect the realistic range of potential human exposures: normally 1 to 5 mg/cm^2 of skin for a solid and up to 10 µL/cm^2 for liquids. The temperature is kept constant because fluctuations could affect the passive diffusion of chemicals. The absorption of a test substance during a given time period (normally 24 hours) is measured by analysis of the receptor fluid and the distribution of the test substance within the skin. However, the guideline is not very specific and additional recommendations for the conduct of dermal absorption studies have

been provided by European Scientific Committee on Consumer Safety (SCCS, 2016). Indeed, for dermal absorption, key factors such as xenobiotic permeability, octanol/water partition coefficient (K_{ow}), the partition coefficient (Log *P*), acid dissociation constant (pK_a), and molecular weight are being used for in silico methods, which have not yet been validated.

Biotransformation The ability of the skin to metabolize chemicals that diffuse through it contributes to its barrier function. Phase I metabolism of skin usually is only a small fraction (approximately 3%) of that in the liver (Rolsted et al., 2008), but it is capable of affecting the outcome of exposure. Biotransformation influences the biological activity of xenobiotics and can result in skin sensitization, carcinogenesis, or other toxicities (Svensson, 2009). While, overall, these activities are normally low, they can be induced (or suppressed). In fact, a significant fraction of the pharmaceuticals used in clinical dermatology are cytochrome P450 inducers, inhibitors, or substrates (Ahmad and Mukhtar, 2004). Well-known xenobiotic inducers of these isozymes include tetrachlorodibenzo-p-dioxin (TCDD), polycyclic aromatic hydrocarbons, and coplanar polychlorinated biphenyls. Exposure to such inducers could influence skin biotransformation, and even sensitize epidermal cells to chemicals that are not good inducers by themselves, a phenomenon observable in vivo and in vitro cell cultures (Walsh et al., 1995).

Biotransformation of a variety of compounds in the skin has been detected, including arachidonic acid derivatives, steroids, retinoids, amines, and polycyclic aromatic hydrocarbons, suggesting that multiple P450 activities are expressed. Using sensitive methodologies, it is now evident that numerous distinct isoenzymes are expressed at varying levels in the skin. Greater than 30 CYP genes have been detected by DNA microarray and real-time polymerase chain reaction (Luu-The et al., 2009; Hu et al., 2010). In addition to the P450 cytochromes, other phase I enzymes expressed in the skin include flavin-dependent monooxygenases, aldehyde dehydrogenases, carboxylesterases, and glutathione peroxidases (Hu et al., 2010). The extent to which phases I and II metabolism influences sensitization to exogenous chemicals may rationalize species differences in the allergic response (Svensson, 2009).

Enzymes participating in phase II metabolism are expressed in skin. Several isozymes, such as UDP glucuronosyltransferase, sulfotransferase, and glutathione *S*-transferase, have been detected in human (Luu-The et al., 2009; Hu et al., 2010) and rodent skin (Oesch et al., 2007). In general, these activities occur at a lower rate than observed in the liver, but exceptions are evident, as in the case of quinone reductase (Khan et al., 1987). Various species express different relative amounts of the various isoenzymes, which could alter resulting target specificities or degrees of responsiveness.

A variety of other metabolic enzyme activities have also been detected in human epidermal cells, including sulfatases, β-glucuronidase, *N*-acetyl transferases, esterases, and reductases (Luu-The et al., 2009; Hu et al., 2010). In addition, the intercellular region of the stratum corneum has catabolic activities, for example, protease, lipase, glycosidase, and phosphatase (Elias, 1992). The epidermis and pilosebaceous units are important drug targets, and are the major sources of biotransformation in the skin. As a result, they are most susceptible to the toxic effects of external xenobiotics. However, other cell types (such as fibroblasts) can also actively biotransform, helping to rationalize observation that organotypic cell cultures containing underlying fibroblasts resemble natural skin to a better extent than those without fibroblasts, especially when using these cell cultures to estimate biotransformation (Gibbs et al., 2007; Luu-The et al., 2009; Hu et al., 2010). The development of these "3D skin models" has stimulated in vitro methods to replace animal testing of cosmetics and other potential skin toxicants.

CONTACT DERMATITIS

Overall, contact dermatitis falls into two major categories: irritant and allergic (Watkins and Maibach, 2009). Both involve inflammatory processes and can have indistinguishable clinical characteristics of erythema (redness), induration (thickening and firmness), scaling (flaking), and vesiculation (blistering) in areas of direct contact with the chemical. Biopsies from affected sites of allergic contact dermatitis reveal a mixed inflammatory cellular infiltrate of lymphocytes, histiocytes and eosinophils, as well as spongiosis (intercellular edema), but often are insufficient to distinguish this conditions from chronic cumulative irritant contact dermatitis (the most frequent subtype) or from other eczematous dermatitides. Indeed, allergic and chronic cumulative irritant contact dermatitis may coexist. For example, cement is alkaline and hygroscopic, producing chronic irritation, and may contain hexavalent chromium, an allergen.

Irritant Dermatitis

Accounting for nearly 80% of all contact dermatitis, irritant dermatitis arises from the direct action of agents on the skin. A chemical in this category is anticipated to give an adverse reaction to anyone if the concentration is high enough, the amount supplied sufficient, and the exposure conditions (frequency and duration) long enough. Irritant dermatitis can be broadly subcategorized as corrosive (chemical burns), acute, and chronic cumulative irritation.

Information on the irritancy of chemicals toward human skin has traditionally been obtained using laboratory animals (mice, rats, rabbits, and guinea pigs). The prototypical methodology has been Draize testing for irritation (Draize et al., 1944), which produces few false negatives (type II errors), but many false positives (type I errors). In this methodology, 0.5 ml (liquid) or 0.5 g (solid) of the target material is applied to a 1×1″ gauze patch onto abraded and intact skin for 24 hours. Six or more albino rabbits or guinea pigs (Hartley) are used and the patches secured to immobilized animals wrapped with an impervious membrane. After 24 hours, the patches are removed and scored for the following endpoints:

1. Erythema and eschar: 0 = none; 1 = barely perceptible; 2 = well-defined; 3 = moderate to severe; 4 = severe w/ or w/out eschar
2. Edema: 0 = none; 1 = barely perceptible; 2 = slight with edges well-defined; 3 = moderate, less than 1 mm; 4 = severe, more than, or equal to 1 mm

The irritancy is determined numerically depending upon the final cumulative scores on abraded and normal skin: less than 0.9 = nonirritating; 1 to 1.9 = mildly irritating, safe with limitations; and more than or equal to 2 = irritating.

For development of new cosmetics, particularly in the European Union, a great need exists for an in vitro system to determine the potential for irritant responses. Since 2004, animal tests are prohibited in the EU for cosmetic products. Animal tests have also been banned for cosmetic ingredients (European Council and Parliament Regulation, 2009). The currently accepted in vitro methods for assessment of the different subtypes of irritant contact dermatitis are reviewed below.

Chemical Burns (Corrosion) A chemical burn produces immediate coagulative necrosis resulting in considerable tissue damage with ulceration and sloughing. Also referred to as a severe 2nd or 3rd degree burn, it does not have a primary inflammatory component. Examples of such include burns from strong acids and alkalies, ethylene oxide, and other severe dermatotoxicants. Examples

Figure 19-2. *Examples of occupational skin toxicity.* (**A**) Burn from ethylene oxide; (**B**) Burn from alkali exposure; (**C**) chronic cumulative irritant contact dermatitis from cutting oil; (**D**) atopic dermatitis; (**E**) allergic contact dermatitis to dichromate; (**F**) phototoxicity from lime juice; (**G**) leukoderma from rubber antioxidants; (**H**) post-inflammatory hyperpigmentation following allergic contact dermatitis due to mercaptobenzothiazole; (**I**) acne from cutting oil; and (**J**) beryllium granulomas. The panels, available at the NIOSH Web site (http://www.cdc.gov/niosh/ocderm1.html), are a small selection from the 140-slide NIOSH program "Occupational Dermatoses—A Program for Physicians" prepared by Drs E. Shmunes, M. M. Key, J. B. Lucas, and J. S. Taylor.

are shown in Figs. 19-2A and B. If the chemical is not quickly and completely removed, damage to the skin may continue and, with increased access to the circulation, systemic injury can occur. Table 19-3 lists some important corrosive agents found in the occupational setting.

Given its severity, in vitro models for corrosion were considered essential given the EU's stance on animal testing. Five validated in vitro alternatives are included in the EU regulations for skin corrosion testing, mainly the TER (rat skin transcutaneous electrical resistance test; OECD TG 430) and tests on reconstructed human epidermis (EpiSkin™, EpiDerm™, SkinEthic™, and epiCS® (Netzlaff et al., 2005; OECD TG 431 and TG 435). In the TER, a liquid (150 μL) or solid ("sufficient to cover surface") target material is applied to rat skin for 24 hours. At that time, electrical resistance is measured and corrosivity assessed as follows: TER more than 5 kΩ, noncorrosive; less than 5 kΩ, corrosive. Unfortunately, TER is not applicable to surfactants or neutral organics. In the reconstructed epidermal models, liquid (500 μL) or solid (500 mg) target material is applied to the reconstructed model and either "breakthrough time" or cell viability is used as an index for corrosivity. Details of these methodologies can be found in the OECD documents and in Almeida et al. (2017).

Acute Irritation Certain chemicals at sufficient concentration can produce an acute irritant response, sometimes called a 1st degree or mild 2nd degree chemical burn. These acute irritants include acid, alkalies, and oxidizing or reducing chemicals that substantially disrupt the cornified layer producing cytotoxicity directly. In 2003, ECVAM started analyzing skin irritation validation models using the previously described "3D models." In 2007, the EpiSkin™ and EpiDerm™ models were approved by ECVAM to replace the in vivo Draize irritation test. A number of in vitro skin irritation models have now been officially validated in the OECD guidelines (OECD TG 439), such as EpiSkin™, EpiDerm™, and SkinEthic™ RHEA. In these systems, 25 μL/cm^2 (liquid) or 25 mg/cm^2 (solid) is applied for 3 hours and cell viability assessed, typically using mitochondrial dehydrogenases to reduce the vital dye MTT. Depending upon the methodology, different viability endpoints are used. A detailed description of these models can be found in the OECD guideline and in Almeida et al. (2017).

Chronic Cumulative Irritant Dermatitis The most common irritation is chronic cumulative from repeated exposures to mild irritants such as soaps, detergents, solvents, and cutting oils. An example from cutting oil is shown in Fig. 19-2C. Currently, there is no

Table 19-3

Selected Chemicals Causing Skin Burns

CHEMICAL	COMMENT
Ammonia	Potent skin corrosive Contact with compressed gas can cause frostbite
Calcium oxide (CaO)	Severe chemical burns Extremely exothermic reaction—dissolving in water can cause heat burns
Chlorine	Liquid and concentrated vapors cause cell death and ulceration
Ethylene oxide	Solutions and vapors may burn Compressed gas can cause frostbite
Hydrogen chloride (HCl)	Severe burning with scar formation
Hydrogen fluoride (HF)	Severe, painful, slowly healing burns from high concentration Lower concentration causes delayed cutaneous injury Systemic absorption can lead to electrolyte abnormalities and death Calcium-containing topical medications and quaternary ammonium compounds are used to limit damage
Hydrogen peroxide	High concentration causes severe burns and blistering
Methyl bromide	Liquid exposure produces blistering and deep burns
Nitrogen oxides	Moist skin facilitates the formation of nitric acid causing severe yellow-colored burns
Phosphorus	White phosphorus continues to burn on skin in the presence of air
Phenol	Extremely corrosive even in low concentrations Systemic absorption through burn sites may result in cardiac arrhythmias, renal disease, and death
Sodium hydroxide	High concentration causes deep burns, readily denatures keratin
Toluene diisocyanate	Severe burns with contact Contact rarely may also result in respiratory and/or skin sensitization

in vitro test to predict chronic cumulative irritation. This typically has been done using human volunteers in what has been termed the Human Repeat Insult Patch Test (HRIPT; McNamee et al., 2008).

In addition to chemical irritants, chronic friction and trauma can mechanically damage the stratum corneum, leading to further loss of its cohesiveness. As a result, the penetration of exogenous chemicals is enhanced at sites of barrier damage. Some chemicals may elicit subsequent toxic effects not evident when the barrier is intact; for example, polyethylene glycol in burn patients (Herold et al., 1982).

The skin at some anatomic sites is more easily irritated than at other sites, which can be demonstrated by measurement of TEWL (Murahata et al., 1986). The eyelids have a thin epidermis and are quite sensitive. Furthermore, the back is more sensitive than the forearm (Zhai et al., 2004). Individuals can vary greatly in their sensitivity to irritant dermatitis, especially the chronic cumulative variety. Although certain genetic contributions to irritant dermatitis have been more clearly defined (Liang et al., 2016), the influence of age and gender remain controversial (reviewed in Belsito, 2005).

Patients with atopic dermatitis (Fig. 19-2D) are especially prone to irritation. The incidence of atopic dermatitis is increasing rapidly in industrialized societies. Reports of its incidence in the pediatric population within these societies vary widely, but generally exceed 20% (Pols et al., 2016). Atopic individuals are not only more sensitive to irritants, but exhibit a propensity to produce specific IgE antibodies to allergens, and typically suffer from hayfever and allergic asthma. These individuals usually have a higher frequency of persistent contact dermatitis than non-atopics (Nethercott and Holness, 1994). Atopic dermatitis has a strong genetic component. In many atopics, defects in the intermediate filament aggregating protein, filaggrin, among other defective genetic barrier genes, are detectable (Palmer et al., 2006; Liang et al., 2016).

Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) is a delayed (T-cell mediated) hypersensitivity reaction (Gell and Coombs Type IV immune response). To induce sensitization through the skin, chemical haptens generally penetrate the lipid barrier and, to be detected by the immune system, attach themselves to carrier proteins. The complete antigens are processed by Langerhans cells (epidermal dendritic cells) and displayed on their surfaces with major histocompatibility complex II molecules. Following migration to the paracortical area of the draining lymph nodes, the Langerhans cells present the processed peptide to naive T-helper cells (Th_0) with specific receptors for the complexed allergen. This antigen presentation leads to the release of a variety of interferons, growth factors, and chemokines, which result in a proliferation of a specific clone of sensitized Th_1 (Belsito, 2008). This phase of the response is termed the induction, during which, over a 1 to 3 week period, memory T-cells are generated and enter the circulation.

Upon subsequent exposure to a specific antigen that has previously been recognized by the immune system, a more vigorous and rapid response occurs due to homing of specific memory T cells to the skin, where they release cytokines and chemokines that attract a variety of other nonspecific inflammatory cells. This phase of the immune response is termed elicitation, which may develop within hours to several days following antigenic exposure (Mark and Slavin, 2006). The "delay" in the response depends upon the potency and amount of allergen applied (Friedmann, 2007). The resultant epidermal spongiosis (intercellular edema) and perivascular dermal infiltrate of lymphohistiocytic cells is characteristic, but not pathognomonic, of ACD.

Thousands of chemicals have been reported to give rise to allergic contact dermatitis, many across a variety of occupations and consumer products. Table 19-4 lists some common contact allergens, four of which, among other potent allergens, are shown in Fig. 19-3. Because most chemicals in the universe are only weakly active or infrequently encountered, much effort has focused on finding the major allergens in the population by systematic patch testing of patients presenting with suspected allergic contact dermatitis. Although not measuring sensitivity in the general population, these results are useful.

Table 19-4

Common Contact Allergens

SOURCE	COMMON ALLERGENS	
Topical medications	Antibiotics Bacitracin Neomycin Polymyxin Aminoglycosides Sulfonamides	Therapeutics Benzocaine Corticosteroids
Personal care products	Preservatives Formaldehyde Formaldehyde releasers • Quaternium-15 • Imidazolidinyl urea • Diazolidinyl urea • DMDM hydantoin • 2-bromo-2-nitropropane-1,3-diol Methylchloroisothiazolone/ Methylisothiazolinone (MCI/MI)	Others Ethylenediamine Lanolin *p*-Phenylenediamine Propylene glycol Fragrances
Plants and trees	Mixtures: • Balsam of Peru • Rosin (colophony)	Defined chemical structure: • Pentadecylcatechols (poison ivy/oak/sumac) • Sesquiterpene lactones • Tuliposide A
Antiseptics	Chlorhexidine Chloroxylenol Dichlorophene Glutaraldehyde	Hexachlorophene Thimerosal (merthiolate) Mercurials Triphenylmethane dyes
Rubber products	Diphenylguanidine Hydroquinone Mercaptobenzothiazole *p*-Phenylenediamine derivatives	Resorcinol monobenzoate Benzothiazolesulfenamides Dithiocarbamates Thiurams
Leather	Formaldehyde Glutaraldehyde	Hexavalent chromium Cobalt
Glues and bonding agents	Bisphenol A/Epichlorohydrin Phenol formaldehyde resins Acrylic monomers Cyanoacrylates	Epoxy resins Toluene sulfonamide resins Urea formaldehyde resins
Metals	Chromium Cobalt	Gold Nickel

The chemicals chosen for patch testing vary with the geographic location to account for local exposures. Additionally, the choice may be directed to specific anatomic sites, such as the foot (Shackelford and Belsito, 2002). Panels may also be adapted to detect emerging trends, as new products appear and others decline in use. Table 19-5 lists the 25 materials most commonly giving positive results in recent patch tests conducted by the North American Contact Dermatitis Group (deKoven et al., 2017). The increasing prevalence of reactions to nickel, especially among younger subjects (Zug et al., 2014), likely reflects increasing exposure, especially through body piercing (Warshaw et al., 2014). A number of agents (nickel, chromium, paraphenylenediamine, and formaldehyde) have shown high prevalence of reactivity for several decades, while others, once thought innocuous, have more recently become recognized as reactive, as evidenced by the recent epidemic of methylisothiazolinone allergy (Urwin and Wilkinson, 2013). The high prevalence of allergic contact dermatitis to metals has raised concern about possible reactions to metallic surgical implants. (Basko-Plluska et al., 2011; Guenther et al., 2016). An example of contact allergy to hexavalent chromium in cement is shown in Fig. 19-2E.

Sensitization to ingredients in topical preparations is a common problem and one that changes as formulations evolve (Pascoe et al., 2010). As with other consumer products, reduction in use of the most prevalent allergenic chemicals, and their replacement by less allergenic substitutes in topicals, is advocated. Caution in using less characterized chemicals as a replacement must be exercised, because their allergenicity may not become evident until they reach large populations of users, as has happened in several prominent cases (Uter et al., 2005). For example, methyldibromo glutaronitrile

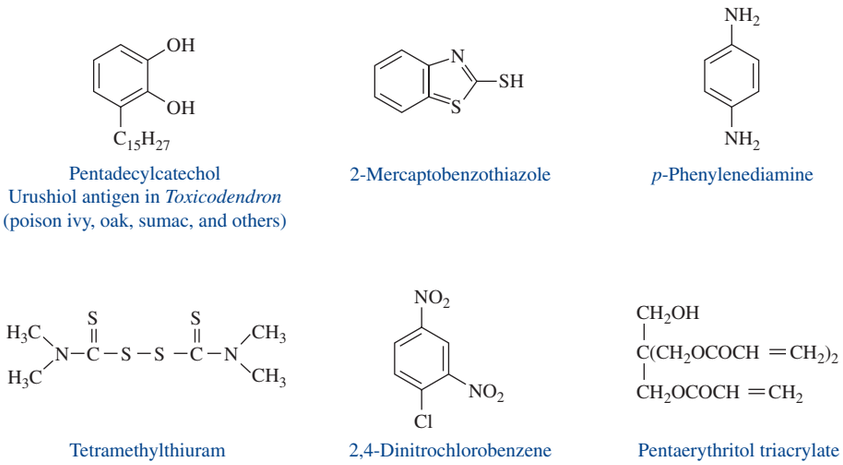


Figure 19-3. *Structural formulas of some potent contact sensitizers.*

Table 19-5

Prevalence of Positive Reactions in Patch Test Patients: The Top 25 Allergens

ALLERGEN	PATIENTS WITH POSITIVE PATCH TEST REACTIONS (%)
Nickel sulfate hexahydrate, 2.5% pet	20.1
Fragrance mix I, 8.0% pet	11.9
Methylisothiazolinone, 0.2% aq	10.9
Neomycin sulfate, 20.0% pet	8.4
Cobalt (ii) chloride hexahydrate, 1.0% pet	7.4
Bacitracin, 20.0% pet	7.4
Myroxylon pereirae resin (balsam of Peru), 25.0% pet	7.2
Formaldehyde, 2.0% aq	7.0
p-Phenylenediamine base, 1.0% pet	7.0
MCI/MI, 0.01% aq	6.4
Fragrance mix II, 14.0% pet	5.7
Lanolin alcohol (Amerchol L 101), 50% pet	5.4
Quaternium-15, 2.0% pet	4.8
Carba mix, 3.0% pet	4.8
Iodopropynyl butylcarbamate, 0.5% pet	4.7
Diphenylguanidine, 1.0% pet	3.8
Methyldibromo glutaronitrile/phenoxyethanol (Euxyl K 400), 2.0% pet	3.7
Oleamidopropyl dimethylamine, 0.1% aq	3.5
Thiuram mix, 1.0% pet	3.1
Propylene glycol, 100.0% aq	2.8
2-Hydroxyethyl methacrylate	2.6
Potassium dichromate, 0.25% pet	2.2
Tixocortol-21-pivalate, 1.0% pet	2.1
2-Bromo-2-nitropropane-1,3-diol (Bronopol), 0.5% pet	2.1
Benzophenone-4, 10.0% pet	2.1

SOURCE: Data from deKoven et al. (2017).

was introduced as a replacement preservative in combination with phenoxyethanol. In initial screens, this combination did not cause allergic contact dermatitis. However, upon widespread use, methyldiboromo glutaronitrile was shown to be a significant contact allergen in Europe (Kynemund Pedersen et al., 2004), and subsequently it has been removed from the European cosmetic market.

Unlike contact irritants, where the response is generally proportional to the applied dose and time, contact allergens can elicit reactions at very small doses. In contrast, at significantly higher doses, saturation of the response, or even inhibition (tolerance), may become evident (Marker and Thomsen, 1986). Nevertheless, careful analysis from human and animal testing (Friedmann, 2007) show that a higher dose confers a greater likelihood of sensitization to a given allergen. Moreover, the dose dependence for the induction of sensitization displays nonlinearity, suggesting that the response is sub-linear, and probably sigmoidal (Friedmann, 2007). These observations have practical implications: as the number of cosmetic preservatives becomes more restrictive due to regulatory activities, especially in the European Union, the likelihood increases that a given biocide will be used in more products and/or at higher concentrations in a given product, and that this higher cumulative exposure will result in sensitization to what otherwise are weak allergens, a phenomenon referred to as the Dillarstone effect.

Immunoregulation Although animal studies have shown strain (presumably genetic) variation in cell-mediated immunity, the evidence for a genetic influence in humans has been slight. Skog (1958) found that 5% of a defined population could not be sensitized to dinitrochlorobenzene and suggested that this was due to inheritance. In another study, significant genetic association with the capacity to become sensitized to *para*-nitrosodimethylaniline was reported (Walker et al., 1967). However, multiple attempts to correlate HLA haplotype and other factors (including T-cell receptor genes) with nickel sensitivity or other contact allergies have yielded conflicting results. In atopic dermatitis, gene variants are thought to contribute to the observed abnormalities in innate and adaptive immune responses (Liang et al., 2016). Nonetheless, definitive evidence of a genetic predisposition to ACD in humans has been meager, probably because of the diverse genetic pool and the limitations of technology.

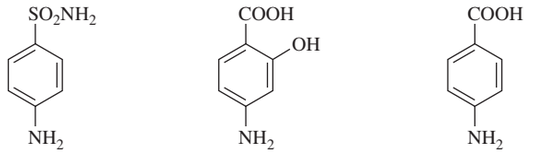
The route of primary sensitization has a profound effect on the subsequent immunologic response. Sulzberger (1929) demonstrated that intracardiac injection of neoarsphenamine induced tolerance rather than sensitization. Tolerance induction has also been reported after primary oral ingestion of allergens (Chase, 1946), as well as after primary epicutaneous application of allergens to areas deficient in HLA-DR-positive Langerhans cells (Elmets et al., 1983). The exact mechanism by which tolerance ensues is controversial, and may depend on the route of exposure (oral, intravenous, epicutaneous, or intraperitoneal) and the precise subset of dendritic cells responsible for antigen presentation (Akbari and Umetsu, 2005). There are data to suggest that the expression of indoleamine 2,3-dioxygenase, an intracellular heme-enzyme that catalyzes oxidative catabolism of indole rings by dendritic cells, results in suppression of T cell responses and promotion of tolerance (Mellor and Munn, 2004). In general, either induction of hapten-specific CD4+/CD25+ or Th_3 suppressor T cells (Elmets et al., 1983; Girolomoni et al., 2004; Cavani, 2005) and/or clonal deletion of the responding T cells (Nossal, 1991) seems responsible for the observed tolerance. Readers interested in a better understanding of the mechanism(s) of tolerance induction are referred elsewhere. (Girolomoni et al., 2004; Akbari and Umetsu, 2005; Cavani, 2005).

Aged individuals have been shown to have various defects in the induction and/or elicitation of ACD (Kwangsukstith and Maibach, 1995). The precise reason is unknown and many aspects of cell-mediated responses are likely involved (Belsito et al., 1987). Experiments in which contact-sensitized aged mice were injected with naive young T cells and subsequently demonstrated normal responses upon antigenic challenge suggest that a failure of T cell amplification signals and/or the generation of sufficient T effector cells may be among the primary deficiencies in aged animals (Belsito and Possick, 1988).

The competency of immune responses in children is also controversial (Kwangsukstith and Maibach, 1995). In the past, it was thought that children rarely developed ACD because of an immature immune system and that patch testing of children with standard concentrations of allergens resulted in a high percentage of irritant reactions (Marcussen, 1963). However, other data suggest that patch testing of children with the allergens commercially available in the United States does not result in increased, and confounding, irritant responses (Rietschel et al., 1990; Zug et al., 2014). Of note, Strauss (1931) was able to sensitize 35 of 48 infants (1 to 4 days old) to *Toxicodendron* oleoresin, suggesting that the apparent hyporesponsiveness of children may be due to limited exposure and not to deficient immunity. Similarly, the effects of gender on the incidence of ACD seem related to the likelihood of exposure (Schubert et al., 1987; Kwangsukstith and Maibach, 1995).

In experimental models and clinical practice, downregulation of ACD has been achieved with ultraviolet radiation (UVB or PUVA), glucocorticoids, and calcineurin inhibitors. Each of these acts somewhat differently. UVB and PUVA significantly decrease the density of epidermal LCs, induce epidermal hyperplasia (making it more difficult for the antigen to reach any remaining epidermal antigen-presenting cells), upregulate IL-10, and induce CD4+/CD25+ regulatory T cells (Belsito, 1989; Aubin and Mousson, 2004). Glucocorticoids also inhibit the type IV reaction at multiple points (Belsito, 1989): epidermal LCs are decreased in number, the production and function of IL-1, IL-2, and IFN-γ are inhibited, and T cell proliferation is decreased. In contrast, cyclosporine A and other macrolactams act primarily to inhibit activation of cNF-AT via inhibition of calcineurin (Bornhövd et al., 2001). Finally, a variety of other pharmacologic agents have been reported to interfere with the induction and/or elicitation of ACD in murine models (Belsito, 1997).

Diagnosis and Testing When a patient exhibits allergic contact dermatitis, finding the responsible chemical is important to avoid continued exposure. For this purpose, patch testing is the gold standard that has been employed and refined since it was first described by Jadassohn (1895). On the washed back of patients who are not exhibiting dermatitis at the sites to be tested, nor using corticosteroids or other immunosuppressive treatments, patches are placed that contain a small amount of the putative allergen. Many of these materials are commercially available at standardized concentrations too low to produce significant irritant reactions, although induction of the allergic response has been reported to occur (Tomb, 1992). Certain chemicals are usually not tested because they induce too strong a response or might produce sensitization (e.g., urushiol from poison ivy). After 2 days, during which time a reaction begins to develop, the patches are removed and the sites of exposure are scored for degree of response. These sites are additionally re-evaluated at any time from 1 to 5 days afterwards (3 to 7 days after the initial application). The relevance of any positive reaction to the patient's actual environment must be considered so that exposure to putative allergens in daily life can be minimized to appropriate chemicals. Interpretation of the results, and resulting modifications in lifestyle, should take into account the



Sulfonamide | *para*-Aminosalicylic acid | *para*-Aminobenzoic acid

Figure 19-4. *Structural formulas of selected para-amino compounds that show cross-reactions in allergic contact sensitization.*

phenomenon of cross-sensitivity, where reactivity to a compound may occur because it shares functional groups that have provoked sensitization with another compound. Fig. 19-4 illustrates this principle with three amine compounds and Table 19-6 lists some common cross reacting chemicals.

Animal testing to predict allergenicity has an extensive history and the prototypical test has been the Guinea Pig Maximization Test by Magnusson and Kligman (1969). In this assay, a chemical is injected intradermally with or without Freund's complete adjuvant. The reaction of the skin to subsequent challenge with a chemical can then be observed and graded. While this approach has been successful, the extrapolation of sensitivity measurements from laboratory animals to humans presented large uncertainties. Therefore, in the last couple decades, the local lymph node assay (LLNA), performed in mice, has become the predominant in vivo animal tool to measure the sensitization capacity of a specific chemical (Basketter et al., 2012). Not only does the LLNA use a smaller number of laboratory animals than the Magnusson-Kligman assay, it also has the capability to rank the potency of allergens from very strong to weak. Because sensitizers can differ in potency over four orders of magnitude, this quantitative assay has been extremely helpful.

As is the case with irritation, the European Union directive eliminating animal use in cosmetic testing has stimulated the development of integrated in vitro and in silico testing strategies, where predictions for potential skin sensitization by a particular material include its physicochemical structure and its performance in various in vitro tests. There are four key biological components in the "adverse outcome pathway" (AOP) for skin sensitization classified as: (1) the molecular initiating event; (2) activation of keratinocytes as detected by gene expression associated with specific cell signaling pathways and inflammatory responses; (3) dendritic cell activation as determined by specific expression of cell surface markers; and (4) T-cell proliferation. There is general agreement in the scientific arena that assessment of only one aspect of the AOP will not provide sufficient information to replace the use of animals for the assessment of chemical allergenicity (reviewed in Maxwell et al., 2014). Rather, information from non-testing methods (in silico data, analogue based "read across" information) and in vitro approaches that evaluate the key events in the AOP will be needed. Information garnered from these approaches will need to be further combined to address the sensitization end point in a weight of evidence framework, which is an area of intense research (Patlewicz et al., 2014; Tollefsen et al., 2014).

As far as addressing the key events in the sensitization AOP, four assays have been endorsed at the OECD level at this time. The first event in the AOP for sensitization can be assessed by the Direct Peptide Reactivity Assay or "DPRA" (Gerberick et al., 2004, 2008; OECD TG 442C), which measures the ability of a chemical to react with peptides, specifically histidine and cysteine. To address the second step of the AOP, keratinocyte activation,

Table 19-6

Common Cross-Reacting Chemicals

CHEMICAL	CROSS REACTOR(S)
Balsam of Peru	Pine resin, cinnamates, benzoates
Bisphenol A	Diethylstilbestrol, monobenzyl ether of hydroquinone
Canaga oil	Benzyl salicylate
Chlorocresol	Chloroxylenol
Diazolidinyl urea	Imidazolidinyl urea
Hydroquinone	Resorcinol
Alkyl hydroxybenzoates	Parabens, hydroquinone, monobenzyl ether of hydroquinone
p-Aminobenzoic acid	*p*-Aminosalicylic acid, sulfonamide
Para-phenylenediamine	*p*-Aminobenzoic acid, aminobenzoate anesthetics
Propyl hydroxybenzoate	Hydroquinone monobenzyl ether
Phenol	Resorcinol, cresols, hydroquinone

KeratinoSens™ has been validated by ECVAM and has accepted OECD guidelines (OECD TG 442D). In the KeratinoSens™ assay, the sensitizing potential of a chemical is assessed using immortalized adherent human keratinocytes (HaCaT cell-line), transfected with a plasmid to quantify luciferase gene induction as a measure of activation of Keap1-NRF2-antioxidant/electrophile response element (ARE)-1. Assessment of the third step in the AOP, dendritic cell activation, can be undertaken using either the ECVAM-validated MUSST or h-CLAT methodologies, the latter of which have OECD guidelines (OECD 442E). In the MUSST assay, expression of CD86 and CD54 on a myeloid dendritic cell line is measured, while h-CLAT measures only CD54 expression. Currently there is no validated methodology to assess the fourth component of the AOP, T-cell activation.

The integration of the results of testing the first three steps of the AOP to predict sensitization and hazard has yet to be resolved. It is an area of active research and various models (Bayesian network, Artificial Neural Network and Weight of Evidence) have been proposed. Currently, the Weight of Evidence or the best "two out of three" approach in assessing sensitization based upon the testing of the first three key events in the AOP has found the greatest success. This was initially proposed by Bauch et al. (2012) and later updated by Natsch et al. (2013) and Urbisch et al. (2015). In this approach, chemicals are identified as non-sensitizers if they are negative in both the DPRA and either the KeratinoSens™ assay or the MUSST/h-CLAT assays. However, using the current approaches does have drawbacks. An issue of significant concern among dermatotoxicologists is the ability of the current in vitro test to detect pre-haptens (abiotically transformed materials; e.g., oxidized in the environment) and pro-haptens (biotically transformed materials via metabolism in the skin). Clearly, one or more of the current methodologies must be adapted to detect pre-and pro-haptens (Patlewicz et al., 2016). Efforts to further improve these systems is already underway as evidenced by the peroxidase peptide reactivity assay "PPRA" (Gerberick et al., 2009; Gerberick, 2016), which looks at identification of pro-haptens via the use of peroxidase in the DPRA.

PHOTOSENSITIVITY

The ultraviolet and visible spectra of solar radiation that reach the earth's atmosphere extend from 290 to 700 nm. Wavelengths beyond this range are either filtered by the earth's atmosphere or are insufficiently energetic to cause cutaneous pathology. Adequate doses of artificially produced UVC (100 to 289 nm) or x-rays (less than 10 nm) can produce profound physical and toxicologic skin changes; however, these would occur only accidentally and more typically in the occupational or laboratory environment. The protective skin pigment, melanin, synthesized by the melanocytes, absorbs a broad range of radiation from ultraviolet B (290 to 320 nm) through the visible spectrum (400 to 700 nm). Other chromophores in the skin include amino acids, particularly tryptophan and tyrosine, and their breakdown products (e.g., urocanic acid), which absorb light in the UVB range. Biologically, the most significant chromophore in the skin is DNA and, as such, damage from radiation can have lasting effects on the genetic information of the target cells, as outlined under the section "Skin Cancer," below.

Phototoxicity

Phototoxic reactions from exogenous chemicals may be produced either by systemic or topical exposures. In acute reactions, the skin can become red and blistered within minutes to hours following ultraviolet light exposure, typically UVA (320 to 400 nm). Occupational examples include coal tar, which, in combination with sunlight, can produce an immediate stinging sensation referred to as "tar smarts." Over time, this damage can result in significant hyperpigmentation of the skin.

Phototoxic chemicals most commonly absorb in the UVA range, thereby assuming a higher energy, excited, triplet state; the subsequent transfer of electrons to other molecules or reduction to more highly reactive-free radicals results in the observed tissue damage (Moan and Peng, 2003; Deleo, 2004). Oxygen dependent photodynamic reactions commonly occur as these excited molecules return to their ground state and transfer their energy to oxygen, forming high reactive singlet oxygen. These reactive products then damage cellular macromolecules, especially unsaturated membrane lipids, which results in cell death. The release of a variety of immune mediators from the dying keratinocytes and the recruitment of inflammatory cells to the skin are the histopathologic signs of phototoxicity. Those chemicals most often associated with phototoxic reactions are listed in Table 19-7.

As opposed to many phototoxins that require oxygen radicals, psoralens are an example of chemicals producing phototoxicity without production of singlet oxygen. Psoralens intercalate within DNA to form covalent adducts, and cross-link when activated by UVA. The result is inhibition of DNA synthesis and repair causing the cells to die. Psoralens can be found in various foods, including lime and celery. Production of photodermatitis in occupational settings where such foods are routinely handled has been reported (Seligman et al., 1987). An example of phototoxicity from furocoumarin present in lime peel is given in Fig. 19-2F. Psoralens in combination with UVA (PUVA) have been used therapeutically to inhibit keratinocyte growth and lymphocyte proliferation in diseases such as psoriasis and cutaneous T-cell lymphoma; however, this modality is infrequently used today because of better treatment options for these diseases, and an enhanced risk of both squamous cell carcinoma and malignant melanoma with prolonged PUVA therapy (Stern, 1992; Stern, 2001).

Nails, an appendage of the skin, may also suffer phototoxic reactions to drugs and, less commonly, to topically applied chemicals. While various types of nail pathology may result, including changes in cell growth and pigmentation, detachment of the various layers of the nail plate from each other (onycholysis) is the most commonly observed effect of phototoxicity. Agents such as coal tar and tetracyclines produce photodynamic onycholysis, which requires generation of oxygen radicals. In contrast, topical or systemic psoralens produce onycholysis by a non-photodynamic mechanism that does not require oxygen (reviewed in Epstein, 1999).

Table 19-7

Selected Phototoxic Chemicals

Furocoumarins
8-Methoxypsoralen
5-Methoxypsoralen
Trimethoxypsoralen
Polycyclic aromatic hydrocarbons
Anthracene
Fluoranthene
Acridine
Phenanthrene
Pyridine
Drugs
Amiodarone
Tetracyclines
Sulfonamides
Sulfonylureas
Nalidixic acid
Thiazides
Phenothiazines
Nonsteroidal anti-inflammatories
Dyes
Anthraquinones
Eosin
Acridine orange
Rose Bengal
Porphyrin derivatives
Hematoporphyrin

Photoallergy

Like phototoxins, most photoallergens respond to light in the UVA range (Table 19-8). Except for the need for photoactivation of the allergen, photoallergy is a typical delayed type hypersensitivity response. The reaction may take hours to days to develop and can persist for several weeks following exposure if the agent is retained within the skin. Use of halogenated salicylanilides in consumer products led to thousands of cases of photoallergy in the 1960s and 1970s. Subsequent to salicylanilides, fragrances, especially musk ambrette, in cosmetics, became conspicuous sources of photoallergy until these materials were banned. Nowadays, sunscreening agents and topical nonsteroidal anti-inflammatory drugs (NSAIDS, e.g., ketoprofen) are the more commonly found photoallergens (European Multicentre Photopatch Test Study (EMCPPTS) Taskforce, 2012). Although a photoallergic response to these agents is rare, they have become widely used as the long-term risks effects of sun exposure and systemic intake of NSAIDS have become appreciated.

Photoallergy is immunopathologically distinguishable from phototoxicity, because the former, like all delayed type hypersensitivities, may result from exposure to a chemical too low to produce

Table 19-8

Selected Photoallergens

Halogenated salicylanilides
Tetrachlorosalicylanilide
Dibromosalicylanilide
Trichlorocarbanilide
Bithionol
Whiteners
Stilbene
Fragrances
Musk ambrette
6-methyl coumarin
Drugs
Ketoprofen
Phenothiazines
Chlorpromazine
Promethazine
Fentichlor
Sunscreens
PABA esters
Benzophenones
Octocrylenes

a phototoxic irritant response, but sufficient to elicit photoallergy. Chemicals causing phototoxicity, because reactivity is generated upon light exposure, may also be photoallergenic. However, this combination is not common.

Among individuals with a history of photosensitivity, 11% exhibited photoallergy (Victor et al., 2010). The diagnosis of photoallergy is best confirmed by photopatch testing, which includes exposure of the allergens to light and a control of nonirradiated allergens. Because the offending agent may not be obvious from the patient's history due to the delay between exposure to the agent and sunlight, a standard panel of photoallergens, as well as the patient's own sunscreen(s) and personal care products containing potentially photoactivated materials, are tested. The methodology for photopatch testing has been extensively reviewed by Honari (2014).

In Vitro Determination of Phototoxicity and Photoallergenicity

One way to assess the ability of a material to cause a phototoxic or photoallergic reaction is its ultraviolet absorption spectrum. If absorption is below the molar extinction coefficient (MEC) of less than 1000 L $\times$ mol^{-1} $\times$ cm^{-1}, then the material has little to no phototoxic or photosensitization hazard (Henry et al., 2009). If absorption occurs, then phototoxicity and photosensitization are both possible. In the reactive oxygen species (ROS) assay, the generation of ROS (superoxide anions and singlet oxygen) following irradiation of the target material by light, while not measuring phototoxicity directly, had 100% sensitivity (with some false positives) in predicting phototoxicity (Haranosono et al., 2014); however, this methodology has not been validated by ECVAM.

According to the OECD, the 3T3 Neutral Red Uptake in vitro phototoxicity test can be used to predict the phototoxic potential of a chemical (OECD TG 432). The 3T3 Neutral Red assay evaluates cytotoxicity in BALB/c 3T3 mouse fibroblasts exposed to the test material in the presence or absence of UVA light, in order to determine the concentration-dependent reduction in Neutral Red Uptake by the cells. Although this method is accepted by the OECD, drawbacks do exist. According to Prieto et al. (2013), the following caveats must be considered: (1) a false negative result can occur since the toxicological hazard of a chemical is primarily due to a mechanism of action not captured by the cell line used; (2) a false positive can occur given a compound's limited bioavailability in vivo due to poor absorption and distribution, or a rapid biotransformation and excretion; and (3) the cell lines have a lack of metabolic capacity, thus some compounds that are metabolized in vivo to biologically active forms may be missed. As a result, intense efforts are underway to evaluate other in vitro phototoxicity test methodologies using photo-exposed "3D skin models" (Portes et al., 2002), as well as a fluorometric ROS assay (Seto et al., 2016).

As opposed to phototoxicity, there is currently no ECVAM-validated or OECD approved guideline for photoallergenicity. However, a number of in vitro testing methodologies utilizing KeratinoSens™ and h-CLAT with the addition of exposure to 5 J/cm^2 of UVA have been undertaken. Tsujita-Inoue et al. (2016) have assessed the second step in the adverse outcome pathway (keratinocyte activation) using the photo-KeratinoSens™ assay. Additionally, Hoya et al. (2009) have evaluated the photo-h-CLAT assay. The development of in vitro systems to assess photosensitization is critical given the ban in Europe in testing cosmetic ingredients in animals, since testing for photoallergenicity in humans is considered unethical because of the potential for the induction of persistent light reactivity, in which the individual remains extremely sensitive to sunlight even in the absence of the initially inducing photoallergen.

As is the case for non-photoactivated allergens, there remains a concern that in vitro testing may miss pre- and pro-photoallergens. To date, two models of photoantigen formation, the photohapten model and the pro-hapten model, have been reported (Tokura et al., 1996; Tokura, 2000). In the induction phase of photoallergy, photoallergens can produce ROS and induce apoptosis in keratinocytes, but the use of either or both biomarkers would detect both photoallergy and phototoxicity, and would not distinguish the two (Onoue and Tsuda, 2006; Kurita et al., 2007). Therefore, other investigators have focused on the in vitro formation of photo-antigens (Lovell and Jones, 2000). In these studies, the photobinding properties of putative photoallergens to human serum albumin have been used, similar to the approach used in the DPRA.

Endogenous Photosensitivities

Endogenous photosensitivity results from both endogenous and exogenous factors (reviewed in Bylaite et al., 2009). In a variety of genetic diseases, such as xeroderma pigmentosum, the cell's ability to repair ultraviolet light induced damage is significantly impaired. Systemic lupus erythematosus, an autoimmune connective tissue disease, features an abnormal sensitivity to UVB; however, the exact reasons are unclear. In hereditary or chemically induced porphyria, enzyme abnormalities disrupt the biosynthetic pathways that produce heme (the building block for hemoglobin, myoglobin, catalases, peroxidases, and cytochromes), leading to accumulation of protoporphyrin precursors throughout the body, including the skin, which are activated by visible light (400 to 410 nm). One such porphyria, porphyria cutanea tarda, can be precipitated by alcohol, estrogens, or certain antibiotics in individuals with hereditary abnormalities in porphyrin synthesis. Pellagra (photodermatitis, diarrhea, dementia, and death), due to alteration in tryptophan metabolism, can be induced by such drugs as isonicotinic acid and hydralazine; the treatment for pellagra is oral niacin (vitamin B3).

Other Responses to Electromagnetic Radiation

The dermatologic toxicity induced by electromagnetic radiation is not limited to the development of phototoxicity or photosensitization (reviewed in Krutmann et al., 2017). Indeed, the most evident acute feature of ultraviolet radiation exposure is erythema (redness or sunburn). The minimal erythema dose (MED), the smallest dose of ultraviolet light that induces an erythematous response, varies greatly from person to person, largely dependent upon skin pigmentation. In the sunburn reaction, vasodilatation, responsible for the color change, is accompanied by the production of a variety of inflammatory mediators from injured keratinocytes. These mediators are responsible for the systemic symptoms that may be associated with sunburn: fever, chills, and malaise. UVB (290 to 320 nm) is the most effective wavelength for inducing erythema in human skin. Substantially greater amounts of UVA reach earth compared to UVB (as much as 100-fold greater); however, UVA's efficiency in generating erythema in humans is about 1000-fold less than that of UVB. Both UVA and UVB have been implicated in the development of melanoma and non-melanoma skin cancers. Because of its wavelengths and greater depth of penetration, UVA is more responsible for some of the long-term effects of sun exposure: wrinkling, thinning (atrophy), and easy bruisability.

Overt pigment darkening is another response to ultraviolet exposure and is accomplished by enhanced melanin production by melanocytes and/or photo-oxidation of melanin. Tanning (increased pigmentation occurring within 3 days of ultraviolet exposure) is to be contrasted with the immediate pigment darkening of photo-oxidation. The tanning response is most readily produced by exposure to UVB and may be induced, along with erythema and DNA repair, by DNA damage. Tanning serves to augment the protective effects of melanin in the skin; however, this protective effect may be insufficient in preventing the damage it induces, especially in fair-skinned individuals (Sheehan et al., 2002).

Chronic exposure to radiation results in a variety of characteristic skin changes (reviewed in Krutmann et al., 2017). These changes accelerate or mimic aging, but the rate depends greatly on the baseline skin pigmentation of the individual. Lighter skin people suffer from chronic sun exposure with a greater frequency than more darkly pigmented individuals. Photo-exposed areas (head, neck, backs of hands, and the sternal area of the chest) are more readily damaged by light. Pigmentary changes, such as freckling and hypomelanotic areas (idiopathic guttate hypomelanosis), wrinkling, telangiectasias (fine dilated superficial blood vessels), pre-cancerous lesions (actinic keratoses and porokeratoses), and malignant skin lesions (basal and squamous cell carcinoma; malignant melanoma) are all consequences of chronic exposure to ultraviolet light. One significant pathophysiologic response of chronic exposure to light is a pronounced decrease in the Langerhans cells. Chronically sun exposed skin may have up to 50% fewer of these cells compared to photo-protected areas (Lim et al., 1983). This decrease may lessen immune surveillance of neoantigens on malignant cells, and may allow for such transformations to proceed unchecked. For these reasons, a tan obtained either naturally or in a tanning salon is not to be recommended (Lim et al., 2011).

Aside from the toxic nature of electromagnetic radiation, natural and environmental exposures to certain bands of light are vital for survival. Ultraviolet radiation is critical for the conversion of 7-dehydrocholesterol to pre-vitamin D3, without which normal endogenous production of vitamin D would not take place. Blue light (420 to 490 nm) can be lifesaving in premature infants due to its ability to photoisomerize bilirubin in the skin. In addition, the toxic effects of ultraviolet light have been exploited for decades through artificial light sources for treatment of skin disorders such as psoriasis.

URTICARIA

The skin is capable not only of delayed type hypersensitivity reactions involving T-cells, but also IgE-mediated reactions resulting in allergic urticaria. For those allergens to which IgE antibodies have been elicited by previous or ongoing systemic or topical exposures, subsequent cutaneous and/or systemic contact can lead to development of hives, typically in minutes, through an immediate, Gell and Coombs type I, hypersensitivity reaction. Hives are raised wheals that usually itch or sting and appear reddish. The symptoms of allergic urticaria result from degranulation of cutaneous mast cells induced by binding of IgE to receptors on the cell surface, leading to release of histamine and other vasoactive substances. Food allergies (Sicherer and Sampson, 2010) and pharmaceuticals (Limsuwan and Demoly, 2010) are major causes of acute allergic urticaria. Some agents (e.g., opiates) can bring about the direct release of histamine from mast cells without antibody mediation, while others (NSAIDs) do so through effects on arachidonic acid metabolism or other uncertain mechanisms. Urticaria lasting longer than 6 weeks, if not attributable to allergen exposure, frequently has an autoimmune basis, that is, the development of autoantibodies to the high infinity IgE receptor. True urticaria generally disappears within hours and, at the most, by 1 day. A hive persisting in a given location longer than 24 hours is more likely related to a non-urticarial process, such as vasculitis. The lifetime prevalence of urticaria has been estimated at 9%, of which nearly 2% occurs chronically (Zuberbier et al., 2010).

Contact urticaria can arise from cutaneous exposure to a plant, animal, or other protein sources (allergic), but can also have a non-allergic basis (Table 19-9). Allergic contact urticaria appears more commonly in atopic individuals and among certain occupational groups, for example, food workers due to proteins in food and the medical profession due to natural rubber latex proteins in gloves (Doutre, 2005). As opposed to the accelerators (carbamates, thiurams, benzothiazoles, and 1,3-diphenylguanidine) and anti-oxidants (*p*-phenylenediamine derivatives) that cause delayed type hypersensitivity, the hevein proteins in latex have caused severe, life threatening reactions and deaths in some individuals following cutaneous or mucosal exposure. In response to the epidemic of latex hypersensitivity, labeling for latex has been required on all medically related products in the United States. Additionally, manufactures have largely eliminated the more highly allergenic hevein proteins (Hev b1, Hev b3, Hev b5, and Hev b6.02) found in natural rubber latex from their products (Koh et al., 2005)

In addition to latex, other allergic contact urticants that result in massive IgE-mediated release of vasoactive agents from the mast cells have been reported. These include certain foods (e.g., nuts, fish, and shellfish) and pharmaceutical products (e.g., penicillins, cephalosporins, sulfonamides, macrolides, vancomycin, tetracyclines, and fluoroquinolones), among others. Insect allergens, such as those present in the stings of bees and wasps, are also known to cause an anaphylactic response.

PIGMENTARY DISTURBANCES

Hypopigmentation

Melanocytes help protect the skin from the harmful effects of ultraviolet light by producing melanin, the production of which

Table 19-9

Substances Reported to Elicit Contact Urticaria

CHEMICALLY DEFINED	CHEMICALLY UNDEFINED
Acetylsalicylic acid	**Animals:**
Acrylic monomer	Amniotic fluid
Alcohols (amyl, ethyl, benzyl, butyl, isopropyl, propyl)	Blood
	Brain
Aliphatic polyamide	Cockroaches
Alpha amylase	Hair
Aminophenazone	Dandruff
Aminothiazole	Intestine
Ammonium	Collagen
Ampicillin	Placenta
Bacitracin	Saliva
Balsam of Peru	Seminal fluid
Benzocaine	Serum
Benzoic acid	**Food:**
Benzophenone	Cheese/milk
Black rubber mix	Eggs
Butylhydroxytoluene (BHT)	Flour
Carba mix	Honey
Cellulose	Rice
Cephalosporins	**Fruits and vegetables:**
Chloramine	Beans
Chloramphenicol	Asparagus
Chlorhexidine	Carrot
Chlorocresol	Olives
Chlorpromazine	Cauliflower
Citric anhydride	Eggplant
Rosin (colophony)	Endives
Cornstarch	Pepper
Denatonium benzoate	Onion
Diethyltoluamide	Spinach
Epoxy resin	Pea
Formaldehyde and its resins	Parsley
Gentamicin	Yucca
Glucocorticoids	Watercress
Hydrolyzed wheat	Latex (cross reaction)
Iodohydroxyquinone	**Grains:**
Lidocaine	Barley
Lindane	Oats
Mechlorethamine	Rye
Menthol	Wheat
Mercaptobenzothiazole	**Meat:**
Methimazole	Beef
Mezlocillin	Chicken
Monoamylamine	Liver
Neomycin	Turkey
Nickel	Lamb
Oleyl amide	**Nuts/Seeds:**
Papain	Almond
Parabens	Peanut
para-aminodiphenylamine	Sesame
para-phenylenediamine	**Seafood:**
Penicillin	Fish
Pentamidine	**Spices:**
Phenylmercury propionate	Cilantro
Phosphorus sesquisulfide	Cumin
Platinum salts	Curry
Propylene glycol	Dill
Polysorbate	Garlic
Polyvinylpyrrolidone	Paprika
Potassium ferrocyanide	**Textiles:** silk, cotton
Promethazine	**Plants and wood:** birch, chrysanthemum, tulip, lichens, teak, mahogany
Propyphenazone	
Proteases	
Reactive paints	
Rhodium	**Cosmetics:** hair gel, henna, hydrolyzed collagen, hydrolyzed wheat, nail polish, perfume, blush
Rifampicin	
Salicylic acid	
Sodium hypochlorite	
Sodium silicate	
Sodium sulfite	
Sorbitan monolaurate	**Miscellaneous:** copy paper chemical, plastic, rubber, tinofix S
Streptomycin	
Sulfur dioxide	
Terpinyl acetate	
Vanilla	
Virginiamycin	
Vinyl pyridine	
Xylanase	
Zinc diethyldithiocarbamate	

SOURCES: Abstracted from Amaro and Goossens (2008); Amin et al. (1997); Burdick and Mathias (1985); Brancaccio and Alvarez (2004); Lahti (1986); von Krogh and Maibach (1982).

results from a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase. As indicated in Table 19-10, a variety of agents can interfere with normal pigmentation to yield either excessive or reduced amounts of melanin. Depigmentation can occur following exposure to phenols, catechols, quinones, and related compounds (McGuire and Hendee, 1971), several of which are shown in Fig. 19-5. Many chemicals inducing leukoderma (depigmentation) do so as a result of their metabolism by tyrosinase to ROS that are toxic to melanocytes. An illustration of depigmentation caused by exposure to monobenzyl ether of hydroquinone, an anti-oxidant once found in rubber gloves, is shown in Fig. 19-2G.

Other causes of toxic leukoderma may occur via effects on melanocytic signaling pathways. In case reports, the cancer chemotherapeutic drug, imatinib mesylate, has been found to give pigment loss in the epidermis, likely through an inhibition of c-kit tyrosine kinase pathway essential for melanocyte function (Arora et al., 2004). Paradoxically, imatinib mesylate may cause hyperpigmentation over the hard palate (Mattsson et al., 2011) and repigmentation of gray hair (Etienne et al., 2002).

In many parts of the world, the use of skin lightening creams containing hydroquinone, corticosteroids or mercurials is common, especially among women. These agents produce their effects by inhibiting tyrosinase activity or expression, but long-term hyperpigmentation, referred to as pseudo-ochronosis, may paradoxically occur. Furthermore, certain of these materials, especially the mercurials, pose the risk of systemic effects, such as nephropathy and neuropathy, if systemically absorbed (Ladizinski et al., 2011).

Pigmentary alterations may also be seen in the nails. Characteristic pigmentation of the nail beds has followed systemic exposure to phenolphthalein, arsenic, silver, anti-neoplastics, antimalarials, and other pharmaceutical agents. For a comprehensive review of drug-induced nail disorders, readers are referred to Valeyrie-Allanore et al. (2007).

Table 19-10

Selected Causes of Cutaneous Pigmentary Disturbances

I. **HYPERPIGMENTATION**
- Ultraviolet light exposure
- Post-inflammatory changes (melanin and/or hemosiderin deposition)
- Hypoadrenalism
- Internal malignancy
- Chemical exposures
  - Coal tar volatiles
  - Anthracene
  - Picric acid
  - Mercury
  - Lead
  - Bismuth
  - Furocoumarins (psoralens)
  - Hydroquinone (paradoxical)
- Drugs
  - Chloroquine
  - Amiodarone
  - Bleomycin
  - Zidovudine (AZT)
  - Minocycline

II. **HYPOPIGMENTATION/DEPIGMENTATION/ LEUKODERMA**
- Post-inflammatory pigmentary loss
- Chemical leukoderma/hypopigmentation
  - Hydroquinone
  - Monobenzyl, monoethyl, and monomethyl ethers of hydroquinone
  - *p*-(*t*-Butyl)phenol
  - Mercaptoamines
  - Phenolic germicides
  - *p*-(*t*-Butyl)catechols
  - Butylated hydroxytoluene
- Vitiligo (autoimmune)

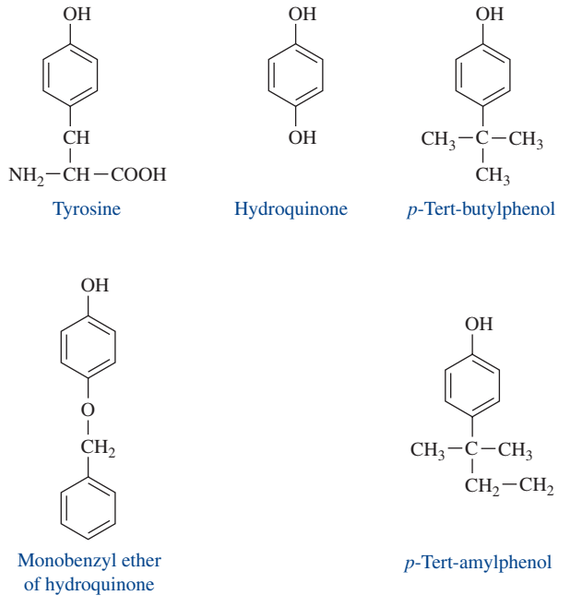


Figure 19-5. *Chemical structure of tyrosine and of selected hypopigmenting and depigmenting agents.*

Hyperpigmentation, termed post-inflammatory, is well-known to result from exposure to phototoxic agents including coal tars, furocoumarin found in certain food such as limes (see Fig. 19-2F), and azo-aniline dyes present in the printing and textile industries. Furthermore, many inflammatory conditions of the skin may result in hyperpigmentation (Fig. 19-2H). Pseudo-hyperpigmentation may also occur due to deposition of materials within the skin (mainly metals such as lead, bismuth, and arsenic). Finally, a number of therapeutic drugs and other xenobiotics may result in hyperpigmentation via their effect on synthesis/release of melanocortin peptides, tyrosinase activity, melanocyte signaling pathways, and other unknown mechanisms.

ACNE

Acne is a common affliction of the pilosebaceous units especially of the face, chest, and back. The condition arises from blockage of the duct leading from the sebaceous gland to the cutaneous surface. This results in retention of sebum and enlargement of the gland. In its most common form (acne vulgaris), hormones associated with puberty lead to high sebum production, hyperproliferation and cornification of the ductal cells, plugging of the orifice, and retention of corneocytes in the follicular lumen (Cunliffe et al., 2004). Proliferation of resident bacteria and inflammation within the blocked pore/sebaceous gland result. Topically, long chain fatty acids can give an acne-like response in animal models, and appear to do so in some individuals exposed to cosmetics. Likely acting in a similar fashion, petroleum products (oils, coal tar) in the workplace can give rise to acneiform eruptions (Ancona, 1986). Insoluble cutting oils used in the machine industry may have this effect, as illustrated in Fig. 19-2I. Such agents, which may contain chlorinated paraffins, stimulate excessive stratification of the ductile cells thus staunching the egress of sebum from the gland.

Chloracne

The most disfiguring form of acne in humans, chloracne, is caused by exposure to halogenated aromatic hydrocarbons. Chloracnegenic chemicals, such as polychlorinated biphenyls (PCBs) and naphthalenes, are often found together with the highly potent polychlorinated dibenzofuran and dioxin contaminants formed during the synthesis of these agents. Chloracne is a rare disease, but still occurs sporadically from industrial and environmental exposures (Gawkrodger et al., 2009; Passarini et al., 2010). It was seen among returning veterans during the Vietnam War following exposure to "Agent Orange." The most recent publicized example has been the poisoning of the Ukrainian President, Viktor Yushchenko; the diagnosis of chloracne and poisoning was made by a Swiss dermatologist, Jean Hilaire Saurat, and his colleagues (Sorg et al., 2009).

The recalcitrant nature of chloracne and its preventability mark it as an important occupational and environmental illness. Typically, blackheads (comedones) and cysts are present behind the ears and about the eyes, on the shoulders, back, and genitalia. As opposed to acne vulgaris, the nose is almost invariably spared. In addition to acne, increased hair in atypical locations (hypertrichosis), hyperpigmentation, brown discoloration of the nails, conjunctivitis, and eye discharge may be present. Because chloracne is a marker of systemic exposure, concurrent effects in the liver and nervous system may accompany the cutaneous findings. Chloracne exhibits progressive degeneration of the sebaceous units with transformation

of the sebocytes to keratinized cells. The prominent hyperkeratosis plugs the follicular canal resulting in acne. A proposed mechanism to account for the observed changes is that chloracnegens shift the differentiation of sebaceous follicular stem cells to that of epidermal stem cells, accounting for the lasting changes (Panteleyev and Bickers, 2006).

Since the original recognition of the syndrome a century ago, effects of chloracnegens on humans have been clearly demonstrated over the last five decades, not only during the Vietnam War, but also through industrial disasters. In 1953, a chemical plant in Ludwigshafen exploded, discharging 2,4,5-trichlorophenol; in 1976, in Seveso, Italy, a reactor explosion liberated tetrachlorodibenzo-*p*-dioxin (TCDD); and in 1968 and 1979 in Japan and Taiwan, respectively, rice cooking oil was contaminated with polychlorinated biphenyls, polychlorinated dibenzofurans, and related polychlorinated aromatic hydrocarbons. Individual poisonings involving remarkably high TCDD body burdens have not been lethal despite severe chloracne (Geusau et al., 2001; Sorg et al., 2009). Skin toxicity can occur within months after exposure and, depending on the severity, may either clear quickly or remain manifest even for decades after exposures cease, reflecting the high lipid solubility of the inciting chemicals. The highly potent TCDD has a half-life in humans of approximately 8 years (Geyer et al., 2002), and chlorinated dibenzofurans and biphenyls may be similarly persistent. Given the persistence of its inducers, the long-term consequences of chloracne have been of particular interest given speculation that individuals developing this condition may be more prone to other adverse health outcomes. So far, however, chloracne has not proven to be a biomarker for cancer (Pesatori et al., 2009).

GRANULOMATOUS DISEASE

Foreign body reactions, isolating invading substances that cannot be readily removed, occur following the introduction of a variety of foreign agents into the skin through injection or subsequent to a laceration or abrasion. These reactions can take three forms: (1) nontoxic (the mere presence of an inert substance in the dermis); (2) toxic, in which the substance induces activations of macrophages; and (3) immunologic, in which T cells are activated. Toxic and immunologic granulomas can produce persistent lesions with abundant inflammatory cells resembling chronic infectious conditions (e.g., tuberculosis, leprosy, Leishmaniasis, etc.), and can present diagnostic challenges (Del Rosario et al., 2005).

The cutaneous response to graphite intradermally injected into the skin via puncture with a "lead pencil" is a common example of the nontoxic granuloma. The resultant "carbon tattoo" may persist as a grayish dot visible on the skin surface for years. However, there is no inflammatory reaction, and, hence, no sequela following this intradermal injection.

Silica or talc (magnesium silicate) is a prime example of an agent causing toxic granulomas. After a latent period of months to years following introduction into the abdominal cavity as a result of the use of powdered surgical gloves, the original large particles desegregate to assume a colloidal state that may incite significant macrophage activation with resultant granuloma formation, which has led to bowel obstruction and other complications. The US Food and Drug Administration (FDA) issued a final rule banning powdered medical gloves beginning on January 19, 2017 (FDA, 2016). Injections of paraffin or mineral oil into the skin have long been known to induce sclerosing lipogranulomas (Heidingsfeld, 1906).

Delayed allergic sensitization (immunologic granulomas) may occur with beryllium. Analogous to the reaction in the lung, skin lesions have been reported in individuals intracutaneously exposed to beryllium. An example of beryllium granuloma of the skin is shown in Fig. 19-2J. Cutaneous gadolinium deposition and fibrosis are seen in the nephrogenic systemic fibrosis syndrome that arises following gadolinium exposure in patients with compromised renal function (Wilford et al., 2010). Metallic mercury and zirconium compounds, formerly used as deodorants, and tattoo dyes (including cobalt, chromium, mercury, and others) can also induce immunologic granulomatous reactions (Kaur et al., 2009) that, in rare cases, can be induced or intensified by intense light (laser) treatment (Tourlaki et al., 2010).

SCLERODERMATOUS SKIN DISORDERS

Scleroderma encompasses a group of rare diseases that involve the hardening and tightening of the skin and connective tissues. In some, scleroderma affects only the skin. But it may also involve structures beyond the skin, including blood vessels and internal organs (heart, lung, kidney, and digestive tract). Signs and symptoms vary, depending on which structures are affected.

Scleroderma results from the overproduction and accumulation of collagen in body tissues. What prompts abnormal collagen production is not precisely known, but the disease appears to be autoimmune. In some genetically susceptible people, symptoms may be triggered by exposure to silica, polyvinyl chloride, epoxy resins, *meta*-phenylenediamine, bleomycin, and denatured rapeseed oil. The most devastating epidemic of "toxic (rapeseed) oil syndrome" occurred in Spain in May of 1981, and is reviewed by Posada de la Paz et al. (2001).

TOXIC EPIDERMAL NECROLYSIS

Toxic epidermal necrolysis (TEN) is a rare life-threatening skin disease with an incidence of one to two cases per million person years. In patients with acquired immune deficiency syndrome (AIDS), the incidence appears to be 1000-fold higher (Harr and French, 2010). At the most severe end of the spectrum of adverse cutaneous hypersensitivity reactions to drugs, this syndrome involves detachment of ≥30% of the epidermal surface from the dermis, commonly accompanied by severe erosions of the mucous membranes. Mucous membrane involvement can result in gastrointestinal hemorrhage, respiratory failure, ocular abnormalities, and genitourinary complications. Even with intensive treatment and prompt identification of causality, it may have a fatality rate of approximately 30%.

Stevens–Johnson syndrome is a milder, but still serious, condition that has been considered by some to fall within the spectrum of TEN; however, many dermatologists consider this a separate entity induced by drugs, viruses, and other often unknown precipitating factors. In Stevens–Johnson syndrome, up to 10% of skin surface area may be affected and there is a proportionally lower fatality rate. The two syndromes may be difficult to separate when 10% to 30% of the skin surface is involved.

TEN is most commonly associated with drugs; however, the disorder has other potential etiologies, including infection, malignancy, and vaccinations. At onset, it may resemble an upper respiratory tract infection (fever, cough, sore throat, and malaise), but prompt diagnosis when the cutaneous lesions develop several days later improves survival. Ongoing exposure to drugs first encountered during the previous month should be stopped, and treatment is initiated similar to that for severe burn patients: fluid and electrolyte replacement, protection from infection, and nutritional assistance. Nearly 200 drugs have been reported to cause this syndrome with the major contributors being the anticonvulsants, NSAIDs, sulfonamides, and allopurinol.

The mechanisms leading to TEN continue to be evaluated, but are not clear. Studies of genetic predisposition have revealed striking correlation between HLAB*1502^{+} in Han Chinese patients and susceptibility to carbamazepine (Chung et al., 2004). The U.S. Food & Drug Administration recommends that Asian patients be screened for this genotype before prescribing carbamazepine (Farkas and Feeney, 2007).

TEN is an immune-related, cytotoxic T cell reaction that causes apoptosis of keratinocytes, which presumably express a foreign antigen. In many ways, it mimics a hypersensitivity reaction, with its characteristic delayed reaction to an initial exposure and a more rapid response following repeated exposure. The proposed immunopathologic pathways leading to TEN include the following: Fas ligand activation on keratinocyte membranes leading to death receptor–mediated apoptosis (Abe et al., 2003); release of destructive proteins (perforin and granzyme B) from cytotoxic T lymphocytes generated following an interaction with cells expressing major histocompatability complex (MHC) class I (Posadas et al., 2002); overproduction of T cell– and/or macrophage-derived cytokines, such as interferon-γ, tumor necrosis factor-α, and various interleukins (Paul et al., 1996; Nassif et al., 2004); and drug-induced secretion of granulysin from cytotoxic T cells, natural killer cells, and natural killer T cells (Chung et al., 2008). In addition to treating the inciting cause (usually discontinuation of a drug), blockage of death receptor ligands using intravenous immunoglobin therapy can be extremely beneficial (Ardern-Jones and Friedmann, 2011).

HAIR

Hair, like the nail, is an "appendage" of the skin. It is a keratinous protein filament that grows from follicles found in the dermis of the skin. The highly structural and organized cortex, or middle layer of the hair, is the primary source of mechanical strength and water uptake. The cortex contains melanin, which gives the hair its color. Hair is one of the defining characteristics of mammals, where it primarily has thermoregulatory and tactile (whiskers) functions. The human body, apart from areas of mucosa and glabrous skin, is covered in follicles which produce thick (terminal) and fine (vellus) hairs. Human hair growth can last from 2 to 6 years and is divided into three phases: (1) anagen (growth) lasting 2 to 6 years; (2) catagen (involution) occurring over 1 month; and telogen (the shedding phase) lasting 3 months.

Toxic effects on hair occur from systemic exposures affecting the anagen phase. The most commonly encountered toxic effect is hair loss (alopecia) and is referred to as anagen effluvium. The principal causes are systemic cancer chemotherapeutics and heavy metals (mercury, bismuth, and thallium). Indeed, thallium salts were previously used in early 20th century for the treatment of ringworm of the scalp (tinea capitis) to induce hair loss and thus cure this fungal infection (Percival, 1930). This use has long ago disappeared due to its narrow therapeutic index and the development of safer and more effective treatment modalities. Kanwar and Narang (2013) have reviewed the multiple etiologies of anagen affluvium.

While loss of hair is more frequently seen as the toxic effect of chemicals on hair, overstimulation of the anagen phase can result in excess hair (hypertrichosis). Acquired, toxic hypertrichosis, may be seen with certain drugs (minoxidil and anabolic steroids), but has also been associated with various cancers and eating disorders. Hexachlorobenzene has also been reported to cause hypertrichosis, although this is usually seen in hexachlorophene-induced porphyria cutanea tarda (see "Endogenous Photosensitivities" section, above), where hypertrichosis is one of the manifestations.

SKIN CANCER

Both ultraviolet radiation and chemical exposures can result in skin cancers. Shortly after the discovery of radioactive elements at the turn of the 20th century, it was observed that x-rays could cause severe burns, squamous cell carcinomas, and basal cell carcinomas of the skin. In addition, the linkage between chemical exposure and scrotal carcinoma was long ago recognized among chimney sweeps (see Potter, 1963 that includes Pott's original 1775 treatise).

UV-Induced Skin Cancer

Most skin cancers in the United States are UV-induced, and may be aggravated by genetic variations in the melanocortin receptor, which are associated with an increased skin cancer risk that is independent of hair type or skin color (Lynde and Sapra, 2010). The most common are the non-melanoma skin cancers, and less commonly malignant melanoma. In data summarized by the Skin Cancer Foundation (Skin Cancer Foundation: Skin Cancer Facts & Statistics, 2017), there were over 5.4 million cases of non-melanoma skin cancer in approximately 3.3 million people in the United States in 2012, the last year in which data was available. It is estimated that one in five Americans will develop skin cancer in the course of a lifetime and that 40% to 50% of Americans who live to age 65 will develop at least one basal or squamous cell carcinoma.

Basal cell carcinoma (BCC) is the most common form of skin cancer with more than 4 million cases diagnosed yearly in the United States. Squamous cell carcinoma is the second most common form with more than 1 million cases per year in the United States. Organ transplant patients, especially those undergoing renal transplantation, are approximately 100 times more likely than the general public to develop squamous cell carcinoma. Actinic keratosis is the most common pre-cancer, affecting more than 58 million Americans (Skin Cancer Foundation: Skin Cancer Facts & Statistics, 2017).

Melanoma is the most lethal form of not only skin cancer, but most other malignancies. One person dies of melanoma every hour. An estimated 87,110 new cases of invasive melanoma will be diagnosed in the United States and 9730 people will die of melanoma in 2017. Thus, although melanoma accounts for less than 1% of skin cancers, it causes most skin cancer-related deaths (Skin Cancer Foundation: Skin Cancer Facts & Statistics, 2017). The vast majority of melanoma is caused by ultraviolet radiation—both natural and artificial (tanning salons). In a UK study, 86% of melanomas could be attributed to UV exposure (Parkin et al., 2011). The estimated 5-year survival rate for patients whose melanoma is detected early is about 98% in the United States. The survival rate falls to 62% when the disease reaches the lymph nodes, and 18% when the disease metastasizes to distant organs (Skin Cancer Foundation: Skin Cancer Facts & Statistics, 2017). The risk for melanoma appears to double in individuals who have had more than five lifetime sunburns (Pfahlberg et al., 2001).

Regular daily use of an SPF 15 or higher sunscreen reduces the risk of developing squamous cell carcinoma by about 40% (Green et al., 1999) and the risk of developing melanoma by 50% (Green et al., 2011). The annual cost of treating skin cancers in the United States is estimated at $8.1 billion: about $4.8 billion for non-melanoma skin cancers and $3.3 billion for melanoma (Guy et al., 2014). In the EU, there have been intense efforts to further elucidate, prevent, and recognize that skin cancer is an occupational disease among outdoor workers (John et al., 2016).

UVB induces pyrimidine dimers and 8-oxoguanine modifications, thereby eliciting mutations in critical genes (Melnikova and Ananthaswamy, 2005; Nishigori, 2006). The p53 tumor suppressor

gene is a major target in which damage occurs and is detectable in most UV-induced squamous cells carcinomas. Because the p53 protein arrests cell cycling until DNA damage is repaired, and may also induce apoptosis, thus, its loss destabilizes the genome of initiated cells and gives them a growth advantage. UV light also has immunosuppressive effects that may help skin tumors survive. Skin cancer incidence is highest in the tropics in pale complexioned individuals, particularly at sites on the head and neck that receive the most intense exposure. Individuals with xeroderma pigmentosa, who are deficient in repair enzymes for pyrimidine dimers, must strictly avoid sun exposure to prevent the occurrence of pre-malignant and malignant lesions at an extremely early age.

Chemically Induced Skin Cancers

As previously mentioned, a landmark epidemiological investigation by Percivall Pott in 1775 connected soot with scrotal squamous cell cancer, which was prevalent among chimney sweeps in England. Since that time, substances rich in polycyclic aromatic hydrocarbons (coal tar, creosote, etc.) have become recognized as skin carcinogens both in humans and animals (Voelter-Mahlknecht et al., 2007). With their variable content of polycyclic aromatic hydrocarbons and nitrosamines, mineral oils have a long history of causing skin cancer in the occupational arena among, for example, metal workers (Tolbert, 1997). Polycyclic aromatic compounds alone are relatively inert chemically, but they can accumulate in cellular membranes and thus perturb function if not removed. They are hydroxylated by a number of cytochrome P450 isoenzymes, primarily 1A1 and 1B1 in epidermal cells, and then conjugated for disposal from the body. Oxidative biotransformation, however, produces electrophilic epoxides that can form DNA adducts. Phenols, produced by rearrangements of the epoxide, can be oxidized further to electrophilic quinones that generate ROS leading to DNA adducts, oxidized bases, and abasic sites (Park et al., 2006). Occupations at risk of skin cancer from exposure to these compounds (e.g., roofing) often involve considerable sun exposure as well. As a major regulator of induction of CYP1A1, and to a lesser extent CYP1B1, the aryl hydrocarbon receptor (AhR) plays a critical role in the response of epidermal cells to polycyclic aromatic hydrocarbons (Swanson, 2004). While the role of AhR polymorphisms in the development of skin cancers has been well demonstrated in murine models (Shimizu et al., 2000; De Souza et al., 2009), screening efforts have not confirmed that this observation is relevant to humans.

Skin Tumor Promotion

As with all cancers, the development of skin cancers can be simplistically viewed as a three-step model: initiation, promotion, and progression. Initiation is a rapid and irreversible event following limited exposure, and the cellular changes persist until cell death. Promotion is reversible and requires frequent exposure to the promoter to enhance survival of the transformed cell. Progression follows sufficient exposure to the promoter and is irreversible, leading to chromosomal rearrangements during mitosis. It is this final trigger to genetic instability that subsequently leads to a cell's malignant potential. In this model, the skin is treated once with a low dose of an initiator, such as a polycyclic aromatic hydrocarbon. However, tumors do not develop unless there is subsequent treatment with a promoter, which must be applied numerous times and at frequent intervals (e.g., twice per week for 3 months). Application of the promoter need not start immediately after initiation, but if it is not continued long enough, or if it is applied before or without the initiator, tumors do not develop. Analysis of the results with this three-stage model can be extrapolated to more complicated (and likely realistic), multi-hit, multi-stage models (Owens et al., 1995)

A number of natural products are tumor promoters, many of which alter phosphorylation pathways. The best studied example, and one of the most potent, is the active ingredient of croton oil, 12-*O*-tetradecanoylphorbol-13-acetate, which transiently stimulates then chronically depletes protein kinase C in epidermis (Fournier and Murray, 1987). A number of other phorbol esters have similar promoting effects.

Another groups of promoting agents, for example, okadaic acid, act as phosphatase inhibitors. Calcium channel modulators and free radical generators can also act as skin cancer promoters. Sensitivity to tumor promotion is an important factor in the relative sensitivity to skin carcinogenesis among different animal species (Poland et al., 1982). Much effort is currently focused on the signal transduction pathway(s) that is (are) perturbed by the treatment with these chemicals. The findings that chromate (a human lung carcinogen) in drinking water enhances tumor yield in mouse skin initiated by ultraviolet light suggest that generation of ROS can be an effective skin cancer promoter, and raises concerns about drinking water standards for hexavalent chromium in the United States (Davidson et al., 2004).

Arsenic

Arsenic is an abundant element near the earth's crust that is encountered routinely in small doses in the air, water, and food. It has long been known to affect human health (Hughes et al., 2011). In earlier times, high exposures occurred from medication (such as Fowler's solution, potassium arsenite, for asthma) and from pesticides (sodium arsenite and lead arsenite), all of which have been phased out in the United States. Around the world, substantial exposures can occur as a result of dust from mining operations, smelting fumes, or from leachate in waterways. Glass manufacturing and food dried using coal with high arsenic content can also contribute to exposures. The environmental burden is increased by disposal of large volumes of wood preserved with chromated copper arsenic, among other sources. Well water derived from geological formations with high arsenic content is now recognized as a major health concern, highlighted by populations in the tens of millions at risk in Bangladesh and India (Tapio and Grosche, 2006). However, even in the United States, arsenic in drinking water remains a concern (Mayer and Goldman, 2016).

Studies in Southwestern Taiwan have shown an association between high arsenic exposure and altered skin pigmentation, hyperkeratosis of the palms and soles, impaired circulation reflecting endothelial cell damage ("black foot" disease), and carcinoma (skin, lung, bladder, liver, and other organs). More recent concentration dependent studies in Bangladesh show hyperpigmentation and keratoses occurring with drinking water concentrations close to 50 ppb (Argos et al., 2011), which was the former U.S. drinking water standard. In 2006, the United States reduced its standard for drinking water to 10 ppb. Furthermore, there appears to be synergism between the toxic effects of arsenic and smoking (lung and bladder), sun exposure (skin), and fertilizer use (liver) (Melkonian et al., 2011).

Due to the observation that even low exposures to arsenic can have adverse health effects, much effort has been devoted to understanding its mechanism of action. Arsenic is a very weak mutagen in bacterial and mammalian cell lines, but it does induce DNA deletions and chromosome aberrations. It appears to act as a co-mutagen by interfering with DNA repair (Rossman et al., 2004).

Consistent with this view, high arsenic exposure from drinking water promotes skin lesions in chewers of betel nut (McCarty et al., 2006), a source of genotoxic nitrosamines that are activated by oral mucosal cytochrome p450 (Miyazaki et al., 2005). The ability of arsenic to inactivate poly-(ADP-ribose) polymerase-1 by binding to its zinc finger motifs may be a significant factor in this regard (Zhou et al., 2011). Generation of ROS in target cells is another proposed mechanism of arsenic's carcinogenicity, and its other pathological effects. The generation of ROS may result from activation of NADPH oxygenases (Cooper et al., 2009), damage to mitochondria (Liu et al., 2005), or, in the case of methylated forms, autoxidation (Kitchin and Ahmad, 2003). Inorganic arsenic is a transplacental carcinogen for various tissues in mice (Waalkes et al., 2004), most likely because it amplifies stem cell compartments, including those in the epidermis (Tokar et al., 2011). Consistent with this observation, arsenite derivatives prevent differentiation and preserve stem characteristics in human keratinocyte culture lines (Reznikova et al., 2010).

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REFERENCES

Abe R, Shimizu T, Shibaki A, et al. Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. *Am J Pathol*. 2003;162:1515–1520.

Ahmad N, Mukhtar H. Cytochrome p450: a target for drug development for skin diseases. *J Invest Dermatol*. 2004;123:417–425.

Akbari O, Umetsu DT. Role of regulatory dendritic cells in allergy and asthma. *Curr Allergy Asthma Rep*. 2005;5:56–61.

Almeida A, Sarmento B, Rodrigues F. Insights on in vitro models for safety and toxicity assessment of cosmetic ingredients. *Int J Pharm*. 2017;519:178–185.

Amaro C, Goossens A. Immunological occupational contact urticaria and contact dermatitis from proteins: A review. *Contact Dermatitis*. 2008;58:67–75.

Amin S, Freeman S, Maibach HI. Systemic toxicity in man secondary to percutaneous absorption. In: Roberts MS, Walters KA, eds. *Dermal Absorption and Toxicity Assessment*. New York: Marcel Dekker; 1998:103–125.

Amin S, Tanglertsampan C, Maibach HI. Contact urticatia syndrome: 1997. *Am J Contact Dermatitis*. 1997;8:15–19.

Ancona AA. Occupational acne. *Occup Med*. 1986;1:229–243.

Ardern-Jones MR, Friedmann PS. Skin manifestations of drug allergy. *Br J Clin Pharmacol*. 2011;71:672–683.

Argos M, Kalra T, Pierce BL, et al. A prospective study of arsenic exposure from drinking water and incidence of skin lesions in Bangladesh. *Am J Epidemiol*. 2011;174:185–194.

Arora B, Kumar L, Sharma A, Wadhwa J, Kochupillai V. Pigmentary changes in chronic myeloid leukemia patients treated with imatinib mesylate. *Annals Oncol*. 2004;15:358–359.

Aubin F, Mousson C. Ultraviolet light-induced regulatory (suppressor) T cells: an approach for promoting induction of operational allograft tolerance? *Transplantation*. 2004;77:S29–31.

Basketter D, Crozier J, Hubesch B, et al. Optimised testing strategies for skin sensitization—the LLNA and beyond. *Regul Toxicol Pharmacol*. 2012;64:9–16.

Basko-Plluska JL, Thyssen JP, Schalock PC. Cutaneous and systemic hypersensitivity reactions to metallic implants. *Dermatitis*. 2011;22:65–79.

Bauch C, Kolle SN, Ramirez T, et al. Putting the parts together: combining in vitro methods to test for skin sensitizing potentials. *Regul Toxicol Pharmacol*. 2012;63:489–504.

Belsito DV. The pathophysiology of allergic contact hypersensitivity. *Clin Rev Allergy*. 1989;7:347–379.

Belsito DV. Patch-testing: after 100 years, still the gold standard in diagnosing cutaneous delayed-type hypersensitivity. In: Kurth R, ed. *Regulatory Control and Standardization of Allergenic Extracts: The Eighth International Paul-Ehrlich-Seminar*. Stuttgart (Germany): Gustav Fischer; 1997:195–202.

Belsito DV. Occupational contact dermatitis: etiology, prevalence, and resultant impairment/disability. *J Am Acad Dermatol*. 2005;53:303–313.

Belsito DV. Contact dermatitis: allergic and irritant. In: Gaspari AA, Tyring SK, eds. *Clinical and Basic Immunodermatology*. London: Springer-Verlag; 2008:171–192: chap. 12.

Belsito DV, Dersarkissian RM, Thorbecke RJ, et al. Reversal by lymphokines of the age-related hyporesponsiveness to contact sensitization and reduced Ia expression on Langerhans cells. *Arch Dermatol Res*. 1987;279:S76–80.

Belsito DV, Possick LE. Age-related changes in allergic contact hypersensitivity: functional T cell deficiencies are primarily responsible. *J Invest Dermatol*. 1988;90:546.

Bornhövd E, Burgdorf WH, Wollenberg A. Macrolactam immunomodulators for topical treatment of inflammatory skin diseases. *J Am Acad Dermatol*. 2001;45:736–743.

Brancaccio RR, Alvarez MS. Contact allergy to foods. *Dermatol Ther*. 2004;17:302–313.

Buchholz BA, Fultz E, Haack KW, et al. HPLC-accelerator MS measurement of atrazine metabolites in human urine after dermal exposure. *Analyt Chem*. 1999;71:3519–3525.

Burdick AE, Mathias CGT. The contact urticaria syndrome. *Dermatol Clin*. 1985;3:71–84.

Busse KL, Maibach HI. Transdermal estradiol and testosterone transfer in man: existence, models, and strategies for prevention. *Skin Pharmacol Physiol*. 2011;24:57–66.

Bylaite M, Grigaitiene J, Lapinskaite GS. Photodermatoses: classification, evaluation and management. *Br J Dermatol*. 2009;161(suppl 3):61–68.

Cavani A. Breaking tolerance to nickel. *Toxicology*. 2005;209:119–121.

Chase MW. Inhibition of experimental drug allergy by prior feeding of the sensitizing agent. *Proc Soc Exp Biol Med*. 1946;61:257–263.

Chung WH, Hung SI, Hong HS, et al. Medical genetics: a marker for Stevens-Johnson syndrome. *Nature*. 2004;428:486.

Chung WH, Hung SI, Yang JY, et al. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. *Nat Med*. 2008;14:1343–1350.

Cooper KL, Liu KJ, Hudson LG. Enhanced ROS production and redox signaling with combined arsenite and UVA exposure: contribution of NADPH oxidase. *Free Radical Biol Med*. 2009;47:381–388.

Cunliffe WJ, Holland DB, Jeremy A. Comedone formation: etiology, clinical presentation, and treatment. *Clin Dermatol*. 2004;22:367–374.

Davidson T, Kluz T, Burns F, et al. Exposure to chromium (VI) in the drinking water increases susceptibility to UV-induced skin tumors in hairless mice. *Toxicol Appl Pharmacol*. 2004;196:431–437.

deKoven JG, Warshaw EM, Belsito DV, et al. North American contact dermatitis group patch test results 2013–2014. *Dermatitis*. 2017;28:33–46.

Deleo VA. Photocontact dermatitis. *Dermatol Ther*. 2004;17:279–288.

Del Rosario RN, Barr RJ, Graham BS, Kaneshiro S. Exogenous and endogenous cutaneous anomalies and curiosities. *Am J Dermatopathol*. 2005;27:259–267.

Desai P, Patlolla RR, Singh M. Interaction of nanoparticles and cell-penetrating peptides with skin for transdermal drug delivery. *Mol Membrane Biol*. 2010;27:247–259.

De Souza VR, Cabrera WK, Galvan A, et al. Aryl hydrocarbon receptor polymorphism modulates DMBA-induced inflammation and carcinogenesis in phenotypically selected mice. *Int J Cancer*. 2009;124:1478–1482.

Doutre MS. Occupational contact urticaria and protein contact dermatitis. *Eur J Dermatol*. 2005;15:419–424.

Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Parmacol Exp Ther*. 1944;82:377–390.

Elias PM. Role of lipids in barrier function of the skin. In: Mukhtar H, ed. *Pharmacology of the Skin*. Boca Raton, FL: CRC Press; 1992:29–38.

Elias PM. Stratum corneum defensive functions: an integrated view. *J Invest Dermatol*. 2005;125:183–200.

Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr Opin Allergy Clin Immunol*. 2009;9:437–446.

Elmets CA, Bergstresser PR, Tigelaar RE, et al. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J Exp Med*. 1983;158:781–794.

Epstein JH. Phototoxicity and photoallergy. *Semin Cutan Med Surg*. 1999;18:274–284.

Etienne G, Cony-Makhoul P, Mahon FX. Imatinib mesylate and gray hair. *N Engl J Med*. 2002;347:446.

European Council and Parliament, Regulation (EC) No. 1223/2009, 30 November, 2009. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1489942612995&uri=CELEX:32009R1223. Accessed March 13, 2017.

European Multicentre Photopatch Test Study (EMCPPTS) Taskforce. A European multicentre photopatch test study. *Br J Dermatol*. 2012;166:1002–1009.

Fan TY, Goff U, Song L, et al. N-Nitrosodiethanolamine in cosmetics, lotions and shampoos. *Food Cosmet Toxicol*. 1977;15:423–430.

Farkas R, Feeney J. Clinical review: Adverse event, Carbamazepine. 2007. Available at: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/016608s098,020712s029,021710_ClinRev.pdf. Accessed March 13, 2017.

Federal Drug Administration. Banned devices; ban powdered surgeon's gloves, powdered patient examination gloves, and absorbable powder for lubricating a surgeon's glove. Federal register: 81 FR 91722, December 19, 2016.

Fisher AA. Unique reactions of scrotal skin to topical agents. *Cutis*. 1989;44:445–447.

Fournier A, Murray AW. Application of phorbol ester to mouse skin causes a rapid and sustained loss of protein kinase C. *Nature*. 1987;330:767–769.

Friedmann PS. The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. *Br J Dermatol*. 2007;157:1093–1102.

Gawkrodger DJ, Harris G, Bojar RA. Chloracne in seven organic chemists exposed to novel polycyclic halogenated chemical compounds (triazoloquinoxalines). *Br J Dermatol*. 2009;161:939–943.

Gerberick F, Aleksic M, Basketter D, et al. Chemical reactivity measurement and the predicitve identification of skin sensitisers. The report and recommendations of ECVAM Workshop 64. *Altern Lab Anim*. 2008;36:215–242.

Gerberick GF. The use of peptide reactivity assays for skin sensitisation hazard identification and risk assessment. *Altern Lab Anim*. 2016;44:437–442.

Gerberick GF, Troutman JA, Foertsch LM, et al. Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. *Toxicol Sci*. 2009;112:164–174.

Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP. Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci*. 2004;81:332–343.

Geusau A, Abraham K, Geissler K, Sator MO, Stingl G, Tschachler E. Severe 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) intoxication: clinical and laboratory effects. *Environ Health Perspect*. 2001;109:865–869.

Geyer HJ, Schramm KW, Feicht EA, et al. Half-lives of tetra-, penta-, hexa-, hepta-, and octachlorodibenzo-p-dioxin in rats, monkeys, and humans: a critical review. *Chemosphere*. 2002;48:631–644.

Gibbs S, van de Sandt JJ, Merk HF, Lockley DJ, Pendlington RU, Pease CK. Xenobiotic metabolism in human skin and 3D human skin reconstructs: a review. *Curr Drug Metab*. 2007;8:758–772.

Girolomoni G, Gisondi P, Ottaviani C, et al. Immunoregulation of allergic contact dermatitis. *J Dermatol*. 2004;31:264–270.

Green A, Williams G, Neale R, et al. Daily sunscreen application and betacarotene supplementation in prevention of basal-cell and squamous-cell carcinomas of the skin: a randomized controlled trial. *Lancet*. 1999;354:723–729.

Green AC, Williams GM, Logan V, Strutton GM. Reduced melanoma after regular sunscreen use: randomized trial follow-up. *J Clin Oncol*. 2011;29:257–263.

Guenther D, Thomas P, Kendoff D, et al. Allergic reactions in arthroplasty: myth or serious problem? *Int Orthop*. 2016;40:239–244.

Guy GP, Machlin SR, Ekwueme DU, Yabroff KR. Prevalence and costs of skin cancer treatment in the U.S., 2002-2006 and 2007-2011. *Am J Prev Med*. 2014;104(4):e69–e74.

Guy RH. Predicting the rate and extent of fragrance chemical absorption into and through the skin. *Chem Res Toxicol*. 2010a;23:864–870.

Guy RH. Transdermal drug delivery. In: Schäfer-Korting M, ed. *Drug Delivery, Handbook of Experimental Pharmacology*. Vol 197. Berlin, Germany: Springer Verlag; 2010b:399–410.

Haranosono Y, Kurata M, Sakaki H. Establishment of an in silico phototoxicity prediction method by combining descriptors related to photoabsorption and photo-reaction. *J Toxicol Sci*. 2014;39:655–664.

Harr T, French LE. Toxic epidermal necrolysis and Stevens-Johnson syndrome. *Orphanet J Rare Dis*. 2010;5:39.

Heidingsfeld ML. Histopathology of paraffin prosthesis. *J Cutan Dis*. 1906;24:513–521.

Henry B, Foti C, Alsante K. Can light absorption and photostability data be used to assess the photosafety risks in patients for a new drug molecule? *J Photchem Photobiol B*. 2009;96:57–62.

Herold DA, Rodeheaver GT, Bellamy WT, et al. Toxicity of topical polyethylene glycol. *Toxicol Appl Pharmacol*. 1982;65:329–335.

Honari G. Photoallergy. *Rev Environ Health*. 2014;29:233–242.

Hoya M, Hirota M, Suzuki M, et al. Development of an in vitro photosensitization assay using human monocyte-derived cells. *Toxicol In Vitro*. 2009;23:911–918.

Hughes MF, Beck BD, Chen Y, Lewis AS, Thomas DJ. Arsenic exposure and toxicology: a historical perspective. *Toxicol Sci*. 2011;123:305–332.

Hu T, Khambatta ZS, Hayden PJ, et al. Xenobiotic metabolism gene expression in the EpiDermin vitro 3D human epidermis model compared to human skin. *Toxicol In Vitro*. 2010;24:1450–1463.

Jadassohn J. Zur Kenntnis der medikamentössen Dermatosen. In: Jarisch A, Neisser A, eds. *Verhandlungen der Deutschen Dermatologischen Gesellschaft* Graz (Austria): V. Kongress; 1895:103–129.

John SM, Trakatelli M, Gehring R, et al. Consensus Report: recognizing non-melanoma skin cancer, including actinic keratosis, as an occupational disease—a call to action. *J Eur Acad Dermatol Venereol*. 2016;30(suppl 3):38–45.

Kanwar AJ, Narang T. Anagen effluvium. *Indian J Dermatol Venereol Leprol*. 2013;79:604–12.

Kao J, Hall J. Skin absorption and cutaneous first pass metabolism of topical steroids: in vitro studies with mouse skin in organ culture. *J Pharmacol Exp Ther*. 1987;241:482–487.

Kaur RR, Kirby W, Maibach H. Cutaneous allergic reactions to tattoo ink. *J Cosmet Dermatol*. 2009;8:295–300.

Khan WA, Das M, Stick S, Javed S, Bickers DR, Mukhtar H. Induction of epidermal NAD(P)H:quinone reductase by chemical carcinogens: a possible mechanism for the detoxification. *Biochem Biophys Res Commun*. 1987;146:126–133.

Kitchin KT, Ahmad S. Oxidative stress as a possible mode of action for arsenic carcinogenesis. *Toxicol Lett*. 2003;137:3–13.

Koh D, Ng V, Leow Y-H, Goh CL. A study of natural rubber latex allergens in gloves used by healthcare workers in Singapore. *B J Dermatol*. 2005;153:954–959.

Krutmann J, Bouloc A, Sore G, Bernard BA, Passeron T. The skin aging exposome. *J Dermatol Sci*. 2017;85:152–161.

Kurita M, Shimauchi T, Kobayashi M, et al. Induction of keratinocyte apoptosis by photosensitizing chemicals plus UVA. *J Dermatol Sci*. 2007;45:105–112.

Kwangsukstith C, Maibach HI. Effect of age and sex on the induction and elicitation of allergic contact dermatitis. *Contact Dermatitis*. 1995;33:289–298.

Kynemund Pedersen L, Agner T, Held E, Johansen JD. Methyldibromoglutaronitrile in leave-on products elicits contact allergy at low concentration. *Br J Dermatol*. 2004;151:817–822.

Ladizinski B, Mistry N, Kundu RV. Widespread use of toxic skin lightening compounds: medical and psychosocial aspects. *Dermatol Clin*. 2011;29:111–123.

Lahti A. Contact urticaria to plants. *Clin Dermatol*. 1986;4:127–136.

Liang Y, Chang C, Lu Q. The genetics and epigenetics of atopic dermatitis—filaggrin and other polymorphisms. *Clin Rev Allergy Immunol*. 2016;51:315–328.

Lim HW, James WD, Rigel DS, Maloney ME, Spencer JM, Bhushan R. Adverse effects of ultraviolet radiation from the use of indoor tanning equipment: time to ban the tan. *J Am Acad Dermatol*. 2011;64:893–902.

Lim HW, Thorbecke GJ, Baer RL, Gigli I. Effect of indomethacin on alteration of ATPase-positive Langerhans cell density and cutaneous sunburn reaction induced by ultraviolet-B radiation. *J Invest Dermatol*. 1983;81:455–458.

Limsuwan T, Demoly P. Acute symptoms of drug hypersensitivity (urticaria, angioedema, anaphylaxis, anaphylactic shock). *Med Clin North Am*. 2010;94:691–710.

Liu SX, Davidson MM, Tang X, et al. Mitochondrial damage mediates genotoxicity of arsenic in mammalian cells. *Cancer Res*. 2005;65:3236–3242.

Lovell WW, Jones PA. Evaluation of mechanistic in vitro tests for the discrimination of photoallergic and photoirritant potential. *Altern Lab Anim*. 2000;28:707–724.

Luu-The V, Duche D, Ferraris C, Meunier JR, Leclaire J, Labrie F. Expression profiles of phases 1 and 2 metabolizing enzymes in human skin and the reconstructed skin models Episkin and full thickness model from Episkin. *J Steroid Biochem Mol Biol*. 2009;116:178–186.

Lynde CW, Sapra S. Predictive testing of the melanocortin 1 receptor for skin cancer and photoaging. *Skin Therapy Lett*. 2010;15:5–7.

Magnusson B, Kligman AM. The identification of contact allergens by animal assay. The guinea pig maximization test. *J Invest Dermatol*. 1969;52:268–276.

Marcussen PV. Primary irritant patch-test reactions in children. *Arch Dermatol*. 1963;87:378–382.

Mark BJ, Slavin RG. Allergic contact dermatitis. *Med Clin North Am*. 2006;90:169–185.

Marker O, Thomsen AR. T-cell effector function and unresponsiveness in the murine lymphocytic choriomeningitis virus infection. I. On the mechanism of a selective suppression of the virus-specific delayed-type hypersensitivity response. *Scand J Immunol*. 1986;24:127–135.

Martin-Bouyer G, Lebreton R, Toga M, Stolley PD, Lockhart J. Outbreak of accidental hexachlorophene poisoning in France. *Lancet*. 1982;1:91–95.

Mattsson U, Halbritter S, Morner Serikoff E, Christerson L, Warfvinge G. Oral pigmentation in the hard palate associated with imatinib mesylate therapy: a report of three cases. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2011;111:e12–e16.

Maxwell G, MacKay C, Cubberley R, et al. Applying the skin sensitisation adverse outcome pathway (AOP) to quantitative risk assessment. *Toxicol In Vitro*. 2014;28:8–12.

Mayer JE, Goldman RH. Arsenic and skin cancer in the USA: the current evidence regarding arsenic-contaminated drinking water. *Int J Dermatol*. 2016;55:e585–e591.

McCarty KM, Houseman EA, Quamruzzaman Q, et al. The impact of diet and betel nut use on skin lesions associated with drinking-water arsenic in Pabna, Bangladesh. *Environ Health Perspect*. 2006;114:334–340.

McGuire J, Hendee J. Biochemical basis for depigmentation of skin by phenolic germicides. *J Invest Dermatol*. 1971;57:256–261.

McKnight RH, Spiller HA. Green tobacco sickness in children and adolescents. *Public Health Rep*. 2005;120:602–605.

McNamee PM, Api AM, Basketter DA, et al. A review of critical factors in the conduct and interpretation of the human repeat insult patch test. *Regul Toxicol Pharmacol*. 2008;52:24–34.

Melkonian S, Argos M, Pierce BL, et al. A prospective study of the synergistic effects of arsenic exposure and smoking, sun exposure, fertilizer use, and pesticide use on risk of premalignant skin lesions in Bangladeshi men. *Am J Epidemiol*. 2011;173:183–191.

Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol*. 2004;4:762–774.

Melnikova VO, Ananthaswamy HN. Cellular and molecular events leading to the development of skin cancer. *Mutation Res*. 2005;571:91–106.

Miyazaki M, Sugawara E, Yoshimura T, Yamazaki H, Kamataki T. Mutagenic activation of betel quid-specific *N*-nitrosamines catalyzed by human cytochrome P450 coexpressed with NADPH-cytochrome P450 reductase in *Salmonella typhimurium* YG7108. *Mutation Res*. 2005;581:165–171.

Moan J, Peng Q. An outline of the hundred-year history of PDT. *Anticancer Res*. 2003;23:3591–3600.

Murahata RI, Crowe DM, Roheim JR. The use of transepidermal water loss to measure and predict the irritation response to surfactants. *Int J Cosmet Sci*. 1986;8:225–231.

Nassif A, Moslehi H, Le Gouvello S, et al. Evaluation of the potential role of cytokines in toxic epidermal necrolysis. *J Invest Dermatol*. 2004;123:850–855.

Natsch A, Ryan CA, Foertsch L, et al. A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *J Appl Toxicol*. 2013;33:1337–1352.

National Institute for Occupational Safety and Health. Workplace Safety & Health Topics: skin exposures & effects. Available at: https://www.cdc.gov/niosh/topics/skin/default.html. Accessed March 13, 2017a.

National Institute for Occupational Safety and Health. Workplace Safety & Health Topics: Skin Notation (SK) Profiles. Available at: https://www.cdc.gov/niosh/topics/skin/skin-notation_profiles.html. Accessed March 13, 2017b.

Nethercott JR, Holness DL. Disease outcome in workers with occupational skin disease. *J Am Acad Dermatol*. 1994;30:569–574.

Netzlaff F, Lehr CM, Wertz PW, Schaefer UF. The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *Eur J Pharm Biopharm*. 2005;60:167–178.

Nishigori C. Cellular aspects of photocarcinogenesis. *Photochem Photobiol Sci*. 2006;5:208–214.

Nossal GJ. Molecular and cellular aspects of immunologic tolerance. *Eur J Biochem*. 1991;202:729–737.

Oesch F, Fabian E, Oesch-Bartlomowicz B, Werner C, Landsiedel R. Drug-metabolizing enzymes in the skin of man, rat, and pig. *Drug Metab Rev*. 2007;39:659–698.

Onoue S, Tsuda Y. Analytical studies on the prediction of photosensitive/phototoxic potential of pharmaceutical substances. *Pharm Res*. 2006;23:156–164.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 428: skin absorption: in vitro method. Available at: http://www.oecd-ilibrary.org/environment/test-no-428-skin-absorption-in-vitro-method_9789264071087-en. Accessed March 13, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 430: in vitro skin corrosion: transcutaneous electrical resistance test method (TER). Available at: http://www.oecd-ilibrary.org/environment/test-no-430-in-vitro-skin-corrosion-transcutaneous-electrical-resistance-test-method-ter_9789264203808-en. Accessed March 13, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 431: in vitro skin corrosion: human skin model test. Available at: http://www.oecd-ilibrary.org/environment/test-no-431-in-vitro-skin-corrosion-human-skin-model-test_9789264071148-en. Accessed March 31, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 432: in vitro 3T3 NRU phototoxicity test. Available at: http://www.oecd-ilibrary.org/content/book/9789264071162-en. Accessed March 13, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 435: in vitro membrane barrier test method for skin corrosion. Available at: http://www.oecd-ilibrary.org/environment/test-no-435-in-vitro-membrane-barrier-test-method-for-skin-corrosion_9789264242791-en. Accessed March 13, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 439: in vitro skin irritation: reconstructed human epidermis test method. Available at: http://www.oecd-ilibrary.org/environment/test-no-439-in-vitro-skin-irritation-reconstructed-human-epidermis-test-method_9789264242845-en. Accessed March 13, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 442C: in chemico skin sensitisation, direct peptide reactivity assay (DPRA). Available at: http://www.oecd-ilibrary.org/environment/test-no-442c-in-

chemico-skin-sensitisation_9789264229709-en. Accessed March 13, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 442D: in vitro skin sensitisation are-nrf2 luciferase test method. Available at: http://www.oecd-ilibrary.org/environment/test-no-442d-in-vitro-skin-sensitisation_9789264229822-en. Accessed March 13, 2017.

Organisation for Economic Co-operation and Development. OECD guidelines for the testing of chemicals, Section 4, Test No. 442E: in vitro skin sensitisation human cell line activation test (h-CLAT). Available at: http://www.oecd-ilibrary.org/content/book/9789264264359-en. Accessed March 13, 2017.

Otberg N, Patzelt A, Rasulev U, et al. The role of hair follicles in the percutaneous absorption of caffeine. *Br J Clin Pharmacol*. 2008;65:488–492.

Owens DM, Spalding JW, Tennant RW, Smart RC. Genetic alterations cooperate with v-Ha-ras to accelerate multistage carcinogenesis in Tg.AC transgenic mouse skin. *Cancer Res*. 1995;55:3171–3178.

Palmer CN, Irvine AD, Terron-Kwiatkowski A, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet*. 2006;38:441–446.

Panteleyev AA, Bickers DR. Dioxin-induced chloracne–reconstructing the cellular and molecular mechanisms of a classic environmental disease. *Exp Dermatol*. 2006;15:705–730.

Park JH, Troxel AB, Harvey RG, Penning TM. Polycyclic aromatic hydrocarbon (PAH) *o*-quinones produced by the aldo-keto-reductases (AKRs) generate abasic sites, oxidized pyrimidines, and 8-oxo-dGuo via reactive oxygen species. *Chem Res Toxicol*. 2006;19:719–728.

Parkin DM, Mesher D, Sasieni P. Cancers attributable to solar (ultraviolet) radiation exposure in the UK in 2010. *Br J Cancer*. 2011;105:S66–S69.

Pascoe D, Moreau L, Sasseville D. Emergent and unusual allergens in cosmetics. *Dermatitis*. 2010;21:127–137.

Passarini B, Infusino SD, Kasapi E. Chloracne: still cause for concern. *Dermatology*. 2010;221:63–70.

Patlewicz G, Casati S, Basketter DA, et al. Can currently available non-animal methods detect pre and pro-haptens relevant for skin sensitization? *Regul Toxicol Pharmacol*. 2016;82:147–155.

Patlewicz G, Kuseva C, Kesova A, et al. Towards AOP application--implementation of an integrated approach to testing and assessment (IATA) into a pipeline tool for skin sensitization. *Regul Toxicol Pharmacol*. 2014;69:529–545.

Paul C, Wolkenstein P, Adle H, et al. Apoptosis as a mechanism of keratinocyte death in toxic epidermal necrolysis. *Br J Dermatol*. 1996;134:710–714.

Percival GH. The treatment of ringworm of the scalp with thallium acetate. *Br J Dermatol*. 1930;42:59–69.

Pesatori AC, Consonni D, Rubagotti M, Grillo P, Bertazzi PA. Cancer incidence in the population exposed to dioxin after the "Seveso accident": twenty years of follow-up. *Environ Health*. 2009;8:39.

Pfahlberg A, Kolmel K-F, Gefeller O. Timing of excessive ultraviolet radiation and melanoma: epidemiology does not support the existence of a critical period of high susceptibility to solar ultraviolet radiation-induced melanoma. *Brit J Dermatol*. 2001;144(3):471–475.

Poland A, Palen D, Glover E. Tumour promotion by TCDD in skin of HRS/J hairless mice. *Nature*. 1982;300:271–273.

Pols DH, Wartna JB, Moed H, et al. Atopic dermatitis, asthma and allergic rhinitis in general practice and the open population: a systematic review. *Scand J Prim Health Care*. 2016;34:143–150.

Portes P, Grandidier MH, Cohen C, Roguet R. Refinement of the Episkin protocol for the assessment of acute skin irritation of chemicals: follow-up to the ECVAM prevalidation study. *Toxicol In Vitro*. 2002;16:765–770.

Posada de la Paz M, Philen RM, Borda AI. Toxic oil syndrome: the perspective after 20 years. *Epidemiol Rev*. 2001;23:231–247.

Posadas SJ, Padial A, Torres MJ, et al. Delayed reactions to drugs show levels of perforin, granzyme B, and Fas-L to be related to disease severity. *J Allergy Clin Immunol*. 2002;109:155–161.

Potter M. Percivall Pott's contribution to cancer research. *Natl Cancer Inst Monogr*. 1963;10:1–13.

Potts RO, Guy RH. Predicting skin permeability. *Pharm Res*. 1992;9:663–669.

Prausnitz MR, Mikszta JA, Cormier M, Andrianov AK. Microneedle-based vaccines. *Curr Top Microbiol Immunol*. 2009;333:369–393.

Prieto P, Cole T, Curren R, et al. Assessment of the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity test method to identify substances not classified for acute oral toxicity (LD50 >2000 mg/kg): results of an ECVAM validation study. *Reg Toxicol Pharmacol*. 2013;65:344–365.

Reznikova TV, Phillips MA, Patterson TJ, Rice RH. Opposing actions of insulin and arsenite converge on PKCdelta to alter keratinocyte proliferative potential and differentiation. *Mol Carcinog*. 2010;49:398–409.

Rietschel RL, Rosenthal LE, Adams RM, et al. Standard patch test screening series used diagnostically in young and elderly patients. *Am J Contact Dermatitis*. 1990;1:53–55.

Riviere JE, Brooks JD. Predicting skin permeability from complex chemical mixtures. *Toxicol Appl Pharmacol*. 2005;208:99–110.

Rolsted K, Kissmeyer AM, Rist GM, Hansen SH. Evaluation of cytochrome P450 activity in vitro, using dermal and hepatic microsomes from four species and two keratinocyte cell lines in culture. *Arch Dermatol Res*. 2008;300:11–18.

Rossman TG, Uddin AN, Burns FJ. Evidence that arsenite acts as a cocarcinogen in skin cancer. *Toxicol Appl Pharmacol*. 2004;198:394–404.

Scheuplein RJ, Blank IH. Permeability of the skin. *Physiol Rev*. 1971;51:702–747.

Schubert H, Berova N, Czernielewski A, et al. Epidemiology of nickel allergy. *Contact Dermatitis*. 1987;16:122–128.

Scientific Committee for Consumer Safety. The SCCS notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 9th revision (SCCS/1514/15, revised 25 April, 2016). Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_190.pdf. Accessed March 13, 2017.

Seligman PJ, Mathias CG, O'Malley MA, et al. Phytophotodermatitis from celery among grocery store workers. *Arch Dermatol*. 1987;123:1478–1482.

Seto Y, Ohtake H, Kato M, Onoue S. Development of fluorometric reactive oxygen species assay for photosafety evaluation. *Toxicol In Vitro*. 2016;34:113–119.

Shackelford K, Belsito DV. The etiology of allergic appearing foot dermatitis: a five year retrospective study. *J Am Acad Dermatol*. 2002;47:715–721.

Sheehan JM, Cragg N, Chadwick CA, Potten CS, Young AR. Repeated ultraviolet exposure affords the same protection against DNA photodamage and erythema in human skin types II and IV but is associated with faster DNA repair in skin type IV. *J Invest Dermatol*. 2002;118:825–829.

Shimizu Y, Nakatsuru Y, Ichinose M, et al. Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proc Natl Acad Sci U S A*. 2000;97:779–782.

Sicherer SH, Sampson HA. Food allergy. *J Allergy Clin Immunol*. 2010;125:S116–S125.

Skin Cancer Foundation: Skin Cancer Facts & Statistics. 2017. Available at: http://www.skincancer.org/skin-cancer-information/skin-cancer-facts. Accessed March 14, 2017.

Skog E. The influence of pre-exposure to alkyl benzene sulphonate detergent, soap and acetone on primary irritant and allergic eczematous reactions. *Acta Derm Venereol (Stockh)*. 1958;38:1–9.

Sorg O, Zennegg M, Schmid P, et al. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) poisoning in Viktor Yushchenko: identification and measurement of TCDD metabolites. *Lancet*. 2009;374:1179–1185.

Stern RS. Risks of cancer associated with long-term exposure to PUVA in humans: current status–1991. *Blood Cells*. 1992;18:91–97.

Stern RS. PUVA Follow up Study: The risk of melanoma in association with long-term exposure to PUVA. *J Am Acad Dermatol*. 2001;44:755–761.

Strauss HW. Artificial sensitization of infants to poison ivy. *J Allergy*. 1931;2:137–146.

Sulzberger MB. Hypersensitiveness to arsphenamine in guinea pigs. *Arch Dermatol*. 1929;20:669–673.

Svensson CK. Biotransformation of drugs in human skin. *Drug Metab Dispos*. 2009;37:247–253.

Swanson HI. Cytochrome P450 expression in human keratinocytes: an aryl hydrocarbon receptor perspective. *Chem Biol Interact*. 2004;149:69–79.

Tapio S, Grosche B. Arsenic in the aetiology of cancer. *Mutat Res*. 2006;612:215–246.

Tokar EJ, Qu W, Waalkes MP. Arsenic, stem cells, and the developmental basis of adult cancer. *Toxicol Sci*. 2011;120:S192–S203.

Tokura Y. Immune responses to photohaptens: implications for the mechanisms of photosensitivity to exogenous agents. *J Dermatol Sci*. 2000;23(suppl 1):S6–S9.

Tokura Y, Nishijima T, Yagi H, Furukawa F, Takigawa M. Photohaptenic properties of fluoroquinolones. *Photochem Photobiol*. 1996;64:838–844.

Tolbert PE. Oils and cancer. *Cancer Causes Control*. 1997;8:386–405.

Tollefsen KE, Scholz S, Cronin MT, et al. Applying Adverse Outcome Pathways (AOPs) to support Integrated Approaches to Testing and Assessment (IATA). *Regul Toxicol Pharmacol*. 2014;70:629–640.

Tomb RR. Patch testing with frullania during a 10-year period: hazards and complications. *Contact Dermatitis*. 1992;26:220–223.

Tourlaki A, Boneschi V, Tosi D, Pigatto P, Brambilla L. Granulomatous tattoo reaction induced by intense pulse light treatment. *Photodermatol Photoimmunol Photomed*. 2010;26:275–276.

Tsujita-Inoue K, Hirota M, Atobe T, Ashikaga T, Tokura Y, Kouzuki H. Development of novel in vitro photosafety assays focused on the Keap1-Nrf2-ARE pathway. *J Appl Toxicol*. 2016;36:956–968.

Urbisch D, Mehling A, Guth K, et al. Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regul Toxicol Pharmacol*. 2015;71:337–351.

Urwin R, Wilkinson M. Methylchloroisothiazolinone and methylisothiazolinone contact allergy: a new 'epidemic'. *Contact Dermatitis*. 2013;68:253–255.

Uter W, Johansen JD, Orton DI, Frosch PJ, Schnuch A. Clinical update on contact allergy. *Curr Opin Allergy Clin Immunol*. 2005;5:429–436.

Valeyrie-Allanore L, Sassolas B, Roujeau JC. Drug-induced skin, nail and hair disorders. *Drug Saf*. 2007;30:1011–1030.

Victor FC, Cohen DE, Soter NA. A 20-year analysis of previous and emerging allergens that elicit photoallergic contact dermatitis. *J Am Acad Dermatol*. 2010;62:605–610.

Voelter-Mahlknecht S, Scheriau R, Zwahr G, et al. Skin tumors among employees of a tar refinery: the current data and their implications. *Int Arch Occup Environ Health*. 2007;80:485–495.

von Krogh G, Maibach HI. The contact urticaria syndrome – 1982. *Semin Dermatol*. 1982;1:59–66.

Waalkes MP, Liu J, Ward JM, Diwan BA. Animal models for arsenic carcinogenesis: inorganic arsenic is a transplacental carcinogen in mice. *Toxicol Appl Pharmacol*. 2004;198:377–384.

Walker FB, Smith PD, Maibach HI, et al. Genetic factors in human allergic contact dermatitis. *Int Arch Allergy Appl Immunol*. 1967;32:453–462.

Walsh AA, deGraffenried LA, Rice RH. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin sensitization of cultured human epidermal cells to carcinogenic heterocyclic amine toxicity. *Carcinogenesis*. 1995;16:2187–2191.

Warshaw EM, Kingsley-Loso JL, deKoven JG, et al. Body piercing and metal allergic contact sensitivity: North American Contact Dermatitis Group data from 2007 to 2010. *Dermatitis*. 2014;25:255–264.

Watkins SA, Maibach HI. The hardening phenomenon in irritant contact dermatitis: an interpretative update. *Contact Dermatitis*. 2009;60:123–130.

Wilford C, Fine JD, Boyd AS, Sanyal S, Abraham JL, Kantrow SM. Nephrogenic systemic fibrosis: report of an additional case with granulomatous inflammation. *Am J Dermatopathol*. 2010;32:71–75.

Zhai H, Fautz R, Fuchs A, Bhandarkar S, Maibach HI. Human scalp irritation compared to that of the arm and back. *Contact Dermatitis*. 2004;51:196–200.

Zhou X, Sun X, Cooper KL, Wang F, Liu KJ, Hudson LG. Arsenite interacts selectively with zinc finger proteins containing C3H1 or C4 motifs. *J Biol Chem*. 2011;286:22855–22863.

Zuberbier T, Balke M, Worm M, Edenharter G, Maurer M. Epidemiology of urticaria: a representative cross-sectional population survey. *Clin Exp Dermatol*. 2010;35:869–873.

Zug KA, Pham AK, Belsito DV, et al. Patch testing in children from 2005 to 2012: results from the North American Contact Dermatitis Group. *Dermatitis*. 2014;25:345–355.

chapter 20

Toxic Responses of the Endocrine System

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INTRODUCTION

Higher animals, including humans, have developed the ability to regulate their internal environment, independent of wide fluctuations in external factors, in the form of endocrine systems. An endocrine system consists of (1) an endocrine gland that secretes a hormone, (2) the hormone itself, and (3) a target tissue that responds to the hormone. The classical definition of a hormone is "a chemical substance produced by a ductless endocrine gland and secreted into the blood, which carries it to a specific target organ to produce an effect." In addition to the humoral communication regulated by endocrine systems, the nervous system also regulates overall bodily functions. The two systems are intimately interconnected and normally work in close concert. One direct point at which the two systems interface involves "neuroendocrine" cells, which are special types of neurons capable of secreting humoral substances (hormones) in response to synaptic input (neurotransmitters).

The hormone-producing glands of humans include the pituitary (hypophysis), the thyroid and parathyroids, the adrenals, the gonads, and the pancreas. The mechanisms by which endocrine glands synthesize, store, and secrete hormones depend on the chemical properties of the hormone. There are primarily three chemical classes of hormones: amino acid derivatives, peptides and proteins, and steroids. The amino acid derivatives include the catecholamines, epinephrine, and norepinephrine (produced in the adrenal medulla), and the thyroid hormones, triiodothyronine (T_3), and thyroxine (T_4). A large number of hormones are peptides or proteins, such as the neurohormones of the posterior pituitary, tropic hormones of the anterior pituitary, and pancreatic hormones. Steroids are produced by the adrenal cortex, the testes, the ovaries, and in pregnancy, the placenta. These hormones are derivatives of cholesterol. In general, designation of a hormone as hydrophilic (peptides and proteins, catecholamines) or hydrophobic (thyroid hormones, vitamin D, steroids) provides information useful in understanding the synthesis, secretion, transport, and target cell mechanism of action of that particular hormone (Table 20-1).

Circulating hormone levels are influenced by the rate of secretion from the endocrine gland, rate of metabolism, and method by which hormone is transported in the blood. The rate of secretion is regulated in the endocrine gland by a variety of physiological factors. The rate of hormonal metabolism or degradation in the bloodstream determines its half-life ($t_{1/2}$) in blood. The cells of the endocrine gland typically contain only a small store of synthesized hormone, although one noted exception is the thyroid that stores

Table 20-1

Groups of Hormones

ROLE	HYDROPHILIC	HYDROPHOBIC (LIPOPHILIC)
Endocrine gland cellular storage	Yes	No
Secretion	Secretory vesicles	Passive diffusion
Transport in blood	Free*	Protein bound
Target organ receptors	Plasma membrane	Nuclear

**Catecholamines are transported on albumin.*

hormone for several months. Therefore, in response to a specific stimulus, the cells synthesize and secrete hormones directly into the bloodstream where they are normally found in very low concentrations (10^{-8} to 10^{-12} M). Although most hormones are carried by the blood, they are not all transported in the same way. Hydrophilic hormones (peptides and proteins) are freely dissolved in plasma and do not require a specialized transport system. In contrast, given the limited aqueous solubilities of hydrophobic hormones (steroids, thyroid hormones), it is not possible for them to exist free in solution in the aqueous environment of the blood so they circulate bound to specialized serum binding proteins or to albumin. The two major functions of serum binding proteins are to (1) solubilize the hormone for transport in the blood, and (2) extend the life of the hormone in circulation ($t_{1/2}$) by protecting the small molecules from enzymatic degradation in the blood stream. The extent to which a hydrophobic hormone is protein bound varies from one hormone to another. However, in all cases, the total hormone pool is predominantly in the bound form. This is important because it is generally accepted that only the pool of free hormone is biologically active. Therefore, binding proteins also provide a large hormone reserve that can be called upon to replenish the free pool. It is the magnitude of the free pool rather than the total pool of hormone that is monitored and adjusted to maintain normal endocrine function.

Hormones elicit a wide variety of biological responses. For virtually all hormones, the initial step in this process is binding of the hormone to a receptor in its target tissue. Those receptors can be located on the plasma membrane (peptide, protein, and catecholamine hormones) or have their action in the cellular nucleus (steroids, thyroid, and vitamin D hormones). The interaction of the hormone with its receptor initiates a chain of intracellular events leading to the physiological response specific to that hormone. For each endocrine system in this chapter, the sections first briefly describe the anatomy and physiology of the normal endocrine gland because such a background is important for understanding the effects of xenobiotics on that gland. This is followed by a description of some effects of environmental chemicals on various aspects of the system's function. Mechanistic information is included whenever possible to aid in the interpretation of findings and to assess their potential for human risk.

PITUITARY GLAND

The pituitary may be divided into four major subdivisions. The pars distalis (adenohypophysis or anterior pituitary) forms the largest subdivision. The pars intermedia forms the thin cellular zone between the adenohypophysis and neurohypophysis. The pars

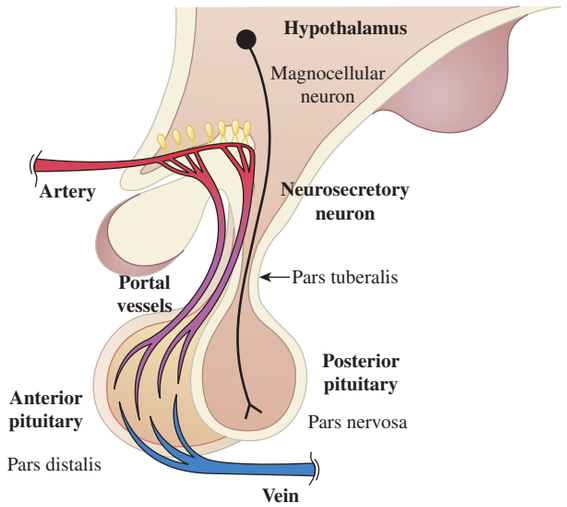

Figure 20-1. *Communication between the hypothalamus and the pituitary.* The anterior pituitary receives endocrine input from the hypothalamus in the form of peptide releasing hormones, which are transported in portal vessels to stimulate release of hormones in the anterior pituitary cells. The posterior pituitary is innervated by axons extending from magnocellular neurons with their cell bodies located in the hypothalamus. Stimulation of these cell bodies results in hormonal secretion in the posterior pituitary (Modified from Hedge et al. [1987].)

tuberalis (stalk) is a very small subdivision, highly vascularized, and is not known to secrete hormones. Finally, the pars nervosa (neurohypophysis or posterior pituitary) forms the second largest subdivision of the pituitary. There are two basic types of communication between the hypothalamus and pituitary (Fig. 20-1). (1) The adenohypophysis receives vascular supply from the median eminence. The communication between the two is endocrine in nature in that peptide hormones are released in nerve endings of tuberoinfundibular neurons, which are neuroendocrine cells located in the median eminence. Once secreted, the peptides are transported in the blood via the pars tuberalis to the anterior pituitary. (2) The neurohypophysis receives neuronal communication from the supraoptic nucleus (SON) and paraventricular nucleus (PVN). The type of communication is neuroendocrine, the cell bodies are located in the SON and PVN, and their axons stretch through the median eminence of the hypothalamus, span the infundibular stalk, and terminate in the posterior lobe. These cells are called magnocellular neurons and are neuroendocrine cells. Therefore, functionally and anatomically, the posterior pituitary is an extension of the hypothalamus. In fact, the hormones produced in the posterior pituitary are actually synthesized in the neuroendocrine cell bodies located in the hypothalamus (SON and PVN).

There are a variety of mechanisms that regulate the rate of release of hormones in the neuroendocrine pathway. Superimposed on endogenous rhythms of hormone release are a number of feedback mechanisms. To maintain appropriate homeostasis, the endocrine organ must constantly monitor systemic hormone concentrations, or some function of it. This awareness is provided in the form of negative feedback loops. By this mechanism, hormones produced in the target organ feed back to reduce hypothalamic peptide and/or anterior pituitary hormone release. For example, high circulating levels of cortisol produced in the adrenal cortex will inhibit corticotropin releasing hormone (CRH) release from the hypothalamus, and adrenocorticotropic hormone (ACTH) release from the pituitary.

Most of the blood supply from the hypothalamus to the pars distalis (adenohypophysis, anterior pituitary) is derived from a portal system (hypothalamo-hypophyseal vessels), which arises from a capillary network in the median eminence, transports down the pituitary stalk, then terminates in the anterior lobe. Blood flowing from the hypothalamus to the pituitary delivers hypothalamic peptides that regulate anterior pituitary hormone secretion. There are six major protein hormones secreted by five distinctive cell types in the anterior pituitary. Table 20-2 provides a summary of some of the characteristics of these five cell types.

Each type of endocrine cell in the adenohypophysis is under the control of a specific releasing hormone from the hypothalamus (Fig. 20-2). These releasing hormones are small peptides synthesized and secreted by tuberoinfundibular neurons of the hypothalamus. They are transported by short axonal processes to the median eminence, where they are released into capillaries and conveyed by the hypophyseal portal system to specific trophic hormone-secreting cells in the adenohypophysis. Each hormone stimulates the rapid release of preformed secretory granules containing a specific trophic hormone. Specific releasing hormones have been identified for thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), adrenocorticotropic hormone (ACTH), and growth hormone (GH). Prolactin (PRL) secretion is stimulated by a number of factors, the most important of which appears to be thyrotropin-releasing hormone (TRH). Dopamine serves as the major prolactin-inhibitory factor to suppress PRL secretion. Another hypothalamic release-inhibitory factor is somatostatin, which inhibits secretion of growth hormone. Regulation of the thyroid gland by TSH and the adrenal cortex by ACTH is discussed in this chapter. Regulation of the gonads by LH and FSH is discussed in Chap. 21.

The pars nervosa (neurohypophysis; posterior pituitary) in the human secretes two important hormones. These are vasopressin, VP (or antidiuretic hormone [ADH]), and oxytocin. They are nonapeptides that share a strong structural homology. ADH plays an important regulatory role in water conservation and maintenance of body fluid osmolality, blood volume, and pressure. ADH (VP) has two major effects corresponding to its two names: (1) it enhances reabsorption of water by the distal tubule and collecting ducts of the kidney producing an antidiuretic effect, which decreases osmotic pressure of the blood, and (2) it causes contraction of vascular smooth muscle producing a generalized pressor effect. Oxytocin stimulates contraction of smooth muscles located in the uterine myometrium to regulate fetal parturition, and in breast alveoli to regulate milk letdown during lactation.

Table 20-2

Pituitary Cell Distribution

CELL TYPE	% CELLS IN PITUITARY	HORMONE SECRETED
Somatotrophs	50	Growth hormone (GH)
Lactotrophs	10–20	Prolactin (PRL)
Corticotrophs	15–20	Adrenocorticotropic hormone (ACTH)
Thyrotrophs	5	Thyroid stimulating
Gonadotrophs	5–15	Luteinizing hormone (LH) Follicle stimulating hormone (FSH)

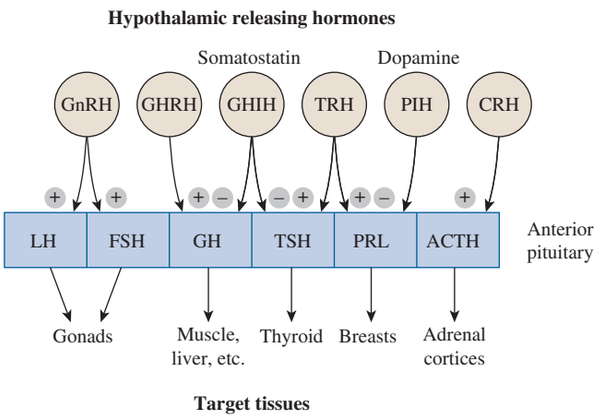

Figure 20-2. *Regulation of anterior pituitary hormone release (rectangles) by hypothalamic releasing hormones (circles).* Major target tissues for the anterior pituitary hormones are indicated. ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; GHIH, growth hormone inhibiting hormone (somatostatin); GHRH, growth hormone releasing hormone; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; PIH, prolactin inhibiting hormone (dopamine); TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone. +, stimulatory; −, inhibitory. (Modified from Hedge et al. [1987].)

Pituitary Toxicity

Few studies have focused on the effects of xenobiotics on the pituitary gland. However, studies consistently show that heavy metals may target pituitary gland structure or function. Cadmium affects many different cell types in the pituitary gland. It affects the lactotrophs by inhibiting prolactin secretion after long-term or acute exposure (Lafuente and Esquifino, 1999). The ability of cadmium to inhibit prolactin secretion may depend on the route of administration and age of the animals. In adult rats, oral and subcutaneous administrations of cadmium inhibit prolactin secretion, whereas only oral administration inhibits prolactin in pubertal animals (Lafuente and Esquifino, 1999). Cadmium also affects the gonadotrophs by inhibiting LH and FSH secretion in male and female rats (Lafuente and Esquifino, 1999; Ronchetti et al., 2016). This effect of cadmium on the gonadotrophs may depend on the route of exposure because oral, but not subcutaneous, administration of cadmium inhibits gonadotropin levels. Further, the ability of cadmium to affect gonadotrophs may differ with age. Studies indicate that the degree of cadmium-induced inhibition of LH and FSH is higher in older rats compared to younger rats (Lafuente and Esquifino, 1999). Similarly, cadmium affects the corticotrophs by altering ACTH levels in a manner that differs with age (Lafuente and Esquifino, 1999). Cadmium exposure increases ACTH levels in rodents exposed during puberty and decreases ACTH levels in animals exposed during adulthood. Further, cadmium may also affect the somatotrophs (Lafuente and Esquifino, 1999). Studies indicate that acute exposure to cadmium decreases circulating GH levels, while treatment for a longer period (14 days) increases circulating GH levels (Lafuente and Esquifino, 1999). One study evaluated the effect of chronic exposure (30 days in drinking water) to cadmium in male Sprague–Dawley rats on circadian release of ACTH, GH, and TSH (Caride et al., 2010a). Compared with controls, cadmium delayed the 24-hour peak of ACTH release and eliminated that of

GH. Interestingly, mean daily circulating levels of ACTH and TSH were increased by the metal. These findings supported that cadmium has chronotoxic effects at the level of the pituitary.

Cadmium is not the only heavy metal that affects the pituitary. Lead and mercury inhibit secretion of LH and FSH from the gonadotropes (Sikka and Wang, 2008). Further, high lead levels have been associated with increases in ADH secretion in case reports (Suarez et al., 1992). Acute arsenic and manganese co-exposure decreases levels of FSH and LH in rats (Adedara et al., 2017). In addition, acute iron overload increases the production of superoxide anions in the female rat pituitary (Rossi et al., 2016).

Environmental contaminants such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) also affect the pituitary gland. PCBs inhibit release of LH and FSH from gonadotrophs (Sikka and Wang, 2008). They also affect the thyrotrophs by inhibiting TSH secretion (Patrick, 2009; Zoeller, 2010). The aryl hydrocarbon agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) reduces expression of GH in mice (Takeda et al., 2014). In contrast, activation of the aryl hydrocarbon receptor with α-naphthoflavone impairs expression of prolactin, but not GH in a pituitary cell line (Moran et al., 2012).

Several pesticides cause adverse effects on the pituitary. The insecticide dimethoate causes pituitary tumors in male and female rats (Reuber, 1984). The organochlorine pesticide methoxychlor increases prolactin levels as well as LH levels (Lafuente et al., 2000). Both dieldrin and endosulfan stimulate prolactin secretion from the pituitary cell line GH3 via a mechanism that involves increasing calcium, which stimulates the release of prolactin (Wozniak et al., 2005). Endosulfan also downregulates expression of mRNA encoding prolactin, LH, GH, and TSH, and it stimulates prolactin and inhibits TSH (Caride et al., 2010b). The pesticide dichlorodiphenyltrichloroethane (DDT) stimulates expression of gonadotropin genes and hormones in a pituitary cell line (Zhou et al., 2014). The herbicide atrazine has been demonstrated in vitro to also have direct pituitary effects by binding to the growth-hormone-releasing hormone receptor and inhibiting GH expression at the mRNA and protein levels (Fakhouri et al., 2010). The fungicide carbendazim increases the levels of LH and FSH in rats (Rama et al., 2014).

Several phytoestrogens affect pituitary cells. Coumestrol reduces pulsatile LH secretion and suppresses the pituitary response to exogenous gonadotropin-releasing hormone (GnRH) in female rats (Dickerson and Gore, 2007; Gore, 2011). Acute genistein exposure suppresses pulsatile LH release in ovariectomized ewes (Dickerson and Gore, 2007). Chronic genistein exposure increases the pituitary size in rodents (Dickerson and Gore, 2007). Equol exposure decreases LH levels in male rats (Loutchanwoot et al., 2013). Isoliquiritigenin decreases proliferation and increases cell death in pituitary explants, likely by a mechanism that involves downregulation of cyclin D2 and E2 and induction of the cyclin-dependent kinase inhibitor Cdkn1a (Weis and Raetzman, 2016).

A variety of chemicals used in industrial processes alter pituitary structure or function (Verma and Rana, 2009). Flame retardants (tetrabromobisphenol A and tetrachlorobisphenol A) increase proliferation in GH3 cells, a pituitary cell line (Kitamura et al., 2002). The plasticizer bisphenol A (BPA) increases GH content in rainbow trout (Aluru et al., 2010), increases LH secretion in adult male rats, and increases GH content and receptors in juvenile rainbow trout (Tohei et al., 2001; Aluru et al., 2010). Prenatal exposure to BPA increases pituitary proliferation and gonadotroph number in female mouse offspring (Brannick et al., 2012). Further, BPA decreases expression of intracellular adhesion molecule-5 (Icam5) in the female neonatal mouse pituitary (Eckstrum et al., 2016). It also increases LH levels in prepubertal female rats (Gámez et al., 2015). The plasticizer 2-ethyl-phthalate stimulates proliferation in rat pituitary cells (Tapella et al., 2017). Further, a mixture of BPA and di(2-ethylhexyl) phthalate (DEHP) increases levels of the LHβ and FSHβ subunits in male rats (Abdel-Maksoud et al., 2015) and a mixture of DEHP and PCB decrease the expression of genes involved in pituitary-gonadal cross talk in male mice (Fiandanese et al., 2016).

2-Mercaptobenzothiazole, which is used in rubber products and can come into contact with drinking water, causes pituitary tumors in chronically exposed rats and mice (Whittaker et al., 2004).

Finally, a few pharmaceutical agents have been linked to changes in the pituitary. Cyanamide, a chemical used in the treatment of alcoholics, increases pro-opiomelanocortin mRNA in the anterior pituitary when coadministered with ethanol (Kinoshita et al., 2001). A safety evaluation of RO2910, a drug for treatment of HIV infection, has shown that it causes hypertrophy in the thyrotrophs (Zabka et al., 2011). The neuroleptic agent sulpiride causes the release of prolactin from the anterior pituitary in the rat and stimulates DNA replication (Capen, 2001). The administration of clomiphene prevents the stimulation of DNA synthesis produced by sulpiride, but does not affect prolactin release from the gland. These findings suggest that the intracellular prolactin content of the pituitary plays a role in the regulation of DNA synthesis through a mechanism mediated by estrogens.

ADRENAL GLANDS

The adrenals are two small glands situated on the superior poles of the kidneys (Fig. 20-3). The major physiological role of the adrenals is management of stress. Each adrenal gland is divided into two morphologically and functionally distinct regions. The adrenal cortex is the more exterior region, and the interior region is the medulla. The cortex synthesizes and secretes steroid hormones that function to regulate salt and fluid balance, glucose homeostasis, and a long-term response to stress. The medulla can be likened to a large postganglionic neuron, and secretes the catecholamines, epinephrine, and norepinephrine, upon stimulation by preganglionic cholinergic sympathetic fibers. The medullary catecholamines combine with norepinephrine from sympathetic nerve endings to mediate a very rapid time frame of response to stress. Because of the anatomic proximity of the two regions of the adrenal glands, blood from the cortex perfuses the medulla. This provides a functional association because cortisol secreted by the cortex stimulates expression of phenylethanolamine-*N*-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine in the medulla.

The adrenals have not been as widely studied in toxicology as other endocrine glands, even though it has been documented to be the most common toxicological target of all (Ribelin, 1984). In fact, the relative frequency of effects on endocrine systems reported from in vivo toxicology studies has been adrenal > testes > thyroid > ovary > pancreas > pituitary > parathyroid, with the adrenal cortex most frequently targeted (Harvey et al., 2007). This relative lack of attention in toxicology is surprising considering the critical role of the adrenals in mediating endocrine homeostasis throughout the body.

ADRENAL CORTEX

As seen in Fig. 20-3, the adrenal cortex can be divided at the cellular level into three separate zones, the zona glomerulosa (outermost),

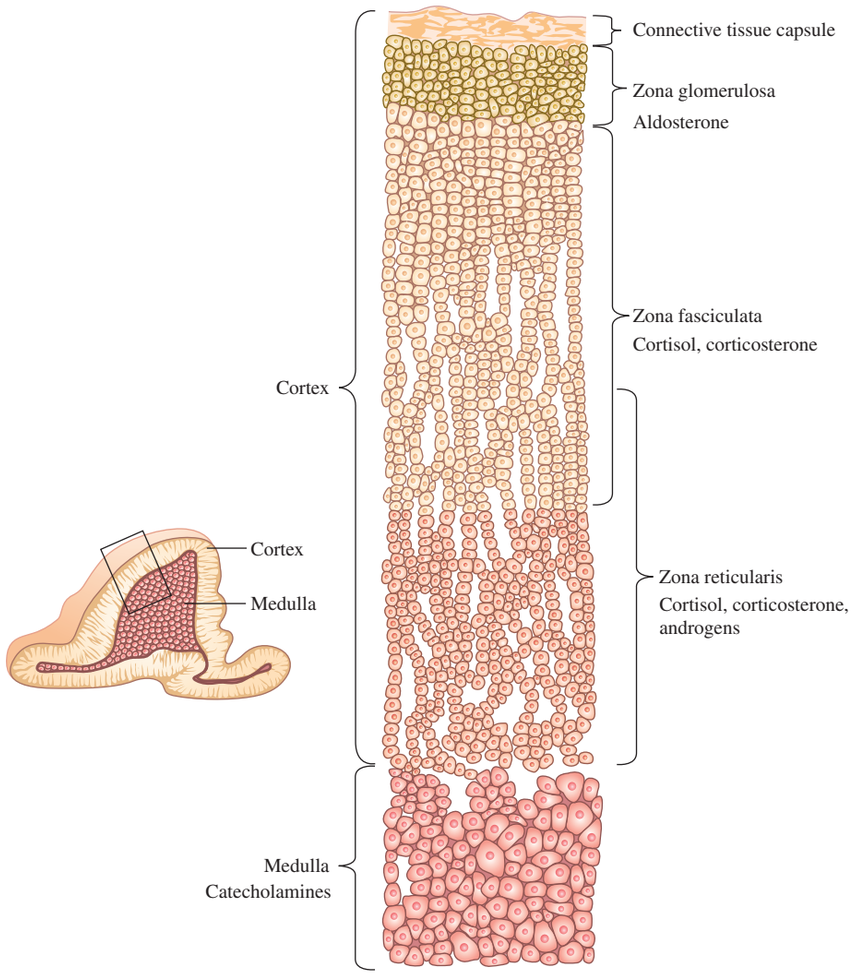

Figure 20-3. Used with permission from Hadley M, Levine J. Endocrinology, 6th Ed., ©2007. Reprinted by permission of Pearson Education, Inc., New York, New York.

the zona fasciculata (intermediate), and the zona reticularis (innermost). The zona glomerulosa is responsible for production of the mineralocorticoid hormone aldosterone. The glucocorticoid hormones, cortisol and corticosterone, are both secreted by the zona fasciculata and, to some extent, the zona reticularis. The zona reticularis also secretes the androgens, dehydroepiandrosterone, and androstenedione. These adrenal androgens are thought to be involved in the onset of puberty and serve a function in postmenopausal women; however, they are of relatively little biological significance under normal circumstances.

The adrenal cortex regulates many physiological functions such as the immune system, inflammation, water and electrolyte balance, and carbohydrate and protein metabolism involving such target organs as the liver, kidney, heart, bone, and nervous system (Harvey, 2010). The adrenal cortex is predisposed to the toxic effects of xenobiotic chemicals for three apparent reasons (Harvey, 2016). First, the adrenocortical cells of most animal species contain large stores of lipids used primarily as substrate for steroidogenesis. Many adrenal cortical toxic compounds are lipophilic and, therefore, can accumulate in these lipid-rich cells. Second, membranes of these cells contain high levels of unsaturated fatty acids that are susceptible to the generation of reactive species such as free radicals via lipid peroxidation. Finally, adrenocortical cells express enzymes involved in steroidogenesis, including those of the cytochrome P450 (CYP) family, which are capable of metabolizing xenobiotic chemicals. A number of toxic xenobiotic chemicals serve as pseudosubstrates for these enzymes and can be metabolized to reactive toxic species causing direct toxicity by covalent interactions with cellular macromolecules, or through lipid peroxidation or the generation of free radicals.

Steroidogenesis

Adrenal steroids are synthesized from cholesterol by specific enzyme-catalyzed reactions that involve a complex shuttling of steroid intermediates between the mitochondria and endoplasmic reticulum. Histologically, adrenal cortical cells are characterized by an abundance of lipid droplets, mitochondria, and smooth endoplasmic reticulum. Lipid droplets contain cholesterol, the precursor substrate for steroid production. The specificity of mitochondrial hydroxylation reactions in terms of precursor acted upon and the position of the substrate that is hydroxylated is confined to a

specific CYP. The common biosynthetic pathway from cholesterol is the formation of pregnenolone, the basic precursor for the three major classes of adrenal steroids (Fig. 20-4).

In the zonae fasciculata and reticularis, pregnenolone is first converted to progesterone by two microsomal enzymes. Three subsequent hydroxylation reactions involve carbon atoms at the 17, 21, and 11 positions. The resulting steroid is cortisol, which is the major glucocorticoid in teleosts, hamsters, dogs, and nonhuman and human primates. Corticosterone is the major glucocorticoid produced in amphibians, reptiles, birds, rats, mice, and rabbits.

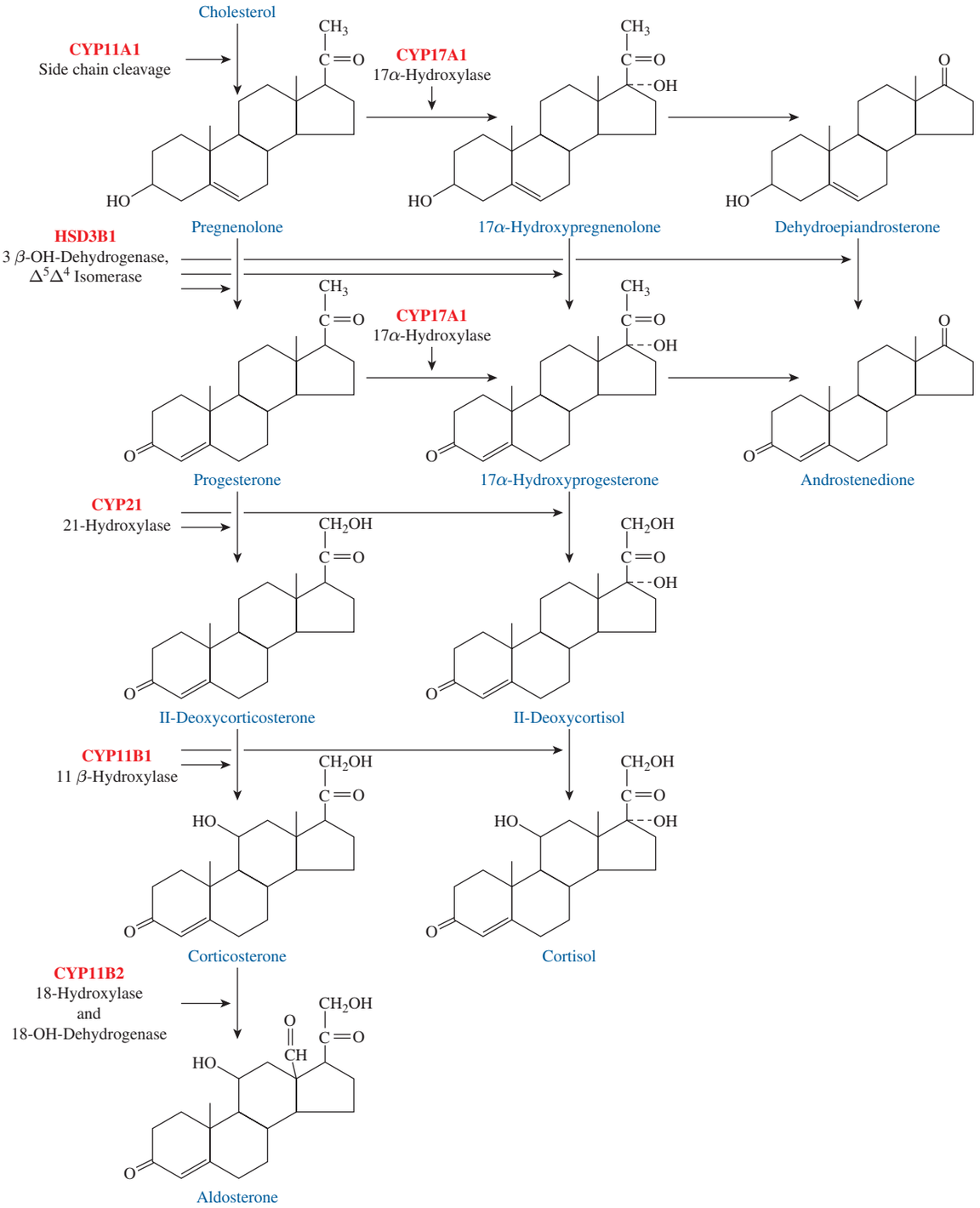

Figure 20-4. Used with permission from Hadley M, Levine J. Endocrinology, 6th Ed., ©2007. Reprinted by permission of Pearson Education, Inc., New York, New York.

It is produced in a manner similar to the production of cortisol, except that progesterone does not undergo 17α-hydroxylation and proceeds directly to 21-hydroxylation and 11β-hydroxylation.

In the zona glomerulosa, pregnenolone is converted to aldosterone by a series of enzyme-catalyzed reactions similar to those involved in cortisol formation; however, the cells of this zone lack the 17α-hydroxyprogesterone that is required to produce cortisol. Therefore, the initial hydroxylation product is corticosterone. The corticosterone is acted on by 18-hydroxylase to form 18-hydroxycorticosterone, which in turn interacts with 18-hydroxysteroid dehydrogenase to form aldosterone. Because 18-hydroxysteroid dehydrogenase is expressed only in the zona glomerulosa, it is not surprising that only this zone has the capacity to produce aldosterone.

Glucocorticoids

To better appreciate the wide-ranging effects of glucocorticoids, the effects of the hormone can be divided into normal physiological and pharmacological effects, since cortisol is therapeutically quite effective and widely prescribed. The physiological effects include (a) hepatic glucose production, gluconeogenesis (liver), (b) protein catabolism (skeletal muscle), (c) fat catabolism (adipose tissue), (d) increased bone resorption, (e) altered mood (CNS), (f) increased gastric acidity (GI tract), and (g) PNMT synthesis (adrenal medulla). Therapeutically, at pharmacological levels the effects of cortisol include (a) preventing vascular collapse during overwhelming stress, (b) providing an anti-inflammatory effect, and (c) invoking immunosuppression.

Adrenocortical Toxicity

The zonae fasciculata and reticularis appear to be the principal targets of xenobiotic chemicals in the adrenal cortex. A number of chemicals shown to cause adrenal toxicity by targeting specific steroidogenic enzymes have been summarized (Harvey, 2016). Classes of chemicals known to be toxic for the adrenal cortex include short chain (three or four carbons) aliphatic compounds, lipidosis-inducers, and amphiphilic compounds (Yarrington et al., 1981, 1985). In general, compounds seen to frequently produce necrosis, particularly in the zonae fasciculate and reticularis, include 7,12-dimethyl benz[*a*]anthracene (DMBA), acrylonitrile, hexadimethrine bromide, polyanetholesulfonate (along with amino-caprionic acid), thioacetamide, and basic polyglutamic acid (Szabo and Lippe, 1989; Colby et al., 1994). By comparison, lipidosis inducers can cause accumulations, often coalescing, of neutral fats, which may be of sufficient quantity to cause a reduction or loss of organellar function and eventual cell destruction. Examples of chemicals that directly target glucocorticoid secretion in the adrenal cortex include dimethoate, ketoconazole, spironolactone, efonidipine, mibefradil, 1-aminobenzotriazole, and various PCBs (Colby et al., 1995; Harvey, 2010). Lesions that are caused may be classified as follows: endothelial damage (e.g., acrylonitrile), mitochondrial damage (e.g., DMNM, *o,p′*-DDD, amphenone), endoplasmic reticulum disruption (e.g., triparanol), lipid aggregation (e.g., aniline), lysosomal phospholipid aggregation (e.g., chlorphentermine), and secondary effects due to embolization by medullary cells (e.g., acrylonitrile). Tricresyl phosphate (TCP) and other triaryl phosphates cause a defect in cholesterol metabolism by blocking both uptake from serum and storage pathways. An inhibition of cytosolic neutral cholesteryl ester hydroxylase (nCEH) by triaryl phosphate results in the progressive accumulation of cholesteryl ester in the form of lipid droplets in the cytoplasm of adrenal cortical cells.

Biologically active cationic amphiphilic compounds produce a generalized phospholipidosis that involves primarily the zonae fasciculate and reticularis and produce microscopic phospholipid-rich inclusions. These compounds affect the functional integrity of lysosomes, which appear ultrastructurally to be enlarged and filled with membranous lamellae of myelin figures. Examples of compounds known to induce phospholipidosis include chloroquine, triparanol, and chlorphentermine.

Adrenocortical toxicity can also involve increased secretion of endogenous glucocorticoids. Compounds that have been shown to cause this include ethanol, chlordecone, carbon disulfide, cannabinoids, cocaine, amitriptyline, and cytotoxic anticancer drugs (Harvey, 2010). Furthermore, pharmacological treatment with glucocorticoid agonists that have been widely used as anti-inflammatory agents can produce symptoms that resemble Cushing's syndrome (Harvey, 2010).

There have been documented cases of iatrogenically (physician-caused) induced adrenocortical toxicity. For example, the anesthetic etomidate and the anticonvulsant aminoglutethimide given as a sedative/antidepressant have been demonstrated to produce significant morbidity and mortality because of their potency as steroidogenic enzyme inhibitors. Etomidate blocks CYP11B1 (CYPβ/18), whereas aminoglutethimide inhibits CYP11A and CYP11B1 and downregulates the ACTH receptor (Johansson et al., 2002; Hinson and Raven, 2006; Harvey, 2016). This iatrogenic suppression of steroidogenesis in the adrenal cortex can result in Addisonian crisis (which can be associated with fatigue, cardiovascular collapse, and death).

In Vitro Testing

In addition to in vivo testing for effects of xenobiotics on the adrenal cortex, much recent work has used an in vitro approach to identify the molecular targets of adrenocortical toxicity. Of particular usefulness has been the human adrenocortical carcinoma–derived NCI-H295R cell line. This cell line expresses all key enzymes necessary for steroidogenesis, and it produces all of the major steroids including progesterone, androgens, estrogens, glucocorticoids, and aldosterone (Harvey et al., 2007; Harvey, 2016). These cells express functional receptors for CRH, angiotensin II, vasoactive intestinal peptide, atrial natriuretic peptide, LH, human chorionic gonadotropin (hCG), tumor necrosis factor, and activin A. They also express functional receptors for ACTH, however, not as well as normal cells. Thus, rather than performing ACTH challenge on these cells, cAMP activators such as forskolin can be used to stimulate corticosterone synthesis (Harvey, 2016). This cell line has proven useful for identification of specific steroidogenic enzymes that are targeted by xenobiotics. One review article provides a useful table listing over 60 compounds that have been shown to induce functional adrenocortical and steroidogenic toxicity along with the specific enzymes targeted (Harvey et al., 2007). Therefore, the H295R cell line is a versatile tool for mechanistic assessment of adrenocortical function and steroidogenesis in general. Because the cell line is derived from a human source, it is also very worthwhile for hazard risk assessment.

Serum Binding Proteins

Cortisol and corticosterone are transported in blood by corticosteroid binding globulin (CBG), also called transcortin. When bound to CBG, the steroid is biologically inactive and cannot have its effects on target tissues, or provide negative feedback to the hypothalamic–pituitary–adrenal (HPA) axis. Thus, a chemical affecting CBG could alter the balance between free and bound hormone,

and impact its availability in target tissues. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been reported to decrease the binding capacity of CBG by a mechanism other than simple displacement of bound glucocorticoid (Harvey, 2010).

Target Tissue Receptors

In addition to direct effects on steroidogenesis, chemically induced changes in adrenal function can result from blockage of the action of adrenocorticoids at peripheral sites. Adrenocortical steroids and HPA axis hormones exert their effects through receptors located in target tissues throughout the body. These receptors can be upregulated or downregulated by the action of xenobiotic compounds. For example, hexachlorobenzene reduces hepatic glucocorticoid receptors in rats (Lelli et al., 2007), and many antisteroidal compounds (antagonists) act by competing with or binding to steroid hormone receptor sites, thereby either reducing the number of available receptor sites or altering their binding affinity. Spironolactone, an antimineralocorticoid, is an example of a peripherally acting adrenocortical hormone antagonist (Los and Colby, 1994).

Neuroendocrine Regulation

The zonae fasciculata and reticularis of the adrenal cortex are under trophic control by the pituitary hormone ACTH, which stimulates them to produce cortisol. The level of output of cortisol by the adrenal is almost entirely determined by the intensity of the ACTH stimulus. The increased cortisol produced then provides long-loop negative feedback on the hypothalamus and anterior pituitary and decreases CRH and ACTH secretion, respectively (Fig. 20-5). Stress is a major factor that can override the negative feedback control system and stimulate cortisol secretion (Harvey, 2016). Persistent exposure of the adrenal cortex to high levels of ACTH during chronic stress can result in adrenocortical hypertrophy. Adrenal androgens are also produced in ACTH-sensitive cells; therefore, abnormal conditions of stimulation of these cells by ACTH, such as Cushing disease, can also raise androgen secretion by the cortex. Conversely, conditions of reduced exposure of the cortex to its tropic hormone ACTH can result in adrenal atrophy.

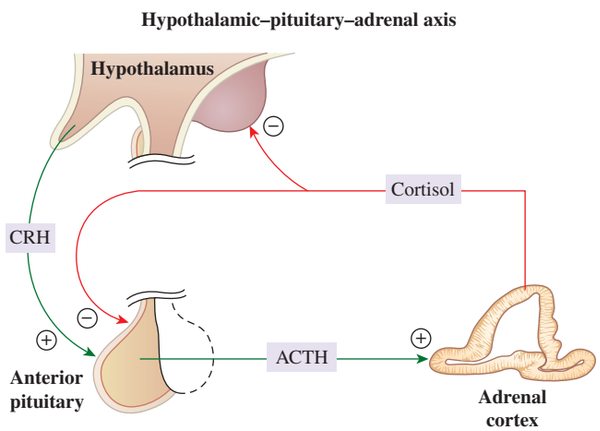

Figure 20-5. *Hypothalamic–pituitary–adrenal axis of regulation.* CRH from the hypothalamus stimulates release of ACTH from the anterior pituitary. ACTH stimulates synthesis and secretion of cortisol from the adrenal cortex. Cortisol provides inhibitory (negative) feedback on the hypothalamus and pituitary. Stress, the major stimulator of the axis, can override the feedback regulatory loop. +, stimulatory; −, inhibitory. (Modified from Hedge et al. [1987].)

Drugs that affect neuroendocrine function can indirectly affect adrenal function. For example, synthetic glucocorticoids, valproic acid, bromocriptine, cyproheptadine, ketanserin, ritanserin, somatostatin analogs, 4′-thio-beta-D-arabinofuranosylcytosine, hexachlorobenzene, alcohol, and caffeine have been shown to suppress ACTH or CRH secretion (Harvey, 2010). ACTH secretion can also be pharmacologically stimulated by, for example, caffeine, methylxanthines, adenosine analogs, 3,4-methylenedioxymethamphetamine, and di-2-ethylhexyl phthalate (Harvey, 2010). Exogenous steroids can disrupt normal function and structure of the adrenal cortex. Agonists will cause negative feedback inhibition of ACTH secretion in the pituitary and will result in atrophy of the zonae fasciculata and reticularis. In contrast antagonists of steroids will block hormone action in target tissues and on negative feedback, which will lead to increased ACTH secretion, and differential hyperplasia of the cortex. Generally, testing of compounds that have a neuroendocrine site of action is only likely to be detected by in vivo studies. The most commonly observed measure of adrenal effects is an increase in adrenal weight (adrenal hypertrophy). As a major role of the adrenal cortex in responding to stress, adrenal hypertrophy occurs in response to elevated ACTH levels that also cause increased cortisol secretion. Additionally, adrenal hypertrophy can be caused by elevated ACTH levels that result from toxicity of adrenocortical steroid synthesizing enzymes. The elevated ACTH in that case would result from loss of negative feedback by steroids on the pituitary. Thus, in cases of adrenal hypertrophy, these two situations can be distinguished by measurement of circulating glucocorticoids. With stress, the levels of steroid will be elevated, whereas with toxicity, they will be very low.

Animal Testing

Special considerations for animal testing in adrenal toxicity were outlined in recent reviews (Harvey et al., 2007; Harvey, 2016). A number of things should to be taken into account. Animal testing of xenobiotics for toxicity often uses the laboratory rodent. However, when screening for effects on adrenal steroidogenesis, the rat may not be the most useful model. This is because rats lack expression of CYP17 and, unlike humans, cannot produce cortisol (Hinson and Raven, 2006). As a result, the major glucocorticoid is corticosterone, and the rat may not detect effects of compounds that interact with CYP17. Aquatic wildlife species are often considered sentinel species for detecting adrenal effects of the environment as risk for other wildlife species as well as humans. This could result from direct exposures to polluted waters or an impact on the food chain as many of these chemicals are known to accumulate and form secondary exposure routes (Harvey, 2016). To avoid increased glucocorticoid levels resulting from the stress of handling, sham dosing should be conducted 7 to 10 days prior to the onset of dosing. If females are used, their stage of estrous cycle needs to be coordinated. Blood samples should be taken at the same time of day and should be completed within 3 minutes of cage disturbance to ensure nonstressed control values. Animal dosing should be via oral gavage (or the route of human exposure) to provide adequate translational relevance. A particularly useful method of testing of chemicals for effects on adrenocortical competence is the ACTH stimulation test in which ACTH is administered subcutaneously following a predetermined period of exposure of test animals to the compound of interest. Blood samples can be collected within 1 to 3 hours for assessment of glucocorticoid levels. A direct impact of the compound on the adrenal gland will be assumed if ACTH fails to elevate corticosterone levels to those seen in controls.

Mineralocorticoids

The adrenals are essential to life, mainly because of the salt-retaining function of the zona glomerulosa, but to a lesser extent, because of the stress management role of the adrenal cortex. Loss of mineralocorticoid production by the cortex results in a life-threatening retention of potassium and hypovolemic shock associated with the excessive urinary loss of sodium, chloride, and water. Aldosterone, the most important mineralocorticoid, participates in the regulation of renal sodium and potassium balance, thereby affecting blood pressure homeostasis. Aldosterone promotes sodium reabsorption and increases the excretion of potassium and hydrogen ions by the kidney (Fig. 20-6). The ratio of the concentrations in the urine of Na^+/K^+ decreases when blood levels of aldosterone increase, and vice versa. All routes of loss of sodium and potassium from the body are controlled by aldosterone. Thus, an increase in the concentration of aldosterone in blood also decreases the Na^+/K^+ ratio of saliva, sweat, and feces.

In theory, chemicals that target steroidogenic enzymes in the pathway to aldosterone secretion could affect both aldosterone and glucocorticoid (StAR, CYP11A1, and CYP21) or only aldosterone synthesis and secretion (CYP11B1; Harvey et al., 2007; Harvey, 2016).

The aldosterone-producing cells of the zona glomerulosa are controlled separately by other tropic substances, but may require permissive concentrations of ACTH. Unlike the cells of the zonae fasciculata and reticularis, regulation of release from the zona glomerulosa is not primarily via ACTH. The renin–angiotensin system is the primary regulator of aldosterone secretion in the adrenal cortex. Renin is a proteolytic enzyme synthesized and secreted in the renal juxtaglomerular apparatus (JGA). Release of renin from the JGA is stimulated by decreases in blood pressure or volume, or reduced plasma sodium. Once released, the enzyme (renin) encounters renin substrate (angiotensinogen) of hepatic origin, which is already in circulation and serves as a prohormone reservoir for angiotensin. In the blood stream, renin cleaves angiotensinogen to become angiotensin I (AgI, a decapeptide), and angiotensin converting enzyme (ACE) further cleaves AgI to produce angiotensin II (AgII). This octapeptide (AgII) is one of the most potent known physiological vasoconstrictors. However, it also acts as a tropic hormone for the zona glomerulosa cells of the adrenal cortex to stimulate synthesis and secretion of aldosterone. Because AgII constricts resistance vessels in the body to increase blood pressure, and decrease vascular capacity, this contributes to a feedback mechanism to decrease secretion of renin by JGA cells. Further, by stimulating aldosterone secretion (long loop feedback), the resulting increase in Na^+ and water retention returns blood volume and osmolality to normal. This further inhibits release of renin, providing a physiological feedback loop of regulation.

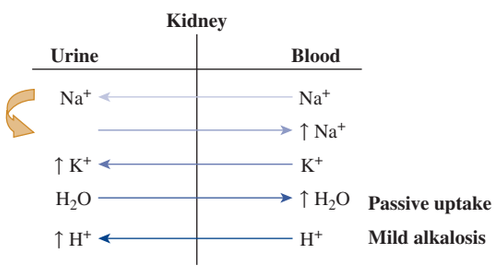

Figure 20-6. *Aldosterone effects on the kidney.* Aldosterone stimulates reuptake of Na^+ from the filtrate while promoting K^+ excretion into the urine. Water (H_2O) is reabsorbed by passive uptake, whereas protons (H^+) are excreted along with K^+.

Fetal Adrenal

The adrenal cortex in the human fetus differs both structurally and functionally from that in the adult. A specialized fetal adrenal cortex exists in primates during late gestation (Garcia-Flores et al., 2017). The cortex is composed of large polyhedral cells that produce abundant cortisol and estrogen precursors. The hormones secreted by the cortex are important for normal development of the fetus, and the steroid precursor dehydroepiandrosterone is converted to estriol by the placenta. The cells of the fetal cortex are produced in the outer cortex and migrate medially, where they undergo hypertrophy and eventually apoptosis. After birth, there is a rapid regression, apoptosis, and lysis of the fetal cortex with dilation of cortical capillaries and replacement by the typical three cortical zones. The fetal adrenal cortex is proportionately much larger than the adult gland because of the large size of the fetal zone (Garcia-Flores et al., 2017). It is important not to misinterpret this as a lesion in neonatal primates since it represents physiological replacement of the fetal cortex with the definitive postnatal adrenal cortex.

X-Zone

Similar to the fetal cortex in primates, the X-zone in the mouse adrenal cortex is also a unique physiological phenomenon. In contrast to the fetal cortex of primates, the X-zone develops postnatally in the inner cortex of mice and is fully formed at weaning. Its function is unknown, but it may be similar to the fetal zone in primates. After weaning, the X-zone degenerates at variable rates, depending on the sex of the mouse. In male mice, the X-zone undergoes degeneration at puberty with accumulation of intracellular fat globules. In females, the zone undergoes slow regression and degeneration during the first pregnancy. As with the fetal zone in primates, it is important not to misinterpret the degeneration associated with regression of the X-zone in mice as a lesion.

ADRENAL MEDULLA

Because it is classified as a specialized postganglionic (sympathetic) neuron, the adrenal medulla is a functional extension of the nervous system and is also called the sympathoadrenal system. Thus, the adrenal medulla comprises a branch of the sympathetic division of the autonomic nervous system. Located in the center of the adrenal gland, the physiological role of the medulla is to help maintain the constant internal environment of the body. The medulla is composed of cells called pheochromoblasts (specialized sympathetic ganglia), also known as chromaffin cells, which are the site of catecholamine synthesis and secretion. Chromaffin cells derive their name from the chromaffin reaction, the formation of a brown color due to oxidation of catecholamine stores (Tischler et al., 2010). These cells are true neuroendocrine cells, which provide a direct interface between the nervous and endocrine systems. That is, sympathetic, cholinergic stimulation of the cell bodies results in secretion of catecholamines, which behave as hormones by entering the circulation and producing true endocrine effects throughout the body. Secretory granules in the chromaffin cells are the site of storage for the catecholamines, which are rapidly released upon receipt of neuronal input from a preganglionic neuron along the splanchnic nerve pathway. As a result of this stimulation, the catecholamines, epinephrine, and norepinephrine are released into the circulation. Peptides are also reported to reside in chromaffin cells. These include chromogranins and the

neuropeptides enkephalin (Schultzberg et al., 1978), neuropeptide Y (NPY; Varndell et al., 1984), substance P (Kuramoto et al., 1985), vasopressin and oxytocin (Hawthorn et al., 1987), galanin (Holgert et al., 1994), and neurotensin (NT; Holgert et al., 1994).

Sympathetic Response

The general functions of the sympathetic division of the autonomic nervous system can be summarized as follows: (a) ensuring reciprocity to counteract and balance the tonic effects of parasympathetic stimulation to visceral structures, (b) assisting in the maintenance of the steady-state functions of the body (digestion, secretion, vasomotor tone, etc.), and (c) assisting in the mobilization of body reserves to meet unusual or emergency situations (fear, fright, injury, etc.). Dr Walter Cannon in 1929 was the first to suggest that activation of the sympathetic nervous system prepares an animal for "fright, fight, or flight."

Catecholamines

The sites of catecholamine synthesis in the body are the CNS, postganglionic sympathetic neurons, and the adrenal medulla. It is the adrenal medulla that is the major site of epinephrine production. In fact, 80% of the medullary output of catecholamines in humans is epinephrine. In the catecholamine biosynthetic pathway, tyrosine is acted on by tyrosine hydroxylase to produce dopa, which is converted to dopamine by dopa decarboxylase. Dopamine in turn is acted on by dopamine β-hydroxylase to form norepinephrine, which is converted to epinephrine by PNMT. Tyrosine hydroxylase is the principal rate-limiting step in catecholamine synthesis. The conversion of tyrosine to dopa then dopamine occurs within the cytosol of chromaffin cells. Dopamine then enters the chromaffin granule, where it is converted to norepinephrine. Norepinephrine leaves the granule and is converted to epinephrine in the cytosol, and epinephrine reenters and is stored in the chromaffin granule. In contrast to the synthesis of catecholamines that occurs in the cytosol, neuropeptides and chromogranin-A proteins are synthesized in the granular endoplasmic reticulum and are packaged into granules in the Golgi apparatus. Release of the catecholamines is stimulated by acetylcholine deriving from preganglionic neurons along the splanchnic nerve. Acetylcholine activates nicotinic and muscarinic cholinergic receptors, which cooperatively stimulate the secretory response by increasing a Ca^{++} flux and activating protein kinase C. This flux involves Ca^{++} from both extracellular influx and intracellular stores. Physiological activators of release include decreased blood pressure, decreased blood glucose, decreased oxygen availability, stress or anxiety, cold, exercise, and postural hypotension. Rather than staining for the chromaffin reaction to identify catecholamine-producing cells, more recently, immunostaining for catecholamine-synthesizing enzymes tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH), and PNMT is used to identify individual cells that produce catecholamines, and whether they synthesize epinephrine (Tischler et al., 2010).

Catecholamines generally affect all tissues. However, the most pronounced effects are on the (a) heart, (b) liver, (c) skeletal muscle, (d) adipocytes, (e) vascular smooth muscle, and (f) bronchial smooth muscle.

Adrenergic Receptors

Catecholamine actions are mediated through interactions with specific classes of adrenergic receptors on target cell membranes. There are two major types of these receptors, known as α and β adrenergic receptors. The α and β classification is further divided into at least two subtypes of each, α_1 and α_2, and β_1 and β_2 (Table 20-3). Each type of receptor has its own unique pattern of distribution throughout the body. The relative number of each receptor type in each target organ determines, in part, the nature of the response of the organ to the catecholamines. For example, α_2 receptors predominate on insulin-secreting pancreatic B cells, whereas β receptors predominate on glucagon-secreting pancreatic A cells. The net effect is that catecholamines inhibit insulin (decreased cAMP production) and stimulate glucagon (increased cAMP production) release so that glucagon can facilitate an antiinsulin effect by increasing blood glucose. Epinephrine and norepinephrine display similar affinities for the different receptor types, with the exception of β_2 receptors that bind epinephrine 10 times greater than norepinephrine. As a result, epinephrine participates more than norepinephrine in mediating its metabolic effects. Therefore, receptor type variation on target tissues contributes to the diversity with which the sympathetic response exerts its specific effects.

General Toxicity

Examples of specific chemicals that target chromaffin cells include toxins that block voltage-gated ion channels (Alvarez et al., 2008), and bacterial toxins, which block exocytosis of secretory granules, thereby preventing catecholamine release (Gasman et al., 1999). The effects of these toxicants on whole animal physiology are relatively unimportant due to their more global and lethal effects on the nervous system. Furthermore, the relevance of the adrenal medulla as a target organ in toxicology is highly dependent on the species, strain, and sex of the animals studied (Tischler et al., 2010). The most common pathological changes seen in the adrenal medulla in toxicological studies involve proliferative lesions classified

Table 20-3

Different Classes of Adrenergic Receptors

RECEPTOR TYPE	SIGNALING PATHWAY	RELATIVE AFFINITY	PHYSIOLOGICAL RESPONSES
α_1	↑ IP_3, DAG	E = NE	Vasoconstriction, uterine contraction, pupil dilation
α_2	↓cAMP	E = NE	Sphincter muscle constriction (GI, bladder), ↓lipolysis, ↓insulin secretion
β_1	↑ cAMP	E = NE	↑heart rate, ↑cardiac output, ↑renin secretion
β_2	↑ cAMP	E >>> NE (10×)	Bronchodilation, vasodilation, uterine relaxation, ↑glycogenolysis, ↑lipolysis, smooth muscle relaxation (GI, bladder), ↑insulin secretion

as nodular hyperplasia, although degenerative changes can also occasionally be observed.

Pheochromocytoma

The adrenal medulla can undergo a series of proliferative changes ranging from diffuse hyperplasia to benign and malignant neoplasms. The latter neoplasms have the capacity to invade locally and metastasize to distant sites. Larger benign adrenal medullary proliferative lesions are designated pheochromocytomas.

Pheochromocytomas are relatively rare in humans (Greim et al., 2009). The incidence is about 1/100,000, and both sexes are affected equally. The peak incidence is ages 40 to 50, and the tumors occur bilaterally in only 10% of the cases. About 10% are malignant, 10% occur outside of the adrenal gland, and 10% are considered to be hereditary (Gimm, 2005). Pheochromocytomas in humans and rats are both composed of chromaffin cells with variable numbers of hormone-containing secretory granules. There appears to be a striking species difference in the response of medullary chromaffin cells to mitogenic stimuli, with rats being very sensitive compared to humans. In humans, pheochromocytomas are uncommon except in patients with inherited clinical syndromes of multiple endocrine neoplasia (MEN). These tumors in rats usually do not secrete excess amounts of catecholamines, whereas human pheochromocytomas episodically secrete increased amounts of catecholamines, leading to hypertension and other clinical disturbances. Further, no data are available to suggest that pheochromocytomas may be inducible in humans. In general, they are usually indolent tumors in humans and mice. These observations have increasingly led regulatory agencies to diminish the need of investigating rat pheochromocytomas for purposes of risk assessment (Tischler et al., 2010).

Proliferative lesions occur in the adrenal medulla with high frequency in many strains of laboratory rats. The incidence of these lesions varies with strain, age, sex, diet, and exposure to drugs and a variety of environmental agents. Studies from the NTP historical database of 2-year-old F344 rats have reported that the incidence of pheochromocytomas was 17.0% and 3.1% for males and females, respectively. Malignant pheochromocytomas were detected in 1% of males and 0.5% of females. In addition to F344 rats, other strains with a high incidence of pheochromocytomas include Wistar, NEGH (New England Deaconess Hospital), Long–Evans, and Sprague–Dawley. Pheochromocytomas are considerably less common in Osborne–Mendel, Charles River, Holtzman, and WAG/Rij rats. Most studies have revealed a higher incidence in males than in females. Crossbreeding of animals with high and low frequencies of adrenal medullary proliferative lesions results in F1 animals with an intermediate tumor frequency. For reasons not well understood, spontaneous pheochromocytomas are largely observed in aging male laboratory rats, but are relatively rare in mice (Greim et al., 2009). The differences between rats and mice have led to the suggestion that the mouse is a more suitable model for humans as regards adrenal medullary risk assessment. These medullary lesions, more frequently observed in males, can be induced by a variety of xenobiotics (Tischler et al., 2010).

Pheochromocytomas in rats differ from those in all other species in that they are common, often bilateral, and can be induced by many chemicals. An extensive listing of chemicals that are effective at producing proliferative lesions in rats and mice has been provided in reviews by Greim et al. (2009) and Tischler et al. (2010). Substances that have been shown in NTP studies to induce pheochromocytoma in rats include metal compounds, halogenated and nonhalogenated aliphatic or aromatic hydrocarbon, aromatics, aromatic amines as well as other dyestuff, pesticides, and pharmaceuticals (Greim et al., 2009). Vitamin D is the most powerful mitogenic stimulus to cause chromaffin cell proliferation in the adrenal medulla in rats (Greim et al., 2009). Because the vitamin D effect has been seen in vivo, but not in vitro, it is thought to result from impaired calcium homeostasis, resulting in hypercalcemia.

In animals, there is no indication as yet that pheochromocytomas are induced by chemicals working through genotoxic mechanisms (Ozaki et al., 2002). Proliferation of chromaffin cells in male rats occurred following exposure to aromatic amines such as *p*-chloroaniline (Chhabra et al., 1991). Rather, proliferation of chromaffin cells in rats can be stimulated by chemicals that induce uncoupling of mitochondrial respiration, hypoxia, disturbances in calcium homeostasis, acute stress, and overfeeding (Greim et al., 2009). Many of the chemicals that induce pheochromocytoma in animals are uncouplers of oxidative phosphorylation, for example, acrylamide (Howland and Alli, 1986) or furan (Mugford et al., 1997).

Unlike in humans, in which they secrete high levels of epinephrine, rat pheochromocytomas are usually characterized by predominant or exclusive production of norepinephrine. Chemicals that induce pheochromocytomas in rats are pharmacologically diverse, and usually stimulate chromaffin cell proliferation by neutrally derived signals, or agents that regulate catecholamine synthesis and release. An important class of compounds that affects the rat adrenal medulla includes sugars and sugar alcohols, such as lactitol, xylitol, and sorbitol (Lynch et al., 1996).

Both nicotine and reserpine have been implicated in the development of adrenal medullary proliferative lesions in rats. Both chemicals act by a shared mechanism, because nicotine directly stimulates nicotinic acetylcholine receptors, whereas reserpine causes a reflex increase in the activity of cholinergic nerve endings in the adrenal. A short dosing regimen of reserpine administration in vivo stimulates proliferation of chromaffin cells in the adult rat, and the mechanism may involve a reflex increase in neurogenic stimulation via the splanchnic nerve.

Roe and Bar (1985) have suggested that environmental and dietary factors may be more important than genetic factors as determinants of the incidence of adrenal medullary proliferative lesions in rats because the incidence of adrenal medullary lesions can be reduced by lowering the carbohydrate content of the diet. Several of the chemicals that increase the incidence of adrenal medullary lesions, such as sugar alcohols, increase absorption of calcium from the gut. Calcium ions as well as cyclic nucleotides and prostaglandins may act as mediators capable of stimulating both hormonal secretion and cellular proliferation. In summary, three dietary factors have been suggested to lead to an increased incidence of adrenal medullary proliferative lesions in chronic toxicity studies in rats (Roe and Bar, 1985). These are excessive intake of (1) food associated with feeding ad libitum; (2) calcium and phosphorus; and (3) other food components (e.g., vitamin D and poorly absorbable carbohydrates), which increase calcium absorption.

Tischler et al. (1995) reported that adult rat chromaffin cells had a marked increase in bromodeoxyuridine (BrdU)-labeled nuclei in vitro following the addition of forskolin (activator of adenylate cyclase) and phorbol myristate (PMA, an activator of protein kinase C), whereas mouse chromaffin cells had a minimal response to the same mitogens. This striking difference in sensitivity to mitogenic stimuli may explain the lower frequency of adrenal medullary proliferative lesions in mice compared to many rat strains. The human adrenal medulla, as in mice, has a low spontaneous incidence of proliferative lesions of chromaffin cells. Human chromaffin cells also failed to respond to a variety of mitogenic stimuli in culture (Tischler and Riseberg, 1993). These findings and others suggest that chromaffin cells of the rat represent an inappropriate model to assess the

potential effects of xenobiotic chemicals on chromaffin cells of the human adrenal medulla.

A relationship exists between adenohypophyseal (anterior pituitary) hormones and the development of adrenal medullary proliferative lesions. For example, the long-term administration of growth hormone is associated with an increased incidence of pheochromocytomas as well as the development of tumors at other sites. Prolactin-secreting pituitary tumors, which occur commonly in many rat strains, also play a role in the development of proliferative medullary lesions. In addition, several neuroleptic compounds that increase prolactin secretion by inhibiting dopamine production have been associated with an increased incidence of proliferative lesions of medullary cells in chronic toxicity studies in rats.

In long-term animal studies, pheochromocytomas often are accompanied by tumors or toxic effects in other organs. They often are seen in cases involving renal, lung, and hepatic toxicity, in addition to endocrine disturbances (Greim et al., 2009). They are associated with the following conditions: hypoxia, uncoupling of oxidative phosphorylation, disturbances of calcium homeostasis, or disturbances of the hypothalamic endocrine axis (Greim et al., 2009). Additionally, pheochromocytomas often develop following treatment with substances that target enzymes in catecholamine synthesis, receptor tyrosine kinase (RET), hypoxia-inducible factor (HIF), succinate dehydrogenase, or fumarate hydratase. As with humans, pheochromocytomas can also occur in mice and rats in conjunction with MEN. This was seen in male rats after administration of propyl gallate (National Toxicology Program, 1982).

In Vitro Testing

A commonly employed cell line used in neurobiology is the PC12 pheochromocytoma line derived from a rat adrenal medullary tumor. These cells respond to nerve growth factor (NGF) by ceasing replication, extending neurites, and increasing a number of neuronal markers. This cell line is widely used in neurotoxicological studies. Corticosterone and glutamate are often used to induce cytotoxicity in this in vitro culture system (Mao et al., 2011; Kritis et al., 2015). One study utilized phenolic acid derivatives and flavonoid extracts of daylily to protect against cytotoxicity caused by these compounds (Tian et al., 2017). However, in using them, it must be considered that rat PC12 cells are representative only of a subset of chromaffin cells, and may differ in some ways from normal and neoplastic chromaffin cells of other species. At this time, there are no human chromaffin cell lines, despite numerous efforts to establish such a cell line (Tischler et al., 2010).

The PC12 cell line has been useful in determining intracellular mechanisms at the molecular level that are involved in chromaffin cell signaling and proliferation. Studies with PC12 cells have shown that substances which inhibit mitochondrial function (cyanide, rotenone) or uncouple oxidative phosphorylation (dinitrophenol, *p*-trifluoromethoxyphenyl hydrazone) stimulate catecholamine secretion in the same way as occurs under hypoxic conditions. This is thought to be dependent upon Ca^{++} influx through voltage-gated channels (Taylor et al., 2000). Proliferation of chromaffin cells can also be induced in vitro by activators of adenylate cyclase that stimulate neuropeptide receptors, as well as stimulators of protein kinase C, which stimulates muscarinic cholinergic receptors.

THYROID GLAND

The thyroid gland consists of two lobes of endocrine tissue located just below the larynx on each side of the trachea with an isthmus connecting the two lobes (Fig. 20-7). A second cell type is also present, the C cells, or parafollicular cells composing the intrafollicular spaces. The C cells synthesize and secrete calcitonin (CT), a hormone involved in calcium homeostasis (see the section "Parathyroid Gland" for more details).

The thyroid secretes two hormones known as thyroxine (T_4) and triiodothyronine (T_3). Both of these hormones are produced in epithelial cells in the basic functional unit of the thyroid known as the follicle (Fig. 20-8). Each follicle consists of a sphere of epithelial cells surrounding a colloidal core. The colloid material is composed of the glycoprotein thyroglobulin (TGB), which acts as a storage depot for T_4 and T_3 (Capen, 2001). In humans, about 3 months of thyroid hormone is stored as TGB in the colloid. This represents the largest reserve of any stored hormone in the body. Generally, the level of secretory activity of follicular cells can be estimated as a direct function of their height. Cells involved in synthesizing thyroid hormone are columnar in shape, whereas quiescent cells are cuboidal (Capen, 2001).

T_4 and T_3 are important regulators of overall metabolism, and their effects are regulated within a long time frame. Essentially all

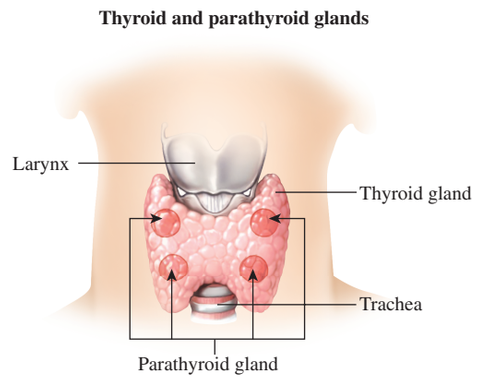

Figure 20-7. *Anatomy of the thyroid and parathyroid glands.* The schematic shows the thyroid gland in humans, which consists of two lobes of endocrine tissue, located just below the larynx on each side of the trachea with an isthmus connecting the two lobes. (Modified from Porterfield [2001].)

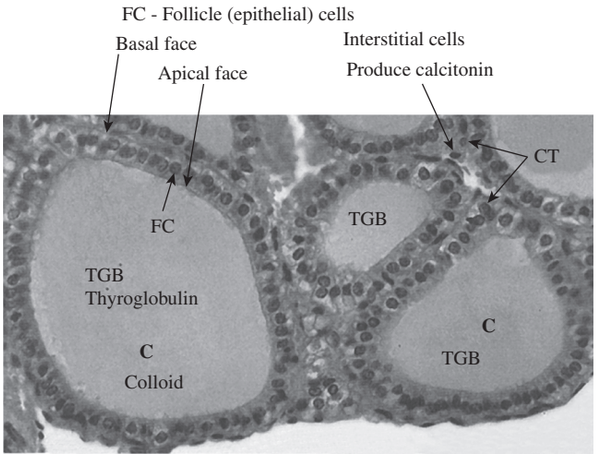

Figure 20-8. *Histology of the thyroid gland.* The micrograph shows thyroid follicles (FC) composed of epithelial cells, the site of thyroid hormone synthesis, with colloid (C), the site of thyroid hormone storage, contained in the follicular lumen. The epithelial cell contains a basal and apical face. The interstitial compartment is composed of C cells, the site of synthesis, and secretion of calcitonin. (Modified from Porterfield [2001].)

tissues are to some degree targets for thyroid hormone. However, the primary target tissues for thyroid hormone include the liver, kidney, heart, brain, pituitary, gonads, and spleen (Zoeller et al., 2007).

Some studies indicate that xenobiotics directly affect the structure of the thyroid gland. For example, some environmental chemicals such as heavy metals and red dye #3 are known to decrease the size of the colloid space (Capen, 2001; Bronnikov et al., 2005). Similarly, iron deficiency reduces the size of follicular cavities in pregnant rats (Hu et al., 2014). These morphological changes are thought to reduce the space required for storing hormones, leading to an impaired ability of the thyroid gland to synthesize and store thyroid hormones. Chemicals such as PCBs are known to alter the appearance of the epithelial cells so that they are hypertrophic or hyperplastic in nature (Capen, 2001; Langer, 2010; Tang et al., 2013), and perchlorate is thought to increase angiogenesis and follicular cell proliferation in fish (Furin et al., 2015). This is thought to lead to excessive thyroid hormone production.

Thyroid Hormone Structure and Synthesis

Thyroid hormones are composed of two modified, covalently linked tyrosine amino acids (Fig. 20-9). Each of the aromatic rings of the tyrosines contains one or two iodides. T_4 contains two iodides on each aromatic ring for a total of four, whereas T_3 contains two iodides on the tyrosine closest to the amino acid moiety (amino and carboxy groups), and one iodide on the outer aromatic ring (Capen, 2001). The iodide is derived from dietary intake and is required for biologic activity. Although the thyroid gland synthesizes and secretes both T_4 and T_3, it primarily releases T_4. In fact, about 90% of thyroid hormone secreted by the thyroid gland is in the form of T_4 in humans.

TGB, a glycoprotein containing large numbers of tyrosine amino acid residues, is synthesized in the epithelial cell and serves as the backbone for thyroid hormone synthesis. Iodine in the form of iodide (I^-) is actively transported into the epithelial cell, where it is oxidized to I_2 by thyroid peroxidase. At the apical membrane, I_2 combines with

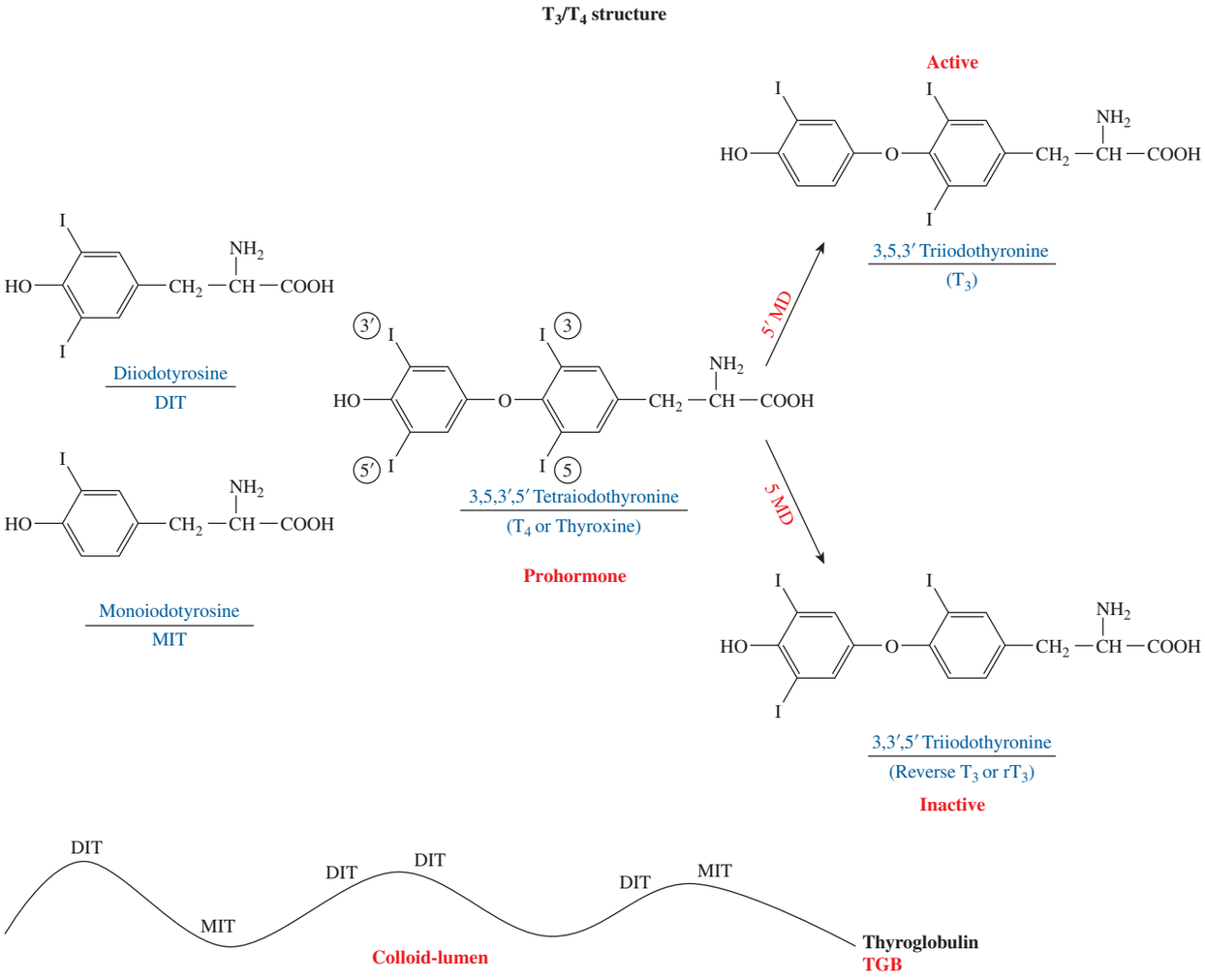

Figure 20-9. *Structure of thyroid hormone (T_3 and T_4).* The schematic shows the structures required to make T_3, T_4, and reverse T_3 (rT_3). At the apical membrane of the follicular cells, I_2 combines with tyrosine residues on thyroglobulin (TGB) to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). Coupling between MIT and DIT occurs such that combined MIT and DIT forms T_3, whereas combined DIT and DIT forms T_4. T_4 from the thyroid gland can be peripherally converted to T_3 (active hormone) or rT_3 (inactive metabolite), then successively deiodinated by the monodeiodinases. (Modified from Hedge et al. [1987].)

tyrosine residues on TGB to form monoiodotyrosine (MIT) and diiodotyrosine (DIT), which remain attached to TGB. Coupling between MIT and DIT occurs such that a combined MIT and DIT forms T_3, whereas a combined DIT and DIT forms T_4 (Capen, 2001).

The iodinated TGB is stored in the follicular lumen as colloid until the thyroid gland is stimulated to secrete hormone. Upon stimulation, iodinated TGB is endocytosed into the epithelial cell and transported in the direction of the basal membrane where lysosomal enzymes hydrolyze peptide bonds to release T_3 and T_4 for passive diffusion into the circulation. Remaining MIT and DIT are recycled in the epithelial cell for synthesis of new TGB (Capen, 2001).

T_4 from the thyroid gland can be peripherally converted to T_3 (active hormone) or reverse T_3 (rT_3, the inactive metabolite) then successively deiodinated by the monodeiodinases. About 40% of circulating T_4 is metabolized to T_3 by 5′-monodeiodinase, and 40% is converted to rT_3 by 5-monodeiodinase (Capen, 2001; Zoeller et al., 2007). Additionally, about two-thirds of T_3 and all rT_3 in circulation are produced from T_4 by peripheral conversion. As a result, the ratio of circulating T_4 and T_3 does not reflect the ratio of these two substances when they were released from the thyroid gland. Based on a variety of observations, it appears that circulating T_4 levels provide a "sink" of prohormone that can serve as a ready supply for peripheral conversion to T_3 (the active form).

Several studies indicate that xenobiotics can interfere with thyroid gland function by adversely affecting the process of thyroid hormone synthesis (Kortenkamp, 2008). For example, environmental chemicals such as perchlorate, chlorate, and bromate inhibit uptake of iodide and thus decrease thyroid hormone synthesis (Crofton, 2008; Steinmaus et al., 2013; Šošić-Jurjević et al., 2014). Triclosan and triclocarban inhibit iodide uptake and thyroperoxidase activity (Wu et al., 2016). Some chemicals such as genistein and daidzein in soy products, thionamides, and substituted phenols can inhibit thyroid peroxidase, blocking incorporation of iodide into TGB (Doerge and Chang, 2002; Delclos and Newbold, 2007). Further, some chemicals may interfere with monodeiodinases, leading to decreased levels of thyroid hormones. Specifically, studies have shown that red dye #3 and propylthiouracil inhibit 5′-monodeiodinase, leading to reduced serum levels of T_3 (Capen, 2001; Crofton, 2008).

Thyroid Hormone Binding Proteins

Once released into the blood, thyroid hormones are rapidly bound to high-affinity serum binding proteins (Capen, 2001). The result is that less than 1% of the T_3 (99.7% bound) and less than 0.1% of the T_4 (99.97% bound) are free in circulation. Only the small unbound fraction of the total hormone pool has access to receptors in target cells, and thus only the unbound fraction can exert biological activity.

There are three types of thyroid hormone binding proteins: thyroid binding globulin (TBG), thyroxine-binding prealbumin (TBPA), and albumin (Capen, 2001). TBG binds about 80% of the thyroid hormones, whereas TBPA and albumin each bind about 10% of the thyroid hormones. Environmental chemicals such as the PCBs and PBDEs are known to displace thyroid hormones from serum binding proteins (Yamauchi and Ishihara, 2006; Patrick 2009; Jugan et al., 2010). The displacement of thyroid hormones from the binding proteins often leads to a rapid decline in serum thyroid hormone levels.

Thyroid Hormone Receptors

Thyroid hormones act by binding to thyroid hormone receptors (TRs; Zoeller, 2005). TRs are members of the nuclear receptor superfamily of ligand-inducible transcription factors. TRs can form homodimers or heterodimers with other nuclear hormone receptors such as the retinoid X receptor (Zoeller, 2005). The homodimers and heterodimers bind to thyroid hormone response elements located in target genes and interact with coactivators and corepressors to regulate transcription. In humans, thyroid hormone receptors are the products of two genes that encode three thyroid hormone receptor isoforms known as TRα, TRβ1, and TRβ2 (Zoeller, 2005). While all three isoforms are present in most tissues, their expression differs spatially and temporally during development (Zoeller, 2005). TRα is abundant in the brain, heart, and immune system, whereas TRβ1 is particularly expressed in the brain, liver, and kidney (Zoeller, 2005).

Environmental chemicals can interfere with thyroid hormone binding to TRs and thyroid hormone–related transcription at multiple levels (Kitamura et al., 2002; Zoeller, 2005; Patrick, 2009; Jugan et al., 2010). First, some chemicals such as PBDEs can bind directly to TRs and induce either agonistic or antagonist effects (Zoeller, 2005). Interestingly, some PBDE congeners have different affinities for TRα and TRβ, whereas some congeners can bind to more than one isoform. Second, some environmental chemicals interfere with thyroid hormone binding to receptors via indirect mechanisms (Zoeller, 2005). In such cases, it is thought that the chemicals exert their effects by promoting coactivators or inhibiting corepressors. For example, BPA impairs thyroid hormone action by inhibiting T_3 binding to TR and by recruiting the nuclear corepressor N-CoR to the TR, resulting in repression of transcription (Zoeller, 2005; Patrick, 2009). Similarly, some PCBs are able to suppress TR/coactivator (SRC-1) complex–mediated transactivation, leading to suppression of TR-mediated transcription (Crofton, 2008). Third, some xenobiotics can interfere with cross talk between TRs and other nuclear receptors. For example, PCBs can partially dissociate the heterodimer TR/retinoic acid receptor from the T_3-response element (Crofton, 2008). BPA suppresses TR transcription by interfering with T3/T4-mediated β3 integrin/c-Src/MAPK/TR-β1 pathways (Sheng et al., 2012). Finally, some chemicals such as BPA, phthalates, and acetochlor can inhibit expression of TRs (Patrick, 2009; Yang et al., 2016).

Thyroid Hormone Clearance

The main pathway for clearance of thyroid hormones from the serum is via conjugation to glucuronic acid or sulfate by phase II enzymes such as glucuronyl transferases (UDPGTs) and sulfotransferases (SULTs), respectively (Patrick, 2009; Zoeller, 2010). The metabolites then are transported across plasma membranes for elimination by transporters, including the multidrug resistance protein 1 and the multidrug resistance–associated protein 2 (MRP2).

A number of studies indicate that some xenobiotics may increase the clearance of thyroid hormones from the serum (McClain et al., 1989). This effect limits the availability of thyroid hormones to act on tissues and often results in symptoms of hypothyroidism (Brouwer et al., 1998; Yamauchi and Ishihara, 2006). For example, coplanar and noncoplanar PCB congeners have been shown to induce UDPGTs and SULTs, resulting in low serum T_4 levels (Crofton, 2008; Patrick, 2009). In contrast, a few studies have shown that xenobiotics such as pentachlorophenol or triclosan may inhibit SULTs, increasing the availability of thyroid hormones to act on tissues (Jekat et al., 1994; Crofton, 2008; Patrick, 2009). Studies have also shown that xenobiotics such as dioxin, rifampicin, and phenobarbital may decrease the transport of thyroid hormones into the brain and liver by inhibiting transporters (Crofton, 2008).

Regulation of Thyroid Hormone Release

Thyroid hormone secretion is regulated by thyroid-stimulating hormone (TSH) (thyrotropin) from the anterior pituitary gland. The rate of release of TSH is under a hypothalamic–pituitary–thyroid regulatory axis involving negative feedback (Fig. 20-10). Specifically, a hypothalamic releasing hormone known as TRH directly stimulates release of TSH from the anterior pituitary gland. TSH then increases secretion of T_4 and T_3 from the thyroid gland. In turn, T_4 and T_3 can feed back to the anterior pituitary to inhibit TSH release and they can feed back to the hypothalamus to inhibit TRH release. Interestingly, most inhibition by thyroid hormones is by T_4, although T_3 can also provide some degree of inhibition. Further, the feedback effect is mediated primarily at the level of the anterior pituitary, but some degree of negative feedback occurs at the level of the hypothalamus.

In addition to these methods of regulating thyroid hormone secretion, there is a circadian rhythm of TRH and TSH release, with a decrease following the onset of sleep. Overall, the thyroid axis responds rapidly at the hypothalamo-pituitary unit, but beyond this level, the system is governed by processes that have extremely long time constants. Therefore, the long half-lives of thyroid hormones in circulation dampen the diurnal rhythm that is obvious in TSH levels. As a result, this rhythm is not reflected in circulating thyroid hormone concentrations. This contributes to the "sluggishness" of the system.

Xenobiotics have been shown to alter the ability of the hypothalamic–pituitary–thyroid regulatory axis to control thyroid hormone levels (Zoeller, 2005; Patrick, 2009). For example, chemicals such as PBDEs may increase TSH levels, leading to increased levels of T_4 and T_3 (Darnerud et al., 2001). Alternatively, xenobiotics may inhibit TRH or TSH levels, leading to decreased levels of T_4 and T_3 (Patrick, 2009).

Physiological Effects

Thyroid hormones influence nearly every tissue in the body, in a variety of ways. In spite of complexities of hormone action on target tissues, it is reasonably accurate to simply view thyroid hormone as the primary determinant of the overall metabolic rate of the body. The effects are exaggerated in states of thyroid excess (hyperthyroidism) or deficiency (hypothyroidism) and produce the clinical and biochemical manifestations of these disorders. In general, thyroid hormone stimulates both anabolic and catabolic biochemical pathways; however, its overriding effect is catabolism (energy mobilization).

Thyroid hormone also produces significant effects on growth and development (Zoeller et al., 2002). It is essential for normal development of the CNS and for maturation of the skeleton (Zoeller et al., 2002; Dickerson and Gore, 2007; Gore, 2011). A deficiency of thyroid hormone in early life leads to a delay in development of the brain in animal models and humans. Brain development is especially dependent on thyroid hormone during the first several months after birth in humans (Zoeller, 2005). If thyroid hormone levels are inadequate during this period, severe irreversible mental retardation in the form of cretinism occurs. Early diagnosis and immediate replacement with thyroid hormones can prevent these effects and are therefore essential.

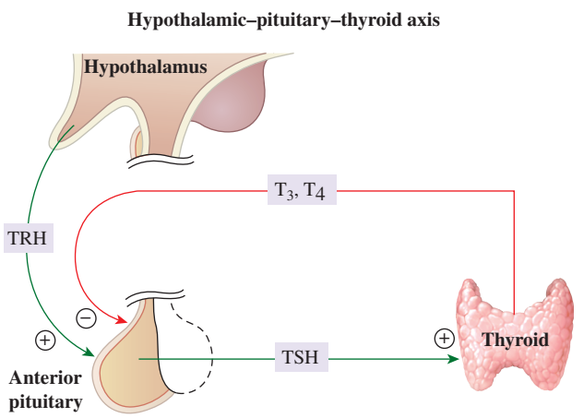

Figure 20-10. *Hypothalamic–pituitary–thyroid axis.* The hypothalamus synthesizes and secretes thyroid-releasing hormone (TRH). TRH travels to the anterior pituitary via the portal plexus and stimulates the thyrotropes to synthesize and secrete TSH. TSH acts on the thyroid gland to stimulate production and/or release of T_3 and T_4. T_3 and T_4 can then exert negative feedback control at the level of the anterior pituitary to inhibit further release of TSH. (Modified from Hedge et al. [1987].)

Thyroid Toxicity

Given the influence of thyroid hormones on numerous tissues in the body, it is not surprising that xenobiotics that affect thyroid hormone levels often cause symptoms of hypothyroidism or hyperthyroidism, or lead to significant impairment in brain development and function. Below are some specific examples of environmental chemicals that have been shown to affect thyroid hormone levels through a variety of mechanisms, resulting in adverse physiological outcomes.

PCBs PCBs are some of the best characterized thyroid-disrupting chemicals (Dickerson and Gore, 2007; Boas et al., 2009; Patrick, 2009; Jugan et al., 2010; Zoeller, 2010; Gore, 2011). PCBs are industrial chemicals that were widely used in capacitors and transformers. They are made of two phenyl rings with varying degrees of chlorination, resulting in 209 different congeners. The production of PCBs was banned in the 1970s, but unfortunately, PCBs are persistent chemicals and thus they are still routinely found in humans and wildlife blood samples and tissues.

PCBs are known to interfere with the thyroid system in a manner that leads to serious neurocognitive effects. Several human studies indicate that PCB exposure in prenatal life is associated with lower full-scale and verbal IQ scores and less short-term and long-term memory and attention in postnatal life (Porterfield, 2000; Zoeller et al., 2002). Rodent studies indicate that prenatal PCB exposure increases hyperactivity. Further, rodent studies indicate that PCB exposure causes hearing disorders because the developing auditory system is sensitive to thyroid hormones (Porterfield, 2000; Zoeller et al., 2002).

Although PCBs may exert negative effects on animals and humans via several mechanisms, the most common pathway is thought to include PCB inhibition of thyroid hormone levels or activity (Patrick, 2009; Gore, 2011). Several studies indicate that PCBs decrease the levels of thyroid hormone by inhibiting thyroid hormone synthesis and/or increasing the metabolism of thyroid hormones by increasing phase II enzymes (Zoeller, 2010). For example, several studies have reported the ability of PCB and other microsomal enzyme inducers to induce UDP-glucuronosyltransferase (UDP-GT) expression (Barter and Klaassen, 1994; Hood et al., 2003). Increased UDP-GT activity decreases serum T_4 levels in rats and mice by increasing glucuronidation and biliary excretion of T_4. Other studies indicate that PCBs interfere with thyroid hormone action by inhibiting the binding of thyroid hormones to binding

proteins or blocking the ability of thyroid hormones to bind to TRs (Zoeller, 2010). In addition, a study in Dutch infants indicates that PCB exposure is associated with negative effects on deiodinase activity (Soechitram et al., 2017).

PBDEs PBDEs are also well-known thyroid-disrupting chemicals (Boas et al., 2009; Jugan et al., 2010; Zoeller, 2010). These chemicals are flame retardants that are used in a variety of products, including electric equipment, clothing, furniture, carpeting, plastics, and paints. PBDEs are not chemically bound to products in which they are used; thus, they can leach from the products into human and wildlife tissues and into the environment over time.

The structure of PBDEs often resembles that of PCBs. Thus, it is not surprising that many of the toxic effects of PBDEs are similar to those elicited by PCBs. Like PCBs, PBDEs have been shown to inhibit thyroid hormone levels and/action, leading to serious neurocognitive deficits. PBDEs inhibit thyroid hormone levels by inducing hepatic phase II enzymes, resulting in increased metabolism of circulating thyroid hormones (Zoeller, 2010). They also can downregulate proteins required for transporting thyroid hormone into target cells and they can bind to TRs, blocking the ability of thyroid hormones to bind to TRs (Zoeller, 2010).

Perchlorate Perchlorate is another thyroid-disrupting chemical (Crofton, 2008; Patrick, 2009; Jugan et al., 2010; Zoeller, 2010). This chemical is widely used as a rocket propellant as well as a chemical in fireworks and airbag deployment systems. Perchlorate is also used in pharmaceutical industries. It is a highly stable and water-soluble compound and thus it is known to persist in the environment, particularly in the water and food supply (Abt et al., 2018). While less is known about the effects of perchlorate on the thyroid system than about PCBs and PDBEs, a few studies indicate that perchlorate exposure inhibits thyroid hormone levels, possibly leading to hypothyroid-like outcomes (Crofton, 2008; Patrick, 2009; Gardell et al., 2016). For example, in a longitudinal study of girls in the United States, exposure to perchlorate along with thiocyanate and nitrate was associated with slow growth in waist circumference and body mass index, but not height (Mervish et al., 2016). In a study on the freshwater fish, threespine stickleback, perchlorate exposure increases lipid droplet numbers, suggesting an obesogenic effect (Gardell et al., 2016). The mechanism of action of perchlorate is thought to be primarily by reducing iodide uptake, which ultimately reduces thyroid hormone synthesis (Patrick, 2009; Schlosser, 2016). It is important to note, however, that the effects of perchlorate on the thyroid gland have not been well studied and the results to date in humans are equivocal.

Pesticides Pesticides such as dichlorodiphenyltrichloroethane (DDT) and hexachlorobenzene (HCB) are known thyroid-disrupting chemicals (Boas et al., 2009; Chiappini et al., 2013; Gore et al., 2015; Campos and Freire, 2016). While both DDT and HCB have been banned in many countries, they persist in the environment due to their long environmental half-lives and thus they may place humans and wildlife at risk for thyroid disorders. Pesticide mixtures containing DDT have been shown to increase thyroid volume and to induce antibodies that attack the thyroid gland, resulting in autoimmune thyroid disease (Crofton, 2008; Boas et al., 2009; Patrick, 2009). Further, DDT has been shown to inhibit TSH receptors, blocking the ability of TSH to induce secretion of T_4 and T_3 and in turn resulting in low circulating T_4 and T_3 levels (Boas et al., 2009; Patrick, 2009). The stable metabolite of DDT known as *p*,*p*′-DDE has been shown to be positively associated with total T_3 and T_4 levels in floricultural workers (Blanco-Muñoz et al., 2016). HCB has been shown to interfere with thyroid function by blocking the ability of thyroid hormones to bind to TR (Crofton, 2008; Boas et al., 2009; Patrick 2009). HCB also causes apoptosis in thyroid cells by reactive oxygen species–mediated ERK1/2 activation (Chiappini et al., 2013). Organochlorine pesticide exposure has been associated with increased TSH and decreased T_4 in a male agricultural population (Piccoli et al., 2016).

Perfluorinated Chemicals Perfluorinated chemicals (PFCs) are a family of chemicals used in many products due to their surface protection properties. Such products include stains and oil-resistant coatings, floor polishes, and insecticides. Some studies have shown that a PFC known as perfluorooctane sulfonate (PFOS) decreases T_4 levels in pregnant dams as well as their pups (Boas et al., 2009). The PFC known as perfluorooctanoic acid (PFOA) has been shown to decrease T_3 levels. PFOA and perfluorononanoic acid exposure were associated with increased T3 and T4 levels in women (Wen et al., 2013; Crawford et al., 2017). While the mechanisms by which perfluorinated chemicals alter thyroid hormone levels are not completely clear, studies suggest that they regulate phase II metabolic enzymes in the liver and increase deiodinases in the thyroid gland (Boas et al., 2009). Further, studies indicate that PFOA and PFOS enter thyroid cells by passive diffusion (Coperchini et al., 2015), which could result in prolonged in vivo exposure to PFCs and lead to damage.

Bisphenol A Bisphenol A (BPA) is a suspected possible thyroid hormone–disrupting chemical (Boas et al., 2009; Zoeller, 2010). It is primarily used in the manufacture of polycarbonate plastics such as those used in baby bottles, toys, and food containers. It is also a component of dental sealants and the linings of food cans. BPA can leach out of products and enter the blood and organs. In fact, recent studies indicate that over 95% of human urine samples contain BPA, indicating continuous and widespread exposure to this chemical. Given the vast exposure of humans to BPA, it is important to consider its potential effects on the thyroid gland. Some laboratory studies have shown that BPA blocks T_3 action by antagonizing the binding of T_3 to its receptor (Zoeller, 2010). Further, some studies have shown that BPA inhibits T_3-mediated gene expression in cell lines (Zoeller, 2010). Studies have also shown that BPA exposure is associated with an increase in anti-thyroperoxidase antibodies in men and women (Chailurkit et al., 2016), a decrease in thyroid volume in school children (Wang et al., 2015), and decreased T_4 levels in men (Sriphrapradang et al., 2013). While the effects of BPA-induced inhibition of thyroid hormone action in humans are not completely understood, several studies suggest that BPA leads to symptoms of hypothyroidism or thyroid resistance syndrome in animal models (Zoeller, 2010; Viguié et al., 2013; Gore et al., 2015). Further, prenatal BPA exposure has been shown to cause attention deficit/hyperactivity-disorder-like symptoms in rodents in postnatal life (Zoeller, 2010) and it has been associated with an increased risk of behavior problems in boys (Evans et al., 2014). These effects are thought to be due, in part, to the BPA-induced inhibition of normal thyroid function, possibly by inhibiting sulfotransferase activity, disrupting thyroid-specific gene expression, and/or suppressing thyroid hormone receptor transcription (Zoeller, 2010; Sheng et al., 2012; Butt and Stapleton, 2013; Gentilcore et al., 2013; Gore et al., 2015).

Phthalates A few recent studies suggest that phthalates may act as thyroid-disrupting agents; however, they are not thought to do so to the same degree as PCBs and PBDEs (Boas et al., 2009; Jugan et al., 2010; Ye et al., 2017). Phthalates are used to improve the flexibility of plastics used in a variety of products, including toys, medical tubing, plastic bottles, and cosmetics. To date, a few small human studies have shown that phthalate exposures may alter the levels of T_4 and T_3 levels in adult men and pregnant women

(Jugan et al., 2010). Interestingly, a by-product of phthalates produced by gram-negative bacteria has been shown to inhibit thyroperoxidase, an enzyme required for thyroid hormone synthesis (Jugan et al., 2010). This results in low thyroid hormone levels and to symptoms of hypothyroidism in humans. A few rodent studies have shown that a phthalate known as di-*n*-butyl phthalate decreases T_4 and T_3 in a dose-dependent manner (Boas et al., 2009; Jugan et al., 2010). Rodent studies have also shown that di(2-ethylhexyl) phthalate reduces thyroid hormone levels in rats (Liu et al., 2015). This likely occurs through a mechanism involving the ability of phthalates to activate the Ras/Akt/TRH receptor pathway (Ye et al., 2017) and/or disrupt the hypothalamic–pituitary–thyroid axis (Dong et al., 2017).

Studies on the consequences of phthalate-induced changes in thyroid hormone levels in humans or rodents are limited at this time. However, one study indicates that gestational exposure to di-*n*-butyl phthalates alters the thyroid hormone profile in rats, leading to defects in sensory motor, craniofacial development, and eye formation (Mahaboob and Radha, 2017). Another study indicates that phthalate exposure is associated with thyroid hormones in children and suggests that this association between phthalate exposure and thyroid hormones may interfere with growth (Wu et al., 2017).

PARATHYROID GLAND

The parathyroid glands are embedded in the surface of the thyroid gland (Fig. 20-7) (Capen and Rosol, 1989). Humans have four parathyroid glands, which are located on the back of side of the thyroid gland. The parathyroid glands are composed of chief cells and oxyphil cells (Capen and Rosol, 1989). The main function of chief cells is to produce parathyroid hormone (PTH), whereas the function of the oxyphil cells is unknown (Capen and Rosol, 1989). In humans, oxyphil cells are absent at birth, appear around puberty, and increase in number with age. Thus, it is thought that they may represent structurally and functionally modified chief cells.

The parathyroid glands are critical for life (Capen and Rosol, 1989). This is largely because PTH helps maintain normal plasma calcium levels (Capen and Rosol, 1989) (Fig. 20-11). Calcium is required in optimal concentrations for many of life's fundamental processes: fertilization, vision, locomotion-muscle contraction, nerve conduction, blood clotting, exocytosis, cell division, and the activity of a number of enzymes and hormones. Therefore, the concentrations of calcium in the cellular and extracellular fluids must be maintained at a constant value. When the parathyroid glands are removed or damaged, PTH levels drop, causing a major drop in circulating calcium levels. In turn, this can lead to tetanic convulsions and death (Capen and Rosol, 1989).

Parathyroid Toxicity

Xenobiotic exposures may alter the structure of the parathyroid gland. In some cases, chemicals such as the anticancer drug L-asparaginase cause death of parathyroid cells (Capen and Rosol, 1989; Capen, 2001). Specifically, studies have shown that L-asparaginase selectively destroys chief cells in rabbits. This results in a reduced size and capacity of the parathyroid to release PTH, which eventually leads to tetany and death. Studies have also shown that sublethal doses of heroin cause degenerative changes in the rat parathyroid gland (Barai et al., 2009). These changes are characterized by cytoplasmic vacuolization, pyknotic nuclei in the chief cells, and dying Golgi complexes and mitochondria.

Many xenobiotic exposures have been shown to increase the size of the parathyroid gland. Lead exposure has been shown to significantly increase parathyroid gland weight (Szabo et al., 1991).

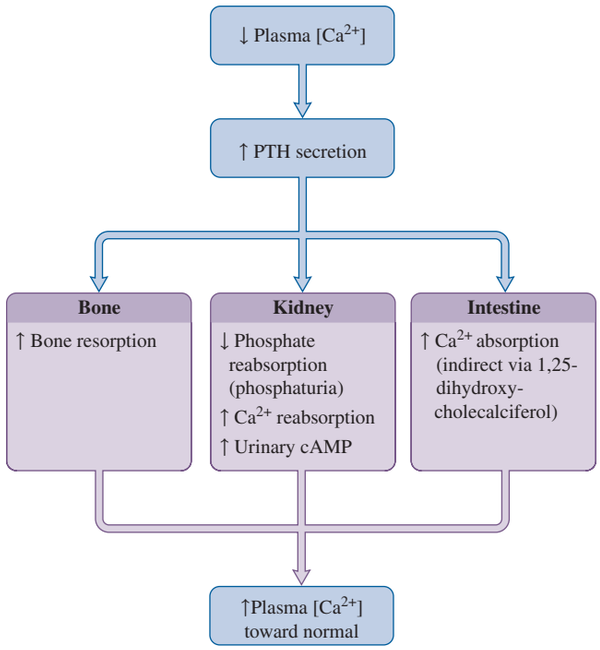

Figure 20-11. *Effects of calcium on target tissues.* Low levels of circulating Ca^{2+} stimulate an increase in secretion of parathyroid hormone (PTH). PTH helps restore normal plasma Ca^{2+} levels by acting on bone, the kidney, and the intestine. (Reproduced with permission from Boron and Boulpaep [2005].)

Some pesticides such as rotenone, malathion, and malaoxon increase proliferation of the chief cells and thus increase the size of the parathyroid gland (Reuber, 1985; Abdo et al., 1988). This scenario often results in parathyroid gland cancer. Further, fungicides such as hexachlorobenzene and bis(tri-*n*-butyltin)oxide, the antifreeze ethylene glycol, the broad-spectrum germicide *o*-benzyl-*p*-chlorophenol, and the diuretic drug hydrochlorothiazide have been shown to induce proliferation and/or adenomas in rat parathyroid glands (Arnold et al., 1986; DePass et al., 1986; Lijinsky and Reuber, 1987; Andrews et al., 1989; Bucher et al., 1990; Wester et al., 1990; National Toxicology Program, 1994). Similarly, irradiation and coumarin exposure induce parathyroid gland adenomas in rodents (National Toxicology Program, 1993). It is unclear whether all of the effects of xenobiotics are due to a primary effect of the chemical on the parathyroid gland or due to effects on the kidney or bones, which in turn alter the parathyroid gland (secondary effects).

PTH Structure and Synthesis

PTH is a polypeptide hormone that is derived from a precursor molecule called preproparathyroid hormone (Potts, 2005) (Fig. 20-12). In humans, preproparathyroid hormone is composed of 115 amino acid residues. The preproparathyroid hormone is cleaved by trypsin-like proteases in the Golgi zone of the chief cells to become proparathyroid hormone, which is 90 amino acids in size. Six amino acid residues are then removed from the proparathyroid hormone by proteases in the Golgi to make PTH. PTH is then packaged into secretory vesicles that migrate to the periphery of the cell, where the hormone is secreted via vesicular exocytosis (Capen, 2001; Potts, 2005). Interestingly, the structure of this hormone is highly conserved among many species, including humans, cows, pigs, dogs, rats, and chickens (Potts, 2005).

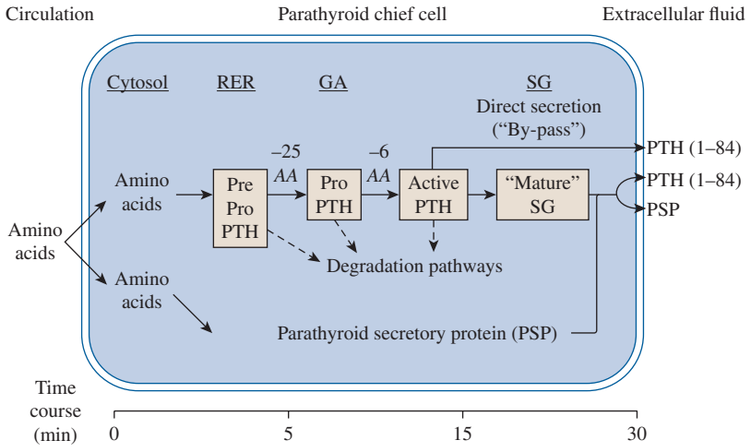

Figure 20-12. *Biosynthesis of PTH.* Active PTH is synthesized as a larger biosynthetic precursor molecule (preproPTH) that undergoes rapid posttranslational processing to proPTH prior to secretion as active PTH (amino acids 1 to 84) from chief cells in the parathyroid glands.

A few studies indicate that xenobiotics may interfere with the normal synthesis of PTH. Metals such as aluminum and cadmium have been shown to inhibit PTH secretion in a variety of species, including humans (Cannata et al., 1983; Kido et al., 1991; Jeffery et al., 1996; Rignell-Hydbom et al., 2009). Similarly, alcohol consumption has been shown to decrease PTH levels in pregnant rats (Keiver and Weinberg, 2003). Lithium, a drug widely used to manage manic-depressive illnesses, has been associated with a rise in PTH levels as well as abnormally high calcium levels (Matsis et al., 1989). Prenatal exposure to nicotine reduces the activity of parathyroid chief cells, leading to hypertrophy and increased levels of calcitonin in parafollicular cells (Abdelhafez et al., 2014). Interestingly, a longitudinal study on Gulf War I veterans showed that depleted uranium exposure was associated with significant decreases in serum levels of PTH as well as significant increases in urinary calcium (Tissandie et al., 2006; McDiarmid et al., 2011). A study on lead exposure in battery manufacturers showed that lead exposure was associated with increased levels of PTH (Dongre et al., 2013). Similarly, lithium treatment was associated with increased levels of parathyroid hormones in patients (McKnight et al., 2012).

PTH Receptors

The PTH receptor is a single G protein–coupled receptor called the PTH/PTHrP receptor (PTHR1; Potts, 2005). This receptor is the primary receptor that mediates the traditional actions of PTH. However, it is thought other receptors exist that bind to portions of PTH or proparathyroid hormone. In humans, the gene for the PTHR1 is located on chromosome 3 and consists of 14 exons. PTHR1 has been extensively localized in bone and kidney cells (Potts, 2005).

Limited information is available about whether xenobiotics bind PTHR1 and exert agonistic or antagonistic actions. However, one study shows that xenobiotics may alter the expression of PTHR1. Specifically, studies have shown that binge alcohol drinking significantly decreases expression of PTHR1 in male rats (Callaci et al., 2009).

PTH Clearance

PTH is primarily metabolized by the liver and kidney (Capen and Rosol, 1989). It is thought that the hormone is cleaved into two major fragments (N-terminus and C-terminus) quite quickly by metabolic enzymes. In cows, for example, the half-life of PTH is estimated to be between 3 and 4 minutes. In dogs, the half-life of PTH is between 5 and 6 minutes, with about 60% of PTH metabolism occurring in the liver.

It is possible that xenobiotics increase the metabolism of PTH and that this leads to abnormally low levels of PTH. Alternatively, it is possible that xenobiotics decrease the metabolism of PTH and that this leads to high levels of PTH. Unfortunately, these issues have not been examined in detail and it is unclear which xenobiotics, if any, directly affect PTH metabolism.

Physiological Effects

The main physiological role of the parathyroid gland is to control circulating calcium levels (Capen and Rosol, 1989; Capen, 2001). Given the importance of maintaining normal levels of circulating calcium, multicellular organisms have evolved a complex system of controls to ensure this constancy. Thus, PTH works in concert with CT and vitamin D (Capen, 2001). CT is secreted from the C cells of the thyroid gland (Capen, 2001). Vitamin D is produced from precursors in the skin, and it is obtained from the diet (Capen, 2001).

PTH serves to increase circulating calcium levels by increasing the release of calcium from bone (Capen, 2001). Bone is remodeled continuously during adulthood by the resorption of old bone by osteoclasts and the subsequent formation of new bone by osteoblasts. These two events are responsible for renewing the skeleton, while maintaining its anatomical and structural integrity. Under normal conditions, bone remodeling proceeds in cycles in which osteoclasts adhere to bone and subsequently remove it by acidification and proteolytic digestion. Shortly after the osteoclasts have left the resorption site, osteoblasts invade the area and begin the process of forming new bone by secreting osteoid (a matrix of collagen and other proteins), which is eventually mineralized (Capen, 2001). In regulating bone remodeling, PTH binds to osteoblasts and increases IL-6 which, along with other cytokines, causes osteoclast differentiation. In turn, this demineralizes bone and releases calcium from the bone into circulation (Capen, 2001).

PTH also serves to increase calcium levels by increasing the tubular reabsorption of calcium by the kidney (Capen and Rosol, 1989). Further, it inhibits the renal reabsorption of phosphate, which aids in increasing the solubility of calcium. PTH also enhances magnesium reabsorption, inhibits bicarbonate ion reabsorption, and blocks exchange of sodium ions by the tubules. These actions of

PTH result in metabolic acidosis, which favors removal of calcium from plasma proteins and bones. In turn, this increases circulating levels of ionized calcium (Capen, 2001).

CT reduces circulating calcium levels by reversing the action of PTH on bone resorption (Capen and Rosol, 1989). When circulating calcium levels become high, CT is secreted by the C cells of the thyroid. CT serves to prevent hypercalcemia by shutting down efflux of calcium from bone. It also negatively regulates PTH to prevent kidney calcification (Capen, 2001).

Vitamin D_3 (cholecalciferol) is a steroid-like compound that is essential for calcium absorption in the gastrointestinal tract. It is derived from cholesterol, and the active form is produced from a precursor 7-dehydrocholesterol. Exposure of the skin to ultraviolet light causes formation of vitamin D, which is biologically inert and must be activated by two sequential hydroxylations (Capen, 2001). The first hydroxylation occurs in the liver, and the second occurs in the kidney. The second conversion is stimulated by PTH activation of 1-hydroxylase enzyme activity. Vitamin D also serves to inhibit PTH actions and build bone.

Some xenobiotics such as pesticides and fungicides can cause excessive PTH secretion by the parathyroid gland and lead to hyperparathyroidism (Capen, 2001). However, it is important to note that most excessive PTH secretion is due to a parathyroid adenoma that is no longer under the negative feedback control of calcium. The traditional symptoms of PTH excess have been characterized as "stones, bones, and groans" for several reasons. First, excessive PTH levels lead to hypercalcemia, hypercalciuria, and hypophosphatemia, which often lead to the formation of renal stones. The constant supply of calcium released via unchecked bone resorption must eventually be excreted, and even though PTH normally elicits calcium reabsorption from the kidney, the consistently elevated calcium exceeds the renal threshold for reabsorption such that calcium leaks into the urine. The hallmark of this disease is abnormally increased bone resorption, leading to severe bone pain.

Xenobiotic exposures such as those to heavy metals may cause low PTH secretion and lead to hypoparathyroidism (Pounds, 1984; Long et al., 1992). This condition leads to a very densely calcified skeleton, hypocalcemia, and hyperphosphatemia. Of great concern is that hypoparathyroidism often leads to tetany and death (Capen, 2001).

Regulation of PTH Release

PTH release is controlled by circulating calcium levels. The cells in the parathyroid gland, kidney, and other cells that respond to calcium possess recognition sites for circulating calcium levels known as calcium sensors or receptors. Recently, the calcium sensor or calcium receptor on the parathyroid cell was cloned and determined to belong to the 7-transmembrane class of G protein–coupled receptors linked to phospholipase C.

When the calcium receptors in the parathyroid gland sense low calcium levels, they stimulate the parathyroid gland to release PTH which functions to raise plasma calcium primarily by stimulating bone resorption and secondarily by enhancing renal calcium reabsorption. Further, PTH stimulates the metabolism in the kidney of vitamin D to its active hormonal form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or 1,25-dihydroxycholecalciferol. This last effect of PTH shifts calcium recovery from the skeletal reserve and the kidney in the acute situation to intestinal absorption mediated by $1,25(OH)_2D_3$ in the chronic situation, thereby sparing mineralized bone.

In general, xenobiotic exposures that interfere with the regulation of PTH could cause problems in controlling calcium levels. As mentioned above, lithium has been shown to increase PTH levels and in turn increase circulating levels of calcium. Depleted uranium exposure has been associated with low PTH and consequently abnormal levels of calcium in the urine (McDiarmid et al., 2011).

ENDOCRINE PANCREAS

The pancreas is an organ that has both endocrine and nonendocrine functions. The acinar or exocrine portion of the pancreas is concerned primarily with the regulation of gastrointestinal function. Scattered among the pancreatic acini are the endocrine units of the pancreas, the Islets of Langerhans. The Islets of Langerhans comprise only 1% to 2% of the weight of the pancreas. The major physiological function of the endocrine pancreas is to serve as the primary homeostatic regulator of fuel metabolism, particularly circulating glucose. Islet cells are sensors of glucose homeostasis (maintaining balance by regulation and counterregulation) that respond to changes in their nutrient and hormonal environment. The hormones secreted by islets have major effects directly on the liver. Three major cell types within the endocrine pancreas are known to produce the hormones involved in this regulation. The most abundant cell type is the beta cell (β), the site of synthesis and secretion of insulin. Glucagon is produced by the alpha cell (α) and the delta cell (δ) is the site of somatostatin synthesis. It is likely that a functional relationship exists between the various cell types of the islets because it is known that both glucagon and somatostatin affect insulin secretion, and that somatostatin also influences glucagon secretion.

Role of the Liver in Glucose Production

Energy for cellular metabolism can be derived from fatty acids (β oxidation) or glucose (glycolysis, TCA cycle) in the blood. The liver is the primary contributor to increasing blood glucose levels. Fig. 20-13 summarizes physiological sources of energy for cellular metabolism.

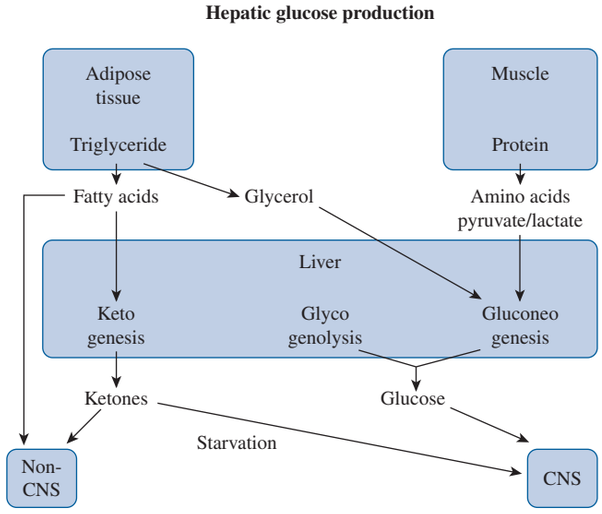

Figure 20-13. *Hepatic production of glucose.* The liver provides most of the circulating glucose in the fasting state by glycogen breakdown (glycogenolysis) and de novo synthesis (gluconeogenesis). Substrates for gluconeogenesis are provided by adipose tissue (glycerol from triglyceride breakdown) and muscle (amino acids from protein breakdown). Fatty acids from triglyceride breakdown are used to produce ketones, which can be used by the CNS for energy as an alternate to glucose during starvation.

Pancreatic Hormones

Insulin The overall effects of insulin are to stimulate anabolic processes (energy storage). Specifically, insulin functions to lower blood levels of glucose, fatty acids, and amino acids and to promote their conversion to the storage form of each: glycogen, triglycerides, and protein, respectively. A number of factors affect the rate of secretion of insulin. The most powerful physiological stimulus is increased circulating blood glucose. In addition, an increase in the concentration of amino acids (especially arginine and leucine) and ketone bodies in blood also increase the rate of secretion of insulin. Glucagon and the gastrointestinal peptides gastrin, secretin, and gastric inhibitory polypeptide also stimulate release of insulin. Conversely, insulin secretion is inhibited by hypoglycemia, epinephrine, and norepinephrine via α_2-adrenergic receptors (inhibition of cAMP production) and somatostatin.

The variety of physiological responses to insulin include (a) increased cellular glucose uptake (in most tissues), (b) lower blood glucose levels, (c) stimulated glycogen synthesis (liver, muscle), (d) stimulated glycerol production (adipose tissue), (e) increased amino acid uptake (liver, muscle), (f) inhibited lipolysis (adipose tissue), and (g) stimulated protein synthesis (replication, transcription, and translation), a mitogenic response. As regards the pathophysiology of insulin, hypersecretion produces hypoglycemia and hyposecretion produces diabetes mellitus.

Glucagon Glucagon is the primary hormone with action counterregulatory to insulin, because it stimulates catabolic processes (energy mobilization) to prevent hypoglycemia. The most powerful physiological stimulus of secretion of glucagon is reduced circulating blood glucose. Thus, as blood glucose levels fall (hypoglycemia), glucagon secretion increases in an attempt to restore normal homeostasis. Conversely, an increase in blood glucose levels inhibits glucagon secretion. In addition to circulating levels of glucose, glucagon secretion is regulated by other factors. The release of glucagon is stimulated by epinephrine and norepinephrine (via β-adrenergic receptors; stimulation of cAMP production), and by the amino acids, arginine, leucine, and alanine (unless accompanied by glucose ingestion). Conversely, glucagon secretion is inhibited by insulin and somatostatin.

The physiological responses to glucagon occur mostly in the *liver* with a stimulation of glycogenolysis, gluconeogenesis (conversion of amino acids and glycerol to glucose), lipolysis, and ketogenesis (over a long time). Additionally, glucagon stimulates the secretion of insulin in pancreatic β cells. Due to the ability of other counterregulatory hormones (epinephrine, growth hormone, and cortisol) to compensate for a deficiency of glucagon, there are no significant pathological conditions associated with abnormal glucagon secretion.

Somatostatin Somatostatin was first isolated from the hypothalamus; its role in regulation of neuroendocrine function is to inhibit secretion of growth hormone in the anterior pituitary. After its identification in hypothalamic tissue, somatostatin was found in other cells of the brain, in various parts of the gastrointestinal tract, and in the δ cells of the pancreas. The generalized function of somatostatin appears to be as a hormone release inhibitor. Its physiological role within the pancreas is unknown; however, it inhibits secretion of insulin and glucagon (paracrine effect), and inhibits its own secretion (autocrine effect).

Interactions of Release

In addition to individual regulation of secretion of insulin and glucagon, the interactions of their release as relates to overall nutrient homeostasis of the individual must be considered. Although glucagon and insulin exert opposing effects on carbohydrate metabolism, they act in concert to preserve normoglycemia in the face of any perturbations that might tend to elevate or lower blood glucose. Many substances that influence insulin secretion also affect glucagon secretion but usually in the opposite direction. Fig. 20-14 shows how insulin and glucagon interact to maintain glucose homeostasis (normoglycemia).

Insulin and glucagon exert opposing effects on various metabolic processes (Table 20-4). Therefore, many investigators like to think of the insulin-to-glucagon ratio in blood as an important determinant of the overall metabolic status. Thus, when there is a high ratio of insulin to glucagon, the effects of insulin dominate, producing a relatively anabolic state. When the ratio of insulin to glucagon is low, a catabolic state exists.

Metabolic Responses in Diabetes

Diabetes mellitus is the result of inadequate insulin action. It is usually manifest in one of two forms. Type 1 (insulin-dependent) results from autoimmune-based destruction of pancreatic β cells. Type 2 involves end-organ insensitivity or resistance to insulin

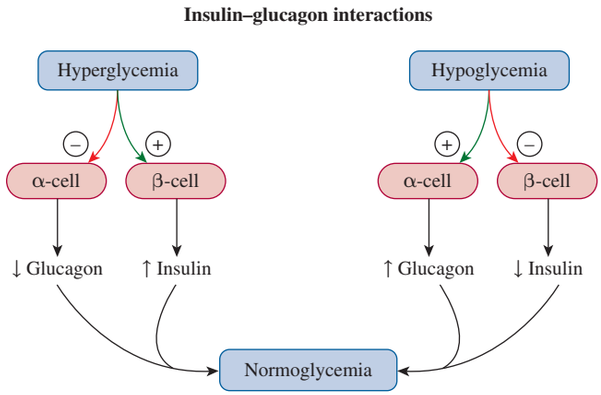

Figure 20-14. *Interactions between insulin and glucagon secretion as regulated by circulating glucose levels.* Hyperglycemia stimulates insulin and inhibits glucagon secretion. Insulin lowers blood glucose to restore normoglycemia. Hypoglycemia inhibits insulin and stimulates glucagon secretion. Glucagon mobilizes glucose from the liver to restore normoglycemia. (Modified from Hedge et al. [1987].)

Table 20-4

Physiological Effects of Insulin and Glucagon

ROLE	INSULIN	GLUCAGON
1. Glucose metabolism	Incr. glucose uptake	Incr. blood glucose
	Decr. blood glucose	
	Incr. glycogen synthesis	Incr. glycogenolysis
	Decr. gluconeogenesis	Incr. gluconeogenesis
2. Fat	Incr. lipogenesis	
	Decr. lipolysis	Incr. lipolysis
3. Protein	Incr. protein synthesis	–

Abbreviations: incr., increased; decr., decreased; –, no effect.

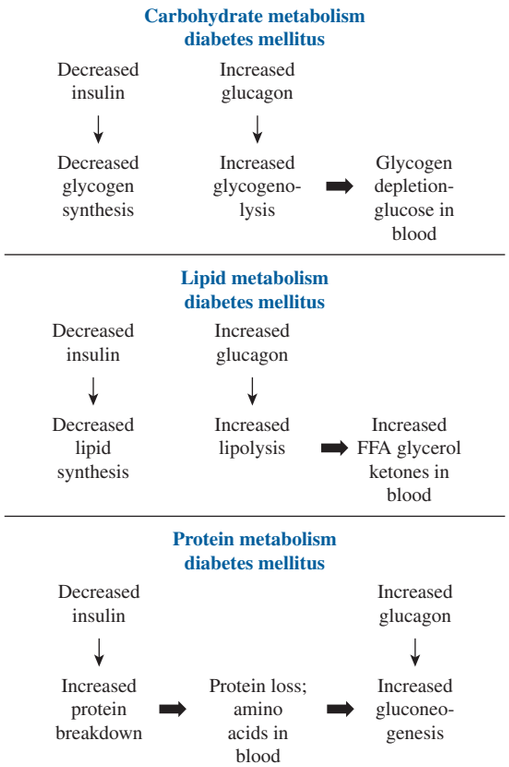

Figure 20-15. *Effects of diabetes mellitus on metabolism.* Decreased insulin (type 1) or insulin action (type 2) inhibits glycogen, lipid, and protein synthesis. Increased glucagon stimulates glycogenolysis, lipolysis, and protein breakdown. Glycogenolysis increases circulating glucose. Increased glycerol and amino acids serve as substrates for gluconeogenesis to further increase circulating glucose.

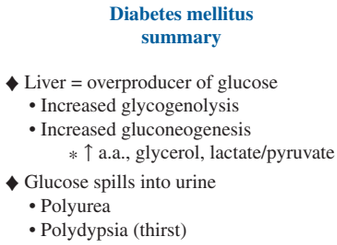

Figure 20-16. *Physiological responses during diabetes mellitus.* Because of increased glycogenolysis and gluconeogenesis stimulated by increased glucagon, the liver becomes an overproducer of glucose. High circulating glucose levels exceed the renal threshold and glucose spills into the urine. This results in polyuria and polydipsia (thirst). a.a., amino acid.

(non-insulin-dependent). In type 2 diabetes, insulin levels also eventually drop due to extended stress placed on pancreatic β cells. As a result of the insufficient physiological insulin action, reduced glucose removal from plasma causes hyperglycemia and a variety of metabolic alterations result. The net effect is that of increased counterregulatory hormone dominance. These effects are summarized in Fig. 20-15. The major clinical effects in poorly controlled diabetes usually relate to progressive deterioration of function in a variety of tissues. Diabetes mellitus constitutes a major public health issue with a worldwide prevalence projected to become 366 million individuals by the year 2030 (Lee et al., 2016). Thus, morbidity and mortality resulting from this disease make diabetes among the most costly of chronic diseases (Fischer, 2010). There is a genetic component associated with diabetes; however, studies in identical twins have suggested there is also most likely an environmental component to the disease. Diet, viral disease, and exogenous chemical substances are thought to trigger a genetic predisposition to its development (Fischer, 2010).

Fig. 20-16 summarizes the clinical consequences of diabetes mellitus in which the liver becomes an overproducer of glucose resulting in glucose excretion from the kidneys. This results not only from lack of insulin action, but increased glucagon secretion (which is insulin-dependent in pancreatic α cells).

Pancreatic Toxicity

The insulin-secreting β cells are particularly sensitive to chemical attack, compared with the glucagon-secreting α and somatostatin-secreting δ cells (Malaisse et al., 1982). Further, the clinical consequences of insulin deficiency are physiologically more severe than those that would result from glucagon deficiency because the other counterregulatory hormones that oppose insulin action can compensate for reduced glucagon regulation. For those reasons, there is relatively little information about chemicals that affect α or δ cells; thus, this section will focus mainly on β-cell effects. Two chemicals that have been widely used to generate animal models of diabetes are alloxan and streptozotocin (STZ). Both of these selectively destroy pancreatic β cells, thereby causing insulin insufficiency (Pisarev et al., 2009; Adeghate et al., 2010; Fischer, 2010). The structures of these chemicals are very different. Alloxan is a cyclic urea derivative, whereas STZ is an *N*-methylnitrosourea containing a deoxyglucose moiety. Each of these, however, can produce β-cell destruction following a single intraperitoneal injection.

The mechanism by which alloxan is thought to have cytotoxic effects involves the generation of reactive species of oxygen, particularly the hydroxyl free radical ($OH^{\bullet}$). Isolated intact pancreatic islets synthesize and secrete insulin in response to high glucose (Fischer, 2010). A short exposure of isolated pancreatic islets to alloxan eliminates glucose-stimulated insulin secretion. There is support for this mechanism because this effect can be completely inhibited by pretreatment of islets with superoxide dismutase (SOD), catalase, diethylenetriaminepentaacetic acid, or dimethylurea (Fischer and Hamburger, 1980a, 1980b). Furthermore, pancreatic islets have been shown to be deficient in the important protective factor, glutathione peroxidase (Malaisse et al., 1982). This probably contributes to the selectivity of alloxan for pancreatic β cells.

Two separate mechanisms are thought to be involved in the effects of STZ on β cells (Fischer, 2010). A single high dose of STZ causes generalized cytotoxicity and cell death, whereas low, multiple doses cause nonlethal cell damage, which results in an immune-based inflammatory response leading to cell death. High-dose exposure to STZ causes alkylation of proteins and toxicity in β cells, where it decomposes to release nitric oxide. Thus, alkylation and nitric oxide–mediated injury could play a role in STZ-induced injury. Additionally, high-dose exposure to STZ causes a decrease in cellular levels of nicotinamide adenine dinucleotide (NAD). Part of the selectivity of STZ for pancreatic β cells is thought to be due to its glucose moiety, which is taken up via the glucose transporter GLUT-2 (Schnedl et al., 1994).

A common target of alloxan and STZ in pancreatic β cells is DNA (Uchigata et al., 1982). There are data to support that DNA damage occurs, poly(ADP-ribose) synthetase is activated, polyadenylation increases, and NAD declines. Pretreatment of rat pancreatic islets with the poly(adenosine diphosphate ribose) inhibitor nicotinamide prior to incubation with alloxan or STZ protects them against inhibition of proinsulin synthesis. However, in rats given

these combined treatments, several months later the animals exhibit islet cell tumors (Yamagami et al., 1985).

Multiple exposure of animals to low doses of STZ demonstrates evidence of an immune system component (Fischer, 2010). There is a progressive series of changes involving infiltrating lymphocytes and macrophages in the islets, which produce inflammatory destruction of β cells 10 to 15 days after the initiation of treatment (Like et al., 1978).

Daily oral administration of the antihistamine–antiserotonin drug cyproheptadine (CPH) to rats causes progressive ultrastructural changes in the endoplasmic reticulum of β cells within 2 to 3 days of treatment (Fischer, 2010). This is accompanied by loss of insulin secretory granules. This is followed by swelling and vesiculation of the ER. The loss of pancreatic insulin appears to be due to a direct effect of CPH on insulin biosynthesis (Miller et al., 1993). However, CPH also inhibits insulin secretion from β cells. This has been attributed to an effect of CPH on calcium influx into β cells.

CPH can be converted in rats to an epoxide metabolite desmethylcyproheptadine-10,11 (DMCPH)-epoxide. This is a major metabolite of CPH found in urine and pancreatic tissue (Fischer, 2010). Decreasing the production of this metabolite using inhibitors of drug metabolism reduces pancreatic insulin depletion caused by CPH (Chow et al., 1988). Thus, DMCPH is likely the most toxic form of the chemical.

Diabetes mellitus was induced in male Wistar rats by STZ injection. Diabetic rats fed a diet supplemented with *Coccinia indica* fruits or leaves showed renoprotective effects as well as an improvement in glucose tolerance (Gurukar et al., 2013).

There appear to be big species differences in β-cell toxicity. Human β-cell destruction has been demonstrated with the rodenticide Vacor (Prosser and Karam, 1978) and during treatment with the drug pentamidine (Bouchard et al., 1982). While humans develop Vacor-induced diabetes, no sensitive animal has been found. Conversely, unlike in rats, STZ, when used as chemotherapy, has not caused diabetes in humans (Fischer, 2010).

Insulin Resistance

Insulin resistance and defective function of pancreatic β cells usually occur sometime before the development of type 2 diabetes. Glucocorticoid treatment induces insulin resistance and enhances insulin secretion in rodents and humans (Rafacho et al., 2010). In that study, dexamethasone treatment of rats for 5 days resulted in hyperinsulinemia at the end of dosing, whereas 10 days following cessation of dexamethasone treatment hyperinsulinemia and insulin resistance had resolved. This provides important information as regards the therapeutic use of glucocorticoids in humans.

In a study investigating nondiabetic residents living near a deserted pentachlorophenol and chloralkali factory in Taiwan, insulin resistance was associated with increased circulating levels of dioxins and mercury (Chang et al., 2011). Furthermore, increased levels of dioxins and mercury combined were associated with even greater insulin resistance. Thus, simultaneous exposure to dioxins and mercury appears to enhance the risk of insulin resistance.

Bisphenol A (BPA), an endocrine disruptor, is a chemical compound in the manufacture of polycarbonate plastics that is widespread in the environment. BPA exposure of pregnant mice resulted in increased insulin, leptin, triglyceride, and glycerol levels and greater insulin resistance in the mothers 4 months postpartum (Alonso-Magdalena et al., 2010). Further, male offspring of the exposed mothers, at 6 months of age, demonstrated reduced glucose tolerance and increased insulin resistance. Thus, BPA may represent a risk factor for diabetes in exposed humans.

In Vitro Testing

Several cell lines are available for testing of insulin secretion. Pancreatic β-cell-derived RINm5F cells which produce insulin were exposed to a combination of the cytokines, IL-1β, TNF-α, and IFNγ to simulate type 1 diabetes mellitus conditions (Gurgul-Convey et al., 2011). This study showed that hydrogen peroxide produced by these cytokines reacted in the presence of trace metal Fe^{++} with nitric oxide to form highly toxic hydroxyl radicals. The authors concluded that pro-inflammatory cytokine-mediated β-cell death is due to nitro-oxidative stress-mediated hydroxyl radical formation in the mitochondria.

Etoposide (VP-16) is an antitumor drug used as a chemotherapeutic agent to treat pancreatic and several other types of cancer. However, a potential cytotoxic effect of etoposide on pancreatic β cells has also been seen (Lee et al., 2016). A study in RIN5F cells cultured with etoposide demonstrated this cytotoxic effect to be mediated via proapoptotic signaling pathways (Lee et al., 2016). These findings indicate a potential for this treatment to produce unwanted diabetogenic side effects. Interestingly, a plant extract of *Coccinia grandis* exerted dose-dependent radical scavenging and antiglycation activities in suspended RIN5F cells cultured with etoposide (Meenatchi et al., 2016).

RINm5F cells were also used to investigate the role of oxidative stress in inorganic arsenic (iAs) exposure (Lu et al., 2011). A number of proapoptotic mitochondrial and cytosolic markers were investigated and found to be elevated during β-cell toxicity. The iAs-induced apoptosis and its cellular signaling events could be reversed by the antioxidant *N*-acetylcysteine. Therefore, it was concluded that iAs-induced oxidative stress causes apoptosis in β cells, by the mitochondria-dependent and ER stress-triggered signaling pathways. Rat-derived INS-1(832/13) cells that secrete insulin in response to glucose were also studied for the effects of exposure to iAs (Fu et al., 2010). Reactive oxygen species derived from glucose metabolism provide a metabolic signal for glucose-stimulated insulin secretion from pancreatic β cells. Exposure of these cells to low levels of iAs increased an antioxidant-mediated response, and dampened glucose-stimulated insulin secretion. These findings support that low levels of arsenic can induce a cellular adaptive oxidative stress response, and disrupt β-cell function. The apoptotic as opposed to oxidative stress effects of iAs between these two studies seem to be disparate. However, two different cell lines were used, and the former study incubated the cells with higher levels of iAs (2 and 5 μM), compared with the latter (0.05 to 0.5 μM). It is possible that oxidative stress induced at the higher levels of iAs exposure (causing cell death) is sufficient to overwhelm that which, at lower levels, plays a regulatory role in insulin secretion (inhibition). This observation suggests that the nature of the cellular response depends on the level of exposure.

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REFERENCES

Abdelhafez AM, Eltony SA, Abdelhameed SY, Elgayar SA. Effect of maternal nicotine/thiocyanate exposure during gestational period upon pituitary, thyroid and parathyroid function/morphology of 1-month-old rat offspring. *J Endocrinol Invest*. 2014;37(5):455–465.

Abdel-Maksoud FM, Leasor KR, Butzen K, Braden TD, Akingbemi BT. Prenatal exposures of male rats to the environmental chemicals

bisphenol A and di(2-ethylhexyl) phthalate impact the sexual differentiation process. *Endocrinology*. 2015;156(12):4672–4683.

Abdo KM, Eustis SL, Haseman J, et al. Toxicity and carcinogenicity of rotenone given in the feed to F344/N rats and B6C3F1 mice for up to two years. *Drug Chem Toxicol*. 1988;11(3):225–235.

Abt E, Spungen J, Pouillot R, Gamalo-Siebers M, Wirtz M. Update on dietary intake of perchlorate and iodine from U.S. food and drug administration's total diet study: 2008–2012. *J Expo Sci Environ Epidemiol*. 2018;28:21–30.

Adedara IA, Abolaji AO, Awogbindin IO, Farombi EO. Suppression of the brain–pituitary–testicular axis function following acute arsenic and manganese co-exposure and withdrawal in rats. *J Trace Elem Med Biol*. 2017;39:21–29.

Adeghate E, Hameed RS, Ponery AS, et al. Streptozotocin causes pancreatic beta cell failure via early and sustained biochemical and cellular alterations. *Exp Clin Endocrinol Diabetes*. 2010;118:699–707.

Alonso-Magdalena P, Vieira E, Soriano S, et al. Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. *Environ Health Perspect*. 2010;118:1243–1250.

Aluru N, Leatherland JF, Vijayan MM. Bisphenol A in oocytes leads to growth suppression and altered stress performance in juvenile rainbow trout. *PLoS One*. 2010;5(5):e10741.

Alvarez YD, Ibanez LI, Uchitel OD, Marengo FD. P/Q Ca^{2+} channels are functionally coupled to exocytosis of the immediately releasable pool in mouse chromaffin cells. *Cell Calcium*. 2008;43:154–164.

Andrews JE, Courtney KD, Stead AG, Donaldson WE. Hexachlorobenzene-induced hyperparathyroidism and osteosclerosis in rats. *Fundam Appl Toxicol*. 1989;12(2):242–251.

Arnold DL, Moodie CA, Collins BT, Zawidzka ZZ, Krewski DR. Two-generation chronic toxicity study with hexachlorobenzene in the rat. *IARC Sci Publ*. 1986;77:405–410.

Barai SR, Suryawanshi SA, Pandey AK. Responses of parathyroid gland, C cells, and plasma calcium and inorganic phosphate levels in rat to sub-lethal heroin administration. *J Environ Biol*. 2009; 30:917–922.

Barter RA, Klaassen CD. Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosytransferase inducers in rats. *Toxicol Appl Pharmacol*. 1994;128:9–17.

Blanco-Muñoz J, Lacasana M, Lopez-Flores I, et al. Association between organochlorine pesticide exposure and thyroid hormones in floriculture workers. *Environ Res*. 2016;150:357–363.

Boas M, Main KM, Feldt-Rasmussen U. Environmental chemicals and thyroid function: an update. *Curr Opin Endocrinol Diabetes Obes*. 2009;16:385–391.

Boron WF, Boulpaep EL, eds. *Medical Physiology*. Philadelphia, PA: Elsevier; 2005.

Bouchard P, Sai P, Reach G, et al. Diabetes mellitus following pentamidine-induced hypoglycemia in humans. *Diabetes*. 1982;31:40–45.

Brannick KE, Craig ZR, Himes AD, et al. Prenatal exposure to low doses of bisphenol A increases pituitary proliferation and gonadotroph number in female mice offspring at birth. *Biol Reprod*. 2012;87(4):82.

Bronnikov VI, Goldyreva TP, Tereshchenko IV. Influence of anthropogenic pollution on thyroid structure in Perm population. *Arkh Patol*. 2005;67(6):18–21.

Brouwer A, Morse DC, Lans MC, et al. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. *Toxicol Ind Health*. 1998;14(1–2):59–84.

Bucher JR, Huff J, Haseman JK, et al. Toxicology and carcinogenicity studies of diuretics in F344 rats and B6C3F1 mice. 1. Hydrochlorothiazide. *J Appl Toxicol*. 1990;10(5):359–367.

Butt CM, Stapleton HM. Inhibition of thyroid hormone sulfotransferase activity by brominated flame retardants and halogenated phenolics. *Chem Res Toxicol*. 2013;26(11):1692–1702.

Callaci JJ, Himes R, Lauing K, Wezeman FH, Brownson K. Binge alcohol-induced bone damage is accompanied by differential expression of bone remodeling-related genes in rat vertebral bone. *Calcif Tissue Int*. 2009;84(6):474–484.

Campos E, Freire C. Exposure to non-persistent pesticides and thyroid function: a systematic review of epidemiological evidence. *Int J Hygiene Environ Health*. 2016;219(6):481–497.

Cannata JB, Briggs JD, Junor BJ, Fell GS, Beastall G. Effect of acute aluminum overload on calcium and parathyroid-hormone metabolism. *Lancet*. 1983;1(8323):501–503.

Capen CC. Toxic responses of the endocrine system. In: Klaassen CD, ed. *Casarett & Doull's Toxicology: The Basic Science of Poisons*. New York: McGraw-Hill; 2001:711–759.

Capen CC, Rosol TJ. Recent advances in the structure and function of the parathyroid gland in animals and the effects of xenobiotics. *Toxicol Pathol*. 1989;17(2):333–345.

Caride A, Fernandez-Perez B, Cabaleiro T, et al. Cadmium chronotoxicity at pituitary level: effects on plasma ACTH, GH and TSH daily pattern. *J Physiol Biochem*. 2010a;66:213–220.

Caride A, Lafuente A, Cabaleiro T. Endosulfan effects on pituitary hormone and both nitrosative and oxidative stress in pubertal male rats. *Toxicol Lett*. 2010b;197:106–112.

Chailurkit LO, Aekplakorn W, Ongphiphadhanakul B. The association of serum bisphenol A with thyroid autoimmunity. *Int J Environ Res Public Health*. 2016;13(11):pii: E1153.

Chang JW, Chen HL, Su HJ, Liao PC, Guo HR, Lee CC. Simultaneous exposure of non-diabetics to high levels of dioxins and mercury increases their risk of insulin resistance. *J Hazard Mater*. 2011;185:749–755.

Chhabra RS, Huff JE, Haseman JK, Elwell MR, Peters AC. Carcinogenicity of *p*-chloroanaline in rats and mice. *Food Chem Toxicol*. 1991;29:119–124.

Chiappini F, Pontillo C, Randi AS, Alvarez L, Kleiman de Pisarev DL. Reactive oxygen species and extracellular signal-regulated kinase 1/2 mediate hexachlorobenzene-induced cell death in FRTL-5 rat thyroid cells. *Toxicol Sci*. 2013;134(2):276–290.

Chow SA, Rickert DE, Fischer LJ. Evidence that drug metabolites are involved in cyproheptadine-induced loss of pancreatic insulin. *J Pharmcol Exp Ther*. 1988;246:143–149.

Colby HD, Abbott B, Cachovic, Debolt KM, Mico BA. Inactivation of adrenal cytochromes P450 by 1-aminobenzotriazole. Divergence of *in vivo* and *in vitro* actions. *Biochem Pharmacol*. 1995;18:1057–1062.

Colby HD, Purcell H, Kominami, Takemori S, Kossor DC. Adrenal activation of carbon tetrachloride: role of microsomal P450 isozymes. *Toxicology*. 1994;94:31–40.

Coperchini F, Pignatti P, Lacerenza S, et al. Exposure to perfluorinated compounds: in vitro study on thyroid cells. *Environ Sci Pollut Res Int*. 2015;22(3):2287–2294.

Crawford NM, Fenton SE, Strynar M, et al. Effects of perfluorinated chemicals on thyroid function, markers of ovarian reserve, and natural fertility. *Reprod Toxicol*. 2017; 69:53–59.

Crofton KM. Thyroid disrupting chemicals: mechanisms and mixtures. *Int J Androl*. 2008;31:209–223.

Darnerud PO, Erikson GS, Johannesson T, Larsen PB, Viluksela M. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environ Health Perspect*. 2001;109(suppl 1):49–68.

Delclos KB, Newbold R. NTP toxicity report of reproduction dose range-finding study of genistein (CAS No. 466-72-0) administered in feed to Sprague–Dawley rats. *Toxic Rep Ser*. 2007;79:C1–C2.

DePass LR, Garman RH, Woodside MD, et al. Chronic toxicity and oncogenicity studies of ethylene glycol in rats and mice. *Fundam Appl Toxicol*. 1986;7(4):547–565.

Dickerson SM, Gore AC. Estrogenic environmental endocrine-disrupting chemical effects on reproductive neuroendocrine function and dysfunction across the life cycle. *Rev Endocr Metab Disord*. 2007;8:143–159.

Doerge DR, Chang HC. Inactivation of thyroid peroxidase by soy isoflavones, in vitro and in vivo. *J Chromatogr B*. 2002;777:269–279.

Dong X, Dong J, Zhao Y, et al. Effects of long-term in vivo exposure to di-2-ethylhexylphthalate on thyroid hormones and the TSH/TSHR signaling pathways in Wistar rats. *Int J Environ Res Public Health*. 2017;14(1):pii: E44.

Dongre NN, Suryakar AN, Patil AJ, Hundekari IA, Devarnavadagi BB. Biochemical effects of lead exposure on battery manufacture workers with reference to blood pressure, calcium metabolism and bone mineral density. *Indian J Clin Biochem*. 2013; 28(1):65–70.

Eckstrum KS, Weis KE, Baur NG, Yoshihara Y, Raetzman LT. Icam5 expression exhibits sex differences in the neonatal pituitary and is regulated by estradiol and bisphenol A. *Endocrinology*. 2016; 157(4):1408–1420.

Evans SF, Kobrosly RW, Barrett ES, et al. Prenatal bisphenol A exposure and maternally reported behavior in boys and girls. *Neurotoxicology*. 2014;45:91–99.

Fakhouri WD, Nunez JL, Trail F. Atrazine binds to the growth hormone-releasing hormone receptor and affects growth hormone gene expression. *Environ Health Perspect*. 2010;118:1400–1405.

Fiandanese N, Borromeo V, Berrini A, et al. Maternal exposure to a mixture of di(2-ethylhexyl) phthalate (DEHP) and polychlorinated biphenyls (PCBs) causes reproductive dysfunction in adult male mouse offspring. *Reprod Toxicol*. 2016;65:123–132.

Fischer LJ. Toxicity to the insulin secreting β-cell. In: McQueen CA, ed. *Comprehensive Toxicology*, Richburg J, Hoyer PB, eds. *Reproductive and Endocrine Toxicology*. 2nd ed. Vol 11. Oxford, UK: Elsevier; 2010:313–337.

Fischer LJ, Hamburger SA. Inhibition of alloxan action in isolated pancreatic islets by superoxide dismutase, catalase, and metal chelator. *Diabetes*. 1980a;29:213–216.

Fischer LJ, Hamburger SA. Dimethyl urea: a radical scavenger that protects isolated pancreatic islets from the effects of alloxan and dihydroxyfumarate exposure. *Life Sci*. 1980b;26:1405–1409.

Fu J, Woods CG, Yehuda-Shinaidman E, et al. Low-level arsenic impairs glucose-stimulated insulin secretion in pancreatic beta cells: involvement of cellular adaptive response to oxidative stress. *Environ Health Perspect*. 2010;118:864–870.

Furin CG, von Hippel FA, Postlethwait JH, et al. Developmental timing of sodium perchlorate exposure alters angiogenesis, thyroid follicle proliferation and sexual maturation in stickleback. *Gen Comp Endocrinol*. 2015;219:24–35.

Gámez JM, Penalba R, Cardoso N, et al. Exposure to a low dose of bisphenol A impairs pituitary-ovarian axis in prepubertal rats: effects on early folliculogenesis. *Environ Toxicol Pharmacol*. 2015; 39(1):9–15.

Garcia-Flores J, Cruceyra M, Canamares M, et al. Sonographic evaluation of fetal adrenal gland in gestational diabetes. *J Ultrasound Med*. 2017; 36(5):999–1007.

Gardell AM, von Hippel FA, Adams EM, et al. Exogenous iodide ameliorates perchlorate-induced thyroid phenotypes in threespine stickleback. *Gen Comp Endocrinol*. 2016;243:60–69.

Gasman S, Chasserot-Golaz S, Vitale N, Bader MF. Bacterial toxins: useful for studying G-proteins implicated in the mechanism of exocytosis in neuroendocrine cells. *Soc Biol*. 1999;193:451–456.

Gentilcore D, Porreca I, Rizzo F, et al. Bisphenol A interferes with thyroid specific gene expression. *Toxicology*. 2013; 304:21–31.

Gimm O. Pheochromocytoma-associated syndromes: genes proteins and functions of RET, VHL and SDHx. *Fam Cancer*. 2005;4:17–23.

Gore AC. Neuroendocrine targets of endocrine disruptors. *Hormones*. 2011;9(1):16–27.

Gore AC, Chappell VA, Fenton SE, et al. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr Rev*. 2015;36(6):E1–E150.

Greim H, Hartwig A, Reuter U, Richter-Reichhelm HB, Thielmann HW. Chemically induced pheochromocytomas in rats: mechanisms and relevance for human risk assessment. *Crit Rev Toxicol*. 2009;39:695–718.

Gurgul-Convey E, Mehmeti I, Lortz S, Lenzen S. Cytokine toxicity in insulin-producing cells is mediated by nitro-oxidative stress-induced hydroxyl radical formation in mitochondria. *J Mol Med*. 2011;89:785–798.

Gurukar MS, Mahadevamma S, Chilkunda ND. Renoprotective effect of *Coccinia indica* fruits and leaves in experimentally induced diabetic rats. *J Med Food*. 2013;16:839–846.

Hadley ME, Levine JE, eds. *Endocrinology.* 6th ed. Upper Saddle River, NJ; Prentice Hall; 2007.

Harvey PW. Toxic responses of the adrenal cortex. In: McQueen CA, ed. *Comprehensive Toxicology*, Richburg J, Hoyer PB, eds. *Reproductive and Endocrine Toxicology*. 2nd ed. Vol 11. Oxford, UK: Elsevier; 2010:265–289.

Harvey PW. Adrenocortical endocrine disruption. *J Steroid Biochem Mol Biol*. 2016;155:199–206.

Harvey PW, Everett DJ, Springall CJ. Adrenal toxicology: a strategy for assessment of functional toxicity to the adrenal cortex and steroidogenesis. *J Appl Toxicol*. 2007;27:103–115.

Hawthorn J, Nussey SS, Henderson JR, Jenkins JS. Immunohistochemical localization of oxytocin and vasopressin in the adrenal glands of rat, cow, hamster and guinea pig. *Cell Tissue Res*. 1987;250:1–6.

Hedge GA, Colby HD, Goodman RL, eds. *Clinical Endocrine Physiology*. Philadelphia, PA: W.B. Saunders; 1987.

Hinson JP, Raven PW. Effects of endocrine-disrupting chemicals on adrenal function. *Best Pract Res Clin Endocrinol Metab*. 2006;20:111–120.

Holgert H, Dagerlind A, Hokfelt T, Lagercrantz H. Neural markers, peptides and enzymes in nerves and chromaffin cells in the rat adrenal medulla during postnatal development. *Brain Res Dev Brain Res*. 1994;83:35–52.

Hood A, Allen ML, Liu Y, Liu J, Klaassen CD. Induction of T_4 UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicol Appl Pharmacol*. 2003;188:6–13.

Howland RD, Alli P. Altered phosphorylation of rat neuronal cytoskeletal proteins in acrylamide induced neuropathy. *Brain Res*. 1986;363:333–339.

Hu X, Teng X, Zheng H, Shan Z, et al. Iron deficiency without anemia causes maternal hypothyroxinemia in pregnant rats. *Nutr Res*. 2014;34(7):604–612.

Jeffery EH, Abreo K, Burgess E, Cannata J, Greger JL. Systemic aluminum toxicity: effects on bone, hematopoietic tissue and kidney. *J Toxicol Environ Health*. 1996;48(6):649–665.

Jekat FW, Meisel ML, Eckard R, Winterhoff H. Effects of pentachlorophenol (PCP) on the pituitary and thyroidal hormone regulation in the rat. *Toxicol Lett*. 1994;71(1):9–25.

Johansson MK, Sanderson JT, Lund BO. Effects of 3-$MeSO_2$–DDE and some CYP inhibitors on glucocorticoid steroidogenesis in the H295R human adrenocortical carcinoma cell line. *Toxicol In Vitro*. 2002;16:131–121.

Jugan ML, Levi Y, Blondeau JP. Endocrine disruptors and thyroid hormone physiology. *Biochem Pharmacol*. 2010;79:939–947.

Keiver K, Weinberg J. Effect of duration of alcohol consumption on calcium and bone metabolism during pregnancy in the rat. *Alcohol Clin Exp Res*. 2003;27(9):1507–1519.

Kido T, Honda R, Tsuritani I, et al. Assessment of cadmium-induced osteopenia by measurement of serum bone Gla protein, parathyroid hormone, and 1-alpha, 25-dihydroxyvitamin D. *J Appl Toxicol*. 1991;11(3):161–166.

Kinoshita H, Jessop DS, Finn DP, et al. Acetaldehyde, a metabolite of ethanol, activates the hypothalamic–pituitary–adrenal axis in the rat. *Alcohol Alcohol*. 2001;36(1):59–64.

Kitamura S, Jinno N, Ohta S, Kuroki H, Fujimoto N. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. *Biochem Biophys Res Commun*. 2002;293(1):554–559.

Kortenkamp A. Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. *Int J Androl*. 2008;31:233–240.

Kritis AA, Stamoula EG, Paniskaki KA, Vavilis TD. Researching glutamate-induced cytotoxicity in different cell lines: a comparative/collective analysis/study. *Front Cell Neurosci*. 2015;9:91.

Kuramoto H, Kondo H, Fujita T. Substance P-like immunoreactivity in adrenal chromaffin cells and intra-adrenal nerve fibers in rats. *Histochemistry*. 1985;82:507–512.

Lafuente A, Esquifino AI. Cadmium effects on hypothalamic activity and pituitary hormone secretion in the male. *Toxicol Lett*. 1999;110:209–218.

Lafuente A, Marquez N, Pousada Y, Pazo D, Esquifino AI. Possible estrogenic and/or antiandrogenic effects of methoxychlor on prolactin release in male rats. *Arch Toxicol*. 2000;74(4–5):270–275.

Langer P. The impacts of organochlorines and other persistent pollutants on thyroid and metabolic health. *Front Neuroendocrinol*. 2010;31(4):497–518.

Lee KI, Su CC, Yang CY, et al. Etoposide induces pancreatic β-cells cytotoxicity via the JNK/ERK/GSK-3 signaling-mediated mitochondria-dependent apoptosis pathway. *Toxicol In Vitro*. 2016;36:142–152.

Lelli SM, Caballos NR, Mazzetti MB, Aldonatti CA, San Martin de Viale LC. Hexachlorobenzene as hormonal disruptor—studies about glucocorticoids: their hepatic receptors, adrenal synthesis and plasma levels on relation to impaired gluconeogenesis. *Biochem Pharmacol*. 2007;73:873–879.

Lijinsky W, Reuber MD. Pathologic effects of chronic administration of hydrochlorothiazide, with and without sodium nitrite, to F344 rats. *Toxicol Ind Health*. 1987;3(3):413–422.

Like AA, Appel MC, Williams RM, Rossini AA. Streptozotocin-induced pancreatic insulitis in mice. Morphologic and physiologic studies. *Lab Invest*. 1978;38:470–486.

Liu C, Zhao L, Wei L, Li L. DEHP reduces thyroid hormones via interacting with hormone synthesis-related proteins, deiodinases, transthyretin, receptors, and hepatic enzymes in rats. *Environ Sci Pollut Res Int*. 2015;22(16):12711–12719.

Long GJ, Rounds JG, Rosen JF. Lead intoxication alters basal and parathyroid hormone-regulated cellular calcium homeostasis in rat osteosarcoma (ROS 17/2.8) cells. *Calcif Tissue Int*. 1992;50(5):451–458.

Los LE, Colby HD. Binding of spironolactone metabolites *in vivo* to renal mineralocorticoid receptors in guinea pigs. *Pharmacology*. 1994;48:86–92.

Loutchanwoot P, Srivilai P, Jarry H. Effects of the natural endocrine disruptor equol on the pituitary function in adult male rats. *Toxicology*. 2013;304:69–75.

Lu TH, Su CC, Chen YW, et al. Arsenic induces pancreatic β-cell apoptosis via the oxidative stress-regulated mitochondria-dependent and endoplasmic reticulum stress-triggered signaling pathways. *Toxicol Lett*. 2011;201:15–26.

Lynch BS, Tischler AS, Capen C, et al. Low digestible carbohydrates (polyols and lactose): significance of adrenal medullary proliferative lesions in the rat. *Regul Toxicol Pharmacol*. 1996;23:256–297.

Mahaboob B, Radha MJ. Gestational di-*n*-butyl phthalate exposure induced developmental and teratogenic anomalies in rats: a multigenerational assessment. *Environ Sci Pollut Res Int*. 2017;24:4537–4551.

Malaisse WJ, Malaisse-Lagae F, Sener A. Determinants of the selective toxicity of alloxan to the pancreatic B cell. *Proc Natl Acad Sci U S A*. 1982;79:927–930.

Mao QQ, Xian YF, Ip SP, Tsai SH, Che CT. Protective effects of peony glycosides against corticosterone-induced cell death in PC12 cells through antioxidant action. *J Ethnopharmacol*. 2011;133:1121–1125.

Matsis PP, Fisher RA, Tasman-Jones C. Acute lithium toxicity—chorea, hypercalcemia, and hyperamylasemia. *Aust N Z J Med*. 1989;19(6):718–720.

McClain RM, Levin AA, Posch R, Downing JC. The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. *Toxicol Appl Pharm*. 1989;99:216–228.

McDiarmid MA, Engelhardt SM, Dorsey CD, et al. Longitudinal health surveillance in a cohort of Gulf War veterans 18 years after first exposure to depleted uranium. *J Toxicol Environ Health A*. 2011;74(10):678–691.

McKnight RF, Adida M, Budge K, et al. Lithium toxicity profile: a systematic review and meta-analysis. *Lancet*. 2012;379(9817):721–728.

Meenatchi P, Purushothaman A, Maneemegalai S. *Antioxidant* antiglycation and insulinotrophic properties of *coccinia grandis* (L.) in vitro: possible role in prevention of diabetic complications. *J Tradi Complement Med*. 2016;7:54–64.

Mervish NA, Pajak A, Teitelbaum SL, et al. Thyroid antagonists (perchlorate, thiocyanate, and nitrate) and childhood growth in a longitudinal study of U.S. girls. *Environ Health Perspect*. 2016;124(4):542–549.

Miller CP, Reape TJ, Fischer LJ. Inhibition of insulin production by cyproheptadine in RINm5F rat insulinoma cells. *J Biochem Toxicol*. 1993;8:127–134.

Moran TB, Brannick KE, Raetzman LT. Aryl-hydrocarbon receptor activity modulates prolactin expression in the pituitary. *Toxicol Appl Pharm*. 2012;265(1):139–145.

Mugford CA, Carfagna MA, Kedderis GL. Furan-mediated uncoupling of hepatic oxidative phosphorylation in Fischer-344 rats—an early event in cell death. *Toxicol Appl Pharmacol*. 1997;144:1–11.

National Toxicology Program. NTP carcinogenesis bioassay of propyl gallate. CAS No 121-79-9 in F344/N rats and B6C3F1 mice (feed study). *Technical Report Series No. 240*. 1982.

National Toxicology Program. NTP toxicology and carcinogenesis studies of coumarin (CAS No. 91-64-5) in F344/N rats and B6C3F1 mice (gavage studies). *Natl Toxicol Program Tech Rep Ser*. 1993;422:1–340.

National Toxicology Program. NTP toxicology and carcinogenesis studies of *o*-benzyl-*p*-chlorophenol (CAS No. 120-32-1) in F344/N rats and B6C3F1 mice (gavage studies). *Natl Toxicol Program Tech Rep Ser*. 1994;424:1–304.

Ozaki K, Haseman JK, Hailey JR, Maronpot RR, Nyska A. Association of adrenal pheochromocytoma and lung pathology in inhalation studies with particulate compounds in the male F344 rat: the National Toxicology Program experience. *Toxicol Pathol*. 2002;30:263–270.

Patrick L. Thyroid disruption: mechanisms and clinical implication in human health. *Altern Med Rev*. 2009;14:326–346.

Piccoli C, Cremonese C, Koifman RJ, Koifman S, Freire C. Pesticide exposure and thyroid function in an agricultural population in Brazil. *Environ Res*. 2016;151:389–398.

Pisarev VB, Snigur GL, Spasov AA, Samokhina MP, Bulanov AE. Mechanisms of toxic effect of streptozotocin on β-cells in the islets of Langerhans. *Bull Exp Biol Med*. 2009;148:937–939.

Porterfield SP. Thyroidal dysfunction and environmental chemicals-potential impact on brain development. *Environ Health Perspect*. 2000;108(suppl 3):433–438.

Porterfield SP. *Endocrine Physiology*. 2nd ed. St. Louis, MO: Mosby; 2001.

Potts JT. Parathyroid hormone: past and present. *J Endocrinol*. 2005;187:311–325.

Pounds JG. Effect of lead intoxication on calcium homeostasis and calcium-mediated cell function: a review. *Neurotoxicology*. 1984;5(3):295–331.

Prosser PR, Karam JH. Diabetes mellitus following rodenticide ingestion in man. *J Am Med Assoc*. 1978;239:1148–1150.

Rafacho A, Quallio S, Ribeiro DL, et al. The adaptive compensations in endocrine pancreas from glucocorticoid-treated rats are reversible after the interruption of treatment. *Acta Physiol*. 2010;200:223–235.

Rama EM, Bortolan S, Vieira ML, Gerardin DCC, Moreira EG. Reproductive and possible hormonal effects of carbendazim. *Regul Toxicol Pharm*. 2014;69(3):476–486.

Reuber MD. Carcinogenicity of dimethoate. *Environ Res*. 1984;34(2):193–211.

Reuber MD. Carcinogenicity and toxicity of malathion and malaoxon. *Environ Res*. 1985;37(1):119–153.

Ribelin WE. The effects of drugs and chemicals upon the structure of the adrenal gland. *Fund Appl Toxicol*. 1984;4:105–119.

Rignell-Hydbom A, Skerfving A, Lundh T, et al. Exposure to cadmium and persistent organochlorine pollutants and its association with bone mineral density and markers of bone metabolism on postmenopausal women. *Environ Res*. 2009;109(8):991–996.

Roe FJC, Bar A. Enzootic and epizootic adrenal medullary proliferative disease of rats: influence of dietary factors which affect calcium absorption. *Hum Toxicol*. 1985;4:27–52.

Ronchetti SA, Novack GV, Bianchi MS, et al. In vivo xenoestrogenic actions of cadmium and arsenic in anterior pituitary and uterus. *Reprod*. 2016;152(1):1–10.

Rossi EM, Marques VB, Nunes Dde O, et al. Acute iron overload leads to hypothalamic–pituitary–gonadal axis abnormalities in female rats. *Toxicol Lett*. 2016;240(1):196–213.

Schlosser PM. Revision of the affinity constant for perchlorate binding to the sodium-iodide symporter based on in vitro and human in vivo data. *J Appl Toxicol*. 2016;36(12):1531–1535.

Schnedl WJ, Ferber S, Johnson JH, Newgard CB. STZ transport and cytotoxicity. Specific enhancement in GLUT-2 expressing cells. *Diabetes*. 1994;43:1326–1333.

Schultzberg M, Lundberg JM, Hokfelt T, et al. Enkephalin-like immunoreactivity in gland cells and nerve terminals of the adrenal medulla. *Neuroscience*. 1978;3:1169–1186.

Sheng ZG, Tang Y, Liu YX, et al. Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism. *Toxicol Appl Pharmacol*. 2012;259(1):133–142.

Sikka SC, Wang R. Endocrine disruptors and estrogenic effects on male reproductive axis. *Asian J Androl*. 2008;10(1):134–145.

Soechitram SD, Berghuis SA, Visser TJ, Sayer PJ. Polychlorinated biphenyl exposure and deiodinase activity in young infants. *Sci Total Environ*. 2017;574:1117–1124.

Šošić-Jurjević B, Filipovic B, Wirth EK, et al. Soy isoflavones interfere with thyroid hormone homeostasis in orchidectomized middle-aged rats. *Toxicol Appl Pharmacol*. 2014;278(2):124–134.

Sriphrapradang C, Chailurkit LO, Aekplakorn W, Ongphiphadhanakul B. Association between bisphenol A and abnormal free thyroxine level in men. *Endocrine*. 2013;44(2):441–447.

Steinmaus C, Miller MD, Cushing L, Blount BC, Smith AH. Combined effects of perchlorate, thiocyanate, and iodine on thyroid function in the National Health and Nutrition Examination Survey 2007–08. *Environ Res*. 2013;123:17–24.

Suarez CR, Black LE III, Hurley RM. Elevated lead levels in a patient with sickle cell disease and inappropriate secretion of antidiuretic hormone. *Pediatr Emerg Care*. 1992;8(2):88–90.

Szabo A, Merke J, Hugel U, et al. Hyperparathyroidism and abnormal $1,25(OH)_2$ vitamin D3 metabolism in experimental lead intoxication. *Eur J Clin Invest*. 1991;21(5):512–520.

Szabo S, Lippe IT. Adrenal gland: chemically induced structural and functional changes in the cortex. *Toxicol Pathol*. 1989;17:317–329.

Takeda T, Taura J, Hattori Y, Ishii Y, Yamada H. Dioxin-induced retardation of development through a reduction in the expression of pituitary hormones and possible involvement of an aryl hydrocarbon receptor in this defect: a comparative study using two strains of mice with different sensitivities to dioxin. *Toxicol Appl Pharmacol*. 2014;278(3):220–229.

Tang JM, Li W, Xie YC, et al. Morphological and functional deterioration of the rat thyroid following chronic exposure to low-dose PCB118. *Exp Toxicol Pathol*. 2013;65(7–8):989–994.

Tapella L, Sesta A, Cassarino MF, et al. Benzene and 2-ethyl-phthalate induce proliferation in normal rat pituitary cells. *Pituitary*. 2017;20(3):311–318.

Taylor SC, Shaw SM, Peers C. Mitochondrial inhibitors evoke catecholamine release from pheochromocytoma cells. *Biochem Biophys Res Commun*. 2000;273:17–21.

Tian H, Yang FF, Liu CY, et al. Effects of phenolic constituents of daylily flowers on corticosterone- and glutamate-treated PC12 cells. *BMC Compl Alt Med*. 2017;17:69.

Tischler AS, Nyska A, Elmore SA. Toxic responses of the adrenal medulla. In: McQueen CA, ed. *Comprehensive Toxicology*, Richurg J, Hoyer PB, eds. *Reproductive and Endocrine Toxicology*. 2nd ed. Vol 11. Oxford, UK: Elsevier; 2010:291–311.

Tischler AS, Riseberg J. Different responses to mitogenic agents by adult rat and human chromaffin cells in vitro. *Endocr Pathol*. 1993;4:15–19.

Tischler AS, Riseberg JC, Gray R. Mitogenic and antimitogenic effects of pituitary adenylate cyclase-activating polypeptide (PACAP) in adult rat chromaffin cell cultures. *Neurosci Lett*. 1995;189:135–138.

Tissandie E, Gueguen Y, Lobaccaro JM, et al. Effects of depleted uranium after short-term exposure on vitamin D metabolism in rat. *Arch Toxicol*. 2006;80(8):473–480.

Tohei A, Suda S, Taya K, Hashimoto T, Kogo H. Bisphenol A inhibits testicular functions and increases luteinizing hormone secretion in adult male rats. *Exp Biol Med (Maywood)*. 2001;226(3):216–221.

Uchigata Y, Yamamoto H, Kawamura A, Okamato H. Protection by superoxide dismutase, catalase, and poly(ADP-ribose) synthetase inhibitors against alloxan- and streptozotocin-induced islet DNA strand breaks and against the inhibition of proinsulin synthesis. *J Biol Chem*. 1982;257: 6084–6088.

Varndell IM, Polak JM, Allen JM, Terenghi G, Bloom SR. Neuropeptide tyrosine (NPY) immunoreactivity in norepinephrine-containing cells and nerves of the mammalian adrenal gland. *Endocrinology*. 1984;114:1460–1462.

Verma Y, Rana SV. Endocrinal toxicity of industrial solvents—a mini review. *Indian J Exp Biol*. 2009;47(7):537–549.

Viguié C, Collet SH, Gayrard V, et al. Maternal and fetal exposure to bisphenol A is associated with alterations of thyroid function in pregnant ewes and their newborn lambs. *Endocrinology*. 2013;154(1):521–528.

Wang N, Zhou Y, Fu C, et al Influence of bisphenol A on thyroid volume and structure independent of iodine in school children. *PLos One*. 2015;10(10):e0141248.

Weis KE, Raetzman LT. Isoliquiritigenin exhibits anti-proliferative properties in the pituitary independent of estrogen receptor function. *Toxicol Appl Pharm*. 2016;313:204–214.

Wen LL, Lin LY, Su TC, Chen PC, Lin CY. Association between serum perfluorinated chemicals and thyroid function in U.S. adults: the National Health and Nutrition Examination Survey 2007–2010. *J Clin Endocrinol Metab*. 2013;98(9):E1456–E1464.

Wester PW, Krajinc EI, van Leeuwen FX, et al. Chronic toxicity and carcinogenicity of bis(tri-*n*-butyltin)oxide (TBTO) in the rat. *Food Chem Toxicol*. 1990;28(3):179–196.

Whittaker MH, Gebhart AM, Miller TC, Hammer F. Human health risk assessment of 2-mercaptobenzothiazole in drinking water. *Toxicol Ind Health*. 2004;20(6–10):149–163.

Wozniak AL, Bulayeva NN, Watson CS. Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca^{2+} fluxes and prolactin release in GH3/B6 pituitary tumor cells. *Environ Health Perspect*. 2005;113(4):431–439.

Wu W, Zhou F, Wang Y, Zhou YK. Exposure to phthalates in children aged 5-7years: associations with thyroid function and insulin-like growth factors. *Sci Tot Environ*. 2017;579:950–956.

Wu Y, Beland FA, Fang JL. Effect of triclosan, triclocarban, 2,2′,4,4′-tetrabromodiphenyl ether, and bisphenol A on the iodide uptake, thyroid peroxidase activity, and expression of genes involved in thyroid hormone synthesis. *Toxicol In Vitro*. 2016;32:310–319.

Yamagami T, Miwa A, Takasawa S, Yamamoto H, Okamoto H. Induction of rat pancreatic B-cell tumors by the combined administration of streptozotocin or alloxan and poly(adenosine diphosphate ribose) synthesis inhibitors. *Cancer Res*. 1985;45:1845–1849.

Yamauchi K, Ishihara A. Thyroid system-disrupting chemicals: interference with thyroid hormone binding to plasma proteins and the cellular thyroid hormone signaling pathway. *Rev Environ Health*. 2006;21(4):229–251.

Yang M, Hu J, Li S, Ma Y, Gui W, Zhu G. Thyroid endocrine disruption of acetochlor on zebrafish (*Danio rerio*) larvae. *J Appl Toxicol*. 2016;36(6):844–852.

Yarrington JT, Huffman KW, Gibson JP. Adrenocortical degeneration in dogs, monkeys, and rats treated with α-(1,4-dioxido-3-methylquinoxalin-2-yl)-*N*-methylnitrone. *Toxicol Lett*. 1981;8:229–234.

Yarrington JT, Loudy DE, Sprinkel DJ, et al. Degeneration of the rat and canine adrenal cortex caused by α-(1,4-dioxido-3-methylquinoxalin-2-yl)-*N*-methylnitrone (DMNM). *Fundam Appl Toxicol*. 1985;5:370–381.

Ye H, Ha M, Yang M,Yue P, Xie Z, Liu C. Di2-ethylhexyl phthalate disrupts thyroid hormone homeostasis through activating the Ras/Akt/TRHr pathway and inducing hepatic enzymes. *Sci Rep*. 2017;7:40153.

Zabka TS, Fielden MR, Garrido R, et al. Characterization of xenobiotic-induced hepatocellular enzyme induction in rats: anticipated thyroid effects and unique pituitary gland findings. *Toxicol Pathol*. 2011;39(4):664–677.

Zhou J, Yang Y, Xiong K, Liu J. Endocrine disrupting effects of dichlorodiphenyltrichloroethane analogues on gonadotropin hormones in pituitary gonadotrope cells. *Environ Toxicol Pharmacol*. 2014;37(3):1194–1201.

Zoeller RT, Dowling ALS, Herzig CTA, et al. Thyroid hormone, brain development, and the environment. *Environ Health Perspect*. 2002;110(suppl3): 355–361.

Zoeller RT, Tan SW, Tyl RW. General background on the hypothalamic–pituitary–thyroid (HPT) axis. *Crit Rev Toxicol*. 2007;37(1–2):11–53.

Zoeller TR. Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals? *Mol Cell Endocrinol*. 2005;242:10–15.

Zoeller TR. Environmental chemicals targeting thyroid. *Hormones*. 2010;9(1):28–40.

chapter 21

Toxic Responses of the Reproductive System

Paul M.D. Foster and L. Earl Gray Jr.

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Acknowledgments

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INTRODUCTION

Any evaluation of toxicity to reproduction will consider those events that may not only have impact on adults' likelihood to have children, but also impact the viability and quality of life of their potential offspring and may even affect later generations. That chemicals can adversely affect reproduction in males and females is not a new concept; one only has to look at the importance of drugs as contraceptives to realize how sensitive the reproductive system can be to external chemical influences that disrupt this process. Of course in these cases, the failure of normal reproduction is a desired outcome in a contraceptive, but unfortunately we have had a number of catastrophes in which such failure has been unintentional. Many of the classic examples in chemical workers, or contamination of groundwater from chemical exposure such as dibromochloropropane (DBCP) or kepone (chlordecone)

have shown the sensitivity of human reproduction to these specific exposures (reviewed by Cannon et al., 1978; Faroon et al., 1995; Winker and Rudiger, 2006). There have been significant improvements in our ability to test for effects of chemicals, agrochemicals, and drugs on reproduction, but unfortunately such adverse episodes continue to occur, for example, the recent reports of the effects of 2-bromopropane in chemical workers (both male and female) in Korea (reviewed by Boekelheide et al., 2004).

Recent trends in human fertility, fecundity—changing social influences (age at which women have their first child), and the knowledge that populations in many western countries are no longer self-sustaining, coupled with the advent of assisted reproductive techniques (ARTs), where according to a recent paper 5.9% of all live births in Denmark used these ARTs (Andersen et al., 2005), all point to the potential for declines in normal human reproduction. Underlying all these issues with human reproductive performance is the concept that exposure to environmental chemicals and drugs may be contributing to these declines.

The advent of the endocrine disruptor debate provided a major impetus to the examination of the methods used in screening and testing for reproductive (and other) toxicity, and highlighted a number of shortfalls, not least in how we should evaluate the latent effects on adults of in utero exposures. However, this chapter will not specifically address the emerging issue of the fetal origins of adult disease as proposed by Barker (see Barker et al., 1989, 1993; Barker, 1995, 1999).

This chapter will take the reproductive cycle (Fig. 21-1) as the biological basis for the description of toxicity to reproduction rather than an encyclopedic approach. The basic biology of the different life stages and processes that are requisites for normal reproduction will be discussed and, where possible, the differences between experimental animals and humans highlighted (see Tables 21-1 and 21-2). These processes will then be placed into perspective by reference to a number of case studies of selected chemicals chosen to illustrate a range of modes of action and how they can perturb reproduction.

Special attention will be focused on endocrine disruption, methods proposed for screening and testing and the selection of chemicals with specific pharmacologies (e.g., estrogen or antiandrogen) to illustrate the types of toxicities produced at different life stages.

The chapter will also provide basic information on testing methodologies for chemicals, pesticides, and drugs, but placed into the reproductive cycle framework. Thus, it will examine the life stages that are exposed and when specific evaluations are undertaken. Lastly, we have included a section on the evaluation of data and weight of evidence, specifically how one looks at concordance of endpoints with examples of the types of profiles observed (based on the examples used previously). We do not believe that we have seen this anywhere before, or in this context, and hopefully this will be a useful tool for students and professionals alike.

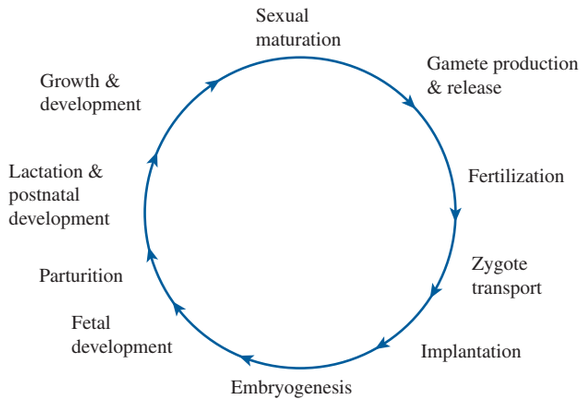


Figure 21-1. *The reproductive cycle.*

Table 21-1

Examples of Reproductive Physiology Similarities Among Humans and Rats

- Steroid hormone control of reproductive function relies on testosterone, dihydrotestosterone, estradiol, and progesterone.
- CNS–hypothalamic secretion of GnRH controls pituitary release and synthesis of FSH and LH.
- FSH and LH regulate germ cell development after puberty, LH surges induce spontaneous ovulation in the female, and LH regulates testis Leydig cell testosterone production.
- Placental support of embryos. Placenta and fetal unit also produce hormones critical for pregnancy maintenance after the first week.
- Hormonal regulation of uterine function and onset of delivery.
- Androgens required to maintain male spermatogenesis and secondary sex characteristics.
- Hormone-dependent mating and other sexually dimorphic behavior. "Rough and tumble" play behavior is sexually dimorphic behavior being imprinted by early androgens.
- Lactation under complex hormonal regulation.
- Dramatic endocrine changes resulting from CNS–HPG maturation responsible for puberty in males and females. Females generally attain puberty at an earlier age than males of the same species.

Abbreviations: CNS, Central nervous system; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HPG, hypothalamic–pituitary–gonadal; LH, luteinizing hormone.

THE REPRODUCTIVE CYCLE

If one considers the purpose of the reproductive system as the production of good quality gametes, capable of fertilization, and producing a viable offspring, which in turn can successfully reproduce, then it is clear that a large number of complex processes need to be orchestrated in a precise, sequential order for optimal performance at different stages of the life cycle of the animals, or humans. Thus following fertilization of an egg by a sperm, the resulting zygote must be transported along the oviduct while maturing into an early embryo. This embryo is then required to implant in the uterus successfully, such that the developing conceptus can differentiate, produce a placenta, and normal embryogenesis and fetal development occur. Once the fetus has completed in utero growth and differentiation, parturition needs to occur at the correct time and the neonate be born and then proceed successfully through the lactation phase of development and be weaned.

When the male and female offspring enter puberty after infancy, they need to acquire sexual characteristics as young adults with mature reproductive systems. Acquisition of sexual maturity involves the generation of gametes by the gonads, which in turn can result in the production of the next generation. For the parental animals, once their reproductive lifespan has finished, the process of reproductive senescence occurs. This myriad of processes all involve complex interplay between tissues and cells, the vast

Table 21-2

Examples in Which the Reproductive Strategy of the Rat Differs From That of the Human

1. The rat is a short (22.5 day) gestation species. Pregnancy in humans is 9 months.
2. The rat placenta lacks aromatase; estrogen is produced during pregnancy by the ovary. Human placental tissue expresses high levels of aromatase.
3. In the rat, sexual differentiation of the reproductive tract is perinatal, whereas central nervous system (CNS) sexual differentiation is a postnatal event, regulated to a great degree by aromatization of testosterone to estradiol (play behavior, an exception, is androgen-dependent in both rats [Hotchkiss et al., 2002, 2003] and humans [Hines, 2003]). In nonhuman primates and presumably humans, more CNS events are prenatal, and androgens are more important than in rats (Goy and Phoenix, 1972; Goy and Resko, 1972; Goy et al., 1988; Hines, 2003).
4. The rat has a 4- to 5-day estrous cycle, with no functional corporus luteum. The estrous cycle can be monitored easily by examining daily cytology. The female rat displays sexual receptivity only during estrus after "lights out" after a proestrus vaginal smear. This behavior is exquisitely dependent on estrogen followed by progesterone. Humans have a menstrual cycle approximately 28 days in duration and do not display periods of peak behavioral estrus during the cycle. Corpora luteal function is sustained for approximately 10 days by mating-induced cervical stimulatory prolactin surges in rats, whereas the human menstrual cycle has a spontaneous luteal phase of 10 to 14 days after ovulation.
5. Male rat sex behavior can be induced by estrogens and involves multiple series of ejaculations in a single mating. Mating involves approximately 10 mounts, with intromission before each ejaculation, followed by a postejaculatory interval before the onset of the next series. In nonhuman primates and presumably humans, male sex behavior is androgen mediated.
6. Both ovaries spontaneously release several ova in response to a luteinizing hormone surge into separate uterine horns, each with a separate cervix in the rat; whereas in women, a single ovum is typically ovulated during each cycle.
7. Pregnancy is easily disrupted by estrogens in rats, but not in humans. Rats, unlike humans, are a litter-bearing species. Most strains used for toxicology testing have litters of 10 to 12 pups. Spontaneous reproductive malformations are very rare in the rats, whereas in humans, some malformations such as cryptorchidism occur in 3% of newborn boys.
8. Spermatogenesis begins at approximately 5 days of age in the rat; the spermatogenic cycle is about 53 days of age, and sperm appear in the epididymis at about 55 days of age. In humans, spermatogenesis begins during puberty at 10 to 14 years of age, and the entire spermatogenic cycle is approximately 75 days in duration.
9. Puberty in the rat (as measured by the age at vaginal opening and the onset of estrous cyclicity) occurs at about 32 days of age in females and 42 days of age (as measured by preputial separation, an androgen-dependent event) in male SD and LE rat strains. In humans, puberty occurs at 9 to 12 years of age in girls, and 10 to 14 years of age in boys.
10. Fertility begins to decline in the female rat at about 6 months of age, especially if never mated and allowed to cycle continuously. Fertility begins to decline in women at about 35 years of age, and at 40 years of age, approximately 50% of women are infertile.

For a review of reproductive physiology, see Knobil and Neill (1994).

majority of these processes being under complex hormonal control that provides the critical signals and precise timing of these events.

Not surprisingly, it is possible to perturb this complex series of events and thus disturb the process and purpose of reproduction. Indeed, all these processes can be targets for the action of specific chemicals that can disturb these events leading to adverse effects on reproduction, such that the normal production of viable offspring cannot occur. It is thus important to consider in an evaluation of reproductive toxicity that while simple yes/no answers as to whether a particular chemical can be a reproductive toxicant are possible, and indeed are used, any description of such toxicity has to be in the context of the life stage of exposure and effect. There are examples of chemicals that can have different effects on reproduction, at different life stages, via different modes of action/mechanisms. Indeed, it might be useful for this particular aspect of toxicity to modify the adage of Paracelsus to "It is the *timing* of the dose that makes the poison." That is, the dose of the toxic chemical and its resultant effects will be dependent on when in the life stage of the organism that the chemical is administered and evaluated.

The subsequent sections will examine some of the hormonal and other control mechanisms that have been deduced from careful physiological studies in experimental animals and humans (see Table 21-3) to provide more information as to where chemicals may produce their effects. Rather than provide a laundry list of chemicals that can produce effects on reproduction, one or two examples will be mentioned to illustrate the variety of processes that can be affected with referral to more detailed references. While reviewing the mammalian reproductive cycle one could start in any position, we have decided to begin with the development of the reproductive system in utero that occurs with the process of sexual differentiation of the embryo and the move forward around the cycle as depicted in Fig. 21-1.

REPRODUCTIVE DEVELOPMENT AND SEXUAL DIFFERENTIATION

During early human development, there is a short period immediately prior to sexual differentiation when the gonad is sexually indifferent and it is not until the seventh week of gestation that male and female morphological characteristics begin to develop. In rodents, the embryo remains sexually indifferent and possesses both male and female reproductive tract primordia until embryonic day 13.5 regardless of its genetic sex.

Gonadogenesis begins with proliferation of the mesodermal (coelomic) epithelium, which invades the underlying mesenchyme, resulting in a longitudinal thickening on the medial side of the mesonephros, known as the gonadal ridge (Byskov, 1986). The invading epithelium begins to form primitive sex cords in the gonadal ridge, which are surrounded by undifferentiated mesenchyme (Pelliniemi, 1975). Primordial germ cells, or primitive sex cells, are first visible in the fourth week in the caudal region of the yolk sac near

Table 21-3

Reproductive Parameters for Various Species

			OVULATION		GESTATION	
SPECIES	AGE AT PUBERTY/ PERIOD	SEXUAL CYCLE DURATION (DAYS)	TIME	TYPE	IMPLANTATION (DAYS)	PARTURITION (DAYS)
Mouse	5–6 weeks	4	2–3 hours	S	4–5	19 (19–21)
Rat	6–11 weeks	4–6	8–11 hours	S	5–6	21–22
Rabbit	6–7 months	Indefinite	10 hours	I	7–8	31 (30–35)
Hamster	5–8 weeks	4	Early estrus	S	5+	16 (15–18)
Guinea Pig	8–10 weeks	16–19	10 hours	S	6	67–68
Ferret	8–12 months	Seasonal	30–36 hours	I	12–13	42
Cat	6–15 months	Seasonal	24–56 hours	I	13–14	63 (52–69)
Dog	6–8 months	9	1–3 days	S	13–14	61 (53–71)
Monkey	3 years	28	9–20 days	S	9	168 (146–180)
Human	12–16 years	27–28	13–15 days	S	7.5	267 (ovulation)

Abbreviations: I, induced; S, spontaneous.

the origin of the allantois and migrate along the hindgut, up the dorsal mesentery and into the gonadal ridges (Eddy et al., 1981). The primordial germ cells divide mitotically during migration and continue to proliferate as they migrate under the underlying mesenchyme and are incorporated into the primary sex cords (Moore, 1982). As the primitive cords begin to form, the mesenchyme is invaded by capillaries. The indifferent gonad now consists of an outer cortex and an inner medulla. In the rodent, formation of the gonadal cords is a rapid process that occurs at gestational day 13 via transitory epithelial cell aggregates along the length of the gonadal ridge (Paranko et al., 1983).

Gonadal differentiation is dependent on signals from the Y chromosome, which contains the genes necessary to induce testicular morphogenesis. One of these signals is the SRY gene, which is the sex-determining region on the short arm of the Y chromosome (Koopman et al., 1990), and acts as a "switch" to initiate transcription of other genes, which contribute to testicular organogenesis. In the absence of the SRY protein, the gonad remains indifferent for a short period of time before differentiating into an ovary.

The first morphological sign of testis formation is the aggregation of primordial germ cells and somatic cells (primitive Sertoli cells). These aggregates develop from the gonadal blastema into plate-like structures, which then develop into simple arches of elongated testicular cords (Paranko et al., 1983). Throughout differentiation, the testicular cords remain connected to the basal portion of the mesonephric cell mass. The cords gradually transform and extend into the medulla of the gonad, where they branch and anastomose to form a network of cords, known as the rete testis (Moore, 1982). A characteristic and diagnostic feature of testicular development is development of a thick fibrous capsule, the tunica albuginea. As this capsule develops, the connection of the prominent testicular (seminiferous) cords with the surface epithelium is disrupted. Gradually the testis separates from the regressing mesonephros, becoming suspended by its own mesentery. Concurrent with testicular cord formation, fetal Leydig cells differentiate from loosely packed, undifferentiated mesenchymal cells in the interstitium (Pelliniemi, 1975). These interstitial Leydig cells produce the male sex hormone testosterone, which induces masculine differentiation of the Wolffian duct and external genitalia. Intratesticular vasculature differentiates in the gonadal mesenchyme along with the growth of epithelial components. A testis-specific distribution of blood vessels is obvious from an early phase of testicular development (Pelliniemi, 1975). See Fig. 21-2 for a diagrammatic representation of sexual differentiation in the human male.

The fetal testis is composed of testicular cords containing supporting immature Sertoli cells and centrally placed spermatogonia, derived from the surface epithelium and primordial germ cells, respectively. These cords are surrounded by a highly vascularized interstitium containing fetal Leydig cells and mesenchyme (Pelliniemi and Niei, 1969). The testicular cords remain without a lumen throughout the fetal period. The seminiferous cords turn into tubules when the Sertoli cells undergo terminal differentiation. This occurs after birth when they finish dividing (roughly at the onset of puberty). They develop tight junctions between adjacent cells, and apical secretion of fluid begins as these cells become

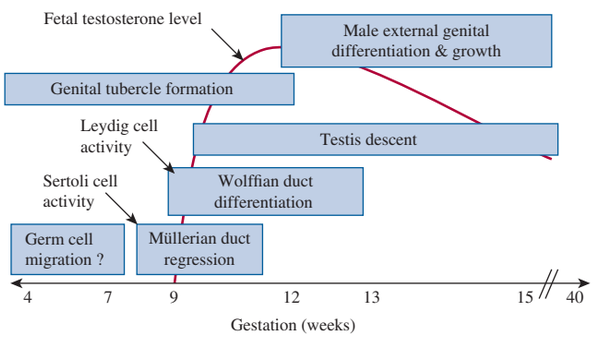


Figure 21-2. *Male sexual differentiation in humans during gestation.* (Reproduced with permission from Klonisch et al., 2004.)

highly polarized. Thus, the lumen forms as Sertoli cells develop their mature phenotype.

In the rodent and human species, fetal testicular androgen production is not only necessary for proper testicular development and normal male sexual differentiation, but also for differentiation of the Wolffian ducts into the epididymides, vasa deferentia, and seminal vesicles (Wilson and Lasnitzki, 1971; Veyssiere et al., 1982; Imperato-McGinley et al., 1992; Barker et al., 1993; Berman et al., 1995; Roy and Chatterjee, 1995; Silversides et al., 1995; Kassim et al., 1997).

Androgens derived from the Leydig interstitial cells stimulate the mesonephric (or Wolffian) ducts to form the male genital ducts, while Sertoli cells produce Müllerian inhibiting substance (MIS or Anti-Müllerian Hormone, AMH), which suppresses development of the paramesonephric (Müllerian) ducts, or female genital ducts.

There is differential maturation of the mesonephric ducts depending on location. Near the testis, some tubules persist and are transformed into efferent ductules, which open into the mesonephric duct, forming the ductus epididymis. Distal to the epididymis, the mesonephric ducts acquire a thickening of smooth muscle to become the ductus deferens, or vas deferens (Moore, 1982).

Development of the external genitalia is similar in the two sexes. In the human, the external genitalia are indistinguishable until the ninth week of gestation, and not fully differentiated until the twelfth week of development. Development of the external genitalia coincides with gonadal differentiation. Early in the fourth week of gestation, the sexually undifferentiated fetus develops a genital tubercle at the cranial end of the cloacal membrane. Labioscrotal (genital) swellings and urogenital (urethral) folds then develop on each side of the cloacal membrane. The genital tubercle then elongates forming a phallus. In response to testicular androgens, the phallus enlarges and elongates forming the penis while the labioscrotal swellings ultimately form the scrotum. At the end of the sixth week of gestation, the urorectal septum fuses with the cloacal membrane dividing the membrane into a dorsal anal and a ventral urogenital membrane. Approximately a week following, these membranes rupture forming the anus and urogenital orifice, respectively (Moore, 1982).

Fetal testicular androgens are responsible for the induction of masculinization of the indifferent external genitalia. The testis remains caudally positioned during the 10th to 15th week until entry into the inguinal canal and transabdominal descent. Testicular descent through the inguinal canal begins in the 28th week, and the testes enter the scrotum by the 32nd week. At birth, the testes reach the bottom of the scrotum (Moore, 1982). There are two critical phases of testis descent, transabdominal and inguinoscrotal, essential to move the testes into the scrotum. Although the precise mechanisms of testicular descent, and causes of cryptorchidism, remain unclear, the insulin-like peptide hormone INSL3 and fetal testicular androgens are known to play a critical role (Klonisch et al., 2004; see Fig. 21-3). Cryptorchidism or undescended testes occur in about 3% of full-term and 30% of preterm males, making it the most common human birth defect (Boisen et al., 2004). However, in a comparative study of the prevalence of cryptorchidism in cohorts of children in Denmark and Finland, a higher prevalence of cryptorchidism was observed in Denmark, with a 9% incidence rate in full-term males reported at birth (Boisen et al., 2004). These data add further evidence to the concept that there is a significant geographical difference in male reproductive health in two neighboring countries, and therefore potential exposure to similar environmental effects. As the major difference was found in the milder forms of cryptorchidism, an environmental rather than a genetic basis for effect is favored. If correct, there is a need to determine the nature of the environmental agents responsible, because similar chemicals may well be implicated in the trends noted in other geographically diverse countries where an increasing frequency of cryptorchidism and testicular cancer has been found (Boisen et al., 2004).

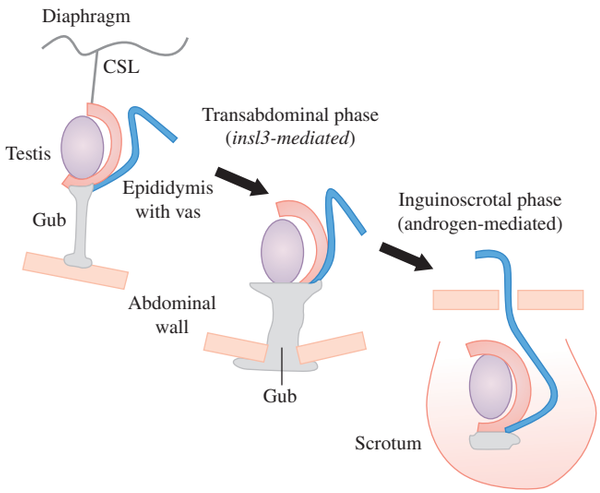


Figure 21-3. *The two distinct phases of testicular descent in mammals.* The first (transabdominal phase) mediated by insl3 from the fetal testis involving the removal of the cranial suspensory ligament (CSL) and development of the gubernaculum (gub). The second (inguinoscrotal phase) being androgen mediated. (Reproduced with permission from Klonisch et al., 2004 and Elsevier Science.)

Thus male, but not female, reproductive tract development is totally hormonally dependent and thus inherently more susceptible to endocrine disruption (see the section "Endocrine Disruption [Including Screening and Puberty]"). It is also of note to mention that the early development and triggers of the testis are mediated by direct hormone action before the establishment of the hypothalamic–pituitary–gonadal (HPG) axis.

GAMETOGENESIS

Formation and production of gametes in mammals begin in early embryonic life with the development of primordial germ cells in the genital ridge and movement of these cells into what will become the gonads (see the section "Reproductive Development and Sexual Differentiation"). The critical feature in the production of gametes is the process of meiosis.

The basic features of meiosis—two cell divisions with no intervening DNA replication results in a halving of the chromosome complement—are conserved throughout evolution. Thus, it is not surprising that the general outline applies to both mammalian males and females. However, the strategies employed are remarkably different between the sexes. The mammalian oocyte begins meiosis during fetal development but arrests part-way through meiosis I and does not complete the first division until ovulation; the second division is completed only if the egg is fertilized (see Fig. 21-4). Oogenesis therefore requires several start and stop signals and, in some species (e.g., the human), may last for more than 10 years. In contrast, male meiosis begins at puberty and is a continuous process, with spermatocytes progressing from prophase through the meiotic second division in little more than a week. This difference in strategy has implications for the action of toxicants and critical time periods when these cells may be vulnerable to attack (see sections on male and female reproductive systems). Critical to this is

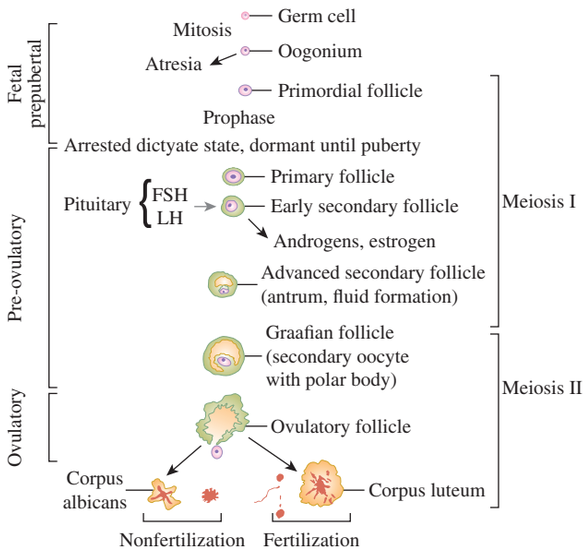


Figure 21-4. *Development of the oocyte.*

the understanding that the complement of oocytes available to the mammalian female is complete at birth, whereas in the male there is significant stem cell (spermatogonial) renewal to maintain the significantly higher number of germ cells available in males.

NEONATAL DEVELOPMENT

At birth, or even late in gestation, male rats display longer anogenital distances (AGD) than do female rats, with neonatal male AGD being more than twice as long as females (Gray et al., 1999a). There are homologous sex differences in humans (Swan et al., 2005; Longnecker et al., 2007). Administration of androgen receptor (AR) antagonists or inhibitors of testosterone synthesis can demasculinize male AGD, whereas androgen administration during this period lengthens female AGD (Gray et al., 1994, 1999a; Wolf et al., 2002; Hotchkiss et al., 2007).

In addition, in many mammalian species, including humans and rats, males of the species engage in more aggressive play than do females (Hines, 2003; Hotchkiss et al., 2003). In rats, play behavior is displayed for a period of a few weeks around 35 days of age and males engage in more rough and tumble aggressive play than do females. This behavior differentiates during neonatal life, and exposure to antiandrogens such as flutamide or vinclozolin shortly after birth demasculinizes this behavior such that treated males engage in female-like play at about 35 days of age. In contrast, neonatal androgen treatments masculinize female rat play behavior such that they will engage in male-like levels of rough and tumble play. In humans, sexually dimorphic play behaviors are displayed fairly early in life. Congenital adrenal hyperplasia (CAH) daughters exposed to high levels of adrenal androgens in utero display male-like play (Hines and Kaufman, 1994). Normally in rats, the development of this behavior differentiates under the influence of a neonatal surge in testosterone in the first few days of pregnancy, after which fetal testis Leydig cells regress and testosterone production declines to very low levels for a few weeks until the emergence of the adult Leydig cells occurs in the testis prior to puberty (Huhtaniemi and Pelliniemi, 1992). In rodents but not humans, interestingly the first wave of spermatogenesis is initiated at about four days of age.

INFANTILE DEVELOPMENT

Later in lactation, during the infantile period of development, several key events occur in the reproductive system of rats including emergence of the nipple buds and areolae in females and maturation of the hypothalamic–pituitary axis. Emergence of the nipple buds is an event most visible around 13 days of age, which is prevented in males by prenatal androgen-induced atrophy of the nipple anlagen. It is noteworthy that male rats with the shortest AGD and highest numbers of female-like nipples have a very high likelihood of displaying reproductive tract lesions such as hypospadias or epididymal agenesis (Barlow et al., 2004; Hotchkiss et al., 2007). Similarly, prenatally androgen-treated females with the longest AGDs and reduced infant nipple numbers are more likely to display reproductive tract malformations (retained male tissues or vaginal agenesis) than less affected females (Hotchkiss et al., 2007). In addition, the effects on AGD are permanent in both sexes (Barlow et al., 2004; Hotchkiss et al., 2004, 2007) with nipple buds being permanent in males.

PUBERTAL DEVELOPMENT

Scientific evidence increasingly supports the concerns about environmentally induced alterations of pubertal events in girls and boys. In addition, experimental animal data are consistent with a potential role of environmental factors in inducing altered pubertal maturation in humans. Puberty is the stage of life when an individual matures from a child, through adolescence to full maturity. The process is marked by dramatic development of hormone-dependent sexual characteristics, somatic growth, and sexual and social behaviors eventually resulting in full sexual maturity and reproductive capacity. The stages of puberty in boys and girls are determined using approaches including the Tanner Stages (Marshall and Tanner, 1969; Marshall and Tanner, 1970) for breast and pubic hair development in girls and gonadal and pubic hair development in boys. Puberty in girls is also assessed using the age at menarche as a marker. Alternatively, increasing levels of estradiol or androgens in the serum prior to the development of some of the early physical markers can be used to determine the onset of puberty in girls and boys, respectively.

Puberty is initiated by activation of the HPG and hypothalamic–pituitary–adrenal (HPA) axes (Ojeda and Heger, 2001; Ojeda et al., 2003) (see Fig. 21-5). At the onset, the HPG axis releases gonadotropin-releasing hormone (GnRH) pulses with increasing frequency and amplitude, which induces complementary pulsatile secretions of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (Ojeda et al., 2003). In turn, LH and FSH stimulate the gonads inducing gonadarche characterized by the onset of gonadal hormone production. In the female, secretion of androgens from theca cells is controlled by LH whereas estradiol production from granulosa cells of maturing follicles prior to ovulation is controlled by FSH. In contrast, in the male, LH stimulates the testicular synthesis and secretion of androgens and insulin-like 3 peptide hormone from the Leydig cells of males. Interestingly, in most mammals including humans and rodents, puberty in the female normally precedes the age of puberty in the male.

In humans, adrenarche, the maturation of adrenal endocrine function, occurs early in pubertal development, resulting in the growth of pubic hair, acne, and other secondary sex traits (Auchus and Rainey, 2004). These physical changes result from increasing adrenal synthesis and secretion of steroids including dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS),

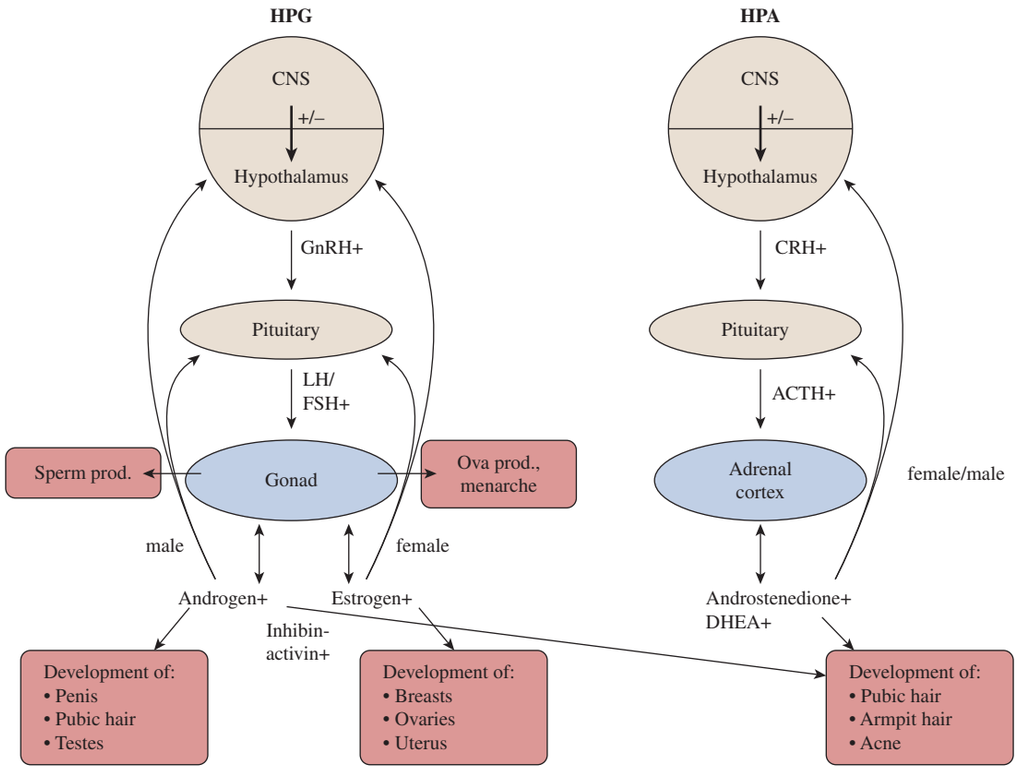


Figure 21-5. *Endocrine control of puberty in males and females.*

and androstenedione, steroids with weak androgenic activities. Adrenarche is independent of gonadarche and typically occurs between 6 and 8 years of age in both sexes. Adrenarche occurs only in primates and is not associated with puberty in all primate species.

Precocious puberty is defined as the onset of sexual traits before 8 and 9 years of age in girls and boys, respectively, whereas puberty is considered as delayed in girls if thelarche (the onset of breast development) is not displayed by 13 years and at 14 years of age in boys when testicular volume of less than 4 mL (Becker and Epperson, 2006; Biro et al., 2006; Den Hond and Schoeters, 2006; Himes, 2006; Muir, 2006; Ojeda et al., 2006; Papathanasiou and Hadjiathanasiou, 2006; Tuvemo, 2006). Delays in boys can occur as a result of either primary hypothalamic–pituitary or gonadal failure, from head trauma or from infection. While the majority of these delays are transient, some cases are associated with gene mutations resulting in either hypogonadotropic hypogonadism or primary gonadal failure.

Currently, the greatest concern is the observation that in the United States and several other countries the age of onset of puberty over the past 40 years has decreased between 0.5 and 1.0 years in girls and the age at menarche has declined by about 0.2 years (Kaplowitz, 2006). Northern European countries have not reported such a trend. Similar trends in male puberty have not been observed in boys.

Some scientists have attributed the trends in pubertal maturation of girls to obesity rates in children. Rapid early weight gain, obesity, and early development have been associated with the development of insulin resistance and an exaggerated adrenarche (Buck Louis et al., 2008). These endocrine alterations, together with elevated leptin levels and enhancement of hormonal activity by conversion of steroids to estrogens by fat cells, could affect the onset and progression of puberty in young obese girls. In addition, the role of environmental factors including endocrine disrupting chemicals (EDCs) in the etiology of this trend also is being investigated. Alterations of puberty in boys and girls can also be of genetic origin. Precocious puberty may result from early-onset of HPG function or via gonadotropin-independent alterations.

Premature thelarche and premature adrenarche are often referred to as pseudoprecocious puberty when the full spectrum of pubertal changes does not occur. Premature thelarche in girls and gynecomastia in boys are known to result from direct exposure to estrogen-containing personal care and "natural" products (Hertz, 1958; Massart et al., 2006; Henley et al., 2007). Although these conditions are usually resolved after termination of exposure, untoward consequences of these conditions may occur with prolonged exposure including shortened stature due to effects of estrogens, the growth plates of the long bones, and sexual–social behavior that is inappropriate for chronological age of the child (Wacharasindhu et al., 2006). Concerns also have been expressed that premature thelarche may enhance the likelihood of developing diseases such as breast cancer and endometriosis.

Numerous human studies have examined the relationships between environmental factors and human puberty. Many studies have shown a positive relation between body fat and onset of the growth spurt, breast development, or menarche (Battaglia et al., 2005; Dunger et al., 2005; Biro et al., 2006; Himes, 2006; McCartney et al., 2007). Environmental exposure to persistent halogenated organic chemicals such as PCBs (Den Hond and Schoeters, 2006), DDT/DDE (Krstevska-Konstantinova et al., 2001; Gladen et al., 2004; Ouyang et al., 2005; Charlier, 2006), brominated flame retardants (Blanck et al., 2000), dioxin (Eskenazi et al., 2000; Warner et al., 2004; Hauser et al., 2005; Wolff et al., 2005), HCB

(Charlier, 2005, 2006), endosulfan (Saiyed et al., 2003), and heavy metals also has been studied for being associated with pubertal alterations, but a consensus about the causative role of these chemicals in altering puberty has not been reached.

Rodent Models of Puberty

Rodents provide important animal models in the study of the genetic and environmental factors that regulate puberty. In rats, GnRH secretion is at low levels during juvenile development until GnRH release is activated by neuronal networks during puberty. During the onset of puberty, there is an increase in the excitatory amino acids and the kisspeptin peptide (Tena-Sempere, 2006) along with decreases in inhibitory neurotransmitters gamma-aminobutyric acid (GABA), and opioid peptides. In addition, several growth factors like epidermal growth factor (EGF)-like ligands and members of the EGF receptor family contribute to glia-to-neuron communication (Ojeda et al., 2003, 2006). While the complete pathway triggering the onset of puberty in rodents has not yet been described, several early components have been identified, including the kisspeptin-GPR-54 regulatory system. Mutations of the GPR54 receptor result in absence of puberty in humans and mice (Semple et al., 2005). In addition, leptin (Vogel, 1996) and insulin-like growth factor 1 (IGF-1) (Jaruratanasirikul et al., 1999) appear to be involved in sustaining pubertal events, once the process has been initiated.

In laboratory rats, the standard landmarks of puberty in the male are the age of male preputial separation (PPS), an androgen-mediated event, and the ages of vaginal opening (VO), an estrogen-mediated event, and first estrus, with VO normally coinciding with the first estrus and the onset of estrous cycles (Goldman et al., 2000). In the laboratory mouse, VO is not a useful indicator of puberty because it is not associated with the ages at first estrus and the onset of estrous cyclicity.

The ages of VO in females and PPS in male rats were included as required endpoints in the 1998 USEPA multigenerational test guidelines (http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-3800.pdf). Furthermore, it is generally agreed among risk assessors that statistically significant alterations in pubertal maturation in rats are adverse effects and these data have been used as a critical effect to establish reference doses in some cases (see the USEPA Vinclozolin Risk Assessment, 2002). Because EDCs share mechanisms of action in common with many of the drugs used to treat altered human pubertal development, it is biologically plausible that these EDCs would also alter pubertal onset in boys and girls, if children were exposed to sufficient levels of these chemicals during this critical stage of life.

Toxicants can alter puberty as a consequence of in utero, lactational, or pubertal exposures. In a multigenerational study, discerning the stage of life when exposure induced the alteration may be challenging, if possible at all, because dosing is not initiated in the parent (F_0) generation, the only generation with in utero and lactational exposure, until well after puberty. Some alternate reproductive test protocols (Gray et al., 1988a) can detect pubertal alterations in the F_0 generation since they initiate exposure right after weaning. In addition, short-term studies using the pubertal male and female rat assays (EDSTAC Final Report, 1998; Gray et al., 2004) can help resolve this uncertainty since exposures are initiated directly to the weanling rat and continued for 20 to 30 days for females and males, respectively. Comprehensive reviews describing the toxicology of puberty in rodents are found elsewhere (Goldman et al., 2000; Stoker et al., 2000; Gray et al., 2004).

Studies using rats demonstrate that body weight at weaning is an important determinant of the age of VO in the female and PPS in the male. However, treatments that reduce growth by 10% or less have little effect on the attainment of the male and female pubertal landmarks. The onset of puberty can be altered in the male and female rat by disrupting the endocrine system at a variety of levels including the entire HPG axis. In addition to effects on the HPG axis, the landmarks of female rat puberty can be altered by the direct action of either estrogens or antiestrogens on VO and other components of the reproductive tract, whereas androgens and antiandrogens can alter male rat pubertal landmarks by acting at the level of the male reproductive tract.

Because estrogen exposure in the peripubertal female rat can accelerate the onset of VO and at the age of the first estrus smear without accelerating the onset of regular estrous cycles, it is useful to monitor all three landmarks of puberty in the female in order to discriminate pseudoprecocious puberty (accelerated age at VO but not the onset of estrous cyclicity) from true precocious puberty (both the ages at VO and the onset of estrous cyclicity are accelerated).

Selected Examples of Chemicals That Alter the Onset of Pubertal Landmarks in Rats After Acute In Utero and/or Lactational Exposures

In utero exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (a single dose of 0.001 mg/kg on gestational day [GD] 15) delays the onset of pubertal landmarks in male and female rats (Gray et al., 1995, 1997b, 1997c; Gray and Ostby, 1995). The mode of action for these effects is not known, but it likely involves permanent organizational effects of TCDD of the reproductive tract during sexual differentiation mediated via aryl-hydrocarbon receptor, AhR. In addition, the treated females display a persistent vaginal thread after puberty.

In utero exposure to the potent alkalating drug busulfan (at 5 and 10 mg/kg sc on GD 15) destroys fetal germ cells, resulting in gonadal atrophy and delayed puberty in both male and female rats (Gray and Ostby, 1998). Some females never displayed VO, or any other pubertal landmark, likely due to a total absence of primary follicles in the ovary.

Prenatal androgen administration can induce agenesis of the lower vaginal canal in females, an effect that could be missed entirely or misconstrued as just a delay in VO unless the females are retained after weaning and thoroughly examined after the normal age at puberty (Hotchkiss et al., 2007). Conversely, prenatal antiandrogen treatments can result in the formation of a lower vaginal "pouch" in treated male rats (Gray et al., 1994).

Administration of estrogens such as ethinyl estradiol or methoxychlor to the dam during gestation and lactation can accelerate VO in female rat offspring. In the higher dosage groups, VO can be detected as early as 10 to 15 days of age, albeit a "pin-hole" like opening (Gray et al., 1989).

Peripubertal administration of EDCs can alter the onset of pubertal landmarks in male (Monosson et al., 1999; Gray et al., 2004) and female rats (Goldman et al., 2000). Androgens play a key role in pubertal maturation in young males, and antiandrogens such as vinclozolin (Monosson et al., 1999), linuron (Gray et al., 1999b), *o,p′*-DDE (Kelce et al., 1995), prochloraz (Blystone et al., 2007), and the phthalates (Gray et al., 1999b) produce measurable delays in this process. For example, vinclozolin treatment delayed pubertal maturation, and retarded sex accessory gland and epididymal growth (at 30 and 100 mg/kg/day) (Monosson et al., 1999). Serum LH (significant at all dosage levels), testosterone, and 5α-androstane,3α,17β-diol (at 100 mg/kg/day) levels were increased. Testis size and sperm production, however, were

unaffected. In contrast, reproductive toxicants such as carbendazim that indirectly alter FSH levels without affecting serum testosterone fail to delay PPS even at dosage levels that cause profound alterations of testicular and hypothalamic–pituitary (FSH secretion) function (Gray et al., 1990).

The ease with which a delay in PPS, a landmark of puberty in the rat, can be measured enables us to use this endpoint to evaluate chemicals for this form of endocrine activity. A "pubertal male assay" including an assessment of PPS is being considered by the USEPA and others (Gray et al., 1997a; Gray, 1998; Stoker et al., 2000) for screening chemicals for endocrine activity, as mandated by 1996 US legislation (the Food Quality Protection Act and Safe Drinking Water Act).

Selected Examples of Chemicals That Alter the Onset of Pubertal Landmarks in Rats After Peripubertal Exposures The "pubertal female rat assay" has been included in the proposed EDSTAC Tier 1 Screening (T1S) battery. In this assay, weanling female rats are dosed daily by gavage for 21 days and the age at VO (puberty) is monitored and the females are necropsied at about 42 days of age (reviewed by Goldman et al., 2000). Vaginal estrous cycles are also determined by daily observation by light microscopy of the cell types present in a vaginal lavage, taken from VO until necropsy. Necropsy measurements include serum thyroid hormones, and uterine and ovarian weight and histology. This assay detects alterations in thyroid hormone status, HPG function, and inhibition of steroidogenesis, estrogens, and antiestrogens. Recently, in studies from different laboratories the pubertal female assay was found to be highly reproducible and very sensitive to certain endocrine activities including estrogenicity, inhibition of steroidogenesis, and antithyroid activity (Gray et al., 2004) (see Table 21-4).

The "pubertal male assay rat" (Monosson et al., 1999; Stoker et al., 2000; Gray et al., 2004) detects alterations of thyroid function, HPG maturation, steroidogenesis, and altered steroid hormone function (androgen). Intact weanling males are exposed to the test substance for about 30 days, the age at puberty is determined and reproductive tissues are evaluated and serum taken for optional hormonal analyses. This assay produced reproducible responses among different labs and was sensitive to androgens, antiandrogens, inhibitors of steroidogenesis, and antithyroid activity. The chemicals studied to date in this assay are listed in Table 21-5.

When antiandrogenic chemicals are administered to juvenile male rats, these chemicals do not induce malformations of the reproductive tract, but pubertal development is delayed. Throughout puberty and into adulthood, the sex accessory glands and other androgen-dependent tissues (i.e., muscles, nervous system) continue to be dependent upon testosterone (T) and 5α-dihydrotestosterone (DHT) for maturation and maintenance of function. Several androgen-dependent events occur during puberty, most notably the separation of the prepuce from the penis (PPS), followed 10 days to 2 weeks later by the appearance of mature sperm in the epididymis (Monosson et al., 1999). During pubertal maturation, there are gradual changes in serum concentrations of testosterone (T) and of its metabolite 5α-androstanediol. In very young rats, 5α-androstanediol is present in greater quantities than T. T concentrations begin to increase, however, as 5α-reductase activity decreases in the Leydig cells, resulting in higher serum T concentrations than androstanediol in older rats. Peak T concentrations generally occur around 50 to 60 days of age.

Table 21-4

Chemicals Evaluated by the US Environmental Protection Agency (EPA) Using the Standardized Pubertal Female Rat Assay in Two Contract Laboratories or Key Studies Performed in Other Laboratories

Studies performed in Laboratory 1 for the EPA
Ethinyl estradiol: positive estrogenic control
Tamoxifen: mixed estrogen agonist–antagonist; successfully identified mixed action
Ketoconazole: inhibits steroidogenesis; caused ovarian histological changes
Methoxychlor: estrogenic pesticide; successfully detected by accelerated puberty
Phenobarbital: alters liver, hypothalamic, pituitary, and ovarian function; delays puberty
Studies performed in Laboratory 2 for the EPA
Methoxychlor: estrogenic pesticide; successfully detected by accelerated puberty
Ketoconazole: inhibits steroidogenesis; caused ovarian histological changes
Bisphenol A: weakly estrogenic plastic monomer; negative for endocrine effects
Propylthiouracil: antithyroid agent; lowered T4, increased thyroid-stimulating hormone, and caused thyroid histological changes at low doses that slightly delayed rat puberty
Fenarimol: fungicide that weakly inhibits aromatase; slight delay in puberty, but lowered T4 and retarded growth
Atrazine: herbicide that alters hypothalamic–pituitary function; delays puberty and growth
Key published studies performed in other laboratories
Methoxychlor: estrogenic pesticide; accelerates puberty (Gray et al., 1989)
Polybrominated diphenyl ether: DE71; antithyroid toxicant; affected thyroid endpoints and delayed puberty (Stoker et al., 2004)
Fadrazole: potent aromatase inhibitor; delayed puberty (Marty et al., 1999)
Antarelix: gonadotropin-releasing hormone antagonist; delayed puberty (Ashby et al., 2002)
Octylphenol: estrogenic surfactant; accelerates female rat puberty (Gray and Ostby, 1998)
DES: estrogenic pharmaceutical; accelerated puberty (Kim et al., 2002)
Tamoxifen: a mixed agonist/antagonistic drug; accelerated puberty (Kim et al., 2002)
ICI 182,780: estrogen receptor (ER) antagonist; delayed puberty (Ashby et al., 2002)
ZM 189,154: ER antagonist; delayed puberty (Ashby et al., 2002)

Pubertal female assay data from contract laboratories (summary available at http://www.epa.gov/scipoly/oscpendo/assayvalidation/meetings.htm).

Table 21-5

Chemicals Evaluated by the US Environmental Protection Agency (EPA) Using the Standardized Pubertal Male Rat Assay in Two Contract Laboratories or Key Studies Performed in Other Laboratories

Studies performed in Laboratory 1 for the EPA
Flutamide: potent antiandrogenic drug; delayed puberty, among other effects
Ketoconazole: inhibits steroidogenesis
Methyltestosterone: androgenic drug; accelerated puberty
Phenobarbital: alters liver, hypothalamic, pituitary, and testis function; delayed puberty, among other effects
Vinclozolin: antiandrogenic fungicide; delayed puberty, among other effects
Dibutyl phthalate: plasticizer that inhibits Leydig cell testosterone function; delayed puberty, among other effects
Studies performed in Laboratory 2 for the EPA
Vinclozolin: antiandrogenic fungicide; delayed puberty, among other effects
Linuron: antiandrogenic herbicide; delayed puberty, among other effects
p,p′-DDE: antiandrogenic pesticide metabolite; delayed puberty, among other effects
Phenobarbital: alters liver, hypothalamic, pituitary, and testis function; delayed puberty, among other effects
Methoxychlor: estrogenic and antiandrogenic pesticide; reduced androgen-dependent tissues weights
Ketoconazole: inhibits steroidogenesis
Atrazine: herbicide, alters hypothalamic pituitary function; delays puberty and growth
Key published studies performed in other laboratories
Vinclozolin: androgen receptor antagonistic fungicide; delayed puberty, increased serum T and luteinizing hormone (Monosson et al., 1999)
Cyproterone acetate: antiandrogenic drug; delayed puberty, among other effects
Polybrominated diphenyl ether, DE71; antithyroid toxicant; affected thyroid endpoints, and delayed puberty (Stoker et al., 2004)
Finasteride: potent inhibitor of enzyme 5 alpha reductase required for dihydrotesterone synthesis; reduced sex gland weight (Marty et al., 2001)

Pubertal female and pubertal male assay data from contract laboratories (summary available at http://www.epa.gov/scipoly/oscpendo/assayvalidation/meetings.htm).

In addition to delaying PPS, some antiandrogens also alter serum LH and T, while others do not. In particular, peripubertal exposure to flutamide (from 23 to 38 days of age) or vinclozolin (from 23 to 55 days of age) causes reductions in prostate, seminal vesicle, and epididymis in rats whereas serum LH and testosterone are increased (Monosson et al., 1999).

SEXUAL MATURITY

Hypothalamic–Pituitary–Gonadal Axis

FSH and LH are glycoproteins synthesized and released from a subpopulation of the basophilic gonadotropic cells of the pituitary gland. Hypothalamic neuroendocrine neurons secrete specific releasing or release-inhibiting factors into the hypophyseal portal system, which carries them to the adenohypophysis, where they act to stimulate or inhibit the release of anterior pituitary hormones. GnRH acts on gonadotropic cells, thereby stimulating the release of FSH and LH. Native and synthetic forms of GnRH stimulate the release of both gonadotrophic hormones.

The neuroendocrine neurons have nerve terminals containing monoamines (norepinephrine, dopamine, and serotonin) that impinge on them. Reserpine, chlorpromazine, and monoamine oxidase (MAO) inhibitors modify the content or actions of brain monoamines that affect gonadotropin production.

In the female (see Fig. 21-6 for structure of the female reproductive tract of the rat and Fig. 21-7 for basic endocrine control), LH acts on thecal cells of the ovary to induce steroidogenesis, particularly the production of progesterone and androgens that are transferred to the granulosa cells which can be stimulated by FSH to produce estradiol. These steroids then provide feedback on the hypothalamus and pituitary to regulate gonadotropin production (see the section "Ovarian Function").

Similarly in the male (see Fig. 21-8 for the basic structure of the male reproductive system and Fig. 21-9 for basic endocrine control), FSH acts primarily on the Sertoli cells, but it also appears to stimulate the mitotic activity of spermatogonia. LH stimulates steroidogenesis in the interstitial Leydig cells. A defect in the function of the testis (in the production of spermatozoa or testosterone) will tend to be reflected in increased levels of FSH and LH in serum because of the lack of the "negative feedback" effect of testicular hormones.

The HPG feedback system is a very delicately modulated hormonal process. Several sites in the endocrine process can be perturbed by different chemicals.

Ovarian Function

Oogenesis Ovarian germ cells with their follicles have a dual origin; the theca or stromal cells arise from fetal connective tissues of the ovarian medulla, and the granulosa cells from the cortical mesenchyme (Fig. 21-4). In women, about 400,000 follicles are present at birth in each human ovary. After birth, many undergo atresia, and those that survive are continuously reduced in number. Any chemical that damages the oocytes will accelerate the depletion of the pool and can lead to reduced fertility in females. About one-half of the numbers of oocytes present at birth remain at puberty; the number is reduced to about 25,000 by 30 years of age. About 400 primary follicles will yield mature ova during a woman's reproductive life span. During the approximately three decades of fecundity, follicles in various stages of growth can always be found. After menopause, follicles are no longer present in the ovary (Knobil and Neill, 1994).

Follicles remain in a primary follicle stage following birth until puberty, when a number of follicles start to grow during each ovarian cycle. However, most fail to achieve maturity. For the follicles that continue to grow, the first event is an increase in size of the primary oocytes. During this stage, fluid-filled spaces appear among the cells of the follicle, which unite to form a cavity or antrum, otherwise known as the Graafian follicle.

Primary oocytes undergo two specialized nuclear divisions, which result in the formation of four cells containing one-half the number of chromosomes (Fig. 21-4). The first meiotic division occurs within the ovary just before ovulation, and the second occurs just after the sperm fuses with the egg. In the first stage of meiosis, the primary oocyte is actively synthesizing DNA and protein in preparation for entering prophase. The DNA content doubles as each of the prophase chromosomes produces its mirror image. Each doubled chromosome is attracted to its homologous mate to form tetrads. The members of the tetrads synapse or come to lie side by side. Before separation, the homologous pairs of chromosomes exchange genetic material by a process known as crossing over. Thus, qualitative differences occur between the resulting gametes. Subsequent meiotic stages distribute the members of the tetrads to the daughter cells in such a way that each cell receives the haploid number of chromosomes. At telophase, one secondary oocyte and a polar body are formed, which are no longer genetically identical.

The secondary oocyte enters the next cycle of division very rapidly; each chromosome splits longitudinally; the ovum and the three polar bodies now contain the haploid number of chromosomes and half the amount of genetic material. Although the nuclei of all four eggs are equivalent, the cytoplasm is divided unequally. The end products are one large ovum and three rudimentary ova (polar bodies), which subsequently degenerate. The ovum is released from the ovary at the secondary oocyte stage; the second stage of meiotic division is triggered in the oviduct by the entry of the sperm.

Although ovarian weight, unlike uterine weight, in the rat does not fluctuate during the estrous cycle, ovarian weight and histology can provide very useful information about the effects of toxicants on the female reproductive system. Ovarian weight can be reduced by either depletion of oocytes or disruption of the HPG axis. Toxicants affect ovarian histology inducing a variety of lesions, including polyovular follicles, oocyte depletion, interstitial cell hyperplasia, corpora albanicans, and absence of corpora lutea. In addition, ovarian tissue can be cultured ex vivo after in vivo treatment or in vitro at different stages of the estrous cycle or during pregnancy to assess their steroidogenic capacity (Berman and Laskey, 1993; Gray et al., 1997a; Calafat et al., 2006).

Case Study—Busulfan The drug busulfan is an alkylating drug used to treat several diseases in humans, including chronic myelogenous leukemia and certain myeloproliferative disorders such as severe thrombocytosis and polycythemia vera. It is also used in combination with other drugs to treat myelofibrosis. Busulfan has been used in very high doses and in combination with other drugs to destroy the bone marrow in preparation for a bone marrow transplant. It may interfere with the normal menstrual cycles in women and block sperm production in men. In addition, busulfan causes ovarian failure and prevents or delays the onset of puberty in girls (http://www.nlm.nih.gov/medlineplus/druginfo/medmaster/a682248.html).

In the rat, busulfan produces a similar profile of reproductive effects, with the most dramatic occurring in utero. In rodents, endocrine function during adulthood can be altered by prenatal exposure to fetal germ cell toxicants. The ovary of the treated female

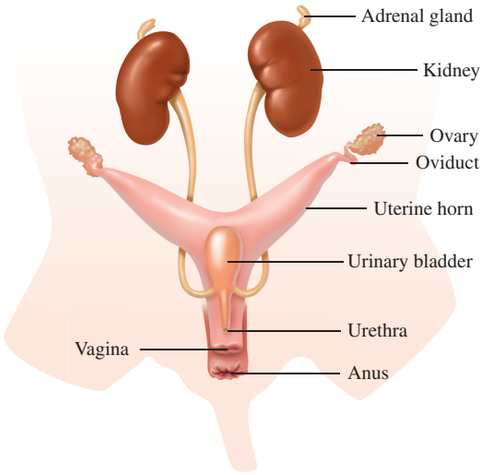


Figure 21-6. *Rat female reproductive system.*

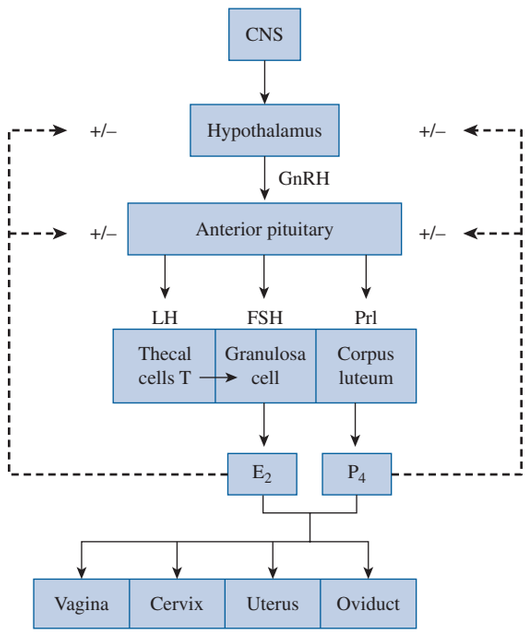


Figure 21-7. *Endocrine control of the female reproductive cycle.* CNS, central nervous system; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; Prl, Prolactin; T, testosterone; E_2, estradiol; P_4, progesterone.

offspring, lacking oocytes, developing follicles (the source of most serum estradiol), or corpora lutea (the major source of progesterone) fails to produce sex hormones. Administration of busulfan (10 mg/kg ip 5 to 7 days before birth) specifically inhibits germ cell development (Hemsworth and Jackson, 1963) in rats. Offspring of rats dosed with 2.5 and 5 mg busulfan per kg on gestational day 14 display permanent reproductive and central nervous system (CNS) alterations (Gray and Ostby, 1998). The most severely affected females do not display estrous cycles or spontaneous sexual behavior as a consequence of this effect. Puberty was markedly delayed in females treated with 10 mg of busulfan. In male progeny, testicular (at 2.5 mg), epididymal (2.5 mg), and ejaculated sperm (5.0 mg) counts, fertility and fecundity (10 mg), measured under continuous

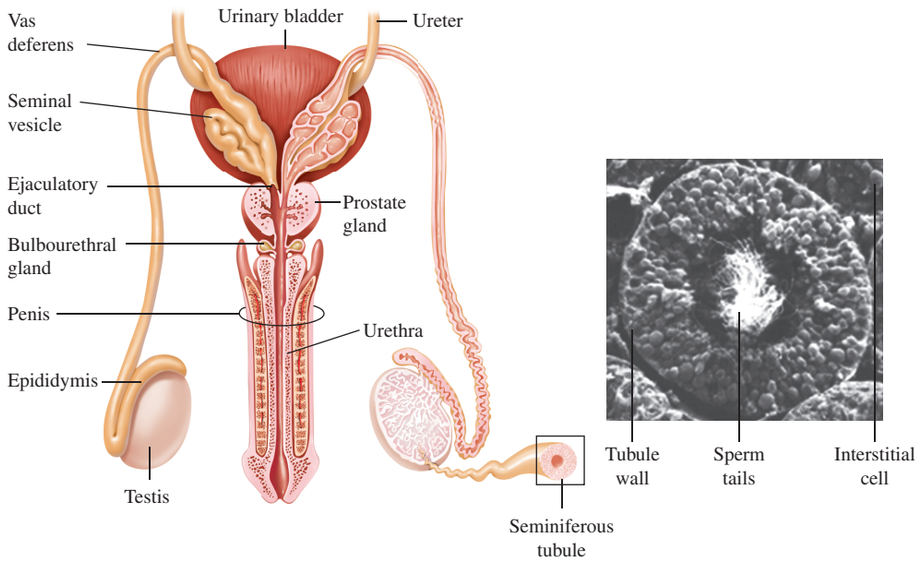


Figure 21-8. *Male reproductive system.*

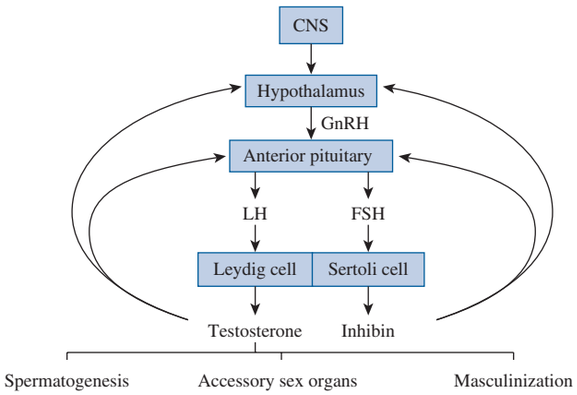


Figure 21-9. *Endocrine control of male reproduction.*

breeding conditions, were reduced. In female progeny, fertility and fecundity (2.5 mg) and ovarian weight (5 mg) were reduced, and the incidence of constant vaginal estrus was increased (10 mg). In addition to these reproductive effects, brain weight was reduced in both sexes of all dosage groups, even though body and other nonreproductive organ weights were only affected in the high-dose group (10 mg). Even though the gonads of both sexes were affected at similar dosage levels, fertility and gonadal hormone production were much more easily disrupted in female than male offspring because the steroid producing cells in the ovary fail to differentiate in the absence of the oocyte. In the male, Leydig cell numbers and steroidogenesis are not so dependent upon normal gametogenesis. Hence, the lack of oocytes in the female progeny results in a lack of estrous cyclicity and the spontaneous display of female mating behavior at proestrus. Technically, busulfan is not considered to be an EDC because the cells that it eliminates, the oocytes, do not display endocrine activity. However, this classification is of little importance because in their absence, ovarian thecal and granulosa cell endocrine functions are severely impaired.

In addition to busulfan, several environmental chemicals have been shown to disrupt ovarian development and oocyte numbers in the rat or mouse. Oral administration of some of the benzidine-based dyes on 8 to 12 days of pregnancy in the mouse or rat produces reproductive effects in the female offspring similar to those seen with the lower dose levels of busulfan, albeit at much higher dose levels (1 g/kg/day) (Gray and Kelce, 1996).

4-Vinylcyclohexene (VCH) is a chemical intermediate used in the production of flame retardants, flavors and fragrances, in the manufacture of polyolefins, and as a solvent and in the manufacture of its diepoxide. Low levels of occupational exposure have been measured during the production and use of 1,3-butadiene. In the mouse, VCH destroys the small preantral follicles in the ovaries (Hoyer and Sipes, 2007) and the monoepoxide metabolites, 1,2-VCH epoxide, 7,8-VCH epoxide, and the diepoxide, VCD, cause preantral follicle loss in rats as well as in mice. Mice are more susceptible to VCH than rats because they are capable of its metabolic bioactivation. Follicle destruction by VCD is selective for primordial and primary follicles and results from upregulating the rate of atresia (apoptosis) through activation of proapoptotic signaling events selectively in the small preantral follicles.

Methoxychlor (MXC) is an organochlorine pesticide and reproductive toxicant that also produces antral follicle atresia, in part by altering apoptotic regulators (Bcl-2 and Bax). MXC directly inhibits follicle growth partly by Bcl-2 and Bax pathways, and increases atresia partly through Bcl-2 pathways (Miller et al., 2005) by inducing oxidative stress (Gupta et al., 2006a, 2006b). A diagrammatic representation of the sites of actions of female reproductive toxicants is presented in Fig. 21-10.

Ovarian Cycle

The cyclic release of pituitary gonadotropins involving the secretion of ovarian progesterone and estrogen is depicted in Fig. 21-11. These female sex steroids determine ovulation and prepare the female accessory sex organs to receive the male sperm. Sperm, ejaculated into the vagina, must make their way through the cervix into the uterus, where they are capacitated. Sperm then migrate into the oviducts, where fertilization takes place. The conceptus then returns from the oviducts to the uterus and implants into the endometrium.

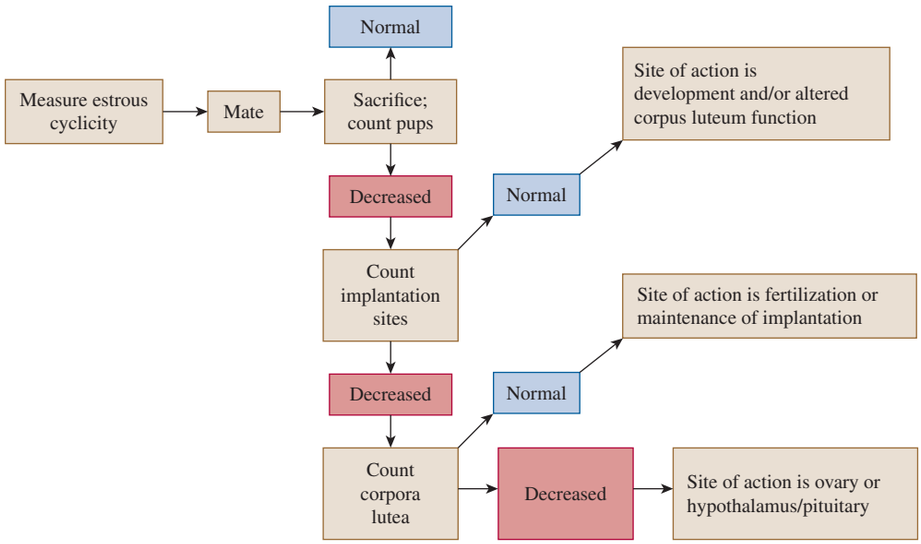


Figure 21-10. *Sites of action for female reproductive toxicants.*

This axis can be disrupted, resulting in infertility at any level of the endocrine system. For example, chemicals that block the LH surge transiently can prevent or delay ovulation resulting in infertility or lower fecundity due to delayed fertilization of ova (Goldman et al., 1993, 1994, 1997; Stoker et al., 1993, 1996, 2001, 2003, 2005; Cooper et al., 1994, 2000).

Postovarian Processes

Female accessory sex organs function to bring together the ovulated ovum and the ejaculated sperm. The chemical composition and viscosity of reproductive tract fluids, as well as the epithelial morphology of these organs, are controlled by ovarian (and trophoblastic) hormones (see Fig. 21-6).

Oviducts The oviducts provide the taxis of the fimbriae, which is under muscular control. The involvement of the autonomic nervous system in this process, as well as in oviductal transport of both the male and female gametes, raises the possibility that drugs known to alter the autonomic nervous system may alter function and therefore fertility. The progression of the fertilized eggs through the oviduct and uterus is under hormonal regulation and chemicals such as the estrogens can stimulate oviductal transport and interfere with uterine endometrial function, precluding implantation (Cummings and Perreault, 1990).

Uterus Uterine endometrium reflects the cyclicity of the ovary as it is prepared to receive the conceptus. The myometrium's major role is contractile. In primates, at the end of menstruation, all but the deep layers of the endometrium are sloughed. Under the influence of estrogens from the developing follicle, the endometrium increases rapidly in thickness. The uterine glands increase in length but do not secrete to any degree. These endometrial changes are called proliferative. After ovulation, the endometrium becomes slightly edematous, and the actively secreting glands become tightly coiled and folded under the influence of estrogen and progesterone from the corpus luteum. These are secretory (progestational) changes (Fig. 21-11). When fertilization fails to occur, the endometrium is shed and a new cycle begins. Only primates menstruate (Knobil and Neill, 1994).

Other mammals have estrous cycles rather than menstrual cycles, see Fig. 21-12 for a diagrammatic comparison of the human menstrual and rat estrous cycles with regard to timing and endocrine control. In the young adult rat, the female has a 4- to 5-day estrous cycle that lacks a functional luteal phase (Knobil and Neill, 1994, Chap. 46; Cooper et al., 1984). During the estrous cycle, the uterus and vagina display remarkable changes in morphology over the brief cycle. Uterine weight and fluid content increase many folds during proestrus under the influence of estrogen. The vaginal cytology also changes daily throughout the estrous cycle, and this can be monitored by examining the cytology of the cells sloughed from the vaginal epithelium into the lumen with daily vaginal lavages.

Uterine weight is a very useful index of estrogenicity in the immature or adult ovariectomized female rats. Because uterine weight and histology fluctuate greatly during the estrous cycle, studies which necropsy females at different stages of the cycle will often be too variable to detect anything but the most profound effects on these endpoints, but nonetheless it can still be a useful endpoint and should be measured. A single vaginal lavage, taken at necropsy, could be used as a covariate to analyze for treatment

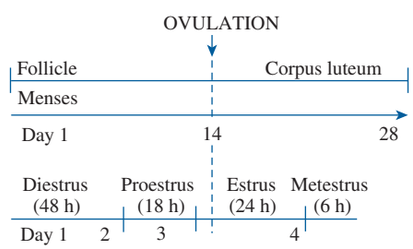


Figure 21-11. *Comparison of the human menstrual cycle with the rat estrus cycle.*

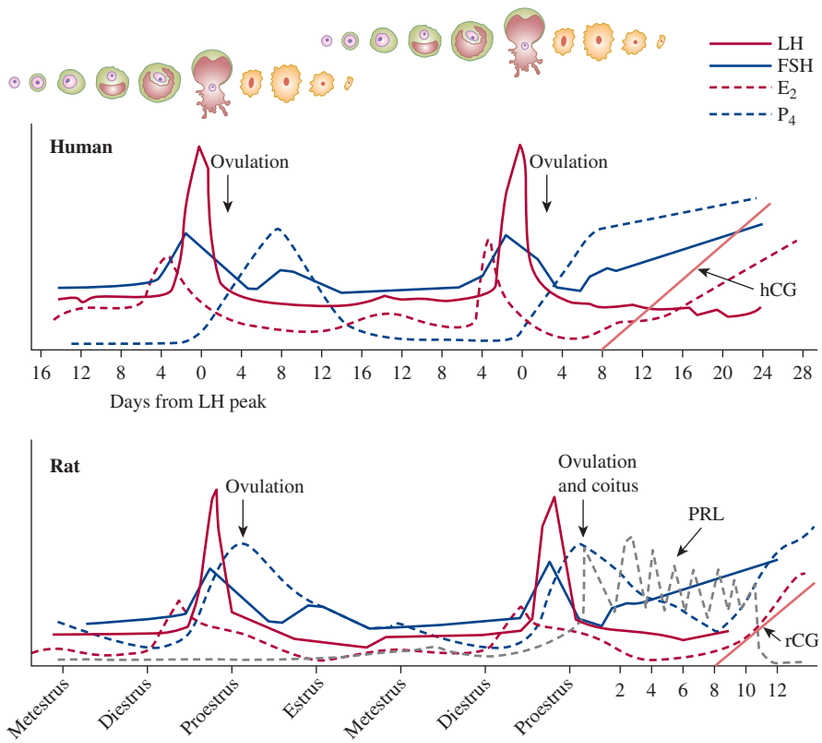


Figure 21-12. *Comparison of the timing of the human and rat cycles.* LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; E_2, estradiol; P_4, progesterone; hCG, human chorionic gonadotropin; rCG, rat chorionic gonadotropin.

effects. It is noteworthy that both uterine weight and ovarian histology provided useful information in a surprising number of cases in the assessment of chemicals in the pubertal female rat assay being validated by the USEPA, OSCP, and EDSP (reports available at http://www.epa.gov/scipoly/oscpendo/).

TESTICULAR STRUCTURE AND FUNCTION

Targets for Toxicity

For the adult male, the overall objective of the reproductive process is the production of gametes capable of fertilization and the production of viable offspring. In considering these processes, there are numerous potential targets for the action of chemicals upon the system (see Fig. 21-13). These would range from the action of dopamine analogs on the hypothalamus interrupting the normal secretion of GnRH to the action of estrogens on the pituitary (and hypothalamus) interfering with gonadotropin (LH and FSH, see Fig. 21-7) production through direct effects on spermatogenesis—where the vast majority of toxicants have their site of action. However, there are specific examples of chemicals that may impair spermatogenesis via an indirect mechanism not specifically related to the HPG axis. So for example, there are a number of examples of nutritional deficits (and overexposures) of critical vitamins and minerals (e.g., vitamin A and zinc) that are essential for normal reproduction. Perturbing the homeostasis of these nutrients can lead to direct effects on spermatogenesis and subsequent issues with fertility. Similarly, chemicals with direct

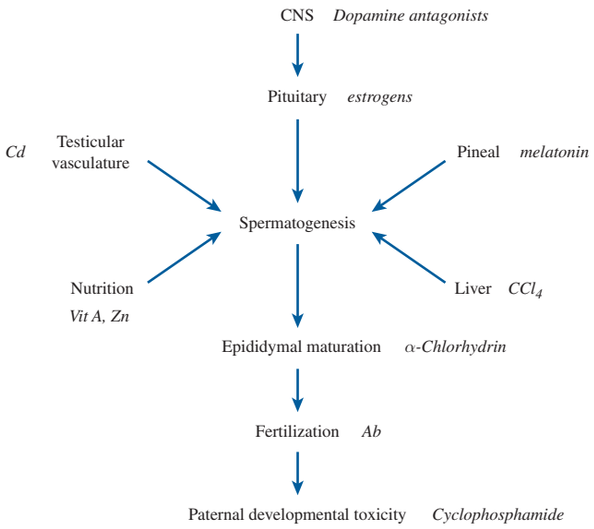


Figure 21-13. *Potential target sites for male reproductive toxicants.* Examples of agents shown in italics.

effects on the liver (e.g., CCl_4) can disturb the normal metabolism of sex steroids leading to changes in clearance (predominantly of glucuronide and sulfate conjugates of hydroxytestosterones in the male), indirectly affecting the HPG axis and exerting effects on male reproduction.

While most experimental rodents normally employed in reproductive toxicity studies do not show marked seasonality in breeding performance, there are dramatic exceptions (e.g., the hamster, where during the breeding season [or under lab conditions of 14 hours light and 10 hours dark] up to 10% of an adult male's body weight may be testicular tissue). In the hamster, markedly changing the light:dark cycle to reduce the hours of daylight can influence melatonin levels and through a cascade of signaling responses cause regression of the testes and concurrent decreases in testicular steroids and mating behavior. This is a normal circumstance in the wild, but care should be taken in laboratory situations to ensure appropriate housing for the conduct of reproduction studies with this species.

The testis also has a finely tuned circulatory system in mammals, termed the pampiniform plexus, designed to shunt the arterial venous blood supply and aid in scrotal cooling. Some chemicals can actually target this structure and the testis circulatory system to induce ischemic shock to the testis again resulting in injury and reduced fertility, with cadmium being an example of an agent that can induce testicular damage via this "indirect" mechanism (Setchell and Waites, 1970).

Testicular Structure and Spermatogenesis

The experimenter should be cognizant of these indirect mechanisms for affecting male reproductive biology; however, the overwhelming number of chemicals known to affect the male reproductive system appears to do so by a direct effect on the testis and an interference with the process of spermatogenesis. In rodents, there is a highly efficient process for the production of sperm in large numbers. A simple cross section of the testis from a rat (see Fig. 21-14), the most common species employed in reproductive toxicity studies, indicates that not all of the seminiferous tubules have an identical morphology. The generalized structure of the rat seminiferous tubule (see Figs. 21-15 and 21-16) shows the organization of a number of different cell types comprising germ cells at different stages of differentiation supported by a somatic cell, the Sertoli cell, that provides structure to the epithelium and numerous support functions (e.g., hormonal signaling, nutrition, fluid provision, etc.) and serves to maintain the haploid germ cells in a protected environment (the so-called blood–tubule barrier) as these would be recognized as foreign by the host immune system. Thus, the seminiferous tubules can be divided into a number of physiological compartments (as indicated in Fig. 21-15) that facilitate the process of spermatogenesis. The boundary of the seminiferous tubule is surrounded by myoepithelial cells that aid in a peristaltic process in moving released spermatozoa present in the lumina of the tubules (at this time these sperm are both nonmotile and nonfertile) along the tubules through the rete testis (into which the seminiferous tubules open—these tubules have both ends attached to the rete) for further progression through the efferent ducts to the epididymis. It is here that a concentration of sperm and changes in their fluid environment occur as the sperm are ushered through the epididymis and acquire motility and fertilizing ability.

Spermatogenesis is an extremely ordered process in the rat (see Fig. 21-17), the spermatogonia have populations that act as the stem cells for the seminiferous tubules and a proportion of these cells then undergo a series of mitotic divisions to increase numbers, move into meiotic prophase, and are then committed to becoming spermatozoa. These germ cells proceed through the various stages of prophase, preleptotene, leptotene, zygotene, and pachytene with increasingly larger cells that undertake DNA replication in preparation for the cells to enter meiosis. The germ cells enter diplotene and then diakinesis with two meiotic divisions, which eventually results in the production of the haploid spermatid population. These initially round cells then undergo an incredible metamorphosis to produce the elongated spermatozoa that mature and are then released into the seminiferous tubule lumen.

The spermatogonial stem cells undergo another round of mitotic division approximately every 13 days in the rat to produce another wave of division and differentiation (see Fig. 21-18). For the rat, the time taken for a spermatogonial stem cell to become a mature spermatozoan is approximately 8 weeks (and 10 weeks for

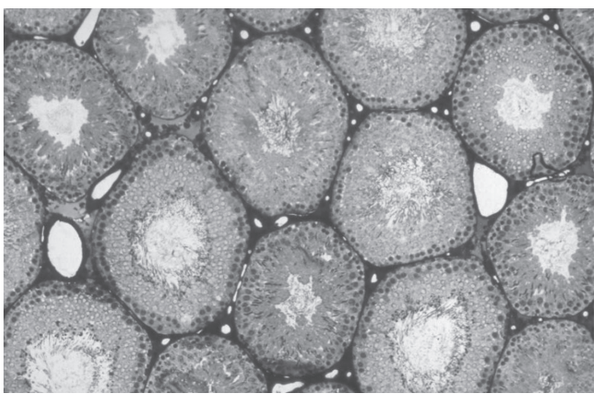


Figure 21-14. *Control testis indicating different cellular associations noted in different seminiferous tubule cross sections.* See also Fig. 21-19.

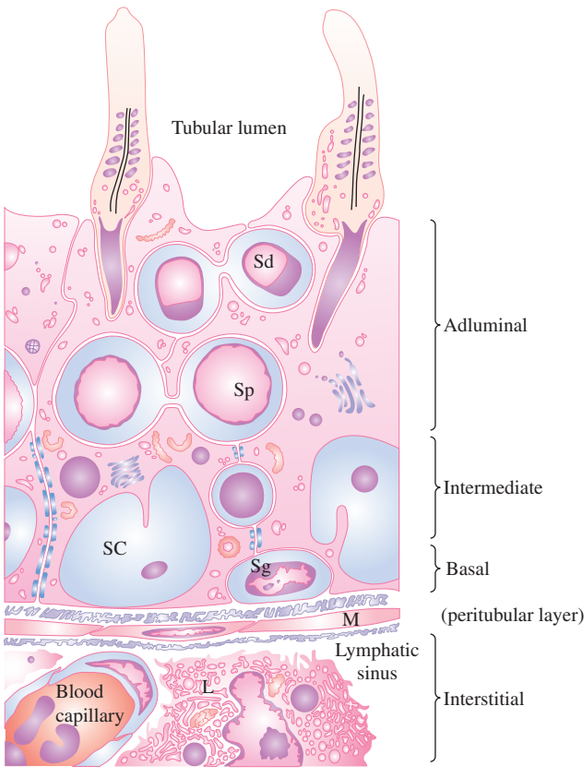


Figure 21-15. *Diagrammatic representation of a portion of a seminiferous tubule showing the cellular arrangements and testicular compartments.* L, Leydig cell; M, myoepithelial peritubular cell; SC, Sertoli cell; Sd, spermatid; Sg, spermatogonium; Sp, spermatocyte. (Reproduced with permission from Foster, 1988.)

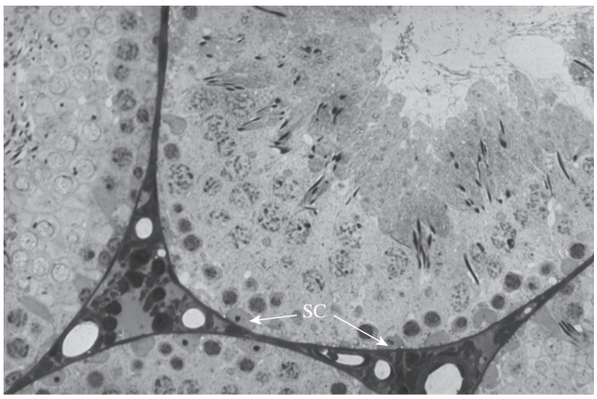


Figure 21-16. *Section of rat seminiferous tubules showing the orientation of different germ cells and Sertoli cells (SC).* Compare with Figs. 21-15 and 21-18.

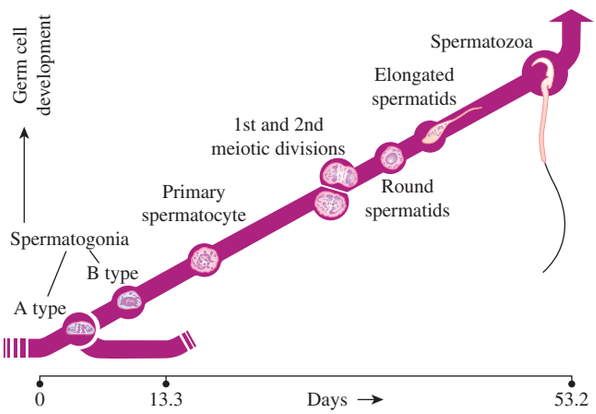


Figure 21-17. *Diagrammatic depiction of spermatogenesis in the rat.* (Reproduced with permission from Foster, 1988.)

humans) with an additional 10 days to 2 weeks for the released sperm to mature in the epididymis and be capable for ejaculation and fertilization of the ovum. One of the consequences of these successive longitudinal waves of differentiation is the highly ordered sequence of events reflected in seminiferous tubular morphology. The most primitive germ cells, the spermatogonia, reside at the periphery of the tubule and as they mature, they move toward the tubule lumen for eventual release (in a process termed spermiation). As can be seen from Fig. 21-18, at any one time the tubule can contain cellular members from four and a half successive spermatogenic cycles, with the most primitive spermatogonia from the current cycle and the most mature, the late spermatids (about to become spermatozoa on release), generated up to 4.5 cycles earlier. Thus when we take a cross section through a seminiferous tubule, this is essentially taking a "snapshot" in time of the various spermatogenic cycles. This also explains why different tubular cross sections have different morphologies, depending on when this "snapshot" is taken because of different cellular associations resulting from different cycles. It is possible for the various species to construct a morphological diagram of the nature of these cellular associations (Fig. 21-19 illustrates this for the rat). The most common system employed for the rat was based on that published by Leblond and Clermont (1952) and describes 14 different patterns of cellular association (or stages—usually depicted by a roman numeral). We know that different biochemical events can go on during the various stages and indeed this can provide clues as to potential mode of action of chemicals that produce stage-specific lesions. Such occurrences do occur regularly (e.g., certain phthalate esters, glycol ethers, antiandrogenic agents, etc.). In the case of a phthalate ester given to an adult rat, there is a marked stage specificity in response that coincides with those stages that have the highest concentrations of FSH receptors (in the Sertoli cells of the tubule) (Creasy et al., 1987). Other Sertoli cell in vitro data indicated that the phthalate ester metabolites responsible for in vivo damage could downregulate Sertoli cell responsiveness to FSH with regard to cAMP production (Lloyd and Foster, 1988), lending support to the establishment of a potential mode of action for this class of chemicals in adult rats.

At spermiation, when the step 19 spermatids are released into the seminiferous tubule lumen, the excess cytoplasm and cellular debris form a residual body and is then phagocytosed by the Sertoli cell. These bodies originate at the luminal surface of the tubule and then as they are resorbed can be seen to move through the Sertoli cell toward the base of the tubule and eventually disappear. Delays in spermiation, that is, step 19 late spermatids being retained beyond Stage VIII of the cycle are one of the more subtle, yet commonly noted testicular lesions—for example, boric acid: an antiseptic, insecticide, and flame retardant (Gray et al., 1990; Chapin and Ku, 1994); dibromoacetic acid: a water disinfection by-product (Linder et al., 1997; Melnick et al., 2007) and hormonal withdrawal (Saito et al., 2000)—and are sometimes accompanied by abnormal residual body formation. Having this "extra" layer of germ cells is a lesion frequently missed by pathologists without a good knowledge and appreciation of the stages of the spermatogenic cycle.

Once sperm are released into the seminiferous tubule lumen and proceed to the epididymis, they can also be the target of toxicant action. Chlorosugars and epichlorohydrin (an organochlorine insecticide) have both been shown to inhibit energy metabolism in sperm that prevents them from functioning normally. The specificity of the response was noted, at least in part, from the specific isoform of lactate dehydrogenase found in testicular germs cells (LDH-X or C4) essential for energy production. We are now learning more about the molecular events that drive fertilization, and it seems likely that here may be a potential target for the action of chemicals to disturb the process. We certainly know of the requirement for the expression of critical cell surface markers by the sperm that facilitate the normal binding of the sperm to the egg surface membrane. Lastly, there is the potential for effects in the male to induce paternally mediated developmental toxicity in the embryo/fetus. While this is not a common occurrence, it has been documented with one or two specific chemicals (e.g., cyclophosphamide—a chemotherapeutic agent [Trasler et al., 1985]).

The number of known environmental chemicals that produce adverse responses in human males is not large. All of these have been shown to induce effects in rodents and especially the rat, although there may be differences in sensitivity based on dose. Interestingly, a number of the classic human testicular toxicants (e.g., DBCP, gossypol) do not seem to produce infertility or testicular toxicity in the mouse and so the rat is more commonly employed as a model for male reproductive toxicity studies. However, this does not imply that all chemicals known to produce injury in the rats would indeed show toxicity in humans. The human does not employ a longitudinal wave for the production of sperm in the testis noted above for the rat, rather cellular associations are organized in a helical fashion that is intrinsically less efficient for sperm production (when estimated on a sperm produced per gram of tissue basis) and thus humans are usually deemed more sensitive, from the risk

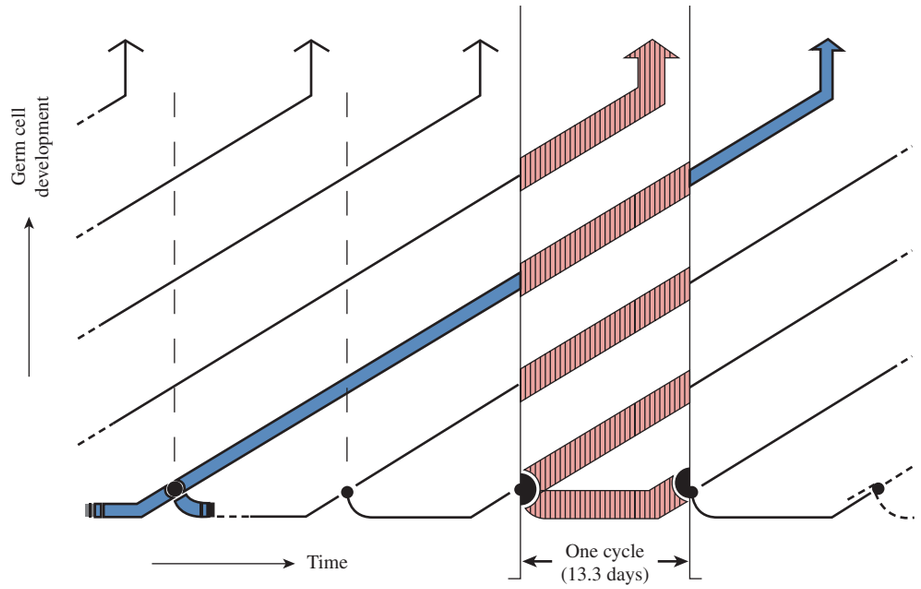


Figure 21-18. *The cyclicity of the seminiferous epithelium of the rat.* The process whereby successive spermatogenic "waves" (see Fig. 21-17) are interrelated. A transverse section of a tubule covering one cycle of the epithelium will contain cellar representatives at different stages of maturity from successive waves. (Reproduced with permission from Foster, 1988.)

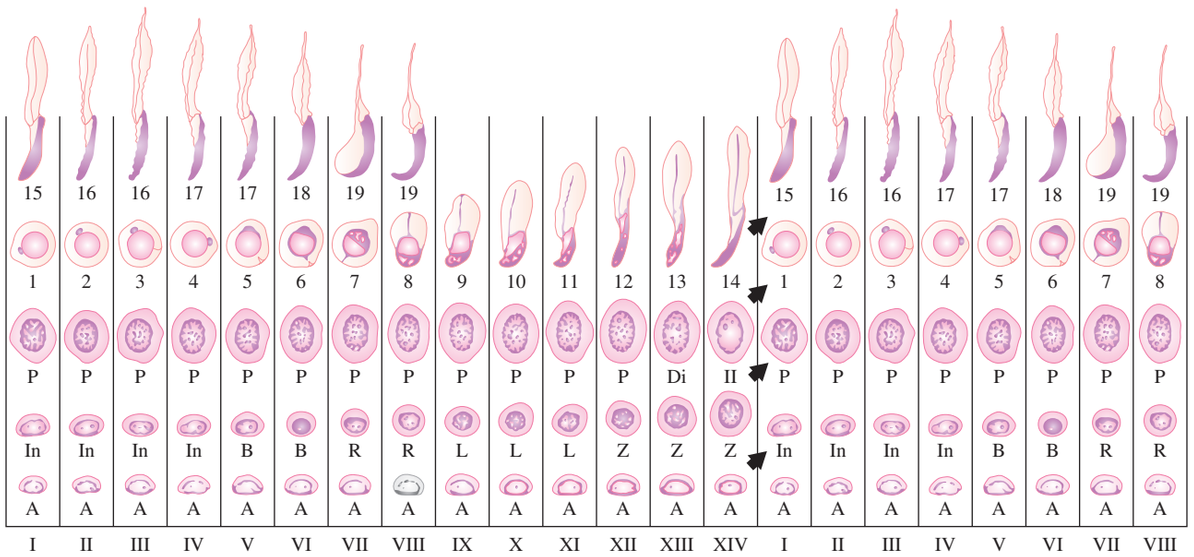


Figure 21-19. *Diagram of the 14 stages of spermatogenesis of the rat testis (after Leblond and Clermont,* 1952*).* Each stage (Roman numeral) contains different cellular associations of spermatogonia (A, IN, or B), spermatocytes (R, resting/preleptotene; L, leptotene; Z, zygotene; P, pachytene; Di, diplotene; II, secondary), and spermatids (Arabic numerals 1–19). (Reproduced with permission from Foster, 1988.)

assessment standpoint, because there are a greater range of values for semen parameters for the human population than the rat and a lesser decrement in sperm number or function is more likely to push larger numbers of men in the population into the infertile range.

Posttesticular Processes

Following the release of mature spermatids from the seminiferous epithelium, the extraneous cytoplasm and organelles form the residual body that is phagocytosed by the Sertoli cell and move from the periphery of the tubule to its base. These nonmotile sperm are moved along the tubules by a peristaltic-like action of the myoepithelial cells of the tubule and eventually emptied into the rete testis.

In rodents, but not humans, a large blood plexus forms over the rete (which is close to the surface and near the pole of the testis) where fluid exchange can take place. Sperm are then moved into the efferent ducts that exit the testis. These ducts are rich in ER

(Oliveira et al., 2004) and the fluid from the seminiferous tubules contains steroid hormones, but is low in overall total protein.

The efferent ducts then empty into the caput (head) of the epididymis that is a single highly coiled tube derived from the Wolffian duct in utero. The epididymis can be divided into three anatomical portions: the caput, corpus (body), and cauda (tail), which has a changing chemical environment and fluid composition from the efferent ducts through to the cauda. The sperm undergo maturation in the caput and corpus and begin to acquire motility, whereas the cauda is principally used for sperm storage, although expression of some critical surface markers does occur that are involved in the process of fertilization. In humans, the passage of sperm takes approximately 6 days and is longer in rats (~10 days). During the movement along the epididymal, tubule fluid is removed by active transport and this stage of the process is one that can be interfered with by toxicants resulting in an inappropriate environment for normal sperm development.

Most mammals possess seminal vesicles and a prostate (in experimental animals, the exception is that dogs do not have seminal vesicles) and the accessory sex organs are depicted in Fig. 21-20. The physiology and anatomy of these organs vary widely among mammalian species, with the rodent having a clear lobular pattern to the prostate that is not seen in humans. These organs are predominantly glandular/secretory in nature and produce much of the seminal plasma for the ejaculation of sperm to survive within the female reproductive tract. Other contributors to the seminal plasma are the epididymis and bulbourethral (Cowper's) glands. The seminal plasma contains many nutrients for sperm motion as well as distinct proteins and ion content. Any disturbance in these components may have an effect on subsequent fertility. For example, a diminution in energy substrates can affect sperm motion characteristics. These "accessory sex organs" are androgen dependent for their function and/or development and are frequently recorded in toxicity studies as indicators of androgen action. Indeed the Hershberger assay, used as part of the EPA's screening battery for endocrine disruptors (see the section "Testing for Reproductive Toxicity"), uses changes in the weights of these organs as indicators of androgen or antiandrogen action in this pharmacological screen.

Semen volume varies considerably among species, ranging from relatively small (1 to 10 mL: in humans) to much larger (~500 mL in boars).

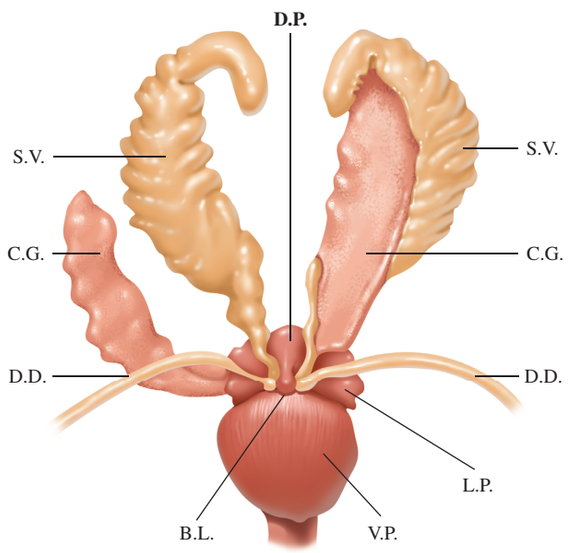


Figure 21-20. *Anatomic relation of components of rodent sex accessory glands.* D.D., ductus deferens; B.L., bladder; V.P., ventral prostate; L.P., lateral prostate; C.G., coagulating gland (also called the anterior prostate); S.V., seminal vesicle; D.P., dorsal prostate. (Reproduced with permission from Hayes, 1982.)

Erection and Ejaculation

These physiological processes are controlled by the CNS but are modulated by the autonomic nervous system. Parasympathetic nerve stimulation results in dilatation of the arterioles of the penis, which initiates an erection. Erectile tissue of the penis engorges with blood, veins are compressed to block outflow, and the turgor of the organ increases. In the human, afferent impulses from the genitalia and descending tracts, which mediate erections in response to erotic psychic stimuli, reach the integrating centers in the lumbar segments of the spinal cord. The efferent fibers are located in the pelvic splanchnic nerves (Andersson and Wagner, 1995).

Ejaculation is a two-stage spinal reflex involving emission and ejaculation. Emission is the movement of the semen into the urethra; ejaculation is the propulsion of the semen out of the urethra at the time of orgasm. Afferent pathways involve fibers from receptors in the glans penis that reach the spinal cord through the internal pudendal nerves. Emission is a sympathetic response produced by contraction of the smooth muscle of the vas deferens and seminal vesicles. Semen is ejaculated out of the urethra by contraction of the bulbocavernosus muscle. The spinal reflex centers for this portion of the reflex are in the upper sacral and lowest lumbar segments of the spinal cord; the motor pathways traverse the first to third sacral roots of the internal pudendal nerves.

Little is known concerning the effects of chemicals on erection or ejaculation (Woods, 1984). Pesticides, particularly the organophosphates, are known to affect neuroendocrine processes involved in erection and ejaculation. Many drugs act on the autonomic nervous system and affect potency (Table 21-5) (see also Buchanan and Davis, 1984; Stevenson and Umstead, 1984; Keene and Davies, 1999). Impotence, the failure to obtain or sustain an erection, is rarely of endocrine origin; more often, the cause is psychological. The occurrence of nocturnal or early-morning erections implies that the neurological and circulatory pathways involved in attaining an erection are intact and suggests the possibility of a psychological cause.

Normal penile erection depends upon the relaxation of smooth muscles in the corpora cavernosa. In response to sexual stimuli, cavernous nerves and endothelial cells release nitric oxide, which stimulates the formation of cyclic guanosine monophosphate (GMP) by guanylate cyclase. The drug sildenafil (Viagra) is used to treat erectile dysfunction; its mechanism of action resides in its ability to selectively inhibit cGMP-specific phosphodiesterase type 5. By selectively inhibiting cGMP catabolism in cavernosal smooth muscle cells, sildenafil restores the natural erectile response (Goldstein et al., 1998; Uckert et al., 2006). In the rat, prenatal exposure to the antiandrogenic fungicide, vinclozolin, induces a significant reduction of erections at all dose levels during the ex copula penile reflex tests in male offspring (Colbert et al., 2005).

Case Studies for Effects on the Male

_m_-Dinitrobenzene *m*-Dinitrobenzene (*m*-DNB), a chemical intermediate, has been extensively studied for its ability to produce rapid deleterious effects on the rat testis since the mid-1980s. The earliest studies using a 10-week dosing regimen (5 day/week by gavage in corn oil) reported effects at 6 mg/kg/day (Linder et al.,

1986), while others have reported testicular injury after a single gavage oral dose of 25 to 50 mg/kg (Blackburn et al., 1988). This paper also showed that the other isomers of DNB (*o*- and *p*-) were without testicular effects when given using the same dosing regimen. Rehnberg et al. (1988) showed that the activity was via a direct mechanism, not secondary to changes in the HPG axis. Foster (1989) showed that 5-day dosing with 5 mg/kg/day produced a minimal-to-moderate testicular lesion within 2 weeks and with 10 mg/kg/day a moderate-to-severe lesion. Testicular weight remained reduced for many weeks after the treatment period with significant dose-related effects on fertility (measured by pregnancy rate and implantation success). Similar studies have shown abnormal sperm function and failure of fertilization in rat in vitro fertilization (IVF) studies (Holloway et al., 1990). Detailed electron microscopic evaluation has shown initial lesions to be present in the Sertoli cells of the testis, which results rapidly in germ cell apoptosis and death. The primary testicular effects on Sertoli cells (see Fig. 21-21) are consistent with the period of infertility noted in the breeding study (see Fig. 21-22) and could also be modeled in Sertoli-germ cell cultures where the *o*- and *p*-isomers were also without effect. A futile redox cycle has been proposed to explain the difference in isomer toxicity in which only *m*-DNB is metabolized to its nitroso-metabolite and further to the hydroxylamine, which is recycled back to the nitroso intermediate to reduce cellular reducing equivalents, such as reduced glutathione GSH (Foster et al., 1987; Cave and Foster, 1990; Ellis and Foster, 1992; Reeve et al., 2002)

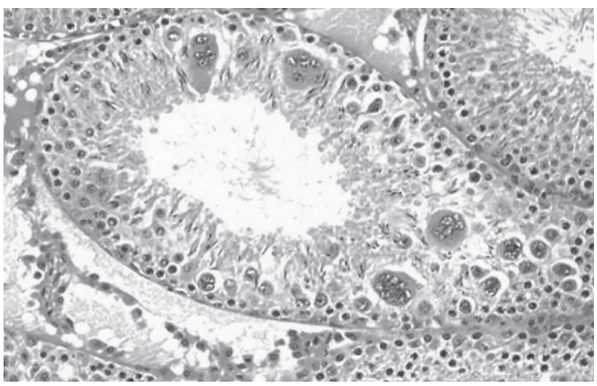


Figure 21-21. *Seminiferous tubules 24 hours after m-dinitrobenzene exposure (25 mg/kg).* Note that multinucleate giant cells and germ cell debris sloughed into the tubular lumen.

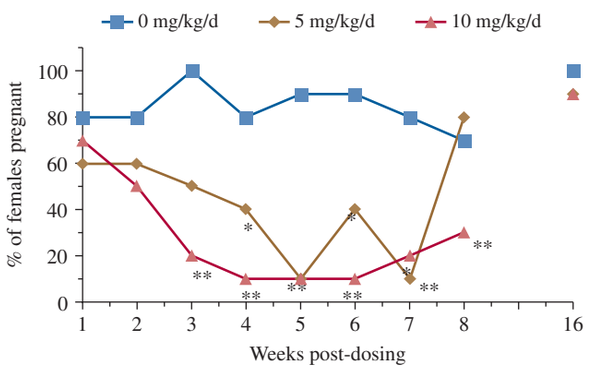


Figure 21-22. *Effect of m-dinitrobenzene on percentage of females pregnant in a serial mating study design.* Note that the range of germ cell types affected consequent to the Sertoli cell injury produced by the compound and the reversibility of the effects after 16 weeks (two spermatogenic waves—see Fig. 21-17). (Reproduced with permission from Foster, 1988.)

Ethylene Glycol Monomethyl Ether Since first published in 1979 (Nagano et al., 1979) that ethylene glycol monomethyl ether (EGME) a water soluble organic solvent can elicit testicular toxicity in the mouse, there has been significant effort expended on trying to understand the mode of action for the induction of these effects. Thus far EGME has been shown to produce testicular toxicity in a wide variety of species, including nonmammals, the major toxicologically used species (rat, mouse, rabbit, and dog) with reasonable evidence that it is likely to have effects in humans should exposure be high enough. A number of studies have described the pathogenesis of the EGME lesion (predominantly in the rat), which seems to be common for the species thus far examined. The earliest features of these studies after a single dose (250 or 500 mg/kg/day) were that there are Sertoli cell vacuoles and swollen germ cell mitochondria, followed by (or concurrent with) a breakdown of the membrane between the Sertoli cell and the pachytene spermatocyte in a spermatogenic stage-specific manner. This is followed quickly (within hours) by the death of (probably those) pachytene spermatocytes (Foster et al., 1983; Creasy and Foster, 1984; Creasy et al., 1986). See Fig. 21-23.

EGME is metabolized to active intermediates; these are methoxyacetaldehyde and methoxyacetic acid (MAA). MAA has a longer half-life and is generally considered the more important of the two. Treating animals with MAA produces identical testicular lesions as that of the parent compound.

The spermatogenic stage-specific effects are on the pachytene spermatocytes immediately before and during meiotic division (so, in stages XII to XIV of the cycle in the rat [see Fig. 21-24], and also the early pachytene spermatocytes, in stages I to IV of the cycle). Dead germ cells can be seen as soon as 12 hours after an effective dose. Continued treatment at a low effective dose seems to affect just the vulnerable cell types, so that a window of missing cells appears downstream from the stage of damage as time progresses (see Fig. 21-24). As with other testis toxicants, higher doses produce a more widespread lesion involving other cell types (Foster et al., 1987), producing immediate (<24 hours) and widespread damage and destruction within the tubules. The Sertoli cell vacuolization regresses after about 12 hours and is not a prominent feature of this lesion as it is with other chemicals like hexanedione, or some of the phthalate esters. Some weak evidence of involvement of this cell

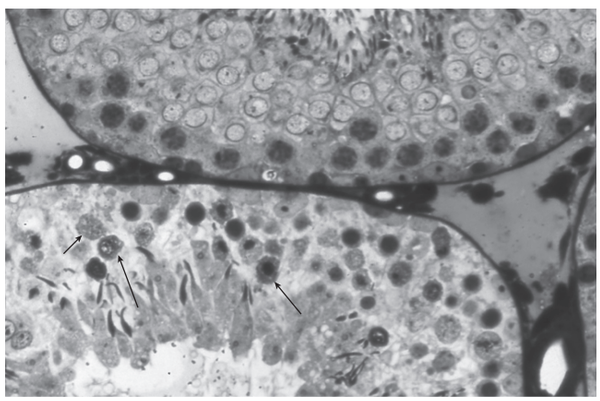


Figure 21-23. *Effect of ethylene glycol monomethyl ether (or its metabolite methoxyacetic acid) 24 hours after a single oral dose (100 mg/kg/d).* Note the damaged spermatocytes (arrows) in lower tubule compared to the upper normal tubule.

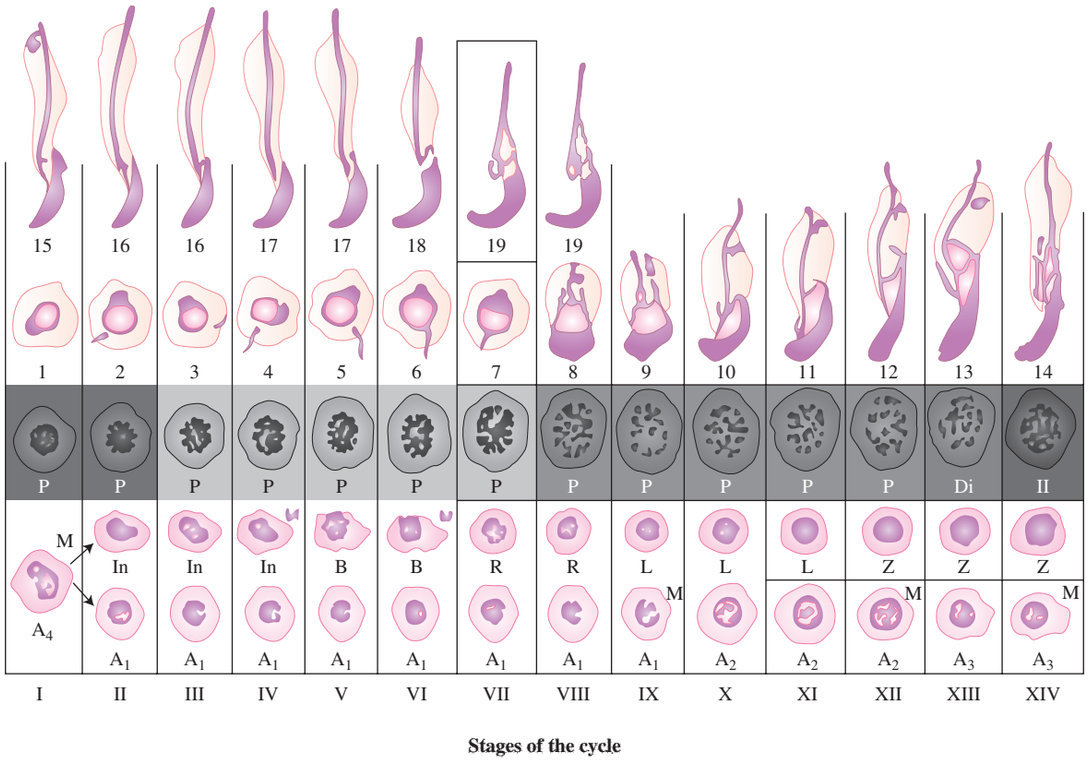


Figure 21-24. *Stage-specific lesions induced by methoxyacetic acid.* Intensity of shading indicates magnitude of effect on spermatocytes. (After Foster et al., 1987.)

type also comes from some in vitro data with isolated seminiferous tubules. In this system, molecules that inhibit transcellular calcium movement block the germ cell death from occurring, supporting a change in Sertoli cell communication to germ cells resulting in the rapid cell death after MAA treatment. Leydig cells do not seem to be pivotally involved in the pathogenesis. The lesion is not characteristic of a low-androgen testicular lesion, and reduced accessory sex organ weights are not a prominent feature associated with the early testicular pathology.

Decrements in fertility in the rat after acute treatment with EGME are entirely consistent with the primary effect on pachytene spermatocyte germ cells, the prominent period of infertility being approximately 5 to 6 weeks after dosing when the pachytene spermatocytes should have matured to become mature sperm capable of fertilization based on the known kinetics of the process (Chapin et al., 1985).

MATING BEHAVIOR IN THE RAT

Female rats are only sexually receptive on the day of proestrus, which is characterized by round nucleated and cornified epithelial cells in the vaginal lavage (Knobil and Neill, 1994). The period of receptivity begins at the onset of the dark phase of their light cycle, terminating later in the evening before the next stage of the cycle, defined as estrus, which is typically characterized by the display of a vaginal lavage of mostly cornified epithelial cells. Serum estradiol rises during proestrus and this is followed by initiation of an LH surge shortly after the onset of the dark phase of the light cycle, which in turn induces ovulation and a brief increase in progesterone. This is normally the only time during which the female is receptive to the male and the display of this behavior can be induced in ovariectomized female rats by a sequence of injections of estradiol for a couple of days prior to mating followed by progesterone administration a few hours before the mating trial is conducted, which must occur during the evening of the rat's diurnal cycle. Male and female rat mating behavior is sufficiently stereotyped that it can be easily quantified to assess the effects of toxicants on these behaviors (Gray et al., 1988b; Gray and Ostby, 1998).

During mating, the female rat displays proceptive behaviors like ear wiggling and darting to induce the male to mount, and when mounted the female is "receptive" displaying a lordosis posture characterized by a raised head and tail and fully arched back. The female maintains this posture for several seconds. In experienced male rats, the latency to the first mount is usually on a few seconds, but inexperienced males may take much longer. When the male rat mounts the lordosing female, he will display a single pelvic thrust that may or may not result in intromission of the penis into the vagina. In rats, there is a rapid dismount after this single pelvic thrust. Within a few seconds, the male mounts again and this series continues until the male ejaculates. Ejaculation is usually identifiable by a slow dismount, after which the male retires for several minutes; a period termed the postejaculatory interval (PIE). During this period, the male produces a 22 kHz vocalization, during which the female does not display proceptive behaviors (Porter et al., 2005). Following the PIE, the series repeats itself several times throughout the evening. At the beginning of the second series, the male dislodges the copulatory plug, formed from the seminal secretions. The copulatory plug normally fits close to the cervix, and it is necessary to facilitate sperm entry into the uterus. It is not unusual to find seven to eight copulatory plugs in the breeding cage the day after mating (Gray et al., 1989). If mating does not occur, then the brief rise in serum progesterone declines by the next day.

In spontaneously ovulating species (e.g., rodents), the endocrine events are comparable with those in the menstrual cycle. In the rabbit, the LH surge and ovulation is a neural reflex produced by copulation (Knobil and Neill, 1994).

Cervix

The mucosa of the uterine cervix does not undergo cyclic desquamation, but there are regular changes in the cervical mucus. Estrogen, which makes the mucus thinner and more alkaline, promotes the survival and transport of sperm. Progesterone makes the mucus thick, tenacious, and cellular. The mucus is thinnest at the time of ovulation and dries in an arborizing, fernlike pattern on a slide. After ovulation and during pregnancy, it becomes thick and fails to form the fern pattern. Disruptions of the cervix may be expressed as disorders of differentiation (including neoplasia), disturbed secretion, and incompetence. Exfoliative cytological (Papanicolaou's stain) and histological techniques are currently used to assess disorders of differentiation. Various synthetic steroids (e.g., oral contraceptives) can affect the extent and pattern of cervical mucus.

Vagina

Estrogen produces a growth and proliferation of vaginal epithelium. The layers of cells become cornified and can be readily identified in vaginal smears. Vaginal cornification has been used as an index for estrogens. Progesterone stimulation produces thick mucus and the epithelium proliferates, becoming infiltrated with leukocytes. The cyclic changes in the vaginal smear in rats are easily recognized. The changes in humans and other species are similar, but less apparent. Analysis of vaginal fluid or cytological studies of desquamated vaginal cells (quantitative cytochemistry) normally reflects ovarian function. However, administration of estrogenic toxicants can induce persistent vaginal cornification in both immature and adult ovariectomized females by acting directly on the cellular dynamics and cell cycling of the vaginal epithelium (Gray et al., 1988b, 1989). Vaginal sampling of cells and fluid might offer a reliable and easily available external monitor of internal function and dysfunction.

FERTILIZATION

Fertilization is the process whereby the genome from one generation is passed to the next to begin the development of a new organism. In mammals, the oocyte is surrounded by two layers: an outer layer of cumulus cells and an inner layer of extracellular matrix termed the zona pellucida (see the review by Hoodbhoy and Dean, 2004). To reach the oocyte, the sperm must penetrate both layers that require high motility, the release of sperm enzymes and the presence of proteins that will facilitate binding of the sperm to the oocyte. Moreover, once fertilization has occurred, mechanisms must be in place to prevent the binding of further sperm to the fertilized oocyte (the zygote). To facilitate these activities, sperm must be capacitated (Hunter and Rodriguez-Martinez, 2004) and the secretion of enzymes (hyaluronidases) allows the sperm to penetrate through the cumulus cells to the zona pellucida. This special extracellular matrix is composed of three glycoproteins and cell surface factors that cause the sperm to release the secretory enzymes present in the acrosome via binding to the specific carbohydrates present in this matrix. The release of these enzymes enables the sperm to penetrate through the zona pellucida to then bind and fuse with the oocyte plasma membrane through specialized proteins to release the genetic material into the oocyte as the male pronucleus, which eventually combines with the genetic material from the female to form the zygote. Once sperm fusion has occurred, a "zona block" is initiated to prevent any further sperm entering through the zona pellucida and fusing with the oocyte membrane. The precise mechanisms of how this occurs have not been fully elucidated (Hoodbhoy and Dean, 2004).

IMPLANTATION

Implantation is an intricately timed event that allows mammals to nourish and protect their young during early development and results from an intimate relationship between the developing embryo and the differentiating uterus (see the diagram in Fig. 21-25). Implantation can only occur when the embryo reaches the blastocyst stage and gains implantation competency and the uterus, through steroid hormone-dependent changes, attains a receptive state. This reciprocal interaction must occur between the blastocyst and uterus together with an increase in uterine vascular permeability at the site of blastocyst attachment. There are four stages that comprise early implantation in mammals: (1) apposition and adhesion of the blastocyst to the uterine lumen, (2) penetration of the epithelium, (3) decidualization of the stromal cells, and (4) trophoblastic invasion into the stromal vasculature. These four stages can vary in length and in precise order depending on the specific species studied and are further reviewed by Schäfer-Somi (2003), Aplin and Kimber (2004), Lee and DeMayo (2004), and Tranguch et al. (2005). The molecular understanding of these physiological events is far from complete, with data being generated mainly from studies in the mouse that have indicated through gene ablation in the uterus, a number of critical factors and cytokines involved in implantation; however, a number of these critical gene products (e.g., VEGF, BMP-2, and wnt-4) have also been shown to produce early embryonic lethality when knocked out. Another important aspect of the implantation process common to many species (and offering an experimental tool to study implantation) is the embryonic diapause (or delayed implantation) evolved as a strategy to ensure proper implantation timing depending on environmental conditions (see review by Lopes et al., 2004). The regulation of this phenomenon varies widely between species ranging from photoperiod through hormonal or nutritional influences.

PLACENTA

The placenta plays a key role in pregnancy, mediating exchanges between the mother and fetus and maternal tolerance of antigens produced by the fetus. There are a huge number of different placental types exhibited by eutherian mammals that exhibit differences in both structure and endocrinology, including significant difference between the major experimental animal species and humans (see reviews by Malassine et al., 2003; Enders and Carter, 2004 and Fig. 21-26). Humans and monkeys possess a hemochorial placenta. Pigs, horses, and donkeys have an epitheliochorial type of placenta, whereas sheep, goats, and cows have a syndesmochorial type of placenta. In laboratory animals (e.g., rat, rabbit, and guinea pig), the placenta is termed a hemoendothelial type. Among the various species, the number of maternal and fetal cell layers ranges from six (e.g., pig, horse) to a single one (e.g., rat, rabbit). Primates, including humans, have three layers of cells in the placenta that a substance must pass across.

Early in implantation the blastocyst comes in contact with the endometrium, and becomes surrounded by an outer layer or syncytiotrophoblast, a multinucleated mass of cells with no discernible boundaries, and an inner layer of individual cells, the

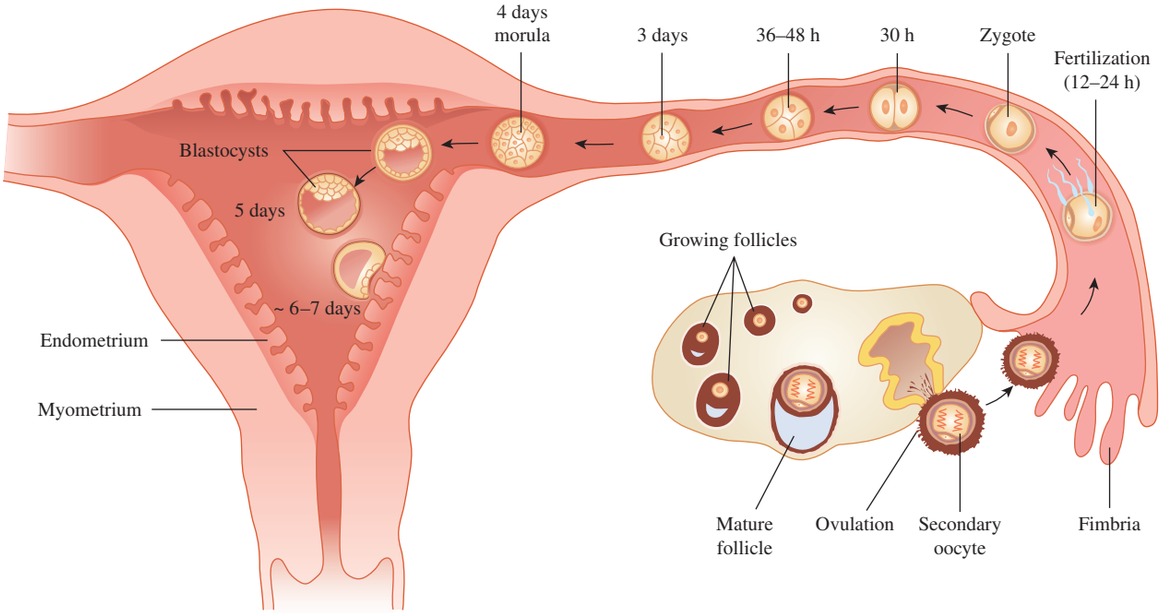


Figure 21-25. *Fertilization and implantation in the female reproductive tract.*

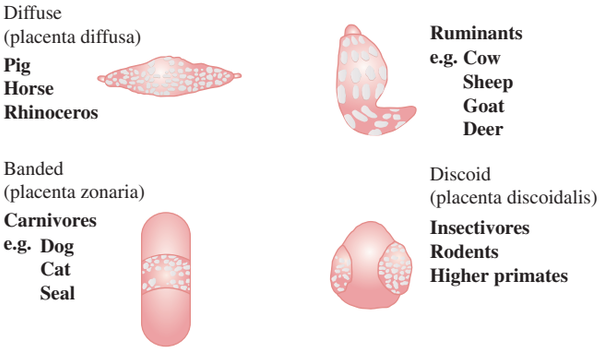


Figure 21-26. *Types of mammalian placentae.*

cytotrophoblast. The syncytiotrophoblast erodes the endometrium, and the blastocyst implants. Placental circulation is then established and trophoblastic function continues. The blastocysts of most mammalian species implant about day 6 or 7 following fertilization. At this stage, the differentiation of the embryonic and extraembryonic (trophoblastic) tissues is apparent.

Trophoblastic tissue differentiates into cytotrophoblast and syncytiotrophoblast cells. The syncytiotrophoblast cells produce chorionic gonadotropin, chorionic growth hormones, placental lactogen, estrogen, and progesterone, which are needed to achieve independence from the ovary in maintaining the pregnancy. Rapid proliferation of the cytotrophoblast serves to anchor the growing placenta to the maternal tissue.

The developing placenta consists of proliferating trophoblasts, which expand rapidly and infiltrate the maternal vascular channels. Shortly after implantation, the syncytiotrophoblast is bathed by maternal venous blood, which supplies nutrients and permits an exchange of gases. Histotrophic nutrition involves yolk sac circulation; hemotrophic nutrition involves the placenta. Placental circulation is established quite early in women and primates and relatively much later in rodents and rabbits. One of the major differences in endocrine placental function between humans and rodents is in the production and regulation of progesterone necessary for the maintenance of pregnancy. In the rodent, the corpus luteum in the ovary has to produce progesterone throughout gestation and is regulated initially by pituitary prolactin secretion and then around midway through gestation by placental lactogens produced by the trophoblast. Thus, hypophysectomy (to remove pituitary influences) in a mouse does not terminate pregnancy after GD 11. In humans, the pituitary gland is not required for the initiation and maintenance of pregnancy, with maintenance of the corpus luteum to produce progesterone dependent on the secretion of human chorionic gonadotropin (hCG) by the trophoblast. There is sufficient progesterone produced by the trophoblast after 8 weeks of gestation in humans to maintain pregnancy even in cases of ovariectomy.

Generally, the placenta is quite impermeable to chemicals/drugs with molecular weights of 1000 Da or more. Because most medications and xenobiotics have molecular weights of 500 Da or less, molecular size is rarely a factor in denying a drug's entrance across the placenta and into the embryo/fetus. Placental permeability to a chemical is affected by placental characteristics including thickness, surface area, carrier systems, and lipid-protein concentration of the membranes. The inherent characteristics of the chemical itself such as its degree of ionization, lipid solubility, protein binding, and molecular size also affect its transport across the placenta. Various transporter families have also been identified in the placenta and can contribute to the passage of xenobiotics across the placenta (see Chap. 5 in this book).

PREGNANCY

In the female rat, during mating repeated cervical stimulation by the penis induces a surge of prolactin from the posterior pituitary within 20 minutes followed by the onset of twice-daily prolactin surges, which persist for an 8- to 13-day period. During this

period, prolactin "rescues" the corpora lutea preventing regression. If sperm transport, fertilization, or implantation is blocked, then the mated female is "pseudopregnant" for about 10 to 13 days (Swingle et al., 1951). During days 6 to 10 of pregnancy, sustained ovarian luteal function also requires pituitary LH. Pregnancy loss, that is full-litter resorption, can be induced at this stage of pregnancy by inhibition of pituitary LH secretion, which in turn causes a reduction in serum progesterone levels (Bielmeier et al., 2004).

Mid-pregnancy of the rat is characterized by hypertrophic development of the corpus luteum, which is dependent upon transition from the early LH and prolactin-dependent phase of pregnancy to one that involves the secretion of hormones by the feto-placental unit (rCG and rPL) that induce rapid growth and differentiation of the corpora lutea, an increase in progesterone synthesis and a doubling in serum progesterone. Doody et al. (1991) examined the levels of expression of mRNA species encoding cholesterol side-chain cleavage cytochrome P-450 (P-450scc), 17α-hydroxylase cytochrome P-450 (P-450 17α), aromatase cytochrome P-450 (P-450AROM), and 3β-hydroxysteroid dehydrogenase (3 β-HSD) in rat ovaries during pregnancy. Expression of P-450scc mRNA is at low, but detectable levels until day 14, thereafter expression increased to high levels (day 14 to 21 of gestation). Levels of P-450 17α mRNA on day 10 of gestation were low and decreased further on days 14 and 17. Expression of 3β-HSD was decreased on day 10, but on days 14, 17, and 21 of gestation high mRNA levels were detectable. Ovarian expression of the three P-450AROM species is also dramatically increased between days 14 and 17 of pregnancy, but declines by day 21.

Because the transition from early to mid-pregnancy in the rat requires hormones from the feto-placental unit, if implantation or uterine decidualization is blocked by a chemical, then the female would resume her estrous cycles and the corpora lutea would regress (Knobil and Neill, 1994). Chemicals that induce whole-litter loss at mid-to-late pregnancy may cause abortions in some of the females, whereas others fail to deliver and appear pregnant for an unusually long period of time. Effects on female fertility due to disruption of hormones during pregnancy may be difficult to detect in standard testing studies if females are mated to similarly treated males or if the chemical affects the reproductive system of both sexes. For example, with chronic di-*n*-butyl phthalate (DBP) administration at high-dose levels induces whole litter loss at mid-pregnancy through reductions in ovarian progesterone production on GD 13. However, ovarian weight is not altered at GD 13 and the onset of female puberty, estrous cyclicity, and mating also were unaffected. This is in contrast to the robust reductions in testis size and histology, sex accessory gland size, and sperm counts in pubertal males treated with DBP at 500 or 1000 mg/kg/day, treated F_0 females only display gross morphological reproductive alterations and reductions in uterine weights during mid-pregnancy. Hence, when female-mediated infertility is affected in this manner, it may only be apparent when treated females are mated to untreated males. Chemicals that reduce serum progesterone production during pregnancy by inhibiting progesterone synthesis also may not alter the estrous cycles in the rat as the rat estrous cycle does not have a functional luteal phase, unlike humans.

Many abortifacients induce pregnancy loss by reducing progesterone levels in the rat. Generally, reducing mid-pregnancy progesterone levels by half or more is sufficient to terminate pregnancy (Carnathan et al., 1987). For example, treating pregnant rats with a prostaglandin F2alpha analogue ICI 81008, which reduces serum progesterone levels during pregnancy, induces full-litter loss in 40% of 300 dams, while 25% had reduced litter sizes and 35% had normal litters (Warnock and Csapo, 1975). In comparison with the 107 control dams, all treated rats with full-litter loss had a drastic reduction in mid-pregnancy plasma progesterone levels. In contrast, those animals with only a partly resorbed litters only had a moderate reduction in progesterone until day 16. In contrast to progesterone, there was no correlation between plasma estradiol-17β levels and the consequences of treatment on pregnancy.

Corpora lutea function during pregnancy also may involve androgens and insulin-like 3 peptide hormone, in concert with lactogenic hormones. Whereas some of the androgenic actions are mediated by conversion of androgens to estrogens, pure androgenic effects also have been implicated (Goyeneche et al., 2002). AR mRNA and protein were expressed throughout gestation in the rat corpus luteum as reported by Goyeneche et al. (2002). In addition, they found that androstenedione, the main circulating androgen in pregnant rats, opposed luteal regression, reduced the number of cells undergoing apoptosis, and enhanced the levels of circulating progesterone.

PARTURITION

Parturition is a complex process involving fetal, placental, and maternal signals, and the precise molecular events controlling this physiological process are not clear. Parturition is best to be thought of as a release from the inhibitory effects of pregnancy on the myometrium of the uterus rather than an active process, although the timing and order of the precise events is an active process. For most mammals, the uterus is held in a quiescent state by high levels of progesterone and it is the decrease of progesterone that provides the trigger for parturition. In humans, this does not seem to be the case (i.e., levels of progesterone do not drop). Some authors have postulated that progesterone receptor inactivity does appear to be related to onset of labor and that local metabolism of progesterone in the cervix and uterus produces a localized decrease in progesterone that initiates labor. The fetus also directly contributes to the onset of parturition by activation of its HPA axis producing increased levels of cortisol. This in turn directly upregulates steroidogenic enzymes (especially CYP 17) in the fetus and placenta, which in turn leads to changes in prostaglandin (particularly PGF2α) and oxytocin production to induce uterine contractions together with the increased activity of cytokines (including NF-κB and IL-1β). In humans, there is no CYP 17 in the placenta, which may be related to the lack of change of progesterone (see reviews by Challis et al., 2005; Mendelson and Condon, 2005; Snegovskikh et al., 2006).

LACTATION

The endocrine control of lactation is one of the most complex physiological mechanisms of human parturition. Mammogenesis, lactogenesis, galactopoiesis, and galactokinesis are all essential to assure proper lactation. Prolactin is the key hormone of lactation and seems to be the single most important galactopoietic (milk synthesis) hormone. Oxytocin, serotonin, opioids, histamine, substance P, and arginine–leucine modulate prolactin release by means of an autocrine/paracrine mechanism, whereas estrogen and progesterone hormones can act at the hypothalamic and adenohypophysial levels. Human placental lactogen and growth factors play an essential role to assure successful lactation during pregnancy, with oxytocin being the most powerful galactokinetic (milk ejection) hormone (Buhimschi, 2004).

SENESCENCE

Reproductive senescence is usually preceded by a dysregulation of the HPG axis. This dysregulation leads to alterations in serum HPG

hormones, accompanied by an upregulation in GnRH, LH, and activin activities and a decrease in steroids in the brain. Receptors for these hormones in the brain are intimately involved in cell proliferation and differentiation in growth and development. In females, reproductive senescence is associated with a transition from regular to irregular estrus (menstrual) cycles leading to acyclicity and ultimately a loss of fertility. GnRH neurons in the brain are affected morphologically and there are changes in GnRH neurosecretion with changes in pulsatility and preovulatory release that are causal in producing acyclicity. Perinatal exposure to toxicants with estrogenic activity can defeminize the HPG axis such that the females are acyclic and infertile while less affected females display the "delayed anovulatory syndrome" and become anovulatory and acyclic at an early age (Gray et al., 1989).

In males, a decrease in androgen is noted in around 20% of fit 60-year-old men, but the value of androgen supplementation is not clear with regard to reproductive senescence. Dysregulation of the HPG axis is also found in aged male rats, with elevations in FSH and LH and decreased testosterone normally found. For reviews on reproductive senescence and possible associations with cognitive function, see the studies of Atwood et al. (2005), Keefe et al. (2006), and Yin and Gore (2006).

ENDOCRINE DISRUPTION (INCLUDING SCREENING AND PUBERTY)

Currently, the potential effects of "endocrine disrupting chemicals" (EDCs) on human health and the proven effects of EDCs on wildlife are a major focus among the scientific community. In 1996, the USEPA was given a mandate under the Food Quality Protection Act and Safe Drinking Water Act to develop test protocols to screen for endocrine effects. The initial impetus for these actions arose from a work session in 1991 on "Chemically Induced Alterations in Sexual Development: The Wildlife/Human Connection" (Colborn, 1994). Recent findings have contributed to these concerns; for example, it has been suggested that in utero exposure to environmental estrogens, antiandrogens or chemicals like phthalates, or 2,3,7,8-TCDD could be responsible for the reported 50% decline in sperm counts in some areas and the apparent increase in cryptorchid testes (Toppari et al., 2001), testicular cancer (Skakkebaek, 2002), and hypospadias (Paulozzi et al., 1997; Canning, 1999; Aho et al., 2000; Toppari et al., 2001; Pierik et al., 2002; Porter et al., 2005; Nassar et al., 2007). The differences in sperm counts between regions are so large that they cannot be explained by methodological biases and "environmental effects are entirely plausible" (Jorgensen et al., 2001, 2002; Giwercman et al., 2006). Indeed, it has been proposed (Skakkebaek, 2002) that the secular deficits in these human reproductive endpoints could be plausibly linked and have an origin in fetal life as the testes are developing. These authors proposed the term testicular dysgenesis syndrome (see Fig. 21-27) to explain the interrelationship of these findings.

Phthalate ester (a group of chemicals used as plasticizers and solvents) exposures have been associated with reduced AGD in boys and lower testosterone levels in men. In females, exposure to EDCs during development could contribute to earlier age at puberty and to increased incidences of endometriosis and breast cancer, for example. The original focus for the discussion of "endocrine disrupters" was research on toxicants reported to possess estrogenic activity, with little consideration given to other mechanisms of endocrine toxicity; mechanisms that, in fact, may be of equal or greater concern. In addition, there has been a great deal of misinformation communicated on issues concerning endocrine disrupters; for example, nonestrogenic chemicals (i.e., phthalates and *p,p′*-DDE) are repeatedly reported to be estrogenic. There is a lack of appreciation for the fact that many endocrine disrupters (i.e., TCDD, EE) are very potent reproductive toxicants. In addition, there has been a tendency to dismiss the wildlife data as correlative, ignoring examples of clear, cause and effect relationships between chemical exposure and reproductive alterations (e.g., DDT metabolite effects on birds, PCB effects on fish, and environmental estrogen effects on domestic animals). There is a lack of recognition that subtle, low-dose reproductive effects seen in laboratory studies with endocrine disrupters will be difficult, if not impossible, to detect in typical epidemiological studies because of high variability normally seen in human reproductive function (e.g., time to fertility, fecundity, and sperm measures), the delayed appearance of the reproductive lesions and a lack of high quality exposure data. There is also a lack of appreciation for the complexity of the multiple mechanisms by which a single chemical can alter the endocrine

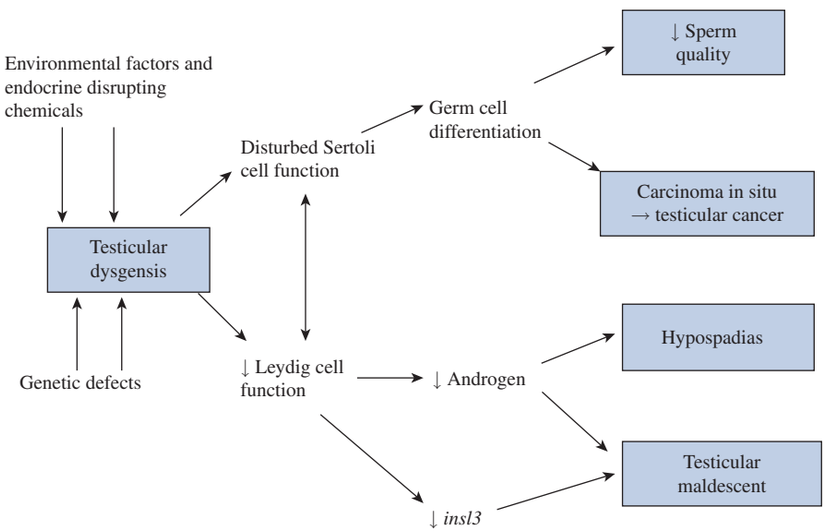


Figure 21-27. *Testicular dysgenesis syndrome.* The hypothesis that events occurring during the development of the testis in fetal life can be related to the secular increases in a number of human reproductive disorders. (Data from Skakkebaek et al., 2002.)

milieu, to say nothing of the complex endocrine alterations induced by mixtures of chemicals.

We are now not only concerned about pesticides and other toxic substances in the environment, but the issue has broadened considerably due to the growing awareness that the list of EDCs present in the environment from human activities includes potent pharmaceutical products and phytosterols. Among these drugs are estrogens, antibiotics, β-blockers, antiepileptics, and lipid-regulating agents. One study reported that the pharmaceuticals found as contaminants of aquatic systems included 36 of 55 pharmaceuticals and five of nine metabolites measured including antiepileptic drugs (Kolpin et al., 1998, 2002, 2004).

In the area of wildlife toxicology and ecosystem health, it is apparent that clear-cut cause and effect relationships exist between exposure to EDCs and adverse effects in several vertebrate classes from fish to mammals. One challenge in interspecies extrapolation is to link laboratory mechanistic studies with EDCs to both individual and population effects in the field.

Are Nonmonotonic Dose–Response Curves (NMDRs) Common After Estrogen or Androgen Signaling Pathway Disruption?

Reports of U-shaped (nonmonotonic), ultra-low-dose effects and nonthreshold effects for EDCs are challenging some of the basic assumptions of risk assessment for noncancer endpoints. While the focus of this debate has centered on the low-dose effects of bisphenol A—a monomer for polycarbonate plastics (Nagel et al., 1997; vom Saal et al., 1998, 2005; Ashby et al., 1999; Owens and Chaney, 2005; Welshons et al., 2006), well-documented U-shaped dose–response curves are known from many other in vitro and some in vivo studies. For example, administration of testosterone produces a well-characterized and reproducible U-shaped dose–response for spermatogenesis (Ewing et al., 1977, 1979, 1981; Robaire et al., 1979) in the intact adult male rat. In vitro studies show that such responses do not always involve multiple mechanisms of action. Several AR ligands, antagonists at low-to-moderate concentrations, became AR agonists at high concentrations. Furthermore, the basic tenet of toxicology from Paracelsus (1564) that "dose alone determines the poison" is too limited for some EDCs because the timing of exposure dictates not only the effect, but also whether the effects are adverse or beneficial. Even when administered during adult life, drugs with EDC-activity can simultaneously have a beneficial effect on one tissue and an adverse effect on another.

Systematic reviews of this issue by the USEPA (EPA, 2013), the European Food Safety Authority (Beausoleil et al., 2016), Gray (Gray and Foster, 2013), Parrott et al. (Parrott et al., 2017), and Coady et al. (Coady et al., 2016) have failed to find a significant body of evidence consistent with NMDRs for the estrogen or androgen pathways leading to toxicity.

In a review of NMDRs for chemicals that induce reproductive effects in males via disruption of the estrogen and androgen signaling pathways, a number of questions were asked related to:

- What is the shape of the dose–response curve over a broad range of doses?
- What is the sensitivity of in vivo endpoints to low doses of chemicals that disrupt A and E signaling pathways?
- If NMDR responses were detected, were these adverse effects and did they occur in the low-dose region of the dose–response curve?
- What is the potential impact of LNT or NMDRC responses on chemical screening and testing for E and A disruption?

Studies were selected for review based on a number of criteria including the measurement of multiple endpoints; that these studies should be reproductive or multigenerational, preferably via the oral route; covered a broad dose range and included six or more dosage levels. Peer-reviewed publications, and regulatory Agency and NTP documents (and large supplemental files), were surveyed. In total, 200 in vivo studies were selected, of which >70 had six or more dose levels roughly equally divided between the androgen and estrogen signaling pathways.

In addition, several research groups have executed studies with male reproductive toxicants to specifically evaluate the shape of the dose–response over a broad dose range, including low-dose levels with endpoints measured across several levels of biological organization (from mRNA for genes to adverse in vivo outcomes). The database included robust dose–response data for chemicals from studies with laboratory animals with several different modes of action including:

- Androgen receptor antagonists: Flutamide (McIntyre et al., 2001; Miyata et al., 2002; Rouquié et al., 2009; Ludwig et al., 2011; Fussell et al., 2015; Sarrabay et al., 2015) and vinclozolin (Schneider et al., 2017).
- Inhibitor of DHT synthesis: Finasteride (Clark et al., 1990; Clark et al., 1993; Bowman et al., 2003).
- Inhibitors of steroid synthesis: Prochloraz (Melching-Kollmuss et al., 2017).
- Di-ortho phthalate esters (Blystone et al., 2010; Mylchreest et al., 1998, 1999, 2000; Andrade et al., 2006a, 2006b).
- Estrogenic drugs: Estradiol (Biegel et al., 1998; Tyl et al., 2008) and ethinyl estradiol (Delclos et al., 2009).
- Phytoestrogens: Genistein (Delclos et al., 2001; Delclos and Newbold, 2007).

Some of the most robust studies in the peer-reviewed literature on the dose-related effects of androgens have been reported in humans, including a wide variety of endpoints in males of different ages (Storer et al., 2003; Woodhouse et al., 2004; Bhasin et al., 2005; Calof et al., 2005; Gray et al., 2005; Coviello et al., 2006; Herbst et al., 2006; Mohr et al., 2006; Sinha-Hikim et al., 2006; Bhasin et al., 2007). NMDR were not evident in these investigations although significant age-related toxicity was noted for adverse effects of high-dose testosterone replacement.

In conclusion, reviews of the scientific literature on NMDR and male reproductive toxicity to date indicate the following: (1) NMDRs are biologically plausible and occur frequently in vitro, but these often occur in vitro at high concentrations of estrogens or androgens that are not relevant in vivo. (2) It appears that NMDRCs are more common (a) in short- versus long-term exposures (Ankley and Villeneuve, 2015) and (b) with upstream, mechanistic events versus downstream phenotypic effects. (3) The shape of the dose–response curve can be affected by several factors, including (but not limited to) (a) life stage, (b) route of exposure, (c) target tissue, (d) species differences in E and A pathways or ADME, (e) gut microbiome, (f) concurrent exposure to other chemicals or nonchemical stressors, and (g) background contaminant levels of ubiquitous chemicals. (4) A few adverse effects of male reproductive toxicants are non-monotonic, but often other effects displaying monotonic responses occur at lower dosage levels. (5) A number of robust multigenerational studies of estrogens and antiandrogens have been executed and NMDRs were uncommon at low dosage levels. (6) Multigenerational test guidelines can be enhanced on a case-by-case basis to improve the sensitivity to low-dose effects (i.e., it is difficult to establish non-monotonicity with only three dose levels and a control).

Known Effects of EDCs in Humans and Animals

The list of chemicals that are known to affect humans, domestic animals, and/or wildlife via functional developmental toxicity or endocrine mechanisms includes 2,3,7,8-TCDD, PCBs and polychlorinated dibenzofurans (PCDFs), methylmercury, EE, alkylphenols, plant sterols, fungal estrogens, androgens, chlordecone, DBCP, *o,p′*-DDD (Mitotane), *o,p′*-DDT, and *p,p′*-DDE (Gray et al., 2001; Gray, 2002). In addition to these xenobiotics, over 30 different drugs taken during pregnancy have been found to alter human development as a consequence of endocrine disruption. These drugs are not limited to estrogens like diethylstilbestrol (DES). EDCs are known to alter human development via several mechanisms besides the estrogen receptor (ER): this includes binding to the AR, or retinoic acid (RAR, RXR) receptors, and by inhibition of steroidogenic enzymes or the synthesis of thyroid hormones. Findings on the effects of background levels of PCBs on the neurobehavioral development of the child have contributed to the concerns about the effects of EDCs on human health via alteration of hormone function including the thyroid (Brouwer et al., 1999; Tan and Zoeller, 2007). In summary, there are several hundred epidemiology studies showing associations among EDCs and adverse human health effects; however, none of these have been sufficiently studied to claim that "cause and effect" exists.

Effects of Drugs on Human Sexual Differentiation In humans (Herbst, 1973; Bardin and Catterall, 1981; Ohyama, 2004; Carmichael et al., 2005) and laboratory rodents (Foster et al., 2001; Fisher, 2004; Gray et al., 2006), exposure to hormonally active chemicals during sex differentiation can produce pseudohermaphroditism. Androgenic drugs such as danazol and methyltestosterone can masculinize human females (i.e., "female pseudohermaphroditism") (Rosa, 1984; Kingsbury, 1985; Schardein, 2000). Laboratory studies demonstrate that these chemicals alter sex differentiation in rodents as well (Duck and Katayama, 1981). The drug aminoglutethimide, which alters steroid hormone synthesis in a manner identical to many fungicides, also masculinizes human females following in utero exposure (LeMaire et al., 1972; Schardein, 2000).

DES provides an unfortunate example of how in utero exposure to a potent endocrine disrupter with estrogenic activity can alter reproductive development in humans (Herbst, 1987; Shaffer, 2000; Veurink et al., 2005). Although a few cases of masculinized females were noted in the late 1950s, most of the effects of DES were not apparent until after the children attained puberty. Transplacental exposure of the developing fetus to DES causes clear cell adenocarcinoma of the vagina, as well as gross structural abnormalities of the cervix, uterus, and fallopian tube. These women are more likely to have an adverse pregnancy outcome, including spontaneous abortions, ectopic pregnancies, and premature delivery (DeCherney et al., 1981). Some of the pathological effects that develop in males following fetal DES exposure appear to result from an inhibition of androgen action or synthesis (underdevelopment or absence of the vas deferens, epididymis, and seminal vesicles) and anti-Müllerian duct factor (persistence of the Müllerian ducts) (Niculescu, 1985). DES may also have caused epididymal cysts, hypotrophic testes, and infertility in males.

Effects of Plant and Fungal Products in Animals and Humans The phytoestrogens and fungal mycotoxins are naturally occurring and widespread in nature. Farnsworth et al. (1975) listed over 400 species of plants that contain potentially estrogenic isoflavonoids or coumestans or were suspected of being estrogenic based on biological grounds (Farnsworth, 1990). Plants contain many other compounds in addition to estrogens that can affect reproductive performance, such as the purported antiandrogenic activity in the oil from saw palmetto (Gordon and Shaughnessy, 2003).

Although most naturally occurring environmental estrogens are relatively inactive, when compared to steroidal estrogens or DES, the phytoestrogen miroestrol is almost as potent as estradiol in vitro and even more potent than estradiol when administered orally (Cain, 1960; Matsumura et al., 2005). In addition, many plant estrogens occur in such high concentrations that they induce reproductive alterations in domestic animals (Adams and Sanders, 1988; Adams, 1990). "Clover disease," which is characterized by dystocia, prolapse of the uterus, and infertility, is observed in sheep grazed on highly estrogenic clover pastures. Permanent infertility (defeminization) can be produced in ewes by much lower amounts of estrogen over a longer time period than are needed to produce "clover disease." In domestic animals, feeds contaminated with the zearalenone-producing fungus (*Fusarium* spp.) induce adverse reproductive effects in a wide variety of domestic animals, including impaired fertility in cows and hyperestrogenism in swine and turkeys (Kuiper-Goodman et al., 1987).

Effects of Organochlorine Compounds in Humans In addition to drugs and plant substances, several pesticides and toxic substances has been shown to alter human reproductive function (listed above). An accidental high-dose in utero exposure to PCBs and PCDFs has been associated with reproductive alterations in boys, increased stillbirths, low birth weight, malformations, and IQ and behavioral deficits (Jacobson and Jacobson, 1997). In addition to the effects associated with this inadvertent exposure, relatively subtle adverse effects were seen in infants and children exposed to relatively low levels of PCBs and PCDFs. The authors reported that the lowest observed adverse effect levels (LOAELs) for developmental neurobehavioral and reproductive endpoints are currently within the range of current human body burdens based on toxicity equivalency factors.

One metabolite of DDT was found to alter adrenal function with sufficient potency to be used as a drug to reduce adrenal androgen production (Knappe et al., 1997). *o,p′*-DDD (mitotane) is used to treat adrenal steroid hypersecretion associated with adrenal tumors. In addition to this usage, lower doses of mitotane restored menstruation in women with spanomenorrhea associated with hypertrichosis.

Occupational Exposures Occupational exposure to pesticides and other toxic substances (i.e., chlordecone and DBCP) in the workplace have been associated with reduced fertility, lowered sperm counts, and/or endocrine alterations in male workers. Workers in Hopewell, VA, exposed to high levels of chlordecone, an estrogenic (Hammond et al., 1979) and neurotoxic (Landrigan et al., 1980) organochlorine pesticide, displayed obvious signs of intoxication, which included severe neurotoxicity and abnormal testicular function (Epstein, 1978; Reich and Spong, 1983). As this cohort was not followed, it is not known if the effects of chlordecone were completely reversible.

It is surprising to learn that occupational exposures to potential EDCs at effective concentrations apparently have not been entirely eliminated from the workplace. A series of publications from about 1990 to 1996 presented documentation of sexual impotence in chemical factory workers exposed to a DES-like stilbene derivative. National Institute of Occupational Safety and Health conducted two studies in response to complaints of impotence and decreased libido among male workers involved in the manufacture of 4,4′-diaminostilbene-2,2′-disulfonic acid (DAS), a key ingredient in the synthesis of dyes and fluorescent whitening agents (Hammond et al., 1987; Grajewski et al., 1996; Quinn et al., 1990;

Smith and Quinn, 1992; Whelan et al., 1996). Both current and former workers had lower serum testosterone levels (Grajewski et al., 1996) and reduced libido (Whelan et al., 1996) as compared to control workers. In addition, duration of employment was negatively correlated with testosterone levels. These studies replicated the observations reported by Quinn et al. (1990) who reported low levels of serum testosterone and problems with impotence in male workers. In a uterotrophic assay, while DAS was only weakly to negligibly estrogenic (Smith and Quinn, 1992), a single sc 30 mg/kg dose of 4-nitrotoluene, a precursor of DAS, increased uterine weights without producing overt toxicity. Samples of DAS from the workplace displayed estrogenic activity.

Environmental Androgens

Androgenic activity has been detected in several complex environmental mixtures. There are pulp and paper mill effluents (PME) from Florida, the Baltic Sea, the Great Lakes, and New Zealand (Parks et al., 2001; Larsson and Förlin, 2002; Ellis et al., 2003). PME effluents from sites on the Fenholloway River in Florida include a chemical mixture that binds to the AR and induces androgen-dependent gene expression in vitro. This mode of action is consistent with the masculinized female mosquitofish (*Gambusia holbrooki*) collected from contaminated sites on the river. Male-biased sex ratios of fish embryos have been reported near a pulp mill in broods of eelpout (*Zoarces viviparus*) in the vicinity of a large kraft pulp mill on the Swedish Baltic coast, suggesting that masculinizing compounds in the effluent were affecting gonadal differentiation and promoting skewed sex ratios. Efforts to date have not conclusively identified chemicals in PME responsible for androgenic activity (Durhan et al., 2002).

Effluents from beef cattle concentrated animal feeding operations (CAFO) from Nebraska and Ohio have been shown to display androgenicity. Orlando et al. (2004) found that CAFO discharge at a site in Nebraska exhibited androgenic activity and found that fish (fathead minnow; *Pimephales promelas*) collected at the site displayed small gonads as compared to fish from a reference site. Durhan et al. (2006) detected the synthetic androgens 17α- and 17β-trenbolone in several water samples from a beef CAFO in Ohio, where trenbolone acetate implants were used to stimulate weight gain and the samples collected from a direct discharge from the feedlot displayed significant androgenic activity in vitro. Complementary laboratory studies revealed that both trenbolone isomers were androgenic in fathead minnows (Ankley et al., 2003) and rats (Wilson et al., 2002). When administered in utero, 17β-trenbolone (TB) masculinized female rat offspring, increased AGD and caused vaginal agenesis and induced male sex accessory tissues in females (Wolf et al., 2002). TB binds to AR with high affinity and induces androgen-dependent gene expression in vitro at concentrations similar to those for DHT (Wilson et al., 2004).

In vivo androgenicity testing using the Hershberger assay shows that TB is as potent as testosterone propionate (TP) in inducing growth of the androgen-dependent levator ani–bulbocavernosus muscles (Wilson et al., 2002) being about 70 times more potent when administered sc versus orally.

Environmental Antiandrogens

Fungicides Vinclozolin and procymidone are two members of the dicarboximide fungicide class that act as AR antagonists (Kelce et al., 1994; Ostby et al., 1999). These pesticides, or their metabolites, competitively inhibit the binding of androgens to AR that leads to an inhibition of androgen-dependent gene expression in vitro and in vivo (Kelce et al., 1997). Peripubertal administration of antiandrogens can alter the onset of pubertal landmarks in the male rat (Monosson et al., 1999). Vinclozolin delays pubertal maturation, and reduces sex accessory gland and epididymal growth and increases serum LH, and testosterone and 5-androstane 3,17-diol levels. In a Hershberger assay using castrated immature testosterone-treated male rats, vinclozolin and procymidone alone or in combination inhibited testosterone-induced growth of androgen-dependent tissues (ventral prostate, seminal vesicles, and levator ani–bulbocavernosus muscles) in a dose-additive fashion (Gray et al., 2001).

Administration of vinclozolin during sexual differentiation demasculinizes and feminizes the male rat offspring such that treated males display female-like AGD at birth, retained nipples, hypospadias, suprainguinal ectopic testes, a blind vaginal pouch, and small to absent sex accessory glands (Gray et al., 1994). In contrast to the phthalates and linuron (see below), even at high dosages (200 mg/kg/day), epididymal hypoplasia was rare and no cases of gubernacular agenesis were noted. At low doses, vinclozolin reduces neonatal AGD and increases the incidence of retained nipples/areolae in infant male rats. In adult life, ventral prostate weight is permanently reduced and male offspring displays permanent female-like nipples (Gray et al., 1999a). Treatment at 50 and 100 mg/kg/day induces hypospadias and other reproductive tract malformations. The most sensitive period of development to the disruptive effects of vinclozolin is GD 16 to 17 with less severe effects seen in males exposed to vinclozolin on GD 14 to 15 and GD 18 to 19. In addition, Hotchkiss et al. (2003) demonstrated that neonatal injection of vinclozolin at 200 mg/kg/day demasculinized aggressive play behavior in male rats at 35 days of age, indicating that CNS sexual differentiation was altered in an antiandrogenic manner.

When procymidone is administered from day 14 of pregnancy to day 3 after birth, AGD is shortened in male pups, and the males display retained nipples, hypospadias, cryptorchidism, cleft phallus, a vaginal pouch, and reduced sex accessory gland size (Ostby et al., 1999). Procymidone also induces fibrosis, cellular infiltration, and epithelial hyperplasia in the dorsolateral and ventral prostatic and seminal vesicular tissues in the offspring when examined as adults.

Prochloraz is a fungicide that disrupts reproductive development and function by several modes of action (Vinggaard et al., 2000, 2002; Noriega et al., 2005). Prochloraz inhibits the steroidogenic enzymes 17,20-lyase and aromatase and it is an AR antagonist. Wilson et al. (2004) found that prenatal prochloraz reduces fetal testis testosterone and increases progesterone production 10-fold on GD 18 without affecting Leydig cell insl3 mRNA levels. Prenatal prochloraz treatment delayed parturition and altered reproductive development in the male offspring in a dose-related manner (Noriega et al., 2005). Treated males displayed reduced AGD and female-like areolas and high-dose males displayed hypospadias, but the epididymides and gubernacular ligaments were relatively unaffected.

Linuron (Herbicide) The herbicide linuron is an AR antagonist. It binds rat and human AR and inhibits DHT-hAR induced gene expression in vitro (Lambright et al., 2000; McIntyre et al., 2000, 2002). In vivo treatment with linuron elicits a positive response in the Hershberger assay for antiandrogens (Lambright et al., 2000). In utero linuron exposure produces dramatic effects in male rat offspring. More than half of the males exposed to 100 mg linuron/kg/day (GD 14 to 18) display epididymal and testicular abnormalities (Gray et al., 1999b) with effects seen at dosage as low as 12.5 mg/kg/day (exposed from GD 10 to 22) (McIntyre et al., 2000). In contrast to the effects of vinclozolin and procymidone, malformed

external genitalia and undescended testes were rarely displayed by linuron-exposed males. Interestingly, the syndrome of effects for linuron is atypical of an AR antagonist and more closely resembles those seen with in utero to phthalates, which inhibit fetal Leydig cell insl3 hormone levels. Wilson et al. (2004) found that fetal testosterone production is significantly reduced in linuron-treated fetal males demonstrating that linuron is antiandrogenic via dual mechanisms of action.

_p,p′_-DDE (Pesticide Metabolite) Kelce et al. (1995, 1997) found that *p,p′*-DDE displayed AR antagonism both in vivo and in vitro. In vitro, *p,p′*-DDE binds to the AR and inhibits androgen-dependent gene expression. In vivo, *p,p′*-DDE delays pubertal development in male rats by about five days at 100 mg/kg/day and inhibits androgen-stimulated tissue growth in the Hershberger assay, which uses castrated immature androgen-treated male rats (Table 21-1). *p,p′*-DDE administered to Long-Evans Hooded and Sprague–Dawley male rats in utero reduces AGD, induces nipples, and permanently reduces androgen-dependent organ weights (Gray et al., 1999b).

Phthalates (Plasticizers and Solvents) The phthalates represent a class of high production volume chemicals that alter reproductive development. While a few in vitro studies suggested that some of the phthalates are estrogenic, DBP injections do not induce an uterotrophic response or estrogen-dependent sex behavior (lordosis) in the ovariectomized adult female rats (Gray, 1998). Likewise, oral DBP or diethylhexyl phthalate (DEHP) treatments fail to accelerate VO or induce constant estrus in the intact female rats. In addition, neither the phthalate diesters nor their monoester metabolites appear to compete significantly with androgens for binding to AR at environmentally relevant concentrations (Parks et al., 2000; Foster et al., 2001; Stroheker et al., 2005). In utero, some phthalate esters alter the development of the male rat reproductive tract at relatively low dosages. Prenatal exposure to DBP, benzyl butyl phthalate (BBP), di-isononyl phthalate (DINP), and DEHP treatment cause a syndrome of effects, including underdevelopment and agenesis of the epididymis and other androgen-dependent tissues and testicular abnormalities (Gray et al., 2000; Foster et al., 2001). Among the antiandrogenic EDCs, the phthalates are unique in their ability to induce agenesis of the gubernacular cords, a tissue whose development is dependent upon the peptide hormone insulin-like peptide-3. Wilson et al. (2004) found that the phthalates reduced both insl3 mRNA and testosterone levels during sexual differentiation of the male rat.

When pregnant SD rats are dosed by gavage with DEHP from GD 8 to day 17 of lactation with 0, 11, 33, 100, or 300 mg/kg/day, in utero exposure induces a low incidence of abnormalities consistent with the "phthalate syndrome" in the 11, 33, and 100 mg/kg/day dose groups along with subtle reductions in reproductive organ weights. In the high-dose group, more than 25% of the males display testicular and/or epididymal abnormalities. Pubertal DEHP treatment alone is sufficient to delay puberty in Long Evans (LE) and SD rats due to lowered testosterone levels.

Prenatal exposure to DBP from day 10 to 22 of gestation produces effects nearly identical to those seen with DEHP, with effects occurring at dosage levels of 50 to 100 mg/kg/day (Mylchreest et al., 1999, 2000). When administered in 4-day periods of gestation (GD 8 to 11, 12 to 15, or 16 to 19), DBP at 500 mg/kg/day was most effective in altering sexual differentiation at GD 16 to 19 (Gray et al., 1999b). When Carruthers and Foster (2005) exposed SD rats to DBP at 500 mg/kg/day for 2-day periods (GD 14 and 15, 15 and 16, 16 and 17, 17 and 18, 18 and 19, or 19 and 20), they also found that the critical window for abnormal development is GD 16 to 18.

DBP also disrupts reproductive function in the rabbit. In rabbits exposed to 400 mg DBP/kg/day in utero (GD 15 to 29), male offspring exhibit reduced numbers of ejaculated sperm, testis weight, and accessory sex gland weight (Higuchi et al., 2003). Additionally, DBP caused a slight increase in histological alterations of the testis, a doubling of abnormal sperm and hypospadias, hypoplastic prostate, and cryptorchid testes with carcinoma in situ-like cells were present in 1/17 DBP-treated male rabbits.

Other studies have explored the structure–activity relationships for different phthalate esters to produce reductions in fetal testicular testosterone production (Furr et al., 2014) that confirmed and extended much of the earlier work indicated above and also included studies on some of the newer, replacement phthalates (which were largely negative in response). The role of PPARα in the response on fetal testosterone production was also explored with known potent ligands (e.g., WY-14643) that were also shown to be inactive. This added to some of the earlier literature (Hannas et al., 2012) that had cast doubt on the role of PPARα in the induction of the male reproductive effects of phthalates. Further studies with the most potent member of the class (di-*n*-pentyl) phthalate indicated the important predictive nature of testosterone production and fetal gene expression information with regard to the induction of subsequent postnatal reproductive tract malformations (Gray et al., 2016).

Environmental Estrogens

Methoxychlor (M) is an estrogenic pesticide that produces a variety of estrogen-like effects on the male and female rats. This pesticide requires metabolic activation in order to display full endocrine activity in vitro. The active metabolites of M bind ER and activate estrogen-dependent gene expression in vitro (Wilson et al., 2005) and in vivo in the female rat; M stimulates a uterotrophic response, accelerates VO and induces constant estrus, reduces ovarian weight lacking corpora lutea and infertility in the female rat (Gray et al., 1989; Chapin et al., 1997). Ovarian function also is altered by M exposure. In the ovariectomized female rat, M also induces estrogen-dependent reproductive and nonreproductive behaviors (Gray et al., 1988b) including female sex behaviors, running wheel activity, and food consumption. Unlike estradiol, M is as effective, or is more effective, when administered orally than when it is injected.

When given to the dam during pregnancy and lactation both male and female offspring are affected, with females being the more sensitive gender with effects ranging from VO at 5 mg/kg/day and above and infertility at 100 mg/kg/day and above. At 50 mg/kg/day F_1 females display irregular estrous cycles and reduced fecundity. F_1 male fertility is unaffected at dose levels up to 200 mg/kg/day, even though they do display permanent reductions in testis and other reproductive organ weights at 50 mg/kg/day and above.

EE is a synthetic derivative of estradiol that is very bioactive orally. This estrogen is in almost all modern formulations of combined oral contraceptive pills. Over time, formulations have decreased the EE dose from as high as 100 μg/day to as low as 20 μg/day. EE is found in many aquatic systems contaminated by sewage effluents, originating principally from human excretion. Along with natural steroidal estrogens, EE plays a major role in causing widespread endocrine disruption in wild populations of fish species and other lower vertebrate species (Jobling and Tyler, 2006).

In the immature SD and Wistar female rats, 0.3 μg/kg/day of EE is effective in inducing uterine weight when given sc, whereas

orally 1.0 μg EE/kg/day stimulates uterine weight (Kanno et al., 2001).

Administration of 0.5 mg EE/kg/day accelerates VO by 5 to 7 days and induces vaginal cornification in LE and SD weanling rats. When administered to the dam during gestation and lactation over a broad dose–response range (0.05 to 50 μg/kg/day), F_1 female LE rats display a variety of reproductive tract lesions including cleft phallus, accelerated VO, and infertility at 5 and 50 μg/kg/day whereas F_1 males are less severely affected. F_1 males did not display any reproductive tract malformations, and seminal vesicle, ventral prostate, and other androgen-dependent organ weights were not affected at any dose whereas testis and epididymal weights were reduced at 50 μg EE/kg/day. In a similar study with the SD rat, EE only affected F_1 females at 50 μg/kg/day and it was reported that they were not infertile and no effects were noted in the male offspring (Sawaki et al., 2003).

EDC Screening Programs

In response to the 1996 legislative mandate for an endocrine screening and testing program, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), which proposed a tiered screening and testing strategy for EDCs in its final report in 1998 (http://www.epa.gov/scipoly/oscpendo/history/finalrpt.htm). The EDSTAC proposal included the following: a process to prioritize chemicals for (a) evaluation and recommendations, (b) screening (Tier 1), and (c) testing (Tier 2) batteries. The recommended screening battery was designed to detect alterations of HPG function; estrogen, androgen, and thyroid hormone synthesis; and androgen receptor (AR) and estrogen receptor (ER)-mediated effects on mammals and other taxa. Based on a "weight-of-evidence" analysis, chemicals positive in Tier 1 would be considered as potential EDCs and subjected to testing (Tier 2). Equivocal effects in Tier 1 could be replicated or evaluated further in additional short-term assays before more extensive Tier 2 testing was initiated. Tier 1 should include assays sensitive enough to detect EDCs, whereas issues of "dose response, relevance of the route of exposure, sensitive life stages, and adversity" would be resolved in the Tier 2 testing phase.

In Vivo Mammalian Assays EDSTAC recommended the laboratory rat as the species of choice for the endocrine screening and testing assays. In vitro assays can produce false-positive responses at high concentrations due to a lack of specificity as assay conditions deteriorate. Although such false-positives can be eliminated by experimental measurement of K_i values, few in vitro screening strategies include K_i determinations to ensure that the effects seen on binding and gene expression assays are the result of competitive inhibition of ER or AR. As a result, a high percentage of screened chemicals that are determined to be "positives" in vitro may not be true receptor ligands. Finally, because in vitro screening assays are unable to integrate the endocrine responses seen in the whole organism, the relationships between endocrine toxicity and other systemic effects cannot be simulated in vitro. To avoid the limitations described above, the EDSTAC proposed three short-term in vivo mammalian assays for the Tier 1 Screening (T1S) Battery: the uterotrophic, Hershberger, and pubertal female rat assays (Gray et al., 1997a, 2002; Gray, 1998). Table 21-6 indicates the range of EDCs tested in validating these assays for use in the screening program

Uterotrophic Assay Estrogen agonists and antagonists are detected in a 3-day uterotrophic assay using subcutaneous administration of the test compound. Based on the evaluation of four variations of the uterotrophic assay protocol in the OECD interlaboratory studies, all of the protocols have produced acceptable responses without regard to rat strain, diet, or housing conditions (Owens and Ashby, 2002; Kanno et al., 2003a, 2003b; Owens et al., 2003, 2006; Owens and Koeter, 2003). The selected uterotrophic assays for estrogens and antiestrogens use either the intact juvenile or the castrated ovariectomized adult/juvenile female rat.

Hershberger Assay The second in vivo assay in T1S, the Hershberger assay, detects antiandrogenic activity simply by weighing androgen-dependent tissues in the castrated male rat (Hershberger et al., 1953; Gray et al., 1997a, 2002; Gray, 1998). In this assay, weights of the ventral prostate, Cowper's glands, seminal vesicle (with coagulating glands and fluids), glans penis, and levator ani/bulbocavernosus muscles are measured in castrated, testosterone-treated (or untreated) male rats after 10 days of oral treatment with the test compound. This assay is very sensitive for detection of androgens and antiandrogens. Other useful endpoints that help reveal the mechanism of action and specificity of the response include weights of the adrenal, liver, and kidney, and measurements of serum (collected by cardiac puncture) levels of testosterone and LH. The Hershberger assay shows high sensitivity and specificity to chemicals with AR-mediated activity. Weakly antiandrogenic pesticides such as *p,p′*-DDE and linuron are easily detected in the Hershberger assay (Lambright et al., 2000; Yamasaki et al., 2003). Chemicals such as finasteride, which inhibit 5α-reductase activity, also are active in this assay. They dramatically reduce male accessory sex gland weight with less effect on the levator ani/bulbocavernosus muscle, which has low levels of this enzyme. Chemicals that are positive in the Hershberger assay often produce adverse effects during puberty and after in utero exposure.

Pubertal Female Rat Assay The third in vivo mammalian/rat assay included in the screening battery is the pubertal female rat assay, which has been in use for nearly two decades (Gray et al., 1988a, 1989). In this assay, weanling female rats are dosed daily by gavage for 21 days while the age at VO (puberty) is monitored. The females are necropsied at about 42 days of age (reviewed by Goldman et al., 2000). Measurements include serum thyroid hormones, uterine and ovarian weight, and histology. This assay detects alterations in thyroid hormone status, HPG function, inhibition of steroidogenesis, estrogens, and antiestrogens, and has been found to be highly reproducible and very sensitive to certain endocrine activities including estrogenicity, inhibition of steroidogenesis, and antithyroid activity.

Alternative Screening Assays Alternative in vivo assays were also discussed by EDSTAC and are being evaluated by the EPA. If they are of sufficient sensitivity, specificity, and relevance, they might replace or augment current T1S assays. However, whether they meet such criteria remains to be determined.

Pubertal Male Rat Assay One promising alternative assay is the pubertal male rat assay (Stoker et al., 2000), which detects alterations of thyroid function, HPG maturation, steroidogenesis, and altered steroid hormone function (androgen). Intact weanling males are exposed to the test substance for approximately 30 days. The age at puberty is determined by measuring the age at PPS, and reproductive tissues are evaluated and serum taken for optional hormonal analyses. The studies conducted on contract for EPA using the pubertal male assay are also presented on the EPA EDSP Web site. This assay produced reproducible responses among different laboratories and was sensitive to androgens and antiandrogens.

In Utero Lactational Assay The EDSTAC recommended that the EPA develop and evaluate an in utero lactational assay due to

Table 21-6

Chemicals Examined in Protocols of the Organization for Economic Cooperation and Development (OECD) and the US Environmental Protection Agency (EPA), Using Standardized Assays with Oral or Subcutaneous Dosing

OECD uterotrophic assay (four protocols—all successfully identified each chemical)
Ethinyl estradiol: positive estrogenic control
Genistein: phytoestrogen
Methoychlor: estrogenic and antiandrogenic pesticide
Nonylphenol: estrogenic "inert" ingredient and surfactant
o,p′-DDT: estrogenic pesticide
Bisphenol A: estrogenic plastic monomer
ZM 189,154: antiestrogenic pharmaceutical
Dibutyl phthalate: negative control; plasticizer that inhibits testis Leydig cells
OECD Hershberger assay (each chemical successfully identified)
Testosterone propionate: androgenic positive control
Trenbolone: androgenic veterinary pharmaceutical
Methyltestosterone: potent androgenic pharmaceutical
Linuron: antiandrogenic herbicide
p,p′-DDE: antiandrogenic pesticide metabolite
Flutamide: potent antiandrogenic pharmaceutical
Finasteride: potent inhibitor of enzyme 5α reductase required for DHT synthesis and full androgen-dependent growth of some sex accessory tissues
Vinclozolin: antiandrogenic fungicide
Procymidone: antiandrogenic fungicide
EPA in utero lactational protocol (partially successful in identifying estrogenicity)
Methoxychlor: estrogenic and antiandrogenic pesticide
EPA execution of adult intact male assay (did not successfully identify endocrine activity of either chemical)
Linuron: antiandrogenic herbicide
Methoxychlor: estrogenic and antiandrogenic pesticide

the unique sensitivity of the fetal reproductive system to disruption by some toxicants. For example, 2,3,7,8-TCDD (dioxin) alters sexual differentiation of male and female rats and hamsters at dosage levels approximately two orders of magnitude below those required to produce adverse effects in pubertal or adult rats (Gray et al., 1997b, 1997c; Stoker et al., 2000). One version of the proposed in utero lactational assay now being evaluated by the EPA takes about 80 days and uses approximately 10 litters per group (120 to 150 pups). In this protocol, androgens and antiandrogens can be detected in approximately 2 to 3 weeks, and EDCs with antithyroid activity can be detected in infant or weanling offspring after 4 to 5 weeks of maternal treatment.

It is important to retain flexibility in the selection of new assays and endpoints for the screening and testing program so that new methods can be used as replacements or to augment the assays if they offer distinct advantages over the current battery of assays. Enhancements to current life cycle and multigenerational tests are also being considered in an effort to improve the quality of the data on EDCs that will be used for risk assessments. Scientists are attempting to minimize animal use by using as few animals as possible in the most precise and sensitive assays, by incorporating sensitive in vitro assays in T1S, and by using quantitative structure–activity relationships (QSAR) models or high throughput prescreening assays in the prioritization of chemicals for use in vivo. It is also possible to avoid unwarranted animal use because chemicals negative in T1S are not subject to T2T. In addition, testing statistical "false positives" can be almost entirely eliminated by ensuring that T1S assay results are replicated in T1.5 before moving to T2T. Attempts to enhance T2T by adding more sensitive endpoints and a more thorough assessment of the animals already on study may also lead to additional reductions in animal use.

Mixtures of EDCs

Humans and wildlife are exposed to mixtures of chemicals, some of which have the potential to disrupt male reproductive development and function throughout the life cycle. Children, the unborn, pregnant mothers, and the elderly are of special concern and exposures occur via an aggregate of routes including the diet, drinking water, inhalation, and through contact with the skin. The human exposome includes mixtures of pesticides, toxic substances, drugs, naturally occurring chemicals in our diet and endogenous hormones. Some of these chemicals are known reproductive toxicants. Some of the chemicals in the exposome include PCBs and DDT that are persistent, bioaccumulative reproductive and developmental toxicants. Even though these mixtures were banned decades ago they still are of concern for effects on fish, wildlife, and humans. The PCBs were responsible for significant decline in some Great Lakes fish populations, and are overtly toxic to mink and interfere with mink reproduction and development. As late as 2011 there were fish consumption advisories for PCBs on more than 1100 bodies of water (>6 million lake acres and 130,000 river miles) in the United States (EPA-820-F-13-058. 2013).

Historically, studies on the effects of mixtures on reproduction development and function in rodents have used different experimental approaches in order to specifically address different questions. One approach has been to study the reproductive effects of an "environmentally relevant" mixtures including

- dilutions of concentrated drinking water samples (Narotsky et al., 2013)
- "realistically proportioned mixtures" of chemicals
  - drinking water (Narotsky et al., 2015)
  - ground water (Chapin et al., 1989)

- mixtures of chemicals and pesticides (Heindel et al., 1995; Christiansen et al., 2012; Isling et al., 2014; Mandrup et al., 2015)
- phytoestrogens (Boberg et al., 2013)
- chemicals found in human samples (Hadrup et al., 2016)
- persistent environmental contaminants (Wade et al., 2002)

In general, the aforementioned studies did not indicate that exposure to "environmentally relevant" levels of these mixtures caused adverse effects on male reproductive development or function. Although the results of these studies can be very useful for the risk evaluation of the specific mixtures they do not lend themselves to predict the toxicity of mixtures of other chemicals or mixtures of the same chemicals at different proportions.

Because we cannot test the toxicity of every mixture in the exposome, another approach is to develop statistical models that accurately predict how untested mixtures will behave. This approach involves mixing chemicals whose individual reproductive toxicity is well characterized to determine what mixture model provides the most accurate prediction of how the chemicals in the mixture interact. If we are exposed to several chemicals at once, at "safe" levels for each chemical, will these chemicals interact in a cumulative manner resulting in adverse effects that can be predicted from the individual chemical dose–response data? In other words, does no effect (chemical a) + no effect (chemical b) =0 (a+b; response addition) or can no effect (chemical a) + no effect (chemical b) = an adverse effect (a+b; dose addition or perhaps synergy).

The response addition model assumes that the chemicals in a mixture will interact independently. Response addition models would predict that exposing a pregnant rat to no-adverse-effect-levels of an androgen receptor antagonist with a chemical that inhibited fetal testosterone synthesis would have little effect on male reproductive tract development. However, it is evident from the scientific literature that response addition models do not accurately predict effects of mixtures of chemicals that disrupt common reproductive targets via diverse mechanisms of toxicity, indicating that excluding chemicals that disrupt reproductive development via diverse mechanisms of toxicity from a cumulative risk group may severely underestimate the potential risk of mixtures. In contrast, dose-addition models more accurately predict the effects of mixtures of chemicals that induce abnormalities in common reproductive tissues (Rider et al., 2008, 2009, 2010).

The following presents a few selected examples of results supporting the use of dose addition as opposed to response addition models to estimate the effects of mixtures of chemicals that disrupt development of common reproductive tissues. For example, in utero administration from gestational day 14 to 18 of 50 mg/kg/day of two equipotent antiandrogenic pesticides vinclozolin and procymidone produced hypospadias in 10% and 0%, respectively, of the resulting male offspring (Gray et al., 2010). When combined, a response addition model would predict a low incidence of hypospadias. However, 95% of the male offspring in the mixture group were malformed. In the mixture, these two antiandrogens did not act independently; they acted in a dose-additive manner as if 100 mg of vinclozolin was administered. Hass et al. (2007) and Metzdorff et al. (2007) reported similar cumulative, dose-additive effects on the reproductive tract of male offspring when three antiandrogens were administered during development. When two equipotent phthalate esters were combined at 500 mg/kg/day, the mixture produced cumulative, dose-additive adverse effects on the epididymis (agenesis), testis, gubernaculum, and external genitalia (hypospadias) (Howdeshell et al., 2008; Gray et al., 2010). For example, neither phthalate induced hypospadias when administered alone, whereas 50% of the male offspring in the mixture group displayed hypospadias (0 + 0 =50). Similarly, administration of a mixture of five phthalate esters also produces effects in the male offspring reproductive tract that can be predicted using dose rather than response addition models (Howdeshell et al., 2015).

The aforementioned studies combined chemicals that acted via common mechanisms of toxicity. However, when chemicals with different mechanisms of toxicity are coadministered in utero, the effects on male rat sexual differentiation are also well predicted by dose-, but not response-addition models (Rider et al., 2008; Christiansen et al., 2009; Rider et al., 2009, 2010; Gray et al., 2010; Hotchkiss et al., 2010; Howdeshell et al., 2016). For example, in utero administration of the AR antagonist procymidone (50 mg/kg/day) with dibutyl phthalate (DBP at 500 mg/kg/day) that inhibits fetal testosterone synthesis induced hypospadias in 1.5% and 0% of the male offspring, respectively. When combined, the incidence of this and several other malformations displayed cumulative dose-additive responses, with 48% of the male offspring displaying hypospadias (1.5 + 0 =48) (Gray et al., 2010).

The above studies administered moderate to high dosage levels of the individual chemicals in the mixture in order to statistically compare the predictive utility of dose- and response-addition models. However, they did not examine, in detail, the effects of low- or very low-dose levels of the mixtures (dose levels well below the respective LOAELs). Recently, experimental studies have begun to examine the behavior of low to very low doses of the effects of antiandrogen mixtures on male rat reproductive development due to concerns that current risk assessment paradigms may be insufficient to protect against combined exposure to endocrine active chemicals at low-dose levels. For example, Schneider et al. (2017) investigated the dose–response relationships of single and combined exposures to three antiandrogens (flutamide, prochloraz, vinclozolin) administered to the dam during gestation and lactation at LOAELs, NOAELs, and acceptable daily levels for each individual substance. They reported that combined exposure at LOAEL levels resulted in antiandrogenic effects in male offspring on AGD, retention of areolae/nipples, delayed preputial separation, and reduced ventral prostate weight in comparison to the individual compounds. A similar study combining 18 chemicals in the mixture was recently reported (Conley et al., 2017).

In summary, a significant preponderance of data from several laboratories studying the effects of mixtures on male rat reproductive development indicates that chemicals that disrupt common target organs like the genitalia, epididymis, or testis are accurately predicted using dose addition models, regardless of the mechanism of toxicity. In contrast, response addition models may severely underestimate the effects of mixtures on the male reproductive tract. Additional studies are warranted to determine how low-dose levels of mixtures behave and also to examine additional pathways beyond those studied to date, which has primarily focused on antiandrogenic chemicals.

TESTING FOR REPRODUCTIVE TOXICITY

Screens and Multigeneration Studies

The testing of xenobiotics for reproductive toxicity has been refined over many decades to provide more comprehensive assessments of a test material's ability to affect the reproductive cycle in laboratory animal models and provide appropriate information to estimate potential risk of exposure to humans. The history and evolution of reproductive toxicity guidelines for pharmaceuticals have recently been reviewed (Collins, 2006). The types of protocols and

guidelines employed depend on the type of chemical being tested and its intended use(s). For example, this would involve extensive and comprehensive testing of pesticides used on food products, where all the population may be exposed, while much more limited information would be required for a specific drug (e.g., used to treat women for menopausal symptoms). In the specific drug case, the experimentation required would be directed at the "target" life stage(s).

It should also be noted that comprehensive testing for reproductive toxicity normally involves the simultaneous exposures of both males and females. Only with specific protocol amendments will the affected sex(es) be determined (e.g., exposure of one sex, or a cross-over mating study design in which treated males and females are mated with the corresponding control animals). The details below apply to the rat, the most common species employed in reproductive toxicity studies. Suitable amendments can be made for other species (the mouse is sometimes used) and nonhuman primates occasionally employed—particularly for testing drugs.

A significant amount of attention has also been focused on the development of "screens" for reproductive toxicity as opposed to the more definitive protocols usually found in testing guidelines. Such a need has arisen primarily for the evaluation of the 80,000 chemicals used in commerce, which have no reproductive toxicity testing information. Thus, the screens currently employed (and particularly the OECD 421 and 422 guidelines) have been developed to prioritize chemicals for more comprehensive testing. Whereas such screening approaches can identify chemicals that have adverse effects on reproductive function, the dosing regimens, endpoints employed, number of dose levels used, etc. serve only to provide a signal, albeit that the basic information is of a quality to set dose levels, or highlight potential areas of issue in more detailed studies. More problematic is what to do with negative data from these screens as these *do not* imply that the chemical tested is without reproductive toxicity, and may still present a risk to humans. Thus, the outcomes from such screens can be summarized as "*a positive response is a positive, but a negative response is a maybe.*" More detailed information on the proposed screening battery for EDCs is provided in the section "Endocrine Disruption (Including Screening and Puberty)."

The most comprehensive assessment of reproductive toxicity would be provided by a protocol that exposes the animal model throughout the reproductive cycle (see Fig. 21-1) and involves the assessment of multiple endpoints at different life stages during this continuous exposure. The protocol and guideline coming closest to this ideal is the multigeneration reproduction study used for the assessment of chemicals, pesticides, and some food additives (OECD 416, EPA OPPTS 870.3800, FDA "redbook" and the NTP Reproductive Assessment by Continuous Breeding [RACB]). These all represent variations on the general theme noted in Figs. 21-28 and 21-29, and the reader is pointed to the specific guidelines for more detailed explanation of the differences. In general, parental (F_0) animals are exposed for approximately 10 weeks prior to mating (based on the duration of the spermatogenic wave of 8 weeks and the passage of sperm through the epididymis and the availability of mature sperm for fertilization; see Fig. 21-16). Exposure continues through mating (and after the mating pairs are separated), gestation, birth, and lactation. Litters may be "standardized" to ensure equal lactational demand on the dams and normalize the growth of pups (litters are usually reduced to four males and four females per litter on PND 4). At weaning, F_1 litters are usually culled to one male and one female that are raised and exposed until adulthood and the exposure continues through the same processes in the second breeding generation, which usually halts at weaning

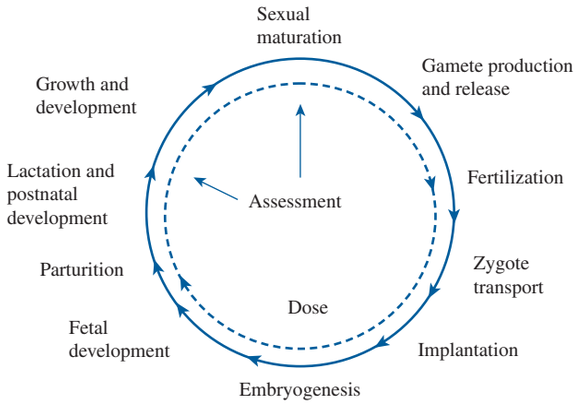


Figure 21-28. *Multigeneration reproduction study.*

of the F_2 pups. These studies normally have at least three dose levels (with the highest dose level designed to induce some toxicity) and at least 20 litters produced per dose group. The RACB study is unusual, in that two to three litters are produced in each generation and in its latest form, four males and females are retained from the last F_1 and F_2 litters to adulthood. These multigeneration studies normally encompass detailed measurements of reproductive performance (number of pregnant females from the number of pairs mated, number of females producing a litter, litter size, number of live pups with their birth weights, and sex). Measurement of growth and analysis of the reproductive organs in the F_0 parental generation are conducted (including specific evaluations of ovarian follicles, estrous cyclicity, and sperm parameters). Similar measurements to those undertaken for the F_0 are made on the F_1 parents, but in addition offsprings are normally carefully examined at birth (and sexually dimorphic endpoints may be collected such as AGD), at weaning, and at puberty (particularly the assessment of VO and time of first estrus in females and balanopreputial separation in males) in addition to the adult measurements of reproductive performance, organ weights, histology, etc. In the EPA multigeneration study design, only 10 adult animals per sex, per group, and per generation are required to have their tissues evaluated histologically. For the OECD 416 design, this number is increased to 20 per sex per group (i.e., each male and female selected for breeding in the F_1 generation).

Generally, only one mating is performed per generation, but in the presence of equivocal data, or when there is a clear indication that effects are becoming more adverse in succeeding generations, then extra litters or generations may be added to the standard designs to clarify potential reproductive toxicity.

Testing for Endocrine Disrupting Chemicals

In a tiered screening and testing approach, only chemicals that display positive reproducible responses in Tier 1 screening (T1S—see the section "Endocrine Disruption [Including Screening and Puberty]") or T1.5 would be evaluated further in full life cycle or multigenerational tests. In Tier 2 testing (T2T), not T1S, issues of dose–response, relevance of the route of exposure, sensitive life stages, and adversity are resolved. For some endocrine activities, the number of sensitive endpoints and F_1 offspring examined in these assays should be expanded on a case-by-case basis.

AGD at birth and nipple/areola retention in infant female and male rats should be included in testing of androgens and

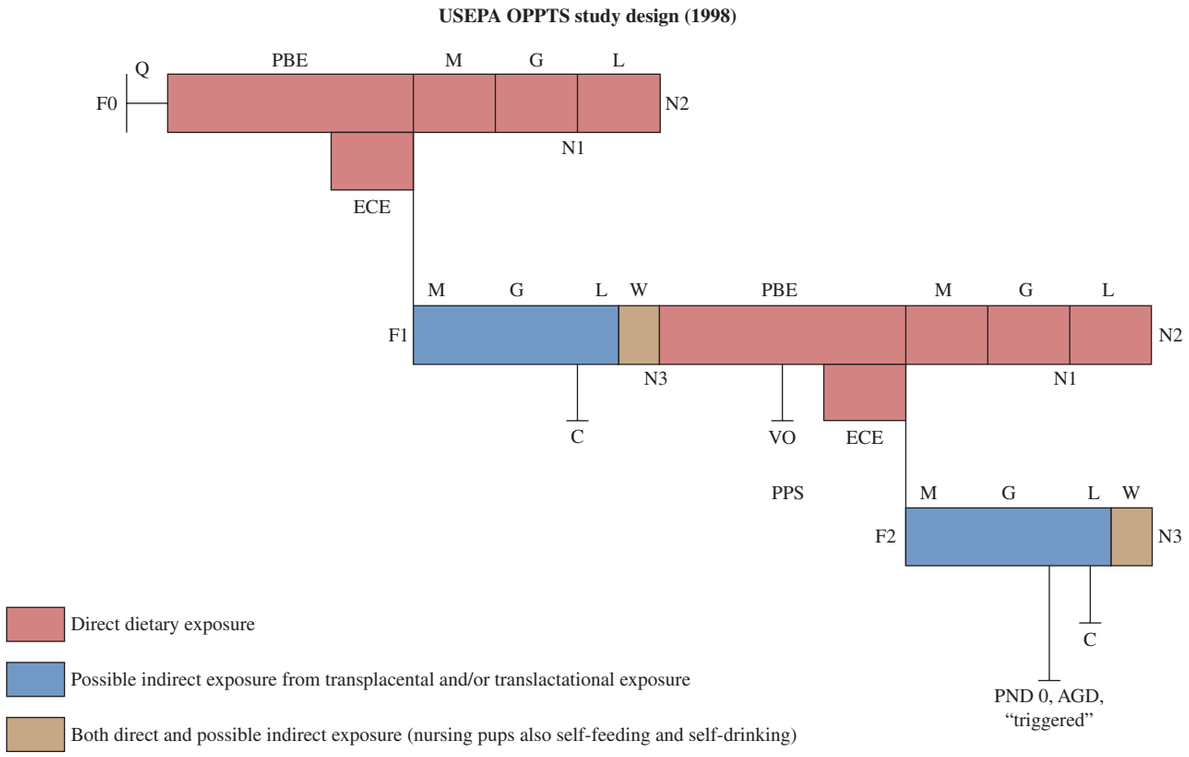


Figure 21-29. *Multigeneration reproduction study as recommended by EPA.* Key for the rat: Q, Quarantine (1 week); PBE, prebreed exposure (10 weeks); M, mating (2 weeks); G, gestation (3 weeks); L, lactation (3 weeks); VO, vaginal opening (evaluated in F_1 females on postnatal day 22 to acquisition); PPS, preputial separation (evaluated in F_1 males on postnatal day 35 to acquisition); W, weaning (postnatal day 21); N1, necropsy of all paternal animals (organ weights, histology, and andrological assessments); N2, necropsy of all maternal animals (organ weights, histology, and ovarian follicle assessments); N3, necropsy of selected weanlings, three/sex/litter, if possible (organ weights); ECE, estrous cyclicity evaluation (3 weeks); C, Cull litters to 10 pups (with equal sex ratio) on postnatal day 4; AGD, anogenital distance measured in F_2 pups on postnatal day 0 if triggered by effects on F_1 reproductive development; N, necropsy.

antiandrogens, respectively, because they are sensitive, potentially permanent effects that are highly correlated with malformations and reproductive organ weight changes later in life (McIntyre et al., 2001). These early alterations constitute part of the antiandrogen-induced developmental syndromes. The syndrome induced by the AR antagonists differs to some degree from that induced by the phthalates, which inhibit the synthesis of fetal testosterone and insulin-like 3 peptide (insl-31) hormones.

Developmental Syndromes and Tailored Testing A careful evaluation of the male rat offspring allows one to distinguish the "phthalate syndrome," in which effects on reproductive development involve a decrease in fetal testicular testosterone and insl-3 peptide hormone biosynthesis (Mylchreest et al., 1999; Mylchreest and Foster, 2000; Parks et al., 2001; Gray et al., 2002; Wilson et al., 2004) from the "AR antagonist syndrome," induced by vinclozolin (Gray et al., 1994, 1999b; Lim et al., 2001) or flutamide (McIntyre et al., 2001). The main distinction between the two syndromes is that the phthalate syndrome includes agenesis of the testis, epididymis, and gubernacular cord. These lesions are rarely seen in the AR antagonist syndrome, even when all of the males display hypospadias. In addition, of all chemicals that interfere with the androgen-signaling pathway in the fetal male rat, only the phthalates affect Leydig cell insl-3 hormone synthesis and cause undescended testes due to gubernacular agenesis (Wine et al., 1997).

In contrast to the antiandrogens, which primarily affect the male offspring (see Table 21-7), in utero exposure to androgenic chemicals has more severe consequences for the female offspring (see Table 21-8). It is important to consider this information when tailoring T2T based on T1S results. For example, when the female rat fetus is exposed to testosterone (Hotchkiss et al., 2007) or the veterinary pharmaceutical trenbolone, agenesis of the vagina and nipples is seen at a low rate in the lower dosage groups. In fact, most of the low-dose effects of androgens in the female offspring (retained prostatic and vesicular tissues and nipple agenesis; Wolf et al., 2002; Hotchkiss et al., 2007) are effects that are likely to be missed in a standard necropsy.

When conducting a multigenerational study, it is important to summarize the data in a manner that clearly delineates the proportion of animals that are affected, indicating that they display any lesion (histological or gross pathology) consistent with the syndrome. In teratology studies, data are typically presented and analyzed in this manner, indicating the number of malformed/number observed on an individual and litter basis, whereas multigenerational studies are frequently presented and analyzed differently, even when clear teratogenic and other developmental responses are noted after birth. Multigenerational protocols are used in T2T because only these protocols expose the animals during all critical stages of development and examine reproductive function of offspring after they mature.

Although the new EPA multigenerational test provides for a comprehensive evaluation of the F_0 or parental generation, a few F_1 animals (offspring with developmental exposure) are examined after maturity to detect anything but the most profound reproductive teratogens (Gray and Foster, 2004). F_0 animals within a

Table 21-7

Detecting Developmental Reproductive Syndromes in Male Rat Offspring

Neonatal-infantile data

1. Anogenital distance at birth (1 to 3 days of age)
2. Areola/nipple retention in infant male rats at 13 to 14 days of age

External necropsy endpoints on all male rat offspring at maturity

1. Body weight any unusual malformations or anomalies, euthanize
2. Shave ventral surface from inguinal region to neck and count nipples and areolas (observer blind to treatment), record position of areolas and nipples
3. Check animals for hypospadias, epispadias, cleft phallus, and measure AGD
4. Note if testes obviously undescended
5. Note if inguinal region soiled with urine
6. Note if prepuce partially or entirely detached from glans penis, especially if a persistent thread of tissue is present along frenulum

Internal endpoints on all male rat offspring at maturity

1. Location of each testis (scrotal, abdominal, and gubernaculum attached to abdominal wall)
2. Gubernacular cords, present or absent, and length in mm if abnormal
3. Note if present, cranial suspensory ligaments
4. Note if testes are small, absent, fluid filled, enlarged, appear infected or other abnormalities
5. Note if epididymides are small, absent, or infected (record region of effects)
6. Note if ventral prostate is small, absent, or infected
7. Note if dorsolateral prostate is small, absent, or infected
8. Note if seminal vesicles are small, absent, infected, or one side larger than the other
9. Note if coagulating glands are small absent, infected, one side larger than the other or detached from seminal vesicles
10. Note if kidneys display hydronephrosis, calcium deposits
11. Note the presence of hydroureter
12. Note the presence of bladder stones or bloody in bladder

Weigh the following organs on all male rat offspring at maturity

1. Each testis individually (examine histology of each testis)
2. Each corpus plus caput epididymis (examine histology of each segment)
3. Each cauda epididymis (examine histology of each segment)
4. Entire seminal vesicle, plus coagulating glands with fluid as a unit, if possible
5. Entire ventral prostate, if possible
6. Each kidney
7. Paired adrenals
8. Liver
9. Levator ani plus bulbocavernosus
10. Cowper's glands as a pair, if possible
11. Glans penis
12. Pituitary
13. Brain

Histology on all male F_1 offspring at maturity

1. Both testes
2. Both epididymides
3. Prostate glands
4. Any grossly abnormal reproductive tissues

Multitude of effects of antiandrogens in male rat offspring that should be evaluated in Tier 2 testing studies displaying this activity in Tier 1 screening or other assays.

dose group typically respond in a similar fashion to the chemical exposure; however, the response to toxicants in utero can vary greatly even within a litter with only a few animals displaying severe reproductive malformations in the lower dosage groups. For example, dose-related adverse reproductive effects are seen in less than 10% of the F_1 offspring treated in utero with lower dosage levels of (a) the phthalate DINP (male reproductive malformations, Gray et al., 2000); (b) 2,3,7,8-TCDD (permanent vaginal threads and ovarian atrophy, Gray et al., 1997c); (c) in utero busulfan (ovarian atrophy and delayed puberty, Gray and Ostby, 1998); (d) DEHP (epididymal and testicular lesions, Gray et al., 2010); (e) linuron (epididymal and testicular hypoplasia, McIntyre et al., 2000); and (f) androgens (vaginal and nipple agenesis, Wolf et al., 2002), Thus, a standard multigenerational protocol that examines only one F_1 animal per sex, per litter after maturity from 20 litters per dosage group, can detect statistically significant alterations only when they are displayed by 25% or more of the offspring. Histopathological alterations must be displayed by 50% or more of the offspring because a histological examination of the reproductive tract is required only in 10 F_1 animals per sex, per dose—far too few to detect anything but the most profound effects on reproductive development. Such an approach would also create uncertainty associated with lowest observed adverse effect levels ("LOAELs") and no observed adverse effect levels ("NOAELs")

Table 21-8

Detecting Developmental Reproductive Syndromes in Female Rat Offspring

Neonatal-infantile data in female rat offspring

1. Anogenital distance at birth (one to three days of age)
2. Areola/nipple agenesis (complete or faint) in infant female rats at 13 to 14 days of age

External necropsy endpoints on all female rat offspring at maturity

1. Body weight any unusual malformations or anomalies, euthanize
2. Shave ventral surface from inguinal region to neck and count nipples and areolas (observer blind to treatment), record position of areolas and nipples
3. Check animals for cleft phallus and measure AGD and position of vaginal opening
4. Note if inguinal region soiled with urine

Internal endpoints on all female rat offspring at maturity

1. Location of ovaries in relationship to kidneys
2. Note if absent, cranial suspensory ligaments
3. Note if ovaries are small, cystic-fluid filled, enlarged, appear infected, or other abnormalities
4. Note if oviducts, uterus, or upper or lower vagina are small, absent, or infected (record region of effects) or fluid-filled
5. Note if ventral prostate tissue is present
6. Note if seminal vesicle tissue is present
7. Note if levator ani/bulbocavernosus muscle tissues are present
8. Note if other male tissues are present
9. Note if kidneys display hydronephrosis, calcium deposits
10. Note presence of hydroureter
11. Note presence of bladder stones or blood in bladder

Weigh the following organs in all female offspring at maturity

1. Paired ovaries (histology)
2. Uterus with fluid (histology)
3. Vagina
4. Each kidney
5. Paired adrenals
6. Liver
7. Pituitary
8. Brain

Histology on all female rat offspring at maturity

1. Both ovaries
2. Uterus
3. Vagina
4. Any suspected male reproductive tissues
5. Any grossly abnormal reproductive tissues

Multitude of effects of androgens in female rat offspring that should be evaluated in Tier 2 testing studies.

identified in multigenerational studies for chemicals that do not include either a complete assessment of all of the endpoints that constitute the syndrome, or those included in the EPA new multigenerational test guidelines.

In "transgenerational" protocols (Gray et al., 1994, 1997c, 1999a, 1999b, 2002; Gray and Ostby, 1995; Gray, 1998; Mylchreest et al., 1998, 2000; McIntyre et al., 2000, 2001), we typically use fewer litters (7 to 10 per dose group), but examine all of the animals in each litter. These protocols actually use fewer animals, but provide enhanced statistical power to detect reproductive effects in the F_1 generation. Additional factors, besides detection of adverse effects at necropsy or during data analysis and interpretation, limit interpretation of data from the standard multigenerational reproduction test. The lifelong exposure of both males and females in the F_1 generation, which allows one to detect effects induced in utero, during lactation, or from direct exposure after puberty, can confound the identification of when the effect was induced (i.e., during adulthood vs. development) or even the affected sex. In studies, where the dosing period normally is terminated near birth or at weaning, precludes misinterpretation of the developmental origin of reproductive effects. Nevertheless, it is clear that transgenerational protocols would not be appropriate for EDCs that induce low-dose alterations in the pubertal or adult animal in the F_0 generation.

Test Design and Numbers of F_1 Animals It is important to reiterate that the endpoints described above, which are sensitive to antiandrogens or androgens in utero (listed in detail in Gray and Foster, 2004), are not sensitive to xenoestrogens or some other EDCs. Thus not all EDCs should be tested similarly to the androgens or antiandrogens. Testing should be tailored based on the pharmacological activity demonstrated in T1S. In addition, the developing fetus is not always the most sensitive life stage. Some EDCs disrupt pregnancy by altering maternal ovarian hormone production in F_0 dams at dosage levels that appear to be without direct effect on the offspring (Gray et al., 1999b). In such cases, the standard EPA multigenerational protocol with minor enhancements would be recommended, or a transgenerational protocol with exposure continued after weaning. The transgenerational or in utero lactational protocols (see Fig. 21-30) fill a gap in the testing program for EDCs that should be used only on a case-by-case basis, as indicated by the results of T1S and any Tier 1 repeat study.

Testing Pharmaceuticals

In the case of pharmaceuticals, it is rare for multigeneration studies to be conducted, because it is not common for all the population to use a specific drug and that exposure to the drug is not necessarily chronic and over many different life stages. Typically, three specific studies are undertaken based on the recommendations of the International Conference on Harmonization of Guidelines S5A: Detection of toxicity to reproduction for medicinal products (see

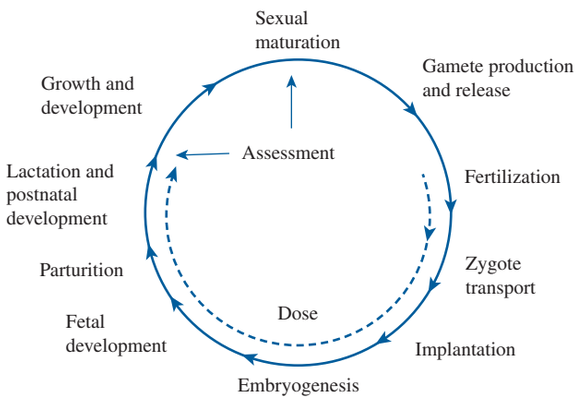


Figure 21-30. *The in utero lactational study design under review by EPA for the assessment of endocrine active agents.*

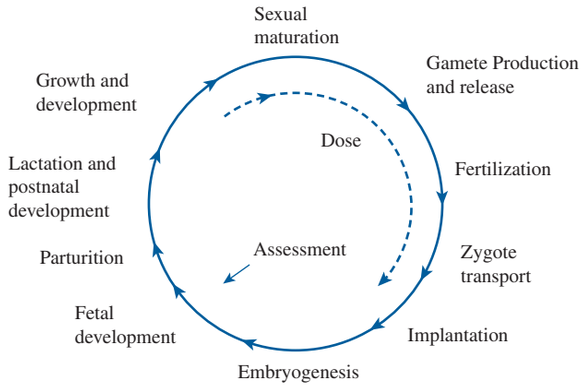


Figure 21-31. *Fertility and early embryonic study.*

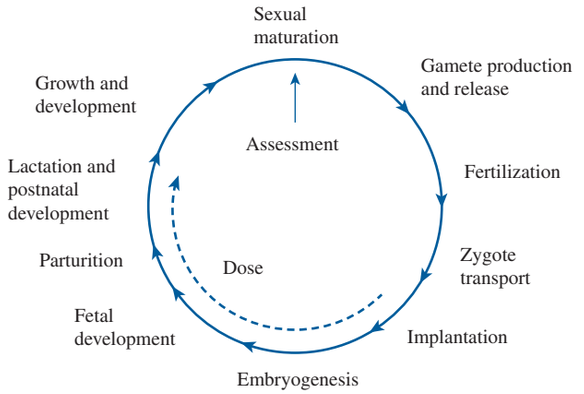


Figure 21-32. *Pre- and postnatal developmental toxicity study.* Dosing is from implantation until the litters are weaned.

Collins, 2006 for specific study descriptions) although any permutation or specific design is open to the investigator to explore specific toxicity based on the pharmacology of the drug tested. The three "most likely" studies are as follows:

1. *A study of fertility and early embryonic development* (see Fig. 21-31). Parental adults are exposed to the test chemical for 2 weeks (females) or 4 weeks (males) prior to breeding and then during breeding. Females then continue their exposure till implantation. Males can be necropsied for the endpoints noted above for the multigeneration studies after pregnancy has been confirmed, and for the pregnant females, necropsy takes place any time after mid-gestation. As with the multigeneration study, reproductive and target organs are weighed and examined histologically, sperm parameters are assessed in males and in females, the uterine implantation sites and ovarian *corpora lutea* are counted, as well as live and dead embryos.

   In this study design, the selection of the dosing regimen for males has been based on pragmatism in attempting to shorten the study, rather than the biology of spermatogenesis in the test species (as employed in the multigeneration study). It is thought that the majority of chemicals that might affect the male should be detectable (by histology) after 4 weeks exposure; however, there are a number of exceptions to this notion. One of the significant advantages of the pharmaceutical guideline approach is that the investigator is encouraged to tailor their testing protocols to reflect the best science and available knowledge. One potential disadvantage is that the basic approach does represent a theoretical "gap" as some chemicals may not manifest a testis histological lesion in 4 weeks and also there is a potential disassociation of structure (testis histology and sperm parameters) from function (male breeding performance).
2. *A study of effects on pre- and postnatal development including maternal function* (see Fig. 21-32). In this study, pregnant females are exposed from implantation until weaning of their offspring (usually PND 21 in the rat). After cessation of exposure, selected offspring (one male and one female per litter) are raised to adulthood and then mated to assess reproductive competence. These animals are observed for maturation and growth (but are not exposed). Puberty indices, as employed in the multigeneration study, are measured. In addition, sensory function, reflexes, motor activity, learning, and memory are also evaluated.
3. *A study of embryo–fetal development* (see Fig. 21-33). This study tests for enhanced toxicity relative to that noted in pregnant females and unlike the previous two studies is normally conducted in two species (typically the rat and rabbit). Exposure occurs between implantation and closure of the hard palate (GD 6 to 15 in the rat), and females are killed just prior to parturition. At necropsy, dams are observed for any affected organs and *corpora lutea* are counted. Live and dead fetuses are counted and examined for external, visceral, and skeletal abnormalities. In the evaluation of prenatal developmental toxicity for a pesticide or other chemical (e.g., OPPTS 870.3700, OECD 414), exposure of the pregnant dams is usually longer and can be continuous throughout pregnancy or, more normally, from implantation until just prior to birth (see Fig. 21-34). Other endpoints measured in the dam and fetuses are identical between the pharmaceutical embryo–fetal toxicity study and that employed for evaluation of an industrial chemical or pesticide.

The FDA Center for Drug Evaluation and Research produced a draft guidance document in 2001 that explored how data should be passed through an integrative process to assess a drug for reproductive toxicity. The tool is used in a tiered manner consisting of three sections: (1) methods applicable to all datasets, (2) approaches applicable only to datasets without evidence of reproductive and developmental toxicity, and (3) approaches applicable to datasets with positive indications of reproductive and/or developmental toxicity. This integration would provide one of three summary risk conclusions that would be applied to the drug label namely—(1) not anticipated to produce reproductive and/or developmental effects above the background incidence for humans when used in accordance with the dosing information on the product label; (2) the drug may increase the incidence of adverse reproductive and/or developmental events, or (3) that the drug is expected to increase the incidence of adverse reproductive and/or developmental effects in humans when used according to the product label.

An examination of the reproductive cycle in a comparison of these three most likely options for FDA studies indicates an obvious gap in the exposure regime for the complete reproductive cycle, namely exposure of weanlings through puberty to adulthood. However, there has been significant interest in the safe use of pharmaceuticals in pediatric populations, and unlike the more standard reproductive and developmental studies, specific studies on juvenile animals are usually performed on a case-by-case basis. The number

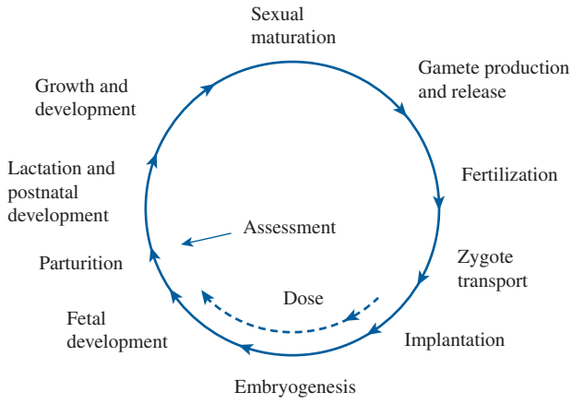


Figure 21-33. *Embryo–fetal developmental toxicity study as used by FDA guidelines.* Dosing starts at implantation and continues to closure of the hard palate (GD 6 to 15 in the rat) with an assessment of fetuses just prior to parturition.

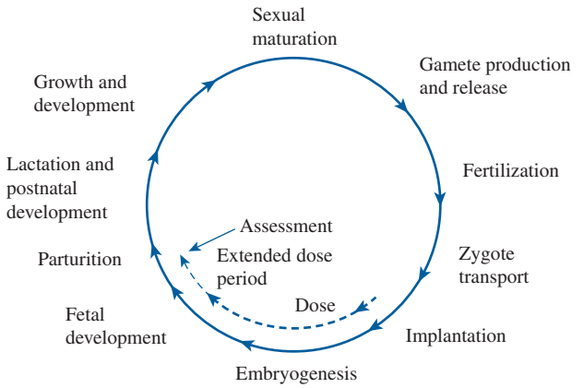


Figure 21-34. *Prenatal developmental toxicity study as used by EPA and OECD.* Note the extended dose period compared with that used in Fig. 21-33.

of these types of studies has also been increasing and whether these could be combined with more standard approaches in a targeted fashion to, for example, evaluate behavioral testing after extended exposure postweaning (as would be conducted in a standard, pre- and postnatal study) or have cohabitation for assessment of fertility and fecundity of the offspring (see reviews by Hurtt et al., 2004; Cappon et al., 2009).

The single generation reproduction study (OECD 415) is used more frequently in Europe where specific testing guidelines for reproductive toxicity may be triggered by the tonnage production of a specific chemical. This design (see Fig. 21-35) has a common F_0 parental exposure period to that used in the multigeneration guideline (OECD multigeneration study—416). Estimates are made of the standard litter parameters and reproductive performance of the F_0 parents, but in this design the study halts at the weaning of the F_1 offspring and no estimate is made of effects on adult F_1 offspring, or on the ability of this generation to reproduce. This study design provides critical information on parental reproductive effects, but has very limited information on the offspring aside from pup number, growth, and survival to weaning, and thus has limited utility in the estimation of transgenerational effects, or postnatal reproductive consequences.

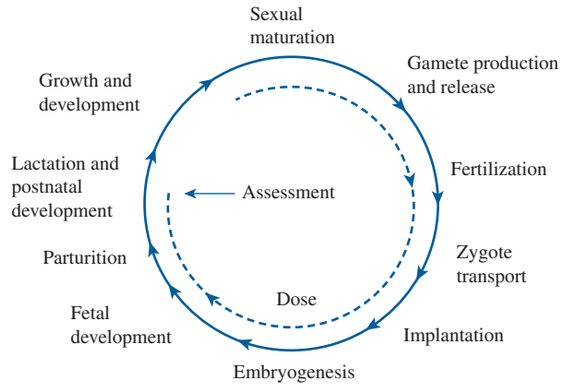


Figure 21-35. *Single generation reproduction study.*

Newer Guidelines and Approaches

The International Life Sciences Institute sponsored a new endeavor looking at how agricultural chemicals might be assessed for toxicity, including reproductive toxicity in a proposed life stage approach (Cooper et al., 2006) using an extended one-generation study (see Fig. 21-36) in the rat. This approach incorporated many of the changes made more recently to testing guidelines (including extra endocrine-related endpoints) and attempted to streamline the testing of agrochemicals for toxicity. The life stages protocol was one of a tiered set of proposed studies, such that all data available could be incorporated into the study design and interpretation of the data. The approach was very laudable, in that it proposed (unfortunately only as an option) incorporation of toxicokinetic data generated during pregnancy and lactation into the study design, as has long been required in drug testing, to aid study design and data interpretation. In addition, endpoints evaluating (at least to some degree) developmental neurotoxicity and developmental immunotoxicity would be measured as a standard, rather than as a triggered option. Its major aim was to reduce the number of animals required and increase the information available on young animals. However, these compromises were not without some flaws compared to the current multigeneration study used for pesticides by EPA or OECD. The study would seek to be a substitute for the current multigeneration study in most instances. The proposal does offer the opportunity to undertake a classical multigeneration study in a second tier, but only if an adverse event was found in the Tier 1 study. Thus, a negative in the extended one-generation study could mean a halt to testing for reproductive toxicity. In particular, the paper recommends the use of the shortened exposure period before breeding the parental animals, similar to that implied in the International Conference on Harmonization of Guidelines (ICH) for fertility and early embryonic development, but it is much harder to justify that pesticides and other agricultural agents, unlike drugs, will not have human exposure at least subchronically and moreover the divorce of reproductive structure (e.g., histopathology of the reproductive organs) from function (e.g., litter parameters) could have serious implications for classification and labeling in some parts of the world.

In Europe, the advent of REACH (Registration, Evaluation, Authorization and Restriction of Chemical substances) was likely to require increased toxicity testing of chemicals at the same time that other efforts were seeking to reduce experimental animal usage. Significant attention was therefore focused on those assays that required (or produced) the largest number of experimental

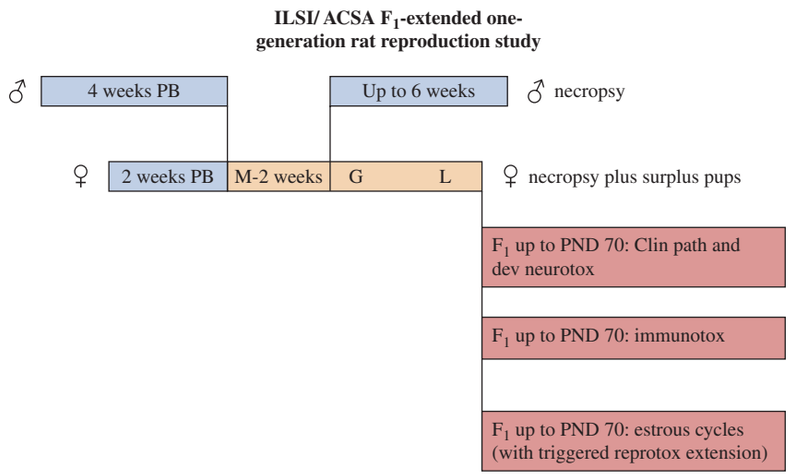


Figure 21-36. *The ILSI/ACSA life stages study for the evaluation of reproduction with developmental immunotoxicity and neurotoxicity.*

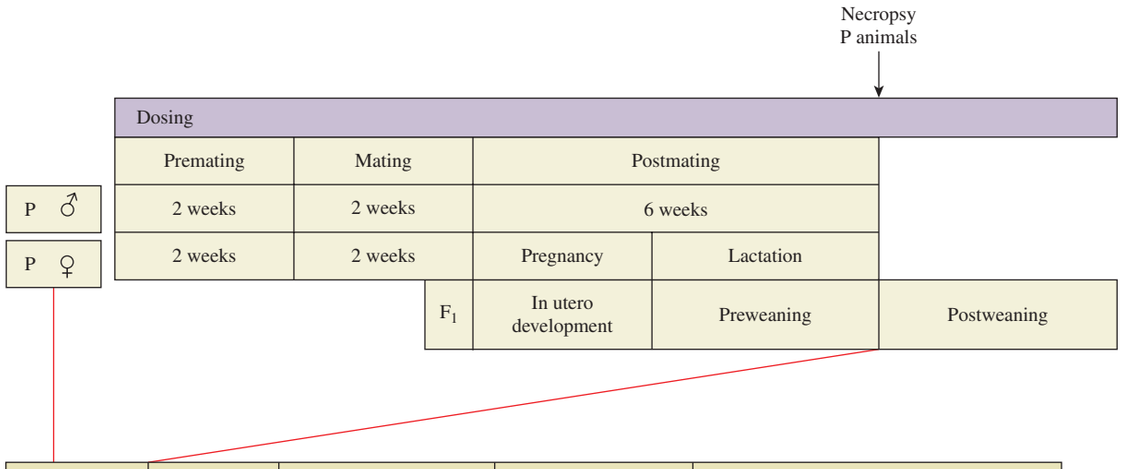
animals—the multigeneration reproduction study and the prenatal developmental toxicity study (usually conducted in two species). The OECD took up the challenge of finding a study that would provide adequate information for the evaluation of reproductive toxicity, but would also reduce animal numbers and has proposed a study (the extended one-generation reproduction study, based on the Cooper et al.'s design) for adoption (see Fig. 21-37; the final draft is available online at http://www.oecd.org/dataoecd/23/10/46466062.pdf). It is proposed to be used for all chemicals (not just pesticides), where a much reduced toxicity database is likely to be available.

As our knowledge of critical windows of exposure has increased, particularly with the increased focus on chemicals that may have endocrine-like activity, the last 15 years has shown the need for a study where there has been a larger focus on the evaluation of potential postnatal adverse outcomes. Thus, there have been updates to standard designs to incorporate more functional endpoints (e.g., sperm and oocyte analysis, vaginal cytology, indices of puberty, and sexual differentiation) to improve the detection of chemicals affecting reproduction and the endocrine status of animals. In particular, in current study designs, the ability to evaluate (both detection and analysis of dose–response) abnormalities of the reproductive tract routinely following in utero exposure to chemicals with endocrine activity was determined to be underpowered by several research groups (McIntyre et al., 2002; Hotchkiss et al., 2008; Blystone et al., 2010). For example, in an evaluation of prenatal developmental toxicity, every fetus is examined for potential abnormalities (typically ~250 fetuses per group) whereas in the multigeneration study, only one male and female pup per litter from a minimum of 20 litters is examined at adulthood for adverse pathological events (i.e., only 40 of the potential 250 animals/group produced). Some recent studies have shown the added value and increased statistical power of evaluating more offspring per litter by retaining them to adulthood, rather than discarding animals already produced, or performing only a gross examination at weaning, when the reproductive organs are not fully differentiated or developed (see Fig. 21-38; Blystone et al., 2010). The NTP has already adopted the improved use of these animals, in its (RACB) study design, by carrying more animals through adulthood for examination, rather than removing them at weaning.

Following a workshop held by the NTP on the evaluation of tumors of the endocrine system in rodent models (Thayer and Foster, 2007), the program adopted a new default paradigm for its rat cancer bioassays that incorporates exposure during the perinatal period (i.e., gestation and lactation). Before embarking on such a study, it would be customary to undertake a preliminary study that evaluated target organ toxicity (for a conventional cancer study, this would be the 90-day toxicity study) and enabled suitable dose levels to be selected for the cancer bioassay. Thus, for a long-term cancer study involving exposure during pregnancy and lactation, a shorter duration study that involved exposure during these critical developmental windows would be required. The NTP has previously used a study that has exposure for 90 days after the pups are weaned. It became apparent that this basic design could easily be adapted to provide more detailed information on reproduction and development, as well as all the necessary information to select dose levels for the cancer study. Thus, there would be a gain in information on a greater array of toxicity endpoints and maximize the utility of the animals that had already been produced and raised to weaning, such that the other, "stand-alone" reproduction and developmental toxicity studies would not be required.

This new NTP design (see Fig. 21-39) employs pregnant animals, with dosing commencing at implantation (gestation day 6 in the rat) and continually exposes the dams throughout gestation and lactation. At weaning (usually postnatal day 21 in the rat), the offspring would be continued to be administered the test article at the same dose level as their respective dam and are subsequently assigned to a number of different cohorts that can be considered as interchangeable "cassettes" that can be included, or not, based on the objectives of the study, or other available information. These cassettes are essentially protocols used on other standard studies and would normally include:

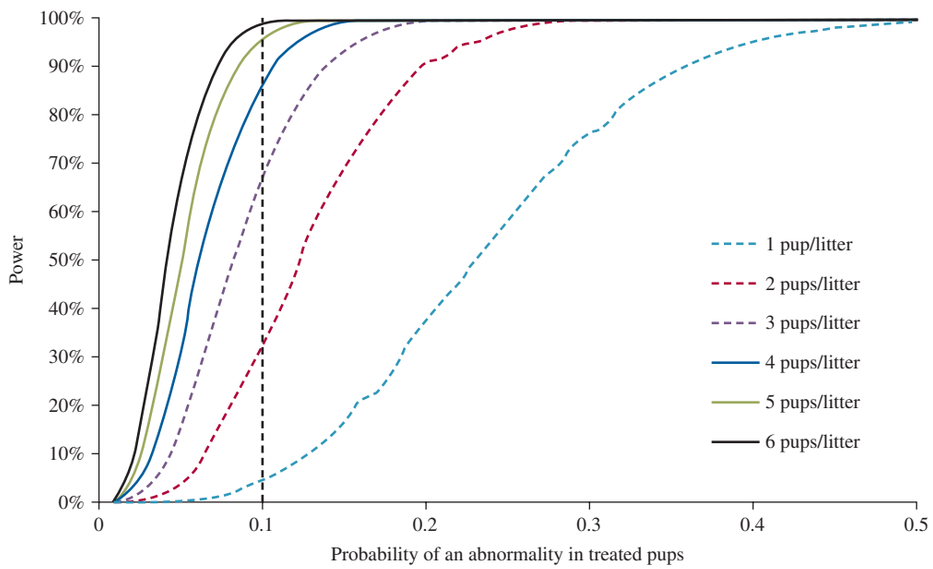
- An evaluation of target organ toxicity, pathology, clinical pathology, etc., similar to a current 90-day toxicity protocol—*a subchronic toxicity cohort*. This would normally require 10 animals to be evaluated per sex, per dose group.
- An evaluation of prenatal developmental toxicity—*a teratology cohort*. One male and female offspring from each litter would be selected and nonsibling matings would be performed in each group on reaching sexual maturity (~PND 110). Just prior to expected delivery, a Cesarean section would be performed on the pregnant dams for a standard evaluation of external, visceral, and skeletal abnormalities of the fetuses.
- An evaluation of breeding performance—*a littering cohort*. One male and female offspring from each litter would be



Parental generation	Cohort	Designation	Animals/cohort	Sexual maturation	Approximate age at necropsy (weeks)
Target is 20 litters per group	1A	Reproductive	20 M + 20 F	Yes	13
	1B	Reproductive	20 M + 20 F	Yes	14 or 20 if triggered
	2A	Neurotoxicity	10 M + 10 F@	Yes	9
	2B	Neurotoxicity	10 M + 10 F@	No	3
	3	Immunotoxicity	10 M + 10 F@	Yes	8
	Surplus	Spares		No	3

@ one per litter and representative of 20 litters in total where possible

Figure 21-37. *Diagrammatic representation of the draft OECD extended one-generation reproduction study.* OECD (2010), OECD Guideline for the testing of chemicals, http://www.oecd.org/chemicalsafety/testingofchemicals/46466062.pdf.



10% incidence detected 4.7% of time with 1 pup; 66.4% 3 pups; 86.5% 4 pups

Figure 21-38. *Power curves for the detection of offspring abnormalities with low background incidence (20 litters, 0% background).* Curves are presented for the probability of detecting an adverse response with increasing numbers of pups retained per litter. Note that with only one pup retained (typically the approach on most reproduction studies) the probability of detecting a 10% response (shown by the *vertical dotted line*) will be only 4.7% for one pup per litter, but 66.4% for three pups per litter and 86.5 for four pups per litter. (From Blystone et al., 2010.)

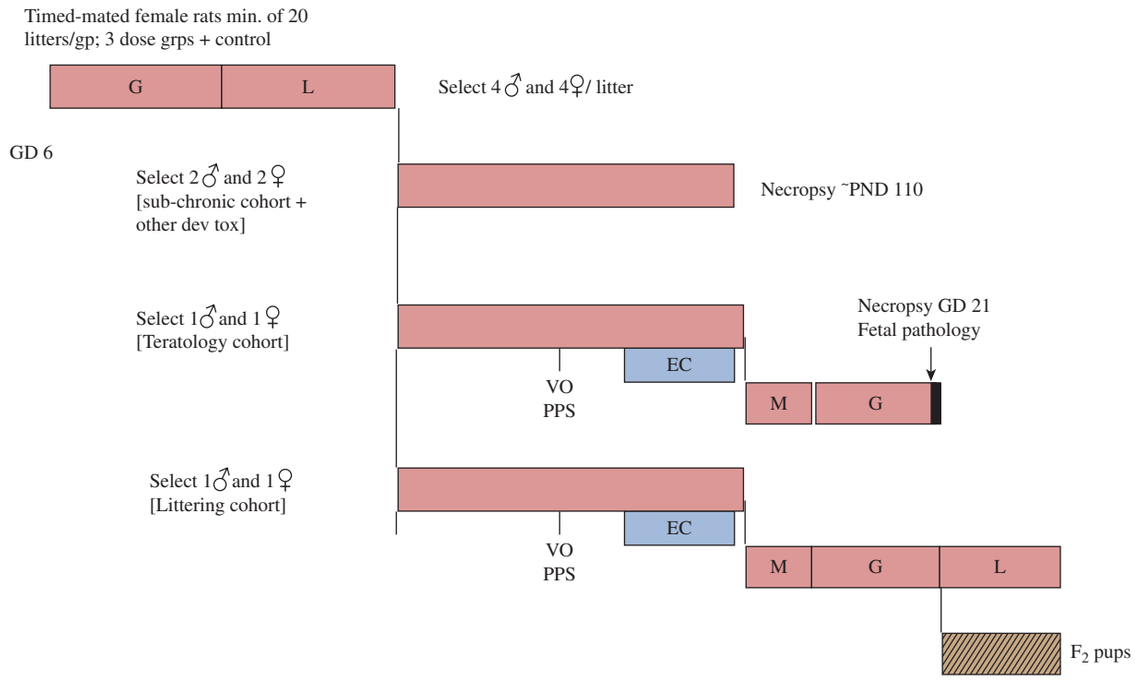


Figure 21-39. *Diagrammatic representation of the NTP modified one generation reproduction study.* Only 10 pups per sex (on reaching adulthood) are required for the subchronic cohort and thus sufficient numbers of animals would be available for evaluations of other developmental toxicity that may include effects on the developing immune or nervous systems. G, Gestation; L, lactation; PND, postnatal day; GD, gestation day; M, mating; VO, vaginal opening; PPS, balanopreputial separation; EC, estrous cyclicity evaluation.

selected and nonsibling matings would be performed in each group on reaching sexual maturity (~PND 110). The pregnant dams would be allowed to deliver their litters and raise them to weaning.

On a specific case basis, other cassettes could be added, or substituted, in the protocol, including an assessment of developmental immunotoxicity and/or developmental neurotoxicity, using other standard guideline protocols.

The study would normally be conducted in the rat with a sufficient number of time-mated animals acquired to ensure a minimum of 20 litters per dose group with normally three dose levels, plus a vehicle control. The normal route of exposure employed on such studies would be oral (e.g., dosed feed, drinking water, or gavage) and treatment would be continuous throughout the study (for gavage, direct dosing of pups may be required at least from PND 12, or as appropriate from toxicokinetic [TK] information). If no other toxicity information is available, a pilot study with a small number of pregnant dams would be required to set dose levels and potentially acquire preliminary TK data in pregnancy and lactation.

This design emphasizes a full evaluation of the F_1 animals in the study. The design uses significantly fewer animals than a RACB or a multigeneration reproduction study and generates important information on both reproduction and postnatal development, together with a pathological evaluation of all the offspring (after PND 4) when they reach adulthood to improve the ability to detect postnatal effects. A major addition will be the information achieved on prenatal developmental toxicity. The teratology and littering cohorts of animals allow the evaluation of fertility and fecundity and importantly, to maintain the relationship between structural changes in the reproductive organs and functional outcomes in the same animals. In addition, the design will be able to maintain a 10-week exposure period prior to mating of the F_1 animals to ensure that any potential male germ cell effects could be reflected in a functional outcome.

The OECD extended one-generation study (Fig. 21-37; OECD 443), while using fewer animals than a typical multigeneration study, does have a number of significant design flaws that have been highlighted in recent publications (Foster 2014, 2017). Some of these issues include: (1) a truncated exposure period before breeding the F_0 animals that will only produce functional effects in males if the chemical under investigation affects epididymal sperm; (2) a failure to routinely breed F_1 animals and so not provide a full characterization of this unique exposure group; (3) the employment of internal triggers to make decisions on when breeding of the F_1 generation, or utilization of specific cohorts of animals, will occur. In particular, the triggers to be employed do not take into account the timing for the production of data from the various components of the study, to make informed decisions on whether to apply triggers, or not; (4) the adverse structural changes will not be related to functional outcomes in the same animals if the triggers are not applied correctly; and (5) the developmental neurotoxicity cohort is underpowered with only 10 animals/sex/group evaluated and has no assessment of learning and memory. Using this proposed OECD design, one could conduct a reproduction study that does not adequately evaluate fertility and fecundity in any generation. Although the EU has endorsed the utility of OECD 443 to replace the multigeneration study (OECD 416) for REACH submission, a recent review of draft implementation (ECHA, 2016) has implied that many of the design shortcomings noted above are required to be corrected—a 10 week pre-exposure period, routinely breeding the F_1 animals, removal of internal triggers, which really brings the 443 study to being more similar to the 416 multigeneration study.

EVALUATION OF TOXICITY TO REPRODUCTION

Concordance of Endpoints

As noted in the section "Testing for Reproductive Toxicity," our standard testing protocols do not allow for discrimination between male and female effects except by some method modification. However, there is built-in redundancy in the number and type of endpoints evaluated in these studies that could indicate male, female, or effects on both sexes during testing. The following examples give an outline of the types of endpoints evaluated typically in rat multigeneration studies (the species most commonly used for reproductive toxicity evaluations).

There are a number of general points that the investigator should note in any estimation of potential reproductive toxicity:

- Adequacy of experimental design and conduct. Was there sufficient statistical power in the evaluation(s)?
- Occurrence of common versus rare reproductive deficits. Biological versus statistical significance.
- Use of historical control data to place concurrent control data into perspective and to estimate population background incidence of various reproductive parameters and deficits.
- Known structure–activity relationships for inducing reproductive toxicity.
- Concordance of reproductive endpoints (e.g., did a decrease in litter size relate to ovarian histology and changes in vaginal cytology?).
- Did the reproductive deficits become more severe with increases in dose? For example, did histological changes at one dose level become decrements in litter size and then reductions in fertility at higher dose levels in any generation?
- Did the reproductive deficits increase in prevalence (more individuals and/or more litters) with dose level in any generation?
- Special care should be taken for decrements in reproductive parameters noted in the F_1 generation (and potentially later generations) that were not seen in the F_0 generation, which may suggest developmental, as well as reproductive, toxicity. Likewise, findings in an F_1 generation animal may (or may not) be reproduced in F_2 offspring. For example, effects in the F_1 generation on reproductive parameters may have resulted in the selection out of sensitive animals in the population, thus not producing F_2 offspring for subsequent evaluation.

The first primary indication of a decrement in reproduction is obtained from the inspection of litter parameters from a breeding and determination of any functional effects. Thus, it is normal to inspect for various treatment groups versus the control, the number of fertile pairings, the mean litter size, pup weight at birth (small litters tend to have higher pup weights), and the sex ratio (to determine if there is a selective effect on any one sex).

In the parental females, there should also be an evaluation of potential maternal toxicity that may or may not impact on the reproductive performance of these animals; these measures are crude and usually restricted to body weight, food consumption, and clinical signs. Gestation length is normally recorded (and this may be shortened or lengthened) together with any signs of dystocia. Chemicals with progestin-like activity may also produce midgestational bleeding observable on the fur/or in the cage. At weaning of the litter (usually PND 21 in the rat), the parental females are usually necropsied (if no further pairings are required) and the uteri examined for implantation sites (which can be compared to the number of offspring produced) and the ovaries examined for corpora lutea and corpora albicans. These data are not as definitive for pre- versus postimplantation loss as in the developmental toxicity study because the female will begin to cycle around postnatal day 15 and have representative corpora lutea from both the pregnancy and the new ovarian cycle(s).

In immature females after weaning, measurements can be made of the onset of puberty (which may be accelerated or delayed) through estimates of the date of VO and first estrus from vaginal smears. Some care needs to be used in the impact of other toxicity on these pubertal parameters, and it is usual to normalize based on bodyweight. However, timing of puberty should not be normalized by body weight at acquisition of puberty (since a delay will invariably mean that the females are larger compared to controls and vice versa for acceleration). Instead, it is more likely that the investigator would employ weight at weaning as a covariate in the analysis of these pubertal endpoints.

As the female matures, estimates will be made of estrous cyclicity particularly if they are normal or abnormal (a rat normally has a 4- to 5-day cycle) and the duration of the cycles (e.g., was persistent estrus or diestrus noted). Vaginal smears are also noted during mating, and the presence of a sperm positive smear would indicate a successful mating that provides at least some surrogate of normal reproductive behavior from the breeding pair.

At necropsy of the adult F_1 female, weights and histopathology would be undertaken on critical organs (e.g., ovaries, uterus, pituitary, and perhaps adrenal as another steroidogenic organ). Histopathology of the ovary is not straightforward and some guidelines require the evaluation of primordial follicle counts in step sections of the ovary. However, such an examination provides only limited information on one type of follicle and it is essential that the pathologists make their evaluation with regard to the normal expected patterns of the different follicular types (from primordial to antral) and when necessary undertake quantitative analysis to confirm an effect on ovarian follicular development.

In the parental males, similar types of evaluation are available to the investigator with regard to potential parental toxicity. Decreases in fertile pairings and litter size may be equally applicable for the detection of a male effect. The presence of a sperm positive smear in the female is indicative that the male has successfully mounted and mated with the female (note the presence of a copulatory plug is not the same because these may be formed from accessory sex organ fluids in the absence of sperm).

Males going through puberty can be estimated using the date of balanopreputial separation of the penis as an index. This androgen-dependent endpoint in the male is also corrected for growth, but the same caveats used for the female around normalization of data would apply to this male measurement. Decrements of ~10% body weight are normally without significant effect on male or female puberty indices (Carney et al., 2004). As the male matures and is bred with a female, observation of the precoital interval (the time between pairing and evidence of mating) can indicate treatment-related effects on mating performance or behavior. However, it should be noted that if the female is at a random stage of estrus when introduced to the male, she may not allow mating until estrus is attained. Thus, delays of more than 4 to 5 days in precoital interval should be carefully examined.

Once a pregnancy has been achieved and there is no requirement for further matings, the parental male is necropsied and organ weights and histological examination conducted usually of the testis, epididymis, prostate, seminal vesicles, and pituitary (and perhaps adrenal as for the female as another steroidogenic organ). Some studies also include other androgen-dependent organ weights such as the levator ani–bulbocavernosus muscle, Cowper's gland, and penis. As with the ovary, histopathological examination of the testis requires some experience and the pathologist must be familiar

with the different stages of the spermatogenic cycle. Although quantitative analysis of stage frequency is rarely required (and not useful in longer term studies), the noting of specific stages that may show a predilection for injury is most helpful, as is an appreciation of the different cellular associations within the seminiferous tubule, which is essential in the detection of more subtle testicular effects.

At necropsy, a number of quantitative estimates can be made of sperm production and function. These can include testicular spermatid counts following homogenization (this endpoint entails destruction of one testis and has to be balanced with the information obtained from histopathology). A sperm sample is also taken from the vas or cauda epididymis for examination of sperm concentration, motility, and morphology, which can be examined using manual methods. More often, computer-assisted sperm analysis is used to evaluate these sperm endpoints and in addition further parameters can be collected, such as forward progressive motility, which is believed more closely related to fertility. Frequently, whole cauda epididymal sperm counts are also evaluated and normalized by weight.

Evaluation of male and female offspring follows the schema outlined above for adults and for animals going through puberty. In addition, other useful endpoints may be included before weaning such as AGD, the presence of nipples and areolae (usually at post natal day 13 before the pup fur has started to grow excessively to prevent examination), and the presence of certain reproductive tract malformations (see Tables 21-7 and 21-8). AGD and nipple retention are sexually dimorphic in the rat and under androgen control. Thus, normally AGD is twice the distance in males than females (this also seems to hold true for humans) and is dependent on levels of circulating DHT. In the normal male rat fetus, the presence of androgen around gestation day 17 causes the anlagen for the nipples to undergo apoptosis and thus males are born without nipples whereas females have the full complement of 12. The presence of an antiandrogen in males or an androgen in females would upset this balance to produce nipple retention in the males and a reduced number in the females reflective of the androgen status of these animals at critical periods of development. Some assessment must also be made of general growth of the offspring (usually bodyweight).

Consistency Across Generations

The F_0 reproductive parameters can differ markedly from those noted in the F_1 and similarly from the F_2 generation in a multigeneration study. Because exposure in a multigeneration study typically starts with the F_0 generation as young adults, critical periods of reproductive development have already taken place. The phthalates (e.g., DBP; Wine et al., 1997) represent a classic example of weak effects noted in the F_0 generation (all the pairs were fertile, but there was a small but significant effect on litter size and pup growth). In the F_1 generation where exposure is from conception to adulthood, when these animals were bred at the same dose, only 19/20 pairings resulted in a litter and thus the effects on reproduction were significantly enhanced in the F_1 generation and illustrate the importance of breeding the F_1 animals to detect functional effects on the offspring due to in utero exposure.

Differences between F_1 and F_2 generations can also arise. Here the exposure duration and critical windows of development are the same. However, since one normally only takes one male and female from each litter to generate the F_1 and F_2 parents, it is distinctly possible that a selection bias can exist (e.g., if sensitive animals did not produce a litter, or the sensitive pups within a litter did not survive, then a pup from this pairing cannot be selected for further generations) and therefore potential effects may decrease in incidence or severity across generations. Other specific effects may increase severity in the F_2 versus the F_1. For example, a selective effect during in utero exposure of F_1 on the mammary gland would show the F_1 population as being normal with regard to reproduction, but when the F_1 animal becomes a parent, it cannot raise the F_2 adequately because of a mammary effect. In this instance, there would be a more profound influence on the F_2 than the F_1 litters.

Graded Effects

In a similar fashion to developmental toxicity, it is important to keep in mind the ability of changes in dose to alter the outcome of reproductive toxicity studies such that it is not just an increase in severity of a reproductive deficit that may be noted with increased dose, but potentially an increased prevalence of a specific effect (on either an individual or litter basis). There may also be an increase in the severity of the type of lesion with the full constellation of the effects only noted at the highest dose level. Thus, for example, a single compound may produce a subtle effect on testis histology at low-dose levels. As the dose level increases, this lesion may become more severe, with pathology noted in the epididymis, which in turn may affect semen parameters that result in a decrease in litter size and as the dose increases further, to a reduction in the number of fertile pairs. Thus, there is a continuum of effects, that may be shown by a chemical producing reproductive toxicity that are interrelated and become important in selecting appropriate effect dose levels and therefore those exposures that maybe without effect.

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REFERENCES

Adams NR. Permanent infertility in ewes exposed to plant oestrogens. *Aust Vet J*. 1990;67:197–201.

Adams NR, Sanders MR. Persistent infertility in ewes after prolonged exposure to oestradiol-17 beta. *J Reprod Fertil*. 1988;84:373–378.

Aho M, Koivisto AM, Tammela TL, Auvinen A. Is the incidence of hypospadias increasing? Analysis of Finnish hospital discharge data 1970–1994. *Environ Health Perspect*. 2000;108:463–465.

Andersen AN, Gianaroli L, Felberbaum R, de Mouzon J, Nygren KG. Assisted reproductive technology in Europe, 2001. Results generated from European registers by ESHRE. *Hum Reprod*. 2005;20:1158–1176.

Andersson KE, Wagner G. Physiology of penile erection. *Physiol Rev*. 1995;75:191–236.

Andrade AJ, Grande SW, Talsness CE, et al. A dose response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): reproductive effects on adult male offspring rats. *Toxicology*. 2006a;228:85–97.

Andrade AJ, Grande SW, Talsness CE, Grote K, Chahoud I. A dose–response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose–response and low dose effects on rat brain aromatase activity. *Toxicology*. 2006b;227:185–192.

Ankley GT, Jensen KM, Makynen EA, et al. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ Toxicol Chem*. 2003;22(6):1350–1360.

Ankley GT, Villeneuve DL. Temporal changes in biological responses and uncertainty in assessing risks of endocrine-disrupting chemicals: insights from intensive time-course studies with fish. *Toxicol Sci*. 2015;144:259–275.

Aplin JD, Kimber SJ. Trophoblast–uterine interactions at implantation. *Reprod Biol Endocrinol*. 2004;2:48.

Ashby J, Owens W, Deghenghi R, Odum J. Concept evaluation: an assay for receptor-mediated and biochemical antiestrogens using pubertal rats. *Regul Toxicol Pharmacol*. 2002;35(3):393–397.

Ashby J, Tinwell H, Haseman J. Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF1 mice exposed in utero. *Regul Toxicol Pharmacol*. 1999;30:156–166.

Atwood CS, Meethal SV, Liu T, et al. Dysregulation of the hypothalamic–pituitary–gonadal axis with menopause and andropause promotes neurodegenerative senescence. *J Neuropathol Exp Neurol*. 2005;64:93–103.

Auchus RJ, Rainey WE. Adrenarche—physiology, biochemistry and human disease. *Clin Endocrinol*. 2004;60:288–296.

Bardin CW, Catterall JF. Testosterone: a major determinant of extragenital sexual dimorphism. *Science (New York, NY)*. 1981;211:1285–1294.

Barker DJ. Fetal origins of coronary heart disease. *BMJ*. 1995;311:171–174.

Barker DJ. Fetal origins of cardiovascular disease. *Ann Med*. 1999;31(suppl 1):3–6.

Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia*. 1993;36:62–67.

Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet*. 1989;2:577–580.

Barlow NJ, McIntyre BS, Foster PM. Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(*n*-butyl) phthalate. *Toxicol Pathol*. 2004;32:79–90.

Battaglia C, De Iaco P, Iughetti L, et al. Female precocious puberty, obesity and polycystic-like ovaries. *Ultrasound Obstet Gynecol*. 2005;26:651–657.

Beausoleil C, Beronius A, Bodin L, et al. Review of non-monotonic dose–responses of substances for human risk assessment. *EFSA Supporting Publications*. 2016;13:1.

Becker AL, Epperson CN. Female puberty: clinical implications for the use of prolactin-modulating psychotropics. *Child Adolesc Psychiatr Clin N Am*. 2006;15:207–220.

Berman DM, Tian H, Russell DW. Expression and regulation of steroid 5α-reductase in the urogenital tract of the fetal rat. *Mol Endocrinol*. 1995;9:1561–1570.

Berman E, Laskey JW. Altered steroidogenesis in whole-ovary and adrenal culture in cycling rats. *Reprod Toxicol*. 1993;7:349–358.

Bhasin S, Parker RA, Sattler F, et al. Effects of testosterone supplementation on whole body and regional fat mass and distribution in human immunodeficiency virus-infected men with abdominal obesity. *J Clin Endocrinol Metab*. 2007;92:1049–1057.

Bhasin S, Woodhouse L, Casaburi R, et al. Older men are as responsive as young men to the anabolic effects of graded doses of testosterone on the skeletal muscle. *J Clin Endocrinol Metab*. 2005;90:678–688.

Biegel LB, Flaws JA, Hirshfield AN, et al. 90-day feeding and one-generation reproduction study in Crl:CD BR rats with 17 beta-estradiol. *Toxicol Sci*. 1998;44:116–142.

Bielmeier SR, Best DS, Narotsky MG. Serum hormone characterization and exogeneous hormone rescue of bromodichloromethane-induced pregnancy loss in the F344 rat. *Toxicol Sci*. 2004;77:101–108.

Biro FM, Khoury P, Morrison JA. Influence of obesity on timing of puberty. *Int J Androl*. 2006;29:272–277; discussion 286–290.

Blackburn DM, Gray AJ, Lloyd SC, Sheard CM, Foster PMD. A comparison of the effects of the three isomers of dinitrobenzene on the testis in the rat. *Toxicol Appl Pharmacol*. 1988;92:54–64.

Blanck HM, Marcus M, Tolbert PE, et al. Age at menarche and tanner stage in girls exposed in utero and postnatally to polybrominated biphenyl. *Epidemiology (Cambridge, Mass)*. 2000;11:641–647.

Blystone CR, Kissling GE, Bishop JB, Chapin RE, Wolfe GW, Foster PM. Determination of the di-(2-ethylhexyl) phthalate (DEHP) NOAEL for reproductive development in the rat: importance of the retention of extra animals to adulthood. *Toxicol Sci*. 2010;116:640–646.

Blystone CR, Lambright CS, Howdeshell KL, et al. Sensitivity of fetal rat testicular steroidogenesis to maternal prochloraz exposure and the underlying mechanism of inhibition: prochloraz reduced fetal testis testosterone. *Toxicol Sci*. 2007;97:512–519.

Boberg J, Mandrup KR, Jacobsen PR, et al. Endocrine disrupting effects in rats perinatally exposed to a dietary relevant mixture of phytoestrogens. *Reproductive Toxicology*. 2013;40:41–51.

Boekelheide K, Darney SP, Daston GP, et al. NTP-CERHR Expert Panel Report on the reproductive and developmental toxicity of 2-bromopropane. *Reprod Toxicol*. 2004;18:189–217.

Boisen KA, Kaleva M, Main KM, et al. Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. *Lancet*. 2004;363:1264–1269.

Bowman CJ, Barlow NJ, Turner KJ, Wallace DG, Foster PMD. Effects of in utero exposure to finasteride on androgen-dependent reproductive development in the male rat. *Toxicol Sci*. 2003;74:393–406.

Brouwer A, Longnecker MP, Birnbaum LS, et al. Characterization of potential endocrine-related health effects at low-dose levels of exposure to PCBs. *Environ Health Perspect*. 1999;107(suppl 4):639–649.

Buchanan JF, Davis LJ. Drug-induced infertility. *Drug Intell Clin Pharm*. 1984;18:122–132.

Buck Louis GM, Gray LE Jr, Marcus M, et al. Environmental factors and puberty timing: expert panel research needs. *Pediatrics*. 2008;121(suppl 3):S192–S207.

Buhimschi CS. Endocrinology of lactation. *Obstet Gynecol Clin North Am*. 2004;31:963–979, xii.

Byskov AG. Differentiation of mammalian embryonic gonad. *Physiol Rev*. 1986;66:71–117.

Cain JC. Miroestrol: an oestrogen from the plant Pueraria mirifica. *Nature*. 1960;188:774–777.

Calafat AM, Brock JW, Silva MJ, et al. Urinary and amniotic fluid levels of phthalate monoesters in rats after the oral administration of di(2-ethylhexyl) phthalate and di-*n*-butyl phthalate. *Toxicology*. 2006;217:22–30.

Calof OM, Singh AB, Lee ML, et al. Adverse events associated with testosterone replacement in middle-aged and older men: a meta-analysis of randomized, placebo-controlled trials. *J Gerontology A Biol Sci Med Sci*. 2005;60:1451–1457.

Canning DA. Hypospadias trends in two US surveillance systems. Rise in prevalence of hypospadias. *J Urol*. 1999;161:366.

Cannon SSB, Veazey JJM, Jackson RRS, et al. Epidemic kepone poisoning in chemical workers. *Am J Epidemiol*. 1978;107:529–537.

Cappon GD, Bailey GP, Buschmann J, et al. Juvenile animal toxicity study designs to support pediatric drug development. *Birth Defects Res B Dev Reprod Toxicol*. 2009;86:463–469.

Carmichael SL, Shaw GM, Laurent C, Croughan MS, Olney RS, Lammer EJ. Maternal progestin intake and risk of hypospadias. *Arch Pediatr Adolesc Med*. 2005;159:957–962.

Carnathan GW, Metcalf LE, Cochrane RL, Nutting EF, Black DL. Relationship between progesterone suppression and pregnancy in rats. *J Pharm Pharmacol*. 1987;39:401–404.

Carney EW, Zablotny CL, Marty MS, et al. The effects of feed restriction during in utero and postnatal development in rats. *Toxicol Sci*. 2004;82:237–249.

Carruthers CM, Foster PM. Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. *Birth Defects Res B Dev Reprod Toxicol*. 2005;74:277–285.

Cave DA, Foster PMD. Modulation of *m*-dinitrobenzene and *m*-nitrosonitrobenzene toxicity in rat Sertoli—germ cell co-cultures. *Fund Appl Toxicol*. 1990;14:199–207.

Challis JR, Bloomfield FH, Bocking AD, et al. Fetal signals and parturition. *J Obstet Gynaecol Res*. 2005;31:492–499.

Chapin R, Dutton S, Ross M, Lamb J. Effects of ethylene glycol monomethyl ether (EGME) on mating performance and epididymal sperm parameters in F344 rats. *Fundam Appl Toxicol*. 1985;5:182–189.

Chapin RE, Harris MW, Davis BJ, et al. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam Appl Toxicol*. 1997;40:138–157.

Chapin RE, Ku WW. The reproductive toxicity of boric acid. *Environ Health Perspect*. 1994;102(suppl 7):87–91.

Chapin RE, Phelps JL, Schwetz BA, Yang RS. Toxicology studies of a chemical mixture of 25 groundwater contaminants. III. Male reproduction study in B6C3F1 mice. *Fundam Appl Toxicol*. 1989;13:388–398.

Charlier C. Endocrine effects of environmental pollutants. *Bull Mem Acad R Med Belg*. 2005;160:301–310.

Charlier C. Effects of environmental pollutants on hormone disturbances. *Bull Mem Acad R Med Belg*. 2006;161:116–124; discussion 124–116.

Christiansen S, Kortenkamp A, Axelstad M, et al. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. *International Journal of Andrology*. 2012;35:303–316.

Christiansen S, Scholze M, Dalgaard M, et al. Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. *Environmental Health Perspectives*. 2009;117:1839–1846.

Clark RL, Anderson CA, Prahalada S, et al. Critical developmental periods for effects on male rat genitalia induced by finasteride, a 5 alpha-reductase inhibitor. *Toxicology and Applied Pharmacology*. 1993;119:34–40.

Clark RL, Antonello JM, Grossman SJ, et al. External genitalia abnormalities in male rats exposed in utero to finasteride, a 5 alpha-reductase inhibitor. *Teratology*. 1990;42:91–100.

Coady K, Matthiessen P, Zahner HM, et al. Endocrine disruption: where are we with hazard and risk assessment? *PeerJ Preprints*. 2016;4:e2580v1.

Colbert NK, Pelletier NC, Cote JM, et al. Perinatal exposure to low levels of the environmental antiandrogen vinclozolin alters sex-differentiated social play and sexual behaviors in the rat. *Environ Health Perspect*. 2005;113:700–707.

Colborn T. The wildlife/human connection: modernizing risk decisions. *Environ Health Perspect*. 1994;102(suppl 12):55–59.

Collins TF. History and evolution of reproductive and developmental toxicology guidelines. *Curr Pharm Des*. 2006;12(12):1449–1465.

Conley JM, Lambright CS, Evans N, Cardon MC, Wilson VS, Gray LE Jr. Oral exposure to a mixture of 18 anti-androgens with varying mechanisms of action at concentrations below individual chemical effect levels produces reproductive tract malformations in the male rat. *The Toxicologist*. 2017.

Cooper RL, Barrett MA, Goldman JM, Rehnberg GR, McElroy WK, Stoker TE. Pregnancy alterations following xenobiotic-induced delays in ovulation in the female rat. *Fundam Appl Toxicol*. 1994;22:474–480.

Cooper RL, Lamb JC, Barlow SM, et al. A tiered approach to life stages testing for agricultural chemical safety assessment. *Crit Rev Toxicol*. 2006;36:69–98.

Cooper RL, Roberts B, Rogers DC, Seay SG, Conn PM. Endocrine status versus chronologic age as predictors of altered luteinizing hormone secretion in the "aging" rat. *Endocrinology*. 1984;114:391–396.

Cooper RL, Stoker TE, Tyrey L, Goldman JM, McElroy WK. Atrazine disrupts the hypothalamic control of pituitary-ovarian function. *Toxicol Sci*. 2000;53:297–307.

Coviello AD, Lakshman K, Mazer NA, Bhasin S. Differences in the apparent metabolic clearance rate of testosterone in young and older men with gonadotropin suppression receiving graded doses of testosterone. *J Clin Endocrinol Metab*. 2006;91:4669–4675.

Creasy DM, Beech LM, Gray TJ, Butler WH. An ultrastructural study of ethylene glycol monomethyl ether-induced spermatocyte injury in the rat. *Exp Mol Pathol*. 1986;45:311–322.

Creasy DM, Beech LM, Gray TJB, Butler WH. The ultrastructural effects of di-*n*-pentyl phthalate on the testis of the mature rat. *Exp Mol Pathol*. 1987;46:357–371.

Creasy DM, Foster PMD. The morphological development of glycol ether-induced testicular atrophy in the rat. *Exp Mol Path*. 1984;40:169–176.

Cummings AM, Perreault SD. Methoxychlor accelerates embryo transport through the rat reproductive tract. *Toxicol Appl Pharmacol*. 1990;102:110–116.

DeCherney AH, Cholst I, Naftolin F. Structure and function of the fallopian tubes following exposure to diethylstilbestrol (DES) during gestation. *Fertil Steril*. 1981;36:741–745.

Delclos KB, Bucci TJ, Lomax LG, et al. Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. *Reproductive Toxicology*. 2001;15:647–663.

Delclos KB, Newbold R. NTP toxicity report of reproductive dose range-finding study of Genistein (CAS No. 446-72-0) administered in feed to Sprague-Dawley rats. *Toxic Rep Ser*. 2007;1–C2.

Delclos KB, Weis CC, Bucci TJ, et al. Overlapping but distinct effects of genistein and ethinyl estradiol (EE(2)) in female Sprague-Dawley rats in multigenerational reproductive and chronic toxicity studies. *Reproductive Toxicology*. 2009;27:117–132.

Den Hond E, Schoeters G. Endocrine disrupters and human puberty. *Int J Androl*. 2006;29:264–271; discussion 286–290.

Doody KJ, Lephart ED, Stirling D, et al. Expression of mRNA species encoding steroidogenic enzymes in the rat ovary. *J Mol Endocrinol*. 1991;6:153–162.

Duck SC, Katayama KP. Danazol may cause female pseudohermaphroditism. *Fertil Steril*. 1981;35:230–231.

Dunger DB, Ahmed ML, Ong KK. Effects of obesity on growth and puberty. *Best Pract Res Clin Endocrinol Metab*. 2005;19:375–390.

Durhan EJ, Lambright C, Wilson V, et al. Evaluation of androstenedione as an androgenic component of river water downstream of a pulp and paper mill effluent. *Environ Toxicol Chem*. 2002;21(9):1973–1976.

Durhan EJ, Lambright CS, Makynen EA, et al. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. *Environ Health Perspect*. 2006;114(suppl 1):65–68.

ECHA. Guidance on Information Requirements and Chemical Safety Assessment. Chapter R.7a: Endpoint specific guidance. Version 5.0. Helsinki, Finland, European Chemicals Agency. 2016:445–454.

Eddy E, Gong D, Fenderson A. Origin and migration of primoridal germ cells in mammals. *Gamete Res.* 1981;4:333–336.

EDSTAC Final Report. 1998. Available at: http://www.epa.gov/endo/pubs/edspoverview/finalrpt.htm.

Ellis MK, Foster PMD. The metabolism of 1,3-dinitrobenzene by rat testicular subcellular fractions. *Toxicol Lett*. 1992;62:201–208.

Ellis RJ, van den Heuvel MR, Bandelj E, et al. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ Toxicol Chem*. 2003;22(7):1448–1456.

Enders AC, Carter AM. What can comparative studies of placental structure tell us?—a review. *Placenta*. 2004;25:S3–S9.

EPA. State of the Science Evaluation: Nonmonotonic Dose–responses as they apply to estrogen, androgen, and thyroid pathways and EPA testing and assessment procedures. 2013.

Epstein SS. Kepone—hazard evaluation. *Science Total Environ*. 1978;9:1–62.

Eskenazi B, Mocarelli P, Warner M, et al. Seveso Women's Health Study: a study of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on reproductive health. *Chemosphere*. 2000;40:1247–1253.

Ewing LL, Desjardins C, Irby DC, Robaire B. Synergistic interaction of testosterone and oestradiol inhibits spermatogenesis in rats. *Nature*. 1977;269:409–411.

Ewing LL, Gorski RA, Sbordone RJ, Tyler JV, Desjardins C, Robaire B. Testosterone–estradiol filled polydimethylsiloxane subdermal implants: effect on fertility and masculine sexual and aggressive behavior of male rats. *Biol Reprod*. 1979;21:765–772.

Ewing LL, Zirkin BR, Chubb C. Assessment of testicular testosterone production and Leydig cell structure. *Environ Health Perspect*. 1981;38:19–27.

Farnsworth NR. The role of ethnopharmacology in drug development. *Ciba Foundation Symp*. 1990;154:2–11; discussion 11–21.

Farnsworth NR, Bingel AS, Cordell GA, Crane FA, Fong HS. Potential value of plants as sources of new antifertility agents II. *J Pharm Sci*. 1975;64(5):717–754.

Faroon O, Kueberuwa S, Smith L, DeRosa C. ATSDR evaluation of health effects of chemicals. II. Mirex and chlordecone: health effects, toxicokinetics, human exposure, and environmental fate. *Toxicol Ind Health*. 1995;11:1–203.

Fisher JS. Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. *Reproduction*. 2004;127:305–315.

Foster PM. Regulatory Forum opinion piece: New testing paradigms for reproductive and developmental toxicity—the NTP modified one generation study and OECD 443. *Toxicol Pathol*. 2014;42:1165–1167.

Foster PM. Influence of study design on developmental and reproductive toxicology study outcomes. *Toxicol Pathol*. 2017;45:107–113.

Foster PM, Cook MW, Thomas LV, Walters DG, Gangolli SD. Differences in urinary metabolic profile from di-*n*-butyl phthalate-treated rats and hamsters. A possible explanation for species differences in susceptibility to testicular atrophy. *Drug Metab Dispos*. 1983;11:59–61.

Foster PM, Mylchreest E, Gaido KW, Sar M. Effects of phthalate esters on the developing reproductive tract of male rats. *Hum Reprod Update*. 2001;7:231–235.

Foster PMD. Testicular organization and biochemical function. In: Lamb JC, Foster PMD, eds. *The Physiology and Toxicology of Male Reproduction*. New York: Academic Press; 1988:7–34.

Foster PMD. Studies on the toxicity of *m*-dinitrobenzene to the testicular Sertoli cell. *Arch Toxicol Suppl*. 1989;13:3–17.

Foster PMD. The cons of the extended 1-generation study protocol: how to get better hazard information and avoid the tyranny of triggers? *Reproduct Toxicol*. 2010a;30:224–224.

Foster PMD. The cons of the extended one—generation study protocol. *Birth Defect Res A Clin Mol Teratol*. 2010b;88:325.

Foster PMD, Lloyd SC, Blackburn DM. Comparison of the in vivo and in vitro testicular effects produced by methoxy-, ethoxy- and *n*-butoxy acetic acids in the rat. *Toxicology*. 1987;43:17–30.

Furr JR, Lambright CS, Wilson VS, Foster PM, Gray LE Jr. A short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation. *Toxicol Sci*. 2014;140:403–424.

Fussell KC, Schneider S, Buesen R, et al. Investigations of putative reproductive toxicity of low-dose exposures to flutamide in Wistar rats. *Arch Toxicol*. 2015;89:2385–2402.

Giwercman A, Rylander L, Hagmar L, Giwercman YL. Ethnic differences in occurrence of TDS—genetics and/or environment? *Int J Androl*. 2006;29:291–297; discussion 304–296.

Gladen BC, Klebanoff MA, Hediger ML, et al. Prenatal DDT exposure in relation to anthropometric and pubertal measures in adolescent males. *Environ Health Perspect*. 2004;112:1761–1767.

Goldman JM, Laws SC, Balchak SK, Cooper RL, Kavlock RJ. Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations. *Crit Rev Toxicol*. 2000;30:135–196.

Goldman JM, Parrish MB, Cooper RL, McElroy WK. Blockade of ovulation in the rat by systemic and ovarian intrabursal administration of the fungicide sodium dimethyldithiocarbamate. *Reprod Toxicol*. 1997;11:185–190.

Goldman JM, Stoker TE, Cooper RL, McElroy WK, Hein JF. Blockade of ovulation in the rat by the fungicide sodium *N*-methyldithiocarbamate: relationship between effects on the luteinizing hormone surge and alterations in hypothalamic catecholamines. *Neurotoxicol Teratol*. 1994;16:257–268.

Goldman JM, Stoker TE, Perreault SD, Cooper RL, Crider MA. Influence of the formamidine pesticide chlordimeform on ovulation in the female hamster: dissociable shifts in the luteinizing hormone surge and oocyte release. *Toxicol Appl Pharmacol*. 1993;121:279–290.

Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA. Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. *N Engl J Med*. 1998;338:1397–1404.

Gordon AE, Shaughnessy AF. Saw palmetto for prostate disorders. *Am Fam Phys*. 2003;67:1281–1283.

Goy RW, Bercovitch FB, McBrair MC. Behavioral masculinization is independent of genital masculinization in prenatally androgenized female rhesus macaques. *Horm Behav*. 1988;22(4):552–571.

Goy RW, Phoenix CH. The effects of testosterone propionate administered before birth on the development of behavior in genetic female rhesus monkeys. *UCLA Forum Med Sci*. 1972;15:193–201.

Goy RW, Resko JA. Gonadal hormones and behavior of normal and pseudohermaphroditic nonhuman female primates. *Recent Prog Horm Res*. 1972;28:707–733.

Goyeneche AA, Calvo V, Gibori G, Telleria CM. Androstenedione interferes in luteal regression by inhibiting apoptosis and stimulating progesterone production. *Biol Reprod*. 2002;66:1540–1547.

Grajewski B, Whelan EA, Schnorr TM, Mouradian R, Alderfer R, Wild DK. Evaluation of reproductive function among men occupationally exposed to a stilbene derivative: I. Hormonal and physical status. *Am J Ind Med*. 1996;29:49–57.

Gray LE. Emerging issues related to endocrine disrupting chemicals and environmental androgens and antiandrogens. In: Hutzinger O. ed. *The Handbook of Environmental Chemistry*. Heidelberg: Springer-Verlag; 2002:209–248.

Gray LE Jr. Tiered screening and testing strategy for xenoestrogens and antiandrogens. *Toxicol Lett*. 1998;102–103:677–680.

Gray LE Jr, Foster PM. Significance of experimental studies for assessing adverse effects of endocrine disrupting chemicals. *Pure Appl Chem*. 2004;75:2125–2141.

Gray LE, Foster PMD. Nonmonotonic dose–response curves and endocrine-disrupting chemicals: fact or falderal? *The Toxicologist*. 2013;132:341.

Gray LE Jr, Furr J, Tatum-Gibbs KR, et al. Establishing the "Biological Relevance" of Dipentyl Phthalate Reductions in Fetal Rat Testosterone Production and Plasma and Testis Testosterone Levels. *Toxicol Sci*. 2016;149:178–191.

Gray LE Jr, Kelce WR. Latent effects of pesticides and toxic substances on sexual differentiation of rodents. *Toxicol Ind Health*. 1996;12: 515–531.

Gray LE Jr, Kelce WR, Monosson E, Ostby JS, Birnbaum LS. Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamsters: reduced ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal androgenic status. *Toxicol Appl Pharmacol*. 1995;131:108–118.

Gray LE Jr, Kelce WR, Wiese T, et al. Endocrine screening methods workshop report: detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. *Reprod Toxicol*. 1997a;11:719–750.

Gray LE Jr, Ostby J. Effects of pesticides and toxic substances on behavioral and morphological reproductive development: endocrine versus nonendocrine mechanisms. *Toxicol Ind Health*. 1998;14:159–184.

Gray LE Jr, Ostby J, Ferrell J, et al. A dose–response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam Appl Toxicol*. 1989;12:92–108.

Gray LE, Ostby J, Furr J, et al. Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update*. 2001;7:248–264.

Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male Rat. *Toxicol Sci*. 2000;58:350–365.

Gray LE Jr, Ostby J, Linder R, Goldman J, Rehnberg G, Cooper R. Carbendazim-induced alterations of reproductive development and function in the rat and hamster. *Fundam Appl Toxicol*. 1990;15: 281–297.

Gray LE Jr, Ostby J, Monosson E, Kelce WR. Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol Ind Health*. 1999a;15(1–2):48–64.

Gray LE Jr, Ostby J, Sigmon R, et al. The development of a protocol to assess reproductive effects of toxicants in the rat. *Reprod Toxicol*. 1988a;2:281–287.

Gray LE Jr, Ostby J, Wilson V, et al. Xenoendocrine disrupters-tiered screening and testing: filling key data gaps. *Toxicology*. 2002;181–182, 371–382.

Gray LE Jr, Ostby JS. In utero 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. *Toxicol Appl Pharmacol*. 1995;133:285–294.

Gray LE Jr, Ostby JS, Ferrell JM, Sigmon ER, Goldman JM. Methoxychlor induces estrogen-like alterations of behavior and the reproductive tract in the female rat and hamster: effects on sex behavior, running wheel activity, and uterine morphology. *Toxicol Appl Pharmacol*. 1988b;96:525–540.

Gray LE Jr, Ostby JS, Kelce WR. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol*. 1994;129:46–52.

Gray LE, Ostby JS, Kelce WR. A dose–response analysis of the reproductive effects of a single gestational dose of 2,3,7,8-tetrachlorodibenzo-*p*- dioxin in male Long Evans Hooded rat offspring. *Toxicol Appl Pharmacol*. 1997b;146:11–20.

Gray LE, Rider C, Wilson V, et al. Cumulative effects of in utero administration to mixtures of reproductive toxicants in the male rat: a systems biology framework. In: Kaap RW, Tyl R, eds. *Target Organ Toxicology*. 3rd ed. Vol Reproductive Toxicology. Raven Press; 2010:332.

Gray LE Jr, Wilson V, Noriega N, et al. Use of the laboratory rat as a model in endocrine disruptor screening and testing. *ILAR J*. 2004;45:425–437.

Gray LE Jr, Wilson VS, Stoker T, et al. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. *Int J Androl*. 2006;29:96–104; discussion 105–108.

Gray LE Jr, Wolf C, Lambright C, et al. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate,

p,p′-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health*. 1999b;15:94–118.

Gray LE, Wolf C, Mann P, Ostby JS. In utero exposure to low doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin alters reproductive development of female Long Evans hooded rat offspring. *Toxicol Appl Pharmacol*. 1997c;146:237–244.

Gray PB, Singh AB, Woodhouse LJ, et al. Dose-dependent effects of testosterone on sexual function, mood, and visuospatial cognition in older men. *J Clin Endocrinol Metab*. 2005;90:3838–3846.

Gupta RK, Miller KP, Babus JK, Flaws JA. Methoxychlor inhibits growth and induces atresia of antral follicles through an oxidative stress pathway. *Toxicol Sci*. 2006a;93:382–389.

Gupta RK, Schuh RA, Fiskum G, Flaws JA. Methoxychlor causes mitochondrial dysfunction and oxidative damage in the mouse ovary. *Toxicol Appl Pharmacol*. 2006b;216:436–445.

Hadrup N, Svingen T, Mandrup K, et al. Juvenile male rats exposed to a low-dose mixture of twenty-seven environmental chemicals display adverse health effects. *PLoS ONE*. 2016;11:e0162027.

Hammond B, Katzenellenbogen BS, Krauthammer N, McConnell J. Estrogenic activity of the insecticide chlordecone (Kepone) and interaction with uterine estrogen receptors. *Proc Natl Acad Sci USA*. 1979;76:6641–6645.

Hammond SK, Smith TJ, Ellenbecker MJ. Determination of occupational exposure to fabric brightener chemicals by HPLC. *Am Ind Hygiene Assoc J*. 1987;48:117–121.

Hannas BR, Lambright CS, Furr J, et al. Genomic biomarkers of phthalate-induced male reproductive developmental toxicity: a targeted RT-PCR array approach for defining relative potency. *Toxicol Sci*. 2012;125:544–557.

Hass U, Scholze M, Christiansen S, et al. Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. *Environmental Health Perspectives*. 2007;115(suppl 1):122–128.

Hauser R, Williams P, Altshul L, et al. Predictors of serum dioxin levels among adolescent boys in Chapaevsk, Russia: a cross-sectional pilot study. *Environ Health*. 2005;4:8.

Hayes AW. *Principles and Methods of Toxicology*. New York: Raven Press; 1982; from previous edition of C&D.

Heindel JJ, Chapin RE, George J, et al. Assessment of the reproductive toxicity of a complex mixture of 25 groundwater contaminants in mice and rats. *Fundam Appl Toxicol*. 1995;25:9–19.

Hemsworth BN, Jackson H. Effect of busulphan on the developing ovary in the rat. *J Reprod Fertil*. 1963;6:229–233.

Henley DV, Lipson N, Korach KS, Bloch CA. Prepubertal gynecomastia linked to lavender and tea tree oils. *N Engl J Med*. 2007;356:479–485.

Herbst AL. Exogenous hormones in pregnancy. *Clin Obstet Gynecol*. 1973;16:37–50.

Herbst AL. The effects in the human of diethylstilbestrol (DES) use during pregnancy. *Princess Takamatsu Symposia*. 1987;18:67–75.

Herbst KL, Calof OM, Hsia SH, et al. Effects of transdermal testosterone administration on insulin sensitivity, fat mass and distribution, and markers of inflammation and thrombolysis in human immunodeficiency virus-infected women with mild to moderate weight loss. *Fertil Steril*. 2006;85:1794–1802.

Hershberger LG, Shipley EG, Meyer RK. Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. *Proc Soc Exp Biol Med*. 1953;83:175–180.

Hertz R. Accidental ingestion of estrogens by children. *Pediatrics*. 1958;21:203–206.

Higuchi TT, Palmer JS, Gray LE Jr, Veeramachaneni DN. Effects of Dibutyl Phthalate in Male Rabbits following in Utero, Adolescent, or Postpubertal Exposure. *Toxicol Sci*. 2003;72:301–313.

Himes JH. Examining the evidence for recent secular changes in the timing of puberty in US children in light of increases in the prevalence of obesity. *Mol Cell Endocrinol*. 2006;254–255;13–21.

Hines M. Sex steroids and human behavior: prenatal androgen exposure and sex-typical play behavior in children. *Ann N Y Acad Sci*. 2003;1007:272–282.

Hines M, Kaufman FR. Androgen and the development of human sex-typical behavior: rough-and-tumble play and sex of preferred playmates in children with congenital adrenal hyperplasia (CAH). *Child Dev*. 1994;65:1042–1053.

Holloway AJ, Moore HDM, Foster PMD. The use of in vitro fertilization to detect reductions in fertility of male rats exposed to 1,3-dinitrobenzene. *Fund Appl Toxicol*. 1990;14:113–122.

Hoodbhoy T, Dean J. Insights into the molecular basis of sperm–egg recognition in mammals. *Reproduction*. 2004;127:417–422.

Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenbergh JG, Gray LE Jr. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague–Dawley rats. *Toxicol Sci*. 2007;96:335–345.

Hotchkiss AK, Ostby JS, Vandenbergh JG, Gray LE Jr. An environmental antiandrogen, vinclozolin, alters the organization of play behavior. *Physiol Behav*. 2003;79:151–156.

Hotchkiss AK, Parks-Saldutti LG, Ostby JS, et al. A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biol Reprod*. 2004;71:1852–1861.

Hotchkiss AK, Rider CV, Blystone CR, et al. Fifteen years after "Wingspread"—environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicol Sci*. 2008;105:235–259.

Hotchkiss AK, Rider CV, Furr J, et al. In utero exposure to an AR antagonist plus an inhibitor of fetal testosterone synthesis induces cumulative effects on F1 male rats. *Reproductive Toxicology*. 2010;30:261–270.

Hotchkiss AK, Ostby JS, Vandenburgh JG, Gray LE Jr. Androgens and environmental antiandrogens affect reproductive development and play behavior in the Sprague–Dawley rat. *Environ Health Perspect*. 2002;110(suppl 3):435–439.

Howdeshell KL, Hotchkiss AK, Gray LE Jr. Cumulative effects of antiandrogenic chemical mixtures and their relevance to human health risk assessment. *Int J Hyg Environ Health*. 2016;220:179–188.

Howdeshell KL, Rider CV, Wilson VS, Furr JR, Lambright CR, Gray LE Jr. Dose addition models based on biologically relevant reductions in fetal testosterone accurately predict postnatal reproductive tract alterations by a phthalate mixture in rats. *Toxicol Sci*. 2015;148:488–502.

Howdeshell KL, Rider CV, Wilson VS, Gray LE Jr. Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats. *Environmental Research*. 2008;108:168–176.

Hoyer PB, Sipes IG. Development of an animal model for ovotoxicity using 4-vinylcyclohexene: a case study. *Birth Defects Res B Dev Reprod Toxicol*. 2007;80:113–125.

Huhtaniemi I, Pelliniemi LJ. Fetal Leydig cells: cellular origin, morphology, life span, and special functional features. *Proc Soc Exp Biol Med*. 1992;201:125–140.

Hunter RH, Rodriguez-Martinez H. Capacitation of mammalian spermatozoa in vivo, with a specific focus on events in the Fallopian tubes. *Mol Reprod Dev*. 2004;67:243–250.

Hurtt ME, Daston G, Davis-Bruno K, et al. Juvenile animal studies: testing strategies and design. *Birth Defects Res B Dev Reprod Toxicol*. 2004;71:281–288.

Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B, Vaughan ED. Comparison of the effects of the 5 alpha-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose–response studies. *Endocrinology*. 1992;131:1149–1156.

Isling LK, Boberg J, Jacobsen PR, et al. Late-life effects on rat reproductive system after developmental exposure to mixtures of endocrine disrupters. *Reproduction*. 2014:147:465–476.

Jacobson JL, Jacobson SW. Evidence for PCBs as neurodevelopmental toxicants in humans. *Neurotoxicology*. 1997;18:415–424.

Jaruratanasirikul S, Leethanaporn K, Pradutkanchana S, Sriplung H. Serum insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-3 (IGFBP-3) in healthy Thai children and adolescents: relation to age, sex, and stage of puberty. *J Med Assoc Thai*. 1999;82:275–283.

Jobling S, Tyler CR. Introduction: the ecological relevance of chemically induced endocrine disruption in wildlife. *Environ Health Perspect*. 2006;114(suppl 1):7–8.

Jorgensen N, Andersen AG, Eustache F, et al. Regional differences in semen quality in Europe. *Human Reprod*. 2001;16:1012–1019.

Jorgensen N, Carlsen E, Nermoen I, et al. East-West gradient in semen quality in the Nordic-Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. *Human Reprod*. 2002;17:2199–2208.

Kanno J, Onyon L, Haseman J, Fenner-Crisp P, Ashby J, Owens W. The OECD program to validate the rat uterotrophic bioassay to screen compounds for in vivo estrogenic responses: phase 1. *Environ Health Perspect*. 2001;109:785–794.

Kanno J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W. The OECD program to validate the rat uterotrophic bioassay. Phase 2: coded single-dose studies. *Environ Health Perspect*. 2003a;111:1550–1558.

Kanno J, Onyon L, Peddada S, Ashby J, Jacob E, Owens W. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dose–response studies. *Environ Health Perspect*. 2003b;111:1530–1549.

Kaplowitz P. Pubertal development in girls: secular trends. *Curr Opin obstet Gynecol*. 2006;18:487–491.

Kassim NM, McDonald SW, Reid O, Bennett NK, Gilmore DP, Payne AP. The effects of pre- and postnatal exposure to the nonsteroidal antiandrogen flutamide on testis descent and morphology in the Albino Swiss rat. *J Anat*. 1997;190:577–588.

Keefe DL, Marquard K, Liu L. The telomere theory of reproductive senescence in women. *Curr Opin obstet Gynecol*. 2006;18:280–285.

Keene LC, Davies PH. Drug-related erectile dysfunction. *Adverse Drug React Toxicol Rev*. 1999;18:5–24.

Kelce WR, Lambright CR, Gray LE, Roberts KP. Vinclozlin and p,p′-DDE alter androgen-dependent gene expression: In vivo confirmation of an androgen receptor-mediated mechanism. *Toxicol Appl Pharmacol*. 1997;142:192–200.

Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray LE Jr. Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol Appl Pharmacol*. 1994;126(2):276–285.

Kelce WR, Monosson E, Gray LE Jr. An environmental antiandrogen. *Recent Prog Horm Res*. 1995;50:449–453.

Kim HS, Shin JH, Moon HJ, et al. Evaluation of the 20-day pubertal female assay in Sprague-Dawley rats treated with DES, tamoxifen, testosterone, and flutamide. *Toxicol Sci*. 2002;67(1):52–62.

Kingsbury AC. Danazol and fetal masculinization: a warning. *Med J Aust*. 1985;143:410–411.

Klonisch T, Fowler PA, Hombach-Klonisch S. Molecular and genetic regulation of testis descent and external genitalia development. *Dev Biol*. 2004;270:1–18.

Knappe G, Gerl H, Ventz M, Rohde W. The long-term therapy of hypothalamic-hypophyseal Cushing's syndrome with mitotane (o,p′-DDD)]. *Deutsche Medizinische Wochenschrift*. 1997;(1946)122:882–886. First published on Langzeit-Therapie des hypothalamisch-hypophysaren Cushing-Syndroms mit Mitotan (o,p′-DDD).

Knobil E, Neill J. *The Physiology of Reproduction*. New York: Raven Press; 1994.

Kolpin DW, Furlong ET, Meyer MT, et al. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. *Environ Sci Technol*. 2002;36:1202–1211.

Kolpin DW, Skopec M, Meyer MT, Furlong ET, Zaugg SD. Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. *Sci Total Environ*. 2004;328:119–130.

Kolpin DW, Thurman EM, Linhart SM. The environmental occurrence of herbicides: the importance of degradates in ground water. *Arch Environ Contam Toxicol*. 1998;35:385–390.

Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature*. 1990;348:450–452.

Krstevska-Konstantinova M, Charlier C, Craen M, et al. Sexual precocity after immigration from developing countries to Belgium: evidence of previous exposure to organochlorine pesticides. *Human Reprod*. 2001;16:1020–1026.

Kuiper-Goodman T, Scott PM, Watanabe H. Risk assessment of the mycotoxin zearalenone. *Regul Toxicol Pharmacol*. 1987;7:253–306.

Lambright C, Ostby J, Bobseine K, et al. Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol Sci*. 2000;56:389–399.

Landrigan PJ, Kreiss K, Xintaras C, Feldman RG, Heath CW Jr. Clinical epidemiology of occupational neurotoxic disease. *Neurobehav Toxicol*. 1980;2:43–48.

Larsson DG, Förlin L. Male-biased sex ratios of fish embryos near a pulp mill: temporary recovery after a short-term shutdown. *Environ Health Perspect*. 2002;110(8):739–742. Erratum in: *Environ Health Perspect*. 110(9):A505.

Leblond CP, Clermont Y. Definition of the stages of the seminiferous epithelium in the rat. *Ann N Y Acad Sci*. 1952;55:548–573.

Lee KY, DeMayo FJ. Animal models of implantation. *Reproduction*. 2004;128:679–695.

LeMaire WJ, Cleveland WW, Bejar RL, Marsh JM, Fishman L. Aminoglutethimide: a possible cause of pseudohermaphroiditism in females. *AmjJ Dis Child*. 1972;124:421–423.

Lim HN, Raipert-de Meyts E, Skakkebaek NE, Hawkins JR, Hughes IA. Genetic analysis of the INSL3 gene in patients with maldescent of the testis. *Eur J Endocrinol*. 2001;144:129–137.

Linder RE, Hess RA, Strader LF. Testicular toxicity and infertility in male rats treated with 1,3-dinitrobenzene. *J Toxicol Environ Health*. 1986;19:477–489.

Linder RE, Klinefelter GR, Strader LF, Veeramachaneni DN, Roberts NL, Suarez JD. Histopathologic changes in the testes of rats exposed to dibromoacetic acid. *Reprod Toxicol*. 1997;11:47–56.

Lloyd SC, Foster PM. Effect of mono-(2-ethylhexyl)phthalate on follicle-stimulating hormone responsiveness of cultured rat Sertoli cells. *Toxicol Appl Pharmacol*. 1988;95:484–489.

Longnecker MP, Gladen BC, Cupul-Uicab LA, et al. In utero exposure to the antiandrogen 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) in relation to anogenital distance in male newborns from Chiapas, Mexico. *Am J Epidemiol*. 2007;165:1015–1022.

Lopes FL, Desmarais JA, Murphy BD. Embryonic diapause and its regulation. *Reproduction*. 2004;128:669–678.

Ludwig S, Tinwell H, Schorsch F, et al. A molecular and phenotypic integrative approach to identify a no-effect dose level for antiandrogen-induced testicular toxicity. *Toxicological Sciences*. 2011;122:52–63.

Malassine A, Frendo JL, Evain-Brion D. A comparison of placental development and endocrine functions between the human and mouse model. *Hum Reprod Update*. 2003;9:531–539.

Mandrup KR, Johansson HK, Boberg J, et al. Mixtures of environmentally relevant endocrine disrupting chemicals affect mammary gland development in female and male rats. *Reproductive Toxicology*. 2015;54:47–57.

Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. *Arch Dis Child*. 1969;44:291–303.

Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in boys. *Arch Dis Child*. 1970;45:13–23.

Marty MS, Crissman JW, Carney EW. Evaluation of the EDSTAC female pubertal assay in CD rats using 17beta-estradiol, steroid biosynthesis inhibitors, and a thyroid inhibitor. *Toxicol Sci*. 1999;52(2): 269–277.

Marty MS, Crissman JW, Carney EW. Evaluation of the male pubertal onset assay to detect testosterone and steroid biosynthesis inhibitors in CD rats. *Toxicol Sci*. 2001;60:285–295.

Massart F, Parrino R, Seppia P, Federico G, Saggese G. How do environmental estrogen disruptors induce precocious puberty? *Minerva Pediatr*. 2006;58:247–254.

Matsumura A, Ghosh A, Pope GS, Darbre PD. Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. *J Steroid Biochem Mol Biol*. 2005;94:431–443.

McCartney CR, Blank SK, Prendergast KA, et al. Obesity and sex steroid changes across puberty: evidence for marked hyperandrogenemia in pre- and early pubertal obese girls. *J Clin Endocrinol Metab*. 2007;92:430–436.

McIntyre BS, Barlow NJ, Foster PM. Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci*. 2001;62:236–249.

McIntyre BS, Barlow NJ, Foster PMD. Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention,

and epididymal malformations that result in subsequent testicular atrophy. *Toxicol Sci*. 2002;65:62–70.

McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PM. Effects of in utero exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat. *Toxicol Appl Pharmacol*. 2000;167:87–99.

Melching-Kollmuss S, Fussell KC, Schneider S, et al. Comparing effect levels of regulatory studies with endpoints derived in targeted anti-androgenic studies: example prochloraz. *Arch Toxicol*. 2017;91:143–162.

Melnick RL, Nyska A, Foster PM, Roycroft JH, Kissling GE. Toxicity and carcinogenicity of the water disinfection byproduct, dibromoacetic acid, in rats and mice. *Toxicology*. 2007;230:126–136.

Mendelson CR, Condon JC. New insights into the molecular endocrinology of parturition. *J Steroid Biochem Mol Biol*. 2005;93:113–119.

Metzdorff SB, Dalgaard M, Christiansen S, et al. Dysgenesis and histological changes of genitals and perturbations of gene expression in male rats after in utero exposure to antiandrogen mixtures. *Toxicol Sci*. 2007;98:87–98.

Miller KP, Gupta RK, Greenfeld CR, Babus JK, Flaws JA. Methoxychlor directly affects ovarian antral follicle growth and atresia through Bcl-2- and Bax-mediated pathways. *Toxicol Sci*. 2005;88:213–221.

Miyata K, Yabushita S, Sukata T, et al. Effects of perinatal exposure to flutamide on sex hormones and androgen-dependent organs in F1 male rats. *J Toxicol Sci*. 2002;27:19–33.

Mohr BA, Bhasin S, Link CL, O'Donnell AB, McKinlay JB. The effect of changes in adiposity on testosterone levels in older men: longitudinal results from the Massachusetts male aging study. *Eur J Endocrinol/European Federation of Endocrine Societies*. 2006;155:443–452.

Monosson E, Kelce WR, Lambright C, Ostby J, Gray LE Jr. Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. *Toxicol Ind Health*. 1999;15:65–79.

Moore KL. *The Developing Human: Clinically Oriented Embryology*. Philadelphia, PA: W. B. Saunders Company; 1982.

Muir A. Precocious puberty. *Pediatr Rev*. 2006;27:373–381.

Mylchreest E, Cattley RC, Foster PM. Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci*. 1998;43:47–60.

Mylchreest E, Foster PM. DBP exerts its antiandrogenic activity by indirectly interfering with androgen signaling pathways. *Toxicol Appl Pharmacol*. 2000;168:174–175.

Mylchreest E, Sar M, Cattley RC, Foster PMD. Disruption of Androgen-Regulated Male Reproductive Development by Di(n- Butyl) Phthalate during Late Gestation in Rats Is Different from Flutamide. *Toxicol Appl Pharmacol*. 1999;156:81–95.

Mylchreest E, Wallace DG, Cattley RC, Foster PM. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyl) phthalate during late gestation. *Toxicol Sci*. 2000;55:143–151.

Nagano K, Nakayama E, Koyano M, Oobayashi H, Adachi H, Yamada T. Testicular atrophy of mice induced by ethylene glycol mono alkyl ethers (author's transl). *Sangyo Igaku*. 1979;21:29–35.

Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ Health Perspect*. 1997;105:70–76.

Narotsky MG, Klinefelter GR, Goldman JM, et al. Comprehensive assessment of a chlorinated drinking water concentrate in a rat multigenerational reproductive toxicity study. *Environmental Science & Technology*. 2013;47:10653–10659.

Narotsky MG, Klinefelter GR, Goldman JM, et al. Reproductive toxicity of a mixture of regulated drinking-water disinfection by-products in a multigenerational rat bioassay. *Environ Health Perspect*. 2015;123:564–570.

Nassar N, Bower C, Barker A. Increasing prevalence of hypospadias in Western Australia, 1980–2000. *Arch Dis Child*. 2007;92(7):580–584.

Niculescu AM. Effects of in utero exposure to DES on male progeny. *J Obstet Gynecol Neonatal Nurs*. 1985;14:468–470.

Noriega NC, Ostby J, Lambright C, Wilson VS, Gray LE Jr. Late gestational exposure to the fungicide prochloraz delays the onset of parturition and causes reproductive malformations in male but not female rat offspring. *Biol Reprod*. 2005;72:1324–1335.

OECD 2010 now: 2011 ref: OECD, Test No. 443: extended one-generation reproductive toxicity study, OECD guidelines for the testing of chemicals,Section 4: health effects, 2011, DOI: doi:10.1787/9789264122550-en.

Ohyama K. Disorders of sex differentiation caused by exogenous hormones. *Nippon Rinsho*. 2004;62:379–384.

Ojeda SR, Heger S. New thoughts on female precocious puberty. *J Pediatr Endocrinol Metab*. 2001;14:245–256.

Ojeda SR, Lomniczi A, Mastronardi C, et al. Minireview: the neuroendocrine regulation of puberty: is the time ripe for a systems biology approach? *Endocrinology*. 2006;147:1166–1174.

Ojeda SR, Prevot V, Heger S, Lomniczi A, Dziedzic B, Mungenast A. The neurobiology of female puberty. *Horm Res*. 2003;60(suppl 3):15–20.

Oliveira CA, Mahecha GA, Carnes K, et al. Differential hormonal regulation of estrogen receptors ERalpha and ERbeta and androgen receptor expression in rat efferent ductules. *Reproduction*. 2004;128:73–86.

Orlando EF, Kolok AS, Binzcik GA, et al. (2004). Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environ Health Perspect*. 2004;112(3):353–358.

Ostby J, Kelce WR, Lambright C, Wolf CJ, Mann P, Gray LE Jr. The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. *Toxicol Ind Health*. 1999;15:80–93.

Ouyang F, Perry MJ, Venners SA, et al. Serum DDT, age at menarche, and abnormal menstrual cycle length. *Occup Environ Med*. 2005;62:878–884.

Owens JW, Ashby J. Critical review and evaluation of the uterotrophic bioassay for the identification of possible estrogen agonists and antagonists: in support of the validation of the OECD uterotrophic protocols for the laboratory rodent. Organisation for Economic Co-operation and Development. *Crit Rev Toxicol*. 2002;32:445–520.

Owens JW, Chaney JG. Weighing the results of differing "low dose" studies of the mouse prostate by Nagel, Cagen, and Ashby: quantification of experimental power and statistical results. *Regul Toxicol Pharmacol*. 2005;43:194–202.

Owens W, Ashby J, Odum J, Onyon L. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dietary phytoestrogen analyses. *Environ Health Perspect*. 2003;111:1559–1567.

Owens W, Koeter HB. The OECD program to validate the rat uterotrophic bioassay: an overview. *Environ Health Perspect*. 2003;111:1527–1529.

Owens W, Zeiger E, Walker M, Ashby J, Onyon L, Gray LE Jr. The OECD program to validate the rat Hershberger bioassay to screen compounds for in vivo androgen and antiandrogen responses. Phase 1: use of a potent agonist and a potent antagonist to test the standardized protocol. *Environ Health Perspect*. 2006;114:1259–1265.

Papathanasiou A, Hadjiathanasiou C. Precocious puberty. *Pediatr Endocrinol Rev*. 2006;3(suppl 1):182–187.

Paranko J, Pelliniemi LJ, Vaheri A, Foidart JM, Lakkala-Paranko T. Morphogenesis and fibronectin in sexual differentiation of rat embryonic gonads. *Differentiation*. 1983;23(suppl):S72–S81.

Parks LG, Lambright CS, Orlando EF, Guillette LJ Jr, Ankley GT, Gray LE Jr. Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol Sci*. 2001;62(2):257–267.

Parks LG, Ostby JS, Lambright CR, et al. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol Sci*. 2000;58(2):339–349.

Parrott JL, Bjerregaard P, Brugger KE, et al. Uncertainties in biological responses that influence hazard and risk approaches to the regulation of endocrine active substances. *Integr Environ Assess Manag*. 2017;13:293–301.

Paulozzi LJ, Erickson JD, Jackson RJ. Hypospadias trends in two US surveillance systems. *Pediatrics*. 1997;100:831–834.

Pelliniemi LJ. Ultrastructure of the early ovary and testis in pig embryos. *Am J Anat*. 1975;144:89–111.

Pelliniemi LJ, Niei M. Fine structure of the human foetal testis. I. The interstitial tissue. *Z Zellforsch Mikrosk Anat*. 1969;99:507–522.

Pierik FH, Burdorf A, Nijman JM, de Muinck Keizer-Schrama SM, Juttmann RE, Weber RF. A high hypospadias rate in The Netherlands. *Human Reprod*. 2002;17:1112–1115.

Porter MP, Faizan MK, Grady RW, Mueller BA. Hypospadias in Washington State: maternal risk factors and prevalence trends. *Pediatrics*. 2005;115:e495–e499.

Quinn MM, Wegman DH, Greaves IA, et al. Investigation of reports of sexual dysfunction among male chemical workers manufacturing stilbene derivatives. *Am J Ind Med*. 1990;18:55–68.

Reeve IT, Voss JC, Miller MG. 1,3-Dinitrobenzene metabolism and GSH depletion. *Chem Res Toxicol*. 2002;15:361–366.

Rehnberg GL, Linder RE, Goldman JM, Hein JF, McElroy WK, Cooper RL. Changes in testicular and serum hormone concentrations in the male rat following treatment with *m*-dinitrobenzene. *Toxicol Appl Pharmacol*. 1988;95(2):255–264.

Reich MR, Spong JK. Kepone: a chemical disaster in Hopewell, Virginia. *Int J Health Serv*. 1983;13:227–246.

Rider CV, Furr J, Wilson VS, Gray LE Jr. A mixture of seven antiandrogens induces reproductive malformations in rats. *Int J Androl*. 2008;31:249–262.

Rider CV, Furr JR, Wilson VS, Gray LE Jr. Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. *Int J Androl*. 2010;33:443–462.

Rider CV, Wilson VS, Howdeshell KL, et al. Cumulative effects of in utero administration of mixtures of "antiandrogens" on male rat reproductive development. *Toxicologic Pathology*. 2009;37:100–113.

Robaire B, Ewing LL, Irby DC, Desjardins C. Interactions of testosterone and estradiol-17 beta on the reproductive tract of the male rat. *Biol Reprod*. 1979;21:455–463.

Rosa FW. Virilization of the female fetus with maternal danazol exposure. *Am J Obstet Gynecol*. 1984;149:99–100.

Rouquié D, Friry-Santini C, Schorsch F, Tinwell H, Bars R. Standard and molecular NOAELs for rat testicular toxicity induced by flutamide. *Toxicological Sciences*. 2009;109:59–65.

Roy AK, Chatterjee B. Androgen action. *Crit Rev Eukaryot Gene Expr*. 1995;5:157–176.

Saito K, O'Donnell L, McLachlan RI, Robertson DM. Spermiation failure is a major contributor to early spermatogenic suppression caused by hormone withdrawal in adult rats. *Endocrinology*. 2000;141:2779–2785.

Saiyed H, Dewan A, Bhatnagar V, et al. Effect of endosulfan on male reproductive development. *Environ Health Perspect*. 2003;111:1958–1962.

Sarrabay A, Hilmi C, Tinwell H, et al. Low dose evaluation of the antiandrogen flutamide following a mode of action approach. *Toxicol Appl Pharmacol*. 2015;289:515–524.

Sawaki M, Noda S, Muroi T, et al. In utero through lactational exposure to ethinyl estradiol induces cleft phallus and delayed ovarian dysfunction in the offspring. *Toxicol Sci*. 2003;75(2):402–411.

Schäfer-Somi S. Cytokines during early pregnancy of mammals: a review. *Anim Reprod Sci*. 2003;75:73–94.

Schardein J. *Chemically Induced Birth Defects*. New York: Marcel Dekker Inc; 2000.

Schneider S, Melching-Kollmuss S, Buesen R, et al. Investigations on the dose–response relationship of combined exposure to low doses of three anti-androgens in Wistar Rats. *The Toxicologist*. 2017;156:1.

Semple RK, Achermann JC, Ellery J, et al. Two novel missense mutations in g protein-coupled receptor 54 in a patient with hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2005;90:1849–1855.

Setchell BP, Waites GM. Changes in the permeability of the testicular capillaries and of the 'blood-testis barrier' after injection of cadmium chloride in the rat. *J Endocrinol*. 1970;47:81–86.

Shaffer NG. DES: a continuing health concern. *Pa Med*. 2000;103:17.

Silversides DW, Price CA, Cooke GM. Effects of short-term exposure to hydroxyflutamide in utero on the development of the reproductive tract in male mice. *Can J Physiol Pharmacol*. 1995;73:1582–1588.

Sinha-Hikim I, Cornford M, Gaytan H, Lee ML, Bhasin S. Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. *J Clin Endocrinol Metab*. 2006;91:3024–3033.

Skakkebaek NE. Endocrine disrupters and testicular dysgenesis syndrome. *Horm Res*. 2002;57(suppl S2):43.

Smith ER, Quinn MM. Uterotropic action in rats of amsonic acid and three of its synthetic precursors. *J Toxico Environ Health*. 1992;36:13–25.

Snegovskikh V, Park JS, Norwitz ER. Endocrinology of parturition. *Endocrinol Metab Clin North Am*. 2006;35:173–191, viii.

Stevenson JG, Umstead GS. Sexual dysfunction due to antihypertensive agents. *Drug Intell Clin Pharm*. 1984;18:113–121.

Stoker TE, Cooper RL, Goldman JM, Andrews JE. Characterization of pregnancy outcome following thiram-induced ovulatory delay in the female rat. *Neurotoxicol Teratol*. 1996;18:277–282.

Stoker TE, Goldman JM, Cooper RL. The dithiocarbamate fungicide thiram disrupts the hormonal control of ovulation in the female rat. *Reprod Toxicol*. 1993;7:211–218.

Stoker TE, Goldman JM, Cooper RL. Delayed ovulation and pregnancy outcome: effect of environmental toxicants on the neuroendocrine control of the ovary(1). *Environ Toxicol Pharmacol*. 2001;9:117–129.

Stoker TE, Jeffay SC, Zucker RM, Cooper RL, Perreault SD. Abnormal fertilization is responsible for reduced fecundity following thiram-induced ovulatory delay in the rat. *Biol Reprod*. 2003;68:2142–2149.

Stoker TE, Laws SC, Crofton KM, Hedge JM, Ferrell JM, Cooper RL. Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols. *Toxicol Sci*. 2004;78(1):144–155.

Stoker TE, Parks LG, Gray LE, Cooper RL. Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. A focus on the EDSTAC recommendations. Endocrine Disrupter Screening and Testing Advisory Committee. *Crit Rev Toxicol*. 2000;30(2):197–252.

Stoker TE, Perreault SD, Bremser K, Marshall RS, Murr A, Cooper RL. Acute exposure to molinate alters neuroendocrine control of ovulation in the rat. *Toxicol Sci*. 2005;84:38–48.

Storer TW, Magliano L, Woodhouse L, et al. Testosterone dose-dependently increases maximal voluntary strength and leg power, but does not affect fatigability or specific tension. *J Clin Endocrinol Metab*. 2003;88:1478–1485.

Stroheker T, Cabaton N, Nourdin G, Regnier JF, Lhuguent JC, Chagnon MC. Evaluation of anti-androgenic activity of di-(2-ethylhexyl)phthalate. *Toxicology*. 2005;208:115–121.

Swan SH, Main KM, Liu F, et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect*. 2005;113:1056–1061.

Swingle WW, Seay P, Perlmutt J, Collins EJ, Barlow G Jr, Fedor EJ. An experimental study of pseudopregnancy in rat. *Am J Physiol*. 1951;167:586–592.

Tan SW, Zoeller RT. Integrating basic research on thyroid hormone action into screening and testing programs for thyroid disruptors. *Crit Rev Toxicol*. 2007;37:5–10.

Tena-Sempere M. KiSS-1 and reproduction: focus on its role in the metabolic regulation of fertility. *Neuroendocrinology*. 2006;83:275–281.

Thayer KA, Foster PM. Workgroup report: National Toxicology Program Workshop on hormonally induced reproductive tumors—relevance of rodent bioassays. *Environ Health Perspect*. 2007;115:1351–1356.

Toppari J, Kaleva M, Virtanen HE. Trends in the incidence of cryptorchidism and hypospadias, and methodological limitations of registry-based data. *Hum Reprod Update*. 2001;7:282–286.

Tranguch S, Daikoku T, Guo Y, Wang H, Dey SK. Molecular complexity in establishing uterine receptivity and implantation. *Cell Mol Life Sci*. 2005;62:1964–1973.

Trasler JM, Hales BF, Robaire B. Paternal cyclophosphamide treatment of rats causes fetal loss and malformations without affecting male fertility. *Nature*. 1985;316:144–146.

Tuvemo T. Treatment of central precocious puberty. *Expert Opin Investig Drugs*. 2006;15:495–505.

Tyl RW, Myers CB, Marr MC, et al. Two-generation reproductive toxicity evaluation of dietary 17beta-estradiol (E2; CAS No. 50-28-2) in CD-1 (Swiss) mice. *Toxicol Sci*. 2008;102:392–412.

Uckert S, Hedlund P, Andersson KE, Truss MC, Jonas U, Stief CG. Update on phosphodiesterase (PDE) isoenzymes as pharmacologic targets in urology: present and future. *Eur Urol*. 2006;50:1194–1207; discussion 1207.

United States Environmental Protection Agency Prevention, Pesticides and Toxic Substances (7508C). EPA 738-R-00-023, October 2000. Reregistration Eligibility Decision (RED), Vinclozolin.

Veurink M, KosterM, Berg LT. The history of DES, lessons to be learned. *Pharm World Sci*. 2005;27:139–43.

Veyssiere G, Berger M, Jean-Faucher C, de Turckheim M, Jean C. Testosterone and dihydrotestosterone in sexual ducts and genital tubercle of rabbit fetuses during sexual organogenesis: effects of fetal decapitation. *J Steroid Biochem*. 1982;17:149–154.

Vinggaard AM, Hnida C, Breinholt V, Larsen JC. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. *Toxicol In Vitro*. 2000;14:227–234.

Vinggaard AM, Nellemann C, Dalgaard M, Jorgensen EB, Andersen HR. Antiandrogenic Effects in vitro and in vivo of the fungicide prochloraz. *Toxicol Sci*. 2002;69:344–353.

Vogel G. Leptin: a trigger for puberty? *Science*. 1996;274:1466–1467.

vom Saal FS, Cooke PS, Buchanan DL, et al. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol Ind Health*. 1998;14:239–260.

vom Saal FS, Nagel SC, Timms BG, Welshons WV. Implications for human health of the extensive bisphenol A literature showing adverse effects at low doses: a response to attempts to mislead the public. *Toxicology.* 2005;212:244–252, author reply 253–244.

Wacharasindhu S, Petwijit T, Aroonparkmongkol S, Srivuthana S, Kingpetch K. Bone mineral density and body composition in Thai Precocious Puberty girls treated with GnRH agonist. *J Med Assoc Thai*. 2006;89:1194–1198.

Wade MG, Foster WG, Younglai EV, et al. Effects of subchronic exposure to a complex mixture of persistent contaminants in male rats: systemic, immune, and reproductive effects. *Toxicol Sci*. 2002;67:131–143.

Warner M, Samuels S, Mocarelli P, et al. Serum dioxin concentrations and age at menarche. *Environ Health Perspect*. 2004;112:1289–1292.

Warnock DH, Csapo AI. Progesterone withdrawal induced by ICI 81008 in pregnant rats. *Prostaglandins*. 1975;10:715–724.

Welshons WV, Nagel SC, vom Saal FS. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology*. 2006;147:S56–S69.

Whelan EA, Grajewski B, Wild DK, Schnorr TM, Alderfer R. Evaluation of reproductive function among men occupationally exposed to a stilbene derivative: II. Perceived libido and potency. *Am J Ind Med*. 1996; 29:59–65.

Wilson JD, Lasnitzki I. Dihydrotestosterone formation in fetal tissues of the rabbit and rat. *Endocrinology*. 1971;89:659–668.

Wilson VS, Bobseine K, Gray LE Jr. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci*. 2004;81(1):69–77.

Wilson VS, Lambright C, Furr J, et al. Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicol Lett.* 2005;146:207–215.

Wilson VS, Lambright C, Ostby J, Gray LE Jr. In vitro and in vivo effects of 17beta-trenbolone: a feedlot effluent contaminant. *Toxicol Sci*. 2002;70:202–211.

Wine RN, Li LH, Barnes LH, Gulati DK, Chapin RE. Reproductive toxicity of di-*n*-butylphthalate in a continuous breeding protocol in Sprague–Dawley rats. *Environ Health Perspect*. 1997;105:102–107.

Winker R, Rudiger HW. Reproductive toxicology in occupational settings: an update. *Int Arch Occup Environ Health*. 2006;79:1–10.

Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA, Gray LE Jr. Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose–response study. *Toxicol Sci*. 2002;65:71–86.

Wolff MS, Britton JA, Russo JC. TCDD and puberty in girls. *Environ Health Perspect*. 2005;113:A17; author reply A18.

Woodhouse LJ, Gupta N, Bhasin M, et al. Dose-dependent effects of testosterone on regional adipose tissue distribution in healthy young men. *J Clin Endocrinol Metab*. 2004;89:718–726.

Woods JS. Drug effects on human sexual behavior. In: Woods NF, ed. *HumanSexuality in Health and Illness*. 3rd ed. St. Louis, MO: Mosby; 1984.

Yamasaki K, Sawaki M, Ohta R, et al. OECD validation of the Hershberger assay in Japan: phase 2 dose response of methyltestosterone, vinclozolin, and *p,p′*-DDE. *Environ Health Perspect*. 2003;111:1912–1919.

Yin W, Gore AC. Neuroendocrine control of reproductive aging: roles of GnRH neurons. *Reproduction*. 2006;131:403–414.

Unit V

Toxic Agents

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chapter 22

Toxic Effects of Pesticides

Lucio G. Costa

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- Metam Sodium
- Sulfur Compounds

Acknowledgments

References

INTRODUCTION

Pesticides can be defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating pests. Pests can be insects, rodents, weeds, and a host of other unwanted organisms (Ecobichon, 2001a). Thus, pesticides occupy a rather unique position among the many chemicals that we encounter daily, in that they are deliberately added to the environment for the purpose of killing or injuring some form of life. Ideally, their injurious action would be highly specific for undesirable targets; in reality, however, many pesticides are not highly selective, and are often toxic to many nontarget species, including humans. Thus, the use of pesticides must minimize the possibility of exposure of nontarget organisms to injurious quantities of these chemicals (Murphy, 1986).

It is not uncommon for people to refer to pesticides as a single unitary class of chemicals, while in fact the term "pesticide" should be equated to that of pharmaceutical drugs. As there are dozens of drugs with different therapeutical indications and different mechanisms of action, several different classes of pesticides exist, with different uses, mechanisms, and, hence, toxic effects in nontarget organisms. The most common classification of pesticides relies on the target species they act on. The four major classes (and their target pests) are those of insecticides (insects), herbicides (weeds), fungicides (fungi, molds), and rodenticides (rodents), and there are also acaricides (mites), molluscicides (snails, other mollusks), miticides (mites), larvicides (larvae), and pediculicides (lice). In addition, for regulatory purposes, plant growth regulators, repellents, and attractants (pheromones) often also fall in this broad classification of chemicals. Furthermore, within each class, several

subclasses exist, with substantially different chemical and toxicological characteristics. For example, among insecticides, one can find organophosphorus compounds, carbamates, organochlorines, pyrethroids, neonicotinoids, and many other chemicals. Even within each of these subclasses, significant differences can exist, as is the case, for example, of organochlorine compounds such as DDT, aldrin, or chlordecone. Thus, detailed knowledge of the toxicological characteristics of each chemical is needed to properly evaluate their potential risks for nontarget species.

The literature pertaining to the chemistry, development, nomenclature, biotransformation and degradation, environmental effects, toxicity in target and nontarget species, and mode of action of pesticides over the past several decades is very extensive, and the reader is referred to the monographs of O'Brien (1967), Ecobichon and Joy (1982), Hayes (1982), Wagner (1983), Matsumura (1985), Costa et al. (1987), Baker and Wilkinson (1990), Dikshith (1991), Hayes and Laws (1991), Chambers and Levi (1992), Satoh and Gupta (2010), and Krieger (2001, 2010), for more in-depth discussions of various aspects of pesticide toxicology.

HISTORICAL DEVELOPMENTS

Pesticides have been used to a limited degree since ancient times. The Ebers Papyrus, written about 1500 BC, lists preparations to expel fleas from the house. The oldest available record is Homer's mention (about 800 BC) that Odysseus burned sulfur "…to purge the hall and the house and the court" (Odyssey XXII, 493–494). Pliny the Elder (AD 23–79) collected in his Natural History many anecdotes on the use of pesticides in the previous three to four centuries (Shepard, 1939). Dioscorides, a Greek physician (AD 40–90), knew of toxic properties of sulfur and arsenic. There are records showing that by AD 900 the Chinese were using arsenic sulfides to control garden insects. *Veratrum album* and *V. nigrum,* two species of false hellebore, were used by the Romans as rodenticides (Shepard, 1939). In 1669, the earliest known record of arsenic as an insecticide in the Western world mentioned its use with honey as an ant bait. Use of tobacco as contact insecticide for plant lice was mentioned later in the same century. Copper compounds were known since the early 1800s to have fungicidal value, and the Bordeaux mixture (hydrated lime and copper sulfate) was first used in France in 1883. Hydrocyanic acid, known to the Egyptians and the Romans as a poison, was used as a fumigant in 1877 to kill museum pests in insect collections, and carbon disulfide has been used as a fumigant since 1854 (Costa, 1987). Even in this century, until the 1930s, pesticides were mainly of natural origins or inorganic compounds. Arsenicals have played a major role in pest control, first as insecticides, then as herbicides. Sulfur has been widely used as a fumigant since the early 1800s, and remains one of the most widely used fungicides as of today. Nicotine has been widely used as an insecticide all over the world, as has been rotenone, used as a fish poison in South America since 1725 (Costa, 1987). Mercuric chloride was used as a fungicide since 1891, slowly replaced by phenylmercury and alkylmercury. Outbreaks of poisoning with the latter compounds (Bakir et al., 1973) have led to a ban of these chemicals.

The period between 1935 and 1950 was characterized by the development of major classes of pesticides, particularly insecticides. In 1939 Paul Mueller found that DDT (dichlorodiphenyltrichloroethane), which had been first synthesized in 1874, acted as a poison on flies, mosquitoes, and other insects. DDT was commercialized in 1942 and was used extensively and successfully for the control of typhus epidemics, and particularly of malaria. Together with DDT, other chlorinated hydrocarbon insecticides were developed. In the early 1940s, scientists in England and France recognized the gamma isomer of hexachlorocyclohexane, commonly known as lindane, which had been first synthesized in 1825 by Faraday, as a highly potent insecticide (Ecobichon, 1982). Starting in the mid-1940s, several other chlorinated insecticides such as chlordane, heptachlor, aldrin, and dieldrin were commercialized. The organophosphorus insecticides were first synthesized in Germany in the late 1930s. Gerhard Schrader, a chemist at I. G. FarbenIndustrie in Germany, is considered the "father" of organophosphorus insecticides. The first one, tetraethylpyrophosphate (TEEP), was brought to the market in 1944, but had little success because of its instability in aqueous solution. Several thousand molecules were synthesized by Schrader, and one (code name E605) was eventually introduced into the agricultural market under the trade name parathion, to become one of the most widely employed insecticides in this class. During those years, compounds of much greater toxicity than parathion were also synthesized as potential chemical warfare agents. Among these, sarin was utilized in 1988 against the Kurds in the Iraq–Iran war, and in a terrorist attack in the Tokyo subway in 1995, while on February 13, 2017, Kim Jong-nam (half-brother of the North Korean leader Kim Jong-un) was killed allegedly with VX (*O*-ethyl s-[2-(diisopropylamino)ethyl] methylphosphothionate). The mechanism of action of organophosphates, i.e. inhibition of acetylcholinesterase, was soon discovered, primarily by knowledge of the effects and mechanism of action of the carbamate physostigmine. This alkaloid had been isolated in 1864 from Calabar beans, the seeds of *Physostigma venenosum,* a perennial plant in tropical West Africa, and its mode of action as a cholinesterase inhibitor was identified in 1926 (Casida, 1964). However, despite the early studies on physostigmine, the carbamate insecticides were introduced only in the early 1950s. Though pyrethrum flower and extracts had been used as insecticides for several centuries, pyrethrins were characterized only between 1910 and 1924 (Casida, 1980). This led then to the development of synthetic pyrethroids, the first of which, allethrin, was followed by several others in the early 1970, particularly because of the work of Michael Elliott in England and of scientists at Sumitomo Chemical Company in Japan (Casida, 2010). Several other classes of insecticides (e.g., avermectins, neonicotinoids, *N*-phenylpyrazoles, and diamides) have also been developed in the past few decades (Casida and Durkin, 2017).

The past 60 years have also seen the development of hundreds of other chemicals used as herbicides, fungicides, and rodenticides (Casida and Durkin, 2017). The development of thioureas, such as ANTU, and of anticoagulants such as warfarin, as rodenticides, dates back to the mid- to late 1940s. A few years later, two important fumigants were introduced, 1,2-dichloropropene and methyl bromide. In the 1950s, phenylureas and chlorophenoxy compounds were developed as herbicides, together with the fungicides captan and folpet. Triazines, chloroacetanilides, and paraquat, all widely used herbicides, came to the market in the 1960s, and so did the important class of dithiocarbamate fungicides, while the herbicide glyphosate was introduced in the mid-1970s.

ECONOMICS AND PUBLIC HEALTH

As with all chemicals, including therapeutic drugs, the use of pesticides must take into consideration the balance of the benefits that may be expected versus the possible risks of injury to human health or degradation of environmental quality. Pesticides play a major role in the control of vector-borne diseases, which represent a major threat to the health of large human populations. Indeed, pesticides of various types are used in the control of insects, rodents, and other pests that are involved in the life cycle of vector-borne

diseases such as malaria, filariasis, yellow fever, viral encephalitis, typhus, and many others (Novak and Lampman, 2001). The history of DDT exemplifies the difficulty in finding a balance between its benefits and the risks, in this case mainly to the environment. When introduced in 1942, DDT appeared to hold immense promise of benefit to agricultural economics and protection of public health against vector-borne diseases. For example, in the Italian province of Latina there were 175 new cases of malaria in 1944, but after a DDT spray control program was initiated, no new cases of malaria appeared by 1949 (Murphy, 1986). Indeed, at the time, the public health benefits of DDT were viewed so great that its discoverer, Paul Mueller, was awarded the Nobel Prize in Medicine in 1948. However, because of its bioaccumulation in the environment and its effects on bird reproduction, DDT was eventually banned in most countries by the mid-1970s. In South Africa, DDT was only banned in 1996 and at the time less than 10,000 cases of malaria were registered in that country. By the year 2000, cases of malaria had increased to 62,000, but with the reintroduction of DDT at the end of that year, cases were down to 12,500 (Maharaj et al., 2005). There are still hundreds of millions of people in the world who are at risk from schistosomiasis, filariasis, and intestinal worm infestations, particularly in Africa and some Asian countries, and these major health problems require a continuous judicious use of pesticides (Novak and Lampman, 2001).

In many parts of the world, excessive loss of food crops to insects or other pests may contribute to possible starvation, and use of pesticides seems to have a favorable cost–benefit relationship (Murphy, 1986). In developed countries, pesticides allow production of abundant, inexpensive, and attractive fruits and vegetables, as well as grains. In this case, cost–benefit considerations are based on economic considerations, particularly with regard to labor costs. Along with insecticides, herbicides and fungicides play a major role in this endeavor. Pests have been estimated to cause 27% to 42% loss in production of major crops around the world, and this would rise to 48% to 83% without crop protection with pesticides (Oerke, 2006). Loss of harvested crops by post-harvest infestation by insects, fungi, and rodents is also a major problem (Ecobichon, 2001a), which is dealt with by the use of fumigants and other pesticides. Pesticides, particularly herbicides, also find useful application in forestry, during reforestation, as well as the clearing of roadways, train tracks, and utilities' rights of way. In the urban setting, pesticides find multiple uses in the home and garden area to control insects, weeds, and other pests (Marsh, 2001; Rust, 2001). It is estimated that more than 75% of households in the United States utilize pesticides (Table 22-1). These could include, for example, chemicals to control termite, cockroach or rodent infestations, herbicides to control weeds in the garden, or insect repellents.

Use of Pesticides

It is commonly believed that there has been a continuous increase in the use of pesticides. While this is certainly true for the period from the 1950s to the 1980s, in the past 30 years or so use of pesticides (as amount of active ingredient) has actually reached a plateau (Table 22-2). This is mostly due to the utilization of more efficacious compounds that require less active ingredient to be applied to obtain the same degree of pest control. Indeed, as recently as 1960 more than 1 kg (2.2 lbs) of a crop protection chemical was applied per hectare (ha), while today the application rates have decreased by almost 100-fold to 10 g/ha (Lamberth et al., 2013). In addition, the introduction of integrated pest management approaches and the increased popularity of organic farming have contributed, at least in the developed countries, to a decrease and/or stabilization of pesticide use.

Table 22-1

Number of U.S. Households Using Pesticides

PESTICIDE TYPE	U.S. HOUSEHOLDS (MILLIONS)	% OF TOTAL HOUSEHOLDS*
Insecticides	59	56
Herbicides	41	39
Fungicides	14	13
Repellents	53	50
Disinfectants	59	56
Any pesticide	78	74

**Based on 105.5 million households and a population of 281.4 million.*
SOURCE: Grube et al. (2011).

Table 22-2

Use and Expenditure on Total Pesticides in the United States in All Market Sectors: 1988–2006

YEAR	USE*	EXPENDITURE†
1988	1,191	7,275
1990	1,201	7,727
1992	1,186	8,385
1994	1,229	10,074
1996	1,229	10,969
1998	1,206	11,416
2000	1,234	11,165
2002	1,206	11,171
2004	1,210	11,905
2006	1,127	11,784

**Millions of pounds of active ingredients. Excludes preservatives, specialty biocides, and chlorine/hypochlorites.*
†Millions of dollars.
SOURCE: Grube et al. (2011).

Expenditures on pesticides have somewhat increased, as new chemicals are more expensive than older ones (Table 22-2). In the United States, almost half of the pesticides used are herbicides, while in other countries, particularly Africa, Asia, and Central America, there is also a substantial use of insecticides (Table 22-3). Because the latter compounds are generally more acutely toxic, they contribute to the still large number of yearly pesticide poisonings (see the section "Human Poisoning"). Table 22-4 shows the most commonly used pesticides in the agricultural, home, and garden, and industry/commercial/government sectors in the United States, while Table 22-5 indicates pesticide use in selected crops in the United States.

Pesticides are often, if not always, used as formulations, in which the active ingredient is present together with other ingredients to allow mixing, dilution, application, and stability. These other ingredients are lumped under the term "inert" or "other" (Tominack, 2000), and may be emulsifiers, solvents, carriers, aerosol propellants, fragrances, and dyes. Though they do not have pesticidal action, such inert ingredients may not always be devoid of toxicity; thus, an ongoing task of manufactures and regulatory agencies is to assure that inert ingredients do not pose any unreasonable risk

Table 22-3

U.S. and World Use of Pesticides: 2007

	UNITED STATES		WORLD	
PESTICIDE	MILLIONS OF POUNDS OF ACTIVE INGREDIENT	%	MILLIONS OF POUNDS OF ACTIVE INGREDIENT	%
Herbicides*	531	47	2096	40
Insecticides	93	8	892	17
Fungicides	70	6	518	10
Others†	439	39	1705	33
TOTAL	1133	100	5211	100

**Includes herbicides and plant growth regulators.*
†Others: rodenticides, fumigants, nematocides, molluscicides, and other chemicals used as pesticides (e.g., sulfur and petroleum oil). Does not include wood preservatives, specialty biocides, and chlorine/hypochlorite.
SOURCE: Grube et al. (2011).

Table 22-4

Most Commonly Used Conventional Pesticide Active Ingredients in the U.S. Market Sectors: 2007 Ranks

PESTICIDE	TYPE	AGRICULTURAL	SECTOR HOME AND GARDEN	INDUSTRY/COMMERCIAL/ GOVERNMENT
Glyphosate	H	1	2	2
Atrazine	H	2	–	–
Metam sodium	Fum	3	–	–
Metolachlor-S	H	4	–	–
Acetochlor	H	5	–	–
Dichloropropene	Fum	6	–	–
2,4-D	H	7	1	1
Methyl bromide	Fum	8	–	–
Chloropicrin	Fum	9	–	–
Pendimethalin	H	10	5	6
Carbaryl	I	–	3	–
MCPP	H	–	4	–
Pyrethroids	I	–	6	–
Malathion	I	–	7	9
Dicamba	H	–	8	–
Trifluralin	H	17	9	–
Pelargonic acid	H	–	10	–
Chlorothalonil	F	12	–	3
MSMA	H	–	–	4
Diuron	H	22	–	5
Triclopyr	H	–	–	7
Copper sulfate	F	–	–	8
Sulfuryl fluoride	Fum	–	–	10

Abbreviations: F, Fungicide; Fum, Fumigant; H, Herbicide; I, Insecticide; MCCP, methylchlorophenoxypropionic acid; MSMA, monosodium methanearsonate.
Shown is rank in each sector.
SOURCE: Grube et al. (2011).

of adverse health effects (Tominack, 2000). Examples that illustrate the potential problems with these "inert" ingredients are those with the insecticides neonicotinoids and the herbicide glyphosate (Defarge et al., 2016; Mullin et al., 2016).

Exposure

Exposure to pesticides can occur via the oral or dermal routes, or by inhalation. From a quantitative perspective, oral exposure lies on the extremes of a hypothetical dose–response curve. High oral doses, leading to severe poisoning and death, are achieved as a result of pesticide ingestion for suicidal intents, or of accidental ingestion, commonly due to storage of pesticides in improper containers. In contrast, chronic low doses are consumed by the general population as pesticide residues in food, or as contaminants in drinking water. Regulations exist to ensure that pesticide residues are maintained at levels below those that would cause any adverse effect (see the section "Regulatory Mandate"). Workers involved in the production, transport, mixing and loading, and application of pesticides, as well as in harvesting of pesticide-sprayed crops, are at highest risk for pesticide exposure. The dermal route is believed in this case to offer the greatest potential for exposure, with a minor contribution of the respiratory route when aerosols or aerial spraying are used. In these latter cases, bystanders or individuals living in proximity of the spraying may also be exposed because of off-target drifts. In the occupational setting, dermal exposure during normal handling or application of pesticides, or in case of accidental spillings, occurs in body areas not covered by protective clothing, such as the face or the hands. Furthermore, deposition of pesticides on clothing may lead to slow penetration through the tissue and/or to potential exposure of others, if clothes are not changed and washed upon termination of exposure. Several methodologies exist to assess exposure by passive dosimetry, such as the use of absorbent cloth or paper patches, of biosensors, or of tracers followed by video imaging (Fenske et al., 1986; U.S. EPA 1999; Chester, 2010). Biological monitoring is also used to quantify the absorbed dose of pesticides. Analysis of body fluids and excreta, usually urine, for parent compound or metabolites, can provide both a quantitative and a qualitative measurement of absorbed dose. The advantage of such approach over passive dosimetry is that it evaluates actual rather than potential absorption, and integrates absorption from all routes of exposure (Sobus et al., 2010). In some cases, modifications of biochemical parameters or a consequence of exposure can be quantified as an indication of both exposure and of a biological effect. This is the case, for example, of measurements of plasma or erythrocyte cholinesterases upon exposure to organophosphorus insecticides (Storm et al., 2000).

Table 22-5

Pesticide Active Ingredient Use in 21 Selected Crops in the United States in 2008*

CROP	PESTICIDE (MILLIONS OF POUNDS)	%
Corn	204	39.9
Soybeans	112	21.7
Potatoes	52	10.1
Cotton	38	7.4
Wheat	23	4.5
Sorghum	14	2.7
Oranges	13	2.5
Peanuts	10	2.0
Tomatoes	10	1.9
Rice	8	1.5
Grapes	8	1.5
Apples	7	1.4
Others†	17	3.2

**Of a total of 516 million pounds, 76% were herbicides, 5.5% insecticides, 5.5% fungicides, and 12.5% other pesticides.*

†Others = barley, grapefruit, lemons, lettuce, peaches, pears, pecans, sugarcane, and sweet corns.

SOURCE: Adapted from Fernandez-Cornejo et al. (2014).

Human Poisoning

Pesticides are not always selective for their intended target species, and adverse health effects can occur in nontarget species, including humans (Calvert et al., 2010). In the general population and in occupationally exposed workers, recurring concerns relate to a possible association between pesticide exposure and increased risk of cancer (Pearce and McLean, 2005; Weichenthal et al., 2010; Silva et al., 2016), or neurodegenerative diseases such as Parkinson disease (Moretto and Colosio, 2013; Hernandez et al., 2016). More recently, the acknowledgment that pesticide standards are based on healthy adults, and thus may not be sufficiently protective of susceptible populations, such as children, has led to new concerns, research, and regulations (NRC, 1993; Colborn, 2006). Evidence that some pesticides may act as endocrine disruptors, possibly contributing to various adverse effects in humans, including cancer and reproductive and developmental toxicity, has also prompted additional concerns and initiatives (Safe, 2000; Bjørlung-Poulsen et al., 2008; Stoker and Kavlock, 2010). Yet, from a global perspective, the major problem with pesticides remains that of acute human poisoning. The World Health Organization (WHO) estimated that there are around 3 million hospital admissions for pesticide poisoning each year that result in around 220,000 deaths (WHO, 1990; Colosio et al., 2010). Most occur in developing countries, particularly in Southeast Asia, and a large percentage is due to intentional ingestion for suicide purposes (Gunnell and Eddleston, 2003). New estimates indicate that there are about 300,000 deaths due to pesticide self-poisoning each year, accounting for one-third of all suicides globally (Bertolote et al., 2006; Gunnell et al., 2007). This percentage can increase to 50% to 90% in some countries of Central and South America (e.g., El Salvador and Peru) or in Asia (e.g. Korea and Taiwan) (Ajdacic-Gross et al., 2008; Chen et al., 2009).

The WHO has recommended a classification of pesticides by hazard, where acute oral or dermal toxicities in rats are considered (IPCS, 2010) (Table 22-6), which is similar to the classification of pesticides according to the "Globally Harmonized System of Classification and Labelling of Chemicals" (GHS). An analysis of commercially available pesticides indicates that, as a class, insecticides are the most acutely toxic. Indeed, among the 86 active ingredients listed in Class IA (Extremely hazardous) and Class IB (Highly hazardous), 51 (59%) are insecticides (IPCS, 2010). Rodenticides are also highly toxic to rats, but do not present the same hazard to humans. Indeed, warfarin, one of the most widely used rodenticides, is the same chemical used as an effective "blood thinner" (anticoagulant) for prevention of stroke and other blood clot–related conditions. Herbicides, again as a class, have generally moderate to low acute toxicity, one exception being paraquat

Table 22-6

WHO-Recommended Classification of Pesticides by Hazard

CLASS		LD_{50} IN RAT (MG/KG BODY WEIGHT) ORAL	DERMAL
Ia	Extremely hazardous	<5	<50
Ib	Highly hazardous	5–50	50–200
II	Moderately hazardous	50–200	200–2000
III	Slightly hazardous	Over 200	Over 2000
U	Unlikely to present hazard	Over 5000	Over 5000

SOURCE: From IPCS (2010).

(which has a low dermal toxicity but causes fatal effects when ingested). Fungicides vary in their acute toxicity, but this is usually low. While this classification has served public health well over the years, it has been recently challenged. In a prospective cohort study in Sri Lanka, Dawson et al. (2010) have pointed out that category II (moderately hazardous; Table 22-6) comprises compounds associated with very different outcomes in humans upon acute exposure for suicidal purposes. For example, the organophosphorus insecticide dimethoate, fenthion, or chlorpyrifos, the pyrethroid insecticide deltamethrin, the phenylpyrazole insecticide fipronil, and the herbicide paraquat are all listed in WHO category II; yet, the mortality rate in this study was significantly variable: 21%, 15%, 8%, 0%, 0%, and 43%, for each indicated pesticide, respectively (Dawson et al., 2010).

Reports of human poisonings worldwide underline particularly the severe acute toxicity of certain anticholinesterase compounds and of paraquat. In Costa Rica between 1980 and 1986, 3330 individuals were hospitalized for pesticide poisoning, and 429 died. Cholinesterase inhibitors (organophosphates and carbamates) caused 63% of hospitalizations and 36% of deaths, while paraquat accounted for 24% of hospitalizations and 60% of deaths. Cholinesterase inhibitors also caused more than 70% of occupational accidents (Wesseling et al., 1993). Of 335 poisoning deaths in Manipal, India, in the 1990s, 70% were due to cholinesterase inhibitors (Mohanty et al., 2004), and similar findings were also reported in Brazil (Caldas et al., 2008). In Sri Lanka between 1986 and 2000, hospital admissions for pesticide poisoning were 12,000 to 20,000 per year, with a 10% fatality rate (Roberts et al., 2003). Organophosphates and the organochlorine insecticide endosulfan (which was banned in 1998) were the compounds most commonly involved. In Malaysia, during the period 1999 to 2001, pesticide poisonings accounted for 17% of all poisoning-related admissions, but 78% of fatalities (Rajasuriar et al., 2007). Similarly, in Korea, during the period 1996 to 2005, deaths due to pesticide poisoning showed a gradual increase, and accounted for 58% of the total poisoning fatalities (Lee et al., 2009a). However, after the ban of paraquat in 2012, overall acute suicidal poisoning with herbicides declined, though there was an increase in the use of glyphosate and glufosinate (Lee et al., 2015). The same pattern of poisoning can be also seen in Western countries. For example, in Greece, the number of poisonings ranged between 1200 and 1700 per year during the period 1988 to 1999. Of these, 40% were due to occupational exposure and 45% to accidental exposure. Organophosphates, carbamates, and paraquat were again involved in the majority of cases (Bertsias et al., 2004). In a 4-year period in Japan, 346 cases of pesticide poisoning were reported; in this case 70% were due to suicide attempts. Again, cholinesterase inhibitors and paraquat were involved in more than 60% of poisonings. Death rate from poisoning with paraquat was over 70%, while it was less than 10% with the herbicide glyphosate or glufosinate (Nagami et al., 2005). Reducing the availability of highly acutely toxic pesticides would be of relevance, as shown by recent data from Germany, where this approach reduced the number of fatal pesticide poisoning from 506 in 1980 to 39 in 2010 (Moebus and Bödeker, 2015). In the State of Louisiana, more than 40% of all pesticide-related hospitalizations during the period 1998 to 2007 involved insecticides (Badaksh et al., 2010). However, from 1995 to 2004, the overall number of pesticide poisonings in the United States has been declining, particularly for anticholinesterase insecticides (−70%), and for paraquat (−79%) (Blondell, 2007). As indicated earlier, while self-poisoning with pesticides for suicidal purposes is very common in some countries (e.g., in China it accounts for about 50% of all suicides; Page et al., 2016), it is the least utilized method in the United States (Ajdacic-Gross et al., 2008).

Regulatory Mandate

The awareness that the misuse of pesticides may pose potential health hazards has led to a realm of regulatory measures to ensure their safe use and the protection of the population. In the United States, the primary authority for pesticide regulation resides with the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Federal Food, Drug and Cosmetic ACT (FFDCA). Under FIFRA, the EPA registers pesticides for use, while under FFDCA, the EPA establishes maximum allowable levels of pesticide residues (tolerances) in foods and animal feeds, which are enforced by other federal agencies (Fenner-Crisp, 2001).

The first legislation passed in the United States was the Federal Insecticide Act of 1910, which only prohibited the manufacture of any insecticide or fungicide that was adulterated or misbranded. FIFRA was originally passed by Congress in 1947, adding the requirement of registration by the Secretary of Agriculture before the sale of insecticides, rodenticides, fungicides, and herbicides. Amendments in subsequent years added other pesticides to FIFRA jurisdiction, and provided the U.S. Department of Agriculture (USDA) with the authority to deny, suspend, or cancel registrations of products. In 1954, Congress amended the FFDCA to require the Food and Drug Administration (FDA) to establish tolerances for pesticides in raw agricultural commodities. This was extended 4 years later to processed foods. In 1970, the primary federal authority for the regulation of pesticides was transferred from USDA and FDA to the newly formed EPA. Between 1970 and 1990, FIFRA was amended several other times to address various issues related to pesticide safety and registration processes.

In 1996, the Food Quality and Protection Act (FQPA) brought important changes to pesticide regulations in the United States that affected both FIFRA and FFDCA. For example, FQPA provided the statutory mandate, under FIFRA, for continuing the expedited consideration of applications for pesticides that may provide reduced risks for human health, nontarget species, and the environment (Fenner-Crisp, 2001). Perhaps more significant were the changes to the FFDCA mandate. In 1958, an amendment of the FFDCA, the

Delaney clause (Section 409), stated that "…no additive shall be deemed safe if it is found to induce cancer when ingested by man or animal or if it is found, after tests which are appropriate for the evaluation of the safety of food additives, to induce cancer in man or animal…." Pesticides were included in this "additive" legislation. Under FQPA, however, pesticide residues are excluded from the definition of food additive, and the Delaney clause no longer applies to residues in food. Thus, tolerances can be set also for carcinogens. FQPA also directs EPA to consider aggregate exposure in the risk assessment process, that is, exposure which occurs from all food uses for a pesticide, as well as from exposures that occur from nonoccupational sources (e.g., drinking water and indoor residential or school use). Additionally, the EPA must consider whether certain pesticides, as well as other substances, may have the same mechanism of toxicity, and if so, carry out a cumulative risk assessment.

Another change introduced by FQPA gives the EPA the mandate to assess special risks of pesticides to infants and children. The Agency must assess aggregate risks based on dietary consumption patterns of children, possible susceptibility of infants and children to pesticides, and cumulative effects of compounds that share the same mechanism of toxicity. In the absence of adequate data, an additional default 10-fold safety factor should be applied to ensure children's safety. Finally, provisions are included for EPA to determine whether certain substances may have endocrine-disrupting effects in humans. Additional regulations concerning pesticides are present in other laws, such as the Safe Drinking Water Act and the Clean Air Act.

Under FIFRA, all pesticides sold or distributed in the United States must be registered by the EPA. Older products, registered before 1984, must undergo a re-registration process. To register a pesticide or a formulated product, a large number of studies (over 140) are required, a process that can take about 10 years and anywhere between $100 and $250 million. The database should include information on product and residue chemistry, environmental fate, toxicology, biotransformation/degradation, occupational exposure and reentry protection, spray drift, environmental impact on nontarget species (birds, mammals, aquatic organisms, plant, and soils), environmental persistence and bioaccumulation, as well as product performance and efficacy. Table 22-7 lists the basic requirements regarding toxicology data needed for new pesticide registration (see also Chap. 2, "Principles of Toxicology"). New tiered approaches to toxicity testing for agricultural chemicals, which would reduce the use of animals and improve the risk assessment process, have been recently proposed (Cooper et al., 2006; Doe et al., 2006).

Other nations, such as Canada, Japan, and most European countries have promulgated legislation similar to that of the United States for registration of pesticides. In the European Union (EU), a number of directives regulate pesticide registration; the active substance must be approved at the EU level, and the formulated products must be authorized at the Member State level. The European Commission Standard Committee on the Food Chain and Animal Health (SCFCAH) authorizes registration of new active substances, based on the initial Draft Assessment Report from a Member State, and a review by the European Food Safety Authority (EFSA). In the EU, tolerances are called Maximum Residue Levels (MRLs) and are set by the European Commission and EFSA. Some developing nations, with a shortage of trained technical, scientific, and legal professionals to develop their own legislation, have adopted the regulatory framework of one or another industrialized nation. The World Health Organization (WHO) provides guidance, particularly with the setting of acceptably daily intake (ADI) values for pesticides (the equivalent of the Reference Dose [RfD] set in the United States by the EPA), through its Joint FAO/WHO Meeting on Pesticide Residues (JMPR). In a few countries, still no legislation has been introduced to curb adverse effects of pesticides on the environment and human health.

Table 22-7

Basic Toxicology Testing Requirements for Pesticide Registration

TEST	ANIMAL SPECIES*
Acute lethality (oral, dermal, inhalation)	Rat, mouse, guinea pig, rabbit
Dermal irritation	Rabbit, rat, guinea pig
Dermal sensitization	Guinea pig
Eye irritation	Rabbit
Acute delayed neurotoxicity	Hen
Genotoxicity studies (in vitro, in vivo)	Bacteria, mammalian cells, mouse, rat, *Drosophila*
Teratogenicity	Rabbit, rodent (mouse, rat, hamster)
Two-four week toxicity study (oral, dermal, inhalation)	Rat, mouse
90-Day toxicity study (oral)	Rat
Chronic toxicity study (oral; 6 months to 2 years)	Rat, dog
Oncogenicity study	Rat, mouse
Reproductive/fertility study	Rat
Developmental neurotoxicity study	Rat

**Substantial efforts are being devoted to develop alternative nonanimal test systems. Nevertheless, only limited in vitro tests (for primary skin irritation) have been validated and accepted by regulatory bodies.*

INSECTICIDES

Insecticides play a most relevant role in the control of insect pests, particularly in developing countries. All of the chemical insecticides in use today are neurotoxicants, and act by poisoning the nervous systems of the target organisms (Casida, 2009) (Table 22-8). The central nervous system (CNS) of insects is highly developed and not unlike that of mammals, and the peripheral nervous system, though less complex, also presents striking similarities (Ecobichon, 2001a). Thus, insecticides are mostly not species-selective with regard to targets of toxicity, and mammals, including humans, are highly sensitive to their toxicity. When selectivity exists, this is often due to differences in detoxication pathways between insects and mammals or, particularly with the newer insecticides, to differential interactions with their target. As a class, insecticides have higher acute toxicity toward nontarget species compared to other pesticides. Some of them, most notably the organophosphates, are involved in a great number of human poisonings and deaths each year. The literature pertaining to the chemistry, development, biotransformation, environmental effects, and toxicity in target and nontarget species of insecticides is extensive, and the reader should refer to the monographs of O'Brien (1967), Brooks (1974), Hayes (1982), Ecobichon (1982), Matsumura (1985), Ford et al. (1986), Clark and Matsumura (1986), Costa et al. (1987), Hayes and Laws (1991), Chambers and Levi (1992), Krieger (2001; 2010), Stevens and Breckenridge (2001a), Gupta (2006), and Satoh and Gupta (2010).

Table 22-8

Molecular Targets of the Major Classes of Insecticides

TARGET	INSECTICIDE	EFFECT
Acetylcholinesterase	Organophosphates Carbamates	Inhibition Inhibition
Sodium channels	Pyrethroids (Type I and II) DDT Dihydropyrazoles	Activation Activation Inhibition
Nicotinic acetylcholine receptors	Nicotine Neonicotinoids	Activation Activation
GABA receptors–gated chloride channels	Cyclodienes Phenylpyrazoles Pyrethroids (Type II)	Inhibition Inhibition Inhibition
Glutamate-gated chloride channels*	Avermectins	Activation
Octopamine receptors†	Formamidines	Activation
Mitochondrial complex I	Rotenoids	Inhibition
Ryanodine receptors	Diamides	Activation

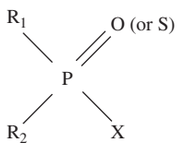
**Found only in insects. In mammals avermectins activate $GABA_A$ receptors.*

†In mammals, formamidines activate α_2-adrenoceptors.

Organophosphorus Compounds

Although a number of organic phosphorus (OP) compounds were synthesized in the 1800s, their development as insecticides only occurred in the late 1930s and early 1940s (Costa, 1988; Gallo and Lawryk, 1991). The German chemist Gerhard Schroeder is credited for the discovery of the general chemical structure of anticholinesterase OP compounds, and for the synthesis of the first commercialized OP insecticide (Bladan, containing TEPP [tetraethyl pyrophosphate] as the active ingredient), and for one of the most known, parathion, in 1944. Since then, hundreds of OP compounds have been made and commercialized worldwide in a variety of formulations. Until as recently as 2000, OPs were about 70% of all insecticide used, but that value has been halved in the following years (e.g., 35% in 2007; Grube et al., 2011). As of 2007, the most commonly used OP in both the agricultural sector and in all other sectors was chlorpyrifos, followed by acephate and malathion (Grube et al., 2011).

The chemistry of OPs has been thoroughly investigated (Chambers et al., 2010a). The general structure of OP insecticides can be represented by



where X is the so-called "leaving group," which is removed when the OP phosphorylates acetylcholinesterase (AChE), and is the most sensitive to hydrolysis; R_1 and R_2 are most commonly alkoxy groups (i.e., OCH_3 or OC_2H_5), though other chemical substitutes are also possible; either an oxygen or a sulfur (in this case the compound should be defined as a phosphorothioate) is also attached to the phosphorus with a double bond. Based on chemical differences, OPs can be divided into several subclasses, which include phosphates, phosphorothioates, phosphoramidates, phosphonates, and others (Chambers et al., 2010a). Fig. 22-1 shows the chemical structures of some commonly used OPs. Most are phosphorothioates, and need to be bioactivated in vivo to their oxygen analogs to exert their toxic action, but some (e.g., dichlorvos, methamidophos, or the nerve agent sarin) have P=O bonds. Most OPs used as insecticides have two methoxy or ethoxy side chains (Fig. 22-1).

Biotransformation The complex array of reactions involved in the biotransformation of OPs in target and nontarget species has been the subject of extensive investigations (Tang et al., 2006; Chambers et al., 2010b). For all compounds that contain a sulfur bound to the phosphorus, a metabolic bioactivation is necessary for their biological activity to be manifest, as only compounds with a P=O moiety are effective inhibitors of AChE. This bioactivation, which consists in an oxidative desulfuration mediated by cytochrome P450 enzymes (CYPs), and occurring mostly but not exclusively in the liver, leads to the formation of an "oxon," or oxygen analog of the parent insecticide (reaction 1 in Fig. 22-2). Multiple CYPs have been shown to activate organophosphorothioates to their oxons, with different substrate specificities; for example, diazinon is activated by human hepatic CYP2C19, while parathion is activated primarily by CYP3A4/5 and CYP2C8, and chlorpyrifos by CYP2B6 (Kappers et al., 2001; Tang et al., 2001; Mutch et al., 2003). Other reactions can also activate OPs; a relevant one is thioether oxidation (formation of a sulfoxide, S=O, followed by the formation of a sulfone, O=S=O) which occurs in the leaving group moiety, and is also catalyzed by CYPs (Costa, 1988). In the case of the OP disulfoton, the sulfoxide and the sulfone are more potent inhibitors of AChE than the parent compound. Several other biochemical reactions are detoxication reactions, as they lead to metabolites of lesser or no toxicity (reactions 2 to 5 in Fig. 22-2). Some, for example, oxidative dearylation or *O*-dealkylation, are mediated by CYPs, and substrate-dependent differences among CYPs also exist in the detoxication process. For example, dearylation of chlorpyrifos is catalyzed primarily by CYP2C19, while that of parathion by CYP2B6 (Tang et al., 2001; Hodgson, 2003). Catalytic hydrolysis by phosphotriesterases, known as A-esterases (which are not inhibited by OPs), also plays an important role in the detoxication of certain OPs (reaction 5 in Fig. 22-5). One example is the enzyme paraoxonase (PON1), which hydrolyzes the oxons of chlorpyrifos and diazinon, and at least in vitro, also of parathion (Li et al., 2000; Costa and Furlong, 2010). Noncatalytic hydrolysis of OPs also occurs when these compounds phosphorylate serine esterases classified as B-esterases that are inhibited by OPs but cannot catalytically hydrolyze them. Examples are the carboxylesterase (CarE) and butyrylcholinesterase (BuChE), in addition to the OP target, AChE. CarE also performs a catalytic hydrolysis of the carboxylic esters of malathion; high levels of expression of CarE in mammals are believed to be a major determinant of their low sensitivity to malathion toxicity. Inhibition of CarE by other compounds (including some OPs) greatly potentiates the toxicity of malathion in mammals (Murphy, 1986). Glutathione *S*-transferases may also contribute to the detoxication of OPs, particularly the methoxy compounds (Abel et al., 2004).

Signs and Symptoms of Toxicity, Mechanism of Action, and Treatment of Poisoning OP insecticides have high acute toxicity, with oral LD_{50} values in rat often below 50 mg/kg, though

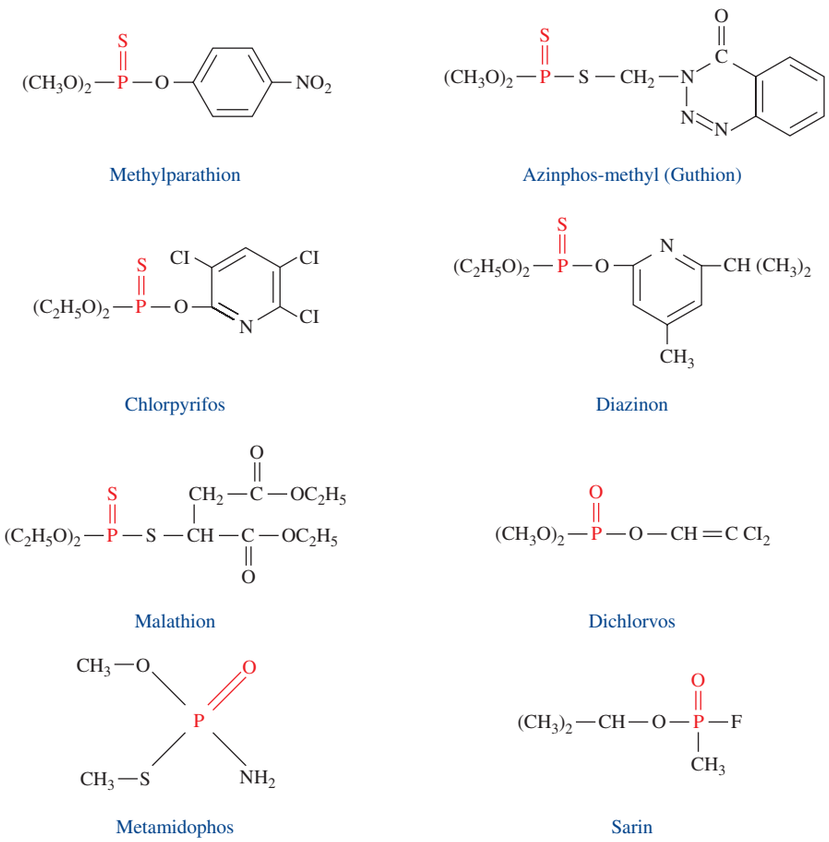


Figure 22-1. *Structures of some organophosphorus insecticides and of the nerve agent sarin.* Note that most compounds used as insecticides are organophosphorothioates (i.e., have a P=S bond), though some (as well as sarin) have a P=O bond and do not require metabolic activation.

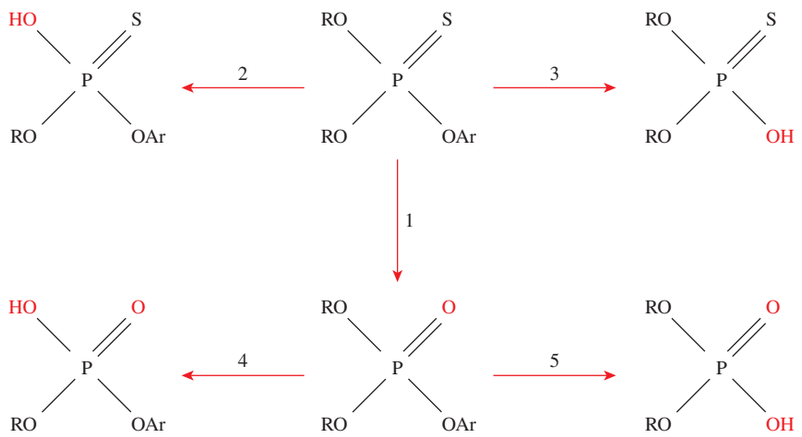


Figure 22-2. *General scheme of biotransformation of dialkyl, aryl phosphorothioate insecticides.* Reaction 1 is the bioactivation by oxidative desulfuration of the parent compound to the active metabolite, the oxon. The other reactions are enzymatic detoxication reactions that yield products that do not inhibit acetylcholinesterase. Reactions 2 and 4 are dealkylation reactions, while reaction 3 is a dearylation. All are mediated by various cytochromes P450. Reaction 5 is a hydrolytic reaction, catalyzed by paraoxonase (PON1). Note that not all reactions occur with every organophosphorus insecticide.

for some widely used compounds (e.g., chlorpyrifos and diazinon) toxicity is somewhat lower due to effective detoxication. One exception is malathion, which has an oral LD_{50} in rat of more than 1 g/kg, due to rapid detoxication by CarE. For several OPs, acute dermal toxicity is also high, with some exceptions being azinphos-methyl and malathion (Murphy, 1986). The primary target for OPs is AChE, a B-esterase whose physiological role is that of hydrolyzing acetylcholine, a major neurotransmitter in the central and peripheral (autonomic and motor-somatic) nervous systems. Acetylcholine released from cholinergic nerve terminals is disposed of solely through hydrolysis by AChE. In fact, in contrast to other neurotransmitters (e.g., norepinephrine or dopamine), it is choline,

Table 22-9

Signs and Symptoms of Acute Poisoning with Anticholinesterase Compounds

SITE AND RECEPTOR AFFECTED	MANIFESTATIONS
Exocrine glands (M)	Increased salivation, lacrimation, perspiration
Eyes (M)	Miosis, blurred vision
Gastrointestinal tract (M)	Abdominal cramps, vomiting, diarrhea
Respiratory tract (M)	Increased bronchial secretion, bronchoconstriction
Bladder (M)	Urinary frequency, incontinence
Cardiovascular system (M)	Bradycardia, hypotension
Cardiovascular system (N)	Tachycardia, transient hypertension
Skeletal muscles (N)	Muscle fasciculations, twitching, cramps, generalized weakness, flaccid paralysis
Central nervous system (M, N)	Dizziness, lethargy, fatigue, headache, mental confusion, depression of respiratory centers, convulsions, coma

Abbreviations: M, muscarinic receptors; N, nicotinic receptors.

the product of acetylcholine hydrolysis by AChE, that is taken up by the presynaptic terminal. Hence, inhibition of AChE by OPs causes accumulation of acetylcholine at cholinergic synapses, with overstimulation of cholinergic receptors of the muscarinic and nicotinic type. As these receptors are localized in most organs of the body, a "cholinergic syndrome" ensues, which includes increased sweating and salivation, profound bronchial secretion, bronchoconstriction, miosis, increased gastrointestinal motility, diarrhea, tremors, muscular twitching, and various CNS effects (Table 22-9). When death occurs, this is believed to be due to respiratory failure as a result of inhibition of respiratory centers in the brainstem, bronchoconstriction, and increased bronchial secretion, and flaccid paralysis of respiratory muscles (Gallo and Lawryk, 1991; Lotti, 2000, 2010). The time interval between exposure and onset of symptoms varies with the route and degree of exposure, and the chemical nature of the OP. The first signs to appear are usually muscarinic, which may or may not be in combination with nicotinic signs. While respiratory failure is a hallmark of severe OP poisoning, mild poisoning, and/or early stages of an otherwise severe poisoning, may display no clear-cut signs and symptoms (Lotti, 2010). Therefore, diagnosis is made through symptom recognition; miosis is observed most often, followed by gastrointestinal symptoms (nausea, vomiting, abdominal pain, vomiting) and hypersalivation.

The interaction of OPs with AChE has been studied in much detail. OPs with a P=O moiety phosphorylate a hydroxyl group on serine in the active (esteratic) site of the enzyme, thus impeding its action on the physiological substrate (Fig. 22-3). The first reaction leads to the formation of a Michaelis complex, while a subsequent reaction leads to phosphorylated AChE (Table 22-10). Rates of these two reactions, which are usually very rapid, indicate the affinity of the enzyme for a given OP. The bond between the phosphorus atom and the esteratic site of the enzyme is much more stable than the bond between the carbonyl carbon of acetate (in acetylcholine) at the same enzyme site. While breaking of the carbon–enzyme bond is complete in a few microseconds, breaking of the phosphorus-enzyme bond can take from a few hours to several days, depending on the chemical structure of the OP. Phosphorylated AChE is hydrolyzed by water at a very slow rate (Fig. 22-3; Table 22-10), and the rate of "spontaneous reactivation" depends on the chemical nature of the R substituents. Reactivation decreases in the order demethoxy, diethoxy, and diisopropoxy (Gallo and Lawryk, 1991). While water is a weak nucleophilic compound, certain hydroxylamine derivatives, known as oximes, can facilitate dephosphorylation of AChE, and are utilized in the therapy of OP poisoning (see below). Reactivation of phosphorylated AChE does not occur once the enzyme–inhibitor complex has "aged" (Fig. 22-3). Aging consists of the loss (by nonenzymatic hydrolysis) of one of the two alkyl (R) groups, and the rate of aging depends on the nature of the alkyl group. When phosphorylated AChE has aged, the enzyme can be considered to be irreversibly inhibited, and the only means of replacing its activity is through synthesis of a new enzyme, a process that may take days.

Upon OP poisoning, prompt treatment is essential. Procedures aimed at decontamination and/or at minimizing absorption depend on the route of exposure. In case of dermal exposure, contaminated clothing should be removed, and the skin washed with alkaline soap (Lotti, 2010). Special attention should be exercised by medical personnel, because passive contamination may occur. In case of ingestion, procedures to reduce absorption from the gastrointestinal tract do not appear to be very effective (Lotti, 2010). The muscarinic receptor antagonist atropine represents the cornerstone of the treatment for OP poisoning, as it prevents the action of accumulating acetylcholine on these receptors (Eddleston and Chowdhury, 2015). Atropine is preferably given intravenously, though the intramuscular route is also effective. The best clinical approach is to administer doses of atropine large enough to achieve evidence of atropinization, that is, flushing, dry mouth, changes in pupil size, bronchodilation, and increased heart rate, which should be maintained for at least 48 hours (Lotti, 2010). Indicative doses of atropine depend on the degree of poisoning and may range from a few milligrams to a few grams (Eddleston and Chowdhury, 2015). Overdosage with atropine is rarely serious in OP poisoned patients (Lotti, 2010). Oximes such as pralidoxime (2-PAM) are also used in the therapy of OP poisoning. 2-PAM contains a positively charged atom capable of attaching to the anionic site of AChE, and facilitates dephosphorylation of the enzyme (Fig. 22-4), thus restoring the catalytic site of AChE to its function. However, this chemical reaction occurs only when the phosphorylated AChE has not undergone aging. Dosing regimens for various oximes depend on the specific compound and the severity of OP poisoning (Lotti, 2010; Eddleston and Chowdhury, 2015). While animal data consistently show a marked positive effect of oximes, several authors reported limited or no efficacy of oximes in the treatment of OP poisoning (Bismuth et al., 1992; Buckley et al., 2005; Worek et al., 2016), and a meta-analysis of several studies of OP-poisoned patients concluded that use of oximes was associated "with either a null effect or possible harm" (Peter et al., 2006). However, inadequate dosing has been held as a major factor for lack of response to oxime therapy, together with the time of administration following OP poisoning, as their therapeutic window is determined by the rate of aging (Johnson et al., 2000; Eddleston et al., 2002). Furthermore, poisoning by OPs bearing two methoxy groups (malathion, methylparathion, dimethoate) is considered to be rather resistant to oxime therapy (Worek et al., 1999a). Though currently used oximes, such as 2-PAM, were developed in

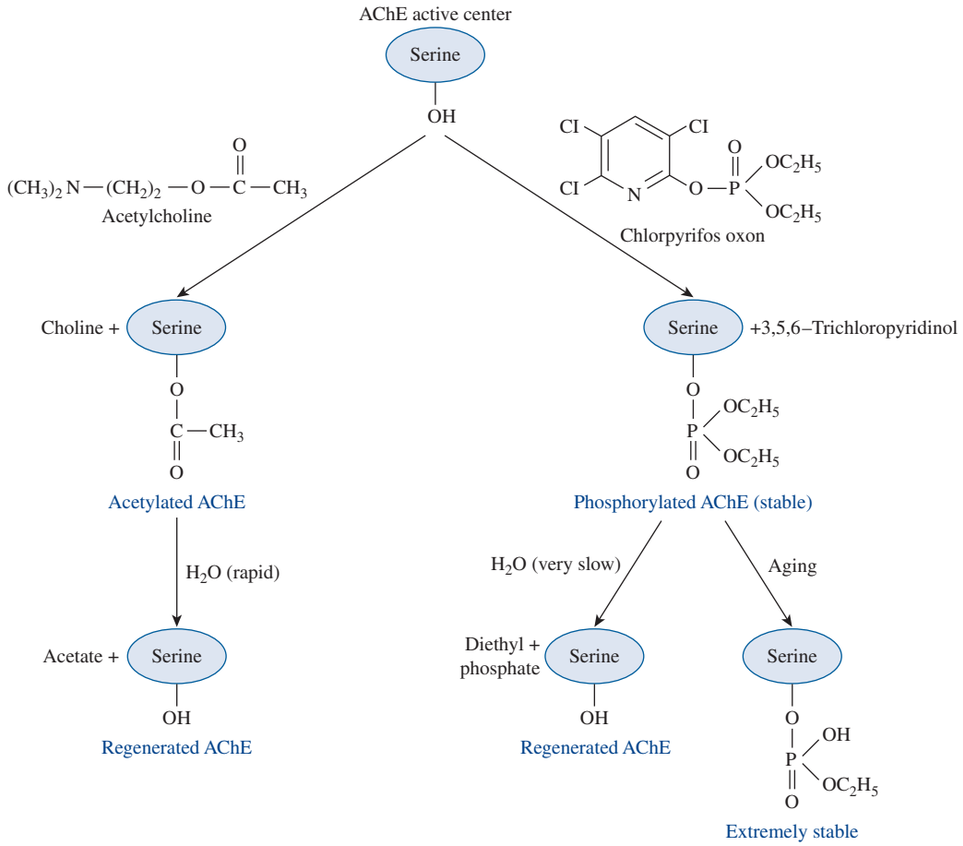


Figure 22-3. *Scheme of hydrolysis of acetylcholine by acetylcholinesterase (AChE) and reaction of chlorpyrifos oxon with AChE (see text for details).*

Table 22-10

Rates of Cholinesterase Inhibition by Carbamate and Organophosphorus Esters

		REACTION RATES	
PARAMETER	KINETIC CONSTANT	CARBAMATE	ORGANOPHOSPHORUS
Complex formation	k_1/k_1	Rapid	Rapid
Inhibition rate	k_2	Variable	Rapid to moderately rapid
Reactivation rate	k_3	Relatively rapid	Slow to extremely slow

SOURCES: Adapted from Ecobichon (2001a, 2001b).

the 1950s, research in this area is still active (Worek et al., 2016). It should also be mentioned that oximes have the potential of forming stable phosphoryl oximes that have themselves anticholinesterase activity, and for this reason, and because of rapid reactivation of carbamylated AChE, they are not indicated in case of poisoning with carbamate insecticides (see the section "Carbamates").

In addition to atropine and oximes, diazepam, to relieve anxiety in mild cases and to reduce muscle fasciculations and antagonize convulsions in the more severe cases, or magnesium sulfate, to reduce acetylcholine release, can also be used in the treatment of acute OP poisoning (Lotti, 2010; Eddleston and Chowdhury, 2015).

Biochemical Measurements In addition to synapses, AChE is also present in red blood cells (RBCs). Additionally, BuChE, also known as pseudo-cholinesterase, is found in plasma. Though the physiological functions of these enzymes in blood are yet to be discovered, activity of both enzymes is usually inhibited upon exposure to OPs, hence their measurements are widely used as an indication of exposure and/or biological effect of OPs. The specificity and usefulness of measurements of RBC AChE and of plasma BuChE, in the case of OP exposure, have been debated for some time (Lotti, 1995). The main issues can be summarized as follows (Costa, 2006): (1) Different OPs may inhibit AChE or BuChE to a different degree (e.g., the oxygen analogs of malathion, diazinon, and chlorpyrifos, and dichlorvos are stronger inhibitors of plasma BuChE than of RBC AChE). (2) There is a high degree of variability of enzyme activity (particularly of BuChE) among individuals, in part due to genetic differences (see the section "Genetic Susceptibility"). This requires establishment of a baseline value for each individual, or in case this is not available, of repeated post-exposure measurements to determine possible changes back toward baseline values. (3) RBC AChE activity is better correlated with target tissue (e.g., brain and diaphragm) AChE than is plasma BuChE activity, as indicated by animal studies (Padilla et al., 1994). There is also a good correlation between the severity of signs and symptoms of poisoning and the degree of inhibition of RBC AChE (Lotti, 2010). (4) Neither measurement is specific for a certain OP, and indeed, other insecticides, such as carbamates, also inhibit AChE and BuChE. (5) A 30%

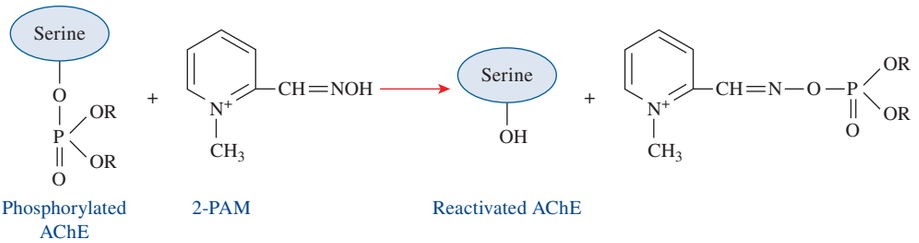


Figure 22-4. *Reactivation of phosphorylated acetylcholinesterase by pralidoxime (2-PAM).* Note that 2-PAM is only effective before the phosphorylated enzyme has undergone the aging reaction.

or greater decrease of plasma BuChE from pre-exposure baseline raises a red flag, and requires health and workplace surveillance, and removal of the worker from the exposure; however, the toxicological significance of such decrease is still much debated (Carlock et al., 1999; U.S. EPA, 2000). Despite these caveats, measurements of plasma BuChE, and particularly of RBC AChE, remain a very valid way to determine exposure to OPs, and early biological effects of OP exposure, to be used as confirmation of diagnosis of OP poisoning, or to monitor occupationally exposed workers. Several methods exist to quantify the activity of these two enzymes (Reiner and Simeon-Rudolf, 2006; Wilson and Henderson, 2007). Whole-blood AChE may also be quantified, considering that only about 10% of the activity is due to the plasma enzyme (Worek et al., 1999b).

Several analytical methods are also available to quantify OPs and their metabolites in body fluids; the parent compound is measured in blood, while metabolites are measured in urine (Lotti, 2010). These measurements are rarely carried out in the clinical setting, but are extensively utilized in epidemiological studies; indeed, determination of metabolite levels in urine is the most practical method to estimate exposure to OPs (Maroni et al., 2000). Such metabolites include alkylphosphate derivatives, as well as chemical residues specific for each compound (the "leaving group"). The alkylphosphates or alkyl-(di)-thiophosphates are the result of metabolism of parent compounds or their oxygen analogs by CYPs or esterases. They are not specific for a certain OP, but are useful to assess exposure to (or internal dose of) several OPs. Some alkylphosphate metabolites of commonly used OPs are shown in Table 22-11. Other metabolites are specific for certain OP compounds; for example, *p*-nitrophenol in urine is an indicator of exposure to parathion or methyl parathion, while 3,5,6-trichloropyridinol is useful to assess exposure to chlorpyrifos or methyl chlorpyrifos (Barr et al., 2005). While quantification of urinary metabolites of OPs have been widely used to assess exposure to OP from occupational, environmental, and dietary sources, caution should be exercised when interpreting results, as dialkylphosphates, and also leaving groups, can be found in the environment, including food and drinks, as a result of OP degradation. This would lead to an overestimate of OP exposure (Lu et al., 2005).

Table 22-11

Examples of Alkylphosphates in Urine

METABOLITE	ORGANOPHOSPHATE
DMP (dimethylphosphate)	Methylparathion, methylchlorpyrifos, dichlorvos, trichlorfon
DEP (diethylphosphate)	Parathion, diazinon, chlorpyrifos
DMTP (dimethylthiophosphate)	Azinphos-methyl (Guthion), fenitrothion
DETP (diethylthiophosphate)	Diazinon, parathion, chlorpyrifos

SOURCE: Adapted from Maroni et al. (2000).

The Intermediate Syndrome A second distinct manifestation of exposure to OPs is the so-called intermediate syndrome, which was first conceptualized by clinicians in Sri Lanka involved in the treatment of suicide attempts (Senanayake and Karalliedde, 1987). The intermediate syndrome is seen in a small subset (20% to 30%) of acute OP poisoning cases, and has been observed following exposure to a large variety of OPs (deBleecker, 2006). The syndrome develops one to several days after the poisoning, during recovery from cholinergic manifestations, or in some cases, when patients are completely recovered from the initial cholinergic crisis. Prominent features of the intermediate syndrome are a marked weakness of respiratory, neck, and proximal limb muscles. Extrapyramidal symptoms may also be present (Detweiler, 2014). Mortality due to respiratory paralysis and complications ranges from 15% to 40%, and recovery in surviving patients usually takes up to 30 days. The intermediate syndrome is not a direct effect of AChE inhibition, and its precise underlying mechanisms are still unknown. The hypothesis that muscle weakness may result from nicotinic receptor desensitization due to prolonged cholinergic stimulation remains the most valid (Lotti, 2010). There is no specific treatment for the intermediate syndrome and intervention is exclusively supportive.

Organophosphate-Induced Delayed Polyneuropathy A few OPs may also cause another type of toxicity, known as organophosphate-induced delayed polyneuropathy (OPIDP). Signs and symptoms of this delayed neurotoxicity include tingling of the hands and feet, followed by sensory loss, progressive muscle weakness and flaccidity of the distal skeletal muscles of the lower and upper extremities, and ataxia (Lotti, 1992; Lotti and Moretto, 2005; Ehrich and Jortner, 2010). These may occur 2 to 3 weeks after a single exposure, when signs of both the acute cholinergic and the intermediate syndromes have subsided. OPIDP can be classified as a distal sensorimotor axonopathy. Neuropathological studies in experimental OPIDP have evidenced that the primary lesion is a bilateral degenerative change in distal levels of axons and their terminals, primarily affecting larger/longer myelinated central and peripheral nerve fibers, leading to breakdown of neuritic segments and the myelin sheaths (Ehrich and Jortner, 2010). OPIDP is not related to AChE inhibition, and indeed one of the compounds involved in several epidemics of this neuropathy, including the so-called Ginger-Jake paralysis in the 1930s in the United States, is TOCP (tri-*ortho*-cresyl phosphate), which is a weak AChE inhibitor. Extensive studies carried out in the past 40 years (Johnson, 1982; Lotti, 1992; Johnson and Glynn, 2001) have identified the target for OPIDP as an esterase, present in nerve tissues as well as other tissues (e.g., lymphocytes), named neuropathy target esterase (NTE). Several OPs, depending on their chemical structure, can

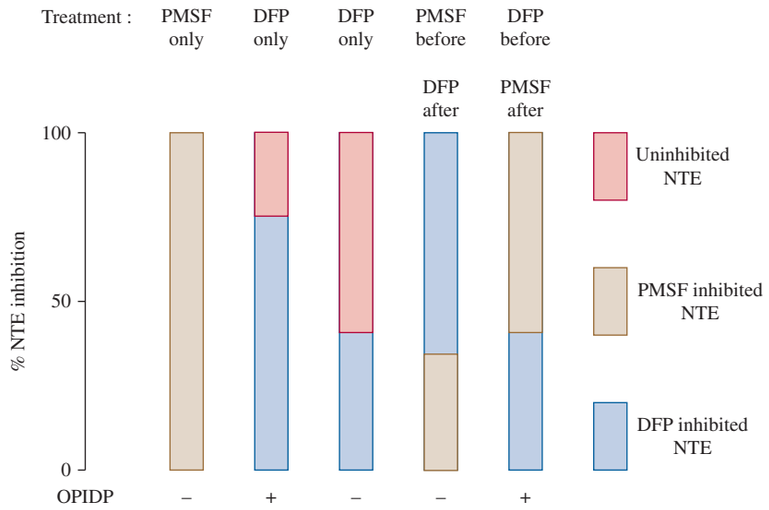


Figure 22-5. *Initiation, protection, and promotion of OPIDP, showing the relationships among timing of administration, dose, NTE inhibition, and clinical response, when DFP (diisopropylfluorophosphate) and PMSF (phenylmethanesulfonyl fluoride) are given alone or in combination.* (From Lotti et al. [1991], with permission.)

inhibit NTE, as do some non-OPs, such as certain carbamates and sulfonyl fluorides. Phosphorylation of NTE by OPs is similar to that observed for AChE. However, only OPs whose chemical structure leads to aging of phosphorylated NTE (by a process analogous to that described for AChE) can cause OPIDP. Other compounds that inhibit NTE but cannot undergo the aging reaction are not neuropathic, indicating that inhibition of NTE catalytic activity is not the mechanism of axonal degeneration, and that the aging of NTE is a key event in the initiation of OPIDP (Johnson and Read, 1987). For OPIDP to be initiated, phosphorylation and subsequent aging of at least 70% of NTE is necessary, and this two-step process occurs within hours of poisoning. When the first clinical signs of OPIDP are evident some weeks later, NTE activity has recovered. Lymphocyte NTE activity has been used in animals and in humans as a marker of NTE in nervous tissue (Lotti and Moretto, 2005).

NTE is a large protein comprising 1327 amino acid residues, and its physiological functions(s) are still unknown. Enzymatically, NTE behaves as a B-esterase, that is, it is inhibited by OPs rather than capable of hydrolyzing them (Wijeyesakere and Richardson, 2010), but also has phospholipase activity, as shown by its ability to hydrolyze phosphatidylcholine to glycerophosphocholine (Zaccheo et al., 2004; Glynn, 2006). It is now established that NTE is a member of a nine-protein family of patatin-like phospholipase domain–containing proteins (PNPLAs), of which NTE is PNPLA6 (Richardson et al., 2013). Mutations of the *PNPLA6* gene have been associated with a number of congenital disorders involving alterations in the central and/or peripheral nervous systems (Hufnagel et al., 2015). Human and mouse NTEs have an overall 39% identity with the Swiss Cheese Protein (Sws) in neurons of *Drosophila,* and a 61% identity in the phospholipase domain, including the active site at serine 966 (Moser et al., 2000). Sws mutant flies show neurodegeneration, glial hyperwrapping, and neuronal apoptosis, and similar changes are also seen in mice in which brain-only NTE has been knocked out, as full NTE deletion is embryo-lethal (Kretzschmar et al., 1997; Akossoglou et al., 2004; Moser et al., 2004). It has been reported that Sws can regulate protein kinase A (by binding and inhibiting the C3 catalytic subunit, PKA-C3), and that exposure of flies to TOCP alters the Sws–PKA–C3 interactions, thereby inhibiting PKA activity and causing neurodegeneration (Wentzell et al., 2014). These findings would support the hypothesis that OPIDP is the result of a toxic gain-of-function of phosphorylated and aged NTE (Lotti and Moretto, 2005; Wijeyesakere and Richardson, 2010). Despite these recent advances, the most crucial issues in the mechanisms of OPIDP development and progression remain obscure; indeed, though reductions in axonal transport have been found to precede overt clinical signs, the exact chain of events occurring between phosphorylation and aging of NTE and axonal degeneration is still unknown.

Several epidemics of OPIDP have occurred in the past, but its occurrence in humans is now rare, since before commercialization OPs must undergo specific neurotoxicity testing in the hen (one of the most sensitive species) to determine whether OPIDP is produced (Moretto, 1999). For this test, high doses of OPs are used, and animals are protected from acute cholinergic toxicity with atropine; clinical, morphological, and biochemical measurements are then carried out. In vitro tests can provide the ratio of relative inhibitory potency toward AChE and NTE, but these have not been accepted by regulatory agencies (Ehrich et al., 1997). A few commercialized OPs (metamidophos, trichlorfon, chlorpyrifos), which initially tested negative in the hen test, have caused OPIDP in humans, mostly as a result of extremely high exposures in suicide attempts (Lotti and Moretto, 2005). The possibility that repeated, low-level exposure to OPs may cause OPIDP is negligible, as the threshold (about 70%) for NTE inhibition and aging would not be reached under these exposure conditions (Wijeyesakere and Richardson, 2010). Though it was once thought that some animal species (e.g., rodents) were insensitive to OPIDP, only mice appear to be somewhat resistant (Veronesi et al., 1991). However, age is an important determinant of susceptibility, with young animals displaying more resistance; in young chicks the threshold for NTE inhibition and aging is more than 90% versus 70% in adult hens (Peraica et al., 1993).

As noted earlier, compounds that inhibit NTE but do not age do not cause OPIDP. When given to experimental animals *before* a neuropathic OP, these compounds exert a protective role by occupying the NTE active site. Surprisingly, however, when given after a neuropathic OP, these compounds have been shown to promote OPIDP (Lotti et al., 1991; Lotti, 2002a) (Fig. 22-5). By borrowing a terminology used for carcinogenic compounds, agents that phosphorylate NTE and age have been named initiators, while NTE inhibitors that do not initiate OPIDP but can promote it have been

named promoters (Lotti, 2002a). It is easy to understand why these latter compounds would be protective when given before an OP initiator; however, why and how they are able to promote OPIDP is still unknown. Promoters can also potentiate axonal degeneration caused by means other than OPs, such as traumatic nerve lesion, or 2,5-hexanedione (Moretto, 2000), and may involve proteins other than NTE itself. Though still mechanistically mysterious, the issue of promotion may have a bearing on risk assessment of potential insecticide mixtures. Indeed, exposure to an initiator at a dose lower than that required to cause (or for causing) OPIDP would nevertheless result in OPIDP if followed by exposure to a promoter.

Genetic Susceptibility Genetically determined variations in biotransformation enzymes or target molecules can modify the response to OPs (Costa, 2001; Povey, 2010). As CYPs are important for the activation and detoxication of OPs, polymorphisms of these enzymes may affect OP metabolism and toxicity. For example, a study of Hodgson (2003) has shown that polymorphic forms of CYP2C19 and CYP3A4 cause differential desulfuration and dearylation of chlorpyrifos. However, the overall influence of CYP polymorphisms on OP toxicity in vivo is not clear at the moment. The liver and plasma A-esterase paraoxonase (PON1), which also detoxifies chlorpyrifos oxon and diazoxon, presents several polymorphisms in the coding and promoter regions, which affect the catalytic efficiency of the enzyme toward different substrates (the Q192R polymorphism), and its level of expression (e.g., the C-108T polymorphism) (Costa et al., 2003, 2013; Costa and Furlong, 2010). Studies in transgenic animal models have clearly indicated that PON1 "status" (encompassing both the Q192R polymorphism and the level of PON1 activity) plays a most relevant role in modulating the acute toxicity of some OPs (Li et al., 2000; Cole et al., 2005) (Fig. 22-6). Evidence that PON1 status may play a role in susceptibility to OPs in humans is also emerging (Furlong et al., 2006; Hoffman et al., 2009; Costa et al., 2013). Genetically engineered PON1s with higher catalytic efficiencies toward different OPs are also being considered as catalytic scavengers to be eventually utilized in case of OP poisoning (Stevens et al., 2008; Costa and Furlong, 2010).

BuChE is present in plasma at high levels, though its physiological function is unclear. BuChE is inhibited by OPs and may guard against their toxicity, as the OP would be unavailable for reaction with its primary target AChE (Costa, 2006). At least 39 genetic variants of BuChE have been identified, with nucleotide alterations in the coding region. Several rare variants are silent, while the most common variants have a reduced activity and are far less efficient scavengers of cholinesterase inhibitors (Lockridge and Masson, 2000; Goodal, 2006; Lockridge et al., 2010). Individuals with such BuChE variants may be predicted to be more susceptible to OP toxicity (Fontoura-da-Silva and Chautard-Friere-Maira, 1996), but this assumption has been challenged and appears to be valid only in the case of OP nerve agents (Lockridge et al., 2016). A number of genetic variants of human AChE have been described (Goodal, 2006), but they are mainly not associated with altered AChE activity (Lockridge et al., 2016).

Long-Term Toxicity While the effects of acute exposure to OPs have been clearly identified and characterized by thousands of animal studies and cases of human poisonings, there is still controversy on possible long-term effects of these insecticides. Acute exposure to high doses of OPs may result, in some cases, in long-lasting adverse health effects (particularly CNS sequelae) as shown in animals, as well as in humans (Rosenstock et al., 1991; Sanchez-Santed et al., 2004). An increased risk of dementia may also be associated with acute OP poisoning (Lin et al., 2015; Sanchez-Santed et al., 2016). More controversial is the possibility that low

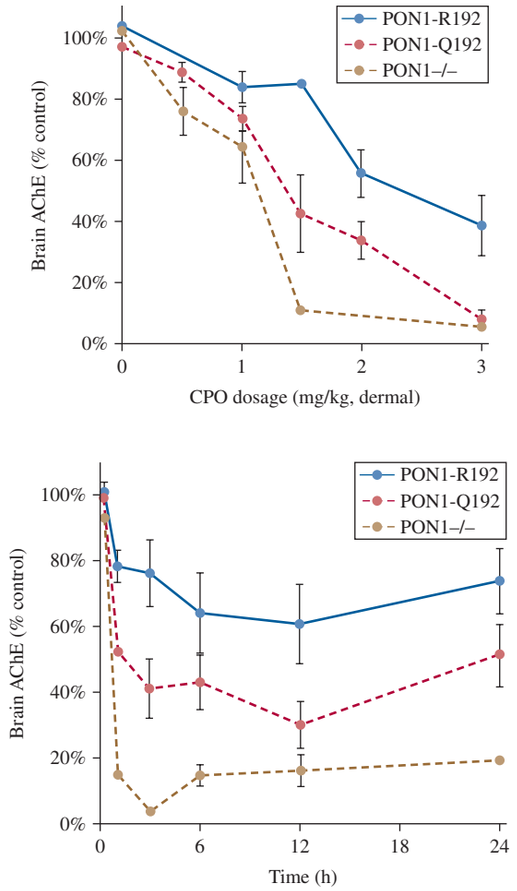


Figure 22-6. *Dose–response (top panel) and time-course (bottom panel) of brain acetylcholinesterase (AChE) inhibition by chlorpyrifos oxon (CPO) in PON1 knockout mice (PON1–/–) or in mice expressing either of the two human $PON1_{R192}$ or $PON1_{Q192}$ transgenes in place of endogenous mouse PON1.* Note that mice lacking PON1 are most sensitive to CPO toxicity, while those expressing human $PON1_{R192}$ are most resistant. Mice expressing human $PON1_{Q192}$ show intermediate sensitivity, despite expressing similar levels of PON1 as $PON1_{R192}$ mice. The experiments indicate the role of PON1 "status" in modulating the toxicity of CPO. (Modified from Cole et al. [2005], with permission.)

exposure to OPs, at doses that produce no cholinergic signs, may lead to long-term adverse health effects, particularly in the central and peripheral nervous systems. Chronic exposure of animals to OPs, at doses that significantly inhibit AChE, but may not be associated with clinical signs, results in the development of tolerance to their cholinergic effects (which is mediated, at least in part, by downregulation of cholinergic receptors), and has been associated with neurobehavioral abnormalities, particularly at the cognitive level (Costa et al., 1982; Prendergast et al., 1998). Evidence describing long-term neuropsychological or neuropsychiatric alterations in humans upon low chronic exposure is contradictory (Daniell et al., 1992; Jamal and Julu, 2002), and several expert reviews tend to conclude that the balance of evidence does not support the existence of clinically significant neuropsychological effects, neuropsychiatric abnormalities, or peripheral nerve dysfunction in humans chronically exposed to low levels of OPs (Ray, 1998; IOM, 2000; Lotti 2002b; Colosio et al., 2003; Moretto et al., 2010). Yet, research in this area, and the ensuing debate, will undoubtedly continue (Muñoz-Quezada et al., 2016).

OPs as a class have not been considered to be mutagenic, and there is little evidence that they may be carcinogenic. However, IARC (International Agency for Research on Cancer, a branch of WHO) recently concluded that four OPs (tetrachlorvinphos, parathion, malathion, and diazinon) are carcinogens (Guyton et al., 2015). The first two, which were classified as possible human carcinogens (Group 2B), are already banned or severely restricted in the United States and the EU. The latter two, which are still widely used, have been classified as probable human carcinogens (Group 2A). For example, the conclusion on malathion was based on limited evidence of carcinogenicity in humans and sufficient evidence in rodents, in addition to some positive genotoxicity results in mammalian systems and endocrine-disrupting effects (Guyton et al., 2015). Of interest is that the U.S. EPA concluded instead that malathion should be classified as a compound with "suggestive evidence of carcinogenicity, but not sufficient to assess human carcinogenic potential" (U.S. EPA, 2016a). The controversy in this area is likely to continue. Immunotoxicity of OPs has been suggested from in vitro or high-dose animal studies, but evidence in humans is lacking. Some OPs have also endocrine-disrupting activities in vitro (Tamura et al., 2003), but in vivo studies, even at dose levels that inhibit brain AChE activity, have not always substantiated these findings (Okahashi et al., 2005).

Developmental Toxicity and Neurotoxicity A report from the National Academy of Sciences highlighted the potential higher exposure of children to pesticides (NRC, 1993), and FQPA indicates that an additional safety factor should be included in the risk assessment process to ensure protection of children, who are presumed to be more sensitive to the effects of toxicants (FQPA, 1996). Experimental data indicate that young animals are more sensitive to the acute cholinergic toxicity of OPs (Costa, 2006; Pope, 2010). This increased sensitivity does not appear to be due to intrinsic differences in AChE, but rather to lower detoxication abilities of young animals. For example, low detoxication by CYPs or PON1 accounts for the age-dependent susceptibility of parathion and chlorpyrifos, respectively (Benke and Murphy, 1975; Mortensen et al., 1996; Costa and Furlong, 2010). In contrast, as discussed earlier, the young appear to be more resistant to OPIDP. In recent years, accumulating evidence has been suggesting that perinatal exposure to OPs may cause developmental neurotoxicity. Studies in rodents indicate that OPs can affect various cellular processes (e.g., DNA replication, neuronal survival, and neurite outgrowth) and noncholinergic pathways (e.g., serotoninergic synaptic functions and the adenylate cyclase system), and cause various behavioral abnormalities (Dam et al., 1998; Jett et al., 2001; Aldridge et al., 2003; Ricceri et al., 2003; Garcia et al., 2005). Such effects are at times seen at dose levels that produce no cholinergic signs of toxicity (Timofeeva and Levin, 2010). These findings, together with results of biomonitoring studies that indicate exposure of children to OPs, particularly in inner cities and farming communities, have led to regulatory restrictions on the use of certain OPs, and to concern for their potential neurotoxic effects in children (Eskenazi et al., 1999; Weiss et al., 2004; Eaton et al., 2008; Muñoz-Quezada et al., 2013), though diverging opinions also exist (Reiss et al., 2015).

Carbamates

Carbamate insecticides have a variety of chemical structures (Fig. 22-7), but all derive from carbamic acid, the majority being *N*-methylcarbamates. They present different degrees of acute oral

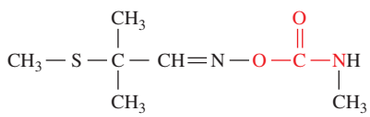
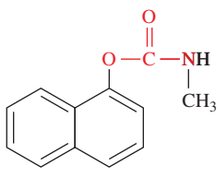
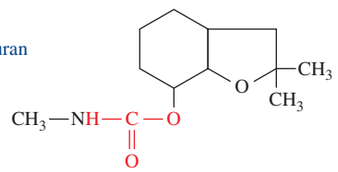
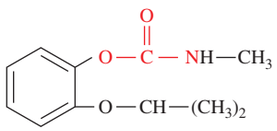
		LD_{50} in rats (mg/kg)		Water solubility (g/L)
		Oral	Dermal	
Aldicarb		0.8	3.2	6.0
Carbaryl		400	>5000	0.7
Carbofuran		10	>1000	0.7
Propoxur		85	~1500	2.0

Figure 22-7. *Structures of some carbamate insecticides, with indication of acute oral and dermal toxicity in the rat, and of water solubility.* Note that carbofuran has been banned in recent years in several countries.

toxicity, ranging from moderate to low toxicity such as carbaryl, to extremely high toxicity, such as aldicarb (Fig. 22-7). Dermal toxicity is lower, but skin penetration is increased by organic solvents and emulsifiers present in most formulations (Ecobichon, 2001b). Carbamates are susceptible to a variety of enzyme-catalyzed biotransformation reactions, and the principal pathways involve oxidation and hydrolysis (Fukuto, 1972; Tang et al., 2006). They do not require metabolic bioactivation, and the metabolites are for the most part devoid of biological activity, though this is not always the case. For example, two metabolites of aldicarb, the sulfoxide and the sulfone, are more potent anticholinesterases than the parent compound (Risher et al., 1987). The mechanism of toxicity of carbamates is analogous to that of OPs, in that they inhibit AChE. However, inhibition is transient and rapidly reversible, because there is rapid reactivation of the carbamylated enzyme in the presence of water (Table 22-10). Additionally, carbamylated AChE does not undergo the aging reaction. The sign and symptoms of carbamate poisoning are the same observed following intoxication with OPs, and include miosis, urination, diarrhea, salivation, muscle fasciculation, and CNS effects (Vale and Lotti, 2015) (Table 22-9). However, differently from OPs, acute intoxication by carbamates is generally resolved within a few hours. Fig. 22-8 shows experimental data on AChE inhibition by propoxur in mice; maximal inhibition is achieved very rapidly, as carbamates are direct AChE inhibitors and do not require metabolic bioactivation, and enzyme activity returns to control levels within 2 hours. Carbamates also inhibit BuChE; the degree of such inhibition varies depending on the specific compound, but is generally modest after moderate exposure (when, however, inhibition of red blood cells and nervous tissue AChE can be substantial) and equivalent to that of AChE after severe exposure (Ecobichon, 2001b).

The transient nature of AChE inhibition following carbamate exposure poses several problems in quantification of its activity. First, measurements should be made shortly (a few hours at most) following exposure; otherwise, even if severe inhibition and symptoms of toxicity were present, the latter would be resolved, and no enzyme inhibition would be detected. Second, particular care should be taken even if blood samples are drawn shortly after exposure, as temperature and time elapsed before the assay would cause reversal of inhibition. The treatment of carbamate intoxication relies on the use of the muscarinic antagonist atropine. Use of oximes is generally not recommended, as 2-PAM has been shown to aggravate the toxicity of carbaryl (Murphy, 1986). Yet, oximes may have beneficial effects in case of other carbamates such as aldicarb (Ecobichon, 2001b), and a current view is that concern over use of oximes in case of carbamate poisoning is unwarranted (Rossman et al., 2009). There are several cases of human poisoning associated with exposure to various carbamates, particularly carbaryl (Cranmer, 1986) and propoxur (Hayes, 1982). Most cases, however, involved aldicarb. This compound, which has a very high acute toxicity, is also highly water soluble (Fig. 22-7). Though, because of this characteristic, it is not registered for use on any fruit or vegetable having high water content, its illegal uses in hydroponically grown cucumbers and in watermelons have led to outbreaks of poisoning (Goes et al., 1980; Goldman et al., 1990). Contamination of drinking water has also been reported (Zaki et al., 1982). Carbamates can inhibit NTE, but since carbamylated NTE cannot age, they are thought to be unable to initiate OPIDP. Additionally, when given before a neuropathic organophosphate, carbamates offer protection against OPIDP (Johnson and Lauwerys, 1969), but when given after, they can promote OPIDP. A few case reports indicate that exposure to very high dosages of methylcarbamates (e.g., carbaryl and carbofuran) may result in a peripheral polyneuropathy similar to OPIDP (Dickoff et al., 1987; Yang et al., 2000). This would imply that aging is not required for OPIDP to develop, or, alternatively, that in these cases, carbamates may have amplified a preexisting subclinical neuropathy. Carefully conducted animal studies would be needed to substantiate this hypothesis.

Subchronic and chronic toxicity studies on carbamate insecticides have been carried out mostly for registration purposes, and their main findings (inhibition of cholinesterases, effects on organ weight and hematological parameters, histopathological changes) are described in detail by Baron (1991). Development of tolerance to some carbamates (propoxur, carbaryl) upon repeated exposure has been observed, and this appears to be due to an induction of microsomal enzymes (Costa et al., 1981). As a class, methylcarbamates are not mutagenic, and there is also no evidence of carcinogenicity. Embryotoxicity or fetotoxicity are observed only at maternally toxic doses (Baron, 1991). Limited evidence suggests that carbamates (e.g., aldicarb) may be more acutely toxic to young animals than to adults (Moser, 1999), possibly because of lower detoxication, but this aspect has not been investigated in the same detail as for OPs.

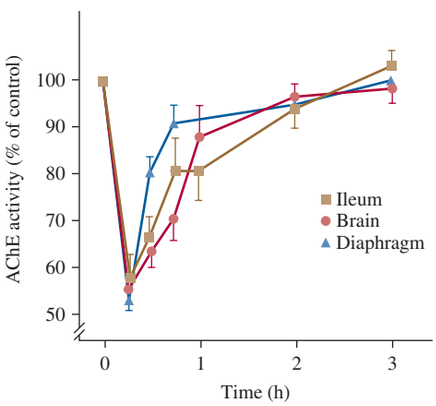


Figure 22-8. *Time-course of acetylcholinesterase (AChE) inhibition in different tissues of mice following administration of a single dose of the carbamate insecticide propoxur (10 mg/kg, i.p.).* Note the near complete recovery of AChE activity within 2 hours after propoxur administration. (From Costa et al. [1981], with permission.)

Pyrethroids

Extracts of the flower heads of *Chrysanthemum cinerariaefolium* were appreciated as insecticides in ancient China and Persia. Reports on their cultivation and commercialization go back to the mid-1800s (Casida, 1980). Pyrethrum extracts contain six insecticidal esters, referred to as pyrethrins, whose structures were elucidated in the early 1900s. However, because pyrethrins were decomposed rapidly by light, synthetic analogs—the pyrethroids—were developed in the 1970s (Casida, 2010). Due to their high insecticidal potency, generally low mammalian toxicity, and lack of environmental persistence, pyrethroids have encountered much success in the past 40 years and now account for about 15% to 20% of the global insecticide market (Soderlund, 2012). Pyrethroids are used widely as insecticides both in the house and in agriculture, in medicine for the topical treatment of scabies and headlice, and in tropical countries for malaria control, in soaked bed nets to prevent mosquito bites, as well as in indoor residual spraying. Pyrethroids are known to alter the normal function of insect nerves by modifying the kinetics of voltage-sensitive sodium channels, which mediate the transient increase in the sodium permeability of the nerve

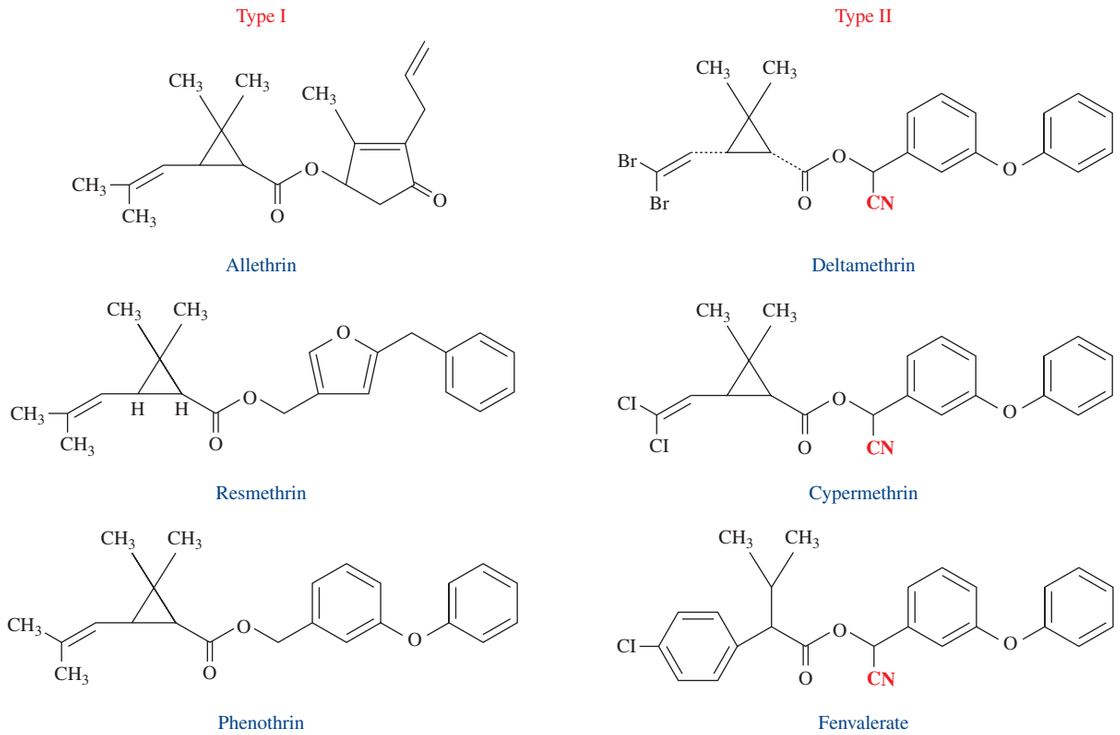


Figure 22-9. *Structures of type I (left) and type II (right) pyrethroid insecticides.* Note that all type II pyrethroids have a cyano (CN) group.

membrane that underlies the nerve action potential (Soderlund, 2012). All pyrethroid insecticides contain an acid moiety, a central ester bond, and an alcohol moiety (Fig. 22-9). The acid moiety contains two chiral carbons; thus, pyrethroids typically exist as stereoisomeric compounds (*trans* and *cis*). Additionally, some pyrethroids also have a chiral carbon on the alcohol moiety, allowing for a total of eight different stereoenantiomers. These chemical considerations are relevant, as pyrethroids' effects on sodium channels, their insecticidal activity, and their mammalian toxicity are stereospecific. The *cis* isomers are generally more toxic than the corresponding *trans* isomers (Casida et al., 1983). The acute oral mammalian toxicity of pyrethroids is generally low. Values of LD_{50} range, for example, from 100 mg/kg (deltamethrin) to 10,000 mg/kg (phenothrin). The relevance of stereo-specificity is underscored by the fact that the LD_{50} for 1R, *trans*-resmethrin is 8000 mg/kg, but that of 1R, *cis*-resmethrin is 100 mg/kg (Casida et al., 1983). The relatively low mammalian toxicity of pyrethroids is confirmed by the fact that despite their extensive worldwide use, there are relatively few reports of human poisonings, and only a dozen deaths (Bradberry et al., 2005). Most deaths occurred following accidental or intentional exposure to pyrethroids. For example, a 45-year-old man died 3 hours after eating beans and cheese prepared using a 10% cypermethrin solution instead of oil (Poulos et al., 1982). He et al. (1989) reported of 573 cases of acute pyrethroid poisoning in China between 1983 and 1988, with some fatalities. The dermal toxicity of pyrethroids is low, because of limited absorption through the skin.

Upon absorption, pyrethroids are very rapidly metabolized through two major biotransformation routes: hydrolysis of the ester linkage, which is catalyzed by hepatic and plasma CarEs and oxidation of the alcohol moiety by CYPs (Miyamoto, 1976; Soderlund and Casida, 1977; Kaneko, 2010). These initial reactions are followed by further oxidations, hydrolysis, and conjugation with sulfate or glucuronide. The relative importance of the hydrolytic or oxidative biotransformation varies from compound to compound, and from isomer to isomer for each pyrethroid. For example, the *trans* isomer of permethrin is more susceptible to hydrolysis by CarE than the *cis* isomer (Soderlund and Casida, 1977; Ross et al., 2006). Type II pyrethroids (see the following section) are less sensitive to hydrolysis. For instance, deltamethrin, a type II pyrethroid containing a cyano group (Fig. 22-9) and present solely as the *cis* isomer, is more extensively metabolized by hepatic CYPs, particularly CYP1A2 and CYP1A1 (V_{max}/K_m = 34.9), than by liver CarE (V_{max}/K_m = 11.5) (Anand et al., 2006a). Though it has been suggested that oxidative metabolism may lead in some cases to bioactivation of certain pyrethroids (Dayal et al., 2003; Ray and Fry, 2006), the current line of evidence would suggest that hydrolytic and oxidative metabolism achieve detoxication of the parent, active compound (Soderlund, 2012). Inhibition of CYPs by piperonyl butoxide (PBO) indeed increases pyrethroid toxicity, and so does inhibition of CarE (Casida et al., 1983). PBO is added to most pyrethroid formulations as a synergist, and the need for an increased use of bed nets treated with both PBO and pyrethroids has been recommended to overcome pyrethroid resistance by malaria-carrying mosquitoes (Churcher et al., 2016). Inhibition of CarE may be of significance, if unauthorized pyrethroid/OP mixtures are utilized (Ray and Forshaw, 2000). In fact, several OPs inhibit CarE activity, and may thus be expected to potentiate pyrethroid toxicity (Choi et al., 2004). Biotransformation of pyrethroids in humans is similar to rats, with ester hydrolysis and oxidation as the predominant metabolic reactions; the latter are mostly carried out by CYP2C9 and CYP3A4 (Kaneko, 2010).

Signs and Symptoms of Toxicity and Mechanisms of Action The acute mammalian toxicity of pyrethroids is well characterized (Soderlund, 2010). Based on toxic signs in rats,

observed after administration of 36 pyrethroids, these compounds have been divided into two classes (Verschoyle and Aldridge, 1980) (Table 22-12). Type I compounds produce a syndrome consisting in marked behavioral arousal, aggressive sparring, increased startle response, and fine body tremor progressing to whole-body tremor and prostration (type I or T [tremor] syndrome). Type II compounds produce profuse salivation, coarse tremor progressing to choreoathetosis, and clonic seizures (type II or CS [choreoathetosis with salivation] syndrome) (Ray and Fry, 2006; Soderlund, 2010, 2012). A key structural difference between type I and type II pyrethroids is the presence only in the latter of a cyano group at the α carbon of the alcohol moiety of the compound (Fig. 22-9). However, certain pyrethroids (e.g., cyphenothrin and flucythrinate) elude such classification, as they produce a combination of the two syndromes (Ray and Fry, 2006; Soderlund, 2012).

Table 22-12

Classification of Pyrethroid Insecticides Based on Toxic Signs in Rats

SYNDROME	SIGNS AND SYMPTOMS	EXAMPLES
Type I (T syndrome)	Aggressive sparring Increased sensitivity to external stimuli Whole-body tremors Prostration	Allethrin Bioallethrin Resmethrin Phenothrin
Type 2 (CS syndrome)	Pawing and burrowing Profuse salivation Coarse tremor Choreoathetosis Clonic seizures	Deltamethrin Fenvalerate Cypermethrin Cyhalothrin

The principal mode of action of pyrethroids in mammals is the same as in insects, that is, disruption of the voltage-gated sodium channels (Narahashi, 1996; Soderlund, 2010, 2012), though secondary targets have also been identified (Breckenridge et al., 2009). Pyrethroids bind to the α subunit of the sodium channel and slow the activation (opening), as well as the rate of inactivation (closing) of the sodium channel, leading to a stable hyperexcitable state. Sodium channels then open at more hyperpolarized potentials, and are held open longer, allowing more sodium ions to cross and depolarize the neuronal membrane (Shafer et al., 2005). In general, type II compounds delay the inactivation of sodium channels substantially longer (more than 10 milliseconds) than do type I compounds (less than 10 milliseconds) (Ray and Fry, 2006). Type I compounds prolong channel opening only long enough to cause repetitive firing of the action potential (repetitive discharge), analogously to DDT (Vijverberg et al., 1982); type II compounds hold the channels open for such long periods that the membrane potential ultimately becomes depolarized to the point at which generation of the action potential is not possible (depolarization-dependent block) (Shafer et al., 2005) (Fig. 22-10). These differences in the time of opening of sodium channels are believed to be at the basis of the differences observed between the T and CS syndromes (Ray and Fry, 2006; Breckenridge et al., 2009).

The higher sensitivity of insects to pyrethroid toxicity, compared to mammals, is thought to result from a combination of higher sensitivity of insect sodium channels, lower body temperature (as pyrethroids show a negative temperature coefficient of action), and slower biotransformation (Ray and Fry, 2006; Costa, 2015). For example, there is a 1000-fold difference in sensitivity to allethrin between the sodium channel in cockroach and rat neurons (Narahashi et al., 2007). Type II pyrethroids, but not type I compounds, also

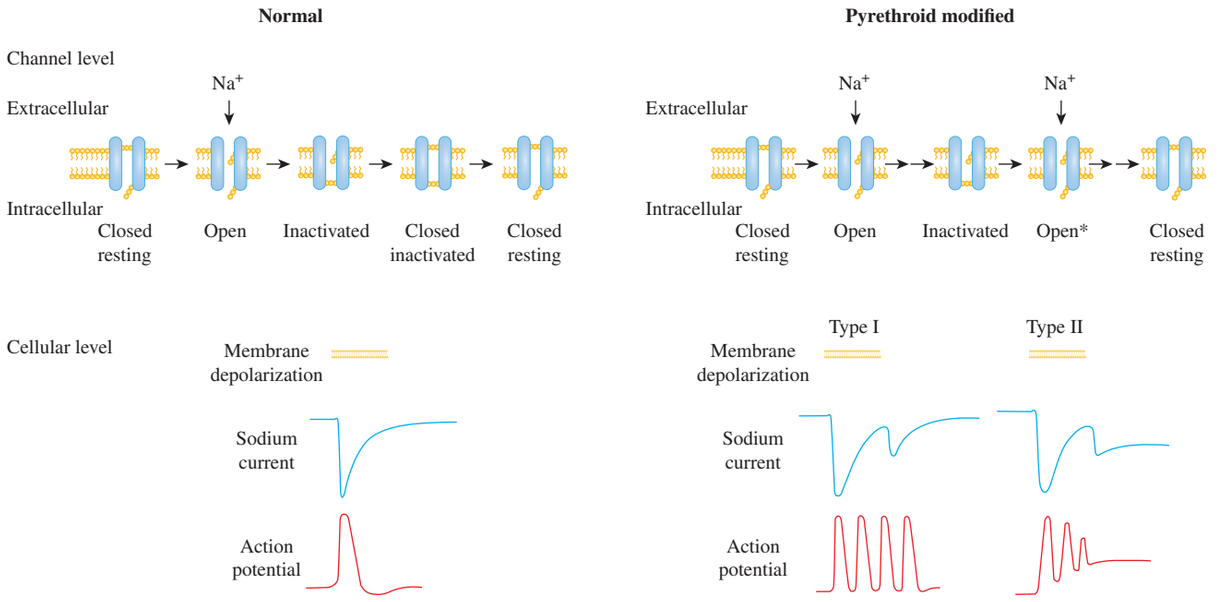


Figure 22-10. *Effect of pyrethroids on neuronal excitability.* Depolarization opens voltage-sensitive sodium channels (VSSCs) allowing Na^+ to enter the cell. To limit Na^+ entry and depolarization length, VSSCs inactivate and return to a "resting" state before reopening (*top left*). Pyrethroids delay inactivation (double arrows between states) of the channel and allow continued Na^+ flux (Open*) (*top right*). Under normal circumstances, depolarization leads to a rapidly inactivating current, and generates a single action potential (*bottom left*). Pyrethroid-modified VSSCs remain open when depolarization ends, resulting in a "tail" current. Type I compounds depolarize the cell membrane above the threshold for action potential generation, resulting in a series of action potential (repetitive firing). Type II compounds cause greater membrane depolarization, diminishing the Na^+ electrochemical gradient and subsequent action potential amplitude, eventually leading to depolarization-dependent block (*bottom right*). (From Shafer et al. [2005], with permission.)

bind to and inhibit $GABA_A$-gated chloride channels (Lawrence and Casida, 1983), albeit at higher concentrations than those sufficient to affect sodium channels (10^{-7} M vs. 10^{-10} M). This effect is believed to contribute to the seizures that accompany severe type II pyrethroid poisoning. However, drugs that enhance GABAergic transmission (e.g., diazepam) have modest effects toward deltamethrin-induced choreoathetosis or seizures (Soderlund, 2010). Type II pyrethroids, such as deltamethrin, also inhibit at low concentration (10^{-10} M) voltage-dependent chloride channels (Forshaw et al., 1993). Because xenobiotics that open these chloride channels, such as ivermectin and pentobarbital, antagonize pyrethroid-induced choreoathetosis and salivation, inhibition of maxi voltage-dependent chloride channels by pyrethroid may contribute to some of the signs associated with the type II poisoning syndrome (Forshaw et al., 2000; Breckenridge et al., 2009; Soderlund, 2010). Other reported targets for pyrethroids include calcium ATPase and voltage-gated calcium channels, which are, however, affected at higher concentrations (10^{-5} M and 10^{-6} M, respectively). The latter interaction may be of interest, as pyrethroids have been shown to increase calcium-dependent neurotransmitter release (Soderlund, 2010). Also of note is the observation that several pyrethroids could stimulate protein kinase C–dependent protein phosphorylation at very low concentrations (10^{-13} M) (Enan and Matsumura, 1993), but whether this interaction is involved in the modulation of sodium and/or chloride channels remains to be determined.

Young animals are more sensitive to the acute toxicity of certain pyrethroids, such as deltamethrin and cypermethrin (Sheets, 2000), most likely because of a lesser capacity for metabolic detoxification (Anand et al., 2006b); however, only minor age-related differences were found for other compounds (Sheets, 2000). Some studies have suggested that certain pyrethroids may cause developmental neurotoxicity, but current evidence has been judged inadequate (Shafer et al., 2005; EFSA, 2009). Furthermore, levels of background pyrethroid exposure (presumably through residues in the diet) in children have been found to be orders of magnitude lower than the corresponding ADI (Heudorf et al., 2004). Also, the use of deltamethrin-impregnated bed nets does not appear to pose any health risk in children and neonates (Barlow et al., 2001), while substantially reducing infant mortality from malaria (Alonso et al., 1991).

Upon occupational exposure, the primary adverse effect resulting from dermal contact with pyrethroids is paresthesia (Flannigan et al., 1985; He et al., 1989). Symptoms include continuous tingling or pricking or, when more severe, burning. The condition reverses in about 24 hours, and topical application of vitamin E has been shown to be an effective treatment. Paresthesia is presumably due to abnormal pyrethroid-induced repetitive activity in skin nerve terminals (Ray and Fry, 2006). Given their widespread use, reports on incidents due to pyrethroid exposure have been increasing over the past decade, though such exposures resulted in minor or no symptoms in most cases (Power and Sudakin, 2007). Cases of mild acute illness with neurological and respiratory symptoms have been reported by flight attendants following aircraft disinfection with permethrin (Sutton et al., 2007). Between 2000 and 2008, a total of 4974 cases of acute pyrethrin/pyrethroid-related illnesses were identified, mostly (85%) of low severity, with respiratory effects being the most common symptoms (Hudson et al., 2014).

Chronic studies with pyrethroids indicate that at high dose levels they cause slight liver enlargement often accompanied by some histopathological changes. There is little or no evidence of teratogenicity and mutagenicity (Miyamoto, 1976; Ray, 1991). An increased rate of lymphoma incidence in rodents has been reported for deltamethrin, but the effect was not dose-dependent (Cabral et al., 1990). Liver tumors were found in rats upon chronic exposure to permethrin, but as the mode of action appears to be due to enzyme induction and increased cell proliferation, it was judged not to be relevant to humans (Osimitz and Lake, 2009). There is no compelling evidence that pyrethroids may act as endocrine disruptors (Kim et al., 2004). Pyrethroids are particularly toxic to fish, but not to birds (Miyamoto, 1976).

Organochlorine Compounds

The organochlorine insecticides include the chlorinated ethane derivatives, such as DDT and its analogs; the cyclodienes, such as chlordane, aldrin, dieldrin, heptachlor, endrin, and toxaphene; the hexachlorocyclohexanes, such as lindane; and the caged structures mirex and chlordecone (Figs. 22-11 and 22-12). From the 1940s to the 1970–1980s, the organochlorine insecticides enjoyed wide use in agriculture, structure insect control, and malaria control programs. Their acute toxicity is moderate (less than that of OPs), but chronic exposure may be associated with adverse health effects particularly in the liver and the reproductive system. Primarily because of ecological considerations, these compounds have been banned in most countries in the past 30 to 40 years. Yet, because of their environmental persistence and high lipophilicity, exposure to these compounds continues, most notably through the diet. Furthermore, some, such as DDT, are being reintroduced in part of the world for malaria control; hence, a discussion of their toxicity has more than historical value.

DDT and Its Analogs DDT (1,1,1-trichloro-2, 2-bis(4-chlorophenyl)ethane) was first synthesized by O. Zeidler in 1874, but its insecticidal activity was discovered only in 1939 by Paul Mueller in Switzerland. Early experiments showed that DDT was effective against a wide variety of agricultural pests, as well as against insects that transmit some of the world's most serious diseases, such as

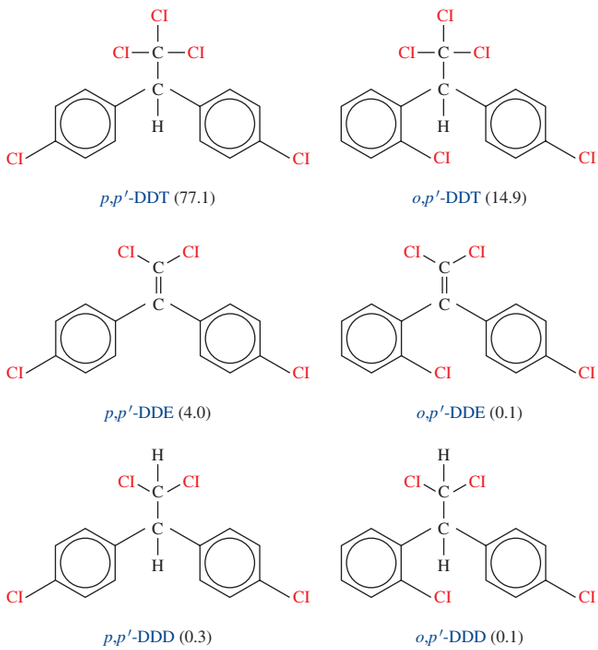


Figure 22-11. *Structures of the organochlorine insecticide p,p′- DDT and its isomers.* The percentage of each isomer present in technical grade DDT is indicated in parenthesis. DDE and DDD also result from biotransformation of DDT.

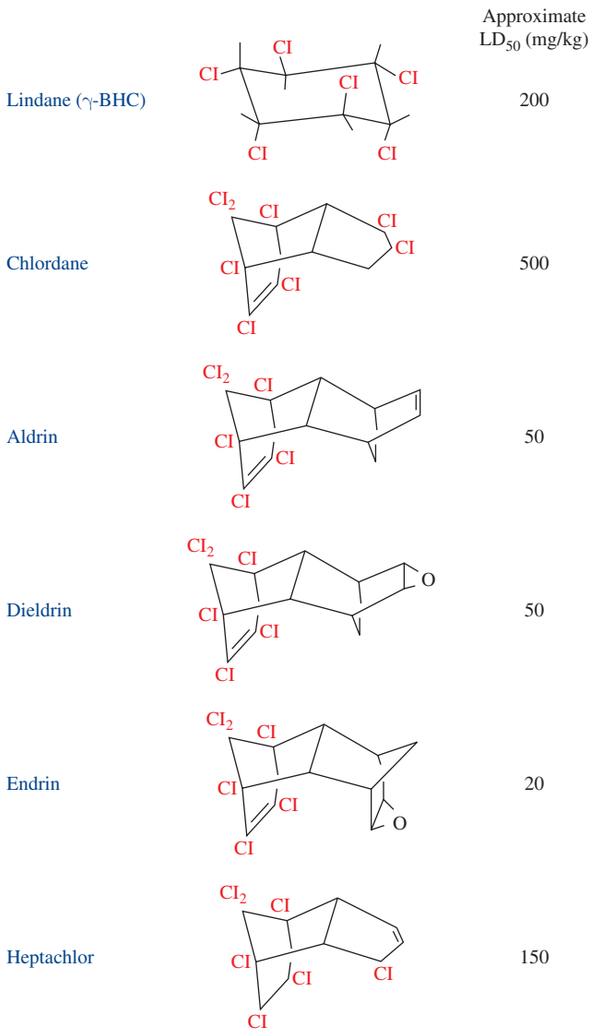


Figure 22-12. *Structure and acute toxicity (oral LD_{50} in rat) of selected organochlorine insecticides of different chemical classes (see text for details).*

typhus, malaria, and yellow fever. In the United States, annual use of DDT rose until 1959 to about 36,000 tons, and then declined gradually until its ban in 1972 (Rogan and Chen, 2005). However, as said, because of its high persistence, global redistribution, and its current use in many parts of the world, a discussion of the toxicology of DDT is still of relevance (Longnecker, 2005). While DDT is the universally accepted common name of the insecticide, technical-trade DDT is a mixture of several isomers (Fig. 22-11), with *p,p′*-DDT being responsible for the insecticidal activity.

DDT has a moderate acute toxicity when given by the oral route, with an LD_{50} of about 250 mg/kg; *p,p′*-DDT is at least 10-fold more toxic than *o,p′*-DDT (Smith, 2010). Dermal absorption of DDT is very limited, resulting in dermal LD_{50} values of more than 1000 mg/kg. In humans, oral doses of 10 to 20 mg/kg produce illness, but doses as high as 285 mg/kg have been ingested accidentally without fatal results. Toxicity from dermal exposure in humans is also low, as evidenced by the lack of significant adverse health effects when thousands of people were liberally dusted with this compound. Upon absorption, DDT distributes in all tissues, and the highest concentrations are found in adipose tissue. DDT is also extensively but slowly metabolized, with DDE, DDD, and DDA (in each case both the *p,p′* and the *o,p′* isomers) being the primary metabolites in humans (Smith, 2010). DDE is also stored in adipose tissue. Excretion is through the bile, urine, and the milk.

Acute exposure to high doses of DDT causes motor unrest, increased frequency of spontaneous movements, abnormal susceptibility to fear, and hypersusceptibility to external stimuli (light, touch, sound). This is followed by the development of fine tremors, progressing to coarse tremors, and eventually tonic–clonic convulsions. Symptoms usually appear several hours after exposure, and death, usually due to respiratory failure, may follow after 24 to 72 hours (Ecobichon and Joy, 1982). Signs and symptoms of poisoning are similar in most animal species, with dogs displaying prominent convulsions. There is no evidence that young animals may be more sensitive than adults to the acute toxicity of DDT, and they may actually be less sensitive (Smith, 2010). In humans, the earliest symptom of poisoning by DDT is hyperesthesia of the mouth and lower part of the face, followed by paresthesia of the same area and of the tongue; this is followed by dizziness, tremor of the extremities, confusion, and vomiting, while convulsions occur only in severe poisoning.

Signs and symptoms of acute poisoning clearly point at the nervous system as the primary target for DDT toxicity (Woolley, 1982). Both in insects and in mammals, DDT interferes with the sodium channels in the axonal membrane by a mechanism similar to that of Type I pyrethroids (Vijverberg et al., 1982). DDT has little or no effect on the resting potential or the rising phase and peak amplitude of the action potential. However, it greatly prolongs the depolarizing (negative) afterpotential of the action potential, and this produces a period of increased neuronal excitability immediately after the spike phase. This, in turn, enhances the probability of repetitive firing, and the insurgence of a "train" of action potentials (Fig. 22-13). Voltage clamp studies have shown that the principal effect of DDT is to slow down the closing of sodium channels once they have opened, while having little or no effect on closed gates. In addition to this effect on sodium channels, DDT also affects ATPases. Though DDT inhibits Na^+, K^+-ATPase, this action would not contribute to its neurotoxic effect (Matsumura and Patil, 1969). Rather, inhibition of a Ca^{2+}-ATPase (an ecto-ATPase, located on the outside of the cell membrane) may be involved in the effects of DDT. As the function of this Ca^{2+}-ATPase is considered to be that of maintaining high external calcium concentrations, its inhibition would lower external calcium and contribute to membrane instability and repetitive firing (Matsumura and Ghiasuddin, 1979). Several neurochemical studies have also shown that DDT exposure alters the levels of some neurotransmitters such as acetylcholine, norepinephrine, and serotonin, as well as of cyclic GMP, but these effects appear to be the results, rather than the cause, of DDT-induced neurotoxicity (Woolley, 1982). Treatment for DDT poisoning focuses on the nervous system. In laboratory animals, phenytoin and calcium gluconate have been found to reduce DDT-induced tremors and mortality, respectively. In humans, in addition to decontamination and supportive treatment, diazepam or phenobarbital may be beneficial to control convulsions, if present.

While acute exposure to DDT is a rare event, chronic exposure has been, and still is, a primary concern. In this regard, an important target for DDT is the liver. DDT and DDE increase liver weight and cause hepatic cell hypertrophy and necrosis, and are potent inducers of CYPs, particularly CYP2B and CYP3A (Smith, 2010; Harada et al., 2016a). DDT, as well as DDE and DDD, have been shown to be carcinogenic in rodents, causing primarily an increase in liver tumors. DDT is not genotoxic in in vitro and in vivo tests. Associations between cancer and DDT have been studied in several

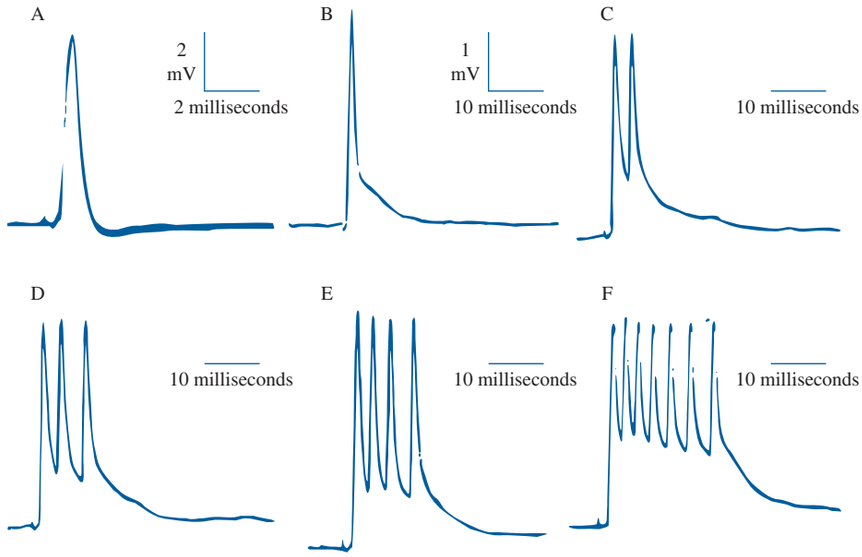


Figure 22-13. *Action potential of a single nerve fiber from the sciatic nerve of the toad Xenopus laevis, before (**A**), and 65, 85, 90, 105, and 135 minutes (**B–F**) after in vitro exposure to 10^{-4} M DDT.* Note the difference in timescale between A and B. The first observable effect is the development of a negative afterpotential (**B**). With time of exposure, repetitive spike discharge is initiated and increased in proportion to the amplitude of the negative afterpotential (**C–F**). (From Van den Bercken [1972], with permission.)

cohorts, and there is evidence, albeit contradictory, of increased risks for liver cancer and non-Hodgkin's lymphoma (Persson et al., 2012; Alavanja et al., 2014). Given the endocrine-disrupting actions of DDT and DDE (see below), particular attention has been devoted to possible associations between exposure to these compounds and hormonally sensitive cancers, and positive associations were found for some (e.g., testicular cancer) but not for others (e.g., breast cancer) (Beard, 2006; McGlynn et al., 2008). DDT is classified as a probable human carcinogen (Group 2A) by IARC (Loomis et al., 2015).

Methoxychlor (2,2-bis (*p*-methoxyphenyl)-1,1,1-trichloroethane) is the *p,p′*-dimethoxy analog of *p,p′*-DDT. Because of its low acute toxicity (LD_{50} in rat = 5000 mg/kg) and short biological half-life, its use greatly expanded following the ban of DDT. Furthermore, methoxychlor is rapidly metabolized, and does not accumulate in tissues. Though convulsions have been reported in dogs after high doses, in the rat depression of the CNS, mild tremors, diarrhea, and anorexia were observed (Smith, 2010). Upon chronic exposure, methoxychlor has been found to be a modest inducer of liver microsomal enzymes, and to cause chronic nephritis, and hypertrophy of kidneys, mammary glands, and uterus. Testicular atrophy and decreased spermatogenesis were also observed. Evidences of mutagenicity and carcinogenicity are inconclusive. Methoxychlor is metabolized by CYP2C19 and CYP1A2 to demethylated compounds, which have estrogenic activity (Smith, 2010); these are likely responsible for the effects caused by methoxychlor on the reproductive system in both male and female animals. Methoxychlor was banned in the EU and in the United States in the early 2000s.

Hexachlorocyclohexanes and Cyclodienes These two families of organochlorine insecticides comprise a large number of compounds that share a similar mechanism of neurotoxic action (Brooks, 2010). Lindane is the γ isomer of benzene hexachloride (BHC; 1,2,3,4,5,6- hexachlorocyclohexane) (Fig. 22-12). BHC is composed of eight stereoisomers, and the γ isomer is the one with insecticidal activity. Cyclodiene compounds include chlordane, dieldrin, aldrin (which is rapidly metabolized to dieldrin), heptachlor, and endrin (Fig. 22-12). Toxaphene, a complex mixture of chlorinated bornanes and camphenes, is also classified as an organochlorine insecticide. All of these compounds were introduced in the late 1940s to early 1950s, and have experienced wide use before being banned in most countries due to their persistence and environmental and human health effects (Costa, 2015). Lindane's use as an insecticide in agriculture has been banned, but it remains available as a scabicide and pediculocide in lotions and shampoos, as a second line of intervention, though in some states (e.g., California) even this use has been banned. The primary use of chlordane has been for termite control, while other compounds were primarily used in agriculture. Endrin was registered for use as a rodenticide to control voles in orchards, a testament to its rather high acute toxicity, compared to most other organochlorines (Fig. 22-12).

Lindane and cyclodienes have moderate to high acute oral toxicity (Fig. 22-12). However, in contrast to DDT, these compounds are readily absorbed through the skin. The primary target for their toxicity is the CNS. Unlike DDT, tremor is essentially absent, but convulsions are a prominent aspect of poisoning. These are due to the ability of these compounds to interfere with γ-aminobutyric acid (GABA)–mediated neurotransmission. GABA is an important neurotransmitter in the mammalian and insect CNS and in the insect neuromuscular junction. GABA receptors are members of the superfamily of ligand-gated ion channels that contain a chloride ionophore; by binding to these receptors, endogenous GABA causes the opening of chloride channels resulting in hyperpolarization of the membrane. Lindane and cyclodienes bind to a specific site (the picrotoxin site) on the chloride channel, thereby blocking its opening and thus antagonizing the "inhibitory" action of GABA (Cole and Casida, 1986; Eldefrawi and Eldefrawi, 1987; Narahashi, 1996). Treatment of acute poisoning is symptomatic; phenobarbital and diazepam can be used as anticonvulsants. Additional reported neurochemical effects of organochlorine insecticides include inhibition of Na^+, K^+-, Ca^{2+}-, and Mg^{2+}-ATPases, and changes in neurotransmitter levels.

As with DDT, these compounds are slowly metabolized, and have a tendency to bioaccumulate in adipose tissue; they are also excreted in milk. Cyclodienes are inducers of microsomal biotransformation enzymes and cause liver enlargement upon chronic exposure (Smith, 2010). They are not genotoxic, but have been shown to act as tumor promoters and to cause liver tumors in mice. In 2015, IARC classified lindane as carcinogenic to humans (Group 1) based on positive human and animal findings (Loomis et al., 2015). As with DDT, they are extremely persistent in the environment, and some have endocrine-disrupting properties. Cyclodienes, and particularly dieldrin, have also been suggested as potential pesticide candidates that may contribute to the etiology of Parkinson disease, given their ability to disrupt dopaminergic neurotransmission (Kanthasamy et al., 2005; Richardson et al., 2006), and the findings of epidemiological studies (Elbaz et al., 2009).

Mirex and Chlordecone These two organochlorine insecticides have a cage-like structure and were introduced in the late 1950s for use against fire ants and leaf-eating insects, respectively. Chlordecone (Kepone) has been the most studied because of one episode that involved 148 workers in a chlordecone producing factory in Hopewell, Virginia, between 1973 and 1975 (Taylor et al., 1978). The primary manifestation of chlordecone toxicity is the presence of tremors, which are observed in animals as well as in humans (Guzelian, 1982). Tremors affected primarily the hands, but fine tremors of the head and the entire body (Kepone shakes) were also observed (Costa, 2015). The exact mechanism of chlordecone neurotoxicity has not been elucidated, but it is believed to involve inhibition of ATPases (both Na^+, K^+- and Mg^{2+}-ATPases), and ensuing inhibition of the uptake of catecholamines (Desaiah, 1982). In contrast to cyclodienes, chlordecone does not cause seizures.

Chlordecone induces hepatic drug metabolizing enzymes, and causes hepatosplenomegaly in rats and humans. It is not mutagenic, but can induce liver tumors in rodents (Smith, 2010). Chlordecone also causes reproductive toxicity in animals, likely by mimicking the effects of excessive estrogens. Low or absent sperm count was found in chlordecone-exposed workers (Taylor et al., 1978; Guzelian, 1982). Chlordecone has been classified among the "dirty dozen" by the Stockholm convention on persistent organic pollutants, and banned worldwide. Adverse effects of this insecticide (particularly developmental toxicity and neurotoxicity) are still being reported in the French West Indies, where chlordecone was banned in 2003 after being extensively used on banana plantations (Costa, 2015; Multigner et al., 2016).

Environmental Ubiquity and Persistence The properties (low volatility, chemical stability, lipid solubility, slow rate of biotransformation, and degradation) that made organochlorine compounds such effective insecticides also brought about their demise because of their persistence in the environment, bioconcentration, and biomagnification in food chains, and the acquisition of biologically active body burdens at higher trophic levels (Ecobichon, 2001a). Sweden banned DDT in 1970, the United States in 1972, and the United Kingdom in 1986. Most other organochlorine insecticides were also banned in the United States during this period; for example, aldrin and dieldrin in 1975, toxaphene in 1982, and chlordane in 1988, while agricultural use of lindane was banned in 2006. These bans occurred largely on the basis of ecological considerations. The extensive use of organochlorines and their environmental persistence made them widespread pollutants. Studies carried out in the Great Lakes region in the United States exemplify the nature and the extent of the problem. Table 22-13 shows the tendency of DDT to bioaccumulate in the food chain. Adverse effects on bird reproduction (e.g., eggshell thinning) were among the first ecological effects to be identified. Particularly compelling was the book by Rachel Carson "Silent Spring" (Carson, 1962), which focused on the bioaccumulation of DDT and its effects on birds (Peakall, 1970). This book and the debate it generated played an important role in the decision to ban DDT and other organochlorines (U.S. EPA, 1975). Monitoring of organochlorine insecticides in fish in the Great Lakes shows extreme high levels in 1970 (e.g. 20,000 ng/g of total DDT, which also includes DDE and DDD), and a progressive decline after the use of these compounds was restricted or banned (Hickey et al., 2006).

Table 22-13
Concentration of DDT in a Lake Michigan Food Chain

	DDT (PPM)
Water	0.000002
Bottom mud	0.014
Fairy shrimp	0.410
Coho salmon, lake trout	3–6
Herring gull	99

SOURCE: From Harrison et al. (1970).

Because of their stability and high lipophilicity, organochlorines are present in adipose tissues of most individuals. For example, DDE, the breakdown product of DDT in the environment and the result of its biotransformation, is an extremely stable compound ($t_{1/2}$ = 7–11 years), which accumulates in fat, and is usually found in human tissue at the highest concentration. Studies in populations from different countries in the 1960s showed that all adipose tissue samples contained DDT at concentrations ranging from 5 to 20 mg/kg (ppm), with DDE accounting for about 60% of total DDT (WHO, 1979). Though concentrations of DDT in humans show a decreasing trend, those of DDE, which is ingested with food, particularly meat and fish, remain more constant or decrease only slightly. Organochlorine insecticides are also excreted in the milk. Though levels have decreased in most countries in the last decades, elevated levels remain in several developing countries (Chao et al., 2006; Bouwman et al., 2012).

Endocrine Disruption An endocrine, or hormone, disruptor can be defined as a xenobiotic that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development, and behavior (Crisp et al., 1998; Stoker and Kavlock, 2010). Several pesticides may fall into this category, and among these, a large number are organochlorine insecticides. The *o,p′*-isomer of DDT, which comprises approximately 15% of the technical grade product (Fig. 22-11), has estrogenic properties, in that it can act as an agonist at estrogen receptors (ER) α and β (Table 22-14). A metabolite of DDT, *p,p′*-DDE, in contrast, inhibits androgen binding to the androgen receptor (Kelce et al., 1995) (Table 22-14). Metabolites of the DDT analog, methoxychlor, are active as ERα agonists and ERβ antagonists and are antiandrogenic. Chlordecone also has estrogenic properties (Guzelian, 1982) (Table 22-14). Other organochlorine compounds with weak estrogenic activity are dieldrin, endosulfan, toxaphene, lindane, and the β isomer of hexachlorocyclohexane (β-BHC). In all cases, the potency of the insecticides or their metabolites is several orders of magnitude less than those of the hormones. Experimental studies have shown that administration of these compounds to

Table 22-14

Potency of DDT and Its Metabolites, and of Chlordecone, at Androgen and Estrogen Receptors

COMPOUND	ANDROGEN RECEPTOR IC_{50} (μM)	ESTROGEN RECEPTOR
p,p′ - DDT	75	>1000
p,p′ - DDE	5	>1000
o,p′ - DDT	95	5
p,p′ - DDD	90	>1000
Chlordecone	125	3
17β-estradiol	0.5	0.002

SOURCE: Adapted from Kelce et al. (1995).

animals can cause hormonally mediated adverse health effects. For example, *p,p′*-DDE had clear antiandrogenic effect when given to fetal, pubertal, and adult male rats (Kelce et al., 1995). Several studies have shown that chlordecone and methoxychlor affect the male reproductive system (Guzelian, 1982; Kavlock, 2001). Of possible relevance is the finding that exposure of rats to methoxychlor during gestation (E8 to E15) induced transgenerational defects in spermatogenic capacity and sperm viability, that is, these abnormalities were also observed in F2 generation animals (Anway et al., 2005). All these findings, though provocative, were obtained at dosage levels that far exceed anticipated environmental exposure. It has been suggested that estrogen equivalents from phytoestrogens present in foods and beverages are several orders of magnitude higher than estrogen equivalents resulting from estrogenic organochlorine insecticide contaminants in food (Safe, 2000). While the association between exposure to organochlorine compounds and possible hormone-related diseases in humans appeared to be weak (Golden et al., 1998; Safe, 2000), the Pine River Statement reviewed 494 studies published between 2003 and 2008 and found that "…DDT and its breakdown product DDE may be associated with adverse health outcomes such as breast cancer, diabetes, decreased semen quality, spontaneous abortion, and impaired development in childhood …" (Eskenazi et al., 2009).

DDT and Public Health: Risk–Benefit Considerations The Stockholm Convention on Persistent Organic Pollutants, ratified in 2004 by 50 countries, outlaws the use of several industrial chemicals (initially 12, the so-called "Dirty Dozen"), including several organochlorine insecticides. However, an exemption clause allows malaria-endemic nations to continue utilizing DDT (an earlier member of the dirty dozen) for indoor residual wall spraying. The United Nations Environment Program estimates that about 25 countries can use DDT under this exemption from [illegible]an. This situation is keeping the debate on the risks and benefits of DDT usage very much alive (Rogan and Chen, 2005; Van den Berg, 2009; Bouwman et al., 2011). On one hand, the environmental and human health effects of DDT are evident; on the other, one has to come to consider the burden of mortality from malaria worldwide. Indeed, there are an estimated 250 million clinical cases of malaria, causing almost one million deaths each year, mostly in children less than 5 years of age, and mostly in sub-Saharan Africa (Van den Berg, 2009; WHO, 2010). Thus, the use of DDT might reduce mortality from malaria and overall infant mortality, if spraying were carried out according to planned schedule, which is not always the case, and if malaria-transmitting mosquitoes do not become resistant to DDT, a problem that in the past has forced switching to other insecticides. The successful results obtained in South Africa in recent years (see the earlier section "Economics and Public Health") would support a continuous use of DDT. However, though indoor residual spraying might not cause the ecological effects that led to the ban of DDT, it would expose humans to amounts of DDT that may cause adverse health effects. In this regard, reproductive and developmental outcomes are of most concern. In particular, preterm births and early weaning (decreased duration of lactation), which can lead to increased infant mortality, have been associated with DDT exposure (Chen and Rogan, 2003; Rogan and Chen, 2005; Forns et al., 2016), though findings are controversial (Roberts et al., 2004). Nevertheless, reported DDT levels in breast milk often exceed the tolerable daily intake and would cause great concern in any developed country (Okankwo et al., 2008). For example in Mozambique, breast milk levels of ΣDDT (i.e., DDT and its metabolites) increased from 3.9 μg/mL in 2002 to 11 μg/mL in 2006, when indoor residual spraying with DDT for malaria control was started (Manaca et al., 2011). High serum levels of DDT have also been found in applicators, when proper personal protective equipment is not utilized (Bouwman et al., 2011). DDT remains a public health intervention that is inexpensive, long-lasting, and effective. Its judicious use should be combined with that of insecticide-treated bed nets to prevent mosquito biting, and with a better availability of therapeutic interventions in affected populations.

Neonicotinoids

Starting in the early 1980s, a new class of insecticides, the neonicotinoids, were developed by chemical modifications of nicotine and of other nicotinic acetylcholine receptor (nAChR) agonists (Casida and Durkin, 2017). Tobacco extracts had been used to repel and kill insects since 1690, and tobacco smoke was also used for fumigation (Ujvary, 2010). Nicotine is an alkaloid extracted from the leaves of tobacco plants, and was used as a free base or as the sulfate salt. Very little nicotine is used currently in the United States, but it is still used as a minor insecticide in some countries. Nicotine is a systemic insecticide effective toward a wide range of insects, including aphids, thrips, and whiteflies (Ujvary, 1999). As the primary component of tobacco used for smoking or chewing, nicotine's pharmacology and toxicology have been thoroughly investigated (Benowitz, 1996; Taylor, 1996). Nicotine exerts its pharmacological and toxic effects in mammals and insects by activating nAChRs. In vertebrates, nAChRs are expressed at neuromuscular junctions, in the peripheral nervous system and in the CNS, while in insects they are confined to the CNS (Eldefrawi and Eldefrawi, 1997). Interaction of nicotine with nAChRs produces initial stimulation followed by protracted depolarization, which results in receptor paralysis. The overall effect is the summation of stimulatory and inhibitory effects of nicotine at all sites expressing nAChRs. At high doses, parasympathetic stimulation and ganglionic and neuromuscular blockade predominate (Matyunas and Rodgers, 2001). Nicotine has a high acute toxicity in vertebrates, with LD_{50}s usually below 50 mg/kg (Ujvary, 2010). Signs and symptoms of poisoning include nausea, vomiting, muscle weakness, respiratory effects, headache, lethargy, and tachycardia. Most cases of poisoning with nicotine occur after exposure to tobacco products, or gum or patches. Workers who cultivate, harvest, or handle tobacco may experience green tobacco sickness, caused by dermal absorption of nicotine.

Neonicotinoids (also referred to as neonics) contain a nitromethylene, nitroimine or cyanoimine groups, and now account for 20% to 25% of the insecticide market (Casida and Durkin, 2017).

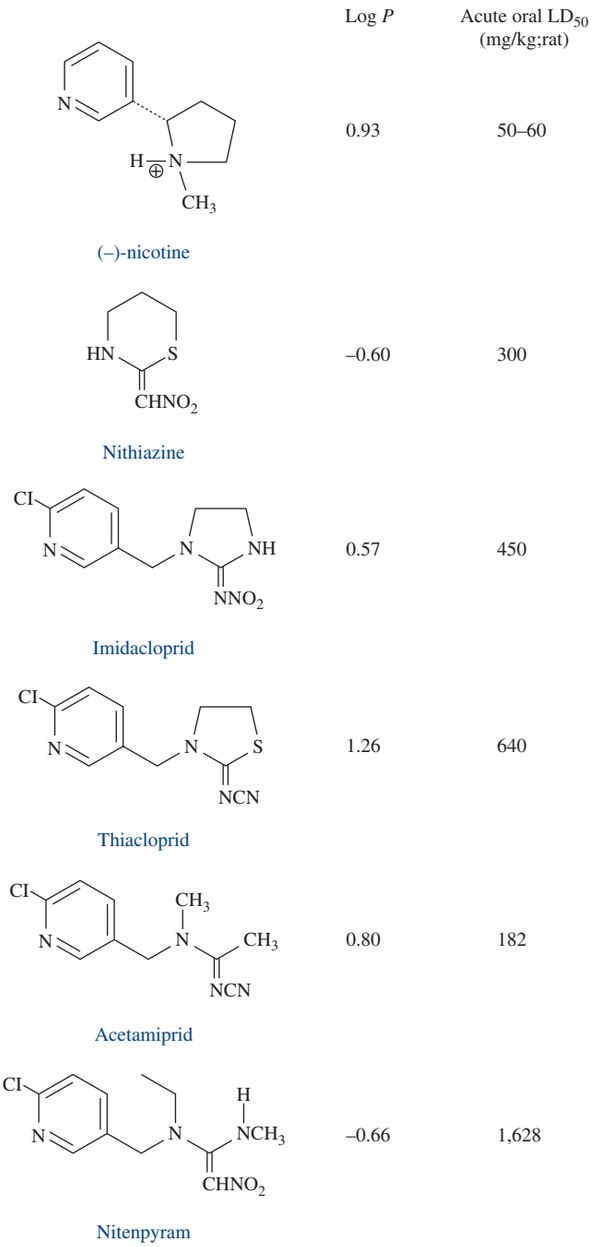


Figure 22-14. *Structures of nicotine and of neonicotinoid insecticides with indication of their acute oral toxicity in rat and their octanol/water partition (P).* (Data are derived from Tomizawa and Casida [2005].)

One of the first compounds synthesized was nithiazine, a nitromethylenyl heterocyclic compound highly toxic toward insects and with low mammalian toxicity, which was not developed commercially because of its photo-instability. Further structure–activity studies led to the development of imidacloprid (introduced in 1991), nitenpyram, acetamiprid, and other neonicotinoid compounds (Matsuda et al., 2001) (Fig. 22-14). The insecticidal activity of neonicotinoids is attributed to activation of postsynaptic nAChRs, which in insects are located exclusively in the CNS. As previously for nicotine, neonicotinoids are used primarily for crop protection as systemic insecticides, but are also effective against fleas in cats and dogs (Schenker et al., 2003). Acute oral toxicity (LD_{50}) of neonicotinoids in rats ranges from 180 to more than 2000 mg/kg (Fig. 22-14), while dermal toxicity is much lower (2000 to 5000 mg/kg), likely because of their low lipophilicity (Tomizawa and Casida, 2005). Signs and symptoms of toxicity in mammals are attributable to stimulation of nAChRs, particularly in the peripheral nervous system, given their poor penetration of the blood–brain barrier (Sheets, 2010). Some neonicotinoids (imidocloprid, thiacloprid) are particularly toxic to birds, other (thiacloprid) to fish. Most neonicotinoids are not mutagenic or carcinogenic, nor teratogenic. Neonicotinoids undergo limited biotransformation in mammals, involving mostly CYP-mediated oxidative reactions (Tomizawa and Casida, 2005; Sheets, 2010).

Neonicotinoids display a high selectivity profile, which is largely attributable to their specificity toward insect versus mammalian nAChRs. The nAChR consists of diverse subtypes assembled in combination from 10 α and 4 β, γ, δ, ε subunits. The most abundant subtypes in the vertebrate nervous system are α4β2 and α7, which are insensitive and sensitive, respectively, to α-bungarotoxin. In insects, neonicotinoids have been shown to bind to at least three pharmacologically distinct nAChRs (Matsuda et al., 2001; Sheets, 2010). Table 22-15 shows the in vitro effects of some neonicotinoids toward insect nAChRs and mammalian α4β2 nAChRs, and compares them to nicotine. Structural features of neonicotinoids that contribute to their selective actions at insect nAChRs have been described (Nakayama and Sukekawa, 1998; Matsuda et al., 2001; Tomizawa and Casida, 2005). Given their wider availability, there is an increasing number of acute neonicotinoid poisonings, primarily due to suicidal attempts; mortality is however low (about 3%), because of their receptor selectivity and low lipophilicity (Phua et al., 2009). Very limited information exists on possible adverse effects of chronic exposure of neonicotinoids in humans, and given their increasing use, investigations in this area are warranted (Cimino et al., 2017). The possibility that some neonicotinoids may exert developmental neurotoxicity has been raised, though the issue is still controversial (EFSA, 2013; Sheets et al., 2016).

In recent years, neonicotinoids have been at the center of a controversy regarding their potential role in the decrease of the bee population. These insecticides are indeed highly acutely toxic to bees (Table 22-16). In addition, they are able to impair the immune system of bees, thereby decreasing the defense mechanisms against infections (Sanchez-Bayo et al., 2016). On the basis of these findings, three neonicotinoids (imidacloprid, clothianidin, and thiamethoxan)

Table 22-15

Specificity of Neonicotinoids for Insect and Vertebrate nAChRs

	IC_{50} (NM)		
INSECTICIDE	INSECT	VERTEBRATE α4β2	SELECTIVITY RATIO
Imidacloprid	4.6	2,600	565
Acetamiprid	8.3	700	84
Thiacloprid	2.7	860	319
Nitenpyram	14.0	49,000	3,500
Flupyradifurone*	2.4	ND	ND
(-)Nicotine	4000	7	0.002

Abbreviation: ND, not determined.
*Butenolide.
SOURCES: From Tomizawa and Casida (2005) and Nauen et al. (2015).

Table 22-16

Acute Toxicity of Neonicotinoids in Bees

COMPOUND	ORAL LD_{50} (μG/BEE)
Imidacloprid	0.004–0.02
Clothianidin	0.004–0.03
Thiamethoxam	0.005–0.03
Dinotefuran	0.02–0.07
Nitenpyram	0.14
Flupyradifurone*	1.2
Acetamiprid	7.1
Thiacloprid	14.6

*Butenolide.
SOURCE: Adapted from Iwasa et al. (2004) and Laurino et al. (2011).

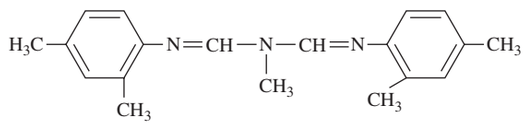
were temporarily banned in the EU in 2013, and a full ban may be foreseen for 2018 to 2020. A new insecticide flupyradifurone has been introduced in 2014 (Nauen et al., 2015). Though it is chemically a butenolide, its mechanism of action is the same as neonicotinoids, that is, activation of the nAChR (Table 22-15), and its toxicity to bees is lower than several but not all neonicotinoids (Table 22-16).

Other Old and New Insecticides

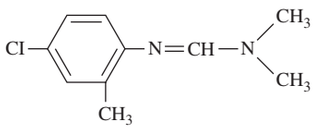
Rotenoids The roots of the East Asian *Derris* plants, particularly *D. elliptica,* and those of *Lonchocarpus utilis* and *L. urucu* in South America contain at least six rotenoid esters, among which the most abundant is rotenone. Rotenone is used as an agricultural insecticide/acaricide, and is one of the few pesticides allowed in organic farming (Isman, 2006). It is rather persistent in food crops after treatment, as indicated by half-life of 4 days in olives (Cabras et al., 2002). Rotenone is very toxic to fish, root extracts were used to paralyze fish for capture and consumption, and rotenone is still used in fishery management. Toxicity of rotenone in target and nontarget species is due to its ability to inhibit, at nanomolar concentrations, the mitochondrial respiratory chain, by blocking electron transport at NADH-ubiquinone reductase, the energy-conserving enzyme complex commonly known as complex I. Insect and fish mitochondria are particularly sensitive to complex I inhibition (Degli Esposti, 1998). Purified rotenone has a high acute toxicity in rodents and dogs, and is less toxic to rabbits and birds (Ujvary, 2010). Poisoning symptoms include initial increased respiratory and cardiac rates, clonic and tonic spasms, and muscular depression, followed by respiratory depression. Acute intoxication in humans is rare; a case report describes a fatal case in a 3.5-year-old girl who ingested an estimated 40 mg/kg of rotenone. Of note is that the label on the insecticide, which was manufactured in France and recommended for external use on animals, had stated "Natural Product—Non Toxic" (De Wilde et al., 1986). An additional case report describes a fatal case in a middle-aged woman after deliberate ingestion of rotenone, at a dosage estimated at 25 mg/kg (Wood et al., 2005).

In recent years, rotenone has received much attention because of its potential role in the etiology of Parkinson disease. An earlier study by Heikkila et al. (1985) showed that stereotaxic administration of rotenone to rats damaged the dopaminergic nigrostriatal pathway, similarly to that observed with MPP^+ (1-methyl-4-phenyl pyridinium, the active metabolite of 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine, MPTP), a known parkinsonism-causing chemical, which is also a complex I inhibitor (Degli Esposti, 1998). More recent studies have shown that administration of rotenone to rats (2 to 3 mg/kg/day for 1 to 5 weeks) caused selective nigrostriatal degeneration, though inhibition of complex I was observed uniformly in brain (Betarbet et al., 2000; Sherer et al., 2003). The finding that rotenone also produced protein inclusions, similar to Lewy bodies, that stained positively for ubiquitin and α-synuclein suggests that the rotenone model for Parkinson disease would be even better than the MPTP model (Betarbet et al., 2002). It has also been shown that an increase in α-synuclein significantly precipitates motor dysfunction and nigrostriatal neurodegeneration in rats exposed to rotenone (Naughton et al., 2017). However, because of the severe systemic toxicity of rotenone, the high variability across and within strains, and reports on nonspecific CNS effects (Lapointe et al., 2004), limitations of the rotenone model have been pointed out (Lapointe et al., 2004; Li et al., 2005). There is no evidence of Parkinson disease-like clinical signs or neurodegenerative pathology in chronic dietary studies (Hollingworth, 2001), and contrasting results have been reported in epidemiological studies of associations between use of rotenone and increased risk of Parkinson disease (Hollingworth, 2001; Li et al., 2005; Tanner et al., 2011). Recently, rotenone has been found to decrease activity of aldehyde dehydrogenase, thus increasing levels of DOPAL (3,4-dihydroxy phenylacetaldehyde), a toxic metabolite of dopamine involved in Parkinson disease pathogenesis (Panneton et al., 2010; Goldstein et al., 2015), providing an alternative mechanism for its potential contribution to this neurodegenerative disease.

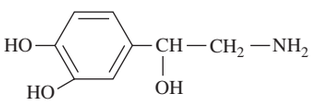
Formamidines Formamidines, such as chlordimeform ((*N*′-(4-chloro-o-tolyl)-*N*,*N*-dimethylformamidine) or amitraz (*N*′-2,4-(dimethyl-phenyl)-*N*,*N*-((2,4-dimethylphenyl) imino) methyl-*N*-methanimidamide), were developed in the early 1960s for use in agriculture and in veterinary medicine as insecticides/acaricides (Hollingworth, 1976). The structures of the most widely used, chlordimeform and amitraz, are shown in Fig. 22-15, together



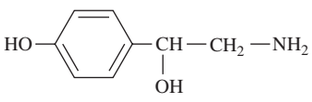
Amitraz



Chlordimeform



Norepinephrine



Octopamine

Figure 22-15. *Structures of the formamidine insecticides/acaricides amitraz and chlordimeform.* Structures of the mammalian neurotransmitter norepinephrine and of the insect neurotransmitter octopamine are also shown.

with the closely related structure of the neurotransmitters norepinephrine and octopamine (the latter is found in insects). In invertebrates, these compounds exert their toxicity by activating an octopamine-dependent adenylate cyclase (Nathanson, 1985). In mammals, symptoms of formamidine poisoning are sympathomimetic in nature, and include hypotension, bradycardia, and sedation (Beeman and Matsumura, 1973; del Pino, et al., 2015). The similarity between insect octopamine receptors and mammalian α_2-adrenergic receptors suggested the latter as a possible target for formamidines. In vivo and in vitro studies have indeed shown that formamidines act as rather selective agonists for α_2-adrenergic receptors (Hsu and Kakuk, 1984; Hsu and Lu, 1984; Costa et al., 1988; Altobelli et al., 2001). Chlordimeform's metabolism plays a most relevant role in its toxicity. The *N*-demethylated metabolite (desmethylchlordimeform) is more acutely toxic than chlordimeform, and displays a more than 400-fold higher potency toward α_2-adrenoceptors (Ghali and Hollingworth, 1985; Costa and Murphy, 1987). In contrast, two other metabolites of chlordimeform, 4-chloro-toluidine and *N*-formyl-4-chloro-*o*-toluidine, are thought to be responsible for the observed haemangioendotheliomas in mice observed in carcinogenicity studies (IPCS, 1998), which has led to the classification of chlordimeform as a probable human carcinogen (Group 2A) by IARC in 1990. Given the increasing evidence of an association between exposure to chlordimeform and 4-chloro-*o*-toluidine and bladder cancer (Popp et al., 1992), chlordimeform was withdrawn from the market in 1992.

In contrast, amitraz is still widely used for the control of ectoparasites in farm animals and crops. Though its acute toxicity is moderate (oral LD_{50} = 100–1600 mg/kg, depending on the animal species), several cases of acute amitraz poisoning have been reported, particularly in Turkey, and most have involved children (Yaramis et al., 2000; Caksen et al., 2003; Proudfoot, 2003; Elinav et al., 2005). Signs and symptoms of poisoning with amitraz mimic those of α_2-adrenergic receptor agonists such as clonidine, and include nausea, hypotension, hyperglycemia, bradycardia, and miosis. A series of acute amitraz poisonings was also reported in South Africa, with CNS depression as the most common clinical sign (Veale et al., 2011). Though α_2-adrenoceptor antagonists such as yohimbine have proved useful as antidotes in animals (Andrade and Sakate, 2003), their usefulness in managing amitraz poisoning in humans has not been evaluated. Amitraz is not genotoxic, but is classified as a possible human carcinogen by the EPA due to some evidence of carcinogenicity in mice (del Pino et al., 2015).

Avermectins The avermectins are macrocyclic lactones, first isolated in 1975 from the fermentation broth of the actinomycete *Streptomyces avermitilis,* which originated from a Japanese soil sample (Campbell, 1989; Fisher and Mrozik, 1992). This fungus synthesizes eight individual avermectins, of which avermectin B_{1a} displays the highest antiparasitic activity. Currently, abamectin (a mixture of 80% avermectin B_{1a} and 20% avermectin B_{1b}) is used as an insecticide, while the semisynthetic derivatives of avermectin B_{1a}, emamectin benzoate and ivermectin, are used as insecticides and for parasite control in human and veterinary medicine, respectively (Stevens and Breckenridge, 2001b). Abamectin is used primarily to control mites, while emamectin benzoate is effective at controlling lepidopteran species in various crops. Ivermectin, obtained by a chemical modification of avermectin B1, is used as an antihelmintic and antiparasitic agent in veterinary medicine, and in humans it has proved to be a very effective treatment for infection of intestinal threadworms, onchocerciasis (river blindness), and lymphatic filariasis (Stevens and Breckenridge, 2001b). The discovery and development of avermectin and of ivermectin led to the 2015 Nobel Prize in Medicine to William Campbell and Satoshi Omura (Molyneux and Ward, 2015). In insects and nematodes, avermectins exert their toxic effects by binding to, and activating, invertebrate-specific glutamate-gated chloride channels at nanomolar concentrations (Lynagh and Lynch, 2012). Acute toxicity of avermectins is high (e.g., oral LD_{50}s in rats of 11 mg/kg [abamectin] or 80 mg/kg [emamectin]), and may be even higher in neonate animals (Stevens and Breckenridge, 2001b). Signs and symptoms of intoxication include hyperexcitability, tremors, and incoordination, followed by ataxia and coma-like sedation. These effects appear to be due to the ability of avermectins to act as allosteric modulators of $GABA_A$ receptor–gated chloride channels in the vertebrate CNS (Fisher and Mrozik, 1992; Casida and Durkin, 2015.).

Avermectins are known to interact with P-glycoprotein, a plasma membrane protein of the ATP binding cassette (ABC) transporters superfamily, whose main function is the ATPase-dependent transport of foreign substances from the cell (Didier and Loor, 1996). The P-glycoprotein-mediated efflux plays an important role in attenuating the neurotoxicity and developmental neurotoxicity of avermectins (Stevens et al., 2010), while P-glycoprotein polymorphisms which may affect its function may increase avermectin neurotoxicity in humans (MacDonald and Gledhill, 2007). Despite the wide use of avermectins, and particularly of ivermectin in Africa and in Central America, there is little evidence of adverse health effects in humans. The major effect following administration of active doses of ivermectin (0.1 to 0.2 mg/kg) is a severe inflammatory response (the Mazzotti reaction), characterized by pruritis, erythema, vesicle, and papulae formation, and attributable to the killing of microfilariae which dislodge from their site of infestation and are transported in the blood and body fluids (Ito, 2013).

Phenylpyrazoles A relatively recent class of insecticides is that of phenylpyrazole derivatives, of which fipronil, commercialized in the mid-1990s, was the first one brought to market. Fipronil is a broad-spectrum insecticide with moderate mammalian toxicity (LD_{50} in rat: oral, 97 mg/kg; dermal, more than 2000 mg/kg), and a high selectivity for target species. Once absorbed, fipronil is extensively metabolized, particularly by CYPs (Wang et al., 2016). Fipronil acts as a blocker of the $GABA_A$-gated chloride channel, but binds to a site different from the picrotoxin binding site used by organochlorine insecticides (Casida and Durkin, 2017). It also has a much higher specificity for insect receptors over mammalian receptors (Hainzl et al., 1998; Narahashi et al., 2007; Wang et al., 2016). Furthermore, in insects fipronil also potently blocks glutamate-activated chloride channels that are not present in mammals (Narahashi et al., 2007). There is no evidence that fipronil is an eye or skin irritant, or has any mutagenic, carcinogenic, or teratogenic effects. A number of human poisonings with fipronil have been reported that resulted from accidental or intentional ingestion. Less than 20% of the patients developed seizures, and all recovered (Mohamed et al., 2004; Lee et al., 2010; Bharathrai et al., 2015).

Diamides These compounds represent a recent class of insecticides with a novel mechanism of action, that is, activation of the ryanodine receptors. Flubendiamide, a phthalic diamide, and chlorantraniliprole, an anthranilic diamide, were introduced in 2008 (Casida, 2015). Ryanodine receptors are a family of calcium channels. Under normal conditions, input from the nervous system activates voltage-gated calcium channels, leading to an increase in intracellular calcium; in turn, this triggers activation of ryanodine receptors located in the muscle sarcoplasmic reticulum, resulting in the release of stored pools of calcium and initiating muscle contraction. Ryanodine, a natural alkaloid present in the shrub *Ryania speciosa*, binds to and blocks these calcium channels, thus

inhibiting muscle contraction. Ryanodine itself had been utilized as an insecticide, but its mammalian toxicity precluded its further use (Satelle et al., 2008). In contrast, diamides bind to a site different from ryanodine, and cause receptor activation in insects by prolonging channel opening (Satelle et al., 2008; Bentley et al., 2010; Casida, 2015). Both flubendiamide and chlorantraniliprole have an extremely favorable toxicological profile (U.S. EPA, 2008), likely due to the difference between insect and mammalian ryanodine receptors, and to the ability of these compounds to selectively activate insect over mammalian ryanodine receptors. Acute toxicity is low (oral LD_{50} = 2000−5000 mg/kg), and there is no evidence of genotoxicity, carcinogenicity, neurotoxicity, and reproductive and developmental toxicity. Resistance to diamides' insecticidal activity has started to emerge, possibly due to mutations in the ryanodine receptor (Troczka et al., 2015).

Bacillus thuringiensis The past decade has seen increasing research and development in the area of biopesticides, that is, pesticides derived from natural materials such as plants, bacteria, and fungi. There are increasing numbers of biopesticides being registered around the world, primarily for use as bioinsecticides, but also as biofungicides or bioherbicides (Glare et al., 2016). Biopesticides are increasingly used as they are effective, can be used in integrated pest management programs and often also in organic productions, and generally have a favorable environmental and toxicological profile (Glare et al., 2016). Biopesticides fall into three major classes: (1) Microbial pesticides, which consist of a microorganism (e.g., a bacterium, fungus or protozoan) as the active ingredient. The most widely used microbial pesticides are subspecies and strains of *Bacillus thuringiensis* (Bt) which act as insecticides. Other microbial pesticides can control different kinds of pests; for example, there are fungi that can control certain weeds and others that can kill specific insects. (2) Plant-incorporated-protectants, which are pesticidal substances that plants produce from genetic material that has been added to the plant. For example, a plant can be genetically manipulated to produce the Bt pesticidal protein. (3) Biochemical pesticides that are naturally occurring substances that control pests by nontoxic mechanisms. Examples are sex pheromones that interfere with mating of insects, or various scented plant extracts that attract insect pests to traps (Sudakin, 2003). Biopesticides have a global market of more than U.S.$2.5 billion, and Bt products comprise 50% of all biopesticides sold. Bt is a soil microorganism, closely related to *Bacillus cereus*, which produces δ-endotoxins, that is, proteins that are selectively toxic to certain insects. Its name comes from the German region of Thuringia, where this strain was found in 1915. Bt-based microbial insecticides were commercialized in France in 1938 and in the United States in 1961. During the stationary phase of its growth cycle, Bt forms spores that contain crystals predominantly comprising one or more Cry and/or Cyt proteins; several hundreds of such proteins have been identified in Bt (Schnepf et al., 1998; van Frankenhuyzen, 2009; Sanahuja et al., 2011; Berry and Crickmore, 2017). After ingestion of Bt by an insect, the crystal proteins are solubilized, and proteolytically processed to active toxins in the insect's midgut. Here they bind to specific receptors in the epithelial cells and insert into the cellular membrane. Next, aggregation of inserted crystal protein occurs, resulting in the formation of pores, which lead to changes in K^+ fluxes across the epithelial cells, and to changes in pH. Ultimately, cells of the midgut epithelium are destroyed by the high pH and by osmotic lysis. Insects eventually die as a result of gut paralysis and feeding inhibition, and subsequent starvation and septicemia (Gringorten, 2001; Bravo et al., 2007). Bt targets primarily leaf-feeding lepidoptera, breaks down rapidly in UV light, and exhibits low mammalian toxicity. The basis for the selective toxicity of Bt is attributed to the fact that crystalline Bt endotoxins require activation by alkalis and/or digestion, conditions absent in the mammalian stomach (Ujvary, 2010). Bt toxins have generally an unremarkable toxicological profile in mammals (McClintock et al., 1995). However, adverse health effects in humans, particularly allergic reactions and infections, have been reported (Ujvary, 2010; Rubio-Infante and Moreno-Fierros, 2016). Bt genes have been expressed in a variety of crop plants, most notably cotton and corn (Sanahuja et al., 2011). Thus, the plant, instead of the Bt bacterium, produces one or more crystal toxins that affect the insect upon feeding. Though concerns about such genetically modified crops remain (Rubio-Infante and Moreno-Fierros, 2016), Bt crops are generally considered to pose no significant risk to the environment and to human health (Mendelsohn et al., 2003). Resistance can develop to Bt toxins that involves alterations in the processing of Cry toxin in the insect's gut, most commonly mutations, down-regulation, or deletion of receptors on the surface of the insect's midgut (Pardo-Lopez et al., 2013).

INSECT REPELLENTS

Insect-transmitted diseases remain a major source of illness and death worldwide, as mosquitoes alone transmit disease to more than 700 million persons annually (Fradin and Day, 2002). Though insect-borne diseases represent a greater health problem in tropical and subtropical climates, no part of the world is immune to their risks. For example, in 1999, the West Nile virus, transmitted by mosquitoes, was detected for the first time in the western hemisphere. In the New York City area, 62 persons infected with the West Nile virus were hospitalized and 7 died (Nash et al., 2001). Other arthropod-borne viral diseases (e.g., equine encephalitis) and tick-borne diseases (e.g., Lyme disease) are also of concern; additionally, other insect bites can be associated with variable adverse health effects, from mild irritation and discomfort to possible allergic reactions. More recently, in the Americas the Zika virus spread by the bite of an infected *Aedes* mosquito (e.g., *A. aegypti*) has been shown to be passed from a pregnant woman to the fetus, causing severe microcephaly and other neurological abnormalities (Hajra et al., 2017). Insect repellents are thus widely used to provide protection toward insect bites (Katz et al., 2008). The best known and most widely used insect repellent is DEET, and a newer compound picaridin is encountering increasing success. Botanical insect repellents based on citronella or oil of eucalyptus, and a biopesticide structurally similar to the amino acid alanine are also commercialized in Europe and the United States (Fradin and Day, 2002).

DEET

DEET (*N*,*N*-diethyl-*m*-toluamide or *N*,*N*-diethyl-3-methylbenzamide) was first developed by the USDA in 1946 for use in the military, and was registered as an insect repellent for the general public in 1957. The EPA estimates that 30% of the U.S. population uses DEET every year. More than 200 formulations exist with varying concentrations of DEET (commonly 4.75% to 40%), which are applied directly to the skin or on clothing. DEET is very effective at repelling insects, flies, fleas, and ticks, and protection time increases with increasing concentrations (Fradin and Day, 2002). The exact repellent mechanism of DEET is still unknown, but it may be related to disturbances of the mosquito antennae that allow the insect to locate humans (Koren et al., 2003). Percutaneous absorption of DEET varies from 7.9% to 59%, depending on the species tested and the conditions of the study (Osimitz and Murphy, 1997). DEET undergoes oxidative biotransformation catalyzed by various CYPs, and is excreted mostly in the urine

(Sudakin and Trevathan, 2003). DEET has low acute toxicity (LD_{50} in rat = 1892 mg/kg (oral) and more than 5000 mg/kg (dermal)) (Schoenig and Osimitz, 2001), and does not appear to pose a significant health concern to humans when used as directed. From 1961 to 2002, eight deaths were reported related to DEET: three resulted from deliberate ingestion, while two were reported following dermal exposure (Tenenbein, 1987; Bell et al., 2002). The remaining three cases were children, age 17 months to 6 years (Zadikoff, 1979). Subchronic toxicity studies in various species did not reveal major toxic effects, with the exception of renal lesions in male rats; these were considered to be reflective of α_{2u}-globulin induced nephropathy, a condition unique to male rats and not occurring in humans (Schoenig and Osimitz, 2001) (see Chap. 14, "Toxic Responses of the Kidney"). No significant effects of DEET were seen in mutagenicity, reproductive toxicity, and carcinogenicity studies. Given the recent Zika virus epidemic, particular attention has been devoted to the use of DEET during pregnancy, and the Center for Disease Control and Prevention has strongly recommended its use given its overall lack of developmental toxicity (Wylie et al., 2016). Acute and chronic neurotoxicity studies also provided negative results (Schoenig et al., 1993; Sudakin and Osimitz, 2010). However, a number of case reports over the past 50 years have indicated possible neurological effects of DEET after high exposures, and most of these were in children (Gryboski et al., 1961; CDC, 1989; Osimitz and Murphy, 1997; Petrucci and Sardini, 2000; Briassulis et al., 2001; Sudakin and Trevathan, 2003). The most common symptoms reported were seizures and encephalopathy. Given that seizure disorders occur in 3% to 5% of children, and almost 30% of children in the United States are utilizing DEET, an association just by chance is certainly possible. Possible mechanism(s) responsible for neurotoxic effects of DEET are unknown, though it has been suggested that DEET's structure is similar to that of nikethamide, a convulsant (Briassulis et al., 2001). It has also been suggested that DEET may disrupt the permeability of the blood–brain barrier, but results are inconclusive (Abdel-Rahman et al., 2002). Overall, given its long-standing and widespread use, DEET appears to be relatively safe when used as recommended (Osimitz and Murphy, 1997; Koren et al., 2003; Wylie et al., 2016). A risk assessment by the Canadian Pest Management Regulatory agency has recommended that toddlers and children, up to 12 years old, should only be exposed to products with up to 10% DEET (Sudakin and Trevathan, 2003), while in all other individuals products with up to 30% DEET can be safely used (Fradin and Day, 2002; Antwi et al., 2008).

Picaridin

Picaridin (1-piperidine carboxylic acid, 2-(hydroxyl-ethyl), 1-methyl propylester) was developed as an alternative to DEET (Patel et al., 2016). Insect repellent formulations (cream, aerosol, wipe) containing 5% to 20% picaridin are highly effective against a variety of arthropod pests, especially mosquitoes, ticks, and flies (Sangha, 2010). Its action in insects is believed to be due to the interaction with specific olfactory receptors of the arthropod (Boeckh et al., 1996). In humans it is absorbed through the skin to a limited degree (90% less than in the rat), and is metabolized via hydroxylation and glucuronidation, before excretion in the urine. The toxicological profile of picaridin is unremarkable. Acute dermal toxicity is low (about 5 g/kg), and NOEL values are about 200 mg/kg/day in dermal subchronic and chronic toxicity studies. There is no evidence of genotoxicity, carcinogenicity, teratogenicity, reproductive toxicity, or neurotoxicity (Sangha, 2010). When used as directed, picaridin-containing formulations are deemed to be safe and effective (Antwi et al., 2008; Charlton et al., 2016).

HERBICIDES

Herbicides are chemicals capable of killing or severely injuring plants. They represent a very broad array of chemical classes and act at a large number of sites of metabolic functions and energy transfer in plant cells (Duke, 1990; Casida, 2009). A classification of herbicides based on the mechanisms by which they exert their biological effects is shown in Table 22-17. Another method of classification pertains to when herbicides are applied: *preplanting* herbicides are applied to the soil before a crop is seeded; *preemergent* herbicides are applied to the soil before the time of appearance of unwanted vegetation; and *postemergent* herbicides are applied to the soil or foliage after the germination of the crop and/or weeds (Ecobichon, 2001a). Herbicides are also divided according to how they are applied to plants: *contact* herbicides are those that affect the plant that was treated, while *translocated* herbicides are applied to the soil or to above-ground parts of the plant, and are absorbed and circulated to distant tissues. Nonselective herbicides will kill all vegetation, while selective compounds are those used to kill weeds without harming the crops. In the past couple of decades, the development of herbicide-resistant crops through transgenic technology has allowed the use of nonselective compounds as selective herbicides (Duke, 2005). A final classification, of relevance to adverse health effects in nontarget species, relies on chemical structures, and this is used in the following sections.

For the past several decades, herbicides have represented the most rapidly growing sector of the agrochemical market, and these compounds now represent almost half of the pesticides used in the United States, and more than one-third of those utilized worldwide (Table 22-3). This can be ascribed in part to movement to monocultural practices, where the risk of weed infestation has increased, and to mechanization of agricultural practices because of increased labor costs. It has been estimated that in the United

Table 22-17

Some Mechanisms of Action of Herbicides

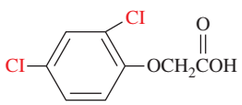
MECHANISM	CHEMICAL CLASSES (EXAMPLE)
Inhibition of photosynthesis	Triazines (atrazine), substituted ureas (diuron), uracils (bromacil)
Inhibition of respiration	Dinitrophenols
Auxin growth regulators	Phenoxy acids (2,4-D), benzoic acids (dicamba), pyridine acids (picloram)
Inhibition of cell division	Chloroacetanilides (alachlor)
Inhibition of protein synthesis	Dinitroanilines
Inhibition of lipid synthesis	Aryloxyphenoxypropionates (diclofop)
Inhibition of specific enzymes	
• Glutamine synthetase	Glufosinate
• Enolpyruvylshikimate -3-phosphate synthetase	Glyphosate
• Acetylase synthase	Sulfonylureas
Cell membrane disruptors	Bipyridyl derivatives (paraquat)

States 70 million workers would be required for weeding to prevent crop yield losses without herbicides (Gianessi and Reigner, 2007). In China, about 1 billion person-days of labor would be required to adequately hand-weed the rice fields (Gianessi, 2013). Indeed, controlling weeds without herbicides represents one of the greatest obstacles to organic crop production, and one of the highest costs. In addition to agriculture, herbicides are also widely utilized in home and garden, in forestry management to facilitate growing of new trees, and to clear roadsides, utilities' rights of way, and industrial areas.

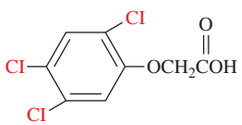
In terms of general toxicity, herbicides, as a class, display relatively low acute toxicity, compared for example to most insecticides. However, there are exceptions, such as paraquat. A number of herbicides can cause dermal irritation and contact dermatitis, particularly in individuals prone to allergic reactions. Other compounds have generated much debate for their suspected carcinogenicity or neurotoxicity. The principal classes of herbicides associated with reported adverse health effects in humans are discussed below.

Chlorophenoxy Compounds

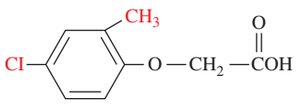
Chlorophenoxy herbicides are characterized by an aliphatic carboxylic acid moiety attached to a chlorine- or methyl-substituted aromatic ring. The most commonly used compound of this class is 2,4-dichlorophenoxyacetic acid (2,4-D), while others are 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; no longer used) and 4-chloro-2-methylphenoxyacetic acid (MCPA) (Fig. 22-16). Chlorophenoxy herbicides are chemical analogs of auxin, a plant growth hormone, and produce uncontrolled and lethal growth in target plants (Casida, 2009). Because the auxin hormone is critical to the growth of many broad leaf plants, but is not used by grasses, chlorophenoxy compounds can suppress the growth of weeds (e.g., dandelions) without affecting the grass. Once absorbed, they selectively eliminate broadleaf plants, due to their larger leaf area and greater absorption. 2,4-D is still one of the most widely used herbicides throughout the world, and is primarily used in agriculture to control weeds in corn and grain, in forestry, and in lawn care practices, with over 120 commercialized formulations (Kennepohl et al., 2010). 2,4,5-T has been withdrawn from use because of concerns that arose from contamination of some formulations with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which can derive from the reaction of two molecules of 2,4,5-trichlorophenol (Fig. 22-17). From 1962 to 1971, a 50:50 mixture of the *n*-butyl esters of 2,4,-D and of 2,3,5-T, known as Agent Orange (from the color of identification bands painted on the storage drums), was extensively used as a defoliant during the Vietnam War, and was found to be contaminated with TCDD to a maximum of 47 μg/g. Exposure of military personnel and of Vietnamese population to Agent Orange has raised concerns on possible long-term health effects, particularly carcinogenicity, endocrine-disrupting effects, and reproductive toxicity that are ascribed to the presence of TCDD (IOM, 2016). Formulations of 2,4-D contain extremely low levels of polychlorinated dibenzo-*p*-dioxins (PCDDs), usually below the limit of detection of 10 ppb (Kennepohl et al., 2010).



2,4-D (2,4-dichlorophenoxy)acetic acid



2,4,5-T (2,4,5-trichlorophenoxy)acetic acid

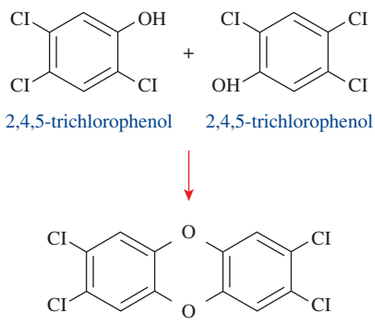


MCPA [(4-cloro-a-tolyl)oxy]acetic acid

Figure 22-16. *Structures of three chlorophenoxy acid herbicides.*

2,4-D is a compound of low to moderate acute toxicity, with oral LD_{50}s in rodents of 300 to 2000 mg/kg. The dog is more sensitive, possibly because of its lower ability to eliminate organic acids via the kidney. Upon oral exposure, 2,4-D is rapidly absorbed, and its salts and esters rapidly dissociate or hydrolyze in vivo, so that toxicity depends primarily on the acid form. It binds extensively to serum albumin, but does not accumulate in tissues, and is excreted almost exclusively through the urine. Ingestion of 2,4-D has caused several cases of acute poisoning in humans, usually at doses above 300 mg/kg, though lower doses have been reported to elicit symptoms. Vomiting, burning of the mouth, abdominal pain, hypotension, myotonia, and CNS involvement including coma are among the clinical signs observed (Bradberry et al., 2000, 2004a). Management of 2,4-D poisoning appears to be aided by urine alkalinization through intravenous administration of bicarbonate (Bradberry et al., 2004a; Proudfoot et al., 2004). The rationale is that ionization of an acid, such as 2,4-D, is increased in an alkaline environment; though, at pH 5.0, practically all 2,4-D would already be ionized, further alkalinization of urine, to pH 7.5 or above, would reduce the nonionized fraction from approximately 0.53% to 0.0017%. As a result, the fraction prone to reabsorption would be more than 300 times lower (Bradberry et al., 2004a), thus diminishing reabsorption and increasing 2,4-D elimination. Nevertheless, it has been recommended that additional studies be conducted to fully validate this approach in case of 2,4-D poisoning (Roberts and Buckley, 2007). Table 22-18 shows an example of the effect of urine alkalinization on 2,4-D renal clearance and plasma half-life.

Dermal exposure is by far the major route of unintentional exposure to 2,4-D in humans. Dermal absorption studies in rats,



2,4,5-trichlorophenol 2,4,5-trichlorophenol

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

Figure 22-17. *Formation of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) during the synthesis of 2,4,5-T because of reaction between two molecules of 2,4,5-trichlorophenol.*

Table 22-18

Effect of Urine Alkalinization on Renal Clearance and Plasma Half-life of 2,4-D

URINE PH	RENAL CLEARANCE (ML/MIN)	HALF-LIFE (HOURS)
5.10–6.5	0.28	219
6.55–7.5	1.14	42
7.55–8.8	9.60	4.7

SOURCE: Adapted from Park et al. (1977).

mice, and rabbit indicate an absorption of 12% to 36%; the absorption in humans, however, is lower (2% to 10%), and is usually less than 6% (Ross et al., 2005). Acute poisoning by 2,4-D via the dermal route is thus uncommon; no reports of systemic toxicity following dermal exposure have been reported for over 20 years, and no fatalities have ever occurred (Bradberry et al., 2004a).

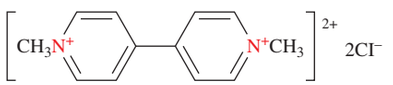
The precise mechanisms of toxicity of chlorophenoxy herbicides have not been completely elucidated, but experimental studies indicate the possible involvement of three actions: (1) cell membrane damage; (2) interference with metabolic pathways involving acetyl-coenzyme A; (3) uncoupling of oxidative phosphorylation (Bradberry et al., 2000). The toxicity of chlorophenoxy herbicides has been summarized in several reviews (Sterling and Arundel, 1986; Munro et al., 1992; Garabrant and Philbert, 2002; Kennepohl et al., 2010). 2,4-D and its salts and esters are not teratogenic in mice, rats, or rabbits, unless the ability of the dam to excrete the chemical is exceeded. There is also no convincing evidence that 2,4-D is associated with human reproductive toxicity (Garabrant and Philbert, 2002). Subchronic and chronic toxicity studies have not provided evidence of immunotoxicity, and there is very limited evidence that 2,4-D may affect the nervous system (Mattsson et al., 1997). There are, however, several case reports suggesting an association between exposure to 2,4-D and neurologic effects ranging from peripheral neuropathy, demyelination and ganglion degeneration in the CNS, reduced nerve conduction velocity, myotonia, and behavioral alterations (Garabrant and Philbert, 2002). 2,4-D-induced hypomyelination upon developmental exposure has also been described (Konjuh et al., 2008)

Numerous in vitro and in vivo studies with 2,4-D indicate that it has very little genotoxic potential (Munro et al., 1992; von Stackelberg, 2013). Long-term bioassays in rats, mice, and dogs provided no evidence to suggest that 2,4-D is a carcinogen in any of these species. While an earlier study in rat reported an increase in the incidence of brain astrocytomas in male animals, only at the highest dose tested (45 mg/kg/day; Serota et al., 1986), it was later argued that the observed tumors were not treatment-related (Munro et al., 1992); furthermore, more recent studies did not replicate this finding (Charles et al., 1996). The chlorophenoxy herbicides have also been extensively studied for possible associations with non-Hodgkin lymphoma or soft-tissue sarcoma, found in a small number of epidemiological studies (Hoar et al., 1986; Munro et al., 1992; Hardell et al., 1994; U.S. EPA, 1994). Garabrant and Philbert (2002) evaluated all cohort and case–control studies available at that time, and concluded that there was no adequate evidence from epidemiological studies to conclude that exposure to 2,4-D is associated with soft-tissue sarcoma, non-Hodgkin lymphoma, or Hodgkin disease. 2,4-D is classified as a Group D agent (not classifiable as to human carcinogenicity) (U.S. EPA, 1997). However, 2,4-D has been recently classified by IARC as possibly carcinogenic to humans (Group 2B) based primarily on limited evidence provided by animal findings (Loomis et al., 2015). It should also be noted that biomonitoring data in the United States and Canada indicate that exposure to 2,4-D in both the general and agricultural populations is well below the noncancer reference dose (Aylward et al., 2010; von Stackelberg, 2013).

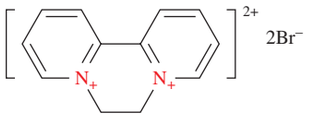
Bipyridyl Compounds

This class of herbicides comprises paraquat and diquat (Fig. 22-18), of which paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is of most toxicological concern, and will be discussed in more detail. Paraquat was first synthesized in the 1800s, but its herbicidal properties were described in 1958, and it was first commercialized in the United Kingdom in 1962 (Lock and Wilks, 2010). Paraquat is a very effective, fast-acting, nonselective contact herbicide used to control broad-leaved weeds and grasses in plantations and fruit orchards, and for general weed control in hundreds of crops (Watts, 2011). Its mode of action as a herbicide is believed to be due to its redox potential, which leads to inhibition of photosynthesis (Dinis-Oliveira et al., 2008). The redox properties of paraquat are also responsible for its mammalian toxicity.

The herbicide that has one of the highest acute toxicity is paraquat; its oral LD_{50} in rat is approximately 100 mg/kg, while guinea pigs, rabbits, and monkeys are more sensitive. Paraquat is more toxic when given by the i.p. route (LD_{50} in rats = 10–20 mg/kg), suggesting that it is not efficiently absorbed from the gastrointestinal tract. Absorption through intact skin is minimal, and inhalation exposure does not occur, as paraquat has no appreciable vapor pressure. Irrespective of the route of exposure, the lung and the kidneys are the organs showing the highest concentrations of paraquat. Only a small fraction of paraquat is metabolized, and the greater part is excreted unchanged in the urine. Thus, the major toxicological concerns for acute paraquat exposure are related to its systemic effects, particularly in the lung, and secondarily, the kidney. Rose et al. (1974) first described an energy-dependent accumulation of paraquat in lung tissue, particularly, but not exclusively, in type II alveolar epithelial cells. The loss of paraquat from lung tissue following in vivo administration is slow. Thus, the basis for the selective toxicity of paraquat to the lung resides in its ability to concentrate in alveolar type II and I cells and Clara cells. Pulmonary concentrations of paraquat can be 6- to 10-fold higher than in plasma (Dinis-Oliveira et al., 2008).



Paraquat
1,1′-dimethyl-4,4′-bipyridylium ion
(dichloride)



Diquat
6,7-diydrodipyridol (1,2-α:2′,1′α)
pyrazidinium (dibromide)

Figure 22-18. *Structures of the bipyridyl herbicides paraquat and diquat, marketed as the dichloride and dibromide salts, respectively.*

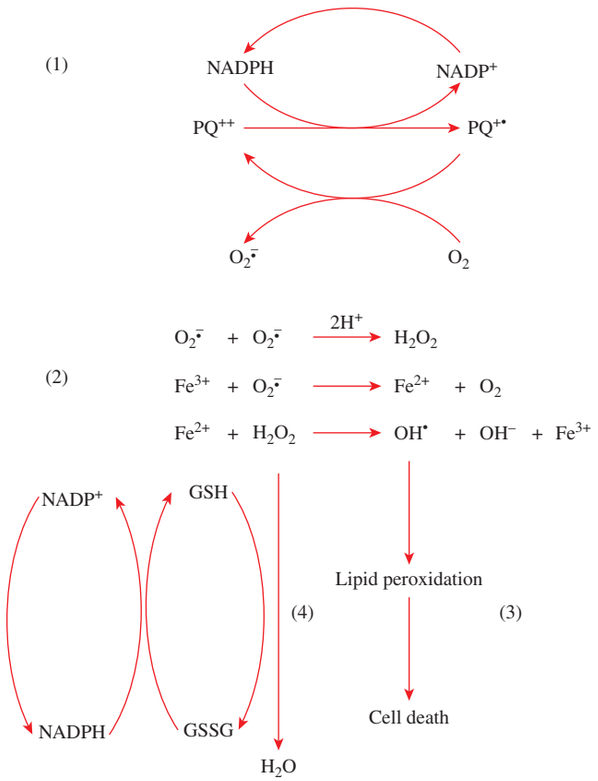


Figure 22-19. *Mechanism of toxicity of paraquat.* (1) Redox cycling of paraquat utilizing NADPH; (2) formation of hydroxy radicals leading to lipid peroxidation (3); (4) detoxication of H_2O_2 via glutathione reductase/peroxidase couple, utilizing NADPH. (Modified from Smith [1987], with permission.)

The mechanism(s) by which paraquat is toxic to cells have been extensively investigated (Autor, 1974; Bus and Gibson, 1984; Smith, 1987). Paraquat can be reduced to form a free radical, which in the presence of oxygen rapidly reoxidizes to the cation, with a concomitant production of superoxide anion ($O_2^{\bullet-}$) (Fig. 22-19). Thus, once paraquat enters a cell, it undergoes alternate reduction followed by reoxidation, a process known as redox cycling (Adam et al., 1990). Superoxide dismutases (SOD) are a family of metalloenzymes that can dismutate superoxide anions to hydrogen peroxide and oxygen. The finding that transgenic mice lacking copper/zinc SOD show marked increased sensitivity to paraquat (Ho et al., 1998) supports a role for superoxide anions in paraquat's cellular toxicity. Nevertheless, superoxide anion itself is unlikely to be the ultimate toxic species. Three hypotheses, which are not mutually exclusive, have been proposed to account for the ensuing cytotoxicity (Lock and Wilks, 2010). The generation of superoxide anion and subsequently of hydroxy radicals would initiate lipid peroxidation, ultimately leading to cell death (Bus and Gibson, 1984). Intracellular redox cycling of paraquat would also result in the oxidation of NADPH, leading to its cellular depletion, which is augmented by the detoxification of hydrogen peroxide formed in the glutathione peroxidase/reductase enzyme system to regenerate GSH (Fig. 22-19). A third hypothesis is that paraquat toxicity is due to mitochondrial damage; however, paraquat does not affect complex I in isolated brain mitochondria (Richardson et al., 2005).

Upon acute exposure to lethal doses of paraquat, mortality may occur 2 to 5 days after dosing, though death can also occur after longer periods (Clark et al., 1966). Damage to alveolar epithelial cells is seen within 24 hours after exposure. Damage progresses in the following 2 to 4 days with large areas of the alveolar epithelium completely lost. This is followed by alveolar edema, extensive infiltration of inflammatory cells into the alveolar interstitium, and finally death due to severe anoxia (Smith and Heath, 1976). Survivors of this first phase, called the destructive phase, show extensive proliferation of fibroblasts in the lung. The second phase, called the proliferative phase, is characterized by attempts by the alveolar epithelium to regenerate and restore normal architecture, and presents itself as an intensive fibrosis (Smith and Heath, 1976). Some individuals who survive the first phase may still die from the progressive loss of lung function several weeks after exposure.

Attempts to develop treatments for paraquat poisoning have focused on prevention of absorption from the gastrointestinal tract, removal from the bloodstream, prevention of its accumulation in the lung, use of free radical scavengers, and prevention of lung fibrosis (Dinis-Oliveira et al., 2008; Lock and Wilks, 2010). Though some approaches have shown promises in vitro or in isolated lung tissue preparations, only the first one, removal of the ingested material by emesis or purgation of the gastrointestinal tract, has been shown to be effective in vivo in animals. Administration of dexamethasone, with induction of P-glycoprotein in several tissues including the lung, and of sodium salicylate, because of its anti-inflammatory properties, has also been shown, in animal studies, to be effective treatments for paraquat poisoning (Dinis-Oliveira et al., 2008).

Since its introduction as a herbicide, there have been thousands of episodes of acute poisoning with paraquat in humans, a large percentage of which were fatal (Casey and Vale, 1994; Wesseling et al., 2001; Lock and Wilks, 2010; U.S. EPA, 2016b). Most cases involved ingestion of a 20% paraquat concentrate solution for suicidal purposes, or resulted from accidental poisoning due to decanting in unlabeled drink bottles or containers. To avoid the latter, in the 1980s, the manufacturers added a blue pigment, a stenching compound, and an emetic substance to the formulation, to make severe unintentional poisoning due to oral intake virtually impossible (Sabapathy, 1995). As said, absorption of paraquat across the human skin is very low (but is increased by damage to skin, and paraquat is a skin irritant), and few cases of paraquat poisoning have been reported following dermal exposure. Signs and symptoms of paraquat poisoning in humans reflect those previously described. A dose of 20 to 30 mg/kg can cause mild poisoning, while 30 to 50 mg/kg can cause delayed development of pulmonary fibrosis, which can be lethal. Higher doses usually cause death within a few days due to pulmonary edema, and renal and hepatic failure (Smith and Heath, 1976). No single therapeutic intervention, among those outlined earlier, has proved efficacious in case of severe acute paraquat poisoning (Bismuth et al., 1982). In rare instances, heart/lung transplant has been used to treat severely paraquat-poisoned patients. The high acute toxicity and the lack of reliable antidotes have led several countries to ban the use of paraquat (e.g., the EU) or to pose restrictions on its use (e.g., the United States) (Watts, 2011).

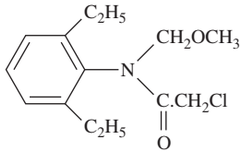
Paraquat has minimal to no genotoxic activity, is not carcinogenic in rodents, has no effect on fertility, is not teratogenic, and only produces fetotoxicity at maternally toxic doses (Lock and Wilks, 2010). Chronic exposure of experimental animals to paraquat affects the same target organs of acute toxicity, that is, the lung and the kidney, and no-effect levels have been established. Under normal use conditions, exposure to paraquat is very low and can be monitored by quantifying paraquat levels in urine, as the compound is mostly excreted unchanged (Lee et al., 2009b). In the late 1970s concern was raised about possible exposure of marijuana smokers to paraquat by inhalation. Paraquat was indeed used to destroy marijuana fields, and residues were then present in the final products (Landrigan et al., 1983). However, no clinical cases were identified.

Chronic paraquat exposure has been suggested as a possible etiological factor in the development of Parkinson's disease. The first suggestion came from a study in the Canadian province of Quebec (Barbeau et al., 1986), and the hypothesis arose from the structural similarity of paraquat to MPP^+ (1-methyl-4-phenylpyridinium ion), the toxic metabolite of MPTP (1-methyl-4-phenyl-1,2,3,5-tetrahydropyridine). MPP^+ itself was initially developed as a possible herbicide, but was never commercialized. It was initially argued that paraquat, being positively charged, cannot easily pass the blood–brain barrier. However, paraquat can be transported into the brain by a neutral amino acid transporter, such as the system L carrier (LAT-1) (Shimizu et al., 2001). Indeed, animal studies have shown that paraquat can cause CNS effects, most notably a neurodegeneration of dopaminergic neurons (McCormack et al., 2002; Fahim et al., 2013). The ability of paraquat to cause oxidative damage through a free radical mechanism may explain the selective vulnerability of dopaminergic neurons, which are per se more susceptible to oxidative damage (McCormack et al., 2005). Paraquat neurotoxicity is also distinct from that of MPTP or rotenone (Richardson et al., 2005). Evidence from human epidemiological studies also suggest an association between paraquat exposure and Parkinson disease with approximately an overall 2.2-fold increased risk (Miller, 2007; Tanner et al., 2011; Moretto and Colosio, 2013; Pezzoli and Cereda, 2013). Nevertheless, animal and human findings have been challenged by some investigators (Li et al., 2005; Berry et al., 2010; Smeyne et al., 2016).

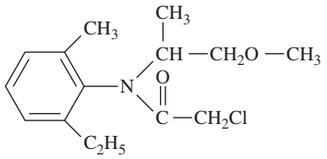
Despite the chemical similarity to paraquat (Fig. 22-18), the herbicide diquat presents a different toxicological profile. Acute toxicity is somewhat lower, with oral LD_{50} in rats of approximately 200 mg/kg (Lock and Wilks, 2001). In contrast to paraquat, it does not accumulate in the lung, and no lung toxicity is seen upon acute or chronic exposure. Upon chronic exposure, target organs for toxicity are the gastrointestinal tract, the kidney, and particularly the eye. Diquat indeed causes a dose- and time-dependent appearance of cataracts in both rats and dogs (Lock and Wilks, 2001). Like paraquat, diquat can be reduced to form a free radical and then reoxidized in the presence of oxygen, with the concomitant production of superoxide anion. This process of redox cycling occurs in the eye and is believed to be the likely mechanism of cataract formation (Lock and Wilks, 2001). A limited number of cases of human poisoning with diquat have occurred. Clinical symptoms include nausea, vomiting, diarrhea, ulceration of mouth and esophagus, decline of renal functions, and neurologic effects, but no pulmonary fibrosis. As for paraquat, therapy for intoxication is directed at preventing absorption and enhancing elimination (Vanholder et al., 1981; Lock and Wilks, 2001). Diquat is not a skin sensitizer, has minimal or no genotoxic activity, is not carcinogenic in rodents, has no effect on fertility, and is not teratogenic.

Chloroacetanilides

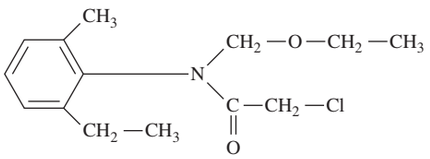
Representative compounds of this class of herbicides are alachlor, acetochlor, and metolachlor (shown in Fig. 22-20), as well as butachlor and propachlor, all registered in the United States between 1967 and 1997. They are used to control herbal grasses and broadleaf weeds in a number of crops, primarily on corn. The herbicidal action of chloroacetanilides is not fully understood, though they have been shown to inhibit the synthesis of proteins and to impact cell division. Chloroacetanilides display moderate to low acute toxicity, with oral LD_{50} in rat ranging from about 600 mg/kg (propachlor) to 2800 mg/kg (metolachlor). Dermal LD_{50} values are usually much higher, indicating a poor absorption of these compounds across the skin (Heydens et al., 2010). Most are nonirritant, or only



Alachlor
2-chloro-2′,6′-diethyl-*N*-
(methoxymethyl) acetanilide



Metolachlor
2-chloro-*N*-(2-ethyl-6-methylpheynyl-*N*-
(2-methoxy-1-methylethyl)-acetamide



Acetochlor
2-chloro-*N*-(ethoxymethyl)-*N*-
2-ethyl-6-methylphenyl)-acetamide

Figure 22-20. *Structures of some chloroacetanilide herbicides.*

slightly irritant to the eye and skin, with the exception of propachlor, which causes severe irritation to the eye (Heydens et al., 2010). Subchronic and chronic toxicity studies, carried out in multiple species (mice, rats, dogs, rabbits), have identified the liver and kidney as principal target organs, and NOAEL (no observed adverse effect level) values have been established (Heydens et al., 2010). Alachlor was found to produce an ocular lesion, termed "progressive uveal degeneration syndrome," in Long–Evans rats (Heydens, 1998). This ocular lesion was not observed in mice, dogs, or other strains of rats. Furthermore, an investigation of similar eye abnormalities in alachlor production workers considered to have the highest alachlor exposure, far exceeding that of farmers or herbicide applicators, provided no evidence of ocular disease (Ireland et al., 1994). Most of the chloroacetanilides do not appear to be teratogenic or to cause reproductive or developmental toxicity; for propachlor, slight effects on the offspring were observed in some studies at the highest dose tested (Heydens et al., 2010).

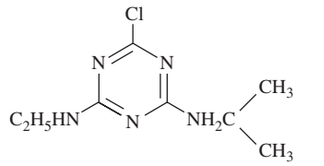
Chloroacetanilides have been extensively tested for genotoxicity in bacterial and mammalian systems in vitro and in animals in vivo, and these studies suggest that these compounds are not genotoxic (Ashby et al., 1996; Heydens et al., 1999, 2010; EFSA, 2004). Yet, these compounds have shown to induce tumors at various sites in rats. Tumors in female mice (lung adenomas), found with alachlor and acetochlor, were considered not to be treatment related (Ashby et al., 1996; Heydens et al., 1999, 2010). In rats, nasal epithelial (olfactory) tumors and thyroid follicular tumors were observed in carcinogenicity studies with alachlor, acetochlor,

and butachlor, while glandular stomach tumors were found with alachlor and butachlor. These findings led to the initial classification of these compounds as probable human carcinogens (Group B2). The discovery of alachlor in well water led to cancellation of its registration in some countries (e.g., in the EU) and to its restriction in others. A series of mechanistic studies in the past two decades has provided evidence that tumors observed in rats may be species-specific, show a threshold, and are not due to genotoxic mechanisms, and hence may not be relevant to humans (Ashby et al., 1996; Heydens et al., 1999, 2010).

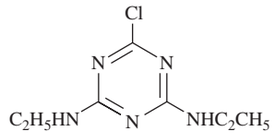
Alachlor and other chloroacetanilides are extensively metabolized in rat, and more than 30 metabolites have been identified in this species, with equal quantities appearing in urine and feces; in mice, alachlor's metabolism is qualitatively similar, but significant quantitative differences are observed between these two species. In contrast, in primates alachlor is metabolized to a limited number of glutathione and glucuronide conjugates, excreted primarily in the kidney, with more than 90% of the dose being excreted in the urine within 48 hours. Furthermore, in rats, alachlor metabolites of intermediate molecular weight undergo biliary excretion and enterohepatic recirculation, while this phenomenon is not observed in primates. Alachlor and other chloroacetanilides undergo demethylation with the release of formaldehyde (Jacobsen et al., 1991), and this compound, known to produce rat nasal tumors upon inhalation exposure, was initially thought to be involved in the nasal carcinogenicity of these herbicides. However, the different nature and distribution of tumors, and additional metabolic considerations, argue against such a hypothesis (Heydens et al., 2010). In rats, alachlor and butachlor are initially metabolized in the liver via the CYP pathway and by glutathione conjugation, and the metabolites undergo enterohepatic circulation and further metabolism in liver and nasal tissue to form the putative carcinogenic metabolite diethyl quinoneimine (DEIQ) (Feng et al., 1990). In the case of acetochlor, the carcinogenic metabolite is EMIQ, an acetochlor quinone imine compound analogous to DEIQ. Quinone imines are electrophilic, deplete glutathione, and exert cellular toxicity by binding to cellular proteins, which is followed by regenerative cell proliferation. Whole-body autoradiography studies have shown that alachlor-derived protein adducts specifically localize in the nasal mucosa of rats, but not in nasal tissue from mice or monkeys. Furthermore, the ability of rat nasal tissue to form DEIQ is much higher (751-fold) than human nasal tissue (Wilson et al., 1995). With regard to rat stomach and thyroid tumors, it is suggested that these result from tissue-specific toxicity leading to compensatory cell proliferation in the fundic mucosa, and to alterations of thyroid stimulating hormone homeostasis, respectively. Both are believed to be threshold-sensitive phenomena, not expected to be relevant to humans (Heydens et al., 2010). Epidemiological investigations in workers involved in the manufacturing of alachlor have not demonstrated any evidence of increased mortality or cancer incidence (Leet et al., 1996). As a result of these studies, alachlor has been reclassified as "likely" at high doses, but "not likely" at low doses, to be a human carcinogen, and a downgrading for acetochlor has also been suggested (Heydens et al., 2010).

Triazines

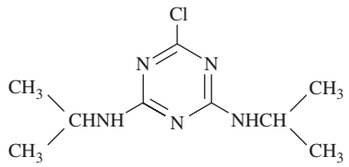
The family of triazine herbicides comprises several compounds (e.g., atrazine, simazine, and propazine; Fig. 22-21) that have been extensively used for the preemergent and postemergent control of broad-leaf weeds and certain grasses for several decades (Breckenridge et al., 2010). Their herbicidal action is due to inhibition of photosynthesis (Casida, 2009). Triazines have low acute oral and dermal toxicity (LD_{50}s = 1–2 g/kg), while chronic toxicity



Atrazine
2-chloro-4(ethylamino)-6-(isopropylamino)-*s*-triazine



Simazine
2-chloro-4,6(ethylamino)-*s*-triazine



Propazine
2-chloro-4,6 bis(isopropylamino)-*s*-triazine

Figure 22-21. *Structures of some triazine herbicides.*

studies indicate primarily a decreased body weight gain as the basis for setting NOAEL values; however, in dogs, cardiotoxicity is seen with atrazine, and this endpoint is used to derive the NOAEL for this compound (Gammon et al., 2005). There is no evidence that triazines are teratogenic, nor developmental or reproductive toxicants (Goodman et al., 2014). The weight of evidence from in vitro and in vivo studies indicates that triazines are not genotoxic (Brusick, 1994), although a possible clastogenic effect of atrazine has been suggested (Taets et al., 1998). Oncogenicity studies found that triazines cause an increased incidence of mammary carcinomas in female Sprague–Dawley rats (Gammon et al., 2005; Breckenridge et al., 2010). Such tumors were not observed in male Sprague–Dawley rats, or in Fischer 344 rats or CD-1 mice of either sex (IARC, 1999; Breckenridge et al., 2010). It is thought that such mammary tumors arise from an endocrine effect that might be expected to show a threshold. In contrast to most rodent species, female Sprague–Dawley rats have a high degree of spontaneous mammary tumors, due their reproductive aging; they display prolonged or persistent estrus associated with high estrogen levels (Eldridge et al., 1996). This is supported by the finding that ovariectomy eliminates mammary tumors in Sprague–Dawley rat that arise both spontaneously and as a result of atrazine administration (Stevens et al., 1999). The possible mechanisms for the effect of atrazine involve an action on pituitary luteinizing hormone, regulated by hypothalamic gonadotropin-releasing hormone, in turn controlled by hypothalamic norepinephrine (Cooper et al., 2000, 2007). Such effect was seen only at high doses of atrazine, but not at low doses, suggesting a threshold response (Breckenridge et al., 2010). Most regulatory agencies have concluded that mammary tumors in female Sprague–Dawley rats are formed via a secondary, hormone-mediated mechanism, of little or no toxicological relevance to humans. Epidemiological studies of triazine herbicides and cancer have provided inconclusive results (Sathiakumar and

Delzell, 1997; Rusiecki et al., 2004), and atrazine has been judged as unlikely to produce cancer in humans (Boffetta et al., 2013).

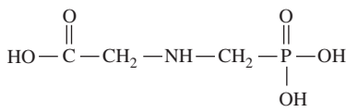
Though exposure to atrazine through residues in food commodities is very low, contamination of groundwater and drinking water is common. The EU banned the use of atrazine in 2004, mainly because it was often detected at levels exceeding the 0.1 mg/L standard for drinking water. Recent publications have reported a possible feminization of frogs, measured in laboratory and field studies, by ppb levels of atrazine (Hayes et al., 2002, 2011). However, other investigators failed to reproduce these findings (Carr et al., 2003; Kloas et al., 2009). It has been argued that the overall weight of evidence shows that atrazine, sometimes at environmentally relevant concentrations, affects molecular/biochemical responses in fish, amphibians, and reptiles; however, these effects are not translated into adverse outcomes in terms of apical endpoints (i.e., changes that are observed in vivo, such as abnormal breeding behavior, physical changes, organ alterations; Van der Kraak et al., 2014). In 2006, the EPA issued a cumulative risk assessment of triazine herbicides, concluding that they posed no harm that would result to the general U.S. population, infants, children, or other consumers (U.S. EPA, 2006). However, atrazine is still under scrutiny for its potential endocrine-disrupting effects. In addition, atrazine and its metabolites have been shown to affect the homeostasis of dopamine, leading to depletion of this neurotransmitter and to disruption of motor functioning (Walters et al., 2015).

Phosphonomethyl Amino Acids

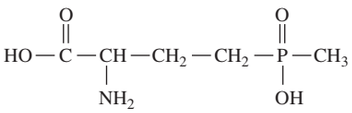
The two compounds of this class are glyphosate (*N*-phosphonomethyl glycine) and glufosinate (*N*-phosphonomethyl homoalanine). Both are broad-spectrum nonselective systemic herbicides used for postemergent control of annual and perennial plants, and are marketed primarily as the isopropylamine salt (glyphosate) or ammonium salt (glufosinate). Though both compounds contain a P=O moiety (Fig. 22-22), they are not organophosphates, but rather organophosphonates, and do not inhibit AChE (Farmer, 2010).

Glyphosate Glyphosate, first commercialized in the mid-1970s, is the most widely used herbicide in the United States (Table 22-4) and worldwide, and the development of transgenic crops that can tolerate glyphosate treatment has expanded its utilization. Glyphosate exerts its herbicidal action by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, responsible for the synthesis of an intermediate in the biosynthesis of various amino acids (Casida, 2009). Although important in plant growth, this metabolic pathway is not present in mammals, providing a high selectivity to this compound. The toxicity profile of technical grade glyphosate is mostly unremarkable (Williams et al., 2000; Farmer, 2010; Bai and Ogbourne, 2016). Oral and dermal LD_{50} are more than 5000 mg/kg; however, given its widespread use, including the home and garden market, accidental or intentional exposures to glyphosate are inevitable. For example, during the period 2001 to 2003, there were over 13,000 reports to the American Association of Poisons Control Centers' Toxic Exposure Surveillance System relating to glyphosate exposure (Bradberry et al., 2004b). There was a major adverse outcome in 18 patients, and 5 died. Several other cases of glyphosate ingestions have been published, with a 10% to 15% mortality rate (Talbot et al., 1991; Lee et al., 2000; Bradberry et al., 2004b; Bai and Ogbourne, 2016). Given the low acute toxicity of glyphosate itself, the attention has focused on its formulation, which contains surfactants to aid its penetration. The most widely used glyphosate product is Roundup®, which is formulated as a concentrate containing water, 41% glyphosate (as isopropylamine salt), and 15% polyoxethyleneamine (POEA). Animal studies suggest that the acute toxicity of this glyphosate formulation is due to the surfactant POEA, which has an oral LD in rat of 1200 mg/kg (Bradberry et al., 2004b). It has been recently suggested that POEA may increase cell permeability to glyphosate (Benachour and Seralini, 2009). Mild intoxication results mainly in transient gastrointestinal symptoms, while moderate or severe poisoning presents with gastrointestinal bleeding, hypotension, pulmonary dysfunction, and renal damage (Talbot et al., 1991). Chronic toxicity studies with glyphosate show failure to gain weight, and hepatic and renal damage at high doses; the NOAEL is more than 500 mg/kg/day (Bai and Ogbourne, 2016). Glyphosate has no teratogenic, developmental, or reproductive effects. Until recently, genotoxicity and carcinogenicity studies in animals were considered negative, and glyphosate was classified as a Group E compound (evidence of noncarcinogenicity in humans) by the EPA (Farmer, 2010). Dietary exposure of the general population has been estimated to be very low, only 1% to 3% of the reference dose (Farmer, 2010). However, in 2015 IARC published a reevaluation of glyphosate and concluded that based on limited evidence in humans, sufficient evidence in animals, and strong evidence of genotoxicity and oxidative stress, glyphosate is a "probable human carcinogen" (Group 2A) (Guyton et al., 2015). In contrast, EFSA concluded that glyphosate is "unlikely to pose a carcinogenic hazard to humans and the evidence does not support classification with regard to its carcinogenic potential" (EFSA, 2015). Conclusions similar to EFSA have been reached by a large panel of experts (Williams et al., 2016). Exposure of the general population appears to be low, always less than the RfD (Solomon, 2016).

Glufosinate Glufosinate is a nonselective contact herbicide that acts by irreversibly inhibiting glutamine synthetase (Ebert et al., 1990; Casida, 2009). Plants die as a consequence of the increased levels of ammonia and deficiency of glutamine, leading to inhibition of photorespiration and photosynthetic processes (Ujvary, 2010). Mammals have other metabolizing systems that can cope with the effects on glutamine synthetase activity to a certain limit. In brain, however, inhibition of more than 10% of this enzyme activity is considered an adverse effect (EFSA, 2005). Glufosinate undergoes very limited metabolism and 80% to 90% is excreted unchanged in the feces (Ujvary, 2010). Glufosinate has relatively low acute toxicity (oral LD_{50} in rat is 1.5 g/kg), and chronic toxicity studies provided a NOAEL of 4.5 mg/kg/day, based on decreased glutamine synthetase activity. There is no evidence of genotoxicity or carcinogenicity, or direct effects on reproductive performance and fertility. Developmental toxic effects were found in rats, rabbits, and mice, but were considered not to be relevant to humans under normal handling or use (Schulte-Hermann et al., 2006; Laugeray et al.,



Glyphosate



Glufosinate

Figure 22-22. *Structures of the phosphomethyl amino acids glyphosate and glufosinate.* Note that though both have a P=O moiety such as organophosphates, these compounds are not acetylcholinesterase inhibitors.

2014). The most commonly used form of glufosinate is as ammonium salt, which is formulated with an anionic surfactant. Several cases of acute human poisoning from glufosinate ammonium–containing products have been reported, particularly in Japan, due to suicidal intent or accidental misuse. Symptoms include gastrointestinal effects, impaired respiration, neurological disturbance, and cardiovascular effects (Koyama et al., 1994; Watanabe and Sano, 1998; Ujvary, 2010). Administration of glufosinate ammonium to mice (75 mg/kg) has been shown to cause repetitive tonic–clonic seizures (Calas et al., 2016). Though glufosinate does not inhibit cholinesterase, a reduction of red blood cell and plasma cholinesterase was found in poisoned patients. As is the case for glyphosate, a role for the surfactant in the acute toxicity has been proposed, particularly with regard to the cardiovascular effects (Ujvary, 2010). In recent years, use of glufosinate has increased, as this herbicide has become an alternative to glyphosate due to the development of glyphosate-resistant weeds. Crop varieties have been genetically engineered to be glufosinate-tolerant.

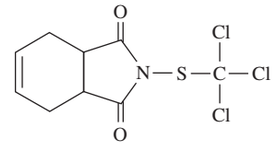
FUNGICIDES

Fungal diseases caused by thousands of species of fungi are virtually impossible to control without chemical application, and even organic farmers utilize a small number of fungicides, such as sulfur, copper, and lime sulfur (Gianessi and Reigner, 2006). In the 1800s, loss of half of a potato or peach crop, or of wine grape by fungal infestation was common, and the use of sulfur, copper sulfate, and lime sulfur drastically improved yield (Gianessi and Reigner, 2006). Fungicidal chemicals are derived from a variety of structures, from simple inorganic compounds, such as copper sulfate, to complex organic compounds. Most fungicides are surface or plant protectants, and are applied prior to potential infection by fungal spores, either to plants or to postharvest crops. Other fungicides can be used therapeutically to cure plants when an infestation has already begun. Still others are used as systemic fungicides that are absorbed and distributed throughout the plant.

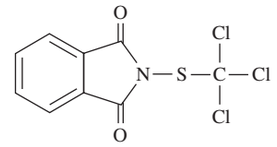
With few exceptions, fungicides have low acute toxicity in mammals. However, some produce positive results in genotoxicity tests and may have carcinogenic potentials, while others are endocrine disruptors. The effects are often associated with the mechanisms by which these compounds act on their targets, the fungi. Some fungicides have been associated with severe epidemics of poisoning and have been banned. For example, methylmercury was associated with poisoning in Iraq, where grains treated with this fungicide were consumed instead of planted (Bakir et al., 1973). Hexachlorobenzene (HCB), used in the 1940–1950s to treat seed grains, was associated with an epidemic of poisoning in Turkey from 1955 to 1959 (Cam and Nigogosyan, 1963). HCB has a high cumulative toxicity and caused a syndrome called black sore, characterized by blistering and epidermolysis of the skin, pigmentation, and scarring. HCB also causes porphyria as well as hepatomegaly and immunosuppression (Ecobichon, 2001a). The main classes of fungicides currently in use are discussed below; additional discussions of fungicide use and toxicity can be found in Hayes (1982), Edwards et al. (1991), and Stevens and Breckenridge (2001a).

Captan and Folpet

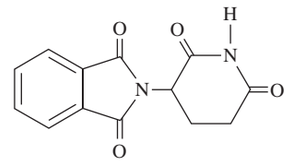
Captan and folpet are broad-spectrum protectant fungicides; together with captafol, which was taken off the market in 1988, they are called chloroalkylthio fungicides, due to the presence of side chains containing chlorine, carbon, and sulfur (Fig. 22-23). Captan and folpet were registered in the United States in the early 1950s.



Captan



Folpet



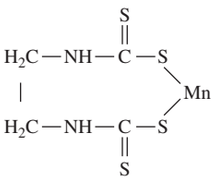
Thalidomide

Figure 22-23. *Structures of the phthalimide fungicides captan and folpet.* The structure of thalidomide is also shown, though phthalimides have been shown not to be teratogenic, despite structural similarities with thalidomide.

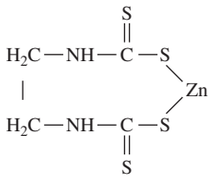
As for most fungicides, these compounds have low acute oral and dermal toxicity (LD_{50} = ~5g/kg), while they are very toxic by the i.p. route (LD_{50} = 40–50 mg/kg). They are potent eye irritants and moderate skin irritants. Dermal absorption is low. Both are extensively and rapidly metabolized in mammals through hydrolysis and thiol interactions, with thiophosgene being a common metabolite (Gordon, 2010). Captan and folpet, as well as thiophosgene, are mutagenic in in vitro tests; however, in vivo mutagenicity tests are mostly negative, possibly because of the rapid degradation of these compounds (Arce et al., 2010). Both fungicides induce the development of duodenal tumors in mice, and on this basis, they were classified by the EPA as probable human carcinogens (Category B2). However, the mode of action for these tumors is thought to be not related to mutagenicity, but instead to be dependent on irritation and cell loss in the intestinal villi, followed by a compensatory increase in proliferation in the crypt compartment (Gordon, 2010). For this reason the EPA changed the classification of captan to "not likely to be a human carcinogen when used according to label directions" (Gordon, 2007). Additionally, tumors observed in rats (renal adenomas and uterine sarcomas) are considered not to be treatment related (Gordon, 2007, 2010). No evidence of increased incidence of cancer was found among applicators exposed to captan over a 9-year period (Greenburg et al., 2008). Similarly, folpet was also shown to be an unlikely carcinogen, for the same reasons as captan (Cohen et al., 2010). Captan and folpet share a common mechanism of toxicity with regard to the development of duodenal tumors in mice, as well as other toxicity endpoints, and are considered for cumulative risk assessment under the FQPA (Bernand and Gordon, 2000). The margin of exposure (MOE) for both captan and folpet is approximately 1,000,000, suggesting that neither should pose a cancer risk for humans. Reentry intervals for farm workers are now based on the potential for eye irritation (Gordon, 2010). Because of their structural similarity to the potent teratogen thalidomide (Fig. 22-23), chloroalkylthio fungicides have been extensively tested in reproductive/developmental studies in multiple species, but no evidence of teratogenicity has been found (McLaughlin et al., 1969).

Dithiocarbamates

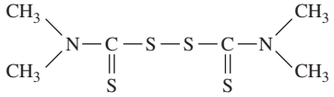
Dithiocarbamates and the related ethylenebisdithiocarbamates are a group of fungicides that have been widely used since the 1940s to control about 400 fungal pathogens in a variety of crops (Stevens and Breckenridge, 2001a). The nomenclature of many of these compounds arises from the metal cations with which they are associated; thus, there are maneb (Mn), ziram and zineb (Zn) (Fig. 22-24), mancozeb (Mn and Zn), and ferbam (Fe). Thiram is an example of dithiocarbamate without a metal moiety (Fig. 22-24). The dithiocarbamates have low to moderate acute toxicity by the oral, dermal, and respiratory route (Hurt et al., 2010). However, chronic exposure is associated with adverse effects that may be due to the dithiocarbamate acid or the metal moiety. These compounds are metabolized to a common metabolite, ethylenethiourea (ETU); on average, 5% to 7.5% of the administered dose is converted to ETU on a weight basis (Hurt et al., 2010). Formation of ETU is responsible for the effects of dithiocarbamates on the thyroid. This compound causes thyroid tumors in rats and mice, which result from the inhibition of the synthesis of the thyroid hormones thyroxine (T4) and triiodothyronine (T3). This leads to elevated serum levels of thyroid-stimulating hormone (TSH), via feedback stimulation of the hypothalamus and the pituitary, and subsequent hypertrophy and hyperplasia of thyroid follicular cells, which progresses to adenomas and carcinomas (Chhabra et al., 1992). Similarly, dithiocarbamates alter thyroid hormone levels and cause thyroid hypertrophy. The hormonal mechanism of thyroid tumors implies a threshold model for hazard assessment. In addition, humans are expected to exhibit a lesser degree of sensitivity to thyroid inhibitors (Hurt et al., 2010). ETU also causes liver tumors in mice, by yet unknown mechanisms, although levels of ETU resulting from fungicide metabolism at maximum tolerated doses are believed to be insufficient to produce hepatic tumors. Neither dithiocarbamates nor ETU are genotoxic in in vitro and in vivo tests.



Maneb
Manganese ethylenebisdithiocarbamate

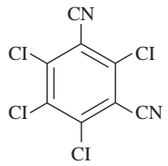


Zineb
Zinc ethylenebisdithiocarbamate

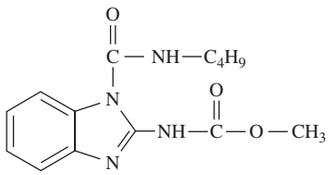


Thiram
Bis(diethylthio-carbamoyl)disulfide

Figure 22-24. *Structures of three dithiocarbamate fungicides.*



Chlorothalonil



Benomyl

Figure 22-25. *Structures of the fungicides chlorothalonil and benomyl.*

Developmental toxicity and teratogenicity are observed with dithiocarbamates and ETU at maternally toxic doses, particularly in rats. These effects are ascribed to an effect of ETU on the thyroid. A key concern with chemicals affecting thyroid functions is their potential developmental neurotoxicity, given the essential role of thyroid hormones in brain development (Chan and Kilby, 2000), and this deserves further investigation. There is also some evidence that dithiocarbamates may cause neurotoxicity by mechanisms not involving ETU. High doses of several of these compounds cause hindlimb paralysis, which is possibly related to the release of the carbon disulfide moiety from a common metabolite, ethylene bisisothiocyanate sulfide (EBIS) (Johnson et al., 1998; Hurt et al., 2010). Chronic exposure to maneb has been associated with parkinsonism, which is likely ascribed to exposure to the manganese moiety, rather than the dithiocarbamate (Ferraz et al., 1988; Meco et al., 1994), particularly when there is coexposure with paraquat (Costello et al., 2009). Maneb has also been shown to produce nigrostriatal degeneration when given in combination with paraquat (Thiruchelvam et al., 2000), and to potentiate the neurotoxicity of MPTP (McGrew et al., 2000). It has been shown that maneb affects dopaminergic neurons by inhibiting mitochondrial functions (Zhang et al., 2003). This fungicide has been withdrawn from the U.S. and the EU markets (Keigwin, 2010).

The structure of dithiocarbamate fungicides resembles that of disulfiram, a compound used therapeutically to produce intolerance to alcohol, by virtue of its ability to inhibit aldehyde dehydrogenase. Interactions of dithiocarbamates with alcohol, leading to elevation in acetaldehyde levels, have been reported (Edwards et al., 1991). As aldehyde dehydrogenase also metabolizes DOPAL, a toxic metabolite of dopamine, inhibition of aldehyde dehydrogenase by dithiocarbamates leads to its accumulation in the striatum, and is considered an important pathogenic mechanism in Parkinson disease (Fitzmaurice et al., 2013).

Chlorothalonil

Chlorothalonil is a halogenated benzonitrile fungicide (Fig. 22-25), first registered in the United States in 1966, and widely used to treat vegetable, ornamental, and orchard diseases (Table 22-4). While oral and dermal toxicities are low (LD_{50} = 5–10 g/kg), it is highly toxic by the intraperitoneal and inhalation routes. It also causes severe irreversible eye lesions in the rabbit, because of its irritant properties, but causes skin irritation only after repeated

dermal applications. Dermal absorption is low, but following oral administration chlorothalonil is rapidly absorbed and metabolized through glutathione conjugation, with excretion occurring primarily through the feces. Chlorothalonil is not mutagenic in in vitro and in vivo tests; however, tumors in the forestomach and the kidney have been found in chronic toxicity studies in both rats and mice, but not in dogs (Parsons, 2010). Such tumors are believed to be due to regenerative hyperplasia, and it is assumed that a threshold can be established for carcinogenicity (Parsons, 2010). Chlorothalonil is not a reproductive or developmental toxicant. Known adverse effects in humans are limited to its irritant effects on the eye and the skin.

Benzimidazoles

Benomyl is the main representative of this class of fungicides (Fig. 22-25). It inhibits fungal growth by inhibiting microtubule assembly in fungi, with minor effects in plants or mammals. Acute toxicity is low, while chronic studies have found effects in the liver, testes, bone marrow, and gastrointestinal tract (Mull and Hershberger, 2001). Allergic contact dermatitis caused by foliar benomyl residues has been reported (O'Malley, 2010). Because of its ability to disrupt microtubule assembly during cell division, benomyl causes chromosomal aberrations (aneuploidy) both in vitro and in vivo, but does not interact directly with DNA. Liver tumors have been observed in chronic oncogenicity studies in mice. The action on dividing cells has also raised concern for benomyl's potential teratogenicity and developmental toxicity. Teratogenic effects were observed following administration of high doses of benomyl and carbendazim (a metabolite of benomyl, which is commercialized as a fungicide, but not in the United States) to rats and zebrafish (Mull and Hershberger, 2001; Kim et al., 2009). However, such effects were not seen in feeding studies in rats or rabbits. Anecdotal evidence suggests that maternal exposure to benomyl may result in anophthalmia in humans, but epidemiological studies did not demonstrate any convincing association (Spagnolo et al., 1994). Benomyl has been shown in animals to affect the male reproductive system (decreased testicular and epididimal weight and reduced sperm count), but the mechanisms underlying these effects remain unclear (Kim et al., 2009). A metabolite of benomyl, *S*-methyl *N*-butylthiocarbamate sulfoxide, is a potent inhibitor of aldehyde dehydrogenase, and administration of benomyl in mice causes a significant increase in the levels of DOPAL in the striatum (Casida et al., 2014), suggesting that benomyl may be among the pesticides considered as etiological factors in Parkinson disease.

Other Old and New Fungicides

Inorganic and Organometal Fungicides Several inorganic and organic metal compounds are, or have been, used as fungicides (Clarkson, 2001). The fungicidal activity of soluble copper salts was discovered as early as 1807, and by 1890 copper sulfate found extensive use, particularly in the formulation known as Bordeaux mixture (copper sulfate and calcium hydroxide). Copper sulfate has overall low toxicity and remains one of the most widely used fungicides. Among organotin compounds, triphenyltin acetate is used as a fungicide, while tributyltin has long been utilized as an antifouling agent. However, because of its adverse ecological effects, particularly on oysters, its use was banned in 2008. It has been replaced in antifouling paints by copper, often in combination with "booster" biocides, such as the fungicides chlorothalonil or zineb, or the herbicide diuron (Dafforn et al., 2011). Triphenyltin has moderate to high acute toxicity, but may cause reproductive toxicity and endocrine disruption (Golub and Doherty, 2004). These effects are believed to be due to activation of RXR (retinoid X receptor) and of PPARγ (peroxisome proliferator–activated receptor γ) by triphenyltin (Kotake, 2012; Harada et al., 2016b). Organic mercury compounds, such as methylmercury, were used extensively as fungicides in the past for the prevention of seedborne diseases in grains and cereals. Given their high toxicity, particularly neurotoxicity, and large episodes of human poisoning (Bakir et al., 1973), their use has since been banned. A discussion of organometal compounds is found in Chap. 23, "Toxic Effects of Metals." Sulfur compounds are among the oldest and still widely used fungicides. As they are often used as fumigants, they are discussed in the section "Fumigants."

Azoxystrobin Azoxystrobin is a broad-spectrum fungicide obtained by chemical modification of strobilurins, antifungal compounds present in the mushroom *Strobilurus tenacellus*, and marketed since 1996. Azoxystrobin is an example of the so-called Qo inhibitor fungicides, which inhibit mitochondrial respiration and energy production by blocking electron transfer at the quinone "outside" site (Qo), ultimately preventing generation of ATP (Casida and Durkin, 2017). Azoxystrobin has low acute toxicity (oral LD_{50} in rat more than 5 g/kg), and a generally unremarkable toxicological profile in mammals (Stevens and Breckenridge, 2001a), though it is toxic to aquatic organisms.

Prothioconazole Prothioconazole is the newest of a series of triazole fungicides, first introduced in the mid-1970s, which are effective against many diseases of wheat, barley, and other crops. They act by blocking the CYP51 sterol 14α-demethylase resulting in depletion of ergosterol, and are also known as inhibitors of sterol biosynthesis (Stevens and Breckenridge, 2001a; Casida and Durkin, 2017). No significant mammalian toxicity has been identified (EFSA, 2007).

RODENTICIDES

Rodents can cause health and economic damages to humans, as they are vectors for several diseases (e.g., plague, endemic rickettsiosis, and spirochetosis), can occasionally bite people, can consume large quantities of postharvest stored foods, and can contaminate foodstuffs with urine, feces, and hair, which may cause diseases. Hence, there is a need to control rodent populations, including rats, mice, and others (e.g., moles and gophers). Limiting their access to feed and harborage, and trapping are two approaches to rodent control; however, rodenticides still play, and will likely continue to play, an important role in this endeavor. To be effective, yet safe, a rodenticide must satisfy several criteria: (a) the poison must be very effective in the target species once incorporated into bait in small quantity; (b) baits containing the poison must not induce bait shyness, so that the animal will continue to eat it; (c) the manner of death must be such that survivors do not become suspicious of its cause; and (d) it should be species-specific, with considerable lower toxicity to other animals (Murphy, 1986; Ecobichon, 2001a; Pelfrene, 2010).

The compounds used as rodenticides comprise a diverse range of chemical structures having a variety of mechanisms of action. The ultimate goal is to obtain the highest species selectivity; in some cases (e.g., norbormide) advantage has been taken of the physiology and biochemistry unique to rodents. With other rodenticides, the sites of action are common to most mammals, but advantage is taken of the habits of the pest animal and/or the usage, thereby minimizing toxicity for nontarget species. Because rodenticides are used in baits that are often placed in inaccessible places, widespread exposures or contaminations are unlikely. However, toxicologic problems can arise from acute accidental ingestions

or from suicidal/homicidal attempts. In particular, poison centers receive thousands of calls every year related to accidental ingestions of rodenticide baits by children, most of which resolve without serious consequences.

Anticoagulants

Following the report of a hemorrhagic disorder in cattle that resulted from the ingestion of spoiled sweet clover silage, the hemorrhagic chemical was identified by University of Wisconsin biochemistry professor Karl Paul Link in 1939 as bishydroxycoumarin (dicoumarol). In 1948, a more potent synthetic congener was introduced as an extremely effective rodenticide; the compound was named warfarin, as an acronym derived from the name of the patent holder, the Wisconsin Alumni Research Foundation (Majerus and Tollefsen, 2006). In addition to their use as rodenticides, coumarin derivatives, including warfarin itself, are used as anticoagulant drugs and have become a mainstay for prevention of thromboembolic disease (Majerus and Tollefsen, 2006). Coumarins antagonize the action of vitamin K in the synthesis of clotting factors (factors II, VII, IX, and X). Their specific mechanism involves inhibition of the enzyme vitamin K epoxide reductase, which regenerates reduced vitamin K necessary for sustained carboxylation and synthesis of relevant clotting factors (Fig. 22-26). The acute oral toxicity (LD_{50}) of warfarin in rats ranges from 50 to 180 mg/kg, while the 90-day LD_{50} has been reported as low as 0.077 mg/kg, indicating that multiple doses are required before toxicity develops (indeed warfarin is referred to as a "multiple dose" rodenticide).

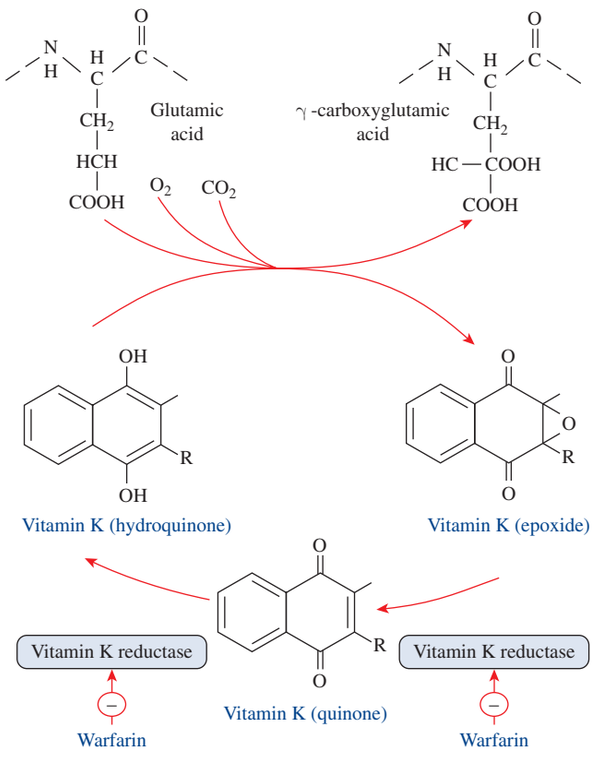


Figure 22-26. *Site of action of the anticoagulant rodenticide warfarin.* Reduced vitamin K (hydroquinone) serves as cofactor for the conversion of glutamic acid to γ-carboxyglutamic acid in the peptide chains of coagulation factors II, VII, IX, and X. During this reaction, vitamin K is oxidized to an epoxide that is then reduced to quinone and hydroquinone by vitamin K reductase, which is inhibited by warfarin.

The appearance of rats resistant to warfarin and to other early anticoagulant rodenticides led to the development of "second generation" anticoagulants. Some are coumarins, such as the "superwarfarins" brodifacoum or difenacoum, while others are indan-1,3-dione derivatives (diphacinone, chlorophacinone). These compounds essentially act like warfarin, but have prolonged half-lives (e.g., brodifacoum 16 to 34 days vs. warfarin 15 to 37 hours), and cause very long-lasting inhibition of coagulation; in addition, they are mostly "single dose" rodenticides (King and Tran, 2015). Some are extremely toxic to most mammalian species; for example, the oral LD_{50} of brodifacoum is about 0.3 mg/kg in rats, rabbits, and dogs (Pelfrene, 2010; King and Tran, 2015). During the period 1987 to 2012, Poison Centers in the United States reported a total of 315,951 exposures to these long-lasting anticoagulant rodenticides; of these, only 2.3% resulted in any effect, and 89% involved children under the age of 6 (King and Tran, 2015). In 2014 almost 80% of all rodenticide poisonings were due to superwarfarins. Because of the potential risks for humans and for nontarget animals, use of these compounds has been restricted to licensed pest control operators. Monitoring of anticoagulant therapy is normally done by measuring prothrombin time (PT) in comparison to normal pooled plasma. Values of INR (international normalized ratio) are then derived, with a target value of 2 to 3. In case of poisoning with an anticoagulant rodenticide, PT is significantly longer, and leads to severe internal bleeding. Hematuria, gingival bleeding, epistaxis, gastrointestinal bleeding, and spontaneous ecchymosis are also observed in case of poisoning with long-acting anticoagulant rodenticides (King and Tran, 2015). When INR is more than 5 to 8, vitamin K can be given as an antidote. In the case of superwarfarins, the early infusion of a lipid emulsion to modify tissue distribution has also proved beneficial (Feinstein et al., 2016).

Bromethalin

Bromethalin is a "single dose" rodenticide, first registered in the United States in the mid-1980s. Its acute toxicity is linked to the ability of certain animal species to metabolize bromethalin to the toxic metabolite desmethylbromethalin. Oral LD_{50} values are approximately 2 mg/kg in rats and cats, but only more than 1000 mg/kg in guinea pigs; however, the LD_{50} of desmethylbromethalin in the latter species is 7.5 mg/kg (Van Lier and Cherry, 1988). Symptoms of poisoning include tremors, convulsions, and prostration, which are seen 2 to 36 hours after exposure. The targets of toxicity for desmethylbromethalin are the mitochondria, where this metabolite, and to a lesser extent the parent compound, uncouples oxidative phosphorylation resulting in decreased ATP synthesis. In the CNS this leads to retention of water causing edema of the myelin sheath (Peterson, 2013). Due to restrictions imposed on superwarfarins, the use of bromethalin, which is readily available for over-the-counter purchase, has increased. Between 1997 and 2014, 126 cases of human exposures to bromethalin have been reported to Poison Centers, with symptoms mostly of gastrointestinal and nervous system nature; there were no moderate, major, or fatal effects in this population, which consisted primarily of children less than 5 years old (Huntington et al., 2016).

Cholecalciferol

This rodenticide is nothing else than vitamin D3, often also used as a dietary supplement. Vitamin D helps the body maintain calcium balance by enhancing its absorption from the gastrointestinal tract and the kidneys. High, toxic doses of cholecalciferol cause an excessive increase in calcium levels, leading to renal, CNS, and muscle alterations (Peterson and Fluegeman, 2013). Key to these effects is

the formation in the kidney of calcitriol (1, 25-dihydroxycholecalciferol), a vitamin D metabolite which exerts its effects of increasing calcium absorption (from the gut) or reabsorption (from the kidney). Several doses of cholecalciferol are needed to obtain the rodenticidal action. Pets are often exposed, while human poisonings are rare.

Zinc Phosphide

Metal phosphides such as aluminum phosphide (AlP), magnesium phosphide (Mg_3P_2), and zinc phosphide (Zn_3P_2) are widely used globally as single-dose rodenticides, and the first two are also utilized as fumigants (Lyubimov and Garry, 2010). Their toxicity is due to the phosphine gas (PH_3) formed on ingestion upon reactions with water or acids as follows:

$$Zn_3P_2 + 6H_20 \rightarrow 3Zn(OH)_2 + 2PH_3;$$

$$Zn_3P_2 + 6H+ \rightarrow 3Zn^{2+} + 2PH_3.$$

Phosphine causes widespread cellular toxicity with injury to the heart, liver, kidney, and nervous system (Sciuto et al., 2016). The mechanism of toxicity involves generation of oxidative stress rather than inhibition of cytochrome *c* oxidase as initially suggested (Proudfoot, 2009; Sciuto et al., 2016). Several cases of human poisoning have been reported with gastrointestinal, cardiovascular, hepatic, and electrolytic balance effects (Ecobichon, 2001a; Proudfoot, 2009). Exposure to phosphides by accidental or intentional ingestion is often lethal, and there are no known antidotes, though recent case reports have indicated beneficial results with *N*-acetylcysteine (Oghabian et al., 2016; Sciuto et al., 2016).

Norbormide

Norbormide was introduced in 1964 as a selective rodenticide, lethal to rats (*Rattus rattus*, *R. norvegicus*), but not to other rodent species (Pelfrene, 2010). Norbormide shows a remarkable selectivity in toxicity; oral LD_{50} in rat is about 5 to 10 mg/kg, while it is more than 2000 mg/kg in mice. In other species, 1000 mg/kg produce no effects. Such species difference in toxicity does not appear to be due to differences in toxicokinetics or biotransformation (Ravindran et al., 2009). Instead, it may be accounted for by differences in response of the peripheral blood vessels to norbormide-induced vasoconstriction. At the cellular level, norbormide causes a rat-specific activation of the mitochondrial permeability transition pore, which requires an intact outer mitochondrial membrane, and is mediated by the 18-kDa translocator protein (TSPO) (Zulian et al., 2011). No cases of human intoxication with norbormide have been reported (Pelfrene, 2010).

Other Compounds

Fluoroacetic Acid and Its Derivatives Sodium fluoroacetate (Compound 1080) is the main representative of this class of rodenticides. Due to its high acute mammalian toxicity (oral LD_{50} in rat = <2 mg/kg), its current use in the United States is mainly in livestock protection collars, though it is still used as rodenticide in some parts of the world (Giannitti et al., 2013). The main targets of toxicity are the CNS and the heart. Fluoroacetate is incorporated into fluoroacetyl-coenzyme A, which condenses with oxaloacetate to form fluorocitrate, which in turn inhibits mitochondrial aconitase. This results in inhibition of the Krebs cycle, leading to lowered energy production, reduced oxygen consumption, and reduced cellular concentration of ATP. Blockage of energy metabolism is believed to account for most signs of toxicity, though some may be due to accumulation of citrate, which is a potent chelator of calcium ions (Pelfrene, 2010). Several cases of human and animal poisoning have been reported, and the estimated lethal dose in humans ranges from 2 to 10 mg/kg. There is no specific antidote for sodium fluoroacetate, and treatment is symptomatic.

Thioureas The discovery of α-naphthylthiourea (ANTU) occurred fortuitously in the mid-1940s in the Psychobiological Laboratory of Curt Richter in Baltimore, where while studying thioureas (favored by geneticists for taste tests because they are so bitter to some people and tasteless to others) he discovered that ANTU was lethal yet tasteless to rodents, while being of low toxicity to humans (Keiner, 2005). Acute oral LD_{50} values are indeed 6 mg/kg in the rat, and 4 g/kg in the monkey. The main target of toxicity is the lung, where ANTU causes marked edema of the subepithelial spaces of the alveolar walls mediated by an inflammatory response. ANTU is believed to be biotransformed to a reactive intermediate that binds to lung macromolecules; however, the exact mechanism of its toxicity is not known. There are no reports of human poisonings with ANTU. In several cases involving a combination of chloralose and ANTU reported in France, symptoms included motor agitation and coma, both characteristic of chloralose poisonings, and pulmonary effects, due to ANTU: all patients recovered (Pelfrene, 2010).

Strychnine Strychnine is an extremely poisonous alkaloid derived from the seeds of *Nux vomica,* which antagonizes the effect of the inhibitory neurotransmitter glycine, and is thus a potent convulsant. It was first registered in the United States as a rodenticide in 1947. Its use, as a single-dose rodenticide, is severely restricted, though it is still used for special situations, for example, management of pocket gophers (Baldwin et al., 2017). Signs of poisoning appear within 1 hour and death occurs by respiratory blockage. Reported lethal doses are 2 to 15 mg/kg in rodents and less than 2 mg/kg in humans (Palatnick et al., 1997).

Red Squill Red squill (sea onion) and its bioactive principal, scilliroside, affect the cardiovascular and central nervous systems and cause emesis; the inability of rodents to vomit explains the rather selective action in these species (Ujvary, 2010). It is no longer used as a rodenticide, mainly because of its limited effectiveness.

Thallium Sulfate Thallium sulfate has been used as a pesticide, most notably as a rodenticide, since the 1920s. Because of its very high acute toxicity (LD_{50} in humans = 8–15 mg/kg), the use of thallium sulfate as a rodenticide has been banned in the United States in 1972, but it is still used in some countries (Clarkson, 2001). Thallium is a mitochondrial poison and has a high affinity for sulfhydryl groups. Main effects of thallium poisoning are peripheral neuropathy, toxic encephalopathy, and alopecia (Li et al., 2015).

FUMIGANTS

A large number of compounds are used for soil or structural fumigation, or for fumigating postharvest commodities. They are active toward insects, mites, nematodes, weed seeds, fungi, or rodents, and have in common the property of being in the gaseous form at the time they exert their pesticidal action. They can be liquids that readily vaporize (e.g., ethylene dibromide), solids that can release a toxic gas on reaction with water (e.g., phosphine released by aluminum phosphide), or gases (e.g., methyl bromide). For soil fumigation, the compound is injected directly into the soil, which is then covered with plastic sheeting or other tarping materials, which are then sealed and kept in place for several days. By eliminating unwanted pests, this treatment enhances the quality of the crops and increases yield. For structural fumigation, the commercial or

residential structure is covered by a tent, fumigated, and then aerated. Fumigation of postharvest commodities, such as wheat, cereals, and fruits, to eradicate pest infestations typically occurs where the commodities are stored (e.g., warehouses, grain elevators, and ship holds). After treatment, mechanical ventilation aerates the commodity until concentration of the fumigant decreases to safe levels.

Compounds used as fumigants are usually nonselective, highly reactive, and cytotoxic. They provide a potential hazard, primarily for applicators, from the standpoint of inhalation exposure, and to a minor degree for dermal exposure or ingestion, in case of solids or liquids. Fumigant residues in food commodities are usually extremely low. Several fumigants used in the past are no longer marketed because of toxicological concerns. These include, for example, carbon disulfide, which is neurotoxic; carbon tetrachloride, a potent hepatotoxicant; 1,2-dibromo-3-chloropropane, a male reproductive toxicant; and ethylene dibromide, a carcinogen. Their toxicity is discussed in other chapters of this book. Some of the most commonly used fumigants are discussed below.

Methyl Bromide

Methyl bromide (CH_3Br) is a broad-spectrum pesticide, used for soil fumigation, commodity treatment, and structural fumigation, and has been the most widely used fumigant for over 70 years. Since the early 1990s, global use of methyl bromide has substantially decreased, because of environmental and toxicological concerns (Ruzo, 2006). With the signing of the Montreal Protocol on Substances That Deplete the Ozone Layer in 1987, the international community initiated a series of steps to reduce emissions of ozone-depleting products, including methyl bromide. As of January 2005, methyl bromide was officially phased out in the United States and in other developed countries (Gullino et al., 2005), while developing countries phased out methyl bromide use in 2015 (Ajwa et al., 2010). However, exemptions to the ban have been granted, for example, in 2014 for use in pre-plant strawberries or in dry-cured hams (Zhao et al., 2016). Concerns on certain toxicological aspects of methyl bromide have also contributed to its decreasing use, and to the search of viable alternatives (Ruzo, 2006). Yet, as of 2007 this compound was still used in the United States (Table 22-4), likely for convenience and economic reasons, and is still used in some countries, such as New Zealand (Bulathsinghala and Shaw, 2014).

The acute toxicity of methyl bromide relates to both its concentration and the duration of exposure, with LC_{50} values in rats of 2833 ppm for 30-minute exposure, and of 302 ppm for an 8-hour exposure (Piccirillo and Piccirillo, 2010). Hundreds of cases of systemic poisoning and dozens of fatalities have resulted over the years from use of methyl bromide as a fumigant (Alexeef and Kilgore, 1983; Herzstein and Cullen, 1990). Acute exposure results in respiratory, gastrointestinal, and particularly neurologic symptoms; the latter include lethargy, headache, seizures, paresthesias, peripheral neuropathy, and ataxia, and are considered to be more relevant than other toxic effects for human risk assessment (Alexeef and Kilgore, 1983; De Haro et al., 1997; Piccirillo and Piccirillo, 2010; de Souza et al., 2013). The mechanism(s) underlying methyl bromide neurotoxicity are not known, though several (e.g., oxidative stress) have been proposed (de Souza et al., 2013; Bulathsinghala and Shaw, 2014). Methyl bromide is positive in several genotoxicity tests in vitro and in vivo. Carcinogenicity studies produced carcinomas in the forestomach of rats following oral ingestion, and increased incidence of adenomas of the pituitary gland in male rats in an inhalation study. Alterations in GSH-mediated detoxication may play a role in these effects (Bulathsinghala and Shaw, 2014). Other studies in rats and mice, however, provided no evidence of carcinogenicity. Methyl bromide is classified by IARC in Group 3 (not classifiable as to its carcinogenicity to humans), given the limited evidence in animals and the inadequate evidence in humans. As methyl bromide is an odorless and colorless gas, another fumigant, *chloropicrin*, which has a pungent odor and causes irritation of the eyes, was often used in conjunction with methyl bromide and other fumigant mixtures to warn against potentially harmful exposures. Chloropicrin has since emerged as an alternative to methyl bromide because its potency and short half-life make it beneficial as a fumigant (Ruzo, 2006). The main adverse effects of chloropicrin are due to its irritant properties. Effects in humans are primarily lacrimation, irritation cough, burning, and chest pains (Oriel et al., 2009).

1,3-Dichloropropene

1,3-Dichloropropene ($C_3H_4Cl_2$), first introduced in 1945, is a soil fumigant, extensively utilized (see Table 22-4) for its ability to control soil nematodes. It has a moderate to high acute toxicity in animals (oral LD_{50} in rats: 130 to 713 mg/kg; dermal LD_{50}: more than 1200 mg/kg; inhalation LD_{50}: approximately 1000 ppm) (Stott et al., 2001). Human fatalities following oral exposure have been reported (Hernandez et al., 1994). 1,3-Dichloropropene is an irritant, and can cause redness and necrosis of the skin. It is extensively metabolized, with the mercapturic acid conjugate being the major urinary metabolite. Data on genotoxicity are contradictory, with positive in vitro, and mostly negative in vivo, results. However, some short-term genotoxicity assays may have been confused by the presence of a mutagen epichlorohydrin, which was historically added as a stabilizing agent (Stott and Gollapudi, 2010). Carcinogenicity studies in rodents have found an increase in benign liver tumors in rats but not in mice after oral administration (Stebbins et al, 2000), and of benign lung adenomas in mice following inhalation exposure (Lomax et al., 1989). Induction of these tumors appears to be due to a nongenotoxic mode of action, possibly by 1,3-dichloropropene functioning as a tumor promoter (Klaunig et al., 2015). The overall toxicology of 1,3-dichloropropene has been reviewed (Stott and Gollapudi, 2010). Though it has been phased out in the EU, 1,3-dichloropropene is one of the most widely used alternatives to methyl bromide for use as a soil nematocide in the United States (Sanchez-Moreno et al., 2009). However, it lacks herbicidal properties, and is often formulated with chloropicrin, which is a better fungicide (Ajwa et al., 2010).

Metam Sodium

Metam sodium ($C_2H_4NNaS_2$), which has been registered in the United States since 1954, is a widely used soil fumigant (Table 22-4), whose toxic action toward soil nematodes, fungi, and weed seeds are due to its hydrolysis product methyl isothiocyanate (MITC). In mammals, metam sodium is metabolized in vivo to carbon disulfide and MITC (Pruett et al., 2001). Acute toxicity is low (e.g., oral LD_{50} in rat = 870 mg/kg), while that of MITC is much higher (oral LD_{50} in rat = 97 mg/kg). Upon chronic exposure in various species, toxic effects in the bladder, kidney, and liver have been reported. Metam sodium is not genotoxic, but because of evidence of carcinogenicity in mice and in male rats (Carlock and Dotson, 2010), it was classified as a probable human carcinogen (B2) by the EPA, and as a compound with "limited evidence of a carcinogenic effect" by the EFSA (EFSA, 2011). In humans, metam sodium can act as a contact sensitizer, inducing allergic dermatitis possibly due to MITC. Main effects of acute exposure to MITC in the vapor state are irritated or burning eyes, nasal and throat irritation, nausea, coughing, and shortness of breath (Dourson et al., 2010; Deguigne et al., 2011). In 1991, because of the derailment of a train car,

approximately 19,000 gallons of metam sodium were accidentally spilled into the Sacramento River in California, causing a large kill of aquatic organisms. Symptoms reported by exposed individuals included headache, eye irritation, nausea, shortness of breath, and dermatitis (Pruett et al., 2001). Metam sodium is increasingly being used as an alternative to methyl bromide (Ruzo, 2006), as it is effective against nematodes, weeds, and fungi (Ajwa et al., 2010).

Sulfur Compounds

Elemental sulfur is considered the oldest of all pesticides, and its pesticidal properties were known to the ancient Greeks as early as 1000 BC (Tweedy, 1981). It is very effective for the control of many plant diseases, particularly fungal diseases, and is still widely used in the United States and worldwide. In agriculture, sulfur finds its major use in grapes and tomatoes, and it can be used in organic farming (Gammon et al., 2010). Sulfur fumigation is also used for preservation of herbs used in traditional Chinese medicine (Jiang et al., 2013). The primary health effect in humans associated with the agricultural use of elemental sulfur is dermatitis (Gammon et al., 2010). In ruminants, excessive sulfur ingestion can cause cerebrocortical necrosis (polioencephalomalacia), possibly due to its conversion by microorganisms in the rumen to hydrogen sulfide (Gammon et al., 2010).

Sulfuryl fluoride (SO_2F_2) is also used as a fumigant, particularly for structural fumigation (for termite control), and for postharvest fumigation of stored commodities. There is no evidence of developmental toxicity, mutagenicity, or carcinogenicity. Upon chronic exposure, the primary effect in multiple species is neurotoxicity, evidenced by microvacuolation in various brain areas; the mechanism is unknown (Eisenbrandt and Hotchkiss, 2010). A recent case report describes the acute poisoning of a family in South Florida after reentering their home that had been fumigated with sulfuryl fluoride. Signs and symptoms included irritation of the eyes, nose, and respiratory tract, nausea and vomiting, and CNS effects (Mulay et al., 2016).

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REFERENCES

Abdel-Rahman A, Shetty AK, Abou-Donia MB. Disruption of the blood–brain barrier and neuronal cell death in cingulate cortex, dentate gyrus, thalamus, and hypothalamus in a rat model of Gulf War syndrome. *Neurobiol Dis*. 2002;10:306–326.

Abel EL, Bammler TK, Eaton DL. Biotransformation of methyl parathion by glutathione *S*-transferases. *Toxicol Sci*. 2004;79:224–232.

Adam A, Smith LL, Cohen GM. An evaluation of the redox cycling potencies of paraquat and nitrofurantoin in microsomal and lung slice systems. *Biochem Pharmacol*. 1990;40:1533–1539.

Ajdacic-Gross V, Weiss MG, Ring M, et al. Method of suicide: international suicide patterns derived from the WHO mortality data base. *Bull World Health Org*. 2008;86:726–732.

Ajwa H, Ntow WJ, Qin R, et al. Properties of soil fumigants and their fate in the environment. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:315–330.

Akossoglou K, Malester B, Xu J, et al. Brain-specific deletion of neuropathy target esterase/Swiss cheese results in neurodegeneration. *Proc Natl Acad Sci U S A*. 2004;101:5075–5080.

Alavanja MCR, Hoffman JN, Lynch CF, et al. Non-Hodgkin lymphoma risk and insecticide, fungicide and fumigant use in the agricultural health study. *PLoS ONE*. 2014;9:e109332.

Aldridge JE, Seidler FJ, Meyer A, et al. Serotoninergic systems targeted by developmental exposure to chlorypyrifos: effects during different critical periods. *Environ Health Perspect*. 2003;111:1736–1743.

Alexeef GV, Kilgore WW. Methyl bromide. *Residue Rev*. 1983;88:101–153.

Alonso L, Lindsay SW, Armstrong JRM, et al. The effect of insecticide-treated bed nets on mortality of Gambian children. *Lancet*. 1991;337:1499–1502.

Altobelli D, Martire M, Maurizi S, et al. Interaction of formamidine pesticides with the presynaptic α_2-adrenoceptor regulating [^{3}H] noradrenaline release from rat hypothalamic synaptosomes. *Toxicol Appl Pharmacol*. 2001;172:179–185.

Anand SS, Bim KB, Padilla S, et al. Ontogeny of hepatic and plasma metabolism of deltamethrin in vitro: role in age-dependent acute neurotoxicity. *Drug Metab Dispos*. 2006b;34:389–397.

Anand SS, Bruckner JV, Haines WT, et al. Characterization of deltamethrin metabolism by rat plasma and liver microsomes. *Toxicol Appl Pharmacol*. 2006a;212:156–166.

Andrade SF, Sakate M. The comparative efficacy of yohimbine and atipamezole to treat amitraz intoxication in dogs. *Vet Hum Toxicol*. 2003;45:124–127.

Antwi FB, Shama LM, Peterson RK. Risk assessment for the insect repellents DEET and picaridin. *Reg Toxicol Pharmacol*. 2008;51:31–36.

Anway MD, Cupp AS, Uzumcu M, et al. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*. 2005;306:1466–1468.

Arce GT, Gordon EB, Cohen SM, et al. Genetic toxicology of folpet and captan. *Crit Rev Toxicol*. 2010;40:546–574.

Ashby J, Kier L, Wilson AGE. Evaluation of the potential carcinogenicity and genetic toxicity to humans of the herbicide acetochlor. *Hum Exp Toxicol*. 1996;15:702–735.

Autor AP. *Biochemical Mechanisms of Paraquat Toxicity*. New York: Academic Press; 1974.

Aylward LL, Morgan MM, Arbuckle TE, et al. Biomonitoring data for 2,4-dichlorophenoxyacetic acid in the United States and Canada: interpretation in a public health risk assessment context using biomonitoring equivalents. *Environ Health Perspect*. 2010;118:177–181.

Badaksh R, Lackovic M, Ratard R. Characteristics of pesticide-related hospitalizations, Louisiana, 1998–2007. *Publ Health Rep*. 2010;125:457–467.

Bai SH, Ogbourne SM. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. *Environ Sci Pollut Res*. 2016;29:18988–19001.

Baker SR, Wilkinson DF, eds. *The Effects of Pesticides on Human Health*. Princeton, NJ: Princeton Scientific Publishing; 1990:438.

Bakir F, Damluji SF, Amin-Zaki L, et al. Methylmercury poisoning in Iraq. *Science*. 1973;181:230–241.

Baldwin RA, Meinerz R, Wimer GW. Novel and current rodenticides for pocket gopher *Thomomys spp.* Management in vineyards: what works? *Pest Manag Sci*. 2017;73;118–122.

Barbeau A, Roy M, Clourier L et al. Environmental and genetic factors in the etiology of Parkinson's disease. *Adv Neurol*. 1986;45:299–306.

Barlow SM, Sullivan FM, Lines J. Risk assessment of the use of deltamethrin on bed nets for the prevention of malaria. *Food Chem Toxicol*. 2001;39:407–422.

Baron RL. Carbamate insecticides. In: Hayes WJ, Laws ER, eds. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 1991:1125–1189.

Barr DB, Allen R, Olsson AO, et al. Considerations of selective metabolites of organophosphorus pesticides in the United States population. *Environ Res*. 2005;99:314–326.

Beard J. DDT and human health. *Science Tot Environ*. 2006;355:78–89.

Beeman RW, Matsumura F. Chlordimeform: a pesticide acting upon amine regulatory mechanisms. *Nature*. 1973;242:273–274.

Bell JW, Veltri JC, Page BC. Human exposures to *N,N*-diethyl-*m*-toluamide insect repellents reported to the American Association of Poison Control Centers 1993–1997. *Int J Toxicol*. 2002;21:341–352.

Benachour N, Seralini GE. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chem Res Toxicol*. 2009;22:97–105.

Benke G, Murphy SD. The influence of age on the toxicity and metabolism of methylparathion and parathion in male and female rats. *Toxicol Appl Pharmacol*. 1975;31:254–269.

Benowitz NL. Pharmacology of nicotine: addiction and therapeutics. *Annu Rev Pharmacol Toxicol*. 1996;36:597–613.

Bentley KS, Fletcher JL, Woodward MD. Chlorantranilipole: an insecticide of the anthranilic diamide class. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2231–2242.

Bernand BK, Gordon EB. An evaluation of the common mechanism approach to the Food Quality Protection Act: Captan and four related fungicides, a practical example. *Int J Toxicol*. 2000;19:43–61.

Berry C, LaVecchia C, Nicotera P. Paraquat and Parkinson's disease. *Cell Death Diff*. 2010;17:1115–1125.

Berry C, Crickmore N. Structural classification of insecticidal proteins: towards in silico characterization of novel toxins. *J Invertebr Pathol*. 2017;142:16–22.

Bertolote JM, Fleischmann A, Eddleston M, et al. Deaths from pesticide poisoning: a global response. *Br J Psychiat*. 2006;189:201–203.

Bertsias GJ, Katonis P, Tzanakakis G, et al. Review of clinical and toxicological features of acute pesticide poisoning in Crete (Greece) during the period 1991–2001. *Med Sci Monitor*. 2004;10:CR622–CR627.

Betarbet R, Sherer TB, Greenamyre JT. Animal models of Parkinson's disease. *BioEssays*. 2002;24:308–318.

Betarbet R, Sherer TB, MacKenzie G, et al. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature Neurosci*. 2000;3:1301–1306.

Bharathrai MY, Venugopal K, Jaligidad K, et al. Fipronil compound consumption presenting as status epilepticus. *Toxicol Int*. 2015;22:165–166.

Bismuth C, Garnier R, Dolly S, et al. Prognosis and treatment of paraquat poisoning: a review of 28 cases. *Clin Toxicol*. 1982;19:461–474.

Bismuth C, Inns RH, Marrs TC. Efficacy, toxicity and clinical use of oximes in anticholinesterases poisoning. In: Ballantyne B, Marrs TC, eds. *Clinical and Experimental Toxicology of Organophosphates and Carbamates*. Oxford, UK: Butterworth-Heinemann; 1992:555–577.

Bjørlung-Poulsen M, Andersen MR, Grandjean P. Potential developmental neurotoxicity of pesticides used in Europe. *Environ Health*. 2008;7:50.

Blondell JM. Decline in pesticide poisonings in the United States from 1995 to 2004. *Clin Toxicol*. 2007;45:589–592.

Boeckh J, Breer H, Geier M, et al. Acylated 1,3-aminopropanols as repellents against bloodsucking arthropods. *Pest Sci*. 1996;48:359–373.

Boffetta P, Adami HO, Berry SC, et al. Atrazine and cancer: a review of epidemiologic evidence. *Eur J Cancer Prev*. 2013;22:169–180.

Bouwman H, Kylin H, Sereda B, et al. High levels of DDT in breast milk: intake, risk, lactation duration, and involvement of gender. *Environ Pollut*. 2012;170:63–70.

Bouwman H, van de Berg H, Kylin H. DDT and malaria prevention: addressing the paradox. *Environ Health Perspect*. 2011;119:744–747.

Bradberry SM, Cage SA, Proudfoot AT, et al. Poisoning due to pyrethroids. *Toxicol Rev*. 2005;24:93–106.

Bradberry SM, Proudfoot AT, Vale JA. Poisoning due to chlorophenoxy herbicides. *Toxicol Rev*. 2004a;23:65–73.

Bradberry SM, Proudfoot AT, Vale JA. Glyphosate poisoning. *Toxicol Rev*. 2004b;23:159–167.

Bradberry SM, Watt BE, Proudfoot AT, et al. Mechanisms of toxicity, clinical features, and management of acute chlorophenoxy herbicide poisoning: a review. *Clin Toxicol*. 2000;39:111–122.

Bravo A, Gill SS, Soberon M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*. 2007;49:423–435.

Breckenridge CB, Elridge JC, Stevens JJ, et al. Symmetrical triazine herbicides: a review of regulatory toxicity endpoints. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1711–1723.

Breckenridge CB, Holden L, Sturgess N, et al. Evidence for separate mechanisms of toxicity for type I and type II pyrethroid insecticides. *Neurotoxicology*. 2009;30S:S17–S31.

Briassulis G, Niarloglou M, Hatzis T. Toxic encephalopathy associated with use of DEET insect repellents: a case analysis of its toxicity in children. *Hum Exp Toxicol*. 2001;20:8–14.

Brooks GT. *Chlorinated Insecticides. Vol I. Technology and Application; Vol. II. Biological and Environmental Aspects*. Cleveland, OH: CRC Press; 1974:249 pp., 197 pp.

Brooks GT. Interactions with the gamma-aminobutyric acid A-receptor: polychlorocycloalkanes and recent congeners and other ligands. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2065–2092.

Brusick DJ. An assessment of the genetic toxicity of atrazine: relevance to human health and environmental effects. *Mutat Res*. 1994;317:133–144.

Buckley NA, Eddleston M, Szinicz L. Oximes for acute organophosphate pesticide poisoning. *Cochrane Database Syst Rev*. 2005;Issue 1: CD005085.

Bulathsinghala AT, Shaw IC. The toxic chemistry of methyl bromide. *Hum Exp Toxicol*. 2014;33:81–91.

Bus JS, Gibson JE. Paraquat: model for oxidant-initiated toxicity. *Environ Health Perspect*. 1984;55:37–46.

Cabral JR, Galendo D, Laval M, et al. Carcinogenicity studies with deltamethrin in mice and rats. *Cancer Lett*. 1990;49:147–152.

Cabras P, Caboni P, Cabras M, et al. Rotenone residues in olives and in olive oil. *J Agric Food Chem*. 2002;50:2576–2580.

Caksen H, Odabas D, Arslan S, et al. Report of eight children with amitraz intoxication. *Hum Exp Toxicol*. 2003;22:95–97.

Calas AG, Perche O, Richard O. Characterization of seizures induced by acute exposure to an organophosphate herbicide, glufosinate ammonium. *Neuroreport*. 2016;27:532–541.

Caldas ED, Rebelo FM, Heliodoro VO, et al. Poisonings with pesticides in the Federal District of Brazil. *Clin Toxicol*. 2008;46:1058–1063.

Calvert GM, Mehler LN, Alsop J, et al. Surveillance of pesticide-related illness and injury in humans. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1313–1369.

Cam C, Nigogosyan G. Acquired toxic porphyria cutanea tarda due to hexachlorobenzene. *J Am Med Assoc*. 1963;183:88–91.

Campbell WC, ed. *Ivermectin and Abamectin*. New York: Springer-Verlag; 1989:363.

Carlock LL, Chen WL, Gordon EB, et al. Regulating and assessing risks of cholinesterase-inhibiting pesticides: divergent approaches and interpretations. *J Toxicol Environ Health Part B, Crit Rev*. 1999;2:105–160.

Carlock LL, Dotson TA. Metam-sodium. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2293–2306.

Carr JA, Gentles A, Smith EE, et al. Response of larval *Xenopus laevis* to atrazine: assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environ Toxicol Chem*. 2003;22:396–405.

Carson R. *Silent Spring*. Boston, MA: Houghton Mifflin; 1962.

Casey P, Vale JA. Deaths from pesticide poisoning in England and Wales: 1945–1989. *Hum Exp Toxicol*. 1994;13:95–101.

Casida JE. Esterase inhibitors as pesticides. *Science*. 1964;146:1011–1017.

Casida JE. Pyrethrum flowers and pyrethroid insecticides. *Environ Health Perspect*. 1980;34:189–202.

Casida JE. Pest toxicology: the primary mechanisms of pesticide actions. *Chem Res Toxicol*. 2009;22:609–619.

Casida JE. Michael Elliot's billion dollar crystals and other discoveries in insecticide chemistry. *Pest Manag Sci*. 2010;66:1163–1170.

Casida JE. Golden age of RyR and GABA-R diamide and isoxazoline insecticides: common genesis, serendipity, surprises, selectivity, and safety. *Chem Res Toxicol*. 2015;28:560–566.

Casida JE, Durkin KA. Novel GABA receptor pesticide targets. *Pest Biochem Physiol*. 2015;121:22–30.

Casida JE, Durkin KA. Pesticide chemical research in toxicology: lessons from nature. *Chem Res Toxicol*. 2017;30:94–104.

Casida JE, Ford B, Jinsmaa Y, et al. Benomyl, aldehyde dehydrogenase, DOPAL, and the cathecholaldehyde hypothesis for the pathogenesis of Parkinson's disease. *Chem Res Toxicol*. 2014;27:1359–1361.

Casida JE, Gammon DW, Glickman AH, et al. Mechanisms of selective action of pyrethroid insecticides. *Annu Rev Pharmacol Toxicol*. 1983;23:413–438.

CDC (Centers for Disease Control and Prevention). Seizures temporarily associated with use of DEET insect repellents – New York and Connecticut. *MMWR*. 1989;38:678–680.

Chambers HW, Meek EC, Chambers JE. Chemistry of organophosphorus insecticides. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010a:1395–1398.

Chambers JE, Levi PE, eds. *Organophosphates: Chemistry, Fate and Effects*. San Diego, CA: Academic Press; 1992:443.

Chambers JE, Meek EC, Chambers HW. The metabolism of organophosphorus insecticides. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010b:1399–1407.

Chan S, Kilby MD. Thyroid hormone and fetal central nervous system development. *J Endocrinol*. 2000;165:1–8.

Chao HR, Wang SL, Lin TC, et al. Levels of organochlorine pesticides in human milk for central Taiwan. *Chemosphere*. 2006;62:1776–1785.

Charles JM, Bond DM, Jeffries TK et al. Chronic dietary toxicity/oncogenicity studies on 2,4- dichlorophenoxyacetic acid in rodents. *Fund Appl Toxicol*. 1996;33:166–172.

Charlton NP, Murphy LT, Parker Cote JL, et al. The toxicity of picaridin containing insect repellent reported to the National Poison Data System. *Clin Toxicol (Phila)*. 2016;54:655–658.

Chen A, Rogan WJ. Nonmalarial infant deaths and DDT use for malaria control. *Emerg Infect Dis*. 2003;9:960–964.

Chen YY, Park NS, Lu TS. Suicide methods used by women in Korea, Sweden, Taiwan and the United States. *J Formos Med Assoc*. 2009;108:452–459.

Chester G: Worker exposure: methods and techniques. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1127–1138.

Chhabra RS, Eustis S, Haseman JK, et al. Comparative carcinogenicity of ethylene thiourea with or without perinatal exposure in rats and mice. *Fundam Appl Toxicol*. 1992;18:405–417.

Choi J, Hodgson E, Rose RL. Inhibitions of trans-permethrin hydrolysis in human liver fractions by chlorpyrifos oxon and carbaryl. *Drug Metab Drug Interact*. 2004;20:233–246.

Churcher TS, Lissenden N, Griffin JT, et al. The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *eLife*. 2016;5:e16090.

Cimino AM, Boyles AL, Thayer KA, et al. Effects of neonicotinoid pesticide exposure on human health: a systematic review. *Environ Health Perspect*. 2017;125:155–162.

Clark DG, McElligott TF, Hurst EW. The toxicity of paraquat. *Br J Ind Med*. 1966;23:126–132.

Clark JM, Matsumura F, eds. *Membrane Receptors and Enzymes as Targets of Insecticidal Action*. New York: Plenum Press; 1986:256.

Clarkson TW. Inorganic and organometal pesticides. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:1357–1428.

Cohen SM, Gordon EB, Singh P, et al. Carcinogenic mode of action of folpet in mice and evaluation of its relevance to humans. *Crit Rev Toxicol*. 2010;40:531–545.

Colborn T. A case for revisiting the safety of pesticides: a closer look at neurodevelopment. *Environ Health Perspect*. 2006;114:10–17.

Cole LM, Casida JE. Polychlorocycloalkane insecticide–induced convulsions in mice in relation to disruption of the GABA-regulated chloride ionophore. *Life Sci*. 1986;39:1855–1862.

Cole TB, Walter BJ, Shih DM, et al. Toxicity of chlorpyrifos and chlorpyrifos oxon in a transgenic mouse model of the human paraoxonase (PON1) Q192R polymorphism. *Pharmacogenet Genomics*. 2005;15:589–598.

Colosio C, Tiramani M, Maroni M. Neurobehavioral effects of pesticides: state of the art. *Neurotoxicology*. 2003;24:577–591.

Colosio C, Vellere F, Moretto A. Epidemiological studies of anticholinesterase pesticide poisoning: global impact. In Satoh T, Gupta RC, eds. *Anticholinesterase Pesticides: Metabolism, Neurotoxicity and Epidemiology*. Hoboken, NJ: Wiley; 2010:343–355.

Cooper RL, Lamb JC, Barlow SM, et al. A tiered approach to life testing for agricultural risk assessment. *Crit Rev Toxicol*. 2006;36:69–98.

Cooper RL, Laws SC, Das PC, et al. Atrazine and reproductive function: mode and mechanism of action studies. *Birth Def Res (pt B)*. 2007;80:98–112.

Cooper RL, Stoker TE, Tyrey L, et al. Atrazine disrupts the hypothalamic control of pituitary-ovarian function. *Toxicol Sci*. 2000;53:297–307.

Costa LG. Toxicology of pesticides: a brief history. In: Costa LG, Galli CL, Murphy SD, eds. *Toxicology of Pesticides: Experimental, Clinical and Regulatory Perspectives*. Heidelberg, Germany: Springer-Verlag; 1987:1–10.

Costa LG. Organophosphorus compounds. In: Galli CL, Manzo L, Spencer PS, eds. *Recent Advances in Nervous System Toxicology*. New York: Plenum Publishing; 1988:203–246.

Costa LG. Pesticide exposure: differential risk for neurotoxic outcomes due to enzyme polymorphisms. *Clin Occup Environ Med*. 2001;1:511–523.

Costa LG. Current issues in organophosphate toxicology. *Clin Chim Acta*. 2006;366:1–13.

Costa LG. The neurotoxicity of organochlorine and pyrethroid pesticides. In: Lotti M, Bleecker MT, eds. *Occupational Neurology*. Vol 131. Handbook of Clinical Neurology Series. Amsterdam: Elsevier; 2015:135–148.

Costa LG, Cole TB, Jarvik GP, et al. Functional genomics of the paraoxonase (PON1) polymorphisms: effect on pesticide sensitivity, cardiovascular disease and drug metabolism. *Annu Rev Med*. 2003;54:371–392.

Costa LG, Furlong CE. Paraoxonase 1: structure, function and polymorphisms. In: Satoh T, Gupta RC, eds. *Anticholinesterase Pesticides: Metabolism, Neurotoxicity, and Epidemiology*. Hoboken, NJ: Wiley; 2010:85–95.

Costa LG, Galli CL, Murphy SD, eds. *Toxicology of Pesticides: Experimental, Clinical and Regulatory Perspectives*. Heidelberg, Germany: Springer-Verlag; 1987:320.

Costa LG, Giordano G, Cole TB, et al. Paraoxonase (PON1) as a genetic determinant of susceptibility to organophosphate toxicity. *Toxicology*. 2013;307:115–122.

Costa LG, Hand H, Schwab BW, et al. Tolerance to the carbamate insecticide propoxur. *Toxicology*. 1981;21:267–278.

Costa LG, Murphy SD. Interaction of the pesticide chlordimeform with adrenergic receptors in mouse brain: an in vitro study. *Arch Toxicol*. 1987;59:323–327.

Costa LG, Olibet G, Murphy SD. Alpha$_2$-adrenoceptors as a target for formamidine pesticides: in vitro and in vivo studies in mice. *Toxicol Appl Pharmacol*. 1988;93:319–328.

Costa LG, Schwab BW, Murphy SD. Tolerance to anticholinesterase compounds in mammals. *Toxicology*. 1982;25:79–97.

Costello S, Cockburn M, Bronstein J, et al. Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the Central Valley in California. *Am J Epidemiol*. 2009;169:919–926.

Cranmer MF. Carbaryl: a toxicological review and risk analysis. *Neurotoxicology*. 1986;7:247–332.

Crisp TM, Clegg ED, Cooper RL, et al. Environmental endocrine disruption: an effects assessment and analysis. *Environ Health Perspect*. 1998;106(suppl 1):11–56.

Dafforn KA, Lewis JA, Johnston EL. Antifouling strategies: history and regulation, ecological impact and mitigation. *Marine Poll Bull*. 2011;62:453–465.

Dam K, Seidler FJ, Slotkin TA. Developmental neurotoxicity of chlorpyrifos: delayed targeting of DNA synthesis after repeated administration. *Dev Brain Res*. 1998;108:39–45.

Daniell WE, Barnhart S, Demers P, et al. Neuropsychological performance among pesticide applicators. *Environ Res*. 1992;59:217–228.

Dawson AH, Eddleston M, Senarathna L, et al. Acute human lethal toxicity of agricultural pesticides: a prospective cohort study. *PLoS Med*. 2010;7(10):e1000357.

Dayal M, Parmar, Dhawan A, et al. Effect of pretreatment of cytochrome P450 (P450) modifiers on neurobehavioral toxicity induced by deltamethrin. *Food Chem Toxicol*. 2003;41:431–437.

De Haro L, Gastaut JL, Jouglard J, et al. Central and peripheral neurotoxic effects of chronic methyl bromide intoxication. *J Toxicol Clin Toxicol*. 1997;35:29–34.

de Souza A, Narvencar KP, Sindhoora KV. The neurological effects of methyl bromide intoxication. *J Neurol Sci*. 2013;15:36–41.

De Wilde AR, Heyndrickx A, Carton D. A case of fatal rotenone poisoning in a child. *J Forensic Sci*. 1986;31:1492–1498.

deBleecker JL. Intermediate syndrome in organophosphate poisoning. In: Gupta RC, ed. *Toxicology of Organophosphate and Carbamate Compounds*. Amsterdam: Elsevier; 2006:371–380.

Defarge N, Takacs E, Lozano VL, et al. Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. *Int J Environ Res Publ Health*. 2016;13:264.

Degli Esposti M. Inhibitors of NADH-ubiquinone reductase: an overview. *Biochim Biophys Acta*. 1998;1364:222–235.

Deguigne MB, Lagarce L, Boels D, et al. Metam sodium intoxication: the specific role of degradation products—methylisocyanate and carbon disulfide—as a function of exposure. *Clin Toxicol (Phila)*. 2011;49:416–422.

del Pino J, Moyano-Cires PV, Anadon MJ, et al. Molecular mechanisms of amitraz mammalian toxicity: comprehensive review of existing data. *Chem Res Toxicol*. 2015;28:1073–1094.

Desaiah D. Biochemical mechanisms of chlordecone neurotoxicity: a review. *Neurotoxicology*. 1982;3:103–110.

Detweiler MB. Organophosphate intermediate syndrome with neurological complications of extrapyramidal symptoms in clinical practice. *J Neurosci Rural Pract*. 2014;5:298–301.

Dickoff DJ, Gerber O, Turovsky Z. Delayed neurotoxicity after ingestion of carbamate pesticide. *Neurology*. 1987;37:1229–1231.

Didier A, Loor F. The abamectin derivative invermectin is a potent P-glycoprotein inhibitor. *Anticancer Drugs*. 1996;7:745–757.

Dikshith TSS, ed. *Toxicology of Pesticide in Animals*. Boca Raton, FL: CRC Press; 1991:255.

Dinis-Oliveira RJ, Duarte JA, Sanchez-Navarro A, et al. Paraquat poisoning: mechanisms of lung toxicity, clinical features, and treatment. *Crit Rev Toxicol*. 2008;38:13–71.

Doe JE, Boobis AR, Blacker A, et al. A tiered approach to systemic toxicity testing for agricultural risk assessment. *Crit Rev Toxicol*. 2006;36:37–68.

Dourson ML, Kahrman-Vincent MJ, Allen BC. Dose response assessment for effects of acute exposure to methyl isocyanate (MITC). *Reg Toxicol Pharmacol*. 2010;58:181–188.

Duke SO. Overview of herbicide mechanisms of action. *Environ Health Perspect*. 1990;87:263–271.

Duke SO. Taking stock of herbicide-resistant crops ten years after introduction. *Pest Manag Sci*. 2005;61:211–218.

Eaton DL, Daroff RB, Autrup H, et al. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit Rev Toxicol*. 2008;38(suppl 2):1–125.

Ebert E, Leist KH, Mayer D. Summary of safety evaluation of toxicity studies of glufosinate ammonium. *Food Chem Toxicol*. 1990;28:339–349.

Ecobichon DJ. Introduction. In Ecobichon DJ, Roy RM, eds. *Pesticides and Neurological Diseases*. Boca Raton, FL: CRC Press; 1982:1–14.

Ecobichon DJ. Toxic effect of pesticides. In: Klaassen CD, ed. *Casarett and Doull's Toxicology. The Basic Science of Poisons*. New York: McGraw-Hill; 2001a:763–810.

Ecobichon DJ. Carbamate insecticides. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001b:1087–1106.

Ecobichon DJ, Joy RM. *Pesticides and Neurological Diseases*. Boca Raton, FL: CRC Press; 1982:281.

Eddleston M, Chowdhury FR. Pharmacological treatment of organophosphorus insecticide poisoning: the old and the (possible) new. *Br J Clin Pharmacol*. 2015;81:462–470.

Eddleston M, Szinicz L, Eyer P, et al. Oximes in acute organophosphorus pesticide poisoning: a systematic review of clinical trials. *QJ Med*. 2002;95:275–283.

Edwards IR, Ferry DG, Temple WA. Fungicides and related compounds. In: Hayes WJ, Laws ER, eds. *Handbook of Pesticide Toxicology*. Vol 3. San Diego, CA: Academic Press; 1991:1409–1470.

EFSA (European Food Safety Authority). Opinion of the Scientific Panel on Plant Health, Plant Protection Product and their Residues on a request from the Commission related to the evaluation of alachlor in the context of Council Directive 91/414/EEC. *EFSA J*. 2004;111:1–34.

EFSA (European Food Safety Authority). Conclusion regarding the peer-review of the pesticide risk assessment of the active substance glufosinate. *EFSA Scientific Report*. 2005;27:1–81.

EFSA (European Food Safety Authority). Conclusion regarding the peer review of the pesticide risk assessment of the active substance prothioconazole. *EFSA J*. 2007;106;1–98.

EFSA (European Food Safety Authority). Scientific opinion of the Panel on Plant Protection Products and their Residues (PPR) on a request from the European Commission on potential developmental neurotoxicity of deltamethrin. *EFSA J*. 2009;921:1–34.

EFSA (European Food Safety Authority). Conclusion of the peer review of the pesticide risk assessment of the active substance metam. *EFSA J*. 2011;9(9):2334.

EFSA (European Food Safety Authority). Scientific opinion on the developmental neurotoxicity potential of acetamiprid and imidacloprid. *EFSA J*. 2013;11:3471 (47 pp).

EFSA (European Food Safety Authority). Conclusion on the peer review of the pesticide risk assessment of the cative ingredient substance glyphosate. *EFSA J*. 2015;13:4302.

Ehrich M, Correll L, Veronesi B. Acetylcholinesterase and neuropathy target esterase inhibitions in neuroblastoma cells to distinguish organophosphorus compounds causing acute and delayed neurotoxicity. *Fundam Appl Toxicol*. 1997;38:55–63.

Ehrich M, Jortner BS. Organophosphorus-induced delayed neuropathy. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1479–1504.

Eisenbrandt DL, Hotchkiss JA. Sulfuryl fluoride. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2245–2258.

Elbaz A, Clavel J, Rathouz PJ, et al. Professional exposure to pesticides and Parkinson's disease. *Ann Neurol.* 2009;66:494–504.

Eldefrawi AT, Eldefrawi ME. Receptors for γ-aminobutyric acid and voltage-dependent chloride channels as targets for drugs and toxicants. *FASEB J*. 1987;1:262–271.

Eldefrawi ME, Eldefrawi AT. Comparative molecular and pharmacological properties of cholinergic receptors in insects and mammals. In: Hedin PA, Hollingworth RM, Masler EP, Miyamoto J, Thompson DG, eds. *Phytochemicals in Pest Control*. Washington, DC: American Chemical Society; 1997:327–338.

Eldridge JC, Stevens JT, Wetzel LT, et al. Atrazine: mechanisms of hormonal imbalance in female SD rats. *Fundam Appl Toxicol*. 1996;42:2–5.

Elinav E, Shapira Y, Ofran Y, et al. Near-fatal amitraz intoxication: the overlooked pesticide. *Basic Clin Pharmacol Toxicol*. 2005;97:185–187.

Enan E, Matsumura F. Activation of phosphoinositide protein kinase C pathway in rat brain tissue by pyrethroids. *Biochem Pharmacol*. 1993;45:703–710.

Eskenazi B, Bradman A, Castorina R. Exposures to children to organophosphate pesticides and their potential health effects. *Environ Health Perspect*. 1999;107(suppl 3):409–419.

Eskenazi B, Chevrier J, Rosas LG, et al. The Pine River Statement: human health consequences of DDT use. *Environ Health Perspect*. 2009;117:1359–1367.

Fahim MA, Shehab S, Nemmar A, et al. Daily subacute paraquat exposure decreases muscle function and substantia nigra dopamine levels. *Physiol Res*. 2013;62:313–321.

Farmer D. Inhibitors of aromatic acid biosynthesis. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1967–1972.

Feinstein DL, Akpa BS, Ayee MA, et al. The emerging threat of superwarfarins: history, detection, mechanisms and countermeasures. *Ann NY Acad Sci*. 2016;1374:111–122.

Feng P, Wilson AGE, McClanahan R et al. Metabolism of alachlor by rat and mouse liver and nasal turbinate tissues. *Drug Metab Dispos*. 1990;18:373–377.

Fenner-Crisp PA. Risk assessment and risk management: the regulatory process. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:681–689.

Fenske RA, Wong SM, Leffingwell JT, et al. A video imaging technique for assessing dermal exposure. II. Fluorescent tracer testing. *Am Ind Hyg Assoc*. 1986;47:771–775.

Fernandez-Cornejo J, Nehring R, Osteen C, et al. Pesticide use in U.S. agriculture: 21 selected crops, 1960–2008. *USDA Econ Informat Bull*. 2014;124:80.

Ferraz HB, Bertolucci PH, Pereira JS, et al. Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. *Neurology*. 1988;38:550–553.

Fisher MH, Mrozik H. The chemistry and pharmacology of avermectins. *Annu Rev Pharmacol Toxicol*. 1992;32:537–553.

Fitzmaurice AG, Rhodes SL, Lulla A, et al. Aldehyde dehydrogenase inhibition as a pathogenetic mechanism in Parkinson's disease. *Proc Natl Acad Sci U S A*. 2013;110:636–641.

Flannigan SA, Tucker SB, Key MM, et al. Synthetic pyrethroid insecticides: a dermatological evaluation. *Br J Ind Med*. 1985;42:363–372.

Fontoura-da-Silva SE, Chautard-Friere-Maira EA. Butyrylcholinesterase variants (BChE and ChE1 loci) associated with erythrocyte acetylcholinesterase inhibition in farmers exposed to pesticides. *Hum Hered*. 1996;46:142–147.

Ford MG, Lunt GG, Reay RC et al. eds. *Neuropharmacology and Pesticide Action*. Chichester, England: Ellis Norwood; 1986:512.

Forns J, Mandal S, Iszatt N, et al. Novel application of statistical methods for analysis of multiple toxicants identifies DDT as a risk factor for early child behavioral problems. *Environ Res*. 2016;151:91–100.

Forshaw PJ, Lister T, Ray DE. Inhibition of a neuronal voltage-dependent chloride channel by the type II pyrethroid, deltamethrin. *Neuropharmacology*. 1993;32:105–111.

Forshaw PJ, Lister T, Ray DE. The role of voltage-gated chloride channels in Type II pyrethroid insecticide poisoning. *Toxicol Appl Pharmacol*. 2000;163:1–8.

FQPA (Food Quality Protection Act). Public Law. 1996;104–170.

Fradin MS, Day JF. Comparative efficacy of insect repellents against mosquito bites. *New Engl J Med*. 2002;347:13–18.

Fukuto TR. Metabolism of carbamate insecticides. *Drug Metab Rev*. 1972;1:117–152.

Furlong CE, Holland N, Richter RJ, et al. PON1 status of farm worker mother and children as a predictor of organophosphate sensitivity. *Pharmacogenet Genom*. 2006;16:183–190.

Gallo MA, Lawryk NJ. Organic phosphorus pesticides. In: Hayes WJ, Laws ER, eds. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 1991:917–1123.

Gammon DW, Aldous CN, Carr WC, et al. A risk assessment of atrazine use in California: human health and ecological aspects. *Pest Manag Sci*. 2005;61:331–355.

Gammon DW, Moore TB, O'Malley MA. A toxicological assessment of sulfur as a pesticide. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1889–1901.

Garabrant DH, Philbert MA. Review of 2,4-dichlorophenoxyacetic acid (2,4-D) epidemiology and toxicology. *Crit Rev Toxicol*. 2002;34:233–257.

Garcia SJ, Seidler FJ, Slotkin TA. Developmental neurotoxicity of chlorpyrifos: targeting glial cells. *Environ Toxicol Pharmacol*. 2005;19:455–461.

Ghali TG, Hollingworth RM. Influence of mixed function oxygenase metabolism on the acute neurotoxicity of the pesticide chlordimeform in mice. *Neurotoxicology*. 1985;6:215–238.

Gianessi LP. The increasing importance of herbicides in worldwide crop production. *Pest Manag Sci*. 2013;69:1099–1105.

Gianessi LP, Reigner NP. The value of herbicides in U.S. crop production. *Weed Technol*. 2007;21:559–566.

Gianessi LP, Reigner NP. The importance of fungicides in U.S. crop production. *Outlook Pest Manag*. 2006;17:2019–213.

Giannitti F, Anderson M, Caspe SG. An outbreak of sodium fluoroacetate (1080) intoxication in selenium- and copper-deficient sheep in California. *Vet Pathol*. 2013;50:1022–1027.

Glare TR, Gwynn RL, Moran-Diez M. Development of biopesticides and future opportunities. *Meth Mol Biol*. 2016;1477:211–221.

Glynn P. A mechanism for organophosphate-induced delayed neuropathy. *Toxicol Lett*. 2006;162:94–97.

Goes AE, Savage EP, Gibbons G, et al. Suspected foodborn carbamate pesticide intoxications with the ingestion of hydroponic cucumbers. *Am J Epidemiol*. 1980;111:254–259.

Golden RJ, Noller KL, Titus-Enrstoff L, et al. Environmental endocrine modulators and human health: an assessment of biological evidence. *Crit Rev Toxicol*. 1998;28:109–227.

Goldman LR, Smith DF, Neutra RR, et al. Pesticide food poisoning from contaminated watermelons in California, 1985. *Arch Environ Health*. 1990;45:229–236.

Goldstein DS, Sullivan P, Cooney A. Rotenone decreases intracellular aldehyde dehydrogenase activity: implications for the pathogenesis of Parkinson's disease. *J Neurochem*. 2015;133:14–25.

Golub M, Doherty J. Triphenyltin as a potential human endocrine disruptor. *J Toxicol Environ Health B Crit Rev*. 2004;7:281–295.

Goodal R. Cholinesterase pharmacogenetics. In: Gupta RC, ed. *Toxicology of Organophosphate and Carbamate Compounds*. Amsterdam: Elsevier; 2006:187–198.

Goodman M, Mandel JS, DeSesso JM, et al. Atrazine and pregnancy outcomes: a systematic review of epidemiological evidence. *Birth Def Res B Dev Reprod Toxicol*. 2014;101:215–236.

Gordon EB. Captan: transition from "B2" to "not likely". How pesticide registrants affected the EPA cancer classification update. *J Appl Toxicol*. 2007;27:519–526.

Gordon EB. Captan and folpet. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1915–1949.

Greenburg DL, Rusiecki J, Koutsos S, et al. Cancer incidence among pesticide applicators exposed to captan in the Agricultural Health Study. *Cancer Causes Control*. 2008;19:1401–1407.

Gringorten JL. Ion balance in the Lepidopteran midgut and insecticidal action of *Bacillus thuringiensis*. In Isheaya I, ed. *Biochemical Sites of Insecticide Action and Resistance*. Heidelberg, Germany: Springer; 2001:167–207.

Grube A, Donaldson D, Kiely T, et al. *Pesticide Industry Sales and Usage: 2006 and 2007 Market Estimates*. Washington, DC: U.S. Environmental Protection Agency; 2011:33.

Gryboski J, Weinstein D, Ordway NK. Toxic encephalopathy apparently related to the use of an insect repellent. *New Engl J Med*. 1961;264:289–290.

Gullino ML, Clini C, Garibaldi A. Life without methyl bromide: the Italian experience in replacing the fumigant. *Commun Agric Appl Biol Sci*. 2005;70:13–25.

Gunnell D, Eddleston M. Suicide by intentional ingestion of pesticides: a continuing tragedy in developing countries. *Int J Epidemiol*. 2003;32:902–909.

Gunnell D, Eddleston M, Phillips Mr, et al. The global distribution of fatal pesticide self-poisoning: systematic review. *BMC Publ Health*. 2007;7:357.

Gupta RC, ed. *Toxicology of Organophosphate and Carbamate Compounds*. Amsterdam: Elsevier; 2006:763.

Guyton KZ, Loomis D, Grosse Y, et al. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. *Lancet Oncol*. 2015;16:490–491.

Guzelian PS. Comparative toxicology of chlordecone (Kepone) in humans and experimental animals. *Annu Rev Pharmacol Toxicol*. 1982;22:89–113.

Hainzl D, Cole LM, Casida JE. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem Res Toxicol*. 1998;11:1529–1535.

Hajra A, Banyopadhyay D, Heise LR, et al. Zika and pregnancy: a comprehensive review. *Am J Reprod Immunol*. 2017;77:e12607.

Harada S, Hiromori Y, Nakamura S, et al. Structural basis for PPARγ transactivation by endocrine-disrupting organotin compounds. *Sci Rep*. 2016b;5:8520.

Harada T, Takeda M, Kojima S, et al. Toxicity and carcinogenicity of dichlorodiphenyltrichloroethane (DDT). *Tox Res*. 2016a;32:21–33.

Hardell L, Eriksson M, Degerman A. Exposure to phenoxyacetic acids, chlorophenols or organic solvents in relation to histopathology, stage, and anatomical localization of non-Hodgkin's lymphoma. *Cancer Res*. 1994;54:2386–2389.

Harrison ML, Loucks OL, Mitchell JW, et al. Systems studies of DDT transport. *Science*. 1970;170:503–508.

Hayes TB, Anderson LL, Beasley VR, et al. Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. *J Steroid Biochem Mol Biol*. 2011;127:64–73.

Hayes TB, Collins A, Lee M, et al. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc Natl Acad Sci U S A*. 2002;99:5476–5480.

Hayes WJ. *Pesticides Studied in Man*. Baltimore, MD: Williams & Wilkins; 1982:672.

Hayes WJ, Laws ER, eds. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 1991;1576.

He F, Wang S, Liu L, et al. Clinical manifestations and diagnosis of acute pyrethroid poisoning. *Arch Toxicol*. 1989;63:54–58.

Heikkila RE, Nicklas WJ, Vyas I, et al. Dopaminergix toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration to rats: implication for the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. *Neurosci Lett*. 1985;62:389–394.

Hernandez AF, Gonzales-Alzaga B, Lopez-Flores I, et al. Systematic reviews on neurodevelopmental and neurodegenerative disorders linked to pesticide exposure: methodological features and impact on risk assessment. *Environ Int*. 2016;92–93:657–679.

Hernandez AF, Martin-Rubi JC, Ballesteros JL, et al. Clinical and pathological findings in fatal 1,3-dichloropropene intoxication. *Hum Exp Toxicol*. 1994;13:303–306.

Herzstein J, Cullen MR. Methyl bromide intoxication in four field workers during removal of soil fumigation sheets. *Am J Ind Med*. 1990;17:321–326.

Heudorf U, Angerer J, Drexler H. Current internal exposure to pesticides in children and adolescents in Germany: urinary levels of metabolites of pyrethroid and organophosphorus insecticides. *Int Arch Occup Environ Health*. 2004;77:67–72.

Heydens WF. Summary of toxicology studies with alachlor. *J Pest Sci*. 1998;24:75–82.

Heydens WF, Lamb IC, Wilson AGE. Chloroacetanilides. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1753–1769.

Heydens WF, Wilson AGE, Kier LD, et al. An evaluation of the carcinogenic potential of the herbicide alachlor to man. *Human Exp Toxicol*. 1999;18:363–391.

Hickey JP, Batterman SA, Chernyak SM. Trends of chlorinated organic contaminants in Great Lakes trout and walleye from 1970 to 1998. *Arch Environ Contam Toxicol*. 2006;50:97–110.

Ho YS, Magnenat JL, Gargano M, et al. The nature of antioxidant defense mechanisms: a lesson from transgenic mice. *Environ Health Perspect*. 1998;106:1219–1228.

Hoar SK, Blair A, Holmes FF, et al. Agricultural herbicide use and risk of lymphoma and soft-tissue sarcoma. *JAMA*. 1986;256:1141–1147.

Hodgson E. In vitro human phase I metabolism of xenobiotics I: pesticides and related compounds used in agriculture and public health, May 2003. *J Biochem Mol Toxicol*. 2003;17:201–206.

Hoffman JN, Keifer MC, Furlong CE, et al. Serum cholinesterase inhibition in relation to paraoxonase 1 (PON1) status among organophosphate-exposed agricultural pesticide handlers. *Environ Health Perspect*. 2009;117:1402–1408.

Hollingworth RM. Chemistry, biological activity and uses of formamidine pesticides. *Environ Health Perspect*. 1976;14:57–69.

Hollingworth RM. Inhibitors and uncouplers of mitochondrial oxidative phosphorylation. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:1169–1261.

Hsu WH, Kakuk TJ. Effects of amitraz and chlordimeform on heart rate and pupil diameter in rats: mediated by alpha$_2$-adrenoceptors. *Toxicol Appl Pharmacol*. 1984;73:411–415.

Hsu WH, Lu ZH. Amitraz-induced delay of gastrointestinal transit in mice: mediated by alpha$_2$-adrenergic receptors. *Drug Develop Res*. 1984;4:655–660.

Hudson NL, Kasner EJ, Beckman J, et al. Characteristics and magnitude of acute pesticide-related illness and injuries associated with pyrethrin and pyrethroid exposures—11 states, 2000–2008. *Am J Ind Med*. 2014;57:15–30.

Hufnagel RB, Arno G, Hein ND, et al. Neuropathy target esterase impairments cause Oliver–McFarlane and Laurence–Moon syndromes *J Med Genet*. 2015;52:85–94.

Huntington S, Fenik Y, Vohra R, et al. Human bromethalin exposures reported to a U.S. statewide poison control system. *Clin Toxicol (Phila)*. 2016;54:277–281.

Hurt S, Ollinger J, Arce G, et al. Dialkyldithiocarbamates (EBDCs). In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1689–1710.

IARC (International Agency for Research on Cancer). Atrazine. *IARC Monogr Eval Carcinog Risks Hum*. 1999;73:59–113.

IOM (Institute of Medicine). *Gulf War and Health.* Vol. 1. Washington, DC: National Academy Press; 2000:408.

IOM (Institute of Medicine). *Veterans and Agent Orange: Update 2014*. Washington, DC: National Academy Press; 2016:1116.

IPCS (International Programme on Chemical Safety). *Chlordimeform. Environmental Health Criteria 1999.* Geneva: World Health Organization; 1998:159.

IPCS (International Programme on Chemical Safety). *The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification: 2009*. Geneva: World Health Organization; 2010:78.

Ireland B, Acquarella J, Farrell T, et al. Evaluation of ocular health among alachlor manufacturing workers. *J Occup Med*. 1994;36:738–742.

Isman MB. Botanical insecticides, deterrents and repellents in modern agriculture and an increasingly regulated world. *Annu Rev Entomol*. 2006;51:45–66.

Ito T. Mazzotti reaction with eosinophilia after undergoing oral ivermectin for scabies. *J Dermatol*. 2013;40:776–777.

Iwasa T, Motoyama N, Ambrose JT, et al. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee *Apis mellifera*. *Crop Protect*. 2004;23;371–378.

Jacobsen NE, Sanders M, Toia RP, et al. Alachlor and its analogues as metabolic progenitors of formaldehyde: fate of *N*-methoxymethyl and other *N*-alkoxyalkyl substituents. *J Agr Food Chem*. 1991;39:1342–1350.

Jamal GA, Julu POO. Low level exposure to organophosphate esters may cause neurotoxicity. *Toxicology*. 2002;181:22–33.

Jett DA, Navoa RV, Beckles RA, et al. Cognitive function and cholinergic neurochemistry in weanling rats exposed to chlorpyrifos. *Toxicol Appl Pharmacol*. 2001;174:89–98.

Jiang X, Huang LF, Zhang SH, et al. Sulfur fumigation, a better or worse choice in preservation of traditional Chinese medicine? *Phytomedicine*. 2013;20:97–105.

Johnson DJ, Graham DG, Amarnath V, et al. Release of carbon disulfide is a contributing mechanism in the axonopathy produced by *N*,*N*-diethyldithiocarbamate. *Toxicol Appl Pharmacol*. 1998;148:288–296.

Johnson MK. The target for initiation of delayed neurotoxicity by organophosphorus esters: biochemical studies and toxicological applications. *Rev Biochem Toxicol*. 1982;4:141–212.

Johnson MK, Glynn P. Neuropathy target esterase. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:953–965.

Johnson MK, Jacobsen D, Meredith TJ, et al. Evaluation of antidotes for poisoning by organophosphorus pesticides. *Emerg Med*. 2000;12:22–37.

Johnson MK, Lauwerys R. Protection by some carbamates against the delayed neurotoxic effects of diisopropyl phosphorofluoridate. *Nature*. 1969;222:1066–1067.

Johnson MK, Read DJ. The influence of chirality on the delayed neuropathic potential of some organophosphorus esters: neuropathic and prophylactic effects of streoisomers of ethyl phenylphosphonic acid (EPN oxon and EPN) correlate with quantities of aged and unaged neuropathy target esterase in vivo. *Toxicol Appl Pharmacol*. 1987;90:103–115.

Kaneko H. Pyrethroid chemistry and metabolism. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1635–1663.

Kanthasamy AG, Kitazawa M, Kanthasamy A, et al. Dieldrin-induced neurotoxicity: relevance to Parkinson's disease pathogenesis. *Neurotoxicology*. 2005;26:701–719.

Kappers WA, Edwards RJ, Murray S, et al. Diazinon is activated by CYP2C19 in human liver. *Toxicol Appl Pharmacol*. 2001;177:68–76.

Katz TM, Miller JH, Hebert AA. Insect repellents: historical perspectives and new developments. *J Am Acad Dermatol*. 2008;58:865–871.

Kavlock RJ. Pesticides as endocrine-disrupting chemicals. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:727–746.

Keigwin RP. Maneb: cancellation order for a certain pesticide registration. *Fed Reg*. 2010;75(73):19967–19968.

Keiner C. Wartime rat control, rodent ecology, and the rise and fall of chemical rodenticides. *Endeavour*. 2005;29:119–125.

Kelce WR, Stone CR, Laws SC, et al. Persistent DDT metabolite *p,p′*-DDE is a potent androgen receptor antagonist. *Nature*. 1995;375:581–585.

Kennepohl E, Munro IC, Bus JS. Phenoxy herbicides (2,4-D). In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1829–1847.

Kim DJ, Seok SH, Baek MW, et al. Benomyl induction of brain aromatase and toxic effects in the zebrafish embryo. *J Appl Toxicol*. 2009;29:289–294.

Kim IY, Shin JH, Kim HS, et al. Assessing estrogenic activity of pyrethroid insecticides using in vitro combination assays. *J Reprod Develop*. 2004;50:245–255.

King N, Tran MH. Long-acting anticoagulant rodenticide (superwarfarin) poisoning: a review of its historical development, epidemiology and clinical management. *Transf Med Rev*. 2015;29:250–258.

Klaunig JE, Gehen SC, Wang Z, et al. Mechanism of 1,3-dichloropropene-induced rat liver carcinogenesis. *Toxicol Sci*. 2015;143:6–15.

Kloas W, Lutz I, Urbatzka R, et al. Does atrazine affect larval development and sexual differentiation of South African clawed frogs? *Ann NY Acad Sci*. 2009;1163:437–440.

Konjuh C, Garcia G, Lopez L, et al. Neonatal hypomyelination by the herbicide 2,4-dichlorophenoxyacetic acid. Chemical and ultrastructural studies in rats. *Toxicol Sci*. 2008;104:332–340.

Koren G, Matsui D, Bailey B. DEET-based insect repellents: safety implications for children and pregnant and lactating women. *Can Med Ass J*. 2003;169:209–212.

Kotake Y. Molecular mechanisms of environmental organotin toxicity in mammals. *Biol Pharm Bull*. 2012;35:1876–1880.

Koyama K, Andou Y, Saruki K, et al. Delayed and severe toxicity of an herbicide containing glufosinate and a surfactant. *Vet Hum Toxicol*. 1994;36:17–18.

Kretzschmar D, Mason G, Sharma S, et al. The Swiss cheese mutant causes glial hyperwrapping and brain degeneration in *Drosophila*. *J Neurosci*. 1997;17:7425–7432.

Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:1908.

Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2342.

Lamberth C, Jeanmart S, Luksch T, et al. Current challenges and trends in the discovery of agrochemicals. *Science*. 2013;341:742–746.

Landrigan PJ, Powell KE, James LE, et al. Paraquat and marijuana-epidemiologic risk assessment. *Am J Public Health*. 1983;73:784–788.

Lapointe N, St-Hilaire M, Martinoli MG, et al. Rotenone induces non-specific central nervous system and systemic toxicity. *FASEB J*. 2004;18:717–719.

Laugeray A, Herzine A, Perche O, et al. Pre- and postnatal exposure to low dose glufosinate ammonium induces autism-like phenotypes in mice. *Front Behav Neurosci*. 2014;8(art. 390):14.

Laurino D, Porporato M, Paletta A, et al. Toxicity of neonicotinoid insecticides to honey bees: laboratory tests. *Bull Insectol*. 2011;64:107–113.

Lawrence LJ, Casida JE. Stereospecific action of pyrethroid insecticides on the γ-aminobutyric acid receptor–ionophore complex. *Science*. 1983;221:1399–1401.

Lee HL, Chen KW, Chi CH, et al. Clinical presentations and prognostic factors of a glyphosate-surfactant herbicide intoxication: a review of 131 cases. *Acad Emerg Med*. 2000;7:906–910.

Lee K, Park EK, Stoecklin-Marois M, et al. Occupational paraquat exposure of agricultural workers in large Costa Rican farms. *Int Arch Occup Environ Health*. 2009b;82:455–462.

Lee SJ, Mulay P, Diebolt-Brown B, et al. Acute illnesses associated with exposure to fipronil-surveillance data from 11 states in the United States, 2001–2007. *Clin Toxicol (Phila)*. 2010;48:737–744.

Lee JW, Hwang IW, Kim JW, et al. Common pesticides used in suicide attempts following the 2012 paraquat ban in Korea. *J Korean Med Sci*. 2015;30:1517–1521.

Lee WJ, Cha ES, Park ES, et al. Deaths from pesticide poisoning in South Korea: trends over 10 years. *Int Arch Occup Environ Health*. 2009a;82:365–371.

Leet T, Acquavella J, Lynch C et al. Cancer incidence among alachlor manufacturing workers. *Am J Ind Med*. 1996;30:300–306.

Li AA, Mink PJ, McIntosh LJ, et al. Evaluation of epidemiologic and animal data associating pesticides with Parkinson's disease. *J Occup Env Med*. 2005;47:1059–1087.

Li S, Huang W, Duan Y, et al. Human fatality due to thallium poisoning: autopsy, microscopy, and mass spectrometry assays. *J Forens Sci*. 2015;60:247–259.

Li WF, Costa LG, Richter RJ, et al. Catalytic efficiency determines the in vivo efficacy of PON1 for detoxifying organophosphates. *Pharmacogenetics*. 2000;10:767–779.

Lin JN, Lin CL, Lin MC, et al. Increased risk of dementia in patients with acute organophosphate and carbamate poisoning. *Medicine*. 2015;94:1–8.

Lock EA, Wilks MF. Diquat. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:1605–1621.

Lock EA, Wilks MF. Paraquat. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1771–1827.

Lockridge O, Duysen EG, Masson P. Butyrylcholinesterase: overview, structure and function. In: Satoh T, Gupta RC, eds. *Anticholinesterase Pesticides: Metabolism, Neurotoxicity and Epidemiology*. Hoboken, NJ: Wiley; 2010:25–41.

Lockridge O, Masson P. Pesticides and susceptible populations: people with butyrylcholinesterase genetic variants may be at risk. *Neurotoxicology*. 2000;21:113–126.

Lockridge O, Norgren RB, Johnson RC, et al. Naturally occurring genetic variants of human acetylcholinesterase and butyrylcholinesterase and their potential impact on the risk of toxicity from cholinesterase inhibitors. *Chem Res Toxicol*. 2016;29:1381–1392.

Lomax LG, Stott WT, Johnson KA, et al. The chronic toxicity and oncogenicity of inhaled technical grade 1, 3-dichloropropene in rats and mice. *Fundam Appl Toxicol*. 1989;12:418–431.

Longnecker MP. Invited commentary: why DDT matters now. *Am J Epidemiol*. 2005;162:726–728.

Loomis D, Guyton K, Grosse Y, et al. Carcinogenicity of lindane, DDT and 2,4-dichlorophenoxyacetic acid. *Lancet Oncol*. 2015;16:891–892.

Lotti M. The pathogenesis of organophosphate neuropathy. *Crit Rev Toxicol*. 1992;21:465–487.

Lotti M. Cholinesterase inhibition: complexities in interpretation. *Clin Chem*. 1995;41:1814–1818.

Lotti M. Organophosphorus compounds. In: Spencer PS, Schaumburg HH, Ludolph AC, eds. *Experimental and Clinical Neurotoxicology*. Oxford, UK: Oxford University Press; 2000:898–925.

Lotti M. Promotion of organophosphate-induced delayed polyneuropathy by certain esterase inhibitors. *Toxicology*. 2002a;181–182:245–248.

Lotti M. Low level exposures to organophosphorus esters and peripheral nerve functions. *Muscle Nerve*. 2002b;25:492–504.

Lotti M. Clinical toxicology of anticholinesterases in humans. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1543–1589.

Lotti M, Caroldi S, Capodicasa E, et al. Promotion of organophosphate-induced delayed polyneuropathy by phenylmethanesulfonyl fluoride. *Toxicol Appl Pharmacol*. 1991;108:234–241.

Lotti M, Moretto A. Organophosphate-induced delayed polyneuropathy. *Toxicol Rev*. 2005;24:37–49.

Lu C, Bravo R, Caltabiano LM, et al. The presence of dialkylphosphates in fresh fruit juices: implications for organophosphorus pesticide exposure and risk assessments. *J Toxicol Environ Health Part A*. 2005;68:209–227.

Lynagh T, Lynch JW. Molecular mechanisms of Cys-loop ion channel receptor modulation by ivermectin. *Front Mol Neurosci*. 2012;5:60.

Lyubimov AV, Garry VF. Phosphine. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2259–2266.

Macdonald N, Gledhill A. Potential impact of ABCB1 (P-glycoprotein) polymorphisms on avermectin toxicity in humans. *Arch Toxicol*. 2007;81:553–563.

Maharaj R, Mthembu DJ, Sharp BL. Impact of DDT re-introduction on malaria transmission in KwaZulu-Natal. *S Afr Med J*. 2005;95:871–874.

Majerus PW, Tollefsen DM. Blood coagulation and anticoagulants, thrombolytic, and antiplatelet drugs. In: Brunton LL, ed. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. New York: McGraw-Hill; 2006:1467–1488.

Manaca MN, Grimalt JO, Sunjer J, et al. Concentration of DDT compounds in breast milk from African women (Manhiça, Mozambique) at the early stages of indoor spraying with this insecticide. *Chemosphere*. 2011;85:307–314.

Maroni M, Colosio C, Ferioli A, et al. Biological monitoring of pesticide exposure: a review. *Toxicology*. 2000;143:5–118.

Marsh RE. Vertebrate pest control chemicals and their use in urban and rural environments. In: Keirger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:251–262.

Matsuda K, Buckingam SD, Kleier D, et al. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci*. 2001;22:573–580.

Matsumura F. *Toxicology of Insecticides*. New York: Plenum Press; 1985:598.

Matsumura F, Ghiasuddin SM. Characteristics of DDT-sensitive Ca-ATPase in the axonic membrane. In: Narahashi T. ed. *Neurotoxicology of Insecticides and Pheromones*. New York: Plenum Press; 1979:245–257.

Matsumura F, Patil KC. Adenosin triphosphatase sensitive to DDT in synapses of rat brain. *Science*. 1969;166:121–122.

Mattsson JL, Charles JM, Yano BL, et al. Single-dose and chronic dietary neurotoxicity screening studies on 2,4-dichlorophenoxyacetic acid in rats. *Fundam Appl Toxicol*. 1997;40:111–119.

Matyunas NG, Rodgers GC: Nicotine poisoning. In: Ford MD, Delaney KA, Ling LJ, Erickson T, eds. *Clinical Toxicology*. Philadelphia, PA: W.B. Saunders, 2001:985–989.

McClintock JT, Schaffer CR, Sjobald RD. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*–based pesticides. *Pestic Sci*. 1995;45:95–106.

McCormack AL, Atienza JG, Johnston LC, et al. Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. *J Neurochem*. 2005;93:1030–1037.

McCormack AL, Thiruchelvam M, Manning-Bog AB, et al. Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol Dis*. 2002;10:119–127.

McGrew DM, Irwin I, Langston JW. Ehtylene-bisdithiocarbamate enhances MPTP-induced striatal dopamine depletion in mice. *Neurotoxicology*. 2000;21:309–312.

McGlynn KA, Quraishi SM, Graubard BI, et al. Persistent organochlorine pesticides and risk of testicular germ cell tumors. *J Natl Cancer Inst*. 2008;100:663–671.

McLaughlin J, Reynaldo EF, Lamar JK, et al. Teratology studies in rabbits with captan, folpet and thalidomide. *Toxicol Appl Pharmacol*. 1969;14:641.

Meco G, Bonifati V, Vanacore N, et al. Parkinsonism after chronic exposure to the fungicide maneb (manganese ethylene-bis-dithiocarbamate). *Scand J Work Environ Health*. 1994;20:301–305.

Mendelsohn M, Kough J, Vituzis Z, et al. Are Bt crops safe? *Nature Biotechnol*. 2003;21;1003–1009.

Miller GW. Paraquat: the red herring of Parkinson's disease research. *Toxicol Sci*. 2007;100:1–2.

Miyamoto J. Degradation, metabolism and toxicity of synthetic pyrethroids. *Environ Health Perspect*. 1976;14:15–28.

Moebus S, Bödeker W. Mortality of intentional and unintentional pesticide poisonings in Germany from 1980 to 2010. *J Public Health Policy*. 2015;36:170–180.

Mohamed F, Senarathna L, Percy A, et al. Acute human self-poisoning with the *N*- phenylpyrazole insecticide fipronil—a $GABA_A$-gated chloride channel blocker. *J Toxicol Clin Toxicol*. 2004;42:955–963.

Mohanty MK, Kumar V, Pastia BK, et al. An analysis of poisoning deaths in Manipal, India. *Vet Hum Toxicol*. 2004;46:208–209.

Molyneux DH, Ward SA. Reflections on the Nobel Prize for Medicine 2015: the public health legacy and impact of avermectin and artemisin. *Trends Parasitol*. 2015;31:605–607.

Moretto A. Testing for organophosphate delayed neuropathy. In Maines MD, Costa LG, Reed DJ, et al. eds. *Current Protocols in Toxicology*. New York: Wiley; 1999:11.5.1–11.5.14.

Moretto A. Promoters and promotion of axonopathies. *Toxicol Lett*. 2000;112–113:17–21.

Moretto A, Colosio C. The role of pesticide exposure in the genesis of Parkinson's disease: epidemiological studies and experimental data. *Toxicology*. 2013;307:24–34.

Moretto A, Tiramani M, Colosio C. Long-term neurotoxicological effectsof anticholinesterases after acute or chronic exposure. In: Satoh T, Gupta RC, eds. *Anticholinesterase Pesticides: Metabolism, Neurotoxicity and Epidemiology*. Hoboken, NJ: Wiley; 2010:97–108.

Mortensen S, Chandra S, Hooper M, et al. Maturational differences in chlorpyrifos–oxonase activity may contribute to age-related sensitivity to chlorpyrifos. *J Biochem Toxicol*. 1996;1:279–287.

Moser M, Li Y, Varpel K, et al. Placental failure and impaired vasculogenesis result in embryonic lethality for neuropathy target esterase-deficient mice. *Mol Cell Biol*. 2004;24:1667–1679.

Moser M, Stempfl T, Li Y, et al. Cloning and expression of the murine sws/NTE gene. *Mech Dev*. 2000;90:279–282.

Moser V. Companion of aldicarb and metamidophos neurotoxicity at different ages in the rat: behavioral and biochemical parameters. *Toxicol Appl Pharmacol*. 1999;157:94–106.

Mulay PR, Clak G, Jackson WL, et al. Acute sulfuryl fluoride poisoning in a family—Florida, August 2015. *MMWR*. 2016;65:698–699.

Mull RL, Hershberger LW. Inhibitors of DNA biosynthesis—mitosis: benzimidazoles. The benzimidazole fungicides benomyl and carbendazim. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:1673–1699.

Mullin CA, Fine JD, Reynolds RD, et al. Toxicological risks of agrochemical spray adjuvants: organosilicone surfactants may not be safe. *Front Public Health*. 2016;4:art 92.

Multigner L, Kadhel P, Rouget F, et al. Chlordecone exposure and adverse effects in French Indies populations. *Environ Sci Pollut Res Int*. 2016;23:3–8.

Muñoz-Quezada MT, Lucero BA, Barr DB, et al. Neurodevelopmental effects in children associated with exposure to organophosphorus pesticides: a systematic review. *Neurotoxicology*. 2013;39:158–168.

Muñoz-Quezada MT, Lucero BA, Iglesias VP, et al. Chronic exposure to organophosphate (OP) pesticides and neuropsychological functioning in farm workers: a review. *Int J Occup Environ Health*. 2016;22:68–79.

Munro IC, Carlo GL, Orr JC, et al. A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. *J Am Coll Toxicol*. 1992;11:559–664.

Murphy SD. Toxic effects of pesticides. In: Klaassen CD, Amdur MO, Doull J, eds. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. New York: Macmillan; 1986:519–581.

Mutch E, Daly AK, Leathart JBS, et al. Do multiple cytochrome P450 isoforms contribute to parathion metabolism in man? *Arch Toxicol*. 2003;77:313–320.

Nagami H, Nishigaki Y, Matsushima S, et al. Hospital-based survey of pesticide poisoning in Japan, 1998–2002. *Int J Occup Environ Health*. 2005;11:180–184.

Nakayama A, Sukekawa M. Quantitative correlation between molecular similarity and receptor-binding activity of neonicotinoid insecticides. *Pestic Sci*. 1998;52:104–110.

Narahashi T. Neuronal ion channels as the target sites of insecticides. *Pharmacol Toxicol*. 1996;78:1–14.

Narahashi T, Zhao X, Ikeda T, et al. Differential actions of insecticides on target sites: basis for selective toxicity. *Hum Exp Toxicol*. 2007;26:361–366.

Nash D, Mostashari F, Fine A, et al. The outbreak of West Nile virus infection in the New York City area in 1999. *New Eng J Med*. 2001;344:1807–1814.

Nathanson JA. Characterization of octopamine-sensitive adenylate cyclase: elucidation of a class of potent and selective octopamine-2 receptor agonists with toxic effects in insects. *Proc Natl Acad Sci U S A*. 1985;82:599–603.

Nauen R, Jeschke P, Velten R, et al. Flupyradifurone: a brief profile of a new butenolide insecticide. *Pest Manag Sci*. 2015;71:850–861.

Naughton C, O'Toole D, Kirik D, et al. Interaction between subclinical doses of the Parkinson's disease associated gene α-synuclein, and the pesticide rotenone precipitates motor dysfunction and nigrostriatal neurodegeneration in rats. *Behav Brain Res*. 2017;316:160–168.

Novak RJ, Lampman RL. Public health pesticides. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:181–201.

NRC (National Research Council). *Pesticides in the Diets of Infants and Children*. Washington, DC: National Academy Press; 1993:386.

O'Brien RD. *Insecticides: Action and Metabolism*. New York: Academic Press; 1967:332.

Oerke EC. Crop losses to pests. *J Agric Sci*. 2006;144:31–43.

Oghabian Z, Afshar A, Rahimi HR: Hepatotoxicity due to zinc phosphide poisoning in two patients: role of *N*-acetylcysteine. *Clin Case Rep*. 2016;4:768–772.

Okahashi N, Sano M, Miyata K, et al. Lack of evidence for endocrine disrupting effects in rats exposed to fenitrothion in utero and from weaning to maturation. *Toxicology*. 2005;206:17–31.

Okankwo JO, Mutshatsi TN, Botha B, et al. DDT, DDE and DDD in human milk from South Africa. *Bull Environ Contam Toxicol*. 2008;81:348–354.

O'Malley M. The regulatory evaluation of the skin effects of pesticides. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:701–787.

Oriel M, Edmiston S, Beauvais S, et al. Illnesses associated with chloropicrin use in California agriculture, 1992–2003. *Rev Environ Contam Toxicol*. 2009;200:1–31.

Osimitz TG, Lake BG. Mode of action analysis for induction of rat liver tumors by pyrethrins: relevance to human cancer risk. *Crit Rev Toxicol*. 2009;39:501–511.

Osimitz TG, Murphy JV. Neurological effects associated with use of the insect repellent *N*,*N*-diethyl-*m*-toluamide (DEET). *Clin Toxicol*. 1997;35:435–441.

Padilla S, Wilson VA, Bushnell PJ. Studies on the correlation between blood cholinesterase inhibition and target tissue inhibition in pesticide-treated rats. *Toxicology*. 1994;92:11–25.

Page A, Liu S, Gunnell D, et al. Suicide by pesticide poisoning remains a priority for suicide prevention in China: analysis of national mortality trends 2006–2013. *J Affect Disord*. 2016;208:418–423.

Palatnick W, Meatherall R, Sitar D, et al. Toxicokinetics of acute strychnine poisoning. *Clin Toxicol*. 1997;35:617–620.

Panneton WM, Kumar VB, Gan Q, et al. The neurotoxicity of DOPAL: behavioral and stereological evidence for its role in Parkinson's disease pathogenesis. *PLoS ONE*. 2010;5:e15251.

Pardo-Lopez L, Soberon M, Bravo A. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiol Rev*. 2013;37:3–22.

Park J, Darrien I, Prescott LF. Pharmacokinetic studies in severe intoxication with 2,4-D and mecoprop. *Proc Eur Soc Toxicol*. 1977;18:154–155.

Parsons PP. Mammalian toxicokinetics and toxicity of chlorothalonil. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1951–1966.

Patel RV, Shaeer KM, Patel P, et al. EPA-registered repellents for mosquitoes transmitting emerging viral disease. *Pharmacotherapy*. 2016;36:1272–1280.

Peakall DB. Pesticides and the reproduction of birds. *Scientific American*. 1970;222:72–78.

Pearce N, McLean D. Agricultural exposures and non-Hodgkin's lymphoma. *Scand J Work Environ Health*. 2005;31(suppl 1):18–25.

Pelfrene AF. Rodenticides. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2153–2217.

Peraica M, Capodicasa E, Moretto A, et al. Organophosphate polyneuropathy in chicks. *Biochem Pharmacol*. 1993;45:131–135.

Persson EC, Graubard BI, Evans AA, et al. Dichlorodiphenyltrichloroethane and risk of hepatocellular carcinoma. *Int J Cancer*. 2012;131:20178–1084.

Peter JV, Moran JL, Graham P. Oxime therapy and outcomes in human organophosphate poisoning: an evaluation using meta-analytic techniques. *Crit Care Med*. 2006;34:502–510.

Peterson ME. Bromethalin. *Top Compan Anim Med*. 2013;28:21–23.

Peterson ME, Fluegeman K. Colecalciferol. *Top Compan Anim Med*. 2013;28:24–27.

Petrucci N, Sardini S. Severe neurotoxic reaction associated with oral ingestion of low-dose diethyltoluamide-containing insect repellent in a child. *Pediatr Emerg Care*. 2000;16:341–342.

Pezzoli G, Cereda E. Exposure to pesticides and solvents and risk of Parkinson's disease. *Neurology*. 2013;80:2035–2041.

Phua DH, Lin CC, Wu Ml, et al. Neonicotinoid insecticides: an emerging cause of acute pesticide poisoning. *Clin Toxicol*. 2009;47:336–341.

Piccirillo VJ, Piccirillo AL. Methyl bromide. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2267–2279.

Pope C. The influence of age on pesticide toxicity. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:819–835.

Popp W, Schmieding W, Speck M, et al. Incidence of bladder cancer is a cohort of workers exposed to 4-chloro-o-toluidine while synthesizing chlordimeform. *Br J Ind Med*. 1992;49:529–531.

Poulos L, Athanaselis S, Coutselinis A. Acute intoxication with cypermethrin (NRDC 149). *J Toxicol Clin Toxicol*. 1982;19:519–520.

Povey AC. Gene–environmental interactions and organophosphate toxicity. *Toxicology*. 2010;278:294–304.

Power LE, Sudakin DL. Pyrethrin and pyrethroid exposure in the United States: a longitudinal analysis of incidents reported to poison centers. *J Med Toxicol*. 2007;3:94–99.

Prendergast MA, Terry AV, Buccafusco JJ. Effects of chronic, low-level organophosphate exposure on delayed recall, discrimination, and spatial learning in monkeys and rats. *Neurotoxicol Teratol*. 1998;20:115–122.

Proudfoot AT. Poisoning with amitraz. *Toxicol Rev*. 2003;22:71–74.

Proudfoot AT. Aluminum and zinc phosphide poisoning. *Clin Toxicol*. 2009;47:89–100.

Proudfoot AT, Krenzelak EP, Vale JA. Position paper on urine alkalinization. *J Toxicol Clin Toxicol*. 2004;42:1–26.

Pruett SB, Myers LP, Keil DE. Toxicology of metam sodium. *J Toxicol Env Health Part B*. 2001;4:207–222.

Rajasuriar R, Awang R, Hashim SBH, et al. Profile of poisoning admissions in Malaysia. *Hum Exp Toxicol*. 2007;26:73–81.

Ravindran S, Hopkins B, Bova S, et al. In vivo metabolism of norbormide in rats and mice. *Environ Toxicol Pharmacol*. 2009;28:147–151.

Ray DE. Pesticides derived from plants and other organisms. In: Hayes WJ, Lawd ER, eds. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 1991:585–636.

Ray DE. Chronic effects of low level exposure to anticholinesterases—a mechanistic review. *Toxicol Lett*. 1998;103:527–533.

Ray DE, Forshaw PJ. Pyrethroid insecticides: poisoning, syndromes, synergies and therapy. *Clin Toxicol*. 2000;38:95–101.

Ray DE, Fry JR. A reassessment of the neurotoxicity of pyrethroid insecticides. *Pharmacol Therap*. 2006;111:174–193.

Reiner E, Simeon-Rudolf V. Methods for measuring cholinesterase activities in human blood. In: Gupta RC, ed. *Toxicology of Organophosphate and Carbamate Compounds*. Amsterdam: Elsevier; 2006:199–208.

Reiss R, Chang ET, Richardson RJ, et al. A review of epidemiologic studies of low-level exposures to organophosphorus insecticides in non-occupational populations. *Crit Rev Toxicol*. 2015;45:531–641.

Ricceri L, Markina N, Valanzano A, et al. Developmental exposure to chlorpyrifos alters reactivity to environmental and social cues in adolescent mice. *Toxicol Appl Pharmacol*. 2003;191:189–201.

Richardson JR, Caudle WM, Wang M, et al. Developmental exposure to diesldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. *FASEB J*. 2006;20:E976–E985.

Richardson JR, Quan Y, Sherer TB et al. Paraquat neurotoxicity is distinct from that of MPTP and rotenone. *Toxicol Sci*. 2005;88:193–201.

Richardson RJ, Hein ND Wijeyesakere SJ, et al. Neuropathy target esterase (NTE): overview and future. *Chem Biol Interact*. 2013;203:238–244.

Risher JF, Miuk FL, Stara JF. The toxicologic effects of the carbamate insecticide aldicarb in mammals: a review. *Environ Health Perspect*. 1987;72:267–281.

Roberts D, Curtis C, Tren R, et al. Malaria control and public health. *Emerg Infect Dis*. 2004;10:1170–1171.

Roberts DM, Buckley N. Urinary alkalinization for acute achlorophenoxy herbicide poisoning. *Cochrane Database Sys Rev*. 2007;1:CD005488.

Roberts DM, Karunarathna A, Buckley NA, et al. Influence of pesticide regulation on acute poisoning deaths in Sri Lanka. *Bull World Health Organiz*. 2003;81:789–798.

Rogan WJ, Chen A. Health risks and benefits of bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT). *Lancet*. 2005;366:763–773.

Rose MS, Smith LL, Wyatt I. Evidence for the energy-dependant accumulation of paraquat into rat lung. *Nature*. 1974;252:314–315.

Rosenstock L, Keifer M, Daniell WE, et al. Chronic central nervous system effects of acute organophosphate pesticide intoxication. *Lancet*. 1991;338:223–227.

Ross JH, Driver JH, Harris SA, et al. Dermal absorption of 2,4-D: a review of species differences. *Regul Toxicol Pharmacol*. 2005;41:82–91.

Ross MK, Borazjani A, Edward CC, et al. Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterase. *Biochem Pharmacol*. 2006;71:657–669.

Rossman Y, Maharovski I, Bentur Y, et al. Carbamate poisoning: treatment recommendations in the setting of a mass casualties event. *Am J Emerg Med*. 2009;27:1117–1124.

Rubio-Infante, Moreno-Fierros L. An overview of the safety and biological effects of *Bacillus thuringiensis* Cry toxins in mammals. *J Appl Toxicol*. 2016;36:630–648.

Rusiecki JA, de Roos A, Lee WJ, et al. Cancer incidence among pesticide applicators exposed to atrazine in the Agricultural Health Study. *J Natl Cancer Inst*. 2004;96:1375–1382.

Rust MK. Insecticides and their use in urban structural pest control. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:243–250.

Ruzo LO. Physical, chemical and environmental properties of selected chemical alternatives for the pre-plant use of methyl bromide as soil fumigant. *Pest Manag Sci*. 2006;62:99–113.

Sabapathy NN. Paraquat formulation and safety management. In: Bismuth C, Hall AH, eds. *Paraquat Poisoning*. New York: Dekker; 1995:335–347.

Safe S. Endocrine disruptors and human health—is there a problem? An update. *Environ Health Perspect*. 2000;108:487–493.

Sanahuja G, Banakar R, Twyman RM, et al. *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol J*. 2011;9:283–300.

Sanchez-Bayo F, Goulson D, Pennacchio F, et al. Are bee diseases linked to pesticides? A brief review. *Environ Int*. 2016;89–90:7–11.

Sanchez-Moreno S, Alonso-Prados E, Alonso-Prados JL, et al. Multivariate analysis of toxicological and environmental properties of soil nematocides. *Pest Manag Sci*. 2009;65:82–92.

Sanchez-Santed F, Canadas F, Flores P, et al. Long-term functional neurotoxicity of paraoxon and chlorpyrifos oxon: behavioral and pharmacological evidence. *Neurotoxicol Teratol*. 2004;26:304–317.

Sanchez-Santed F, Colomina MT, Herrero Hernandez E. Organophosphate pesticide exposure and neurodegeneration. *Cortex*. 2016;74:417–426.

Sangha GK. Toxicology and safety evaluation of the new insect repellent picaridin (Saltidin). In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2219–2230.

Satelle DB, Cordova D, Cheek TR. Insect ryanodine receptors: molecular targets for novel pest control chemicals. *Invert Neurosci*. 2008;8:107–119.

Sathiakumar N, Delzell E. A review of epidemiologic studies of triazine herbicides and cancer. *Crit Rev Toxicol*. 1997;27:599–612.

Satoh T, Gupta RC, eds. *Anticholinesterase Pesticides: Metabolism, Neurotoxicity and Epidemiology*. Hoboken, NJ: Wiley; 2010:625.

Schenker R, Tinembart O, Humbert-Droz E, et al. Comparative speed of kill between nitenpyram, fipronil, imidacloprid, selamectin and cythioate against adult *Ctenocephalides felis* (Bouché) on cats and dogs. *Vet Parasitol*. 2003;112:249–254.

Schnepf E, Crickmore N, Van Rie J, et al. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev*. 1998;62:775–806.

Schoenig GP, Hartnagel RE, Schardein JL, et al. Neurotoxicity evaluation of *N,N*-diethyl-*m*-toluamide (DEET) in rats. *Fund Appl Toxicol*. 1993;21:355–365.

Schoenig GP, Osimitz TH. DEET. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001:1439–1459.

Schulte-Hermann R, Wogan GN, Berry C, et al. Analysis of reproductive toxicity and classification of glufosinate-ammonium. *Reg Toxicol Pharmacol*. 2006;44:S1–S76.

Sciuto AM, Wang BJ, Martens ME, et al. Phosphine toxicity: a story of disrupted mitochondrial metabolism. *Ann NY Acad Sci*. 2016;1374:41–51.

Senanayake N, Karalliedde L. Neurotoxic effects of organophosphate insecticides. An intermediate syndrome. *New Engl J Med*. 1987;316:761–763.

Serota DG, et al. *Combined Chronic Toxicity and Oncogenicity Study in Rats with 2,4-D Acid*. Unpublished Report 2184–102. Vienna, Australia: Hazleton Laboratories; 1986.

Shafer TJ, Meyer DA, Crofton KM. Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environ Health Perspect*. 2005;113:123–136.

Sheets LP. A consideration of age dependent differences in susceptibility to organophosphorus and pyrethroid insecticides. *Neurotoxicology*. 2000;21:57–64.

Sheets LP. Imidacloprid: a neonicotinoid insecticide. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2055–2064.

Sheets LP, Li AA, Minnema DJ, et al. A critical review of neonicotinoid insecticides for developmental neurotoxicity. *Crit Rev Toxicol*. 2016;46:153–190.

Shepard HH. *The Chemistry and Toxicology of Insecticides*. Minneapolis, MN: Burgess; 1939:383.

Sherer TB, Kim JH, Betarbet R, et al. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and α-synuclein aggregation. *Exp Neurol*. 2003;179:6–16.

Shimizu K, Ohtaki K, Matsubara K, et al. Carrier-mediated processes in blood–brain barrier penetration and neural uptake of paraquat. *Brain Res*. 2001;906:135–142.

Silva JF, Mattos IE, Luz LL, et al. Exposure to pesticides and prostate cancer: systematic review of the literature. *Rev Environ Health*. 2016;31:311–327.

Smeyne RJ, Breckenridge CB, Beck M, et al. Assessment of the effects of MPTP and paraquat on dopaminergic neurons and microglia in the substantia nigra pars compacta of C57BL/6 mice. *PLoS ONE*. 2016;11:e0164094.

Smith AG. Toxicology of DDT and some analogues. In: Krieger R, ed. *Hayes's Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press, 2010:1975–2032.

Smith LL. Mechanism of paraquat toxicity in the lung and its relevance to treatment. *Human Toxicol*. 1987;6:31–36.

Smith LL, Heath D. Paraquat. *CRC Crit Rev Toxicol*. 1976;4:411–445.

Sobus JR, Morgan MK, Pleil JD, et al. Biomonitoring: uses and considerations for assessing nonoccupational human exposures to pesticides. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1021–1036.

Soderlund DM: Toxicology and mode of action of pyrethroid insecticides. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1665–1686.

Soderlund DM. Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances. *Arch Toxicol*. 2012;86:165–181.

Soderlund DM, Casida JE. Effects of pyrethroid structure on rates of hydrolysis and oxidation by mouse liver microsomal enzymes. *Pestic Biochem Physiol*. 1977;7:391–401.

Solomon KR. Glyphosate in the general population and in applicators: a critical review of studies on exposures. *Crit Rev Toxicol*. 2016;46(suppl 1):21–27.

Spagnolo A, Bianchi F, Calabro A, et al. Anophthalmia and benomyl in Italy: a multicenter study based on 940,615 newborns. *Reprod Toxicol*. 1994;8:397–403.

Stebbins KE, Johnson KA, Jeffries TK, et al. Chronic toxicity and oncogenicity studies of ingested 1,3-dichloropropene in rats and mice. *Reg Toxicol Pharmacol*. 2000;32:1–13.

Sterling TD, Arundel AV. Health effects of phenoxy herbicides. *Scand J Work Environ Health*. 1986;12:161–173.

Stevens J, Breckenridge CB, Wright J. The role of P-glycoprotein in preventing developmental and neurotoxicity: avermectins- A case study. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2093–2110.

Stevens JT, Breckenridge CB. Crop protection chemicals. In: Hayes AW, ed. *Principles and Methods of Toxicology*. Philadelphia, PA: Taylor & Francis; 2001a:565–648.

Stevens JT, Breckenridge CB. The avermectins: insecticidal and antiparasitic agents. In: Krieger R, ed. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2001b:1157–1167.

Stevens JT, Breckenridge CB, Wetzel LT et al. A risk characterization for atrazine: oncogenicity profile. *J Toxicol Environ Health*. 1999;56:69–109.

Stevens RC, Suzuki SM, Cole TB, et al. Engineered recombinant human paraoxonase 1 (rHuPON1) purified from *Escherichia coli* protects against organophosphate poisoning. *Proc Natl Acad Sci U S A*. 2008;105:12780–12784.

Stoker TE, Kavlock RJ. Pesticides as endocrine-disrupting chemicals. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:551–569.

Storm E, Rozman KK, Doull J. Occupational exposure limits for organophosphate pesticides based on inhibition of red blood cell acetylcholinesterase. *Toxicology*. 2000;150:1–29.

Stott WT, Gollapudi BB. 1,3-Dichloropropene. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2281–2292.

Stott WT, Gollapudi BB, Rao KS. Mammalian toxicity of 1,3-dichloropropene. *Rev Environ Contam Toxicol*. 2001;168:1–42.

Sudakin DK: Biopesticides. *Toxicol Rev*. 2003;22:83–90.

Sudakin DK, Osimitz T. DEET. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:2111–2125.

Sudakin DK, Trevathan WR. DEET: a review and update of safety and risk in the general population. *J Toxicol Clin Toxicol*. 2003;41:831–830.

Sutton PM, Vergara X, Beckman J, et al. Pesticide illness among flight attendants due to aircraft disinfection. *Am J Ind Med*. 2007;50:345–356.

Taets C, Aref S, Rayburn AL. The clastogenic potential of triazine herbicide combinations found in potable water supplies. *Environ Health Perspect*. 1998;106:197–201.

Talbot AR, Shiaw MH, Huang JS, et al. Acute poisoning with a glyphosate surfactant herbicide (Roundup): a review of 93 cases. *Hum Exp Toxicol*. 1991;10:1–8.

Tamura H, Yoshikawa H, Gaido KW, et al. Interaction of organophosphate pesticides and related compounds with the androgen receptor. *Env Health Perspect*. 2003;111:545–552.

Tang J, Cao Y, Rose RL, et al. Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse and rat liver microsomes. *Drug Metab Dispos*. 2001;29:1201–1204.

Tang J, Rose RL, Chambers JE. Metabolism of organophosphorus and carbamate pesticides. In: Gupta RC, ed. *Toxicology of Organophosphate and Carbamate Compounds*. Amsterdam: Elsevier; 2006:127–143.

Tanner CM, Kamel F, Ross GW, et al. Rotenone, paraquat and Parkinson's disease. *Environ Health Perspect*. 2011;119:866–872.

Taylor JR, Selhorst JB, Houff SA, et al. Chlordecone intoxication in man. I. Clinical observations. *Neurology*. 1978;28:626–630.

Taylor P. Agents acting at the neuromuscular junction and autonomic ganglia. In: Hardman JG, Limbird LE, ed. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. New York: McGraw-Hill; 1996:177–197.

Tenenbein M. Severe toxic reactions and death following the ingestion of diethyltoluamide-containing insect repellents. *J Am Med Ass*. 1987;258:1509–1511.

Thiruchelvam M, Richfield EK, Baggs RB, et al. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. *J Neurosci*. 2000;20:9207–9214.

Timofeeva OA, Levin ED. Lasting behavioral consequences of organophosphate pesticide exposure during development. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:837–846.

Tominack RL. Herbicide formulations. *Clin Toxicol*. 2000;38:129–135.

Tomizawa M, Casida JE. Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu Rev Pharmacol Toxicol*. 2005;45:247–268.

Troczka BJ, Williams AJ, Williamson MS, et al. Stable expression and functional characterization of the diamondback moth ryanodine receptor G4946E variant conferring resistance to diamide insecticides. *Sci Rep*. 2015;5:14680.

Tweedy BW. Inorganic sulfur as a fungicide. *Residue Rev*. 1981;78:43–68.

Ujvary I. Nicotine and other insecticidal alkaloids. In: Yamamoto I, Casida JE, eds. *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*. Tokyo: Springer-Verlag; 1999:29–69.

Ujvary I. Pest control agents from natural products. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:119–229.

U.S. EPA (U.S. Environmental Protection Agency). *DDT: A review of Scientific and Economic Aspects of the Decision to Ban its Use as a Pesticide*. Washington, DC: U.S. EPA; 1975:300.

U.S. EPA (U.S. Environmental Protection Agency). *A SAB Report: Assessment of Potential 2,4-D Carcinogenicity. Review of Epidemiological and Other Data on Potential Carcinogenicity of 2,4-D*. Washington, DC: U.S. EPA; 1994.

U.S. EPA (U.S. Environmental Protection Agency). *Carcinogenicity Peer Review (4th) of 2,4- Dichlorophenoxyacetic Acid (2,4-D)*. Washington, DC: U.S. EPA; 1997.

U.S. EPA (U.S. Environmental Protection Agency). *Exposure Factors Handbook*. EPA/600/C-99/001, February. Washington DC: U.S. EPA; 1999.

U.S. EPA (U.S. Environmental Protection Agency). *The Use of Data on Cholinesterase Inhibition for Risk Assessments of Organophosphorus and Carbamate Pesticides*. Washington, DC: U.S. EPA; 2000:50. Available at: http://www.epa.gov/pesticides/trac/science/cholin.pdf.

U.S. EPA (U.S. Environmental Protection Agency). *Triazine Cumulative Risk Assessment and Atrazine, Simazine and Propazine Decisions*. Washington, DC: U.S. EPA; 2006.

U.S. EPA (U.S. Environmental Protection Agency). *Pesticide Fact Sheet: Flubendiamide*. Washington, DC: U.S. EPA; 2008:65.

U.S. EPA (U.S. Environmental Protection Agency). *Malathion: Human Health Draft Risk Assessment for Registration Review*. Washington, DC: U.S. EPA; 2016a:258.

U.S. EPA (U.S. Environmental Protection Agency). *EPA Takes Strong Steps to Prevent Poisonings and Protect Workers from Paraquat*. Press release, March 23, 2016. Washington, DC: U.S. EPA; 2016b.

Vale A, Lotti M. Organophosphorus and carbamate insecticide poisoning. In: Lotti M, Bleecker ML, eds. *Occupational Neurology*. Vol 131. Handbook of Clinical Neurology Series. Amsterdam: Elsevier; 2015:149–168.

Van den Bercken J. The effect of DDT and dieldrin on myelinated nerve fibers. *Eur J Pharmacol*. 1972;20:205–214.

Van den Berg H. Global status of DDT and its alternatives for use in vector control to prevent disease. *Environ Health Prespect*. 2009;117:1656–1663.

Van der Kraak GJ, Hosmer AJ, Hanson ML, et al. Effects of atrazine in fish, amphibians, and reptiles: an analysis based on quantitative weight of evidence. *Crit Rev Toxicol*. 2014;44(supl 5):1–66.

van Frankenhuyzen K. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. *J Invert Pathol*. 2009;101:1–16.

Van Lier RBL, Cherry LD. The toxicity and mechanism ofaction of bromethalin: a new single-feeding rodenticide. *Fund Appl Toxicol*. 1988;11:664–672.

Vanholder R, Colardyn F, De Reuck J et al. Diquat intoxication: report of two cases and review of the literature. *Am J Med*. 1981;70:1267–1271.

Veale DJ, Wium CA, Muller GJ. Amitraz poisoning in South Africa: a two year survey (2008–2009). *Clin Toxicol (Phila)*. 2011;49:40–44.

Veronesi B, Padilla S, Blackmon K, et al. Murine susceptibility to organophosphorus-induced delayed neuropathy (OPIDN). *Toxicol Appl Pharmacol*. 1991;107:311–324.

Verschoyle RD, Aldridge WN. Structure–activity relationships of some pyrethroids in rats. *Arch Toxicol*. 1980;45:325–329.

Vijverberg HPM, van der Zalm JM, van den Bercken J. Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves. *Nature*. 1982;295:601–603.

von Stackelberg K. A systematic review of carcinogenic outcomes and potential mechanisms from exposure to 2,4-D and MCPA in the environment. *J Toxicol*. 2013;2013; 371610.

Wagner SL. *Clinical Toxicology of Agricultural Chemicals*. Park Ridge, NJ: Noyes Data Corporation; 1983:306.

Walters JL, LAndsell TA, Lookingland KJ, et al. The effects of gestational and chronic atrazine exposure on motor behaviors and striatal dopamine in male Sprague–Dawley rats. *Toxicol Appl Pharmacol*. 2015;289:185–192.

Wang X, Martinez MA, Wu Q, et al. Fipronil insecticide toxicology: oxidative stress and metabolism. *Crit Rev Toxicol*. 2016;46:876–899.

Watanabe T, Sano T. Neurological effects of glufosinate poisoning with a brief review. *Hum Exp Toxicol*. 1998;17:35–39.

Watts M. *Paraquat*. Gelugor, Malaysia: Pesticide Action Network Asia and the Pacific; 2011:44.

Weichenthal S, Moase C, Chan P. A review of pesticide exposure and cancer incidence in the Agricultural Health Study cohort. *Environ Health Perspect*. 2010;118:1117–1125.

Weiss B, Amler S, Amler SW. Pesticides. *Pediatrics.* 2004;113:1030–1036.

Wentzell JS, Casar M, Kretschmar D. Organophosphate-induced changes in the PKA regulatory function of swisscheese/NTE lead to behavioral deficits and neurodegeneration. *PLoS ONE*. 2014;9:e87526.

Wesseling C, Castillo L, Elinder CG. Pesticide poisonings in Costa Rica. *Scand J Work Environ Health*. 1993;19:227–235.

Wesseling C, van Wendel De Joode B, Ruepert C, et al. Paraquat in developing countries. *Int J Occup Environ Health*. 2001;7:275–286.

WHO (World Health Organization). *DDT and its Derivatives. Environmental Health Criteria 9*. Geneva: WHO; 1979:194.

WHO (World Health Organization). *Public Health Impact of Pesticides Used in Agriculture*. Geneva: WHO; 1990.

WHO (World Health Organization). *World Malaria Report 2010*. Geneva: WHO; 2010:238.

Wijeyesakere SJ, Richardson RJ. Neuropathy target esterase. In: Krieger R, ed. *Hayes' Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 2010:1435–1455.

Williams GM, Aardema M, Acquavella J, et al. A review of the carcinogenic potential for glyphosate by four independent expert panels and comparison to the IARC assessment. *Crit Rev Toxicol*. 2016;46(suppl 1):3–20.

Williams GM, Kroes R, Munro IC. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Reg Pharmacol Toxicol*. 2000;31:117–165.

Wilson AGE, Lau H, Asbury KJ, et al. Metabolism of alachlor by human nasal tissue. *Fundam Appl Toxicol*. 1995;15:1398.

Wilson BW, Henderson JD. Determination of cholinesterase in blood and tissue. *Curr Protoc Toxicol*. 2007;12.13.1–12.13-16.

Wood DM, Alsahaf H, Streete P, et al. Fatality after deliberate ingestion of the pesticide rotenone: a case report. *Crit Care*. 2005;9:R280–R284.

Woolley DE. Neurotoxicity of DDT and possible mechanisms of action. In: Prasad KN, Vernadakis A, eds. *Mechanisms of Action of Neurotoxic Substances*. New York: Raven Press; 1982:95–141.

Worek F, Diebold C, Eyer P. Dimethylphosphoryl-inhibited human cholinesterases: inhibition, reactivation and aging kinetics. *Arch Toxicol*. 1999a;73:7–14.

Worek F, Mast U, Kiderlen D, et al. Improved determination of acetylcholinesterase activity in human whole blood. *Clin Chim Acta*. 1999b;288:73–90.

Worek F, Thiermann H, Wille T. Oximes in organophosphate poisoning: 60 years of hope and despair. *Chem Biol Interact*. 2016;259(pt B):93–98.

Wylie BJ, Hauptman M, Woolf AD, et al. Insect repellant during pregnancy in the era of the Zika virus. *Obstet Gynecol*. 2016;128:1111–1115.

Yang PY, Taso TC, Lin JL, et al. Carbofuran-induced delayed neuropathy. *J Toxicol Clin Toxicol*. 2000;38:43–46.

Yaramis A, Soker M, Bilici M. Amitraz poisoning in children. *Hum Exp Toxicol*. 2000;19:1–3.

Zaccheo O, Dinsdale D, Meacock PA, et al. Neuropathy target esterase and its yeast homologue degrade phosphatidylcholine to glycerophosphocholine in living cells. *J Biol Chem*. 2004;279:24024–24033.

Zadikoff CM. Toxic encephalopathy associated with use of insect repellents. *J Pediatr.* 1979;95:140–142.

Zaki MM, Moran D. Harris D. Pesticide in groundwater: the aldicarb story in Suffolk County, New York. *Am J Public Health*. 1982;72:1391–1395.

Zhang J, Fitsanakis VA, Gu G, et al. Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *J Neurochem*. 2003;84:336–346.

Zhao Y, Abbar S, Amoah B, et al. Controlling pests in dry-cured ham: a review. *Meat Sci.* 2016;111:183–191.

Zulian A, Sileikyte J, Petronilli V, et al. The translocator protein (peripheral benzodiazepine receptor) mediates rat-selective activation of the mitochondrial permeability transition by norbormide. *Biochim Biophys Acta*. 2011;1807:1600–1605.

chapter 23

Toxic Effects of Metals

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Major Toxic Metals

- Arsenic
  - *Toxicokinetics*
  - *Toxicity*
  - *Carcinogenicity*
  - *Treatment*
- Beryllium
  - *Toxicokinetics*
  - *Toxicity*
  - *Carcinogenicity*
- Cadmium
  - *Exposure*
  - *Toxicokinetics*
  - *Toxicity*
  - *Carcinogenicity*
  - *Treatment*
- Chromium
  - *Toxicokinetics*
  - *Toxicity*
  - *Carcinogenicity*
- Lead
  - *Exposure*
  - *Toxicokinetics*
  - *Toxicity*
  - *Carcinogenicity*
  - *Treatment*
- Mercury
  - *Global Cycling and Ecotoxicology*
  - *Exposure*
  - *Toxicokinetics*
  - *Toxicity*
  - *Sensitive Subpopulations*
  - *Treatment*
- Nickel
  - *Toxicokinetics*
  - *Toxicity*
  - *Carcinogenicity*
  - *Treatment of Nickel Toxicity*

Essential Metals with Potential for Toxicity

- Cobalt
  - *Toxicokinetics*
  - *Essentiality*
  - *Toxicity*
- Copper
  - *Toxicokinetics*
  - *Essentiality*
  - *Toxicity*
  - *Hereditary Disease of Copper Metabolism*
  - *Treatment*
- Iron
  - *Toxicokinetics*
  - *Essentiality and Deficiency*
  - *Toxicity*
  - *Treatment*
- Magnesium
  - *Toxicokinetics*
  - *Essentiality and Deficiency*
  - *Toxicity*
- Manganese
  - *Toxicokinetics*
  - *Toxicity*
- Molybdenum
  - *Toxicokinetics*
  - *Essentiality and Deficiency*
  - *Toxicity*
- Selenium
  - *Toxicokinetics*
  - *Essentiality and Deficiency*
  - *Toxicity*
- Trivalent Chromium
  - *Essentiality*
- Zinc
  - *Toxicokinetics*
  - *Essentiality and Deficiency*
  - *Toxicity*

Metals Related to Medical Therapy

- Aluminum
  - *Toxicokinetics*
  - *Toxicity*
  - *Treatment*
- Bismuth
  - *Toxicokinetics*
  - *Toxicity*
  - *Treatment*
- Gallium
  - *Toxicokinetics*
  - *Toxicity*
- Gold
  - *Toxicokinetics*
  - *Toxicity*
- Lithium
  - *Toxicokinetics*
  - *Toxicity*
- Platinum
  - *Toxicokinetics*
  - *Toxicity*

Minor Toxic Metals

- Antimony
  - *Toxicity*
- Barium
  - *Toxicity*
- Cesium
  - *Toxicity*
- Fluorine
  - *Toxicity*
- Palladium
  - *Toxicity*
- Silver
  - *Toxicity*
- Tellurium
  - *Toxicity*
- Thallium
  - *Toxicity*
  - *Treatment*

Tin
Toxicity
Titanium
Toxicity
Uranium
Toxicity
Vanadium
Toxicity
Acknowledgments
References

INTRODUCTION

What Is a Metal?

What defines a "metal" is not always obvious and the differences between metallic and nonmetallic elements may be subtle (Vouk, 1986). Metals are typically defined by physical properties of the element in the solid state, but can vary widely with the metallic element. Current development of nano-sized metal particles revealed additional unrealized behaviors of metals and their interactions with biological systems. General metal properties include high reflectivity (luster), high electrical conductivity, high thermal conductivity, and mechanical ductility and strength. A characteristic of metals of toxicological importance is that they exhibit variable oxidation states and may react in biological systems by losing one or more electrons to form cations (Vouk, 1986). In the periodic table, within a group there is often a gradual transition from nonmetallic to metallic properties going from lighter to heavier atoms (e.g., Group IVa transitions from carbon to lead). Various names are applied to subsets of metallic elements including alkali metals (e.g., lithium and sodium), the alkaline earth metals (e.g., beryllium and magnesium), the transition (or "heavy") metals (e.g., chromium and nickel), and the metalloids (e.g., arsenic and antimony), the latter of which show characteristics of metals and nonmetals.

Over 75% of the elements in the periodic table are metals and several more are considered metalloids. This chapter discusses metals and certain metal complexes or molecules that have been reported to produce significant toxicity and disease in humans. The discussion includes major toxic metals (e.g., lead and cadmium), essential metals (e.g., zinc and copper), medicinal metals (e.g., platinum and bismuth), and minor toxic metals including metals of technological significance (e.g., indium and uranium). This chapter will also discuss toxic metalloids (e.g., arsenic and antimony) and certain nonmetallic elemental toxicants (e.g., selenium and fluoride). An overview of *toxic effects of metals* is shown in Fig. 23-1.

Metals as Toxicants

Humankind's use of metals has been critical to the progress and success of human civilization. It is difficult to imagine an advanced civilization without extensive use of metals and metal compounds. Eras of human development are defined by the development of specific metals, such as the many copper alloys of the Bronze Age and advancements in the Iron Age. However, metals are unique among pollutant toxicants in that they are all naturally occurring and, in many cases, are ubiquitous to some level within the human environment. Thus, regardless of how safely metals are used in industrial

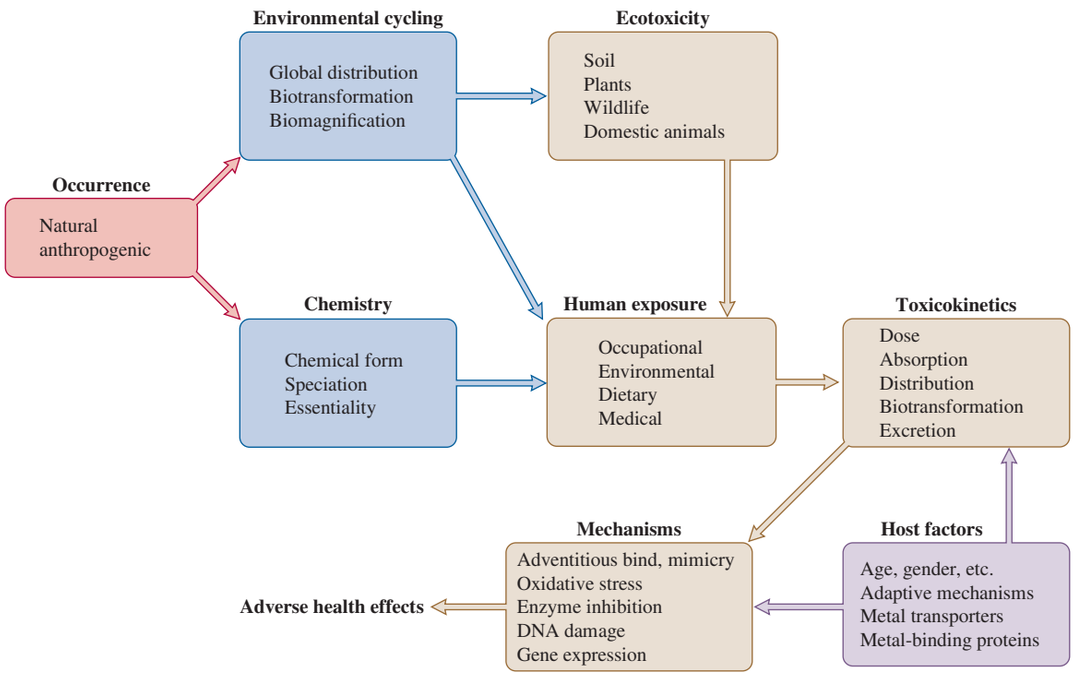

Figure 23-1. *Overview of metal toxicology.*

processes or consumer endpoint products, some level of human exposure is inevitable. Furthermore, life evolved in the presence of metals and organisms have been forced to utilize and manage the potential toxicities of these omnipresent elements. Evolution of complex biological systems required incorporating elemental metals into structural and enzymatic processes, such that many metals are essential to biological processes and life. With essentiality there is intentional bioaccumulation involving safe transport, storage, and coordination that limits toxicity while providing essential functions. Genetic absence or excess metal concentrations overwhelming of metal chaperones and coordination systems causes even essential metals to be toxic. In addition, nonessential metals with close physical properties to essential metals utilize the transport, chaperoning, and coordination systems to access biological systems and cause toxicity. This can also account for bioaccumulation of toxic metals without known biological function.

Metals differ from other toxic substances because, as elements, they are neither created nor destroyed by human endeavors. What human industry and civilization generally do accomplish is to concentrate metals in the biosphere. The anthropogenic contribution to the levels of metals in air, water, soil, and food is well recognized. Human use of metals can also alter the chemical form or speciation of an element and thereby impact toxic potential. Production of metal nanoparticles has created many novel metal properties including unique toxicities. Unfortunately, substantial waste is produced in commercialization of metals and too often the waste and metal products are only sparingly recycled. These factors combine to make metals persistent and potentially more toxic in the human environment.

Due to their very early use, metals are one of the oldest classes of toxicants known to humans. The oldest documented environmental/occupational exposure to metals is that of the Tyrolean Neolithic mummy called Ötzi (c. 3359 to 3105 BC) with copper and high arsenic enrichment in hair follicles (Bolt, 2012). Ötzi was exposed through the early production of bronze tools and weapons at the beginning of the Bronze Age where bronze was produced by combining molten copper with other alloying metals and metalloids. Human use of lead probably started prior to 2000 BC, when abundant supplies were obtained from ores as a by-product of smelting silver. Arsenicals and mercurial compounds have been used in Chinese medicines for thousands of years. Prehistoric skulls were painted with mercury containing cinnabar and the mining of cinnabar and isolation of mercury have long been recognized for producing neurotoxicity. The first description of abdominal colic in a man who extracted metals is credited to Hippocrates in 370 BC. Arsenic and mercury are discussed by Theophrastus of Erebus (370 to 287 BC), and Pliny the Elder (AD 23 to 79). Arsenic-containing pigments (e.g., orpiment and realgar) were used in early decoration of Egyptian tombs and inorganic arsenic was the "secret poison" for assassinations. Mercury assumed almost a mystical stature in early science and was a large focus of alchemy. Modern technological advances since the industrial revolution produced new anthropogenic exposures to metals, such as cadmium, uranium, and rare metals (e.g., cerium and thallium) and metal mixtures (e.g., gallium-arsenide). Cadmium was first recognized in the early 1800s, and it was much later before the metal was widely used and its toxicity recognized. The toxicological importance of some of the rarer or lesser used metals has increased with new applications, such as chemotherapies and microelectronics, or other emerging technologies.

Historically, metal toxicology often concerned acute or overt, high-dose effects, such as abdominal colic from lead or the bloody diarrhea and uropenia after mercury exposures. Due to advances in our understanding of toxic potential of metals, and the accompanying improvements in industrial hygiene and stricter environmental standards, acute high-dose effects of metals are now uncommon or relegated to intentional overdose in the developed world. However, unregulated recycling of massive amounts of electronic wastes in the developing world presents one example of substantial exposure to toxic metals, especially in children. Advanced technologies for quantifying exposures and realization of the broad range of pathogenic effects have shifted the focus of metal toxicology to the contribution of subtler, chronic, low-dose effects in disease promotion. These might include a level of effect that is not cytotoxic, but impairs important but highly complex indices of an affected individual's performance or health, such as lower than expected IQs due to low level, childhood lead exposure, or chronic disease from naturally occurring exposures to arsenic. Metal exposures contribute to the etiology of diseases of essentially all organ systems including neurological dysfunction and degeneration, cardiovascular and metabolic diseases, lung disease and increased infectivity, renal disease, skeletal/muscular disorders, and reproductive dysfunction. Several metals are listed by International Agency for Research on Cancer (IARC) as known or probable carcinogens promoting a range of cancer types (Straif et al., 2009). While metal or metalloid exposures are more frequently being associated with chronic diseases, proving causation and toxic mechanisms in humans remains challenging, as etiology of multifactorial diseases (e.g., cancer or cardiovascular disease) is complex and real-world exposures rarely occur in the context of a single toxic metal or agent. Animal or cellular/molecular models are helpful in proving both causation and mechanisms, but the metals and novel metal materials as a class of toxicants still present many challenges in toxicological research.

The elemental nature of metals impacts their bioabsorption, biotransformation, and toxicity. In contrast to chemical toxicants, it is not possible to detoxify metals by destructive metabolism to subcomponents of lesser toxicity as they are nondegradable, elemental atomic species. This level of indestructibility combined with frequent bioaccumulation contributes to the high concern for metals as toxicants. However, these concerns can be mitigated by changes in the physical properties of the metals, such as change in valence, oxidation/reduction, or anabolic metabolism that render metals inert for biological reactions, incapable of entry into biological systems or cells, or more readily excreted to limit toxicity. Most elemental metals tend to form ionic bonds with biological macromolecules. However, biological conjugation to form organometallic compounds can occur for various metals (Dopp et al., 2004; Drobna et al., 2010), particularly with metalloids, such as arsenic (Li et al., 2017), that show mixed carbonaceous and metallic qualities. The redox capacity of a given metal or metallic compound should also be considered as part of its metabolism and mechanisms for both toxification and detoxification.

Movement of Metals in the Environment

Metals comprise the majority of the Earth's crust and are redistributed naturally in the environment by both geological and biological cycles. The global water cycle is the major natural contributor to transport of metals from the crust to the biosphere and global distribution. Rainwater entering surface and groundwater aquifers releases soluble metals from rocks and ores. Surface water runoff and exchange of groundwater with surface waters, as well as human pumping of water from aquifers, release metals into the biosphere and rivers that eventually transport these substances to the ocean to be precipitated as sediment or taken up into forming rainwater, reinitiating the natural water cycle. Biological cycles moving

metals include biomagnification by microorganisms, plants, and animals resulting in incorporation and amplification in food cycles. Human activities, including pumping of ground water, mining, farming, and fossil fuel burning, shorten the residence time of metals in ore deposits and exponentially facilitates amplification of metals in the biosphere. Acquisition of groundwater with naturally occurring high levels of crustal elements such as arsenic, fluoride, selenium, radon, or thallium causes major health problems in many parts of the world. Human mobilization also creates non-naturally occurring metallic compounds through smelting of ores or manufacturing processes that are beyond natural energy processes.

Increased distribution of metals and metal compounds in the environment raises concern for ecotoxicological impacts. Human industry greatly enhances metal distribution in the global environment by discharge to soil, water, and air, as exemplified by the 200-fold increase in lead content of Greenland ice since the onset of the industrial revolution. Mining operations continue to supply a range of industrially important metals, but often at a cost of mobilizing large amount of toxic waste, as with the contamination of many communities with mercury from artisanal and small-scale gold mining (Drace et al., 2016). Due to global cycling and biomagnification through extensive food chains, mercury and mercurial metabolites are found far from points of discharge of elemental mercury, such as refining, fossil fuel burning, and artisanal gold mining. The elemental mercury is biomethylated by microbial actions and biomagnification through aquatic food chains is found even in isolated and pristine areas of the world (see Fig. 23-5). Reports of metal intoxication are common in plants, aquatic organisms, invertebrates, fish, sea mammals, birds, and domestic animals and the ecotoxicity of specific metals is discussed in each individual section below.

Routes of Exposure and Absorption of Metals

The main routes of human exposure to metals include oral ingestion, inhalation, dermal contact, and rarely through parenteral routes (Tchounwou et al., 2012). Oral ingestion of naturally occurring metals comes primarily from drinking water drawn from contaminated groundwater sources (e.g., arsenic, fluoride, manganese, and iron) and food. Metals accumulate in various foods, especially those grown on lands or soils naturally containing or anthropogenically contaminated with metals. For example, leafy vegetables such as spinach or tobacco accumulate large amounts of cadmium from the soil in which they are grown. Rice and some other grains concentrate hazardous levels of arsenic, cadmium, and copper (Meharg et al., 2009; Carey et al., 2012; Hosseini Koupaie and Eskicioglu, 2015). Many metals such as iron, zinc, lead, chromium, manganese, nickel, copper, and cobalt accumulate in sea animals including zooplanktons and fish (Achary et al., 2017). Mercury bioamplification through the aquatic food chain and ingestion of fish is the major route of human exposure (Siedlikowski et al., 2016). However, the form of the metal found in food and water is important when assessing toxic risk as that may be inert, as in the case of organic arsenicals found in shellfish and ocean fish and trivalent chromium found in drinking water. The absorption of metals through the gastrointestinal tract depends on the metal solubility, pH of the environment, secreted ligands, rate of transport through the mucous layer, and the presence and density of specific transport proteins in the gut wall (Whitehead et al., 1996).

Occupational exposures to metals and newly synthesized metal nanoparticles (e.g., cobalt ferrite, cesium, and cadmium core) primarily enter the human body through inhalation and secondarily via oral ingestion (Horev-Azaria et al., 2013; Ahmad and Zhou, 2017). Smelting operations and metal manufacturing pose significant risks for metals and combined exposure to metals, as has been realized since the first alloying of copper in the Bronze Age. Inhalation of metal-containing fumes generated by welding and related processes may result in the clinical syndrome known as "metal fume fever" that presents with nonspecific complaints including influenza-like symptoms, fever, shaking chills, arthralgias, myalgias, headache, and malaise (Greenberg and Vearrier, 2015). This usually resolves and is benign; however, a significant portion of those afflicted develop severe symptoms or death (Greenberg and Vearrier, 2015). Many elemental metals such as chromium, copper, iron, cadmium, aluminum, arsenic, and beryllium exert the respective health effects after inhalation and absorption in the respiratory tract (Leikauf, 2002; Jaishankar et al., 2014). Hexavalent chromium is predominantly a lung carcinogen due to its kinetics of uptake into the lung epithelium before being reduced to trivalent chromium that is trapped in the intracellular space (De Flora, 2000; Park et al., 2004). Arsenic is unique in posing an equivalent risk for lung cancers regardless of being ingested or inhaled due to its ample distribution throughout the body (Smith et al., 2009). Again, the form of the metal is essential for posing a toxic risk as many valences and oxidative states of the metals are inert or cannot enter cells to cause biological effects. Hexavalent chromium is the only form that causes lung cancers, because Cr(0) or Cr(III) are inert or outcompeted for cellular uptake. In the processing of nickel, it is predominantly soluble nickel and nickel subsulfide that can be taken into the lung epithelium with nickel subsulfide being the main carcinogenic form of the metal (Dunnick et al., 1995; Oller et al., 1997). In addition to specific metal transporters that move the metals from apical to basolateral surfaces for absorption into the blood stream, soluble metals are absorbed through the alveoli and follow water between epithelial cells (Williams et al., 2000). Insoluble forms are coughed out or moved by the mucociliary tree into the gastrointestinal tract for excretion.

Dermal exposures are usually limited due to poor uptake of the small, charged metal ions across the impervious and lipophilic epidermis. However, corrosive forms of metals such as strong acids (e.g., chromic acid) or bases (e.g., metal hydroxides) cause dermal and mucus membrane corrosion and chemical burns. Exogenous factors that increase dermal exposure include dose, vehicle, molecular volume, counter ion, valence, protein reactivity and tissue deposition, solubility, and pH. Endogenous factors include age of the skin, anatomical site, homeostatic control, skin layer, and the importance of oxidation and reduction of xenobiotics on the skin (Hostynek, 2003). The pharmaceutical and cosmetic industries take advantage of the low dermal permeability of metals or nanoparticles of metals in making metal-containing dermal preparations that catalyze the detoxification of damaging UV radiation in the dead outer skin layers.

Finally, metals introduced therapeutically either by injection/infusion or implantation can pose toxic risk with either overdose or, in the case of orthopedic prostheses, immune or foreign body reactions. Degradation products of cobalt-, titanium-, and chrome/nickel-based implants evoke pathologic allergic foreign body reactions, lymphocyte proliferation, and allergic rejection reactions (Hallab et al., 2001; Ring et al., 2016). In rare cases, metal implants have caused systemic metal toxicity (Bradberry et al., 2014; Fox et al., 2016).

Distribution of Metals in the Body

The distribution of metals to various tissues or organs depends on the rate of blood flow to the organs and organ-specific properties for accumulation of metals (Williams et al., 2000). As with their actions at the molecular level, many nonessential, toxic metals distribute and accumulate in the body by mimicking and competing with essential

metals of similar physical character. Metals disrupt metabolic functions by accumulating in organs or by displacing vital nutrients or metals (Singh et al., 2011). The majority of lead (94%) accumulates in the bone and teeth with a nonuniform distribution, while 99% of blood lead is associated with red blood cell (ATSDR, 2007c). In contrast, after 2 to 4 weeks of exposure, arsenic accumulates in keratin-rich tissues such as hair, nails, and skin (ATSDR, 2016).

Metal Transporters and Metal-Binding Proteins

Ionic metals are charged and as such do not readily cross lipid membranes. Thus, they must be absorbed into the body and in cells by transport through selective and relatively nonselective ion channels. All cells possess mechanisms for metal ion homeostasis that involve a balance between uptake and efflux systems. The importance of metal transporters in human diseases is well illustrated by Menkes and Wilson diseases, which are caused by genetic mutations in copper-transport protein genes *ATP7A* and *ATP7B*, respectively. Mutation of *ATP7A* causes copper deficiency (Menkes), while mutation of *ATP7B* results in copper overload (Wilson) (see Fig. 23-7). The 30 different transporters and channels for zinc (the third most abundant transition element in the body) exemplify the breadth of metal transporters and their complex regulation of metal ion concentrations in the circulation, in cells, and within cellular compartments. These proteins include transporters and channels that bring Zn into cells and organelles (14 Zips and 3+ TRPs) and 10 Znts that move Zn out of the cytoplasm (Cousins, 2010; McCormick et al., 2014). Zip4 channels found throughout the gastrointestinal track are essential for uptake of dietary Zn and the majority of the other proteins are critical for regulating Zn actions in 100 to 300 enzymatic reactions essential for health. Magnesium (the fourth most abundant metal in the body) has seven transporters and channels that are distributed differentially throughout the body. One, the TRPM7 channel, is a signal-generating receptor, as well as a transcellular membrane transporter (Brandao et al., 2013; Krapivinsky et al., 2014). Activation in response to Mg stimulates TRPM7 kinase activity and Zn-dependent cleavage of a C-terminal kinase that translocates to the nucleus for epigenetic regulation of chromatin structure (Krapivinsky et al., 2014). The extracellular and intracellular concentrations of all of the essential metals are tightly controlled by their transporters and chaperone proteins such that these proteins become rate-limiting regulators of metal action and toxicity.

Many of the transporters are promiscuous in transporting a number of similarly sized and valenced cations. This provides an axis for nonessential, toxic metals to have cellular access and opportunity to cause toxicity. Toxicity also occurs when toxic metals or excessively high concentrations of essential metals outcompetes an essential metal for transport and action. For example, elemental nickel will cause cardiotoxicity when the nickel is in sufficient concentration to block calcium from entering the cardiomyocytes through L-type channels. In contrast, metal transporters are also important for cellular resistance to metals or metalloids (Rosen, 2002). For instance, enhanced efflux via multidrug resistance protein pumps limits arsenic toxicity and is involved in acquired tolerance to arsenic (Liu, 2010). Decreased influx via reduced calcium G-type channels is involved in acquired tolerance to cadmium (Leslie et al., 2006). A full review of metal transporter and channel biology is beyond the scope of this chapter, and examples of their importance to metal toxicity are discussed for the individual metals.

Metal ions are rarely free during distribution and transport, because free metal is reactive. Thus the free concentration is tightly regulated to provide appropriate homeostasis and desired function (e.g., calcium-dependent cell signaling) without toxicity. Metal chaperones are a class of proteins and small molecules that prevent metal ions from roaming freely within the circulation and in cells. Transferrin is a glycoprotein that binds most of the ferric iron in plasma and helps transport iron across cell membranes where it is delivered to ferritin, the primary cellular iron storage protein. Transferrin also transports aluminum and manganese and may serve as a general metal detoxicant protein, because it binds many toxic metals including cadmium, zinc, beryllium, and aluminum. Ceruloplasmin is a ferroxidase enzyme that is the major copper-carrying protein in blood and helps convert ferrous iron to ferric iron, which then binds to transferrin. Ceruloplasmin also stimulates iron uptake by a transferrin-independent mechanism. Its importance is demonstrated by genetic reduction in expression causing Wilson's disease, a copper storage disease with iron overload.

Chaperones are also responsible for delivering metals into metalloproteins, such as the approximately 1000 Zn and many Cu-containing enzymes in humans (Kambe et al., 2016; Matson Dzebo et al., 2016). For example, after ceruloplasmin releases Cu^{+} ions to the membrane-bound Cu importer CTR1, it is released to the Cu chaperone ATOX1 in the cytoplasm for delivery to cytoplasmic metal-binding domains of ATP7A and two homologous multidomain P1B-type ATPases located in the trans-Golgi network (Matson Dzebo et al., 2016). These ATPases transfer Cu ions through the membrane to the lumen of the Golgi where Cu loading of target proteins occurs. In addition to ATOX1, Cu is chaperoned to Cu/Zn superoxide dismutase (SOD1) by the Cu chaperone for Cu/Zn superoxide dismutase or binds the Cu chaperone for cytochrome *c* oxidase (COX17) for delivery into the mitochondria and incorporation in cytochrome *c* oxidase (COX; Matson Dzebo et al., 2016). Disruption of any of these proteins has profound consequences on enzyme production, cytoplasmic antioxidant activity (SOD1), and energy metabolism (COX). As discussed above, the 30 zinc channels and transporters chaperone zinc across membranes and through specific cellular compartments. There are many other examples of similar chaperones for essential metals and these chaperones are again a target of toxicity if they are overwhelmed by a competing metal or bind an incorrect metal. Examples include systems to keep redox active iron, manganese, and nickel from damaging organelles and cells (Aguirre and Culotta, 2012).

Metallothioneins are an important class of metal chaperones whose expression is induced by metal exposure and that play an important role in essential metal homeostasis and metal detoxification (Isani and Carpene, 2014). Twenty of the 60 amino acids in the metallothioneins are highly conserved cysteines essential for binding and coordinating Zn and Cu, as well as Cd, Mg, and any other d10 electron configuration metals (Isani and Carpene, 2014). Expression of metallothioneins is highly induced by a variety of metals acting on the metal transcription factor-1 (MTF-1; Laity and Andrews, 2007; Isani and Carpene, 2014). Other stimulants include oxidative stress, heat shock, and exposure to chemotherapeutic agents. The high induction of metallothioneins in the kidney by Cd creates a sink for sequestering Cd, such that its biological half-life in humans approaches 30 years. However, this is also the source of renal toxicity when Cd overwhelms the sequestering chaperone.

Protein binding of metals is essential for homeostatic mechanisms and toxicities. In general, the actions of metals cannot be accounted for by stochastic molecular interactions and instead rely on specific protein binding and coordination in highly structured three-dimensional protein binding sites. With essential metals, the binding and coordination result in enzyme catalytic sites capable of efficient shuttling of electrons. Many of these catalytic sites coordinate transition metals, such as iron, copper, manganese, and

molybdenum, with binding to negatively charged histidine imidazoles, cysteine sulfur, or combinations of the two. As seen with the metallothioneins, many of the metal ions have great affinity for cysteines, although the metals differ in their affinity to monocysteine thiols, as seen with orthovanadate and organic arsenicals, and cysteine or cysteine/histidine coordination pockets, as with zinc and trivalent inorganic arsenic (Beyersmann and Hartwig, 2008). The valence, charge structure, radius, and physical properties all contribute to specificity in binding.

Excretion of Metals

Metals are excreted through fecal, biliary, and urinary elimination, as well as through sweat and storage in hair and nails. Several metals including lead, cadmium, arsenic, inorganic mercury, organic mercury, iron, manganese, magnesium, chromium, zinc, copper, nickel, cobalt, tin, and aluminum are excreted through bile and urine with variability to the amount of metals excreted through either route (Ishihara and Matsushiro, 1986). As discussed above, factors that affect excretion of metals include induction of methionine and chaperone proteins (Jaw and Jeffery, 1989). Essential calcium is suggested to decrease bone deposition of lead and invariably increases its excretion and deposition to other target organs (Gochfeld, 1997). In contrast, highly soluble metals that have low protein affinity, such as inorganic arsenic, urinary excretion is the main route of bulk excretion. Arsenic is somewhat unique in being metabolized by a highly conserved As(III) *S*-adenosylmethionine methyltransferase (AS3MT) to monomethylated and dimethylated metabolites that are excreted in the urine more readily than the parent compound (Drobna et al., 2009; Chen et al., 2011a; Li et al., 2017). The more efficient excretion of the arsenic metabolites is protective in humans as *AS3MT* polymorphisms coding for enzyme with decreased catalytic activity increase disease risk (Kuo et al., 2015; Li et al., 2017). Metals, such as cadmium, lead, and mercury, are known to be excreted through the skin where the excretion rate might be higher than urinary excretion (Sears et al., 2012). Hair and nails sequester metals in keratin matrices to reduce toxicity, and this provides important media for monitoring long-term exposure to metals (Laohaudomchok et al., 2011; Calderon et al., 2013; Grashow et al., 2014).

Biomarkers of Metal Exposure

Biomarkers of exposure, toxicity, and susceptibility are important in assessing the level of concern with metal intoxication. Exposure biomarkers, such as concentrations in blood urine, nails, and hair, have long been used for metals. Techniques in molecular toxicology have greatly expanded the possibilities for biomarkers. Thus, in the case of chromium, DNA–protein complexes may serve as a biomarker of both exposure and carcinogenic potential. The capacity for expression of genes that potentially play protective roles against metal toxicity, such as metallothioneins and hemeoxygenase, shows promise as markers of both effect and susceptibility. The use of such biomarkers may aid in identifying particularly sensitive subpopulations.

Estimates of the relationship of exposure level to toxic effects for a particular metal adhere to the fundamental principles of dose–response relationships. However, the dose of a metal is a multidimensional concept and is a function of time, as well as concentration. The most toxicologically relevant definition of dose is the amount of active metal within cells of target organs. The toxic form is often presumed to be the free metal, but free metal concentrations are technically difficult or impossible to precisely determine in vivo. In addition, protein binding of metals produces biological effects and the kinetics of binding and sequestering of metals is also very difficult to quantify.

A critical indicator of retention of a metal is its biological half-life, or the time it takes for the body or organ to excrete half of an accumulated amount. The biological half-life varies according to the metal as well as the organ or tissue. For example, the biological half-lives of cadmium in the kidney and lead in the bone are 20 to 30 years, whereas half-lives for some metals, such as arsenic or lithium, are only a few hours to days. The half-life of lead in the blood is only a few weeks, as compared with the much longer half-life in the bone and other tissues. As mentioned, hexavalent chromium is predominantly a lung carcinogen due to its kinetics of uptake and retention in the lung epithelium (De Flora, 2000; Park et al., 2004). Due to tight binding to the airway epithelial cell DNA, distribution to the circulation is limited and thus blood and urine levels poorly reflect toxicologically relevant chromium concentrations. After inhalation of mercury vapor, at least two half-lives describe the retention in the brain, one on the order of a few weeks and the other measured in years. Differences in distribution and retention half-lives greatly complicate the selection of the optimal matrix for assessing functional lead levels (Barbosa et al., 2005). Continued, chronic metal exposure clearly complicates retention kinetics.

The blood, urine, hair, and nails are the most accessible tissues for quantifying metal exposure. The hair and nail content should be considered excreted metal as it is irreversibly sequestered away from biological action. Results from single measurements may reflect recent exposure, long-term, or past exposure depending on retention time in the particular tissue. Blood and urine concentrations usually, but not always, reflect recent exposures and correlate with acute adverse effects. An exception is urinary cadmium, which may reflect kidney damage related to renal cadmium accumulation over several decades. The hair can be useful in assessing variations in exposure to metals over the period of its growth. Analyses can be performed on segments of the hair, so that metal content of the newest growth can be compared with past exposures. Hair levels of mercury are a reliable measure of exposure to methylmercury. For most other metals, however, the hair is not a reliable tissue for quantifying exposure because of metal deposits from external contamination that complicate analysis (e.g., Zn from shampoo). In contrast, finger nails and toenails have been reliably used for measuring chronic exposures and provide ample material for quantifying multiple metals when using sensitive instrumentation, such as inductively coupled plasma mass spectrometry (ICP-MS) (Goulle et al., 2009; Grashow et al., 2014). However, often only total metal levels can be quantified in nails and it is not possible to identify different metal and metalloid species. This is important for exposures to environmental metalloids, such as arsenic, that are found in many different forms and are metabolized in the body. For arsenic and other metal speciation, it is essential to use blood or urine and couple HPLC separation of the arsenicals prior to ICP-MS quantification (Carlin et al., 2016).

Chemical Mechanisms of Metal Toxicology

A uniform chemical mechanism of toxic action for all metals is implausible because of the great variation in chemical properties and toxic endpoints. Chemically, metals in their ionic form can be very reactive and can interact with biological systems in a large variety of ways. In this regard, a cell presents numerous biologically active metal-binding sites in proteins and nucleic acids. Such adventitious binding is an important chemical mechanism by which exogenous metals exert toxic effects that result in steric rearrangement that impairs the function of biomolecules (Kasprzak, 2002). As discussed above, binding and coordination of essential metals

in proteins are critical for enzymatic activity and binding of inappropriate toxic metals in these enzymes are a major mechanism for disrupting normal homeostasis. Binding of genotoxic metals (e.g., chromium and methylmercury) disrupts DNA structure, promotes ineffective DNA repair, and is mutagenic (Kasprzak, 2002).

Mimicry of essential metals provides a major mechanism for toxic metals to produce their effects. In this regard, the toxic metals bind to physiological sites normally reserved for essential elements. The unique physical and chemical properties of essential metals contribute to control and function of a large range of key metabolic and signaling pathways (Kasprzak, 2002; Cousins et al., 2006). Through mimicry, the toxic metals gain access to and disruption of the important actions of the essential metal and critical metal-mediated cellular functions. For example, mimicry and replacement of zinc is a mechanism of toxicity for cadmium, copper, and nickel. Thallium mimicking potassium and manganese mimicking iron are critical factors in their toxicity. Mimicry of arsenate and vanadate for phosphate allows for cellular transport of these toxic elements, whereas selenate, molybdate, and chromate mimic sulfate and can compete for sulfate carriers and in chemical sulfation reactions (Bridges and Zalups, 2005). Organometallic compounds also act as mimics of biological chemicals, as, for example, with methylmercury, which is transported by amino acid or organic anion transporters (Bridges and Zalups, 2005). Indeed, molecular or ionic mimicry at the level of transport is often a key event in metal toxicity.

Metals in their ionic form can be very reactive and form DNA and protein adducts in biological systems. For example, once hexavalent chromium enters the cell it is reduced by various intracellular reductants to give reactive trivalent chromium species that form DNA adducts or DNA–protein cross-links, events likely to be important in chromium genotoxicity (Zhitkovich, 2005). Metals also induce an array of aberrant gene expression, which, in turn, produces adverse effects. For example, nickel can induce the expression of Cap43/NDRG1, under the control of the hypoxia-inducible transcription factor (HIF-1), which is thought to play a key role in nickel carcinogenesis (Costa et al., 2005). An array of aberrant hepatic gene expression occurs in adult mice after in utero arsenic exposure, which could be an important molecular event in arsenic hepatocarcinogenesis (Liu et al., 2006).

Free ionic transition metals (e.g., iron, copper, manganese, and molybdenum) produce significant toxicity through one electron reduction with generation of free electrons and subsequent formation of reactive oxygen species (ROS). As discussed, tight coordination of these metals in enzymes catalyzes efficient electron flow and transfer. However, when in excess or unbound, ionic metals catalyze formation of cell membrane damaging oxygen-centered lipid radicals and peroxides, as well as incomplete reduction of molecular oxygen through Haber–Weiss and Fenton reactions with superoxide and H_2O_2.

$$Fe^{2+} + O_2 \rightarrow (FeO_2)^{2+}(\text{oxidant}) \rightarrow \text{Lipid}$$

$$\text{Lipid}\bullet + O_2 \rightarrow \text{LipidOO}\bullet$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH-$$

The resultant lipid or hydroxyl radicals react indiscriminately with macromolecules and are capable of damaging proteins and DNA (Galaris and Pantopoulos, 2008). In addition to oxygen-based radicals, damaging carbon- and sulfur-based radicals also occur. In contrast, lower levels of generated lipid and hydrogen peroxides are signal generating and affect gene regulation through protein interactions. Signal-generated low levels of ROS, such as H_2O_2, are second messengers for many receptor-mediated vasoactive and mitogen responses, and high ROS levels from respiratory bursts or mitochondrial injury overwhelm cellular antioxidant defenses, promote mitochondrial calcium leak, and damage or kill cells. Chromium is an example of a metal that generates metal and oxygen radical species during reduction from its hexavalent to trivalent form. However, the oxygen-centered radicals are not required for chromium to damage DNA and proteins (Zhitkovich et al., 2001; Zhitkovich, 2005; Reynolds et al., 2012). Metals or metalloids, such as arsenic, that cannot undergo one electron reduction and the Haber–Weiss reaction are still capable of increasing intracellular ROS, but do so through protein binding and activation of signaling cascades (Samikkannu et al., 2003; Straub et al., 2008, 2009).

Molecular Responses to Metal Exposure

Exposure to essential metals produces homeostatic signaling for normal tissue maintenance and regeneration while nonessential metals signal for homeostatic disruption or directly damage macromolecules and tissues. The results include inappropriate activation of growth or metabolic signaling, loss of essential enzymatic activity, direct structural damage, and if severe, cellular apoptosis and/or necrosis. Disruption of normal cell signaling through inappropriate growth factor signaling, promotion of maladaptive stress responses, and cell senescence can be as detrimental in disease promotion as cell death, especially in chronic diseases such as cancers or cardiovascular disease where there is little or no evidence of cell death.

The ability of metals to affect gene expression and generate pathogenic cell phenotypes is well-documented. However, the mechanistic role of metal-induced changes in gene expression in the etiology of human disease has only recently begun to be elucidated. Modern genomic technologies have identified hundreds to thousands of genes whose levels of expression are affected after exposure to excess essential and nonessential metals. The intended consequence of metal activation of gene expression is often to protect the organism from metal-induced damage as metal exposure is associated with increased expression of genes encoding proteins that remove the metal from the cell via chelation or increased export; reduce the level of oxidative stress; and repair the metal-induced intracellular damage. However, the inappropriate activation of gene expression after metal exposure can contribute to a variety of human pathologies.

There is no unifying mechanism for metal-activated cell signaling or dysregulated gene expression. Aside from response to direct protein damage from metal radicals or indirect ROS generation, metals initiate cell signaling by activating cell receptors. Targeted receptors include both tyrosine kinase receptors (e.g., epidermal growth factor receptors [EGFR]) (Simeonova et al., 2002; Andrew et al., 2009; Tong et al., 2015)) and G protein–coupled receptors (Straub et al., 2009; Garciafigueroa et al., 2013; Klei et al., 2013). However, the activation differs from tightly regulated activation by normal endogenous ligands. The most plausible mechanism for metal activation of receptors and downstream kinases in the receptor signaling pathways is by causing allosteric change through binding to crucial structural cysteine motifs (Ahmadibeni et al., 2007). Signal amplification is the inherent property of cell receptors and this allows for a relatively small amount of toxic metal in chronic exposures to affect broad programs of genetic and epigenetic change that ultimately generate pathogenic cell phenotypes. These broad signaling programs include activation of the pleiotropic mitogen-activated protein kinase (MAPK) cascades that can lead to enhanced cell proliferation at low metal concentrations or cell death at higher

concentrations depending on the MAPK family member (e.g., Erk, JNK, or p38). Similarly, metal signaling activates the Src family kinases (Barchowsky and O'Hara, 2003; Ahmadibeni et al., 2007; Nemec et al., 2010) that regulate programs of cell proliferation. The kinases are intermediates in amplifying signals for metal-induced transcriptional programmatic change that include the stress-responsive activation of activator protein-1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), metal transcription factor-1 (MTF-1), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), hypoxia inducible factors (HIF-1α and HIF-2α), and the signal transducer and the signal activator of transcription (STAT) family. Again, the genes driven by these transcriptional programs often code for cell protection as seen with the Nrf2 pathway inducing phase II metabolism and antioxidant proteins, the HIF factors increasing expression of proteins that oppose hypoxia, and MTF-1 induction of metallothioneins to sequester metals. However, due to overlapping effects of different metals on the transcriptional programs, it is difficult to predict the cell phenotypes induced by exposure to mixed metals. For example, chromium exposure will prevent arsenic from activating the Nrf2 protective program to reduce antioxidant genes (O'Hara et al., 2006). Chromium will directly activate the Src family kinase Fyn to stimulate STAT1 suppression of nickel-induced HIF-1α and NF-κB signaling (Nemec and Barchowsky, 2009; Nemec et al., 2010). Likewise, the activity of a single transcription factor can be influenced by a variety of metals through multiple signaling pathways. For example, the activity of NF-κB is affected by copper, arsenic, vanadium, chromium, cadmium, mercury, lead, or any other metal that can induce intracellular oxidative stress (Barchowsky et al., 1996; Chen and Shi, 2002; Valko et al., 2006; Thevenod and Lee, 2013).

Metal-induced epigenetic regulation contributes to the overall transcriptional reprograming in metal-induced adaptive and pathogenic responses (Salnikow and Zhitkovich, 2008; Martinez-Zamudio and Ha, 2011; Chervona and Costa, 2012; Ryu et al., 2015) that may increase disease risk (Vilahur et al., 2015). Epigenetic changes include posttranslational modification of histones and DNA methylation to convert cytosine to 5-methylcytosine (Ryu et al., 2015; Bommarito et al., 2017). The ability of nickel, cadmium, arsenic, and chromium to induce cancer has been linked to metal-inducible epigenetic changes (Arita and Costa, 2009). Exposure during fetal development may greatly enhance chronic toxic potential of metals as epigenomic changes in imprinted genes, in fetal epigenomic changes, including altered global DNA methylation, gene-specific CpG methylation, and altered microRNA expression during development have been associated with adverse birth outcomes and later-life disease risk (Burris et al., 2016; Bommarito et al., 2017).

Factors Impacting Metal Toxicity

The standard factors that impact the toxic potential of all chemicals apply to the metals as well. Exposure-related factors include dose, route of exposure, duration, and frequency of exposure. Because metals can be highly reactive, the portal of entry is often initially the organ most affected, as with the lung after hexavalent chromium inhalation.

Host-based factors that can impact metal toxicity include age at exposure, gender, and capacity for biotransformation. Metal impacts should be considered throughout the entire range of life stages. Fetal-stage toxicity of metals is well-documented, as with methylmercury, and many metals are teratogenic. For many inorganics, there is no impediment to transplacental transport, as with lead or arsenic, and human fetal blood lead levels (BLL) are similar to maternal levels. In addition to overt teratogenic toxicities, fetal and perinatal exposures result in epigenetic reprograming of development that can lead to enhanced risk of adult disease. Developing stem cells, as well as rapidly proliferating and differentiating cells, are important pathogenic (Burris et al., 2016) and carcinogenic (Tokar et al., 2011c) targets in the fetus. Several metals (e.g., arsenic, nickel, lead, and chromium) are transplacental carcinogens in rodents. It is quite clear that children are more sensitive to metal intoxication due to developmental stage and continued rapid growth (e.g., neurotoxicity of lead). Due to mimicry, lead exposure is four to five times greater in developing children than adults as they absorb larger amounts of calcium, magnesium, manganese, and strontium to build bones, muscle, and neural tissues. Child pica behavior presents additional exposure concerns as lead and other toxic metals are concentrated in ingested dirt, paint chips, and dusts. Elderly persons are also believed to be generally more susceptible to metal toxicity than younger adults due to overall degeneration of kidneys and other organs involved in metal distribution (Bridges and Zalups, 2017a). Recognition of factors that influence toxicity of a metal is important in determining risk, particularly in susceptible subpopulations.

As discussed above, the chemical form and nature of the metals directly affect their toxic potential. The oxidation state and valence of the metal greatly affect its competition for uptake and mechanism of interaction with cellular macromolecules. Metabolism by microbes or mammals affects distribution, site of action, and excretion. For instance, methylmercury is a potent neurotoxin, whereas the inorganic mercurials primarily attack the kidney, and methylated arsenicals are potentially more potent than inorganic arsenic, but are excreted more readily (Bridges and Zalups, 2017b).

Lifestyle factors such as smoking or alcohol ingestion may have direct or indirect impacts on the level of metal intoxication. For instance, tobacco smoke contains many toxic metals, such as cadmium and arsenic, and smoking may double the lifetime burden of cadmium in nonoccupationally exposed individuals. Other components of tobacco smoke may also influence pulmonary effects, as, for instance, with metals that are lung carcinogens or promote pulmonary disease. Arsenic exposures synergize with tobacco smoke to increase risk of cardiovascular and lung disease, as well as cancer (Parvez et al., 2013; Nigra et al., 2016). Alcohol ingestion may influence toxicity by altering diet, reducing essential mineral intake, and altering hepatic iron deposition. Alcohol can also synergize with metals, such as arsenic, through amplification of interacting signal transduction pathways (Klei and Barchowsky, 2008) that may promote cancers (Wang et al., 2012). The composition of the diet can significantly alter gastrointestinal absorption of various dietary metals. In addition, susceptibility to the toxic effects of metals can be altered by dietary constituents competing at sites of metal action (e.g., selenium in diet can reduce arsenic effects on metabolic enzymes [Walton et al., 2003; Argos et al., 2013]) or by inducing antioxidant or metabolic adaptive responses (e.g., induction of Nrf2 program of phase II metabolizing enzymes).

Adaptive mechanisms are critical to susceptibility to the toxic effects of metals, and organisms have a variety of ways in which they can adapt to otherwise toxic metal insults. Typically, adaptation is acquired after the first few exposures and can be long-lasting or transient after exposure ceases. Adaptation can be at the levels of uptake and excretion or, with some metals, through long-term storage in a toxicologically inert form (e.g., hair and nails). For instance, enhanced arsenic efflux is involved in acquired tolerance to the metalloid on the cellular level (Liu et al., 2001). Conversely, intentional sequestration of toxic metals is another adaptive tactic and examples of such long-term storage include lead inclusion bodies, which form in various organs and contain protein-immobilized

lead in a distinct cellular aggresome. These bodies are thought to be protective by limiting the level of free, and therefore toxic, lead within the cell, and the inability to form such bodies clearly increases the chronic toxic effects of lead, including carcinogenesis (Waalkes et al., 2004). Similarly, cadmium exposure causes the overexpression of MT that will sequester cadmium and reduce its toxicity as an adaptive mechanism (Isani and Carpene, 2014). Metal exposure can also induce a cascade of molecular/genetic responses that may reduce toxicity, such as with metal-induced oxidative stress responses (Valko et al., 2006). It is clear that acquired metal adaptation, although allowing immediate cellular survival, may in fact be a potential contributing factor in long-term toxicity (Waalkes et al., 2004). For instance, acquired self-tolerance to cadmium- or arsenic-induced apoptosis may actually contribute to eventual carcinogenesis by allowing survival of damaged cells that would otherwise have been eliminated (Hart et al., 2001; Pi et al., 2005).

Therapeutic Use and Toxicity of Metals

Metals and metal compounds have a long and rich history of pharmacological use. Metallic compounds, largely because of their potential toxicity, have been used in chemotherapeutic settings for millennia. Traditional Chinese medicines, usually complex mixtures, were made with toxic metals, such as mercury, as intentional ingredients (Liu et al., 2008). Arsenic and arsenicals have been a part of Chinese medicine for approximately 5000 years and Ehrlich's *magic bullet* (arsphenamine) for syphilis and infectious diseases was a mixture of organoarsenicals. Prior to arsphenamine, syphilis was often treated with mercury. Arsenicals were the workhorse therapeutic agents of the 1800s, although they caused as many untoward effects as cures. Inorganic arsenic trioxide remains a frontline drug for treating promyelocytic leukemia (Chen et al., 2011b). Today, many metallic chemicals remain valuable pharmacological tools, as exemplified by the highly effective use of platinum compounds in cancer chemotherapy. Other examples of medicinal metals used today include aluminum (antacids and buffered analgesics), bismuth (peptic ulcer), lithium (mania and bipolar disorders), and gold (arthritis). Metallic compounds find their way into a variety of pharmacological preparations as active or inactive ingredients.

Treatment of metal poisoning is sometimes used to prevent, or even attempt to reverse, toxicity. Metal chelation has made a resurgence in the treatment of cardiac diseases (Solenkova et al., 2014; Lamas et al., 2016) with the therapeutic strategy of giving metal chelators that complex the metal and enhance its excretion (Kosnett, 2015). Most chelators are not specific and will interact with a number of metals, eliminating more than the metal of concern and possibly essential metals. In addition, the vast array of biological metal ligands is a formidable barrier to chelator efficacy as is the chelator's water/fat solubility (Kosnett, 2015). Metal chelation therapy should be considered a secondary alternative to toxic metal exposure reduction or even prevention. Chelator therapy can be used for many different metals including lead, mercury, iron, and arsenic.

MAJOR TOXIC METALS

Arsenic

Arsenic (As) is a toxic and carcinogenic metalloid. The word *arsenic* is from the Persian word *Zarnikh*, as translated to the Greek *arsenikon*, meaning "yellow orpiment." Arsenic has been known and used since ancient times as the *poison of kings and the king of poisons*. The element was first isolated in about 1250, but arsenicals have been used since ancient times as drugs and even today are very effective against acute promyelocytic leukemia (Chen et al., 2011b). Arsenic exists in the trivalent and pentavalent forms and is widely distributed in nature. The most common inorganic trivalent arsenic compounds are arsenic trioxide and sodium arsenite, while common pentavalent inorganic compounds are sodium arsenate, arsenic pentoxide, and arsenic acid. Important organoarsenicals include arsanilic acid, arsenosugars, and several methylated forms produced as a consequence of inorganic arsenic biotransformation by various organisms, including humans. Arsine (AsH_3) is an important gaseous arsenical. Industrial and military toxic arsenicals include phenylarsine oxide, roxarsone, and lewisite.

Occupational exposure to arsenic occurs in the manufacture of pesticides, herbicides, and other agricultural products. Exposure to arsenic fumes and dusts occurs in smelting industries, especially copper smelting (Enterline et al., 1995; IARC, 2012; ATSDR, 2016). Environmental arsenic exposure mainly occurs from arsenic-contaminated drinking water, which can be very high depending on the subsurface geology (IARC, 2012; ATSDR, 2016; Carlin et al., 2016). Arsenic in drinking water is generally from natural sources. Due to high prevalence in groundwater, it is estimated that at least 150,000 individuals daily drink water contaminated with more than 10 ppb (USEPA and WHO MCL; Carlin et al., 2016). Food, especially seafood and rice, may contribute significantly to daily arsenic intake (Carlin et al., 2016; Mantha et al., 2017). Arsenic in seafood is largely in an organic form called arsenobetaine that is much less toxic than the inorganic forms (ATSDR, 2016).

Toxicokinetics Inorganic arsenic is well absorbed (80% to 90%) from the gastrointestinal tract, distributed throughout the body, often metabolized by methylation, and then excreted primarily in urine (Hughes et al., 2011; ATSDR, 2016). Trivalent inorganic arsenic and trivalent methylated metabolite absorption from the gastrointestinal track and movement into cells throughout the body occur through aquaglyceroporins 7 and 9 (Liu, 2010). As these channels are primarily water and glycerol channels, trivalent arsenicals can freely distribute throughout the body with the volume of distribution of water. Pentavalent inorganic and methylated arsenic species resembles phosphate and must compete with mM amounts of phosphate for cell entry. Low-solubility arsenic compounds (e.g.,, arsenic trioxide, arsenic selenide, lead arsenide, and gallium arsenide) are absorbed less efficiently after oral exposure. Skin is a potential route of exposure to industrial levels of arsenic, and systemic toxicity has been reported in persons having dermal contact with solutions of inorganic arsenic (Hostynek, 2003), but the relevance of this to today's exposure paradigms is limited. Airborne arsenic is largely trivalent arsenic oxide and deposition in airways and absorption of arsenicals from lungs is dependent on particle size and chemical form (ATSDR, 2016).

Excretion of absorbed arsenic is mainly via the urine. The whole-body biological half-life of ingested arsenic is about 10 hours, and 50% to 80% is excreted into the urine over 3 days (Kenyon et al., 2005; ATSDR, 2016). The biological half-life of methylated arsenicals is in the range of 30 hours (Hughes et al., 2005). Arsenic has a predilection for skin and is excreted by desquamation of skin and in sweat, particularly during periods of profuse sweating. It also concentrates in forming fingernails and hair bound to keratins. Arsenic exposure produces characteristic transverse white bands across fingernails (Mees' line), which appear about 6 weeks to 2 months after the onset of symptoms of arsenic toxicity (Patel et al., 2011). Arsenic in the fingernails and hair has been used as a biomarker for exposure, including both current and past exposures, while blood and urinary arsenic is a good indicator for current exposure.

Methylation of inorganic arsenic species is protective in enhancing excretion, but enhances chronic toxicity with methylarsenite (MMA) and dimethylarsenite (DMA) being as or more potent in the cell as arsenite (Hughes et al., 2011; Li et al., 2017). High proportions of MMA and low proportions of DMA in urine are related to increased risks of cancer and cardiovascular disease, whereas low levels of MMA and high levels of DMA are linked to adiposity and the incidence of diabetes (Cosselman et al., 2015). Some animal species even lack arsenic methylation capacity, perhaps as an adaptation mechanism. Fig. 23-2 illustrates the biotransformation of arsenic. Arsenate (As^{5+}) is rapidly reduced to arsenite (As^{3+}) by arsenate reductase (presumably purine nucleoside phosphorylase). Arsenite is then sequentially methylated to form monomethylarsonic acid and dimethylarsinic acid (DMA^{5+}) by arsenite methyltransferase (AS3MT) using *S*-adenosylmethionine (SAM) as a methyl group donor (Hughes et al., 2011; Li et al., 2017). In humans, urinary arsenicals are composed of 10% to 30% inorganic arsenicals, 10% to 20% MMA, and 55% to 76% DMA (IARC, 2012; Cosselman et al., 2015). However, large variations in arsenic methylation occur due to factors such as age and sex. There are genetic polymorphisms in arsenic metabolic genes (Engstrom et al., 2013; Li et al., 2017) and the role of these variants in promoting disease is currently under investigation. Arsenic metabolism also changes through the course of pregnancy, reflected in higher urinary excretion of DMA and lower urinary levels of inorganic arsenic and MMA, which may have toxicological impact on the developing fetus (Hopenhayn et al., 2003).

Toxicity

Acute Poisoning Ingestion of large doses (70 to 180 mg) of inorganic arsenic can be fatal. Symptoms of acute intoxication include fever, anorexia, hepatomegaly, melanosis, cardiac arrhythmia, and, in fatal cases, terminal cardiac failure. Acute arsenic ingestion can damage mucous membranes of the gastrointestinal tract, causing irritation, vesicle formation, and even sloughing. Sensory loss in the peripheral nervous system is the most common neurological effect, appearing at 1 to 2 weeks after large doses and consisting of Wallerian degeneration of axons, a condition that is reversible if exposure is stopped. Anemia and leucopenia, particularly granulocytopenia, occur a few days after high-dose arsenic exposure and are reversible. Intravenous arsenic infusion at clinical doses in the treatment of acute promyelocytic leukemia may be significantly or even fatally toxic in susceptible patients, and a few sudden deaths have been reported. Approximately 30% of patients undergoing arsenic chemotherapy experience life-threatening re-entrant arrhythmias caused by arsenic impairment of cardiac inwardly rectifying potassium channels (Ficker et al., 2004; Cubeddu, 2016). Arsenic cancer therapies are also associated with noncardiogenic pulmonary edema and severe pulmonary leukocytosis (Camacho et al., 2000; Briasoulis and Pavlidis, 2001). Acute exposure to a single high dose can produce encephalopathy, with signs and symptoms of headache, lethargy, mental confusion, hallucination, seizures, and even coma (ATSDR, 2007a).

Arsine gas, generated by electrolytic or metallic reduction of arsenic in nonferrous metal production, is a potent hemolytic agent, producing acute symptoms of nausea, vomiting, shortness of breath, and headache accompanying the hemolytic reaction. Exposure to arsine is fatal in up to 25% of the reported human cases and may be accompanied by hemoglobinuria, renal failure, jaundice, and anemia in nonfatal cases when exposure persists (Pullen-James and Woods, 2006).

Chronic Toxicity The skin is a major target organ in chronic inorganic arsenic exposure. In humans, chronic exposure to arsenic induces a series of characteristic changes in skin epithelium. Diffuse or spotted hyperpigmentation and, alternatively, hypopigmentation can first appear between 6 months and 3 years with chronic exposure to inorganic arsenic (ATSDR, 2007a). Palmar-plantar hyperkeratosis usually follows the initial appearance of arsenic-induced pigmentation changes within a period of years (ATSDR, 2007a). Susceptibility to skin lesions is increased by folic acid and vitamin B deficiencies due to impaired methylation of ingested inorganic arsenic (Gamble et al., 2006; Ahsan et al., 2007). Skin cancer that presents as in situ squamous carcinoma that is often indistinguishable from Bowen disease is common with protracted high-level arsenical exposure (ATSDR, 2007a; IARC, 2012).

Cardiovascular disease and coronary artery disease in particular are the noncancer diseases most strongly associated with environmental arsenic exposures (Moon et al., 2012; NRC, 2014; Nigra et al., 2016). Several highly powered prospective epidemiological studies have confirmed the association even with low to moderate levels of arsenic exposure (Chen et al., 2011c; Moon et al.,

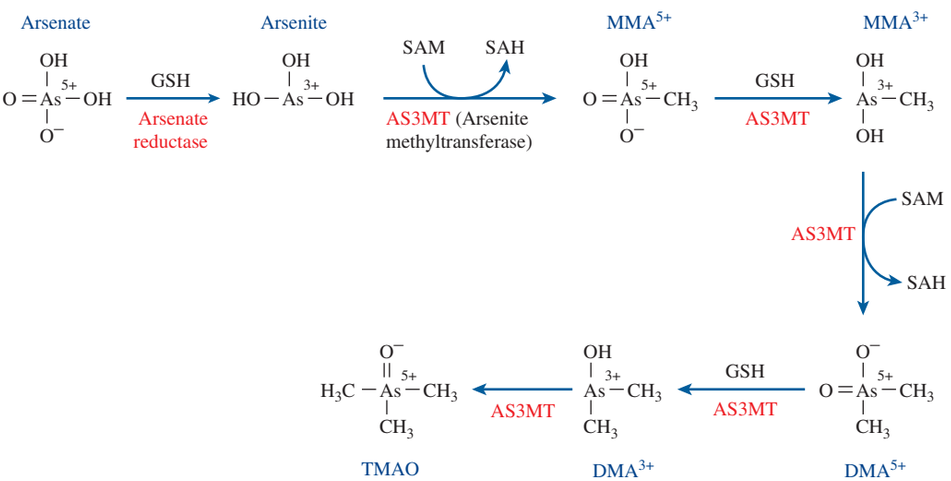

Figure 23-2. *Arsenic metabolism.* AS3MT, arsenic methyltransferase (Cyt19); DMA^{3+}, dimethylarsinous acid; DMA^{5+}, dimethylarsinic acid; GSH, reduced glutathione; MMA^{3+}, monomethylarsonous acid; MMA^{5+}, monomethylarsonic acid; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; TMAO, trimethylarsenic oxide.

2013; D'Ippoliti et al., 2015; James et al., 2015). The dominant form of disease stems from the atherogenic potential of arsenic and its propensity to enhance both vessel disease and prolongation of the cardiac Q-T interval (Chen et al., 2013; Nigra et al., 2016). Atherosclerotic mouse models have shown increased atherosclerosis after even moderate arsenic exposures and that smooth muscle cell remodeling and macrophage lipid metabolism are the prime pathogenic targets (Lemaire et al., 2011, 2015). In addition, mouse models have also demonstrated that low to moderate levels of exposure cause perivascular fibrosis and loss of perivascular matrix integrity, especially in the heart (Soucy et al., 2005; Hays et al., 2008). Peripheral vascular disease has been observed in persons with chronic exposure to inorganic arsenic in the drinking water in Taiwan (Wang et al., 2007). It is an arteriosclerosis manifested by acrocyanosis and Raynaud's phenomenon and may progress to endarteritis and gangrene of the lower extremities (black foot disease). Studies have shown an association between high arsenic exposure in Taiwan and Bangladesh and an increased risk of diabetes mellitus (Navas-Acien et al., 2006; Tseng, 2008). Finally, the vascular endothelium is highly sensitive to arsenic exposures with low-level exposure promoting proliferation, angiogenesis, and vessel remodeling, whereas high exposures cause cell death and loss of vessels (Soucy et al., 2003, 2005; Straub et al., 2008).

Chronic exposure to arsenic is associated with a range of nonmalignant respiratory effects including respiratory symptoms, chronic obstructive pulmonary disease (COPD), and respiratory disease mortality (von Ehrenstein et al., 2005; Smith et al., 2006; Parvez et al., 2013). Susceptibility to the respiratory effects of arsenic is enhanced by in utero exposure (Rahman et al., 2011; Ramsey et al., 2013b; Steinmaus et al., 2016) and co-exposure to tobacco smoke (Parvez et al., 2013). In addition, arsenic exposure is associated with increased respiratory tract infections (Rahman et al., 2011; Farzan et al., 2016) and chronic lung infections, including pulmonary tuberculosis (Smith et al., 2011). Prospective epidemiologic studies indicate that arsenic causes chronic loss of lung defenses, such as secretion of CC16 protein from airway cells (Parvez et al., 2010). Animal studies suggest that low to moderate levels of arsenic in utero exposures of mice to low to moderate concentrations decrease immune gene expression and promotes inflammatory protein expression (Kozul et al., 2009b; Ramsey et al., 2013a) that may make mice more susceptible to airway infections (Kozul et al., 2009a). In keeping with reduced lung defenses, as in the heart, arsenic compromises lung matrix, wound repair, and barrier function (Hays et al., 2008; Lantz et al., 2009; Petrick et al., 2009; Sherwood et al., 2013).

Liver injury, characteristic of long-term or chronic arsenic exposure, manifests itself initially as jaundice, abdominal pain, and hepatomegaly (Mazumder and Dasgupta, 2011). Liver injury may progress to cirrhosis and ascites, even to hepatocellular carcinoma (Liu and Waalkes, 2008; Straif et al., 2009; IARC, 2012).

Arsenic is a peripheral neurotoxicant; it produces a clinical picture of severe polyneuropathy after severe poisoning (ATSDR, 2007a). Occupational exposures, as in copper smelters, or repeated exposure to low levels of inorganic arsenic produce peripheral neuropathy (ATSDR, 2007a; Sinczuk-Walczak et al., 2010). This neuropathy usually begins with sensory changes, such as numbness in the hands and feet, but later may develop into a painful "pins and needles" sensation. Both sensory and motor nerves can be affected, and muscle tenderness often develops, followed by weakness, progressing from proximal to distal muscle groups. Sensory-motor dysfunction is pronounced in exposed children (Parvez et al., 2011), suggesting that in utero or early development exposures increase susceptibility. Histological examination reveals a dying-back axonopathy with demyelination, and effects are dose-related (ATSDR, 2007a). Arsenic exposures, especially in utero and during early developmental exposures, also impair central cognition and memory behaviors (Wasserman et al., 2011; Tyler and Allan, 2014; Wasserman et al., 2014). In addition, studies in mice suggest that the impact of in utero and perinatal exposures on cognition may be less in females who respond to arsenic with greater antioxidant adaptation (Allan et al., 2015).

Arsenic, especially with chronic early-life exposure, is immunotoxic and potentially increases risk of infections and inflammatory-like diseases during childhood and in adulthood (ATSDR, 2016; Ferrario et al., 2016). As mentioned above, this immunosuppressive potential is prevalent in respiratory tract infections (Kozul et al., 2009a; Rahman et al., 2011; Ramsey et al., 2013a; Steinmaus et al., 2016), and enhancement of a global inflammatory state may underlie the etiology of neural, cardiovascular, metabolic, and cancer disease promotion. For example, arsenic exposures alter transcriptional programing in circulating macrophages to enhance atherogenesis that underlies arsenic-promoted cardiovascular disease (Lemaire et al., 2014). In addition to increasing inflammation, arsenic may increase allergy and autoimmune diseases (Ferrario et al., 2016). Further impacts on the circulation include hematologic consequences of chronic exposure to arsenic that interferes with heme synthesis and increase in urinary porphyrin excretion, a biomarker for arsenic exposure (Ng et al., 2005).

Mechanisms of Toxicity The trivalent compounds of arsenic are thiol-reactive, and thereby activate or inhibit enzymes or alter protein structure by reacting with proteinaceous thiol groups. Pentavalent arsenate mimics phosphate and can inhibit phosphotransfer reactions, such as mitochondrial oxidative phosphorylation, when present in concentrations that are stoichiometrically competitive. Arsine gas is formed by the reaction of hydrogen with arsenic, and is a potent hemolytic agent (ATSDR, 2007a, 2016).

In addition to these basic modes of action, several mechanisms have been proposed for arsenic toxicity and carcinogenicity. Arsenic and its metabolites stimulate oxidant production through activation of NADPH oxidases and inference with mitochondrial respiration. Transition between trivalent and pentavalent oxidation states occurs through two electron transfer and thus arsenic is not capable of directly generating reactive oxygen species. Instead, reaction with critical cysteines in receptors and signaling enzymes that stimulate NADPH oxidase ROS generation (Straub et al., 2008, 2009; ATSDR, 2016). The oxidants generated are second messengers in signal transduction cascades leading to activation of tyrosine and serine/threonine kinase pathways for enhanced proliferation, cell turnover, and potential transformation (Simeonova and Luster, 2002; Andrew et al., 2009; NRC, 2014; ATSDR, 2016). Signaling through oxidant activation of the Nrf2 transcriptional program produces adaptation to arsenic-promoted stress with broad expression of antioxidant capacity and phase II metabolism genes (Lau et al., 2013; Sinha et al., 2013). Arsenic exposures produce longer term phenotypic change through a range of epigenetic effects including altered DNA methylation, histone modification, altered miRNA expression, and genomic instability (Rossman and Klein, 2011; ATSDR, 2016; Bailey et al., 2016; Howe and Gamble, 2016). Some mechanisms, however, may be cell type or organ specific, as there is emerging evidence that arsenic impacts target tissue stem cells in various ways to facilitate oncogenic change or impair tissue metabolism and regeneration (Tokar et al., 2011c; Bailey et al., 2016; Zhang et al., 2016).

Carcinogenicity The carcinogenic potential of arsenic was recognized over 110 years ago by Hutchinson, who observed an

unusual number of skin cancers occurring in patients treated for various diseases with medicinal arsenicals (IARC, 2012). IARC and NTP (IARC, 2012; NTP, 2016b) have long classified arsenic as a known human carcinogen, mostly associated with tumors of the skin, lung, and urinary bladder, and possibly the kidney, liver, and prostate (Straif et al., 2009; IARC, 2012). The mode of action for arsenic-induced cancers remains unresolved and is likely to be multifactorial. Resolution of a mode of action has been complicated by the difficulty in confirming the carcinogenicity of inorganic arsenic in experimental animals (States et al., 2011; Tokar et al., 2011c). Unlike many carcinogens, arsenic is not a mutagen in bacteria and acts weakly in mammalian cells, but can induce chromosomal abnormalities, aneuploidy, and micronuclei formation (IARC, 2012; ATSDR, 2016). Arsenic can also act as a co-mutagen and/or co-carcinogen (Rossman et al., 2002; IARC, 2012; ATSDR, 2016). It is clear that inorganic arsenic and its metabolites are not directly genotoxic, as arsenic does not react directly with DNA. Instead, reaction with thiols in critical proteins produces transcriptional and epigenetic changes that promote cancers and tumor growth (Cohen et al., 2013; NRC, 2014). Arsenic inhibits a number of DNA repair mechanisms (Hartwig et al., 2002; Kitchin and Wallace, 2008; Zhou et al., 2014) and downregulates expression of some DNA repair genes (Andrew et al., 2003; IARC, 2012). Trivalent arsenicals can bind to cysteines in zinc coordination pockets of DNA binding proteins to replace the zinc, inhibit the protein's activity, and promote genomic instability (Hartwig et al., 2002; Zhou et al., 2011; IARC, 2012). Inhibition of the tumor suppressor p53 and promotion of maladaptive antioxidant and antiapoptotic responses may underlie the co-carcinogenic properties of arsenicals (IARC, 2012; NRC, 2014). As discussed, arsenic signaling for increased ROS generation accounts for a large portion of its tendency to promote cell proliferation at lower concentrations and DNA damage at higher concentrations that interfere with mitochondrial respiration (IARC, 2012; NRC, 2014; ATSDR, 2016).

Arsenic-induced skin cancers include basal cell carcinomas and squamous cell carcinomas, both arising in areas of arsenic-induced hyperkeratosis (Davis et al., 2000; IARC, 2012). The basal cell cancers are usually only locally invasive, but squamous cell carcinomas, resembling Bowen disease, may have distant metastases. In humans, the skin cancers often, but not exclusively, occur on areas of the body not exposed to sunlight (e.g., on palms of hands and soles of feet) and often occur as multiple primary malignant lesions. Animal models have shown that arsenic acts as a rodent skin tumor co-promoter with 12-*O*-teradecanoyl phorbol-13-acetate in v-Ha-*ras* mutant Tg.AC mice (Germolec et al., 1998) or as a co-carcinogen with UV irradiation in hairless mice (Rossman et al., 2004).

The majority of arsenic-related cancer deaths result from lung cancer with in utero exposures increasing the risk of lung cancer later in life (Smith et al., 2006; Tokar et al., 2011b; IARC, 2012; ATSDR, 2016; Bailey et al., 2016; Steinmaus et al., 2016). Based upon a meta-analysis, Ferreccio et al. (2013) and Begum et al. (2015) estimated about 4.51 additional lung cancer cases per 100,000 people for a maximum contamination level of 10 µg/L of arsenic in drinking water and enhanced risk with co-exposure to tobacco smoke. Again, it is likely that arsenic is not the primary carcinogen, but synergizes with the carcinogenic potential of tobacco smoke constituents. Arsenic is unique among the metals and chemical carcinogens, because arsenic-promoted lung cancer is independent of route of exposure with oral ingestion and inhalation giving equivalent increases in risk of disease (Smith et al., 2009).

The unique kinetics of arsenic and its methylated trivalent metabolites greatly increase risk for transitional cell carcinoma of the bladder (Tokar et al., 2011a; IARC, 2012; Cohen et al., 2013). The metabolites are readily excreted into the urine and the enhanced concentration promote cyclic cell death and epithelial regeneration that ultimately results in cellular transformation (Cohen et al., 2013). However, metabolism of inorganic arsenite is not required to produce carcinogenic responses in the bladder epithelium, as hyperplasia and transformation is increased in mice lacking *As3mt*, relative to wild-type controls (Chen et al., 2011a). Inorganic arsenic also forms into intracellular cytoplasmic granules in the bladder epithelium (Yokohira et al., 2011). Bladder cancer risk is up to six-fold higher in arsenic-exposed females relative to males, and the association of arsenic exposure with bladder cancer risk is greatly enhanced in smokers or ever-smokers (Ferreccio et al., 2013). It appears that most renal cancers associated with arsenic exposure are transitional cell carcinomas and are thought to arise from the urothelial cells (IARC, 2012).

Treatment For acute arsenic poisoning, treatment is symptomatic, with particular attention to fluid volume replacement and support of blood pressure. The oral chelator penicillamine or succimer (2,3-dimercaptosuccinic acid [DMSA]) is effective in removing arsenic from the body. Dimercaptopropanesulfonic acid (DMPS) has also been used for acute arsenic poisoning with fewer side effects (Aposhian and Aposhian, 2006). However, for chronic poisoning, chelator therapy has not proved effective in relieving symptoms (Rahman et al., 2001; Liu et al., 2002) except for a limited preliminary trial with DMPS (Mazumder, 2005). The best current strategy for preventing chronic arsenic poisoning is by reducing exposure.

Beryllium

Beryllium (Be), an alkaline earth metal, was discovered in 1798. The name beryllium comes from the Greek *beryllos*, a term used for the mineral beryl. Beryllium alloys are used in automobiles, computers, sports equipment, and dental bridges. Pure beryllium metal is used in nuclear weapons, aircraft, x-ray machines, and mirrors. Beryllium compounds are divalent and human exposure to beryllium and its compounds occurs primarily in beryllium manufacturing, fabricating, or reclaiming industries. Individuals may also be exposed to beryllium from implanted dental prostheses. The general population is exposed to trace amounts of beryllium through the air, food, and water, as well as from cigarette smoke (IARC, 2012; ATSDR, 2015a).

Toxicokinetics The primary route of exposure to beryllium compounds is inhalation. After being deposited in the lung, beryllium is slowly absorbed into the blood. In patients accidentally exposed to beryllium dust, serum beryllium levels peak about 10 days after exposure with a biological half-life of 2 to 8 weeks (ATSDR, 2015a). Gastrointestinal and dermal absorption of beryllium is low (less than 1%), but incidental oral exposure to soluble beryllium compounds or exposure through damaged skin may significantly contribute to total body burden (Deubner et al., 2001). Most of the beryllium circulating in the blood is bound to serum proteins, such as prealbumins and globulins. A significant part of the inhaled beryllium is stored in the bone and lungs. More soluble beryllium compounds are distributed to the liver, lymph nodes, spleen, heart, muscle, skin, and kidney. Elimination of absorbed beryllium occurs mainly in the urine and only to a minor degree in the feces (ATSDR, 2015a). Because of the long residence time of beryllium in the skeleton and lungs, its biological half-life is over 1 year (ATSDR, 2015a). Beryllium persists in the lungs even decades after exposure causing increased potential for a sustained inflammatory response (Sawyer and Maier, 2011; ATSDR, 2015a).

Toxicity

Skin Effects Exposure to soluble beryllium compounds may result in conjunctivitis and papulovesicular dermatitis of the skin, which is likely an inflammatory response to beryllium (ATSDR, 2015a). Beryllium exposure may also cause a delayed-type hypersensitivity reaction in the skin, which is a cell-mediated immune response. If insoluble beryllium-containing materials become embedded under the skin, a chronic granulomatous lesion develops, which may be necrotizing and ulcerative. Beryllium fluoride patch test may in itself be sensitizing, which has been replaced by the use of 1% beryllium sulfate (ATSDR, 2015a; Fontenot et al., 2016). Furthermore, human leukocyte antigen (HLA)-DRArg74 (HLA-DR3) is linked to beryllium sensitization, but not chronic beryllium disease (Hultman and Pollard, 2015). However, the HLA-DRPheb47 gene is a susceptibility marker for beryllium hypersensitivity in individuals lacking HLA-DPGlu69 (Amicosante et al., 2005).

Acute Chemical Pneumonitis Inhalation of beryllium can cause a fulminating inflammatory reaction of the entire respiratory tract, involving the nasal passages, pharynx, tracheobronchial airways, and the alveoli. In the most severe cases, it produces acute fulminating pneumonitis (ATSDR, 2015a). This occurs almost immediately after inhalation of aerosols of soluble beryllium compounds, particularly the fluoride, during the ore extraction process. Fatalities have occurred, although recovery is generally complete after a period of several weeks or even months.

Chronic Granulomatous Disease Berylliosis, or chronic beryllium disease (CBD), was first described among fluorescent lamp workers exposed to insoluble beryllium compounds, particularly beryllium oxide. Granulomatous inflammation of the lung, along with dyspnea on exertion, cough, chest pain, weight loss, fatigue, and general weakness, are the most typical features (ATSDR, 2015a). Impaired lung function and hypertrophy of the right heart are also common. Chest x-rays show miliary mottling. Histologically, the alveoli contain small interstitial granulomas resembling those seen in sarcoidosis. In severe cases CBD may be accompanied by cyanosis and hypertrophic osteoarthropathy (ATSDR, 2015a). Beryllium sensitization after initial exposure can progress to CBD (Newman et al., 2005). As the lesions progress, interstitial fibrosis increases, with loss of functioning alveoli, impairment of effective air-capillary gas exchange, and increasing respiratory dysfunction. CBD involves an antigen-stimulated, cell-mediated immune response. HLA, T cells, and pro-inflammatory cytokines (TNF-α and IL-6) may be involved in the pathogenesis of CBD (Fontenot et al., 2016; McKee and Fontenot, 2016). The fact that certain small percentage of individuals exposed to beryllium develop CBD irrespective of the low level of exposure suggests a genetic factor in the pathogenesis of the disease (Laulicht et al., 2015). Polymorphisms in the DPB1 locus in the HLA-D region of chromosome 6, specifically glutamic acid at position 69, is associated with beryllium sensitivity and CBD (Laulicht et al., 2015; Fontenot et al., 2016). The genetic variant of the HLA-DPB1 gene generates a protein with a profound negative charge that increases the affinity to beryllium and thus increases the susceptibility to beryllium sensitization and CBD (Silveira et al., 2012).

Carcinogenicity A number of epidemiology studies in U.S. beryllium workers found that death due to lung cancer was increased, along with increased incidence of respiratory diseases (IARC, 2012; ATSDR, 2015a). The increase in lung cancers is linked to high exposure levels that occurred prior to stricter exposure regulations introduced in the 1950s. The likelihood of lung cancer was greater in workers with acute beryllium disease than in those with CBD (Gordon and Bowser, 2003; ATSDR, 2015a). Beryllium has been classified as a human carcinogen (IARC, 2012; NTP, 2016b).

Experimental studies confirmed carcinogenic potential of beryllium compounds by inhalation. For example, a single, short (less than 48-minute) exposure to 410 to 980 mg/m^3 beryllium metal aerosol induced lung tumors in rats 14 months after exposure. Chronic beryllium sulfate inhalation (13 months, 0.034 mg Be/m^3) resulted in 100% lung tumor incidence in rats (Gordon and Bowser, 2003). Injection of beryllium compounds also induced osteosarcomas in rabbits (IARC, 2012). Beryllium compounds are negative in bacterial mutation assays. In mammalian cells, soluble beryllium compounds show weak mutagenic potential, but can induce malignant transformation. The ability of beryllium compounds to produce chromosomal aberrations is controversial, and appears to depend on the compound, dose, and experimental conditions (Gordon and Bowser, 2003). The carcinogenic mechanism of beryllium is not yet clear. Several molecular events can occur including oncogene activation (K-*ras*, c-*myc*, c-*fos*, c-*jun*, and c-*sis*), and tumor suppressor gene dysregulation (p53, p16), but mutations in *p53* or K-*ras* are not evident. Beryllium-induced lung tumors show hypermethylation of p16 leading to loss of expression, and have decreased expression of genes associated with DNA repair. Finally, there is an obvious controversy in the carcinogenic effect between soluble and insoluble beryllium, thereby suggesting that the two forms of the metal have different pathological effects (Strupp, 2011).

Cadmium

Cadmium (Cd) is a toxic transition metal that was discovered in 1817 as an impurity of "calamine" (zinc carbonate) for which it is named (from the Latin *cadmia*). Until recently the industrial use of cadmium was quite limited, but now it has become an important metal with many uses. About 75% of cadmium produced is used in batteries, especially nickel–cadmium batteries. Because of its noncorrosive properties, cadmium has been used in electroplating or galvanizing alloys for corrosion resistance. It is also used as a color pigment for paints and plastics, in solders, as a barrier to control nuclear fission, as a plastic stabilizer, and in some special application alloys. This metal is typically found in ores with other metals, and is commercially produced as a by-product of zinc and lead smelting, which are sources of environmental cadmium. Cadmium ranks seventh on the ATSDR priority list of toxic substances and is the fourth most important metal (ATSDR, 2012a).

Exposure Cadmium is ubiquitous in the environment and exposure to the general population occurs mainly through ingestion of plant-derived foods and certain types of seafood, as well as inhalation of tobacco smoke and industrial emissions (Jarup and Akesson, 2009; ATSDR, 2012a). Many leafy plants readily accumulate cadmium from soil. Both natural and anthropogenic sources of cadmium contamination occur for soil, including fallout of industrial emissions, some fertilizers, soil amendments, and use of cadmium-containing water for irrigation, all resulting in a slow but steady increase in the cadmium content in vegetables over the years (Jarup and Akesson, 2009; ATSDR, 2012a). Shellfish accumulate relatively high levels of cadmium (1 to 2 mg/kg), and animal liver and kidney can have levels higher than 50 μg Cd/kg. Cereal grains such as rice and wheat, and tobacco concentrate cadmium to levels of 10 to 150 μg Cd/kg. With nearby industrial emission, air can be a significant source of direct exposure or environmental contamination (Jarup and Akesson, 2009). Total daily cadmium intake from all sources in North America and Europe ranges from 10 to 30 μg Cd per day. Of this, about 10% or less is retained (ATSDR, 2012a).

Tobacco leaves accumulate high levels of cadmium from the soil making cigarette smoking a major nonoccupational source of cadmium exposure. Smoking is thought to roughly double the lifetime body burden of cadmium (Satarug et al., 2010; ATSDR, 2012a).

Historically, levels of cadmium in the workplace have dramatically improved with the appreciation of its potential toxicity in humans, development of safety restrictions, and improved industrial hygiene. Inhalation is the dominant route of exposure in occupational settings (ATSDR, 2012a). Airborne cadmium in the present-day workplace environment is generally 5 μg/m^3 or less and occupational standards range from 2 to 50 μg/m^3 (ATSDR, 2012a). Occupations potentially at risk from cadmium exposure include those involved with refining zinc and lead ores, iron production, cement manufacture, and industries involving fossil fuel combustion, all of which can release airborne cadmium. Other occupations include the manufacture of paint pigments, cadmium–nickel batteries, and electroplating (ATSDR, 2012a).

Toxicokinetics Gastrointestinal absorption of cadmium is limited to 5% to 10% of a given dose. Cadmium absorption can be increased by dietary deficiencies of calcium or iron and by diets low in protein. In the general population, women have higher blood cadmium levels than men, possibly due to increased oral cadmium absorption because of relatively low iron stores in women of childbearing age. Indeed, women showing low serum ferritin levels have twice the normal rate of oral cadmium absorption (ATSDR, 2012a; Kim et al., 2015). It has recently been shown that rats on an iron-deficient diet have an increased absorption of cadmium, which correlated with the upregulation of the iron transporter, DMT, which transports both iron and cadmium (Ryu et al., 2004). Absorption of cadmium after inhalation is generally greater, ranging from 10% to 60%, depending on the specific compound, site of deposition, and particle size (Prozialeck and Edwards, 2010; ATSDR, 2012a). For instance, 5% to 50% of cadmium fumes, as generated in cigarette smoke, may be absorbed. As much as 100% of cadmium eventually reaching the alveoli can be transferred to blood (Satarug and Moore, 2004; ATSDR, 2012a).

The relationship of cadmium metabolism and toxicity is shown in Fig. 23-3. Once absorbed, cadmium is very poorly excreted and only about 0.001% of the body burden is excreted per day. Both urinary and fecal excretory routes are operative (Satarug and Moore, 2004; ATSDR, 2012a). The half-life of cadmium in humans is estimated to be more than 26 years and half-lives of several months up to several years have been calculated in rodents and other mammals (ATSDR, 2012a). Cadmium is transported in blood by binding to albumin and other higher-molecular-weight proteins. It is rapidly taken up by tissues and is primarily deposited in the liver and to a lesser extent in the kidney. Transport into cells is mediated through calcium channels (Leslie et al., 2006) and through molecular mimicry (Bridges and Zalups, 2005). In the liver, kidney, and other tissues, cadmium induces the expression of metallothioneins (MT) that are responsible for cellular retention (Klaassen et al., 1999). Cadmium is stored in the liver primarily as cadmium–MT. Cadmium–MT may be released from the liver and transported via blood to the kidney, where it is reabsorbed and degraded in the lysosomes of the renal tubules. This releases cadmium to induce more cadmium–MT complex or cause renal toxicity. Cadmium excretion in urine increases relative to body burden (ATSDR, 2012a). Cadmium is nephrotoxic, and when renal pathology is present the urinary excretion of cadmium is increased due to decreased renal absorption of filtered cadmium (Zalups and Ahmad, 2003).

Toxicity Acute, high-dose cadmium toxicity in humans is now a rare event. Acute cadmium toxicity from the ingestion of high concentrations of cadmium in the form of heavily contaminated beverages or food causes severe irritation to the gastrointestinal epithelium. Symptoms include nausea, vomiting, and abdominal pain. Inhalation of cadmium fumes or other heated cadmium-containing materials may produce acute pneumonitis with pulmonary edema. Inhalation of large doses of cadmium can be lethal for humans (ATSDR, 2012a). Acute cadmium toxicity depends on solubility of cadmium compounds (ATSDR, 2012a). For instance, with acute inhalation exposures, the more soluble cadmium chloride, oxide fume, and carbonate are more toxic than the relatively

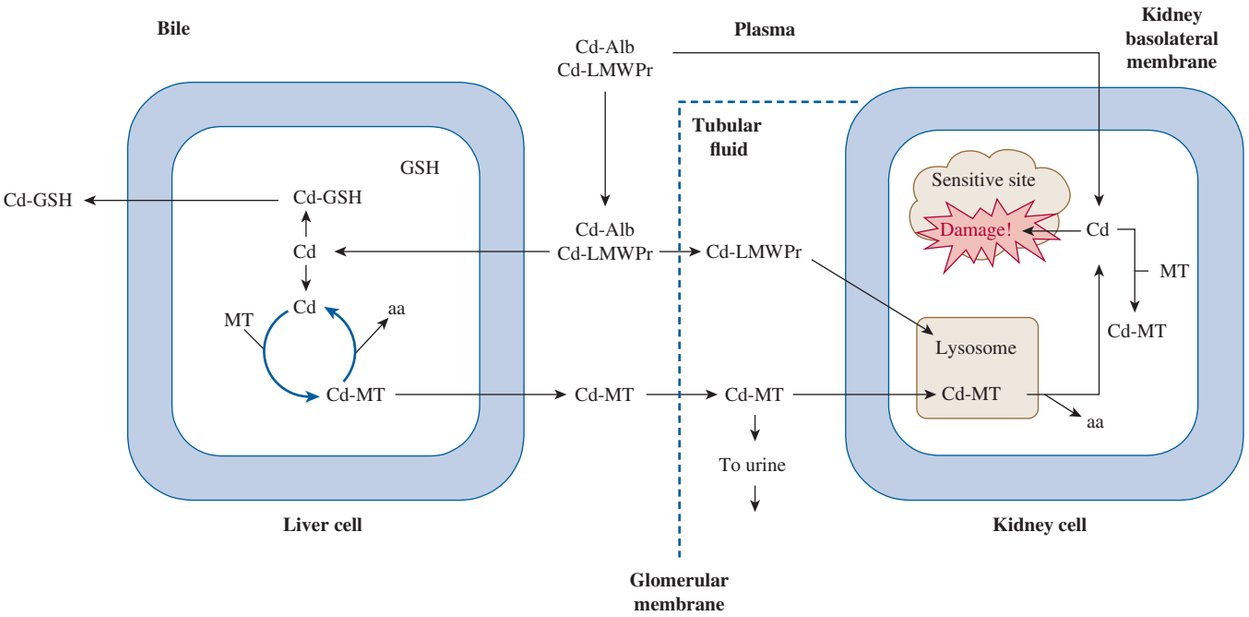

Figure 23-3. *Cadmium transport, protein binding, and toxicity.* aa, amino acids; Cd-Alb, Cd–albumin; Cd-LMWPr, Cd associated with low-molecular-weight proteins; GSH, glutathione; MT, metallothionein.

less soluble sulfide (Klimisch, 1993). The major long-term toxic effects of low-level cadmium exposure are renal injury, obstructive pulmonary disease, osteoporosis, and cardiovascular disease (Jarup and Akesson, 2009; ATSDR, 2012a). Cancer is primarily a concern in occupationally exposed groups. The chronic toxic effects of cadmium are clearly a much greater concern than the rare acute toxic exposures.

Nephrotoxicity Cadmium is toxic to tubular cells and glomeruli, markedly impairing renal function. Pathologically, these lesions consist of initial tubular cell necrosis and degeneration, progressing to an interstitial inflammation and fibrosis. There appears to be a critical concentration of cadmium in the renal cortex that, once exceeded, is associated with tubular dysfunction. This concentration depends on the individual, and chronic cadmium nephropathy is seen in about 10% of the population at renal concentrations of about 200 μg/g and in about 50% of the population at about 300 μg/g (Jarup and Akesson, 2009). Because of the potential for renal toxicity, there is considerable concern about the levels of dietary cadmium intake for the general population. In fact, it is thought that upwards of 7% of the general population may have significant cadmium-induced kidney alterations due to chronic exposure with kidney cadmium levels as low as 50 μg/g (Jarup and Akesson, 2009).

Cadmium-induced renal toxicity is reflected by proteinuria as a result of renal tubular dysfunction. The predominant proteins include β_2-microglobulin, *N*-acetyl-β-D-glucosaminidase (NAG), and MT, as well as retinol-binding protein, lysozyme, ribonuclease, α_1-microglobulin, and immunoglobulin light chains (Chen et al., 2006; Prozialeck and Edwards, 2010). The presence of larger proteins, such as albumin and transferrin, in the urine after occupational cadmium exposure suggests a glomerular effect as well. The pathogenesis of the glomerular lesion in cadmium nephropathy is not well understood (Prozialeck and Edwards, 2010). Urinary excretions of proteins and cadmium have been used as biomarkers for cadmium exposure.

The induction of MT by cadmium and the subsequent sequestration of cadmium as the cadmium–MT complex likely protect tissues from cadmium toxicity. However, if cadmium–MT complex is injected, it is acutely nephrotoxic (Nordberg, 2004). This led to the hypothesis that the cadmium–MT complex was responsible for chronic cadmium nephropathy. In this scenario, cadmium–MT released from the liver would be filtered by the kidney and reabsorbed in proximal tubule cells, where it is degraded releasing locally high levels of "free" cadmium (Fig. 23-3). Nephrotoxicity in normal rats after liver transplantation from cadmium-exposed rats supported this hypothesis (Chan et al., 1993). However, MT-null mice, which are unable to produce the major forms of MT, are hypersensitive to chronic cadmium nephropathy (Klaassen et al., 1999), suggesting that cadmium nephropathy is not necessarily mediated through the cadmium–MT complex. Kidney pathology from a single injection of cadmium–MT also differs greatly from that induced by chronic oral inorganic cadmium exposure (Liu et al., 1998). Inorganic cadmium can be taken into the kidney from the basolateral membrane, and is more toxic than cadmium–MT to cultured renal cells (Bridges and Zalups, 2005; Prozialeck and Edwards, 2010). It is likely that inorganic cadmium can bind to other low-molecular-weight proteins or other complexes for renal uptake, and these complexes can contribute to chronic cadmium nephropathy (Zalups and Ahmad, 2003).

Chronic Pulmonary Disease Cadmium inhalation is toxic to the respiratory system in a fashion related to the dose and duration of exposure (ATSDR, 2012a). Cadmium-induced obstructive lung disease in humans can be slow in onset, and results from chronic bronchitis, progressive fibrosis of the lower airways, and accompanying alveolar damage leading to emphysema. Pulmonary function is reduced with dyspnea, reduced vital capacity, and increased residual volume. The pathogenesis of these lung lesions is not completely understood, but can be duplicated in rodents (ATSDR, 2012a). The chronic effects of cadmium on the lung clearly increased the mortality of cadmium workers with high exposure.

Skeletal Effects Occupational cadmium exposure is a well-recognized cause for renal tubular dysfunction associated with hypercalciuria, renal stone formation, osteomalacia, and osteoporosis (Kazantzis, 2004). The long-term consumption of cadmium-contaminated rice caused Itai-Itai disease, which occurred mostly in multiparous elderly women and was characterized by severe osteomalacia and osteoporosis, resulting in bone deformities and concomitant renal dysfunction. Vitamin D deficiency and perhaps other nutritional deficiencies are thought to be cofactors in Itai-Itai disease (Kazantzis, 2004; Nordberg, 2009). Issues with loss of bone density, height loss, and increased bone fractures have now been reported in populations exposed to far lower levels of environmental cadmium than Itai-Itai victims (Kazantzis, 2004). Cadmium affects calcium metabolism, at least partially through renal dysfunction, and excess excretion of calcium often occurs in the urine. The skeletal changes are possibly related to a loss or decrease of calcium absorption, and interference with the actions of parathyroid hormone, disruption of collagen metabolism, and impairment of vitamin D activity (Lagerkvist et al., 1988), although the effects on vitamin D activity are debated (Engstrom et al., 2009). Cadmium may also act directly on bone, and animal studies have shown the metal stimulates osteoclast activity, resulting in the breakdown of bone matrix. Cadmium in bone interferes with calcification and bone remodeling (Kazantzis, 2004). In accord with human victims of Itai-Itai, multiparity in mice enhances the osteotoxicity of cadmium (Wester et al., 1981).

Cardiovascular Effects Some epidemiological evidence suggests cadmium may be an etiologic agent for cardiovascular disease including hypertension, although these associations are not observed in all studies (Jarup and Akesson, 2009; Julin et al., 2013; Larsson and Wolk, 2015). There is strong association between cadmium exposure and peripheral vascular disease and cadmium may partially mediate the negative effect of smoking on peripheral artery disease (Navas-Acien et al., 2004). Animal and cell culture experiments indicate that low-level cadmium induces endothelial cell angiogenesis, but that higher, environmentally relevant levels may directly damage the vascular endothelium (Prozialeck et al., 2008). Additional studies indicate that cadmium may be toxic to myocardium (Kopp et al., 1982), although the relevance of these results to humans is not clear.

Neurotoxicity There are only limited data from animals and humans that cadmium can be neurotoxic (Jarup and Akesson, 2009; ATSDR, 2012a). Studies in humans have suggested a relationship between abnormal behavior and decreased intelligence in children and adults exposed to cadmium, but are typically complicated by exposure to other toxic metals. Furthermore, the blood–brain barrier severely limits cadmium access to the central nervous system, and a direct toxic effect appears to occur only with cadmium exposure prior to blood–brain barrier formation (young children), or with blood–brain barrier dysfunction under certain pathological conditions. Additionally, the choroid plexus epithelium may accumulate high levels of cadmium, reducing access to other areas of the central nervous system (Zheng, 2001).

Reproductive Health Effects Increasing evidence indicates that cadmium exposure may be associated with multiple adverse

women's reproductive health outcomes (Pollack et al., 2014). In general, women have a higher body burden of cadmium than men, likely due to higher absorption of cadmium in women with low iron stores (ATSDR, 2012a; Vilahur et al., 2015). Pregnant women appear to accumulate more cadmium than nonpregnant women (Vilahur et al., 2015). Cadmium tends to accumulate in the placenta rather than crossing the placental barrier and consequently the strongest epidemiological evidence indicates an association between cadmium and preeclampsia (Pollack et al., 2014). There is additional suggestive support for association with negative fertility-related outcomes. In addition, prenatal exposure to cadmium is associated with adverse effects on birth outcomes, child health, and development with associations with epigenetic effects of cadmium interference with de novo DNA methyltransferases (Kippler et al., 2013; Vilahur et al., 2015).

Carcinogenicity Cadmium compounds are considered to be human carcinogens (IARC, 2012; NTP, 2016b). In humans, occupational respiratory exposure to cadmium has been most clearly associated with lung cancer (IARC, 2012; NTP, 2016b). Early human studies also indicated a possible link to cancer of the prostate, which has not been confirmed by more recent work (Chen et al., 2016a; NTP, 2016b), despite evidence that the prostate can be a target of cadmium carcinogenesis in rats (Waalkes, 2003). Both the kidney and pancreas accumulate high concentrations of cadmium, and exposure to cadmium may be associated with human renal and pancreatic cancer (Straif et al., 2009; IARC, 2012).

Multiple rodent studies have confirmed that inhalation of various cadmium compounds will lead to lung cancer (Waalkes, 2003; IARC, 2012; NTP, 2016b). Lung tumors can also be produced by systemic cadmium exposure in mice (Waalkes, 2003). Beyond the lung, in rodents cadmium can produce a variety of tumors, including malignant tumors at the site of repository injection (e.g., subcutaneous). Compounds such as cadmium chloride, oxide, sulfate, sulfide, and cadmium powder produce local sarcomas in rodents after subcutaneous or intramuscular injections. A single injection can be effective, but multiple injections of cadmium at the same site cause more aggressive sarcomas that show a higher rate of local invasion and distant metastasis. The relevance of injection site sarcoma production to human cancer is unclear. Cadmium also induces tumors of the testes, specifically benign Leydig cell tumors, but this is likely due to a high-dose mechanism involving acute testicular necrosis, degenerative testicular atrophy, and subsequent overstimulation by luteinizing hormone, factors very likely of limited relevance in humans (Waalkes, 2003). Other studies found that cadmium exposure can induce tumors of the pancreas, adrenals, liver, kidney, pituitary, and hematopoietic system in mice, rats, or hamsters. Cadmium can be carcinogenic in animals after inhalation or oral administration or by various injection routes (Waalkes, 2003). Various studies indicate that zinc administration will generally block cadmium carcinogenesis, whereas dietary zinc deficiency can enhance the response (Waalkes, 2003; IARC, 2012; NTP, 2016b). The mechanisms of cadmium carcinogenesis are poorly understood (Waalkes, 2003) and it is evident that direct DNA damage or genotoxicity has a minor role (Hartwig, 2013). Plausible mechanisms that are not mutually exclusive include aberrant gene expression and epigenetic regulation, inhibition of DNA damage repair, inhibition of apoptosis, and interference with normal cellular redox status and homeostasis (Waisberg et al., 2003; Hartwig, 2013).

Treatment At the present time, there is no effective clinical treatment for cadmium intoxication. In certain cases (Itai-Itai disease, osteomalacia) vitamin D is prescribed, although its effects have not been satisfactory (Nordberg, 2009). In experimental systems, some chelators can reduce acute cadmium-induced mortality, but chelation therapy for cadmium generally results in significant adverse effects.

Chromium

Chromium (Cr) was named from the Greek word "chroma" meaning color, because of the many colorful compounds made from it. It is part of the mineral crocoite (lead chromate), and the element was first isolated in 1798. Most naturally occurring chromium is found in the trivalent state in chromite ores, which are generally refined to ferrochromium or metallic chromium for use in industrial processes. Because trivalent chromium (Cr^{3+}) has been suggested to be an essential trace nutrient important for glucose metabolism, it will be discussed separately in the section "Essential Metals with Potential for Toxicity."

Hexavalent chromium (Cr^{6+}) is rarely found in nature and is formed as a by-product of industrial processes. Most chromite ores are processed to sodium dichromate, a hexavalent chromium compound, which is used as an oxidizing agent in stainless steel production and welding, chromium plating, ferrochrome alloys and chrome pigment production, and tanning industries (ATSDR, 2012b). Hexavalent chromium is a human carcinogen and produces a variety of toxic effects (Carlier et al., 1991; ATSDR, 2012b; IARC, 2012). Chromium in ambient air originates primarily from industrial sources, particularly ferrochrome production, ore refining, and chemical processing. Chromium fallout is deposited on land and water, and, eventually, in sediments. Widespread industrial uses have increased chromium levels in the environment. The hexavalent chromium compounds are also toxic to ecosystems, and microbial and plant variants occur that adapt to high chromium levels in eco-environment (Cervantes et al., 2001). Up to 38% of drinking water supplies in California have detectable levels of hexavalent chromium, but little is known about the health effects from environmental exposures (Sedman et al., 2006; Smith and Steinmaus, 2009; Sun et al., 2015). Cobalt–chromium alloy hip replacement can increase blood levels of chromium and cause systemic toxicities (Bradberry et al., 2014; Madl et al., 2015).

Toxicokinetics Absorption of hexavalent chromium compounds is higher (2% to 10%) than that of trivalent chromium compounds (0.5% to 2%) (ATSDR, 2012b). Inhaled chromium compounds are absorbed in the lung via transfer across alveolar cell membranes. Dermal absorption depends on the chemical form, vehicle, and integrity of the skin. Concentrated potassium chromate may cause chemical burns to the skin and facilitate absorption. Hexavalent chromium readily crosses cell membranes via sulfate and phosphate transporters, while trivalent chromium compounds form octahedral complexes making entry into cells difficult (Standeven and Wetterhahn, 1989; ATSDR, 2012b). Once in the blood, hexavalent chromium is taken up by erythrocytes, while trivalent chromium is only loosely associated with erythrocytes. Chromium compounds are distributed to all organs of the body, with high levels in the liver, spleen, and kidney. Particles containing chromium can be retained in the lungs for years. Absorbed chromium is excreted primarily in urine. The half-life for excretion of oral potassium chromium is about 35 to 40 hours (Sedman et al., 2006; ATSDR, 2012b).

Once hexavalent chromium enters cells, it is reduced intracellularly by ascorbic acid, glutathione, and/or cysteine, ultimately to trivalent chromium. It is thought that the toxicity of hexavalent chromium compounds results from damage to cellular components during this process, including the generation of free radicals and the formation of DNA adducts (Zhitkovich, 2005; Reynolds et al., 2012; Sun et al., 2015).

Toxicity Toxic effects have been attributed primarily to airborne hexavalent chromium compounds in industrial settings (Gibb et al., 2000a, 2000b). Hexavalent chromium is corrosive and may cause chronic ulceration and perforation of the nasal septum, as well as chronic ulceration of other skin surfaces (ATSDR, 2012b). It elicits allergic contact dermatitis among previously sensitized individuals, which is a type IV allergic reaction inducing skin erythema, pruritus, edema, papule, and scars. The prevalence of chromium sensitivity is less than 1% among the general population and up to 4% in occupational settings (Shelnutt et al., 2007). Occupational exposure to chromium may be a cause of asthma (Bright et al., 1997; Antonini et al., 2004). Accidental ingestion of high doses of hexavalent chromium compounds may cause acute renal failure characterized by proteinuria, hematuria, and anuria, but kidney damage from lower level chronic exposure is equivocal (ATSDR, 2012b).

Carcinogenicity Hexavalent chromium compounds are classified as *known to be human carcinogens* by the National Toxicology Program (NTP, 2016b). Occupational exposure to hexavalent chromium compounds, particularly in the chrome production and pigment industries, is associated with increased risk of lung cancer (Gibb et al., 2000b) and hexavalent chromium–containing compounds are considered to be human carcinogens (IARC, 2012). An association of hexavalent chromium in the drinking water with stomach cancer has also been suggested (Sedman et al., 2006; Smith and Steinmaus, 2009), but the evidence that chromium causes stomach cancer or other cancers is inconclusive (IARC, 2012).

Hexavalent chromium compounds are genotoxic; a review of more than 700 sets of short-term genotoxicity test results with 32 chromium compounds revealed 88% of hexavalent chromium compounds were positive, as a function of solubility and bioavailability to target cells (De Flora, 2000). Trivalent chromium compounds were generally non-genotoxic, probably because trivalent chromium is not readily taken up by cells. However, once hexavalent chromium enters cells, it is reduced by various intracellular reductants to create reactive chromium species and it is likely that trivalent chromium is the ultimate DNA damaging species (Zhitkovich, 2005; Reynolds et al., 2012; Sun et al., 2015). During the reduction process, various genetic lesions can be generated, including chromium–DNA adducts, DNA–protein cross-links, DNA–chromium intrastrand cross-links, DNA strand breaks, and oxidized DNA bases (O'Brien et al., 2003; Macfie et al., 2010). Hexavalent chromium compounds are mutagenic, causing base substitutions, deletions, and transversions in bacterial systems, and hypoxanthine guanine phosphoribosyltransferase, *supF* mutations, etc. in mammalian mutagenesis systems (O'Brien et al., 2003).

Hexavalent chromium compounds also react with other cellular constituents during the intracellular reduction process. They can cause the generation of reactive oxygen radicals, inhibit protein synthesis, and arrest DNA replication. Hexavalent chromium can disrupt the p53 signaling pathway, alter the ATM/ATR cell cycle checkpoints, induce apoptosis, and interfere with DNA damage repair (Zhitkovich, 2005; Salnikow and Zhitkovich, 2008). All these effects could well play an integrated role in chromium carcinogenesis (O'Brien et al., 2003; Sun et al., 2015). Hexavalent chromium can also directly react with and activate cell signaling enzymes, such as Src family kinases, to stimulate signal cascades leading to transcriptional repression of important cellular protectant proteins (O'Hara et al., 2003; Nemec and Barchowsky, 2009; Nemec et al., 2010).

Lead

Lead (Pb) has been used by humans for at least 7000 years, because it is ubiquitous, easy to extract, highly malleable, and ductile. In the early Bronze Age, lead was used with antimony and arsenic. Lead's elemental symbol, Pb, is an abbreviation of its Latin name *plumbum*. Lead in lead compounds primarily exists in the divalent form. Metallic lead (Pb^0) is resistant to corrosion and can combine other metals to form various alloys. Organolead compounds are dominated by Pb^{4+}. Inorganic lead compounds are used as pigments in paints, dyes, and ceramic glazes. Organolead compounds were widely used as gasoline additives until the late 1980s causing global increases in atmospheric lead. Lead alloys are used in batteries, shields from radiation, water pipes, and ammunition. Environmental lead comes mainly from human activity and is listed as a top toxic substance (ATSDR, 2007c, 2015b). The phasing out of leaded gasoline and the removal of lead from paint, solder, and water supply pipes have significantly lowered blood lead levels in the general population. Lead exposure in children remains a major health concern and lead contamination from aging water supply infrastructure is a major public health concern in a majority of U.S. cities and municipalities. Lead is not biodegradable and ecotoxicity of lead remains a concern (Bellinger et al., 2013). For instance, the leaded fish sinkers or pellets lost in the bottom of lakes and riverbanks can be mistaken for stone and ingested by birds causing adverse effects including death (De Francisco et al., 2003).

Exposure Blood lead levels in the general population of the United States have been decreasing over the past three decades as regulations regarding lead paint, leaded fuels, and lead-containing plumbing materials have reduced exposure (ATSDR, 2007c). Occupational exposure to lead occurs for workers in the lead smelting and refining industries, battery manufacturing plants, glass manufacturing, steel welding or cutting operations, construction, rubber products and plastics industries, printing industries, firing ranges, radiator repair shops, and other industries requiring flame soldering of lead solder (ATSDR, 2007c). In these occupations, the major routes of lead exposure are inhalation and ingestion of lead-bearing dusts and fumes.

Lead-containing paint in older housing and aging water infrastructure are primary sources of lead exposure in the general population and in children (Needleman, 2004; Bellinger, 2011; Brown and Margolis, 2012). Major environmental sources of lead for infants and toddlers up to 4 years of age is hand-to-mouth transfer of lead-containing paint chips or dust from floors of older housing (Needleman, 2004; Bellinger, 2011). Lead in household dust can also come from outside of the home and may be related to lead in neighborhood soil (von Lindern et al., 2003; von Lindern et al., 2016). A major route of exposure for the general population is from food and water (Brown and Margolis, 2012). Dietary intake of lead has decreased dramatically in recent years, and for infants, toddlers, and young children is less than 5 μg/day (Manton et al., 2005). Herbal medicines could be potential sources of lead exposure (Levin et al., 2008). Certain Ayurvedic herbal products contained lead levels ranging up to 37 mg/g and over 55 cases of lead poisoning have been related to the ingestion of herbal medicines (Patrick, 2006). A review by the EPA in 2004 found lead levels in 71% of the water systems in the United States showed less than 5 μg Pb/L (ppb) (ATSDR, 2007c). Only 3.6% exceeded the action level of 15 ppb. Lead in urban air is generally higher than that in rural air. Air lead in rural areas of eastern United States is typically 6 to 10 ng/m^3 (ATSDR, 2007c). Blood lead levels and x-ray fluorescence (XRF) analysis of lead in bone are the primary biomarkers for exposure.

Toxicokinetics Adults absorb 5% to 15% of ingested lead and usually retain less than 5% of what is absorbed. Children absorb 40% to 50% of ingested lead with 32% retention (ATSDR, 2007c). Lead absorption can be enhanced by low dietary zinc, manganese,

iron, and calcium, especially in children (ATSDR, 2007c; Wu et al., 2011). Airborne lead is a minor component of exposure. Lead absorption by the lungs depends on the form (vapor vs. particle), particle size, and concentration. About 90% of lead particles in ambient air that are inhaled are small enough to be retained. Absorption of retained lead through alveoli is relatively efficient.

Lead in blood is primarily (about 99%) in erythrocytes bound to hemoglobin; only 1% of circulating lead in serum is available for tissue distribution. Lead is initially distributed to soft tissues such as the kidney and liver, and then redistributed to the skeleton and hair. The half-life of lead in the blood is about 30 days. The fraction of lead in bone increases with age from 70% of body burden in childhood to as much as 95% in adulthood, with a half-life of about 20 years. Lead released from bones may contribute up to 50% of the lead in blood, and can be a significant source of endogenous exposure. Bone lead release may be important in adults with accumulated exposure and in women due to bone resorption during pregnancy, lactation, and menopause, and from osteoporosis (Gulson et al., 2003; Silbergeld and Patrick, 2005; ATSDR, 2007c). Lead crosses the placenta, so that amounts in cord blood generally correlate with maternal blood concentration, but is often slightly lower. Lead accumulation in fetal tissues, including brain, is proportional to maternal blood levels (Gulson et al., 2003; Bellinger, 2011)

The major route of excretion of absorbed lead is the kidney. Renal excretion of lead is usually through glomerular filtrate with some renal tubular resorption. Fecal excretion via biliary tract accounts for one-third of total excretion of absorbed lead (ATSDR, 2007c). Physiological-based pharmacokinetic (PBPK) models have been developed for lead risk assessment. The O'Flaherty model is a model for children and adults (Fleming et al., 1999). The integrated exposure uptake biokinetic (IEUBK) model was developed by the EPA for predicting blood lead level in children (Mickle, 1998). The Leggett model allows simulation of lifetime exposures and can be used to predict blood lead in both children and adults (ATSDR, 2007c).

Toxicity Lead can induce a wide range of adverse effects in humans depending on the dose and duration of exposure. The toxic effects range from inhibition of enzymes to the production of severe pathology or death (ATSDR, 2007c). Children are most sensitive to effects in the central nervous system (Bellinger, 2011; Wu et al., 2011), while in adults, peripheral neuropathy and hypertension are concerns (ATSDR, 2007c; Navas-Acien et al., 2007). Other target tissues include the gastrointestinal, immune, skeletal, and reproductive systems. Effects on heme biosynthesis provide a sensitive biochemical indicator even in the absence of other detectable effects.

Neurological, Neurobehavioral, and Developmental Effects in Children Clinically overt lead encephalopathy may occur in children with high exposure to lead, probably at blood lead level of 70 μg/dL or higher (ATSDR, 2007c). Symptoms of lead encephalopathy begin with lethargy, vomiting, irritability, loss of appetite, and dizziness, progressing to obvious ataxia, and a reduced level of consciousness, which may progress to coma and death. The pathological findings at autopsy are severe edema of the brain due to extravasations of fluid from capillaries in the brain. This is accompanied by the loss of neuronal cells and an increase in glial cells. Recovery is often accompanied by sequelae including epilepsy, mental retardation, and, in some cases, optic neuropathy and blindness (ATSDR, 2007c; Bellinger, 2011; NTP, 2012).

The most sensitive indicators of adverse neurological outcomes are psychomotor tests or mental development indices, and broad measures of IQ. Most studies report a 2- to 4-point IQ deficit for each μg/dL increase in BLL within the range of 5 to 35 μg/dL (NTP, 2012). The Centers for Disease Control and Prevention set the goal of eliminating more than or equal to 10 μg/dL BLL in children by 2020 (CDC, 2012). However, effects of lead on IQ may occur below this level (Bellinger, 2011; Budtz-Jorgensen et al., 2013). Recent studies found that deficits in cognitive and academic skills could occur with BLL less than 5.0 μg/dL (Budtz-Jorgensen et al., 2013). A study of a cohort of children from pregnancy to 10 years of age found that lead exposure around 28 weeks of gestation is a critical period for later child intellectual development, and lead's effect on IQ occurs with first few micrograms of BLL (Schnaas et al., 2006; NTP, 2012). In addition, an association of childhood BLL with cognitive function and socioeconomic status in adults has been recently established (Bellinger, 2017; Reuben et al., 2017).

Lead can affect the brain by multiple mechanisms. It may act as a surrogate for calcium and/or disrupt calcium homeostasis (Marchetti, 2014). Lead affects virtually every neurotransmitter system in the brain, including glutamatergic, dopaminergic, and cholinergic systems (Marchetti, 2014). The stimulation of protein kinase C through mimicking calcium may alter blood–brain barrier and inhibition of cholinergic modulation of glutamate-related synaptic transmissions (Marchetti, 2003). All these systems play a critical role in synaptic plasticity and cellular mechanisms for cognitive function, learning, and memory (NTP, 2012).

Neurotoxic Effects in Adults Adults with occupational exposure may demonstrate abnormalities in a number of measures in neurobehavior with cumulative exposures resulting from BLL more than 40 μg/dL, whereas encephalopathy occurs with exposures above 100 μg/dL (ATSDR, 2007c; Bellinger, 2011). Peripheral neuropathy is a classic manifestation of lead toxicity in adults. More than a half-century ago, foot drop and wrist-drop characterized the house painter and other workers with excessive occupational exposure to lead but are rare today. Peripheral neuropathy is characterized by segmental demyelination and possibly axonal degeneration. Motor nerve dysfunction, assessed clinically by electrophysiological measurement of nerve conduction velocities, occurred with BLL as low as 40 μg/dL (Goyer, 1993).

Hematologic Effects Lead has multiple hematologic effects, ranging from increased urinary porphyrins, coproporphyrins, δ-aminolevulinic acid (ALA), and zinc protoporphyrin to anemia (ATSDR, 2007c). The heme biosynthesis pathway and the sites of lead interference are shown in Fig. 23-4. The most sensitive effects of lead are the inhibition of δ-aminolevulinic acid dehydratase (ALAD) and ferrochelatase. ALAD catalyzes the condensation of two units of ALA to form porphobilinogen (PBG). Inhibition of ALAD results in accumulation of ALA. Ferrochelatase catalyzes the insertion of iron into the protoporphyrin ring to form heme. Inhibition of ferrochelatase results in accumulation of protoporphyrin IX, which takes the place of heme in the hemoglobin molecule and, as the erythrocytes containing protoporphyrin IX circulate, zinc is chelated at the site usually occupied by iron. Erythrocytes containing zinc protoporphyrin are intensely fluorescent and may be used to diagnose lead exposure. Feeding lead to experimental animals also raises heme oxygenase activity, resulting in increases in bilirubin formation. Anemia only occurs in very marked cases of lead toxicity, and is microcytic and hypochromic, as in iron deficiency. The changes in ALAD in peripheral blood and excretion of ALA in urine correlate with BLL and serve as early biochemical indices of lead exposure (ATSDR, 2007c). Genetic polymorphisms have been identified for alleles of the *ALAD* gene that may affect the toxicokinetics of lead (Montenegro et al., 2006). However, no

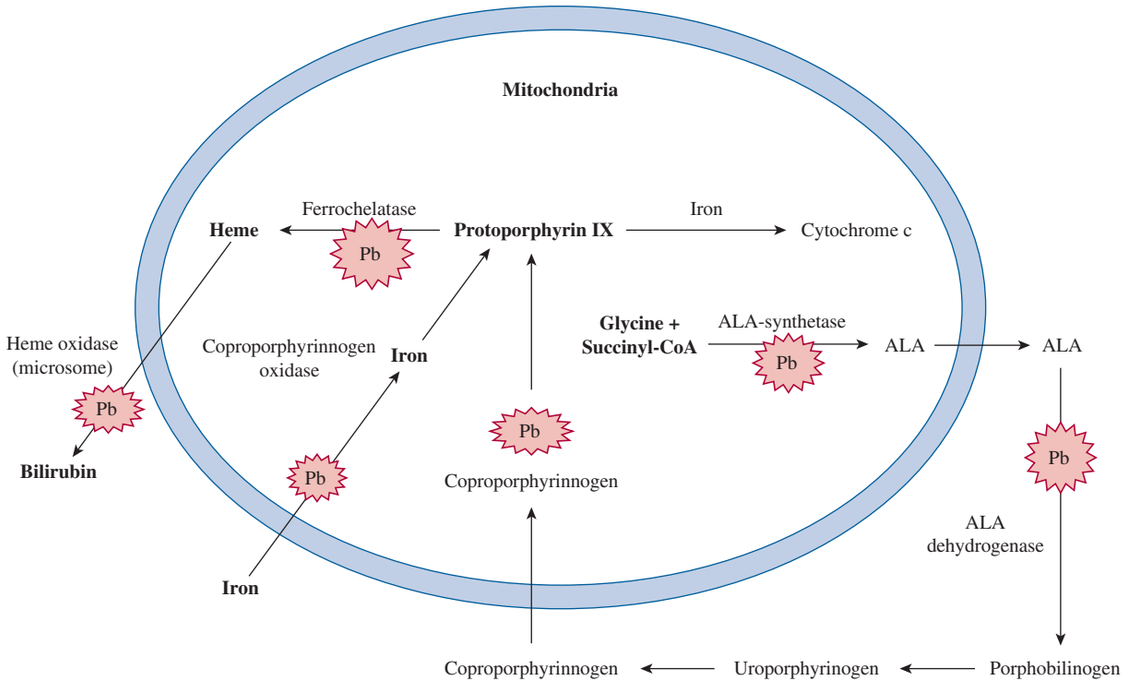

Figure 23-4. *Lead interruption of heme biosynthesis.* ALA, δ-aminolevulinate; Pb, sites for lead effects. The major lead inhibition sites are ALA dehydrogenase and ferrochelatase.

firm evidence exists for an association between *ALAD* genotype and susceptibility to lead toxicity at background exposures (van Bemmel et al., 2011).

Renal Toxicity Acute lead nephrotoxicity consists of proximal tubular dysfunction and can be reversed by treatment with chelating agents. Chronic lead nephrotoxicity consists of interstitial fibrosis and progressive nephron loss, azotemia, and renal failure (Goyer, 1993). However, occurrence of lead nephrotoxicity is mostly associated with very high level exposure and has declined (Goyer, 1993). A characteristic microscopic change is the presence of intranuclear inclusion bodies. By light microscopy the inclusions are dense, homogeneous, and eosinophilic with hematoxylin and eosin staining. The bodies are composed of a lead–protein complex. The protein is acidic and contains large amounts of aspartic and glutamic acids with little cystine. The inclusion bodies are a form of aggresome accumulating large amounts of lead in a relatively inert, nontoxic state. MT-null mice cannot form inclusion bodies after lead treatment and are hypersensitive to lead-induced nephropathy and carcinogenesis, suggesting that lead inclusion body formation requires MT as a participant (Waalkes et al., 2004). In fact, MT is found on the outer surface of lead inclusion bodies, indicating that it may transport the metal to the forming inclusion (Waalkes et al., 2004). Lead nephrotoxicity impairs the renal synthesis of heme-containing enzymes in the kidney, such as heme-containing hydroxylase involved in vitamin D metabolism causing bone effects (ATSDR, 2007c). Again these are effects of high level exposures, and the association of chronic kidney disease with environmental or general population exposures remains controversial (Bellinger, 2011; Evans and Elinder, 2011).

Effects on Cardiovascular System There is evidence of a causal relationship between lead exposure and hypertension (ATSDR, 2007c; Navas-Acien et al., 2007; NTP, 2012). This includes sufficient evidence that both BLL and bone lead levels are positively associated with general hypertension and hypertension in pregnancy (Silbergeld and Patrick, 2005; NTP, 2012). However, bone lead, a more reliable marker of chronic exposure, is more consistently associated with both hypertension and cardiovascular mortality (NTP, 2012). Analysis of data from the NHANES II for the U.S. population, including BLL and blood pressure measurements in a general population (5803 people aged 12 to 74), found a correlation between BLL at relatively low levels and blood pressure (Harlan, 1988). Further investigation with a systematic review of human data indicated a causal relationship between lead and hypertension (Navas-Acien et al., 2007). Elevated blood pressure is more pronounced in middle age than at young age (ATSDR, 2007c).

The pathogenesis of lead-induced hypertension is multifactorial. Lead interferes with endogenous nitric oxide generation and subsequent cGMP-dependent vasorelaxation, possibly through lead-induced ROS generation. Lead may mimic calcium in activating renin secretion in the renin–angiotensin–aldosterone system, as well as increase sympathetic to promote hypertension. Lead alters calcium-activated contraction and functioning of vascular smooth muscle cells, including increased contractility by decreasing Na^+/K^+-ATPase activity and stimulating the Na^+/Ca^{2+} exchange pump. Lead may also increase secretion of vasoconstrictive ligands, such as endothelin and thromboxane (ATSDR, 2007c). It is likely that renal dysfunction caused by lead contributes to its cardiovascular effects, although it is also true that the cardiovascular effects and hypertension contribute to renal dysfunction (NTP, 2012).

Immunotoxicity In 2012, the NTP concluded that there was limited evidence that low BLL (less than 10 mg/dL) are associated with adverse immune outcomes in children and inadequate

evidence for adults (NTP, 2012). Higher exposures in workers has demonstrated a stronger association with immune dysfunction (ATSDR, 2007c). The developing immune system is sensitive to toxic effects of lead (Dietert, 2008; Bellinger, 2011). A hallmark of lead-induced immunotoxicity is a pronounced shift in the balance in T-helper cell function toward Th2 responses at the expense of Th1 functions, resulting in elevated IgE levels (Leifer and Dietert, 2011). Increased IgE levels and inflammatory cytokines were found in lead-exposed neonatal rodents, and there is an association between BLL and elevated IgE levels in children (Luebke et al., 2006; NTP, 2012). In experimental animals, lead has been shown to target macrophages and T cells, especially $CD4^+$ T cells. In occupational exposure, lead-associated immunologic changes include altered T-cell subpopulations, reduced immunoglobulin levels, and reduced polymorphonuclear leukocyte chemotactic activity (Luebke et al., 2006; ATSDR, 2007c; NTP, 2012)

Bone Effects Lead has an extremely long half-life in bone, accounting for over 90% of the body lead in adults (ATSDR, 2007c; NTP, 2012). It can affect bone by interfering with metabolic and homeostatic mechanisms including parathyroid hormone, calcitonin, vitamin D, and other hormones that influence calcium metabolism, as lead substitutes for calcium in bone. It is known to affect osteoblasts, osteoclasts, and chondrocytes and has been associated with osteoporosis and delays in fracture repair (Carmouche et al., 2005). In children exposed to lead, a higher bone mineral density (BMD) was observed (Campbell et al., 2004). This may be due to accelerated bone maturation through inhibition of parathyroid hormone–related peptide, may ultimately result in lower peak BMD in young adulthood, and might predispose subjects to osteoporosis later in life (Campbell et al., 2004; ATSDR, 2007c). A positive association between lead exposure and dental caries in children has been shown in a number of studies. Lead is deposited in teeth, inhibits mineralization of enamel and dentine, and affects metabolism of the cells in the dental pulp (ATSDR, 2007c). Lead in bone is recognized as a potential source for exposure to other tissues when bone is mobilized, as during pregnancy (Silbergeld and Patrick, 2005).

Other Effects Lead colic is a major gastrointestinal symptom of severe lead poisoning, and is characterized by abdominal pain, nausea, vomiting, constipation, and cramps (ATSDR, 2007c). It is rarely seen today, except in intentional poisoning. Lead-induced gametotoxic effects have been demonstrated in both male and female animals (Goyer, 1993).

Carcinogenicity Lead and lead compounds are reasonably anticipated to be human carcinogens based on limited evidence of carcinogenicity from studies in humans and sufficient evidence of carcinogenicity from studies in experimental animals (NTP, 2016b). The association of lead exposure with increased human cancer risk was strengthened by recent studies (ATSDR, 2007c), and inorganic lead compounds were recently reclassified as probably carcinogenic to humans while organic lead compounds were considered not classifiable as to human carcinogenicity (IARC, 2006). A study of a cohort of 20,700 workers co-exposed to lead and engine exhaust found a 1.4-fold increase in the overall cancer incidence and a 1.8-fold increase in the incidence of lung cancer among those who ever had elevated BLL (Anttila et al., 1995). Another epidemiological study of 27,060 brain cancer cases and 108,240 controls who died of nonmalignant disease in the United States from 1984 to 1992 provides evidence for a potential link between occupational exposure to lead and brain cancer (Cocco et al., 1998). Analysis of eight principal studies with well-documented lead exposures suggests associations of lead exposure with increased lung and stomach cancers (Steenland and Boffetta, 2000). However, workers were not exposed to lead alone, and exposures to other potential carcinogens such as arsenic, cadmium, and engine exhausts could confound these interpretations. Lead does not appear to be directly genotoxic in vivo or in vitro, and lead may interact with other toxicants to facilitate chemical carcinogenesis (Silbergeld, 2003).

Lead is a nephrocarcinogen in adult rodents (IARC, 2012). Lead-induced renal tumors also occur after perinatal exposure in the absence of the extensive chronic nephropathy (Waalkes et al., 1995). MT-null mice, which do not form lead inclusion bodies, are hypersensitive to lead-induced proliferative lesions of the kidney (Waalkes et al., 2004) and develop testicular teratomas (Tokar et al., 2010) compared with similarly exposed wild-type mice.

Several mechanisms have been proposed for lead-induced carcinogenesis, including regenerative repair, inhibition of DNA synthesis or repair, generation of ROS with oxidative damage to DNA, substitution of lead for zinc in transcriptional regulators, interaction with DNA-binding proteins, and aberrant gene expression (Silbergeld, 2003).

Treatment Chelation therapy is warranted in workmen with BLL more than 60 μg/dL. For children, criteria have been established (Laraque and Trasande, 2005) that may serve as guidelines to assist in evaluating the individual case with potential health effects. The oral chelating agent DMSA (also called succimer) has advantages over EDTA in that it can be given orally and is effective in temporarily reducing BLL. However, DMSA neither improves long-term BLL in children nor reduces brain lead levels beyond the cessation of lead exposure alone (Smith and Strupp, 2013; Andersen and Aaseth, 2016). A recent study shows that DMSA lowered BLL in children, but had no detectable benefit on learning and behavior (Dietert et al., 2004). Chelation therapy is nonetheless still recommended for children (Warniment et al., 2010). Treatment of organic lead poisoning is symptomatic.

Mercury

Mercury (Hg) was named after the Greco-Roman god known for swift flight. Also called quicksilver, metallic mercury is in liquid state at room temperature. The symbol Hg was derived from the Latinized Greek *hydrargyrum*, meaning "water" and "silver." Mercury was known in ancient times from approximately 1500 BC. By 500 BC mercury was used to make amalgams with other metals. Mercury vapor (Hg^0) is much more hazardous than the liquid form. Mercury binds to other elements (such as chlorine, sulfur, or oxygen) to form inorganic mercurous (Hg^{1+}) or mercuric (Hg^{2+}) salts. This metal can form a number of stable organometallic compounds by attaching to one or two carbon atoms. Methylmercury (CH_3Hg^+, or MeHg) is the toxicologically most important organic form (ATSDR, 2013a). Mercurial compounds have characteristic toxicokinetics and health effects that depend on oxidation state and associated organic species (ATSDR, 2013a).

Global Cycling and Ecotoxicology Mercury exemplifies movement of metals in the environment (Fig. 23-5). Atmospheric mercury, in the form of mercury vapor (Hg^0), is derived from natural degassing of the Earth's crust and through volcanic eruptions as well as from evaporation from oceans and soils. Anthropogenic sources are estimated to contribute two-thirds of the total atmospheric mercury (Chen et al., 2016b; Gonzalez-Raymat et al., 2017). These contributions include emissions from metal mining and smelting (mercury, gold, copper, and zinc), coal combustion, municipal incinerators, and chloralkali industries. Mercury vapor is

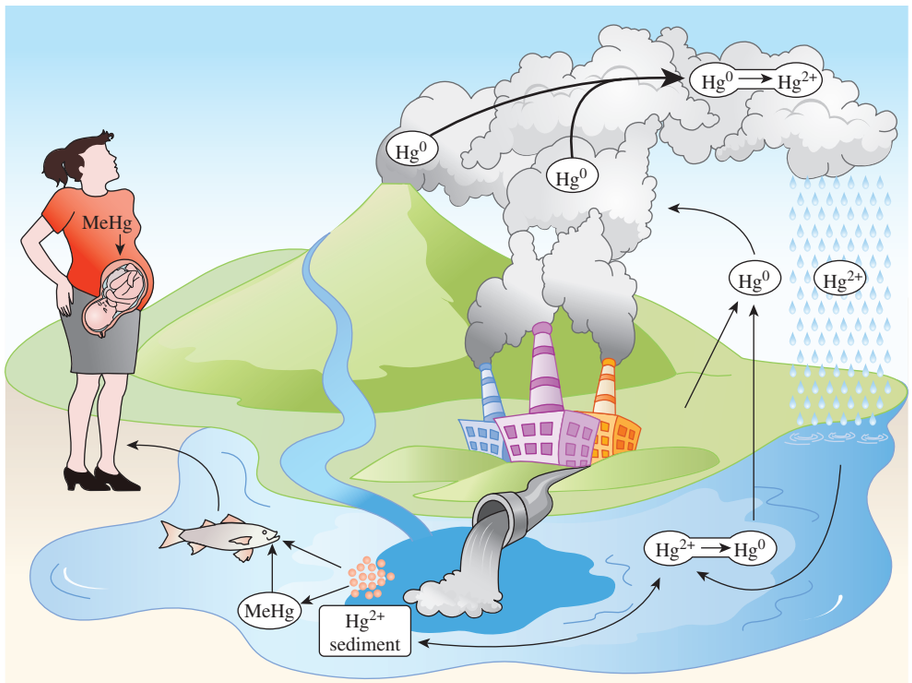

Figure 23-5. *The movement of mercury in the environment.* In nature, mercury vapor (Hg^0), a stable monoatomic gas, evaporates from the Earth's surface (both soil and water) and is emitted by volcanoes. Anthropogenic sources include emissions from coal-burning power stations and municipal incinerators. After approximately 1 year, mercury vapor is converted to soluble form (Hg^{2+}) and returned to the Earth by rainwater. It may be converted back to the vapor by microorganisms and reemitted into the atmosphere. Thus, mercury may recirculate for long periods. Mercury attached to aquatic sediments is subjected to microbial conversion to methylmercury, starting with plankton, then herbivorous fish, and finally ascending to carnivorous fish and sea mammals. This biomethylation and biomagnification result in human exposure to methylmercury through consumption of fish, and pose the health risk to humans, especially the developing fetus.

a chemically stable monatomic gas and its residence time in atmosphere is about 1 year. Thus, mercury is globally distributed even from point resources. Eventually it is oxidized to a water-soluble inorganic form (Hg^{2+}), and returned to the Earth's surface in rainwater. The metal may then be reduced back to mercury vapor and returned to the atmosphere, or it may be methylated by microorganisms present in sediments of bodies of fresh and ocean water (Chen et al., 2016b). This natural biomethylation reaction produces methylmercury (MeHg). Methylmercury enters the aquatic food chain starting with plankton, then herbivorous fish, and finally ascending to carnivorous fish and sea mammals. On the top of the food chain, tissue mercury can rise to levels 1800 to 80,000 times higher than levels in the surrounding water. This biomethylation and bioconcentration result in human exposure to methylmercury through consumption of fish (Magos and Clarkson, 2006; Chen et al., 2016b). Organomercurial compounds are generally more toxic than inorganic mercury to aquatic organisms, aquatic invertebrates, fish, plants, and birds. Organisms in the larval stages are generally more sensitive to toxic effects of mercury (Magos and Clarkson, 2006; Chen et al., 2016b).

Exposure

Dietary Exposure Consumption of fish is the major route of exposure to methylmercury. Unlike the case of polychlorinated biphenyls, which are also deposited in fat, cooking the fish does not lower the methylmercury content. Inorganic mercury compounds are also found in food. The source of inorganic mercurial is unknown but the amounts ingested are far below known toxic levels. Mercury in the atmosphere and in drinking water is generally so low that they do not constitute an important source of exposure to the general population (ATSDR, 2013a; Chen et al., 2016b).

Occupational Exposure Inhalation of mercury vapor can occur from the working environment, as in the chloralkali industry, where mercury is used as a cathode in the electrolysis of brine. Occupational exposure may also occur during manufacture of a variety of scientific instruments and electrical control devices, and in dentistry where mercury amalgams are used in tooth restoration. In the processing and extraction of gold, large quantities of metallic mercury are used to form an amalgam with gold. The amalgam is then heated to drive off the mercury, resulting in a substantial atmospheric release (ATSDR, 2013a; Drace et al., 2016). This extraction process is highly prevalent in artisanal and small-scale gold mining that is often not regulated and, thus, poses a significant environmental justice issue (Drace et al., 2016).

Medicinal Exposure Mercury was an important constituent of drugs for centuries and was used as an ingredient in diuretics, antiseptics, skin ointments, and laxatives. These uses have largely been replaced by safer drugs. Thimerosal contains the ethylmercury radical attached to the sulfur group of thiosalicylate (49.6% mercury by weight as ethylmercury), and has been used as a preservative in many vaccines since 1930s, although its use in children's vaccines was discontinued in 2001 over concerns that children may be exposed to excessive levels of mercury. The use of mercury amalgam in dental restoration releases mercury vapor in the oral cavity and can result in increased mercury body burden. Although the

potential health effects of amalgams have been fiercely debated, the amounts are low compared with occupational exposure (Magos and Clarkson, 2006).

Accidental Exposure Fatal mercury poisonings come mainly from accidental exposure. Elemental mercury spills can occur in many ways, such as from broken elemental mercury containers, medicinal devices, barometers, and melting tooth amalgam fillings to recover silver. Inhalation of large amount of mercury vapor can be deadly (Magos and Clarkson, 2006; ATSDR, 2013a). Oral ingestion of large amounts of inorganic mercury chloride has also been lethal in suicide cases (Magos and Clarkson, 2006; ATSDR, 2013a). A well-known organomercurial poisoning episode was from consumption of fish contaminated with methylmercury from industrial waste in Minamata, Japan (Ekino et al., 2007). Consumption of grains and rice treated with methylmercury or ethylmercury as fungicides to prevent plant root diseases in Iraq and China also led to a significant number of poisonings (Clarkson, 2002). Contact with even a small amount of dimethylmercury (CH_3CH_3Hg) can penetrate laboratory gloves, resulting in rapid transdermal absorption, delayed cerebella damage, and death (Nierenberg et al., 1998; Blayney, 2001).

Toxicokinetics

Mercury Vapor Mercury vapor is readily absorbed (about 80%) in the lungs, rapidly diffuses across alveolar membranes into the blood, and distributes to all tissues in the body due to its high lipid solubility. Once the vapor has entered cells, it is oxidized to divalent inorganic mercury by tissue and erythrocyte catalase. A significant portion of mercury vapor crosses the blood–brain barrier and placenta before it is oxidized by erythrocytes, and thus shows more neurotoxicity and developmental toxicity compared with administration of inorganic mercury salts that cross membranes less rapidly. After mercury vapor undergoes oxidation, its deposition resembles inorganic mercury. Approximately 10% of mercury vapor is exhaled within a week of exposure, and that converted to inorganic mercury is excreted mainly in urine and feces, with a half-life of 1 to 2 months (Magos and Clarkson, 2006; ATSDR, 2013a). Liquid metallic mercury, such as that swallowed from a broken thermometer, is only poorly absorbed by the gastrointestinal tract (0.01%), is not biologically reactive, and is generally thought to be of little or no toxicological consequence.

Inorganic Mercury Inorganic mercury is poorly absorbed from the gastrointestinal tract. Absorption ranges 7% to 15% of ingested dose, depending on the inorganic compound (ATSDR, 2013a). A small portion of absorbed inorganic mercury is formed by reduction in tissues and exhaled as mercury vapor. The highest concentration of inorganic mercury is found in the kidney, a major target. Renal uptake of mercury salts occurs through two routes: from luminal membranes in renal proximal tubule in the form of the cysteine *S*-conjugates (Cys-*S*-Hg-*S*-Cys) or from the basolateral membrane through organic anion transporters (Bridges and Zalups, 2005). Inorganic mercury salts do not readily pass blood–brain barrier or placenta and are mainly excreted in urine and feces, with a half-life of about 2 months.

Methylmercury Methylmercury is well absorbed from the gastrointestinal tract. About 95% of methylmercury ingested from fish is absorbed. It is distributed to all tissues in about 30 hours (Magos and Clarkson, 2006; ATSDR, 2013a). About 10% of absorbed methylmercury is distributed to the brain and 5% remains in the blood. The concentration in erythrocytes is 20 times that in plasma. Methylmercury is bound to thiol-containing molecules such as cysteine (CH_3Hg-*S*-Cys), which mimic methionine to cross the blood–brain barrier and placenta through the neutral amino acid carrier. Methylmercury readily accumulates in hair, and although concentrations are proportional to that in blood, they are about 250-fold higher. Thus, hair mercury is often used as an indicator of exposure. Detoxification of Hg^{2+} and MeHg species can also occur by complexing with glutathione, and the GSH–mercury complexes facilitate systemic excretion efflux from cells, as well as elimination by the liver and kidneys (Martinez-Finley and Aschner, 2014). Methylmercury undergoes extensive enterohepatic recycling, which can be interrupted to enhance fecal excretion. Methylmercury is slowly metabolized to inorganic mercury by microflora in intestine (about 1% of the body burden per day). In contrast to inorganic mercury, 90% of the methylmercury is eliminated from the body in the feces, and less than 10% is in the urine, with a half-life of 45 to 70 days (Clarkson et al., 2007; ATSDR, 2013a).

The disposition of ethylmercury is similar to methylmercury. The major differences include that the conversion to inorganic mercury in body is much faster for ethylmercury, which can result in renal injury. The mercury levels in brain are lower for ethylmercury than those for methylmercury. The half-life for ethylmercury is only 15% to 20% of that for methylmercury (Clarkson et al., 2007).

Toxicity

Mercury Vapor Inhalation of mercury vapor at extremely high concentrations may produce an acute, corrosive bronchitis and interstitial pneumonitis and, if not fatal, may be associated with central nervous system effects such as tremor or increased excitability (Magos and Clarkson, 2006). With chronic exposure to mercury vapor, the major effects are on the central nervous system. Early signs are nonspecific, and this condition has been termed the *asthenic-vegetative syndrome* or *micromercurialism*. Identification of the syndrome requires neurasthenic symptoms and three or more of the following clinical findings: tremor, enlargement of the thyroid, increased uptake of radioiodine in the thyroid, labile pulse, tachycardia, dermographism, gingivitis, hematologic changes, or increased excretion of mercury in urine. The triad of tremors, gingivitis, and erethism (memory loss, increased excitability, insomnia, depression, and shyness) has been recognized historically as the major manifestation of mercury poisoning from inhalation of mercury vapor. Sporadic instances of proteinuria and even nephrotic syndrome may occur in persons with exposure to mercury vapor, particularly with chronic occupational exposure. The pathogenesis is probably immunologically similar to that occurring after exposure to inorganic mercury (Magos and Clarkson, 2006). Mercury vapor release from amalgam is in general too low to cause significant toxicity (Magos and Clarkson, 2006).

Inorganic Mercury Kidney is the major target organ for inorganic mercury (Magos and Clarkson, 2006; ATSDR, 2013a). Although a high dose of mercuric chloride is directly toxic to renal tubular cells, chronic low-dose exposure to mercury salts may induce rare immunologic glomerular disease (Magos and Clarkson, 2006). However, the nephrotic syndrome is not rare in African women who have used skin lightening creams containing ammoniated mercury. All the African women with the nephrotic syndrome had, at the time of use, 90 to 250 mg Hg/L in their urine (Barr et al., 1972; Niu et al., 2017).

Methylmercury The major human health effect from exposure to methylmercury is neurotoxicity. Clinical manifestations of neurotoxicity include paresthesia (a numbness and tingling sensation around the mouth and lips) and ataxia, manifested as a clumsy, stumbling gait, and difficulty in swallowing and articulating words. Other signs include neurasthenia (a generalized sensation

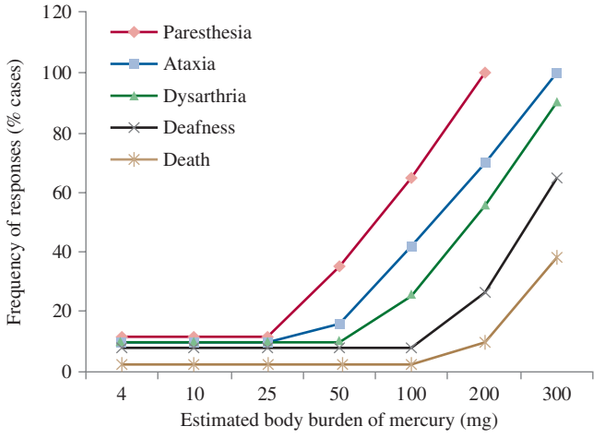

Figure 23-6. *A dose–response simulation of estimated methylmercury body burden and the onset and frequency of symptoms from Iraq epidemic poisoning in 1970s.*

of weakness), vision and hearing loss, and spasticity and tremor (Magos and Clarkson, 2006; ATSDR, 2013a). These may finally progress to coma and death. Neuropathological observations have shown that the cortex of the cerebrum and cerebellum are selectively involved with focal necrosis of neurons, lysis and phagocytosis, and replacement by glial cells. These changes are most prominent in the deeper fissures (sulci), as in the visual cortex and insula. The overall acute effect is cerebral edema, but with prolonged destruction of gray matter and subsequent gliosis, cerebral atrophy results (Magos and Clarkson, 2006). A study of the Iraq epidemic of methylmercury exposure has provided dose–response estimates of the body burden of mercury required for the onset and frequency of symptoms (Bakir et al., 1973) (Fig. 23-6).

Mechanism of Toxicity High-affinity binding of divalent mercury to sulfhydryl groups of proteins in the cells is an important mechanism for producing nonspecific cell injury or even cell death. A number of general mechanisms of toxicity have been observed after mercury exposures. Although mercury does not participate in Fenton-like reactions, oxidative stress plays a significant role in mercury toxicity. Reduced glutathione concentrations and antioxidant enzymes have been reported in mice exposed to methylmercury (Stringari et al., 2008; Franco et al., 2009). Genes associated with oxidative stress have been found to be upregulated by inorganic mercury exposure using microarray studies in yeast and human cells (Kawata et al., 2007; Jin et al., 2008; Hwang, 2012; Martinez-Finley and Aschner, 2014). In vitro exposures to inorganic or methylmercury also affected the MAPK signaling pathway, although this action might be indirect and mediated by mercury-induced ROS (Kim et al., 2002; Kim and Sharma, 2005; Haase et al., 2011). Low-level exposures impair the neurodevelopment by affecting neural progenitor cell proliferation, differentiation, and apoptosis, as well as impairing key signaling molecules (Rac1, Cdc42, and RhoA) critical to migration of cerebrocoritcal neurons (Martinez-Finley and Aschner, 2014). Methylmercury has also been shown to disrupt microtubules in neurites and in neonatal mice (Johansson et al., 2007; Ferraro et al., 2009). Both inorganic and methylmercury damage mitochondria and disrupt intracellular calcium homeostasis (Freitas et al., 1996; Cambier et al., 2009; Nesci et al., 2016).

Sensitive Subpopulations Early life stages are particularly vulnerable to mercury intoxication (Counter and Buchanan, 2004; Diez, 2009). In Minamata, Japan, pregnant women who consumed fish contaminated with methylmercury manifested mild or minimal symptoms, but gave birth to infants with severe developmental disabilities, raising initial concerns for mercury as a developmental toxicant. Methylmercury crosses the placenta and reaches the fetus, and is concentrated to a level in fetal brain at least five to seven times that of maternal blood (Clarkson, 2002). Prenatal methylmercury exposure at high levels can induce widespread damage to the fetal brain. However, the observed effects from low-level exposures are inconsistent (Counter and Buchanan, 2004; Martinez-Finley and Aschner, 2014). In the Seychelles Children Development Study, a group with significant methylmercury exposure from a diet predominantly of fish was studied for adverse developmental effects. These children were examined six times over 11 years using extensive batteries of age-appropriate developmental endpoints, but no convincing associations were found except for delayed walking (Davidson et al., 2006). The National Research Council reviewed the epidemiological studies relating in utero methylmercury exposure and fetal neurological development. It concluded that the current EPA reference dose (RfD) for methylmercury of 0.1 μg/kg/day or 5.8 μg/L cord blood is scientifically justifiable for protection of human health (NRC, 2000). The RfD is equivalent to 12 ppm methylmercury in maternal hair.

The safety of thimerosal (ethylmercury) used in childhood vaccines has also received extensive attention. A recent review indicates that thimerosal is safe at the doses used in vaccines, except for a potential for local hypersensitivity (Magos and Clarkson, 2006). Steps have been rapidly taken to remove thimerosal from vaccines in the United States by switching to single-dose vials that do not require preservatives. Nonetheless, the World Health Organization concluded that it is safe to continue using thimerosal in vaccines, which is important for developing countries where it is essential to use multidose vials (Magos and Clarkson, 2006).

Although the use of mercury amalgam in children can contribute to mercury exposure, the level of exposure is too low to cause significant toxicological effects (DeRouen et al., 2006; Magos and Clarkson, 2006).

Acrodynia has occurred in children chronically exposed to inorganic mercury compounds in teething powder and diaper disinfectants, as well as to organomercurials. It is characterized by pink hands and feet (also called pink disease). These subjects are photophobic and suffer from joint pains (Clarkson, 2002).

Treatment Therapy for mercury poisoning should be directed toward lowering the concentration of mercury at the critical organ or site of injury. For the most severe cases, particularly with acute renal failure, hemodialysis may be the first measure, along with administration of chelating agents for mercury, such as cysteine, EDTA, BAL, or penicillamine. Caution should be taken to avoid inappropriate use of chelating agents in putative mercury poisoning patients (Risher and Amler, 2005). Chelation therapy is not very helpful for alkyl mercury exposure. Biliary excretion and reabsorption by the intestine can be interrupted by oral administration of a nonabsorbable thiol resin, which can bind mercury and enhance fecal excretion (Clarkson, 2002).

Nickel

Nickel (Ni) has been in use since ancient times. However, because the ores of nickel were easily mistaken for ores of silver, a more complete understanding of nickel and its specific use came with

more contemporary times. In 1751, nickel was first isolated from the ore kupfernickel (niccolite) from which it derives its name. Nickel is used in various metal alloys, including stainless steels, and in stainless steel welding, electroplating, batteries, pigments, catalysts, and ceramics. Major properties of nickel alloys include strength, corrosion resistance, and good thermal and electrical conductivity. Occupational exposure to nickel occurs by inhalation of nickel-containing aerosols, dusts, or fumes, as well as dermal contact in workers engaged in nickel production (mining, milling, refinery, etc.) and nickel-using operations (melting, electroplating, welding, nickel–cadmium batteries, etc.) (ATSDR, 2005a; NTP, 2016b). Nickel, like many other metals, is ubiquitous in nature, and the general population is exposed to low levels of nickel in air pollution (Huggins et al., 2004; Ito et al., 2016), cigarette smoke, water, and food. Nickel has various oxidation states but the 2+ oxidation state is the most prevalent form in biosystems. The major soluble nickel compounds are nickel acetate, nickel chloride, nickel sulfate, and nickel nitrate. The important water-insoluble nickel compounds include nickel sulfide, nickel subsulfide, nickel oxide, nickel carbonyl, and nickel carbonate (ATSDR, 2005a; NTP, 2016b).

Toxicokinetics Inhalation of nickel is the most important route toxicologically. Inhaled nickel particles are deposited in the respiratory tract and, as with all inhaled particles, the site of deposition depends on the particle size. Large particles (5 to 30 μm) deposit in the nasopharyngeal area via impaction, smaller particles (1 to 5 μm) enter the trachea and bronchiolar region by sedimentation, and particles smaller than 1 μm enter the alveolar space. About 25% to 35% of the inhaled nickel that is retained in the lungs is absorbed into the blood. The insoluble nickel particles can be taken up into cells by phagocytosis. When applied or in contact with skin, the rate of absorption depends on the rate of penetration into the epidermis, which differs for different chemical forms of nickel. In humans, about 27% of a single oral dose of nickel in drinking water is absorbed, depending on the compound, whereas only about 1% is absorbed when nickel is given with food. Intestinal nickel absorption occurs through calcium or iron channels, or by the divalent metal transport protein-1 (ATSDR, 2005a; Saito et al., 2016).

The main transport proteins of nickel in blood are albumin, histidine, and α_2-microglobulin. Nickeloplasmin and MT also can bind and transport nickel. After inhalation exposure, nickel is distributed to the lungs, skin, kidneys, liver, pituitary, and adrenals. The half-life of nickel is 1 to 3 days for nickel sulfate, 5 days for nickel subsulfide, and more than 100 days for nickel oxide (ATSDR, 2005a). Absorbed nickel is excreted into urine. Urinary nickel correlates closely with exposure to airborne levels of insoluble nickel compounds. Thus, urinary nickel may serve as a suitable measure of current nickel exposure.

The marked differences in carcinogenic activities of various nickel compounds may be due to differences in delivery of nickel ion to specific cells and subcellular target molecules. For example, injection of animals with crystalline nickel subsulfide or crystalline nickel sulfide results in a high incidence of tumors at the site of injection sites, although tumors are generally not observed in animals similarly injected with soluble nickel sulfate (ATSDR, 2005a; IARC, 2017). The crystalline nickel particles can be actively phagocytized and apparently deliver larger quantities of nickel ions into the nucleus of local cells compared with water-soluble nickel compounds that diffuse away from the site (Kasprzak et al., 2003; Costa et al., 2005). Several water-soluble nickel compounds can, however, produce local malignant tumors when repeatedly injected into the peritoneal cavity of rats, suggesting repeated exposures to the target cells may be required (IARC, 2017).

Essentiality of nickel in higher organisms is questionable, although nickel may be nutritionally essential for some plants, bacteria, and invertebrates. Nickel deficiency syndromes have not been reported in humans and nickel-dependent enzymes or cofactors are unknown (Denkhaus and Salnikow, 2002).

Toxicity

Contact Dermatitis Nickel-induced contact dermatitis is the most common adverse health effect from nickel exposure and is found in 10% to 20% of the general population. It can result from exposure to airborne nickel, liquid nickel solutions, or prolonged skin contact with metal items containing nickel, such as coins and jewelry. Nickel sensitization usually arises from prolonged contact with nickel or exposure to a large dose of nickel. The resulting dermatitis is an inflammatory reaction mediated by type IV delayed hypersensitivity (ATSDR, 2005a).

Nickel Carbonyl Poisoning Metallic nickel combines with carbon monoxide to form nickel carbonyl ($Ni[CO]_4$), which decomposes to nickel and carbon monoxide on heating to 200°C (the Mond process) (NRC, 2008). This reaction provides a convenient and efficient method for nickel refining. However, nickel carbonyl is extremely toxic, and can cause acute toxicity. Intoxication begins with headache, nausea, vomiting, and epigastric or chest pain, followed by cough, hyperpnea, cyanosis, gastrointestinal symptoms, and weakness. The symptoms may be accompanied by fever and leukocytosis. The more severe cases can progress to pneumonia, respiratory failure, and eventually to cerebral edema and death.

Carcinogenicity Nickel is a respiratory tract carcinogen in nickel-refining industry workers (NTP, 2016b; IARC, 2017). Risks are highest for lung and nasal cancers among workers heavily exposed to nickel. Because the refining of nickel in some plants that were studied involved the formation of nickel carbonyl, it was believed for a time that nickel carbonyl was the principal carcinogen. However, multiple additional epidemiological studies of workers in other refineries suggest that the source of the increased risk is the mixture of nickel compounds (NTP, 2016b; IARC, 2017). Studies often involve a complex mixture of the metal and several of its compounds making separate carcinogenic assessment challenging (NTP, 2016b; IARC, 2017). Nickel sulfate and combinations of nickel sulfides and oxides encountered in nickel refining are considered to cause human cancer (NTP, 2016b; IARC, 2017). Human studies have also shown a strong association between primary exposure to water-soluble nickel compounds in the nickel-refinery industry and elevated cancer risk (Grimsrud and Andersen, 2010, 2012). Nonetheless, controversy remains about the role of soluble nickel compounds in human cancer causation based on a biokinetic hypothesis that they are unable to deliver sufficient nickel to critical local targets because they are soluble (Grimsrud and Andersen, 2010, 2012; Goodman et al., 2011). Metallic nickel is considered *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals (NTP, 2016b).

Studies with rats and some with mice have shown that inhaled or intratracheal instilled nickel subsulfide or nickel oxide produces lung tumors, including carcinoma, in a dose-related fashion and causes adrenal gland tumors (NTP, 2016b; IARC, 2017). Injection of various nickel compounds (generally water insoluble) at various sites (subcutaneous, intramuscular, intrarenal, etc.) causes local tumors (primarily sarcomas) in laboratory animals often in a dose-related fashion (NTP, 2016b; IARC, 2017). The relevance of routes to human exposure situations is debatable, but such data are used to assess carcinogenic potential. Nickel monoxides, hydroxides,

and crystalline sulfides are also considered to be carcinogenic in animals based on studies finding injection site tumors and/or lung tumors after intratracheal instillations (NTP, 2016b; IARC, 2017). Metallic nickel produces injection site tumors and lung tumors after intratracheal instillation (NTP, 2016b; IARC, 2017). Rodent carcinogenesis studies of soluble nickel compounds have also yielded positive results in rodents (Kasprzak et al., 2003; NTP, 2016b). For instance, water-soluble nickel acetate is a complete transplacental carcinogen for the rat pituitary and initiator of kidney tumors in the rat (Diwan et al., 1992). The water-soluble nickel compounds, nickel chloride, nickel sulfate, and nickel acetate, produced local mesotheliomas or sarcomas when given by repeated intraperitoneal injection to rats, suggesting repeated exposure to soluble salts is required (IARC, 2017). In two strain A mouse studies, multiple intraperitoneal injections of nickel acetate increased lung adenocarcinoma incidence (one study) and lung tumor multiplicity (both studies; IARC, 2017). However, many rodent studies using soluble nickel compounds have been negative (Sivulka, 2005; Goodman et al., 2011).

Mechanism for Nickel Carcinogenesis The carcinogenicity of nickel is thought to be due to the generation of ionic nickel in target cells at sites that are key for carcinogenesis (NTP, 2016b). This has allowed consideration of these compounds as a single group (NTP, 2016b). Ionic nickel is thought to be the active and genotoxic form of the metal, and there is no reason to suspect that the mechanisms by which nickel causes cancer in experimental animals would differ from humans (Sivulka, 2005; NTP, 2016b).

Carcinogenic nickel particles that are phagocytized and deliver large quantities of nickel ions into the nucleus are generally not mutagenic but are clastogenic (Costa et al., 2005). In this way insoluble nickel compounds can produce specific chromosomal damage, notable in the heterochromatic long arm of the X chromosome that suffers regional decondensation, frequent deletions, and other aberrations (Costa et al., 2005). Nickel compounds also produce chromosomal abnormalities such as sister chromatid exchange, especially in heterochromatin, micronuclei formation in human lymphocytes, microsatellite mutations in human lung cancer cells, and mutations in renal cells (Kasprzak et al., 2003). Many studies in vitro and in vivo indicate a variety of soluble and insoluble forms of nickel cause genetic damage, including DNA damage, cell transformation, and DNA repair disruption (NTP, 2016b). The redox activity of nickel may produce ROS that could attack DNA directly (NTP, 2016b).

Epigenetic Effects A broad spectrum of epigenetic effects occurs with nickel and includes alterations in gene expression resulting from perturbed DNA methylation and posttranslational histone modification (Sun et al., 2013). A notable nickel-inducible gene is Cap43/NDRG1, under the control of the hypoxia-inducible factor-1α (HIF-1α). During tumor development, HIF-1α facilitates angiogenesis and regulates numerous genes including glucose transport and glycolysis, which are essential for tumor growth. A correlation of overexpression of Cap43 with the neoplastic state of the cells was noted (Costa et al., 2005). Another nickel-induced gene amplification is the *Ect2* gene. The Ect2 protein is overexpressed in nickel-transformed cells, which can cause microtubule disassembly and cytokinesis, and may contribute to morphological changes in cells (Clemens et al., 2005). Nickel produces low but measurable ROS in cells and depletes cellular glutathione. Oxidative DNA damage, oxidative protein damage, and lipid peroxidation, as well as inhibition of DNA repair enzymes can be observed after nickel exposure (Kasprzak et al., 2003; Valko et al., 2005, 2006).

Treatment of Nickel Toxicity Sodium diethylcarbodithioate (DDTC) is the preferred drug for nickel treatment. Disulfiram, another nickel-chelating agent, has been used in nickel dermatitis and in nickel carbonyl poisoning. Other chelating agents, such as D-penicillamine and DMPS, provide some degree of protection from clinical effects (Blanusa et al., 2005).

ESSENTIAL METALS WITH POTENTIAL FOR TOXICITY

Cobalt

Cobalt (Co) is a relatively rare, ferromagnetic transition metal first isolated in the 1730s. The name cobalt comes from the German word *kobalt*, which is derived from *kobold* meaning "goblin," a name applied by miners of the time to cobalt ore, which was thought to be worthless and poisonous. Cobalt is usually not mined alone and tends to be produced primarily as a by-product of copper and nickel mining. It is useful in various alloys, where it provides corrosion and wear resistance, and in cemented carbides ("hard" metals). It is used in permanent magnets, as a paint or varnish dryer, in catalysts, and in production of pigments (ATSDR, 2004b). Cobalt is essential as a metal cofactor coordinated in vitamin B_{12}, but excess exposure induces a number of adverse health effects (Leyssens et al., 2017).

Toxicokinetics The toxicokinetics and possible adverse health effects of inorganic cobalt compounds have been reviewed (De Boeck et al., 2003; ATSDR, 2004b; Leyssens et al., 2017). Cobalt absorption depends on the compound. Less than 5% of an oral dose of cobalt oxide is absorbed, whereas about 30% of an oral dose of cobalt chloride is absorbed in rodents. Oral absorption of cobalt varies widely in humans, and it is estimated to be between 5% and 45%. Increasing doses of cobalt results in a decreasing proportional absorption, so increased cobalt levels tend not to cause significant accumulation. Absorption of inhaled cobalt compounds appears to be relatively effective in humans and animals. About 80% of absorbed cobalt is excreted in urine, and about 15% is excreted in feces. The liver, kidneys, adrenals, and thyroid have relatively high concentrations. The normal levels in human urine and blood are less than 2.0 and 0.2 to 0.5 μg/L, respectively. Cobalt in blood is largely associated with red blood cells.

Essentiality Cobalt is an essential nutrient, in small amounts, to mammals, including humans. The essential form of cobalt is cobalamin, a cobalt-containing tetrapyrrolic ring and critical component of vitamin B_{12}. Vitamin B_{12} is required for the production of red blood cells and in the prevention of pernicious anemia. The recommended daily intake of vitamin B_{12} for adults is 2.4 μg/day, which contains 0.1 μg of cobalt (IOM, 2000b) Insufficient natural levels of cobalt in the diet of sheep and cattle result in cobalt deficiency disease, characterized by anemia and loss of weight or retarded growth.

Toxicity The toxic reactions to Co exposure primarily depend on its chemical form. In the occupational and environmental settings, exposure is predominantly to Co metal particles. In the medical setting (e.g., metal on metal [MoM] hip implants), exposure to Co nanoparticle wear products, as well as Co ions occurs (Leyssens et al., 2017). Occupational inhalation of cobalt-containing dust may cause respiratory irritation at air concentrations between 0.002 and 0.01 mg/m^3. Higher concentrations may be a cause of "hard metal" pneumoconiosis, a progressive form of pulmonary interstitial fibrosis. This disease is observed in workers exposed to cobalt–tungsten carbide but not observed with exposure to cobalt alone (ATSDR,

2004b; Leyssens et al., 2017). Occupational dermal exposure is sometimes associated with an allergic dermatitis.

In the cardiovascular system, cobalt can be erythropoietic when most mammals, including humans, ingest excessive amounts. Chronic oral administration of high levels of cobalt for the treatment of anemia can cause goiter, and epidemiological studies suggest that the incidence of goiter is higher in regions containing increased levels of cobalt in water and soil (Leyssens et al., 2017). The goitrogenic effect has been elicited by the oral administration of 3 to 4 mg/kg to children in the course of treatment of sickle cell anemia. Intravenous exposure to cobalt can cause increased blood pressure, slowed respiration, tinnitus, and deafness due to nerve damage. Severe cardiomyopathies were one of the first observed toxicities of cobalt (more than 10 mg/day), especially in isolated incidence of excessive consumption of beer containing cobalt as a foaming agent (Weber, 1998; Packer, 2016). Autopsy findings in such cases have found a 10-fold increase in the cardiac levels of cobalt (Packer, 2016). The clinical emergence of cobalt cardiomyopathy seems to require the coexistence of one or more cofactors, particularly a low-protein diet, thiamine deficiency, alcoholism, and hypothyroidism (Packer, 2016; Leyssens et al., 2017). However, there are increasing reports of cobalt leaching from metal prostheses or cobalt containing nanoparticle wear products causing fatal cardiomyopathies (Fox et al., 2016; Packer, 2016). In animals, myocardial degeneration can be produced by cobalt injection. Chronic cobalt exposure targets mitochondrial enzymes in complex II and complex III, with modest effects on respiration and a minimal lowering of ATP levels (Clyne et al., 2001; Packer, 2016). These exposures cause pronounced decreases in mitochondrial manganese superoxide dismutase levels in rat hearts, which may contribute to oxidative myocardial injury (Clyne et al., 2001). In addition, cobalt-stabilized HIF-1α increases expression of endothelin-1, which would signal for cardiac hypertrophy and remodeling (Kakinuma et al., 2001).

Based on experimental studies in animals, cobalt sulfate has been classified as *reasonably anticipated to be carcinogenic in humans* (NTP, 2016b). In rats and mice, inhalation of cobalt sulfate induces lung tumors, including carcinomas (Bucher et al., 1999). Repository injections or implantation of various cobalt compounds can produce local sarcomas in rodents (IARC, 2006). Incidence of adrenal gland tumors was also increased in female rats exposed to cobalt sulfate (IARC, 2006; NTP, 2016b). The mechanisms by which cobalt produces cancer are not fully defined but potentially include inhibition of DNA repair and formation of DNA-damaging ROS, and through alterations of important cellular functions by replacing other essential metal ions (ATSDR, 2004b; IARC, 2006; NTP, 2016b).

Cobalt–tungsten carbide powders and hard metals are *reasonably anticipated to be human carcinogens* based on limited evidence of carcinogenicity from human studies and supporting evidence from studies on mechanisms of carcinogenesis (IARC, 2006; NTP, 2016b). No studies examining the carcinogenicity of cobalt–tungsten carbide powders or hard metals in animals have been identified. Potential carcinogenic mechanisms include the release of cobalt ions, increased production of ROS with resultant oxidative stress response, and by causing cytotoxicity, genotoxicity, inflammation, and apoptosis (NTP, 2016b).

Copper

Copper (Cu) has been used for many centuries. By 2000 BC, "bronze" or copper–tin alloys were in wide use in Europe. In Roman times, copper became known as *cerium* because so much of it was mined in Cyprus, and eventually Anglicized into *copper*. Copper is an essential element widely distributed in nature. Food, beverages, and drinking water are major sources of exposure in the general population. The Recommended Dietary Allowance (RDA) of copper varies according to age, and pregnancy and lactation. Daily intake of copper in adults is 0.9 mg/day, in children between 0.2 and 0.9 mg/day, and in pregnant or lactating women 1.0 to 1.3 mg per day (IOM, 2001). The average daily intake of copper from food is about 1.2 to 1.7 mg/day (Chambers et al., 2010).

Copper exposure in industry is primarily from inhaled particulates in mining or metal fumes in smelting operations, welding, or related activities. In the general population, exposure to elevated levels of copper can occur via metal leaching from copper plumbing or copper-lined cookware (ATSDR, 2004c). Excess copper in the water represents a significant risk factor to the aquatic environment, producing endocrine disruption and other toxic effects in fish (Handy, 2003; Arnold et al., 2009).

Toxicokinetics Approximately 55% to 75% of an oral dose of copper is absorbed from the gastrointestinal tract, primarily in the duodenum. Intestinal copper absorption can be reduced by zinc, iron, molybdate, and fructose (IOM, 2001). As discussed earlier, once absorbed, copper is rarely free, and is bound to ceruloplasmin in the blood. Ceruloplasmin releases Cu^+ ions to the membrane-bound Cu importer CTR1 for transfer to the chaperone ATOX1 and delivery to cytoplasmic metal-binding domains of ATP7A and two homologous multi-domain P1B-type ATPases located in the trans-Golgi network (Matson Dzebo et al., 2016). The copper is then available for coordination in proteins as they are synthesized by the Golgi. The importance of metal transporters in human diseases is well illustrated by Menkes and Wilson diseases, which are caused by genetic mutations in copper-transport protein genes *ATP7A* and *ATP7B*, respectively. Mutation of *ATP7A* causes copper deficiency (Menkes), while mutation of *ATP7B* results in copper overload (Wilson) (see Fig. 23-7). Although all tissues and cells contain copper, the primary sites for copper storage include the liver, which accounts for more than 80% of the stored copper, and brain. The majority of the copper is bound to the chaperones or incorporated into enzymes (Stern, 2010; Matson Dzebo et al., 2016). The amount of copper in the body is maintained at homeostatic levels mainly through control of excretion, although copper binding to hepatic MT may act as a form of intracellular storage. In mammals, the major route of excretion for excess copper is via the feces, with very little copper excreted into the urine. The bile is the major route of excretion from the liver. Bile secretion, enterohepatic recirculation, and intestinal reabsorption all help to maintain copper homeostasis (ATSDR, 2004c).

Essentiality Copper is an essential component of several metalloenzymes, including type A oxidases and type B monoamine oxidases. Of the type B oxidases, cytochrome *c* oxidase (COX) is probably the most important because it catalyzes a key reaction in energy metabolism, and inherited mutational defects can result in severe pathology in humans (Hamza and Gitlin, 2002; Matson Dzebo et al., 2016). Of the type A oxidases, lysyl oxidase (LOX) plays a major role in the formation and repair of extracellular matrix by oxidizing lysine residues in elastin and collagen, thereby initiating the covalent cross-linkage (Finney et al., 2014). Copper/zinc superoxide dismutase (SOD) is present in most cells, particularly of the brain, thyroid, liver, lung, and blood, and helps protect from oxygen toxicity by reducing superoxide radicals to hydrogen peroxide (Valko et al., 2006). Disruption of copper binding in any of these proteins has profound consequences on enzyme production (LOX), cytoplasmic antioxidant activity (SOD1), and energy metabolism (COX).

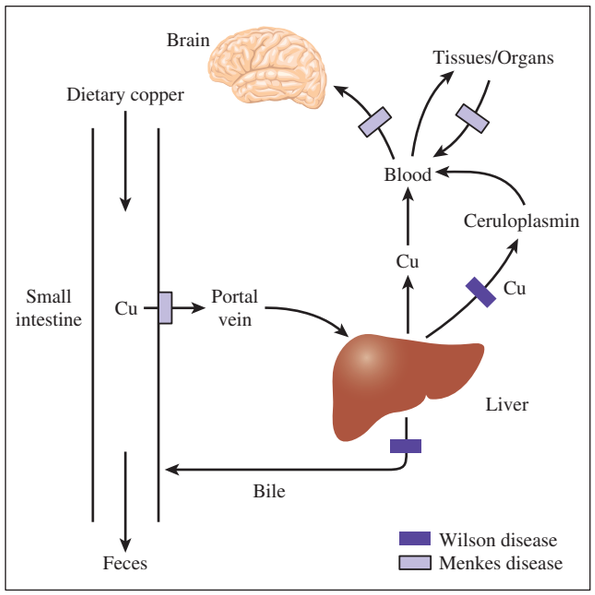

Figure 23-7. *Pathways of copper in the body and defects in Menkes and Wilson diseases.* Copper is absorbed by the enterocytes of the small intestine and transported across the basolateral membrane of enterocytes into the portal circulation. The latter process is defective in Menkes disease patients and results in accumulation of copper in the enterocytes and overall copper deficiency in the body. Most of the newly absorbed copper is normally taken up by the liver. In cases of copper overload, excess copper is excreted in the bile and this process is blocked in Wilson disease, as is the delivery of copper to ceruloplasmin, the principal copper carrier in the blood. Other low-molecular-weight proteins such as Cu–metallothionein and Cu–histidine are also proposed to be important sources of copper to tissues. The transport of copper to the brain is blocked in patients with Menkes disease, leading to the severe neurological abnormalities. (Adapted from Mercer [2001], with permission from Elsevier.)

Copper deficiency is uncommon in humans, but can occur as a result of malnutrition, overdose of molybdenum, or excessive consumption of zinc (Maret and Sandstead, 2006). However, as mentioned earlier, Menkes disease is a rare genetic disorder caused by poor copper transport. Copper deficiency manifests clinically by hypochromic, microcytic anemia that is refractory to iron supplementation and predisposes to infection. More recently, a series of case reports has implicated acquired copper deficiency in the etiology of adult-onset progressive myeloneuropathy and in the development of severe blood disorders including myelodysplastic syndrome (Goodman et al., 2009). This deficiency is sometimes accompanied by bone abnormalities. Less frequent manifestations are hypopigmentation of the hair and hypotonia. Biomarkers of copper deficiency include low serum and urine copper levels, ceruloplasmin concentration, and copper-dependent enzyme activities (IOM, 2001).

Toxicity The upper safe limit for daily copper consumption is estimated to be 10 mg Cu/day (IOM, 2001). The most commonly reported adverse health effects of excess oral copper intake are gastrointestinal distress. Nausea, vomiting, and abdominal pain have been reported shortly after drinking solutions of copper sulfate or beverages stored in containers that readily release copper (IOM, 2001; ATSDR, 2004c). Ingestion of drinking water with more than 3 mg Cu/L will produce gastrointestinal symptoms. Ingestion of large amounts of copper salts, most frequently copper sulfate, may produce hepatic necrosis and death. Animal and human epidemiological studies have not found any relation between copper exposure and cancer (Tully et al., 2000).

Hereditary Disease of Copper Metabolism

Menkes Disease This is a rare sex-linked genetic defect in copper metabolism resulting in copper deficiency in male infants. It is characterized by peculiar hair, failure to thrive, severe mental retardation, neurological impairment, connective tissue dysfunction, and death usually by 3 to 5 years of age. The majority of the pathologies associated with Menkes disease can be linked to deficiencies in copper-containing proteins (Kaler, 2013; Matson Dzebo et al., 2016). Bones are osteoporotic with flared metaphases of the long bones and bones of the skull. There is extensive degeneration of the cerebral cortex and of white matter. The gene responsible for Menkes disease, *ATP7A*, belongs to the family of ATPases and is a copper transporter (Fig. 23-7). Deficiency in this copper transporter in Menkes disease blocks copper transport across the basolateral membrane of intestinal cells into the portal circulation, resulting in accumulation of copper in the enterocytes and systemic copper deficiency in the body. The transport of copper to the brain is also blocked, causing severe neurological abnormalities. Animal models for copper deficiency support the importance of adequate copper intake during embryogenesis and early development (Shim and Harris, 2003). Supplementation with copper-histidine has been shown to delay disease progression and successfully increase life span (Kodama et al., 2011).

Wilson Disease This is an autosomal recessive genetic disorder of copper metabolism characterized by the excessive accumulation of copper in the liver, brain, kidneys, and cornea (Kaler, 2013; Matson Dzebo et al., 2016; Roberts and Socha, 2017). Serum ceruloplasmin is low and serum copper not bound to ceruloplasmin is elevated. Urinary excretion of copper is high. Clinical abnormalities of the nervous system, liver, kidneys, and cornea are related to copper accumulation. Patients with Wilson disease have impaired biliary excretion of copper, which is believed to be the fundamental cause of the hepatic copper overload. Wilson disease is associated with hepatic diseases ranging from mild hepatitis to acute liver failure. There is also an increased incidence of hepatocarcinoma in patients with Wilson disease (Roberts and Socha, 2017). Neurological symptoms associated with Wilson disease include dystonia, tremor, dysarthria, and psychiatric disturbances. Genetic studies have identified the defect in copper transport as mutations of the Wilson disease locus (WND) on chromosome 13, encoding P-type ATPase (*ATP7B*) (Kaler, 2013; Matson Dzebo et al., 2016). There appear to be several polymorphisms of the defect, which may explain the clinical variability in the disorder. Diagnosis may be suspected with elevated serum copper but must be confirmed by liver biopsy and elevated liver copper (normally 15 to 55 μg/g vs. more than 250 μg/g in Wilson disease).

Hereditary Aceruloplasminemia This is the autosomal recessive genetic disorder of copper-binding protein ceruloplasmin, associated with the iron overload syndrome (Kono, 2013). Clinical signs and symptoms, which result from iron overload in the brain, include mental confusion, memory loss, dementia, cerebellar ataxia, altered motor function, retinal degeneration, and diabetes. Ceruloplasmin-null mice accumulate iron predominantly in organs of the reticuloendothelial system (Shim and Harris, 2003). In these mice, hematologic indices and serum iron are abnormal by 10 weeks of age, with profound iron overload in the spleen and liver. Hepatic copper deposition is also approximately doubled in these mice. However, neurodegeneration and diabetes are not observed in these mice (Shim and Harris, 2003).

Indian Childhood Cirrhosis (ICC) This is a disorder occurring in young children characterized by jaundice due to an insidious and progressive liver disease. Two distinguishing features are a widespread brown orcein staining (indicating copper) and intralobular hepatic fibrosis progressing to portal cirrhosis and chronic inflammation (Nayak and Chitale, 2013; Patra et al., 2013). The etiology is debated, but investigators in India and other countries where sporadic cases and series of endemics ICC-like diseases occur support an etiology of hepatotoxic injury from ingested copper in genetically susceptible infants (Nayak and Chitale, 2013). It is plausible that both ICC and non-Indian CC (NICC) can be linked to exposures from copper-containing fluid storage vessels and specific gene abnormalities (Drouin et al., 2000; Mukherjee et al., 2005; Patra et al., 2013).

Treatment Treatment for Wilson disease and other diseases of copper overload includes copper chelators and supplementation with zinc salts. Clinical improvement can be achieved by chelation of copper with D-penicillamine, Trien (triethylenetetramine 2HCl), zinc acetate, and tetrathiomolybdate. The combination of tetrathiomolybdate and zinc acetate is more effective (Brewer and Askari, 2005; Kodama et al., 2011; Roberts and Socha, 2017).

Iron

Iron (Fe) is a highly abundant transition metal that came into use around 4000 BC. An early source of iron was from fallen meteorites and the name may derive from the Etruscan word *aisar* that means "the gods." Iron is an essential metal for erythropoiesis and a key component of hemoglobin, myoglobin, heme enzymes, metalloflavoprotein enzymes, and mitochondrial enzymes. Physiologically, iron mainly exists as the ferrous (2+) and ferric (3+) forms. Toxicological considerations are important in terms of iron deficiency, accidental acute exposures, and chronic iron overload due to idiopathic hemochromatosis or as a consequence of excess dietary iron or frequent blood transfusions (Weinberg, 2010; Boshuizen et al., 2017; Gammella et al., 2017).

Toxicokinetics Iron metabolism is regulated by a complex series of events that maintain homeostasis, mainly involving absorption, storage, and excretion (Coffey and Ganz, 2017; Gammella et al., 2017). Heme iron from meat, poultry, and fish is highly bioavailable. Nonheme iron absorption is much less efficient in humans and is influenced by its solubility and by other dietary factors, such as ascorbic acid which enhances uptake. Nonheme iron is absorbed in the duodenum, where acidic gastric secretions enhance iron solubility. Soluble inorganic iron is taken up in the proximal duodenum by divalent metal transporter protein 1 (DMT1), which couples the transport of Fe^{2+} to a proton gradient (Coffey and Ganz, 2017). The higher efficiency of heme uptake suggests a receptor-mediated mechanism; however, the identity of the receptor is unresolved (Coffey and Ganz, 2017). Iron taken up at the apical membrane of enterocytes is either sequestered within the iron storage protein ferritin or exported into the portal circulation through ferroportin—a receptor for hepcidin, the master regulator of systemic iron homeostasis (Coffey and Ganz, 2017; Gammella et al., 2017). Hepcidin is essential for regulating export of iron from enterocytes, as well as release from ferritin stores in hepatocytes and macrophages. Its expression and activity is inhibited by iron levels (Coffey and Ganz, 2017; Gammella et al., 2017). Ferroportin exports Fe^{2+} that must be oxidized by copper-containing ferroxidases, such as ceruloplasmin and hephaestin, before being loaded onto transferrin for systemic delivery to cells (Coffey and Ganz, 2017; Gammella et al., 2017). A β_1-globulin produced in the liver, transferrin, delivers iron to tissues by binding to transferrin receptor-1 on the cell membrane, followed by endocytosis. Intracellular iron homeostasis is regulated by a complex coordination of iron trafficking and iron storage involving an iron response element/iron regulatory protein system and antioxidant response elements (Coffey and Ganz, 2017; Gammella et al., 2017). The human body contains about 3 to 5 g of iron. About two-thirds of body iron is in hemoglobin, 10% is in myoglobin and iron-containing enzymes, and the remainder is bound to iron storage proteins such as ferritin and hemosiderin, stored in liver and reticuloendothelial cells in the spleen and bone marrow. Iron stores serve as a reservoir to supply cellular iron needs, mainly for hemoglobin production. Erythrocyte destruction and production are responsible for most iron turnover. Humans cannot directly excrete iron, so regulation of iron levels are mainly for uptake. The major route of excretion of iron is elimination of heme-containing proteins into the gastrointestinal tract and eventually the feces, as well as elimination of porphyrins in the urine.

Essentiality and Deficiency Iron deficiency is the most common nutritional deficiency worldwide, affecting infants, young children, and women of childbearing age and is considered a major public health issue (Boshuizen et al., 2017; Coffey and Ganz, 2017; Soliman et al., 2017). The critical period for iron deficiency in children is between the ages of 6 months and 2 years. The major manifestation of iron deficiency is anemia with microcytic hypochromic red blood cells. Other effects of iron deficiency include impaired psychomotor development and intellectual performance, decreased resistance to infection, adverse pregnancy outcomes, and possibly increased susceptibility to lead and cadmium toxicity. Oral ferrous sulfate is the treatment of choice for iron deficiency.

Toxicity Acute iron poisoning from accidental ingestion of iron-containing dietary supplements is the most common cause of acute toxicity. It most often occurs in children. This type of poisoning decreased after the introduction of childproof lids on prescription medicines and vitamin supplements. Severe toxicity occurs after the ingestion of more than 0.5 g of iron or 2.5 g of ferrous sulfate. Toxicity occurs about 1 to 6 hours after ingestion. Symptoms include abdominal pain, diarrhea, and vomiting. Of particular concern are pallor, metabolic acidosis, liver damage, and cardiac collapse. Death may occur in severely poisoned children within 24 hours. Supportive therapy and iron chelation with deferoxamine (also known as desferrioxamine) should be used as soon as possible (Andersen and Aaseth, 2016).

Chronic iron toxicity from iron overload in adults is a relatively common problem. Hereditary hemochromatosis is an insufficiency of hepcidin caused by mutations in genes regulating the protein's expression (Coffey and Ganz, 2017). Without the negative regulation of iron uptake, the inadequate levels of hepcidin promote excessive iron absorption, excessive mobilization of macrophage iron stores, saturation of transferrin with iron, and increased serum non-transferrin bound iron (Coffey and Ganz, 2017). *Hemochromatosis* refers to excessive deposition of iron that causes organ damage, often resulting in fibrosis. *Hemosiderosis* refers to increased iron stores in the form of hemosiderin. The body iron content can increase 20 to 40 g, up to 10 times higher than normal levels. Liver iron overload from hereditary hemochromatosis appears to be associated with an increased risk for hepatocellular carcinoma, as well as with other malignancies (Papanikolaou and Pantopoulos, 2005). Oxidant stress would be a presumable carcinogenic mode of action. β-thalassemia is a common genetic disorder of hemoglobin synthesis that occurs most frequently in people from Mediterranean countries, North Africa, the Middle East, India, Central Asia, and Southeast Asia. The iron overload results from impaired β-globin

production and chronic anemia that expands the erythroblast population. This leads to feedback suppression of hepcidin expression and thus increased iron absorption and tissue iron loading. The iron overload is exacerbated by the need for frequent blood transfusions in these patients. In addition, patients who receive high-dose chemotherapy in preparation for stem cell transplantation often have decreased transferrin levels, increased total serum iron content, and a pronounced rise in non-transferrin bound iron (Boshuizen et al., 2017). Inhalation of iron oxide fumes or dust by workers in hematic mines (mainly Fe_2O_3), steel workers, and welders may produce siderosis (nonfibrotic), and in some cases silicosis (fibrotic) in the lung, with increases in total body iron (Doherty et al., 2004).

Tissue injury from excessive iron may cause cirrhosis, diabetes, cardiomyopathy, atherosclerosis, and endocrine failure (Papanikolaou and Pantopoulos, 2005; Berdoukas et al., 2015; Kontoghiorghe and Kontoghiorghes, 2016; Coffey and Ganz, 2017; Guo et al., 2017). In addition, several neurodegenerative disorders are associated with aberrant iron metabolism in the brain, such as neuroferritinopathy, aceruloplasminemia, and manganism (Papanikolaou and Pantopoulos, 2005; Farina et al., 2013). Much of the tissue injury results from ROS generation as the excess free iron is the quintessential Fenton catalyst (Berdoukas et al., 2015; Boshuizen et al., 2017). In addition, injuries are worsened when damaged tissue becomes hypoxic and necrotic, and additional iron is released from intracellular stores.

Treatment Desferrioxamine is the chelator of choice for the treatment of acute iron intoxication and chronic iron overload. Deferoxamine therapies have also been shown to be effective in reducing iron overloads caused by thalassemias, defects in red blood cell synthesis (Andersen and Aaseth, 2016; Kontoghiorghe and Kontoghiorghes, 2016). However, chelation therapy comes with many risks and complications and proposed alternatives include using transferrin infusions (Boshuizen et al., 2017). Cancer chemotherapy patients with iron overload have responded well to transferrin supplementation with reduced non-transferrin bound iron and ROS generation (Boshuizen et al., 2017)

Magnesium

Magnesium (Mg) was recognized as an element in 1755. The name originates from the Greek word for a district in Thessaly called *Magnesia*. Magnesium is a nutritionally essential metal that plays a key role in a wide range of important fundamental cellular reactions (Herroeder et al., 2011; Grober et al., 2015). Nuts, cereals, seafood, and meats are good dietary sources of magnesium. The drinking water content of magnesium increases with hardness of the water. Magnesium citrate, oxide, sulfate, hydroxide, and carbonate are widely taken as dietary supplements, antacids, or cathartics. Magnesium hydroxide, or milk of magnesia, is one of the universal antidotes for poisoning. Topically, the sulfate is also used to relieve inflammation. Parenteral administration of magnesium sulfate has been carried out in the treatment of seizures associated with eclampsia of pregnancy and acute nephritis. Magnesium is analgesic and is increasingly being used as an adjuvant in anesthesia (Herroeder et al., 2011; Eizaga Rebollar et al., 2017; Rodriguez-Rubio et al., 2017).

Toxicokinetics Ingested magnesium is absorbed mainly in the small intestine. The colon also absorbs some magnesium. Calcium and magnesium are competitive with respect to absorption, and excess calcium will partially inhibit magnesium absorption. Serum magnesium concentrations are remarkably constant at 10 mM. Magnesium is excreted into the digestive tract by the bile and in pancreatic and intestinal juices. Approximately 60% to 65% of the total body magnesium is in the bone, 27% in muscle, 6% to 7% in other organs, and only 1% is in extracellular fluid. Of the magnesium filtered by the glomeruli, about 95% is reabsorbed, an important factor in maintaining homeostasis.

Essentiality and Deficiency Magnesium is a cofactor of many enzymes. In the glycolytic cycle, there are seven key enzymes that require divalent magnesium. Magnesium-containing enzymes are involved in the citric acid cycle and in β-oxidation of fatty acids and MgATP is essential for most energy and phosphotransfer reactions. Deficiency may occur as a complication of various disease states such as malabsorption syndromes, renal dysfunction, and endocrine disorders. Magnesium deficiency in humans causes neurological depression, neuromuscular irritability, frank tetany, and even convulsions (Grober et al., 2015; Serefko et al., 2016). Magnesium deficiency induces an inflammatory syndrome (Mazur et al., 2007), and is a risk factor for diabetes mellitus, hypertension, hyperlipidemia, and ischemic heart diseases (Ueshima, 2005). Supplementation of magnesium, by either intravenous or oral administration, is beneficial.

Toxicity The toxic effects of hypomagnesemia are more common than hypermagnesemia (Grober et al., 2015). In industrial exposures, no ill effects are produced with a twofold increase in serum magnesium, although concurrent increases occur in serum calcium. Inhaled freshly generated magnesium oxide can cause metal fume fever, similar to that caused by zinc oxide. In nonoccupationally exposed individuals, toxicity can occur when magnesium-containing drugs, usually antacids, are ingested chronically by persons with serious renal failure. The toxic effects may progress from nausea and vomiting to hypotension, electrocardiograph abnormalities, central nervous system effects, coma, and systolic cardiac arrest (Herroeder et al., 2011; Grober et al., 2015). Magnesium toxicity can sometimes be counteracted with calcium infusion.

Manganese

Manganese (Mn) was in use in prehistoric times. Paints that were pigmented with manganese dioxide can be traced back 17,000 years. The pure element was isolated in 1774 and named after the Latin *magnes*, meaning "magnet." Manganese is the third most abundant element in the human body and is essential for many metabolic, cellular, and structural functions. Manganese metalloenzymes include arginase, glutamine synthetase, phosphoenolpyruvate decarboxylase, and manganese SOD (Peres et al., 2016). Manganese is also a cofactor for a number of enzymatic reactions. It exists in many valences but the divalent cation is by far the predominant species within cells. Divalent manganese may be oxidized to the more reactive and toxic trivalent form. The major source of manganese intake is from food. Vegetables, grains, fruits, nuts, and tea are rich in manganese. Daily manganese intake ranges from 2 to 9 mg and the adequate intake is 2.3 and 1.8 mg/day for adult men and women, respectively (IOM, 2001; ATSDR, 2012c). As with many essential metals, both deficiency and excess caused by high environmental or occupational exposures promote toxicity, such as neurocognitive deficits (Peres et al., 2016; Vollet et al., 2016).

Occupational exposures to high concentrations of manganese can occur in a number of settings, including manganese dioxide mines and smelters (ATSDR, 2012c). Significant exposure can also occur in factories making manganese steel alloys, electrical coils, batteries, glass, and welding rods, and during production of potassium permanganate ($KMnO_4$) (ATSDR, 2012c). The industrial use of manganese has expanded in recent years as a ferroalloy in

the iron industry and as a component of alloys used in welding. Environmental exposures are often associated with manganese-based organometallic pesticides, maneb and mancozeb, as well as in communities located in close proximity to manganese industrial facilities (ATSDR, 2012c; Haynes et al., 2015). Manganese intoxication has been reported after ingestion of contaminated water and infant formula (Wasserman et al., 2011; ATSDR, 2012c; Vollet et al., 2016). There is current interest in the toxicology of manganese-containing fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT). In addition, manganese compounds, such as mangafodipir, are increasingly used as MRI enhancers in clinical imaging techniques (Peres et al., 2016; Vollet et al., 2016).

Toxicokinetics Approximately 1% to 5% of ingested manganese is normally absorbed. Interactions between manganese and iron, as well as other divalent elements, occur and impact the toxicokinetics of manganese especially after oral exposure (Farina et al., 2013; Vollet et al., 2016). Iron and manganese can compete for the same binding protein in serum (transferrin) and the same transport systems (DMT1) (Peres et al., 2016). Inhalation of particulate manganese may result in direct transfer to brain tissue via the olfactory system (Peres et al., 2016). Within the plasma, manganese is largely bound to γ-globulin and albumin, with a small fraction bound to transferrin. Manganese concentrates in mitochondria, so that tissues rich in these organelles, such as the pancreas, liver, kidneys, and intestines, have the highest concentrations of manganese. Manganese disrupts mitochondrial function to increase oxidative stress (Peres et al., 2016). It readily crosses the blood–brain barrier and accumulates in specific brain regions (Peres et al., 2016). Manganese is eliminated in the bile and reabsorbed in the intestine and the principal route of manganese excretion is with the feces (Peres et al., 2016; Vollet et al., 2016). Biliary excretion is poorly developed in neonates and exposure during this period may result in increased delivery of manganese to the brain and other tissues (Peres et al., 2016). Urinary levels of manganese are a poor biomarker of exposure, because primary homeostatic biliary excretion and a short half-life result in only a small amount being excreted in the urine (Vollet et al., 2016).

Toxicity Chronic manganese-induced neurotoxicity (manganism) is of great concern and the brain is considered the most sensitive organ to manganese. Manganism affects the release of dopamine from dopaminergic neurons, the same neurons affected by Parkinson disease. While both conditions lead to some similar neurological effects, effects on dopaminergic neurons are not the same, also causing distinct behavioral effects (Peres et al., 2016). Neurotoxicity due to inhalation of airborne manganese ranging from 0.027 to 1 mg Mn/m^3 has been reported in a number of occupational settings. One of the earliest descriptions of Mn overexposure was in an occupational study of Chilean miners who presented with a condition referred to as "locura manganica" or manganese madness (Vollet et al., 2016). Overt manganism occurs in workers exposed to aerosols containing extremely high levels of manganese (more than 1 to 5 mg Mn/m^3). Neurotoxicity also occurs after ingestion of manganese-contaminated water (1.8 to 14 ppm; Peres et al., 2016). Manganism is associated with elevated brain levels of manganese, primarily in those areas known to contain high concentrations of nonheme iron, such as the substantia nigra, basal ganglia, caudate–putamen, globus pallidus, and subthalamic nuclei (Farina et al., 2013; Peres et al., 2016). Early manifestations of manganese neurotoxicity include headache, insomnia, memory loss, muscle cramps, and emotional instability. Initial outward symptoms progress gradually and are mainly psychiatric. As exposure continues and the disease progresses, patients may develop prolonged muscle contractions (dystonia), decreased muscle movement (hypokinesia), rigidity, hand tremor, speech disturbances, and festinating "cock-walk" gait. These signs are associated with damage to dopaminergic neurons that control muscle movement (Peres et al., 2016). Specialized T1-weighted magnetic resonance brain imaging of manganism patients indicates high levels in the basal ganglia and especially in the globus pallidus (Peres et al., 2016).

Inhalation of manganese-containing dust in certain occupational settings can lead to an inflammatory response in the lung. Symptoms of lung irritation and injury may include cough, bronchitis, pneumonitis, and, occasionally, pneumonia (ATSDR, 2012c). Men working in plants with high concentrations of manganese dust show an incidence of respiratory disease that is 30 times greater than normal. Manganese exposure also alters cardiovascular function in animals and humans, as evidenced by abnormal electrocardiogram and the inhibition of myocardial contraction. Manganese dilates blood vessels and induces hypotension (Jiang and Zheng, 2005). Liver cirrhosis is a major contributing factor for hepatic encephalopathy, often associated with increased manganese levels in the brain (Peres et al., 2016).

Interactions between manganese and iron play a role in manganese toxicity (Farina et al., 2013). The coaccumulation of iron with manganese in the globus pallidus raises the concern that iron may be a contributing factor facilitating neuronal cell loss during manganese intoxication (Peres et al., 2016). Chronic exposure to manganese alters iron concentrations in blood and cerebrospinal fluid, presumably due to manganese–iron interaction at certain iron–sulfur-containing proteins, which regulate iron homeostasis. Serum parameters associated with iron metabolism, such as ferritin, transferrin, and total-iron-binding capacity, are significantly altered (Peres et al., 2016). High levels of total iron and iron-associated oxidative stress, decreased ferritin, and abnormal mitochondrial complex-1 have been repeatedly reported in postmortem samples of substantia nigra from manganism patients (Farina et al., 2013; Peres et al., 2016).

There is insufficient evidence that manganese is a carcinogen in animals or humans (ATSDR, 2012c).

Molybdenum

Molybdenum (Mo) was first separated from lead and graphite in 1778. The name "molybdenum" was derived from Greek *molybdos* meaning "lead-like." As an essential element, molybdenum acts as a cofactor for four enzymes in humans: sulfite oxidase, xanthine oxidase, aldehyde oxidase, and mitochondrial amidoxime reductase (Schwarz, 2016). Molybdenum exists in five oxidation states but the predominant species are Mo^{4+} and Mo^{6+}. Molybdenum concentration in food varies considerably depending on the local environment and molybdenum is added in trace amounts to fertilizers to stimulate plant growth. The human requirement for molybdenum is low and easily provided by a common U.S. diet. The recommended dietary allowance (RDA) for molybdenum is 45 μg/day (IOM, 2001).

The most important mineral source of molybdenum is molybdenite (MoS_2) (ATSDR, 2017b). The industrial uses of this metal include the manufacture of high-temperature-resistant steel alloys for gas turbines and jet aircraft engines and in the production of catalysts, lubricants, and dyes. Ammonium tetrathiomolybdate is used as a molybdenum-donating copper chelator in treatment of Wilson disease and is often used as a model molybdenum compound in animal toxicity studies (ATSDR, 2017b).

Toxicokinetics Water-soluble molybdenum compounds are readily absorbed when ingested. In animals, gastrointestinal

absorption varies between 75% and 95%. In humans, absorption of molybdenum after oral intake varies from 28% to 77% (ATSDR, 2017b). Once absorbed, molybdenum rapidly appears in blood and most tissues. The highest molybdenum concentrations are found in the kidneys, liver, and bones. Very little molybdenum appears to cross the placenta. When elevated exposure is ceased, tissue concentrations quickly return to normal levels. Molybdenum metabolism is related to copper and sulfur. Exposure to molybdenum decreases intestinal absorption of copper and sulfate, and impairs the sulfation of chemicals (ATSDR, 2017b). Excretion, primarily via the urine, is rapid and 36% to 90% of a dose of molybdenum is excreted in urine in experimental animals. In humans, the urinary excretion ranges from 17% to 80% of the total dose (ATSDR, 2017b). Very little (less than 1%) of molybdenum excretion is via bile. When consuming a low-molybdenum diet, the intestinal molybdenum absorption and tissue uptake are increased, while urinary excretion is decreased to reduce the molybdenum loss. With high dietary intake, urinary excretion can be dramatically increased to help eliminate excess molybdenum (ATSDR, 2017b).

Essentiality and Deficiency Molybdenum deficiency consists of disturbances in uric acid metabolism and sulfite metabolism. Molybdenum cofactor (Moco) deficiency is a pleiotropic genetic disorder characterized by the loss of the molybdenum-dependent enzymes sulfite oxidase, xanthine oxidoreductase, and aldehyde oxidase, due to mutations in the genes involved with Moco biosynthesis. This rare human genetic metabolic disorder is characterized by severe neurodegeneration resulting in early childhood death (Schwarz, 2016).

Toxicity Molybdenum is of low toxicity. Chronic exposure to excess molybdenum in humans is characterized by high uric acid levels in serum and urine. A gout-like syndrome has been observed in inhabitants exposed to high levels of environmental molybdenum or among workers exposed to molybdenum in a copper–molybdenum plant (ATSDR, 2017b). When inhaled, both metallic molybdenum and sparingly soluble molybdenum trioxide have been reported to cause pneumoconiosis and pulmonary deficits (ATSDR, 2017b). Molybdenosis is a form of molybdenum poisoning that produces a disease in ruminants similar to copper deficiency (Raisbeck et al., 2006). In many ways molybdenum toxicity resembles copper deficiency. Treatment with supplemental copper can often reverse the adverse effects of excess molybdenum (Raisbeck et al., 2006). Conversely, treatment of Wilson disease with molybdenum compounds reduces copper burden (Weiss and Stremmel, 2014). Molybdenum treatment may also be beneficial for angiogenesis, inflammation, and other disorders associated with excess copper (Jurowska et al., 2016).

Selenium

Selenium (Se) was discovered in 1817, and named after the Greek word *selene* meaning moon. Although technically a nonmetal, certain forms have metal-like properties. Selenium is an essential element found in selenoproteins and deficiency is recognized in humans and animals (Wrobel et al., 2016). It is also toxic and high doses cause overt selenium poisoning (*selenosis*)(ATSDR, 2003b; Wrobel et al., 2016). The availability and the toxic potential of selenium compounds are related to their chemical forms and, most importantly, to solubility. Selenium occurs in nature and biological systems as selenate (Se^{6+}), selenite (Se^{4+}), selenide (Se^{2+}), and elemental selenium (Se^{0}) (Fairweather-Tait et al., 2011).

Foods are a good source of selenium. Seafood (especially shrimp), meat, milk products, and grains provide the largest amounts in the diet (Fairweather-Tait et al., 2011; Wrobel et al., 2016). Levels of selenium in river water vary depending on environmental and geologic factors. Combustion of coal and other fossil fuels, as well as mining and processing of coal with high selenium content are the primary sources of soil, water, and airborne selenium compounds (ATSDR, 2003b; Fairweather-Tait et al., 2011; Chen et al., 2014; Rowe, 2014). Occupational exposure comes from selenium refining operations, metal smelting, and milling operations, incineration of rubber tires, and municipal waste (ATSDR, 2003b).

Toxicokinetics Orally administered selenite, selenate, and selenomethionine are readily absorbed, often greater than 80%, whereas elemental selenium and selenides are virtually insoluble and poorly absorbed (ATSDR, 2003b). Because of their insolubility, these forms may be regarded as an inert selenium sink. Monogastric animals have higher intestinal absorption than ruminants, probably because selenite is reduced to an insoluble form in rumen. Selenium is distributed systemically bound to plasma selenoprotein P (SePP), the major circulating transport form of selenium that accounts for the majority of selenium in plasma and is responsive to changes in level of dietary exposure (Fairweather-Tait et al., 2011). Selenium accumulates in many tissues, with the highest accumulation in the liver and kidney. It is transferred through the placenta to the fetus, and it also appears in milk. Levels in milk are dependent on dietary intake. Selenium in red blood cells is associated with glutathione peroxidase and is about three times more concentrated than in plasma. Selenium is primarily eliminated in urine and feces. In cases of acute exposure to toxic concentrations of selenium, significant amounts are eliminated in expired air, causing the characteristic "garlic breath" (Fairweather-Tait et al., 2011; Wrobel et al., 2016).

Selenium metabolism is well regulated in order to meet several metabolic needs (Fig. 23-8). Inorganic selenium and selenocysteine undergo stepwise reduction to the key intermediate hydrogen selenide, which is either transformed to selenophosphate for selenoprotein synthesis or excreted into breath or urine after being transformed into methylated metabolites of selenide. Selenophosphate is involved in the synthesis of selenocystein tRNA according to the UGA code for the selenocysteine residue (Schweizer and Fradejas-Villar, 2016). The translation of selenoprotein mRNA requires *cis*-acting sequences in the mRNA and transacting factors dedicated to selenocysteine incorporation (Schweizer and Fradejas-Villar, 2016; Wrobel et al., 2016). Selenoprotein synthesis is highly dependent on selenium availability (Fairweather-Tait et al., 2011; Schweizer and Fradejas-Villar, 2016).

Essentiality and Deficiency Selenium is notable for its actions in antioxidant systems through involvement in over 20 selenoproteins (Schweizer and Fradejas-Villar, 2016; Wrobel et al., 2016). For instance, glutathione peroxidase is the selenium-dependent enzyme that reduces peroxides using glutathione, and thereby protects membrane lipids, proteins, and nucleic acids from damage by oxidants or free radicals. The enzyme thioredoxin reductase is another selenium-dependent enzyme that has an important role in body's defense against oxidative damage. SePP is both the main transporter of selenium and is the major plasma selenoprotein. It also serves as an antioxidant in the extracellular space (Wrobel et al., 2016). Selenium W is a low-molecular-weight selenoprotein and may have redox functions (Wrobel et al., 2016). Iodothyronine deiodinases are selenoproteins contributing to systemic or local thyroid hormone homeostasis (Wrobel et al., 2016). Selenium content in endocrine tissues (thyroid, adrenals, pituitary, testes, and ovary) is higher than that in many other organs. Hormones and

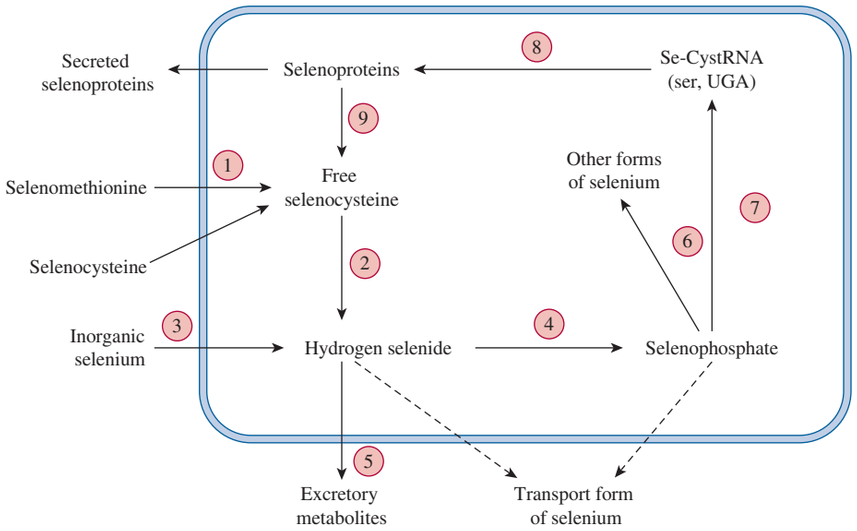

Figure 23-8. *Selenium metabolism pathways.* (1) The transsulfuration pathway; (2) selenocysteine β-lyase; (3) reduction by glutathione; (4) selenophosphate synthetase; (5) methylation; (6) replacement of sulfur in tRNA by selenium; (7) replacement of oxygen in serine to produce selenocysteine; (8) decoding of UGA in mRNA with insertion of selenocysteine into primary structure of protein; and (9) proteolytic breakdown of proteins. The origin and identity of the transport form for selenium are unknown, as indicated by the broken lines.

growth factors also regulate expression of selenoproteins (Wrobel et al., 2016).

The most extensively documented deficiency of selenium in humans is Keshan disease. This endemic cardiomyopathy was first discovered in Keshan County in China where there are very low concentrations of selenium in the soil and food (Fairweather-Tait et al., 2011). Keshan disease patients show very low plasma selenium levels. This deficiency occurs most frequently in children under 15 years of age and in women of childbearing age and is characterized by various degrees of cardiomegaly and cardiac decompensation. Deficiency of selenium also occurs in domestic animals and rodents. Selenium supplementation reduces these adverse effects (Fairweather-Tait et al., 2011).

Kashin–Beck disease is an osteoarthropathy found in areas where combined deficiency of selenium and iodine occurs with elevated exposure to mycotoxin and fulvic acids (Fairweather-Tait et al., 2011). Selenium deficiency is a major contributing factor in this disease. Other potential effects of selenium deficiency include immune dysfunction, and susceptibility to cancer or infectious/inflammatory diseases (Fairweather-Tait et al., 2011; Wrobel et al., 2016). Established tolerable upper intake limits of selenium in adults are 200 to 300 μg/day (IOM, 2000a). Metabolic balance studies in adults indicate about 50 to 70 μg/day is required to maintain selenium balance and presumably to satisfy selenium requirements (IOM, 2000a; Fairweather-Tait et al., 2011). Data indicate that daily intake less than 20 μg can cause Keshan disease.

Toxicity Acute selenium toxicity in humans is rare. Intentional or accidental ingestion of a large dose of sodium selenate or sodium selenite can be life-threatening. Symptoms of fatal selenium intoxication include nausea and vomiting, followed by pulmonary edema and rapid cardiovascular collapse (ATSDR, 2003b; Fairweather-Tait et al., 2011). Chronic selenium toxicity (*selenosis*) can occur with environmental exposure when the intake exceeds the excretory capacity. Effects are mainly dermal and neurological including hair and fingernail loss, tooth discoloration, numbness, paralysis, and occasional hemiplegia. Selenosis occurred in several villages in China where people were exposed to very high selenium in food (Fairweather-Tait et al., 2011). Intoxication from environmental selenium has also been noted in people residing in Venezuela and South Dakota. Selenium toxicity in animals was recognized in South Dakota when the livestock that had been grazed in areas with high soil selenium developed *alkali disease* and *blind staggers*. In a study of people living in this area, poor dentition, a yellowish discoloration of the skin, skin eruptions, and diseased fingernails and toenails were found (Fairweather-Tait et al., 2011).

Selenium has various bioinorganic interactions, which may affect the toxicity of selenium or other metals. It forms insoluble complexes with various metals, as, for example, with arsenic. Selenium complexation can increase the biliary excretion of various metals. Selenium forms complexes with copper, and toxicity of either selenium or copper is influenced by the intake of the other elements. The methylation of selenium can influence other methylation reactions, and can reduce arsenic metabolism and toxicity (Chen et al., 2009; Zwolak and Zaporowska, 2012). Selenium prevents the toxic effects of cadmium and can reduce the toxic effects of methylmercury (Zwolak and Zaporowska, 2012). The mechanisms for these interactions are only partially understood, but their occurrence influences the determination of safe and toxic levels of selenium for the general population.

Selenium and selenium compounds are not considered to be human carcinogens (ATSDR, 2003b). However, selenium sulfide is considered *reasonably anticipated to be a human carcinogen* based on multiple positive rodent studies where tumors of the lung or liver were produced after exposure via stomach tube (NTP, 2016b). In contrast, epidemiological studies have linked low blood selenium levels with increased cancer risk, as well as decreased cancer risk in areas with high soil levels of selenium (ATSDR, 2003b; Wrobel et al., 2016). Selenium supplementations appear to decrease human cancer rates, especially for prostate cancer (Fairweather-Tait et al., 2011; Wrobel et al., 2016). Increasing selenium content of forage crops has been shown to be beneficial in reducing cancer risk. Some experimental evidence supports a role for selenium in reduction of spontaneous tumors or tumors formed by organic carcinogens in rats and mice (Mosior et al., 1992).

Trivalent Chromium

Essentiality The essentiality of trivalent chromium (Cr^{3+}), a naturally occurring trace nutrient, has been controversial as it has been difficult to find populations that are deficient in the metal. There is evidence to suggest that Cr^{3+} may serve as a cofactor for insulin in glucose metabolism by enhancing insulin signaling (Vincent, 2013; Bailey, 2014). However, the failure to identify the responsible biomolecules that bind Cr^{3+} and their mode of action, particularly a postulated species named glucose tolerance factor, resulted in the essentiality of chromium being questioned (Vincent, 2013). While there are suggestions that tissue levels of chromium are reduced among diabetic individuals, recent meta-analysis of a large body of clinical trials of Cr^{3+} supplementation to improve glucose metabolism in diabetic have shown no beneficial effects (Bailey, 2014). Nonetheless, the adequate intake of Cr^{3+} is now proposed as 35 and 25 μg/kg/day for men and women, respectively (IOM, 2001). Chromium picolinate remains a top-selling nutritional supplement that contains milligram quantities of Cr^{3}. In addition to finding no clear benefit of the supplement, there are no demonstrated toxic effects of consuming high levels of supplemented Cr^{3+} (IOM, 2005; Vincent, 2013).

Zinc

Zinc (Zn) was named from German *zink* meaning tin. It has been used since ancient times in alloys and medicines. An essential metal, zinc deficiency results in severe health consequences. In contrast, zinc toxicity is relatively uncommon and occurs only at very high exposure levels. Zinc is ubiquitous in the environment and is present in most foodstuffs, water, and air. The major route of zinc intake is through the diet, the contents of which vary from 5.2 to 16.2 mg Zn/day (IOM, 2001; ATSDR, 2005b). The recommended daily allowance (RDA) for zinc in children less than 3 years old is 3 mg/day; this value increases to 8 mg/day for children between 4 and 13 years old. For men more than 14 years old the RDA is 11 mg/day, while for women it is 8 to 9 mg/day, which increases to 12 to 13 mg/day during pregnancy (IOM, 2001).

Occupational exposure to dusts and fumes of metallic zinc occurs in zinc mining and smelting. The zinc content of substances in contact with galvanized copper or plastic pipes may be high. Many countries regulate workplace levels of zinc oxide fume and dust, and OSHA has set an average legal limit of 1 mg/m^3 for zinc chloride fumes and 5 mg/m^3 for zinc oxide (dusts and fumes) in workplace air during an 8-hour workday, 40-hour work week (ATSDR, 2005b).

Toxicokinetics The absorption of zinc from the gastrointestinal tract is homeostatically regulated. About 20% to 30% of ingested zinc is absorbed. Zinc uptake from the intestinal lumen involves passive diffusion and a carrier-mediated process through zinc-specific transmembrane transporters such as ZnT-1. Intestinal absorption of zinc can be reduced by dietary fiber, phytates, calcium, and phosphorus, while amino acids (tryptophan), picolinic acid. and prostaglandin E_2 can enhance zinc absorption. Zinc is the third most abundant element in the human body with total levels in the range of 1.5 to 3 g, most of which is found in muscle (60%), bone (30%), skin/hair (8%), liver (5%), and pancreas (3%). It is involved in over 300 enzymatic reactions essential for health and is responsible for tertiary and quaternary protein structure required for protein and protein/DNA interactions. As discussed above, there is little free zinc in the body and 30 different transporters and channels regulate its distribution. These transporters and channels include those that bring Zn into cells and organelles (14 Zips and 3+ TRPs) and 10 Znts that move Zn out of the cytoplasm (Cousins, 2010; McCormick et al., 2014; Hojyo and Fukada, 2016). Zip4 channels found throughout the gastrointestinal track are essential for uptake of dietary Zn. The highest concentrations of zinc are found in the prostate, pancreas, liver, and kidney. In plasma, zinc concentration is about 1 mg/L, and is bound to albumin (60% to 80%), which represents the metabolically active pool of zinc. The remainder is bound to α_2-macroglobulin and transferrin. Zinc is excreted in both urine and feces. The concentration of zinc in the plasma is not a sensitive indicator of zinc status and does not reflect the dose–response relationship between zinc levels in the body and effects at various target sites. Zinc ions are involved as intercellular and intracellular messengers, and the homeostasis of zinc has to be tightly controlled. The most reliable index of zinc status is the determination of zinc balance, using a U-shaped homeostatic model to analyze the relationship between intake and excretion (ATSDR, 2005b).

Zinc is an effective inducer of metallothionein synthesis and when metallothionein is saturated in intestinal cells zinc absorption is decreased (Baltaci et al., 2018). Metallothionein is also an important storage depot for cellular zinc. Liver metallothionein concentration is influenced by hormonal factors, including adrenocorticotropic hormone and parathyroid hormone, and various stimuli that impact zinc metabolism. The high concentration of zinc in the prostate is probably related to the rich content of zinc-containing enzyme acid phosphatase.

Essentiality and Deficiency As mentioned there are more than 300 catalytically active zinc metalloenzymes and 2000 zinc-dependent transcription factors (Hojyo and Fukada, 2016; Liang et al., 2016). Zinc participates in a wide variety of metabolic processes, supports a healthy immune system, and is essential for normal growth and development during pregnancy, childhood, and adolescence. Zinc deficiency is related to poor dietary zinc intake, dietary phytate (inositol hexakisphosphate) intake, chronic illness, or oversupplementation with iron or copper (Prasad, 2004). Symptoms of zinc deficiency include growth retardation, appetite loss, alopecia, diarrhea, impaired immune function, cognitive impairments, dermatitis, delayed healing of wounds, taste abnormalities, and impaired sexual function (Prasad, 2004). *Acrodermatitis enteropathica* is a rare autosomal recessive disorder involving zinc deficiency that can appear after weaning from breast or formula feeding. The deficiency is due to mutations in a zinc-specific transporter that is highly expressed in the intestine. The disease is characterized by periorificial and acral dermatitis, alopecia, and diarrhea (Hojyo and Fukada, 2016; Ogawa et al., 2016). Zinc supplementation, alone or with other micronutrients, is recommended for zinc-deficient children, especially in developing countries.

Therapeutic uses of zinc include the treatment of acute diarrhea in infants with severe zinc deficiency, the treatment of common cold by its antiviral and immunomodulatory effects, therapy for Wilson disease to help reduce copper burden and to induce MT, and the prevention of blindness in age-related macular degeneration (Prasad et al., 1994).

Toxicity Acute zinc toxicity from excessive ingestion is uncommon, but gastrointestinal distress and diarrhea have been reported after ingestion of beverages standing in galvanized cans. After inhalation of zinc oxide, and to a lesser extent other zinc compounds, the most common effect is "metal fume fever" characterized by fever, chest pain, chills, cough, dyspnea, nausea, muscle soreness, fatigue, and leukocytosis. Acute inhalation of high levels of zinc chloride as in the military use of "smoke bombs" results in more pronounced damage to the mucous membrane including interstitial

edema, fibrosis, pneumonitis, bronchial mucosal edema, and ulceration. Chronic intakes of zinc (150 to 450 mg/day) have been associated with low copper status, altered iron function, and reduced immune function. Recently, excessive use of denture adhesive cream has been recognized as a potential source for zinc toxicity. These individuals ingest 350 to 1200 mg of zinc per day (Hedera et al., 2009). After long-term exposure to lower doses of zinc (60 mg/day), symptoms generally result from a decreased dietary copper absorption, leading to early symptoms of copper deficiency, such as decreased erythrocyte number or decreased hematocrit (IOM, 2001; Trumbo et al., 2001).

Neuronal Toxicity Zinc has dual effects in the brain. As an essential cofactor for numerous enzymes and proteins, zinc deficiency may alter activity of the antioxidant enzyme Cu–Zn SOD, resulting in excess free radicals that are damaging to cell membranes (Valko et al., 2005). A genetic abnormality of Cu–Zn SOD may be the basis of a familial form of amyotrophic lateral sclerosis (Beckman et al., 2001). Zinc can also act as a neurotransmitter for normal brain functions (Wright and Baccarelli, 2007). It modulates the solubility of β-amyloid in the brain and protects against β-amyloid toxicity, but excess zinc may trigger neuronal death that is independent or synergistic with the toxic effect of β-amyloid (Greenough et al., 2013). In contrast, excess zinc released by oxidants can act as a potent neurotoxin (Nuttall and Oteiza, 2014). Synaptically released zinc might contribute to excitotoxic brain injury, and the release of excess, toxic free zinc into the brain that occurs during excitotoxic brain injury could be a factor that sets the stage for the later development of Alzheimer disease (Wright and Baccarelli, 2007; Nuttall and Oteiza, 2014).

Pancreatic Toxicity Because large amounts of zinc accumulate in secretory granules of pancreatic islet β-cells, zinc released under certain conditions can affect the function or survival of islet cells and cause β-cell death. Excess dietary zinc is associated with damage to exocrine pancreas (Maret, 2017). A single, high-dose injection of zinc increases plasma α-amylase activity and can produce fibrosis and necrosis of pancreatic exocrine cells, but does not affect the islets of Langerhans cells (Cai et al., 2005).

Zinc and Carcinogenicity Epidemiological studies of workers in electrolytic zinc and copper refining industries have not found an increased incidence of cancer associated with occupational zinc inhalation. Oral zinc supplementation does not appear to have a significant effect on cancer incidence (ATSDR, 2005b). In contrast, zinc deficiency may be associated with increased risk of cancer in humans (Pan et al., 2017). Zinc supplementation could decrease oxidative stress and improve immune function, which may be a possible mechanism for its cancer preventive activity (Prasad, 2004). In experimental animals, zinc prevents cadmium-induced testicular cancer, but facilitates cadmium-induced prostate tumors (Waalkes, 2003).

METALS RELATED TO MEDICAL THERAPY

Aluminum

Aluminum (Al) is the third most abundant element in the Earth's crust after oxygen and silicon. Elemental aluminum was first identified in 1827. Due to its high reactivity, aluminum is not found in the free state in nature. Chemical compounds of aluminum occur typically in the trivalent state (Al^{3+}). As a hard trivalent ion, aluminum binds strongly to oxygen donor ligands such as citrate and phosphate. The chemistry of aluminum compounds is complicated by a tendency to hydrolyze and form polynuclear species, many of which are sparingly soluble (Willhite et al., 2014).

Aluminum has many uses, mainly in the form of alloys, and finds use in packing, construction, transportation, electrical applications, and beverage cans. Aluminum compounds are also used as food additives. Human exposure to aluminum comes primarily from food and secondarily from drinking water. Occupational exposures to aluminum occur during mining and processing, as well as in aluminum welding. The levels of exposure can vary greatly according to the type of industry and hygiene conditions (Riihimaki and Aitio, 2012; Caito and Aschner, 2015). Inhalation of aluminum-containing dust particles is of health concern.

Aluminum exists predominantly in forms that are innocuous to humans and most species. However, acidic conditions, such as acid rain or dry acid deposition, can dramatically increase the amount of aluminum in ecosystems, resulting in well-described destructive effects on plants, fish, and other wildlife.

Toxicokinetics Aluminum is poorly absorbed after either oral or inhalation exposure and is not absorbed dermally. Inhalation of particulate aluminum may result in direct transfer to brain tissue via the olfactory system (Riihimaki and Aitio, 2012). Less than 1% of aluminum in the diet is absorbed. Absorption from the gut depends largely on pH and the presence of complexing ligands, particularly carboxylic acids, through which aluminum becomes absorbable. For example, intestinal absorption is enhanced in the presence of citrate. Biological speciation is also of major importance in distribution and excretion of aluminum in mammals (Riihimaki and Aitio, 2012; Caito and Aschner, 2015). In plasma, 80% to 90% of aluminum binds to transferrin, an iron transport protein with receptors in many tissues. The transferrin pathway is also considered a mechanism for aluminum transport across the blood–brain barrier. The lung, liver, and bone have the highest concentrations of aluminum. Aluminum is removed from blood by the kidneys and excreted in urine. In patients with impaired renal function, tissue aluminum concentrations can increase and are associated with encephalopathy and osteomalacia (ATSDR, 2008).

Aluminum compounds can alter absorption of other elements in the gastrointestinal tract. For instance, aluminum inhibits fluoride absorption and may decrease the absorption of calcium and iron compounds and salicylic acid, which, in turn, may affect the absorption of aluminum (ATSDR, 2008). The binding of phosphorus by aluminum in the intestinal tract can lead to phosphate depletion and, potentially, osteomalacia. Aluminum interacts with calcium in bone and kidney, resulting in aluminum osteodystrophy (Malluche et al., 2008). Aluminum may also alter gastrointestinal tract motility by inhibition of acetylcholine-induced contractions, which is probably why aluminum-containing antacids often cause constipation.

Toxicity Acute aluminum toxicity is rare. Most cases of aluminum toxicity in humans are observed in patients with chronic renal failure, or in persons exposed to aluminum in the workplace, with the lung, bone, and central nervous system as major target organs. Aluminum affects similar target organs in animals and can produce developmental effects.

Lung and Bone Toxicity Occupational exposure to aluminum dust can produce lung fibrosis in humans, but this effect is probably due to lung overload caused by excessive deposition of dust (DeVoto and Yokel, 1994). Osteomalacia has been associated with excessive intake of aluminum-containing antacids in otherwise healthy individuals. This is assumed to be due to interference with intestinal phosphate absorption. Osteomalacia also can occur in uremic patients exposed to aluminum in the dialysis fluid. In these patients, osteomalacia may be a direct effect of aluminum on bone mineralization as bone levels are high (Willhite et al., 2014).

Neurotoxicity Aluminum is neurotoxic to experimental animals, with wide species and age variations. In susceptible animals, such as rabbits and cats, aluminum toxicity is characterized by progressive neurological impairment resulting in death associated with status epilepticus (Rubio et al., 2010). The most prominent early pathological change is the accumulation of neurofibrillary tangles (NFTs) in large neurons, proximal axons, and dendrites of neurons of many brain regions. This is associated with loss of synapses and atrophy of the dendritic tree. Not all species show this reaction to aluminum. For instance, rats fail to develop NFTs or encephalopathy and monkeys develop NFTs only after more than a year of aluminum infusion. Impairment of cognitive and motor function and behavioral abnormalities are often observed. While studies in animals have provided some insights into the mechanisms of the neurotoxicity of aluminum in experimental models, the relationship to any human disease is still uncertain.

Dialysis Dementia This is a progressive, neurological syndrome reported in patients on long-term intermittent hemodialysis for chronic renal failure (Kandimalla et al., 2016). The first symptom in these patients is a speech disorder followed by dementia, convulsions, and myoclonus. The disorder, which typically arises after 3 to 7 years of dialysis treatment, may be due to aluminum intoxication. The aluminum content of brain, muscle, and bone increases in these patients. Sources of the excess aluminum may be from oral aluminum hydroxide commonly given to these patients or from aluminum in dialysis fluid derived from the tap water used to prepare the dialysate fluid. The high serum aluminum concentrations may be related to increased parathyroid hormone levels that are due to low blood calcium and osteodystrophy common in patients with chronic renal disease. The syndrome may be prevented by avoiding the use of aluminum-containing oral phosphate binders and by monitoring of aluminum in the dialysate.

Alzheimer Disease A possible relationship between aluminum and Alzheimer disease has been a matter of speculation for decades (Kandimalla et al., 2016). The basis for this relationship is the finding of increased aluminum levels in Alzheimer diseased brains and neurofibrillary lesions in experimental animals, and the fact that aluminum is associated with various components of the pathological lesions in Alzheimer disease brain tissue. However, elevated aluminum levels in Alzheimer disease brains may be a consequence and not a cause of the disease. The reduced effectiveness of the blood–brain barrier in Alzheimer disease might allow more aluminum into the brain. Also, recent studies have raised the possibility that the staining methods in earlier studies may have led to aluminum contamination (Bondy, 2010; Kandimalla et al., 2016). Furthermore, the NFTs seen in aluminum encephalopathy differ structurally and chemically from those in Alzheimer disease. Epidemiological studies examining the role of aluminum exposure in Alzheimer disease arrive at conflicting conclusions. An examination of 20 epidemiological studies concluded that there is not enough evidence to support a primary causative role of aluminum in Alzheimer disease, and aluminum does not induce Alzheimer pathology in vivo in any species, including humans (WHO, 1997). However, there is increasing evidence suggesting a link between aluminum in the brain and other neurodegenerative diseases (Bondy, 2010; Kandimalla et al., 2016).

Treatment Chelation therapy for aluminum, mostly in dialyzed and/or uremic patients, resembles that for iron overload, with deferoxamine and deferiprone (Blanusa et al., 2005).

Bismuth

Bismuth (Bi) is a metal with a stable valence of 3+. The name *bisemutum* is from German *Wismuth*, perhaps from the term *weiβe Masse*, for "white mass." It was confused in early times with tin and lead due to its resemblance to those elements. Bismuth was shown to be distinct from lead in 1753. The solubility of most bismuth salts is low but can be affected by pH and the presence of sulfhydryl or hydroxy-containing ligands. The significance of environmental and occupational bismuth exposure is unclear. Human exposure to bismuth generally is due to medicinal use.

Trivalent insoluble bismuth salts such as bismuth subnitrate, subcarbonate, and subgallate are used for various gastrointestinal disorders including diarrhea, flatulence, constipation, cramps, and dyspepsia (Salvador et al., 2012). Colloidal bismuth subcitrate, bismuth subsalicylate, and ranitidine bismuth citrate are widely used to treat peptic ulcer and *Helicobacter pylori*–associated gastritis (Alkim et al., 2017). Outdated medicinal applications include the use of bismuth salts for treatment of syphilis, malaria, warts, stomatitis, and infections of the upper respiratory tract (Salvador et al., 2012; Keogan and Griffith, 2014). A potential application includes the use of bismuth subnitrate to prevent cisplatin nephrotoxicity, probably due to a specific induction of renal MT (Kondo et al., 2004), although this does not appear to have been applied in practice. The use of α-particle-emitting bismuth compounds as radiotherapeutic agents and as antitumor agents shows some promise (Morgenstern et al., 2011; Keogan and Griffith, 2014).

Toxicokinetics Most bismuth compounds are insoluble and poorly absorbed from the gastrointestinal tract or when applied to the skin. The three widely used compounds, colloidal tripotassium dicitrato bismuthate, bismuth subsalicylate, and ranitidine bismuth citrate, are all poorly absorbed (less than 1%) (Tillman et al., 1996). The highest concentrations of bismuth are found in kidneys, and to a lesser extent in the brain, liver, and bone (Larsen et al., 2005). Passage of bismuth into the amniotic fluid and fetus has been demonstrated. Bismuth is cleared from the body through urine and feces (Irgolic et al., 1977). Traces of bismuth can be found in milk and saliva. The elimination half-life is reported to be about 21 days, depending on bismuth compounds.

Toxicity Bismuth-containing medications are consumed worldwide and the risk of bismuth-related toxicity in the general population is relatively low. The main target organs for bismuth toxicity are the kidney, brain, and bone (Tillman et al., 1996; Larsen et al., 2005; Salvador et al., 2012). Acute renal injury is related to very high doses of bismuth, or to oral intake of organic bismuth compounds such as bismuth sodium triglycocollamate or thioglycollate, particularly in children. The tubular epithelium is the primary site of toxicity, where bismuth produces degeneration of renal tubular cells and nuclear inclusion bodies composed of bismuth–protein complex analogous to those found with lead exposure. A large single oral dose of colloidal bismuth subcitrate damages the proximal tubes, but the damage is reversible in both humans and animals (Leussink et al., 2002).

Treatment The most effective treatment of bismuth toxicity is to discontinue bismuth intake. Chelation therapy using dimercaprol (BAL), DMSA, and DMPS reduces the bismuth concentration in most organs (especially the kidney and liver) and increases elimination of bismuth in urine. BAL was the only chelator effective in lowering brain bismuth concentrations (Slikkerveer and de Wolff, 1989).

Gallium

Gallium (Ga) has a very low melting point with the main valence state of 3+ (gallic), although the 2+ (gallous) form can also form stable gallium compounds. Gallium, stemming from *Gallia*

meaning Gaul or France, was predicted to exist prior to being discovered in 1875 by its characteristic spectrum. Gallium is of interest because of the use of radiogallium as a diagnostic tool for the localization of bone lesions. Nonradioactive gallium nitrate has been used as an antitumor agent and in the treatment of hypercalcemia (Chitambar, 2012). Gallium is obtained as a by-product of copper, zinc, lead, and aluminum refining and is used in high-temperature thermometers, as a substitute for mercury in arc lamps, as a component of metal alloys, and as a seal for vacuum equipment (White and Shine, 2016). Gallium arsenide is a widely used semiconductor material. Gallium is the only metal other than mercury that is liquid at or near room temperature.

Toxicokinetics Gallium salts are sparingly absorbed from the gastrointestinal tract, but accumulation in tissues can be observed after repeated administrations (Chitambar, 2016; White and Shine, 2016). The oral bioavailability is improved with gallium complexes such as gallium maltolate. Gallium accumulates mainly in the bone, inflammatory lesions, and tumors, as well as in the liver, spleen, and kidney. It binds to plasma transferrin and enters cells by iron transport mechanisms (Chitambar, 2016). Urine is the major route of gallium excretion with lesser amounts in the feces (Chitambar, 2012, 2016).

Toxicity The trivalent gallium cation biologically resembles ferric iron. It affects cellular acquisition of iron by binding to transferrin and interacts with the iron-dependent enzyme ribonucleotide reductase, resulting in decreased dNTP pools and inhibition of DNA synthesis (Chitambar, 2016, 2017). The abundance of transferrin receptors and ribonucleotide reductase renders tumor cells susceptible to the cytotoxicity of gallium. Use of bolus intravenous injection of gallium nitrate in the treatment of lymphoma and bladder cancer is limited by potential nephrotoxicity, which can be reduced by slow continuous infusion over several days (Chitambar, 2017). Gallium nitrate is an effective treatment for cancer-related hypercalcemia and diseases associated with accelerated bone loss including myeloma, bone metastasis, Paget disease, and osteoporosis. It accumulates in metabolically active regions of bone and favorably alters the mineral properties to enhance hydroxyapatite crystallization and reduce mineral solubility. Gallium inhibits osteoclastic bone resorption without poisoning the osteoclast cells, yielding a skeletal system with increased calcium and phosphate content and improved strength (Tanaka, 2004; Chitambar, 2010). Adverse effects may include nausea, vomiting, and anemia. Less frequent are gallium-induced neurological, pulmonary, and dermatologic effects.

Acute and chronic toxicity to the lung (including alveolar and bronchioalveolar tumors), testes, and kidney is associated with exposures of gallium arsenide in animals, although the role of the individual metals in this response is unclear (Chitambar, 2010). There is little information regarding adverse effects of gallium compounds after occupational exposure, but renal toxicity was the primary side effect of gallium nitrate treatment in human clinical trials (White and Shine, 2016) and there is significant renal toxicity of gallium arsenide in rats (Fowler et al., 2005).

Gold

Gold (Au) was well known and highly valued since prehistoric times. It is widely distributed but usually in small quantities. The metal has a number of industrial uses because of its electrical and thermal conductivity. Monovalent organogold salts (e.g.,, auranofin, aurothioglucose, and gold sodium thiomalate) are used for the treatment of rheumatoid arthritis and infectious diseases (Faa et al., 2018). Monovalent and trivalent coordinated gold complexes have antitumor potential (Faa et al., 2018). Colloidal gold or gold nanoparticles are used to label subcellular structures for electron microscopy, carriers for drugs, and photothermal agents (Hornos Carneiro and Barbosa, 2016; Faa et al., 2018).

Toxicokinetics Gold salts are poorly absorbed from the gastrointestinal tract. Water-soluble, therapeutic gold compounds are readily absorbed after intramuscular injection, and peak concentrations in blood are reached in 2 to 6 hours. Gold is initially bound to serum albumin, and then distributes to various tissues. With continued therapy, the concentration of gold in synovium of affected joints is 10 times that of the muscle, bone, or fat. Gold deposits are also found in the macrophages, kidney, liver, testes, and skin. About 60% to 90% of gold is excreted via the kidney while 10% to 40% occurs via biliary excretion into feces. Chelators such as dimercaprol may increase the excretion of gold. Gold has a long biological half-life, and elevated tissue and blood levels can be demonstrated for months after cessation of treatment.

Toxicity Contact dermatitis is the most frequently reported toxic reaction to gold and is sometimes accompanied by stomatitis, probably involving an allergic mechanism (Chen and Lampel, 2015). Gold-induced allergic responses include delayed hypersensitivity, formation of intracutaneous nodules and immunogenic granuloma, as well as the occurrence of eczema (Chen and Lampel, 2015). The use of gold in the form of organic salts to treat rheumatoid arthritis may be complicated by the development of proteinuria and the nephrotic syndrome, which morphologically consists of immune complex glomerulonephritis, with granular deposits along the glomerular basement membrane and in the mesangium (Schiff and Whelton, 2000). The pathogenesis of the immune complex disease is not certain, but gold may behave as a hapten and generate the production of antibody complexes for the glomerular deposits.

Gold nanoparticles are being developed as therapeutic agents that may reduce the toxic side effects of traditional gold compounds (Hornos Carneiro and Barbosa, 2016). However, the margin of safety for these nanoparticles is yet to be established and in vivo and in vitro studies suggest that toxicity is related to the shape or physical properties of the nanomaterial (Hornos Carneiro and Barbosa, 2016).

The health risks associated with gold mining, especially miners who used elemental mercury to amalgamate and extract gold, have been reviewed (Eisler, 2003; Drace et al., 2016). Gold miners have increased frequency of pulmonary diseases, including tumors, and increased prevalence of infectious diseases. Mercury intoxication associated with gold mining activities is well documented (Eisler, 2003; Drace et al., 2016).

Lithium

Lithium (Li) is an alkali metal and one of the lightest metallic elements. It was discovered in 1817, and the name was derived from the Greek *lithos* for stone. Lithium is used in batteries, alloys, catalysts, photographic materials, and the aerospace industry. Lithium hydride produces hydrogen on contact with water and is used in manufacturing electronic tubes, in ceramics, and in chemical analysis. Lithium carbonate and lithium citrate are widely used for mania and bipolar disorders. In this regard, lithium is active possibly through its effects on signal transduction, such as phosphoinositide hydrolysis, glycogen synthase kinase-3, and neurotropic cascades (Oruch et al., 2014). Topical applications of lithium succinate are still used in the treatment of seborrheic dermatitis (Mayser and Schulz, 2016).

Toxicokinetics Lithium is readily absorbed from the gastrointestinal tract, with peak therapeutic levels at 30 minutes to 3 hours postingestion. It is not bound to plasma proteins, but associates with red blood cells. Lithium is distributed to total body water with higher levels in the kidney, thyroid, and bone, as compared with other tissues. Excretion is chiefly through the kidneys with 80% of the filtered lithium reabsorbed. The usual elimination half-life is 12 to 27 hours, but it may rise to nearly 60 hours if renal function is compromised. Lithium can substitute for sodium or potassium on several membrane-bound transport proteins (Oruch et al., 2014). It enters cells via the amiloride-sensitive sodium channel or the Na/H^+ exchanger. The greater part of lithium is retained in the cells, perhaps at the expense of potassium. In general, it may be competing with sodium at certain sites, such as in renal tubular reabsorption (Trepiccione and Christensen, 2010).

Toxicity From the industrial point of view, except for lithium hydride, lithium salts are not considered hazardous nor toxic. Lithium hydride is intensely corrosive, may produce burns on the skin because of the formation of hydroxides, and is explosive. Intoxications related to lithium exposure are mainly related to its medicinal (Oruch et al., 2014), as the therapeutic index of lithium is very narrow. In this regard, 0.7 to 1.2 mmol/L is considered an adequate therapeutic blood level, while blood levels only threefold higher commonly result in severe symptoms such as seizures and coma. The toxic responses to lithium include neuromuscular changes (tremor, muscle hyperirritability, and ataxia), central nervous system disorders (blackout spells, epileptic seizures, slurred speech, coma, psychosomatic retardation, and increased thirst), cardiovascular disturbances (cardiac arrhythmia, hypertension, and circulatory collapse), gastrointestinal symptoms (anorexia, nausea, and vomiting), and renal damage (albuminuria and glycosuria). The renal lesions are believed to be due to temporary hypokalemic nephritis. Long-term sequelae from acute lithium poisoning include cognitive losses such as impaired memory, attention and executive functions, and visuospatial deficits.

Chronic lithium nephrotoxicity and interstitial nephritis may occur with long-term exposure even when lithium levels remain within the therapeutic range. Lithium nephrotoxicity primarily targets distal and collecting tubes, with a higher incidence of proteinuria and associated glomerular pathology (Markowitz et al., 2000). Chronic lithium-induced neurotoxicity, nephritis, and thyroid dysfunction may occur, especially in susceptible patients with identifiable clinical risk factors such as nephrogenic diabetes insipidus, older age, abnormal thyroid function, and impaired renal function (Oruch et al., 2014).

Acute lithium overdose produces neurological sequelae and cardiac toxicity, which can be fatal. The toxicity may be treated by the administration of diuretics (amiloride) and lowering of blood levels via hemodialysis. Treatment with diuretics must be accompanied by replacement of water and electrolytes (Oruch et al., 2014).

Platinum

Platinum (Pt) is a malleable, ductile, silvery-white noble metal. Naturally occurring platinum and platinum-rich alloys have been known for a long time. The first European reference to platinum appeared in 1557 and the element was isolated in 1741. In platinum compounds, the maximum oxidation state is 6+, while the 2+ and 4+ valences are most stable. Platinum is found in nature either in the metallic form or in a number of mineral forms in various ores. Environmental platinum levels are very low. Platinum compounds are used as automobile catalysts, in jewelry, in electronics, and in dental alloys. Platinum coordination complexes are very important antitumor agents.

Toxicokinetics After a single inhalation exposure, most of the inhaled platinum is rapidly cleared from the lungs by mucociliary action, swallowed, and excreted in the feces, with half-life of about 24 hours. A small portion is detected in the urine, indicating very little platinum is absorbed. After intravenous administration of clinical doses, the drug has an initial elimination half-life in plasma of 25 to 50 minutes. More than 90% of the platinum in the blood is covalently bound to plasma proteins. After administration of the main metallochemotherapeutic form, *cis*-dichlorodiammine platinum(II) (cisplatin), high concentrations are found in the kidney, liver, intestine, spleen, and testes, but there is poor penetration into the brain. Only a small portion of the drug is excreted by the kidney during the first 6 hours. By 24 hours up to 25% is excreted, while by 5 days up to 43% of the administered dose is recovered in the urine (Cohen and Lippard, 2001; Dugbartey et al., 2016).

Toxicity Platinum can produce profound hypersensitivity reactions in susceptible individuals (Makrilia et al., 2010; Sabin and Grammer, 2012). The signs of hypersensitivity include urticaria, contact dermatitis of skin, and respiratory distress, ranging from irritation to an asthmatic syndrome, after exposure to platinum dust. The skin and respiratory changes are termed *platinosis*. They are mainly confined to persons with a history of industrial exposure to soluble compounds such as sodium chloroplatinate, although cases resulting from the wearing of platinum jewelry have been reported (Forte et al., 2008). The complex salts of platinum may act as powerful allergens, particularly ammonium hexachloroplatinate and hexachloroplatinic acid. Platinum salt sensitization may persist for years after cessation of exposure (Cristaudo et al., 2005). Halogeno complex salts of platinum are potent allergens provoking Type I allergic symptoms in platinum refining workers. Skin prick tests can detect sensitization at an early stage and are the mainstay of surveillance programs (Cristaudo et al., 2005; Merget et al., 2017).

Antitumor Effects of Platinum Complexes The platinum-coordinated complexes are important antitumor agents, including cisplatin, carboplatin, and oxaliplatin (Cohen and Lippard, 2001; Dilruba and Kalayda, 2016; Ndagi et al., 2017). They are routinely administered, often in combination with other anticancer drugs, in the treatment of a wide spectrum of malignancies, especially epithelial cancers. Platinum complexes are neutral and have a pair of *cis*-leaving groups. Low intracellular chloride concentrations favor hydrolysis of chloride-leaving groups in cisplatin to yield a positively charged molecule, which then reacts with DNA and proteins, forming both intrastrand and interstrand DNA cross-links with guanine and/or adenine (Dilruba and Kalayda, 2016; Ndagi et al., 2017). In tumor cells, the replication of DNA is impaired due to cisplatin-induced DNA cross-links, while in normal cells, guanine is repaired before replication. The formation of DNA adducts with platinum compounds is also responsible for cytotoxicity and both efficacy and cytotoxicity are affected by genetic variance in DNA repair enzymes (Dilruba and Kalayda, 2016; Perez-Ramirez et al., 2017).

Carcinogenic Effects of Platinum Complexes Although cisplatin has antitumor activity in humans, it is considered *reasonably anticipated to be a human carcinogen* (NTP, 2016b). Cisplatin is clearly carcinogenic in rodents (NTP, 2016b). In fact, in mice deficient in metallothionein, cisplatin can induce liver carcinoma at clinically relevant doses (Waalkes et al., 2006). Cisplatin is a strong mutagen in bacterial systems and causes chromosomal aberrations in cultured hamster cells and a dose-dependent increase in sister chromatid exchanges.

Toxicities of Platinum Antitumor Complexes Cisplatin is a nephrotoxin, which often compromises its usefulness as a therapeutic agent (Dugbartey et al., 2016). Platinum compounds with antitumor activity produce proximal and distal tubular cell injury, mainly in the corticomedullary region, where the concentration of platinum is highest. Associated with cisplatin nephrotoxicity is the risk for electrolyte abnormalities (Dugbartey et al., 2016). Cisplatin is also associated with cumulative peripheral sensory neuropathy, ototoxicity due to irreversible damage of the hair cells in Corti organ, and significant nausea and vomiting (Dilruba and Kalayda, 2016). Bone marrow suppression, manifested as anemia, neutropenia, and thrombocytopenia, is relatively common during treatment with platinum complexes, especially when given in combination with fluorouracil. Second- and third-generation platinum compounds, such as carboplatinum and oxaliplatinum, given at standard chemotherapeutic doses, have lower toxicity profiles (Dilruba and Kalayda, 2016; Ndagi et al., 2017).

MINOR TOXIC METALS

Antimony

Antimony (Sb) is a metalloid that belongs to the same periodic group as arsenic. It was recognized in antiquity (3000 BC or earlier) in various compounds for its fine casting qualities, and the description of its isolation appeared in 1540. The name antimony may have come from the Greek words "anti" and "monos," which means "opposed to solitude" as it was thought never to exist in its pure form. Most antimony compounds are of trivalent and pentavalent states. Antimony has many uses including in alloys, and in production of fireproofing chemicals, ceramics, glassware, and pigments. It is used medically in the treatment of the parasitic diseases: schistosomiasis and leishmaniasis (Sundar and Chakravarty, 2010; Nigra et al., 2016). Occupational antinomy exposure comes from industrial emissions (Perez and Tang, 2015; Nigra et al., 2016; ATSDR, 2017a). In addition to living in close proximity to industrial sources of antimony, water and food are the major routes for environmental exposure (ATSDR, 2017a).

The disposition of antimony in the body resembles that of arsenic and antimony can negatively impact arsenic metabolism and excretion in co-exposures (Nigra et al., 2016). Most antimony compounds are absorbed from the lung and the gastrointestinal tract. The major sites of antimony accumulation are the liver, kidney, lung, spleen, and blood (ATSDR, 2017a). The accumulation of antimony in blood may be due to high affinity of trivalent antimony for erythrocytes. In humans and rodents, pentavalent antimony is only sparingly reduced to the trivalent form, and evidence of antimony methylation in mammals is low (ATSDR, 2017a). The pentavalent form is predominantly excreted in urine, whereas trivalent antimony is conjugated to GSH and is excreted via the bile and found mainly in feces (ATSDR, 2017a).

Toxicity Most information about antimony toxicity has been obtained from industrial experiences (Nigra et al., 2016; ATSDR, 2017a). Occupational exposures are usually by inhalation of dust containing antimony compounds, such as the pentachloride, trichloride, trioxide, and trisulfide (Sundar and Chakravarty, 2010; ATSDR, 2017a). Acute toxicity from the pentachloride and trichloride exposures includes rhinitis and, in severe exposures, even acute pulmonary edema. Chronic exposure by inhalation of other antimony compounds results in rhinitis, pharyngitis, trachitis, and, over the longer term, bronchitis and eventually pneumoconiosis with obstructive lung disease and emphysema (ATSDR, 2017a). Transient skin eruptions (antimony spots) may occur in workers with chronic exposure (Sundar and Chakravarty, 2010). Antimony compounds are cardiotoxic and may enhance cardiovascular disease and hypertension (Perez and Tang, 2015; Nigra et al., 2016). Cardiomyopathies and pancreatitis are the dose-limiting untoward effects of antimony compounds used in antiparasitic therapies (Sundar and Chakravarty, 2010). Trivalent forms of antimony appear more toxic and may produce cardiotoxicity involving arrhythmias and myocardial damage, although the evidence of atherosclerosis from industrial exposure to antimony is significant but not strong (Nigra et al., 2016).

The chemicotoxicological similarity between arsenic and antimony has prompted research on mutagenic and carcinogenic potential of antimony compounds. Antimony trioxide is considered an animal carcinogen (NTP, 2016a; ATSDR, 2017a), but human data on antimony are difficult to evaluate with the frequent co-exposure to arsenic and other metals (ATSDR, 2017a). Antimony compounds are generally negative in nonmammalian genotoxicity tests, whereas mammalian tests usually give positive results for trivalent but negative results for pentavalent antimony compounds. The in vivo potential of antimony to induce chromosome aberrations or genotoxicity appears inconsistent (ATSDR, 2017a).

The metal hydride of antimony, stibine (SbH_3), is a highly toxic gas that can be generated when antimony is exposed to reducing acids or when certain batteries are overcharged (ATSDR, 2017a). High-purity stibine is also used in the production of semiconductors and, like arsine (AsH_3), causes hemolysis.

Barium

Barium (Ba) is an alkaline earth metal found in the environment in the 2+ oxidation state. It was first identified in 1774, and named from the Greek word *barys* meaning "heavy." Barium and barium compounds (barium carbonate, sulfate, chloride, and hydroxide) are used in ceramics, electronics, insecticides, rodenticides, pigments, and as x-ray contrast media. Barium is relatively abundant in nature and is found in plants and animal tissue. Some foods, such as Brazil nuts, pecans, and seafood, may contain high amounts of barium. Barium from natural sources may exceed Federal government standards in some freshwater, although the amount found in food and water usually is not high enough to be a health concern. Occupational exposure to barium primarily occurs in workers who inhale barium sulfate from working with the ore, barite, and barium carbonate dust during mining and manufacturing (ATSDR, 2007b).

Insoluble barium sulfate is not absorbed from the gastrointestinal tract, and is nontoxic to humans. Consequently, it is commonly used as a contrast dye for radiological imaging of the gastrointestinal tract. Ingested soluble compounds are absorbed, but vary greatly and are affected by many factors such as age, duration, dose, and amounts of other elements in the intestine (ATSDR, 2007b). Aerosols of soluble barium compounds are well absorbed in the lung. Bone and teeth are the major sites of barium deposition, containing up to 90% of the body burden. The remainder of barium in the body is found in soft tissues, such as the lung, aorta, brain, heart, spleen, liver, and pancreas (ATSDR, 2007b). Once filtered by the glomeruli, barium is reabsorbed by the renal tubules with only small amounts appearing in the urine. The major route of barium excretion is the feces. The elimination half-life is about 1 to 2 weeks.

Toxicity Occupational poisoning by barium is uncommon, but a benign pneumoconiosis (*baritosis*) may result from inhalation of barium sulfate (barite) dust or barium carbonate (ATSDR, 2007b). This is not incapacitating and is usually reversible with cessation of exposure. Intentional or accidental poisoning from ingestion of an acute toxic dose (over 200 mg) of soluble barium salt results in

intractable vomiting, severe diarrhea, and gastrointestinal hemorrhage (ATSDR, 2007b). Cardiac arrest is often the cause of death. Profound hypokalemia and muscle weakness progressing to flaccid paralysis are the hallmarks of barium poisoning (ATSDR, 2007b). The mechanism of toxicity probably involves the blocking of calcium-activated potassium channels responsible for cellular efflux of potassium in cardiomyocytes and muscle. The progressive muscle weakness seen in barium intoxication in humans could be due to barium-induced hypokalemia rather than a direct effect on muscles, which is not observed in experimental animals. Treatment with intravenous potassium appears beneficial.

After long-term exposure to barium, nephrotoxicity has been observed in rats and mice. Animal studies designed to assess cardiovascular function have not found significant alterations. Barium compounds are not considered carcinogenic in rodents and there is no evidence of carcinogenicity in humans (ATSDR, 2007b).

Cesium

Cesium (Cs) is a soft silvery-gold alkali metal discovered in 1860, and named from the Latin word caesius meaning "sky blue." Similar to mercury and gallium, metallic cesium exists in the liquid state at slightly above room temperature, and is used in vacuum tubes and in atomic clocks. Cesium compounds only have the 1+ oxidation state, and are used as catalysts in inorganic chemistry, in pharmaceuticals, as well as in scintillation counters. ^{137}Cesium is a by-product of nuclear reactions and is used in radiation therapy.

The Chernobyl nuclear plant accident in 1986 resulted in a large release of cesium into the atmosphere, which then spread as radioactive fallout into the soil, rivers, and lakes, causing serious ecological problems in Northern Europe. The transfer of radiocesium into the food chain and into sheep and reindeer may contribute to human cesium exposure and these ecological issues remain in some areas even decades after the original accident (Jacob et al., 2009).

Most cesium compounds are water soluble and are well absorbed through inhalation, ingestion, or skin contact. Once in the blood, cesium is rapidly distributed throughout the body, with higher concentrations in kidneys, skeletal muscle, liver, and red blood cells (Leggett et al., 2003). Cesium can cross the placenta and appears in milk. It mimics potassium for cellular transport. Urinary excretion is the primary route of elimination of cesium from the body. The biological half-life of cesium is variable, ranging from 50 to 150 days. A physiologically based kinetic model has been developed to describe the distribution and retention of cesium in humans (Leggett et al., 2003).

Toxicity

Radioactive Cesium Exposure to radioactive cesium (^{134}Cs and ^{137}Cs) is of much greater human health concern than exposure to nonradioactive cesium. The initial symptoms after radioactive cesium exposure include nausea, vomiting, and diarrhea. Local skin blistering is common when there is significant dermal contact. With continued exposure to radioactive cesium, adverse neurological and developmental effects can be observed. Symptoms can eventually progress to bone morrow suppression, infection, hemorrhage, and even death (ATSDR, 2004a).

Nonradioactive Cesium Compounds Stable cesium (^{133}Cs) compounds are relatively less toxic. High-dose exposure can cause irritation to the gastrointestinal tract and to the eye (ATSDR, 2004a). High-dose ingestion of cesium salts as homeopathic remedies for cancer treatment has raised concerns with a number of clinical side effects related to electrolyte imbalance. In these therapies, total cesium intakes of 6 g/day may produce severe hypokalemia, hypomagnesemia, prolonged QTc interval, episodes of polymorphic ventricular tachycardia, with or without torsade de pointes, and even acute heart arrest (Melnikov and Zanoni, 2010). Prussian blue has been approved in the treatment of cesium poisoning (Thompson and Callen, 2004).

Fluorine

Fluorine (F) was isolated in 1886 by Henri Moissan for which he was awarded the 1906 Nobel Prize in Chemistry. Fluorides are organic and inorganic compounds containing the nonmetallic element fluorine. Fluoride is an essential component for normal mineralization of bones and dental enamel, and it has been widely used to reduce the prevalence and severity of dental caries in children and adults. Fluorosilicic acid and sodium fluorosilicate have been used in water fluoridation since 1940s. Adequate intake level is about 0.05 mg/kg/day in adults but is much lower for infants (ATSDR, 2003a). Toxicologically important fluoride compounds include hydrogen fluoride and sodium fluoride. The major sources of fluoride intake in the general population are water, food, and fluoride-containing dental products. However, excessive intake of fluorides has been observed from environmental sources, including from drinking water with naturally high fluoride (Meenakshi and Maheshwari, 2006), or from exposure to indoor fluoride air pollution from the use of high fluoride-containing coal (ATSDR, 2003a; Jha et al., 2011).

Fluorides are readily absorbed (75% to 90%) from the gastrointestinal tract (ATSDR, 2003a; Jha et al., 2011). Once absorbed, fluorides are rapidly distributed throughout the body. Approximately 99% of the fluoride in the body is found in bones and teeth. Fluoride is incorporated into bone by replacing the hydroxyl ion in hydroxyapatite to form hydroxyfluorapatite. Fluoride in bone can be remobilized slowly as a result of the ongoing process of bone remodeling, especially in the young children. Fluoride is readily transferred across the placenta but poorly transferred to breast milk. Fluorides are mainly excreted in urine. Plasma and urine fluoride levels are related to fluoride intake and are a biomarker for excess exposure, while hair, fingernails, and tooth enamel are indicators of long-term response (ATSDR, 2003a; Jha et al., 2011).

Toxicity

Dental Fluorosis Excessive fluoride intake from water during the period of enamel formation in children can cause dental fluorosis. In its mild form, dental fluorosis is characterized by white, opaque areas on the tooth surface. In its severe form, it is manifested as yellowish brown to black stains and severe pitting of the teeth. Dental fluorosis incidence and severity in some instances can be decreased by the cessation of water fluoridation (Clark et al., 2006). Inappropriate use of fluoride toothpaste and fluoride supplements in young children is a risk factor for dental fluorosis (Browne et al., 2005). Dental fluorosis is more common and severe in areas of endemic environmental fluorosis (Meenakshi and Maheshwari, 2006).

Skeletal Fluorosis Long-term exposure to very high oral doses of fluoride or occupational exposure to cryolite dusts can result in skeletal fluorosis. Cases of skeletal fluorosis are predominantly found in developing countries and are associated with high fluoride intake coupled with malnutrition (ATSDR, 2003a; Meenakshi and Maheshwari, 2006; Patel et al., 2017). Skeletal fluorosis does not usually manifest symptomatically until the disease attains an advanced stage. Fluoride is mainly deposited in the neck, knee, pelvic, and shoulder joints and/or bones, which makes it difficult

to move or walk. The symptoms of skeletal fluorosis are similar to arthritis, and early on include sporadic pain, back stiffness, burning-like sensation, pricking and tingling in the limbs, muscle weakness, and chronic fatigue. These symptoms are associated with abnormal calcium deposits in bones and ligaments. In the advanced stage, symptoms include osteoporosis in long bones, and bone outgrowth. The vertebrae may fuse together and eventually the victim may be crippled (crippling skeletal fluorosis), a disability often accompanied by kyphosis (humpbacked) or lordosis (arched back).

There is a suggestion that excess fluorine in drinking water is associated with osteosarcomas (ATSDR, 2003a); however, most modern studies have not found this association (Levy and Leclerc, 2012; Archer et al., 2016). Other health effects include respiratory tract, skin, and eye irritation after inhalation exposure to hydrogen fluoride or fluorine gas. Gastrointestinal symptoms occur with excess ingestion of fluoride (ATSDR, 2003a). Chronic endemic fluorosis may also lead to muscle fiber degeneration, low hemoglobin levels, skin rashes, neurological manifestations, compromised immunity, and endocrine effects.

Palladium

Palladium (Pd) belongs to the platinum group metals. It was discovered in 1803, and named after the asteroid Pallas. Palladium occurs together with other platinum group metals (platinum, rhodium, ruthenium, iridium, and osmium) at a very low concentration in the Earth's crust, and is recovered as a by-product of refining nickel, platinum, and other base metals. Palladium compounds commonly exhibit oxidation state of 2+, although compounds with oxidation state 4+ are observed. Organopalladium compounds also exist. Palladium is used in automobile catalysts, in dentistry (for crowns and bridges), in electrical appliances, and in jewelry and coinage. Environmental palladium levels are increasing, but exposure in the general population is low. Dental alloys and work in metal refining or catalyst manufacture can be major sources of palladium exposure (Thyssen and Menne, 2010; Faurschou et al., 2011).

Palladium chloride is poorly absorbed from the gastrointestinal tract or from subcutaneous injection sites. After intravenous administration of palladium compounds, palladium is distributed to the kidney, liver, spleen, lung, and bone. In cells, palladium compounds likely complex with amino acids, proteins, DNA, and other macromolecules. Orally administered palladium is poorly absorbed and eliminated in feces, whereas intravenous palladium is mainly eliminated in the urine. Half-lives range from 5 to 12 days.

Toxicity Palladium sensitization is a health concern, as very low doses are sufficient to cause allergic reactions in susceptible individuals (Thyssen and Menne, 2010; Faurschou et al., 2011). Persons with known nickel allergy may be especially susceptible. Contact dermatitis is a main manifestation of palladium sensitivity, unlike that with platinum. Immediate hypersensitivity (type I) reactions to palladium have been reported in refinery workers sensitized to platinum (Faurschou et al., 2011). Palladium sensitization is often from dental alloys or jewelry. Occupational exposure to palladium salts may cause skin and eye irritation, and occasionally asthma (Thyssen and Menne, 2010; Faurschou et al., 2011).

There is little evidence that palladium compounds are carcinogenic. These compounds are negative in bacterial mutagenicity tests and in micronucleus test in human peripheral lymphocytes. Tetraammine palladium hydrogen carbonate induced a clastogenic response to lymphocytes in vitro but did not produce positive results in the micronucleus test (WHO, 2002). Some organopalladium complexes and nanoparticles have been shown to have antitumor potential similar to that of cisplatin (Dumas and Couvreur, 2015).

Silver

Silver (Ag) is a rare, naturally occurring element found as a soft, "silver"-colored metal. It was known since ancient times and was separated from lead as early as 4000 BC. The chemical symbol for silver, Ag, is from its Latin name *argentum*. Metallic silver and 1+ oxidation forms are common. Silver metal is used for jewelry, silverware, electronic equipment, and dental filling. Soluble silver salts (e.g., silver nitrate and silver sulfide) have been used to treat bacterial infection. Silver halide is used in the manufacture of photographic plates, while silver sulfadiazine is used in the treatment of burns. Occupational exposure occurs mainly from inhalation of silver fumes and dusts in a number of settings (ATSDR, 1990; Drake and Hazelwood, 2005). There is no essentiality for silver, and dietary intake is in the range of 0.4 to 27 μg/day (Hadrup and Lam, 2014), which is much less than the silver intake from medicinal uses. For drinking water disinfection, WHO permissible level is 0.1 mg Ag/L (WHO, 2014).

One of the recent uses of silver is in silver nanoparticles (AgNP), materials with sizes ranging from 1 to 100 nm, containing 20 to 15,000 atoms of silver. Because of their antimicrobial activity, AgNP are used in a variety of consumer products including medical devices, disinfectants, appliances, textiles, and water treatment (Hadrup and Lam, 2014; Wang et al., 2015). The primary routes of exposure for AgNP include lung, skin, and gastrointestinal tract (Hadrup and Lam, 2014; WHO, 2014; Wang et al., 2015). It has been reported that levels of AgNP more than 20 mg/kg i.v. is toxic (Shoults-Wilson et al., 2011).

Sliver compounds can be absorbed orally, by inhalation, and through damaged skin. Ingested silver compounds are absorbed at a level of less than 18%, and only 2% to 4% is retained in tissues (Hadrup and Lam, 2014). Metallic silver and insoluble silver compounds are not readily taken up by the body, and pose minimal health risk (Drake and Hazelwood, 2005). Toxicity from particulate or colloidal silver comes from released ionic silver (Hadrup and Lam, 2014). Silver is widely distributed to most tissues including the muscle, cerebellum, spleen, duodenum, heart, lung, liver, and kidney (Hadrup and Lam, 2014). It is not clear whether silver crosses the blood–brain barrier, although several studies indicate accumulation in specific areas of the brain (Hadrup and Lam, 2014). Autopsy findings after silver treatment of burn victims indicate the highest levels occur in the skin, gingiva, cornea, liver, and kidneys. Silver is predominantly excreted in the feces with only minimal amounts being eliminated in the urine (Hadrup and Lam, 2014).

Toxicity Silver and silver nanoparticles are relatively nontoxic (Hadrup and Lam, 2014; Munger et al., 2014; WHO, 2014; Wang et al., 2015), and have wide use in therapeutics and consumer products. Large oral doses of silver nitrate or acetate may cause severe gastrointestinal irritation due to its caustic action (Wang et al., 2015). Lesions of the kidneys and lungs and arteriosclerosis have been attributed to both industrial and medicinal exposures (Hadrup and Lam, 2014). Deposition of silver in renal glomerular basement membranes after high-level exposure may produce hypertension and possible cardiac complications (Hadrup and Lam, 2014; Gonzalez et al., 2016). At lower exposure levels, more subtle effects of impaired endothelial function and inhibition of vascular endothelial growth factor action result in impaired angiogenesis and vasorelaxation (Abukabda et al., 2016; Gonzalez et al., 2016). The respiratory tract may be affected in severe cases of silver intoxication. Chronic bronchitis has also been reported to result from medicinal use of colloidal silver (ATSDR, 1990). Silver has very limited genotoxic effects and is not considered a carcinogen.

Prolonged exposure to silver compounds is accompanied with skin discoloration (argyria) or the eyes (argyrosis), which is a local

or systemic effect observed in individuals that are occupationally exposed to AgNP, such as people who ingested a AgNP suspension, or patients who have been treated with nAg-containing burn dressings (Drake and Hazelwood, 2005; Wang et al., 2015) . The affected area becomes bluish-gray or ash gray. This is most prominent in the areas of the body exposed to sunlight, as light acts as a catalyst by triggering the photoreduction of these compounds to form metallic silver, similar to the process of developing a photographic negative.

Chelating therapy and dermal abrasion are ineffective in removing silver deposits from the body and there is no effective treatment for argyria. Argyria can be considered a mechanism for detoxication of silver by sequestering it in the tissues as nontoxic silver–protein complexes or silver sulfide (ATSDR, 1990; Drake and Hazelwood, 2005).

Tellurium

Tellurium (Te) is a metalloid chemically related to selenium and sulfur. Discovered in the 1780s, tellurium is named after the Latin word for earth (*tellus*). It is naturally found as the telluride of gold or combined with various other metals. Produced most often as a by-product of electrolytic copper refining, tellurium is used as an additive to improve metallurgic characteristics of copper, steel, and lead alloys (Gerhardsson, 2015). Tellurium vapor is used in "daylight" lamps and as a semiconductor in combination with other metals. It is also used in explosives, specialized glass, and thermoelectric and electronic devices. It has long been used as an antimicrobial agent and new nanoparticle forms are being developed as novel therapeutic agents (Zare et al., 2017).

The biochemistry and toxicity of inorganic and organometallic tellurium compounds have been reviewed (Zare et al., 2017). Soluble tetravalent tellurates, absorbed into the body after oral administration, are reduced to tellurides, partly methylated, and then exhaled as dimethyl telluride. The dimethylated form is responsible for the characteristic garlic odor in persons intoxicated by tellurium compounds. Food is the main source of tellurium for the general population, while in industrial exposure inhalation would predominate. Some organometallic tellurium compounds are absorbed through the skin. Respiratory absorption data are limited, but inhaled tellurium compounds are likely well absorbed. After oral exposure of tellurides, 10% to 20% of the ingested dose is absorbed (Gerhardsson, 2015). The kidney, bone, and liver accumulate tellurium and it is estimated that bone stores may have a half-life of up to 2 years or more (Gerhardsson, 2015). The urine and bile are the principal routes of excretion. Tellurium crosses the blood–brain barrier and the placenta.

Toxicity Of toxicological importance are elemental tellurium, the gases hydrogen telluride and tellurium hexafluoride, and the water-soluble sodium or potassium tellurites and tellurates (Gerhardsson, 2015). Many organometallic forms of tellurium exist. Tellurates and tellurium are of generally low toxicity, but tellurites are typically more toxic. Acute intoxication by inhalation in occupational exposures results in sweating, nausea, a metallic taste, and garlic smelling breath. In fact, garlic breath is an indicator of exposure to tellurium by dermal, inhalation, or oral routes. The cases of tellurium intoxication reported from industrial exposure do not appear to have been life-threatening. Two deaths occurred within 6 hours of accidental poisoning by mistaken injection of sodium tellurite (instead of sodium iodine) into the ureters during retrograde pyelography (Gerhardsson, 2015; Zare et al., 2017). The victims had garlic breath, renal pain, cyanosis, vomiting, stupor, and loss of consciousness. The amount of sodium telluride injected was about 2 g.

Acute systemic effects of tellurium toxicity in rats include listlessness, decreased locomotor activity, somnolence, anorexia, weight loss, gastrointestinal disturbances, changes in fur, and occasionally epilation and hind leg paralysis (Gerhardsson, 2015). Chronic exposure to high doses of tellurium dioxide produces renal and hepatic injury (Gerhardsson, 2015).

There are no data on human or animal carcinogenicity of tellurium; however, there are studies indicating an anticarcinogenic effect of tellurium (Gerhardsson, 2015; Zare et al., 2017). Tellurium compounds are genotoxic and/or mutagenic in hamster fibroblasts, fungus and bacteria (Degrandi et al., 2010), rat astrocytes (Roy and Hardej, 2011), human blood cells (Santos et al., 2009), and human promyelocytic cells (Santos et al., 2009). In most cases, exposure to these compounds led to oxidative stress and DNA damage, and apoptosis was the predominant mechanism of cell death.

Thallium

Thallium (Tl) is one of the most toxic metals. Thallium (from the Greek *thallos* meaning "a green shoot or twig") was discovered in 1861. The thallium ion has a similar charge and ion radius as the potassium ion, and its toxic effects may result from interference with the biological functions of potassium. In addition, thallium disrupts mitochondrial function leading to increases in intracellular oxidative stress and apoptosis (Hanzel and Verstraeten, 2009).

Thallium is obtained as a by-product of the refining of iron, cadmium, and zinc, and is used as a catalyst in alloys, and in optical lenses, jewelry, low-temperature thermometers, semiconductors, dyes, pigments, and scintillation counters. It has been used medicinally as a depilatory. Thallium compounds, chiefly thallous sulfate, were used as rat poisons and insecticides (ATSDR, 1992). Once the commonest sources of human thallium poisoning, the use of thallium as rodenticides or insecticides is now banned (ATSDR, 1992; Peter and Viraraghavan, 2005). Industrial poisoning is a special risk in the manufacture of fused halides for the production of lenses and windows. Naturally high thallium concentration in soils and consequent uptake into edible plants in Southwest Guizhou, China, caused locally endemic chronic thallium poisoning (Xiao et al., 2012).

Thallium is absorbed through the skin and gastrointestinal tract. The highest concentrations after thallium poisoning are in the kidney. After the initial exposure, large amounts are excreted in urine during the first 24 hours, but after that urinary excretion becomes slow and the feces become an important route of excretion. The half-life of thallium in humans has been reported to range from 1 to 30 days and depends on the initial dose. Thallium undergoes enterohepatic recirculation. Prussian blue, the most commonly used antidote, is given orally to break the enterohepatic recycling by trapping thallium secreted into bile and carrying it into the feces (WHO, 1996). Thallium can transfer across the placenta and is found in breast milk, and may cause toxicity in the offspring (WHO, 1996).

Toxicity The triad of gastroenteritis, polyneuropathy, and alopecia is regarded as the classic syndrome of thallium poisoning (WHO, 1996). Other signs and symptoms also occur depending on the dose and length of exposure. The estimated acute lethal dose in humans is 10 to 15 mg/kg. Death is due mainly to renal, central nervous system, and cardiac failure within a few days to 2 weeks (WHO, 1996; Peter and Viraraghavan, 2005).

Alopecia is the best known effect of thallium poisoning. Depilation begins about 10 days after ingestion and complete hair loss can occur in about 1 month. Other dermal signs may include palmar erythema, acne, anhydrosis, and dry scaly skin due to toxic effects of thallium on sweat and sebaceous glands. After oral

ingestion of thallium, gastrointestinal symptoms occur, including nausea, vomiting, gastroenteritis, abdominal pain, and gastrointestinal hemorrhage. Neurological symptoms usually appear 2 to 5 days after acute exposure, depending on age and the level of exposure. A consistent and characteristic feature of thallium intoxication in humans is the extreme sensitivity of the legs, followed by the "burning feet syndrome" and paresthesia. Central nervous system toxicity is manifest by hallucinations, lethargy, delirium, convulsions, and coma. The acute cardiovascular effects of thallium are initially manifested by hypotension and bradycardia due to direct effects of thallium on sinus node and cardiac muscle. This is followed by hypertension and tachycardia due to vagal nerve degeneration. In severe cases, cardiac failure occurs.

Major symptoms of chronic thallium poisoning include anorexia, headache, and abnormal pain. Other toxic effects of thallium include fatty infiltration and necrosis of the liver, nephritis, pulmonary edema, degenerative changes in the adrenals, and degeneration of the peripheral and central nervous system. In severe cases, alopecia, blindness, and even death have been reported as a result of long-term systemic thallium intake (WHO, 1996; Peter and Viraraghavan, 2005).

A review on thallium poisoning during pregnancy in humans gives a range of fetal effects from severe toxicity to normal development. The only consistent effect identified is a trend toward prematurity and low birth weight in children exposed to thallium during early gestation (Hoffman, 2000). It may be teratogenic, especially with regard to cartilage and bone formation, but most of the evidence comes from birds and not mammals.

Treatment Therapy for thallium intoxication combines forced diuresis, use of activated charcoal, prevention of reabsorption by administration of Prussian blue, and administration of potassium ferric hexacyanoferrate (WHO, 1996). Prussian blue is the recommended drug of choice in acute thallium poisoning (Hoffman, 2003).

Tin

Tin (Sn) is a silver-white metal. The name tin derives from the Anglo-Saxon, *tin*, through the Latin, *stannum*. Tin is one of the earliest metals known and was used as a component of bronze from antiquity. Because of its hardening effect on copper, tin was used in bronze implements as early as 3500 BC. However, the pure metal was not used until 600 BC. Metallic tin can combine with chloride, sulfur, or oxygen to form inorganic tin compounds (stannous, Sn^{2+}; and stannic, Sn^{4+}). Tin can also bind with carbon to form a number of toxicologically important organotin compounds including dimethyltin, dibutyltin, dioctyltin, triphenyltin, and tricyclohexyltin (ATSDR, 2005c). Currently, tin is used in the manufacture of various alloys, such as bronze and brass, for fabricating window glass and in solders, but was previously widely used in food packaging. Stannic chlorides are used in dyeing textiles. Organic tin compounds have been used in fungicides, bactericides, and slimicides, as well as in plastics as stabilizers. The average daily intake of tin from all sources is about 4.0 mg, considerably lower than the 17 mg estimated in previous decades, thanks to better food packaging technology (Blunden and Wallace, 2003). Organotin compounds are ubiquitous contaminants in the environment. Bioconcentration in aquatic organisms and ecotoxicity are dependent on the bioavailability of the particular compounds. Some tin compounds, especially organotins, show high bioavailability and may pose adverse effects toward aquatic ecosystems (Okoro et al., 2011; Fang et al., 2017).

Inorganic tin compounds are poorly absorbed after oral, inhalation, or dermal exposures. For example, only 3% of stannous and less than 1% of stannic compounds are absorbed from the gastrointestinal tract (Rudel, 2003). The majority of an oral dose of inorganic tin is excreted in the feces, while only a small portion of absorbed tin is eliminated via urine (Rudel, 2003). The organotin compounds, particularly trimethyltin and triethyltin compounds, are better absorbed than inorganic tins (Rudel, 2003; Okoro et al., 2011; Fang et al., 2017). The tissue distribution of tin from these organometallic compounds shows the highest concentration in the bone, liver, kidney, and lung, with smaller amounts in the muscle, spleen, heart, or brain. Tetraethyltin, triethyltin, and diethyltin undergo dealkylation to ethyltin compounds, whereas tributyltin is dealkylated to di- and monobutyltin compounds. Phenyltin compounds undergo dearylation, mainly by microsome monooxygenase and P450 enzymes (ATSDR, 2005c).

Toxicity Metallic tin and inorganic tin compounds are relatively nontoxic. Ingestion of food items contaminated with high levels of inorganic tins may cause acute gastroenteritis, while chronic inhalation of inorganic tins (e.g.,, stannic oxide dust or fumes) may lead to benign nonfibrotic pneumoconiosis called *stannosis* (ATSDR, 2005c). Some organic tin compounds are highly neurotoxic, particularly triethyltin and trimethyltin, and cause encephalopathy and cerebral edema (Aschner and Aschner, 1992; Rohl et al., 2009). Toxicity declines as the number of carbon atoms in the chain increases. Trimethyltin produces degenerative lesions in the hippocampus and associated structure of the limbic system in primates and rodents. The lesions are characterized by neuron cell apoptosis with astrocyte swelling and gliosis. Microglia and astrocyte activation with the production of pro-inflammatory cytokines may well contribute to the lesion. Triethyltin produces cerebral edema in experimental animals (ATSDR, 2005c; Rohl et al., 2009).

Triphenyltin, tributyltin, dibutyltin, and dioctyltin compounds produce immunotoxicity in experimental animals, characterized by thymic atrophy, and suppression of T-cell-mediated immune response (ATSDR, 2005c; Ueno et al., 2009). Exposure of pregnant animals to organotin compounds such as tributyltin and triethyltin may induce developmental and endocrine-disrupting effects (ATSDR, 2005c).

Experimental studies have failed to find convincing evidence of carcinogenicity, mutagenicity, or teratogenicity of inorganic tin compounds. There is inadequate information to assess carcinogenic potential of organotin compounds in animals, and they are considered not classifiable as to human carcinogenicity (ATSDR, 2005c). Studies of genotoxicity of organotin compounds have given mixed results depending on the specific compound and test system. Triphenyltin compounds are potential chromosome mutagens, inducing production of micronuclei and sister chromatid exchange in Chinese hamster cells (ATSDR, 2005c).

Titanium

Discovered in 1791, titanium is named for the *Titans* of Greek mythology. Most titanium compounds are in the 4+ oxidation state (titanic), but the 3+ oxidation state (titanous) and 2+ compounds can occur. Titanium can form organometallic compounds. Because of its resistance to corrosion, inertness, and tensile strength, titanium has many metallurgic applications, and finds use in aircraft, armor plating, naval ships, missiles, and as a component of surgical implants and prostheses. Titanium dioxide (TiO_2), the most widely used titanium compound, is used as a white pigment in paints, paper, toothpaste, and plastics, as a food additive to whiten flour, in dietary products and confections, and as a whitener in cosmetics (Lomer et al., 2004). Titanium complexes (such as titanium diketonate and budotitane) and titanocene complexes (such as titanocene

dichloride) have undergone clinical trials as cancer chemotherapeutics (Cini et al., 2017). Titanium tetrachloride ($TiCl_4$) is used to make titanium metal and other titanium-containing compounds. Occupational exposures to $TiCl_4$ can be of toxicological concern (ATSDR, 1997, 2014).

Approximately 3% of an oral dose of inorganic titanium is absorbed. The majority of the absorbed dose is excreted in the urine while unabsorbed titanium is excreted in the feces. The normal urinary concentration of titanium has been estimated at 10 μg/L (ATSDR, 1997; Loza-Rosas et al., 2017), and the estimated total body burden of titanium is about 15 mg. As a result of inhalation exposure, titanium accumulates in the lungs where it remains for long periods of time. Lung burdens increase with age and vary according to geographic location. Concentrations of titanium in the liver (8 μg/g) and kidney (6 μg/g) are similar. Titanium may circulate in plasma bound to transferrin (Loza-Rosas et al., 2017), which is thought to be a mediator for delivery of titanium to tumor cells (Desoize, 2004).

Toxicity Occupational inhalation exposure to $TiCl_4$ can produce mild to severe pulmonary injury because it undergoes rapid hydrolysis on contact with water to form hydrochloric acid, titanium oxychloride, and TiO_2. Occupational exposure to titanium dioxide occurs during production. TiO_2 is classified as a nuisance particulate with a threshold limit value of 10 mg/m^3. Inhalation and instillation of titanium particles coated with alumina and/or amorphous silica produce mild and reversible pulmonary effects. The base pigment-grade and/or nanoscale TiO_2 particles produce minimal pulmonary toxicity, regardless of particle size and surface area (Sayes et al., 2007; Warheit and Donner, 2015). In general, TiO_2 has been considered toxicologically inert regardless of route of exposure.

Epidemiological studies in humans have not found the association of titanium exposure with increased risk of lung cancer and chronic respiratory diseases. Exposure of rats to TiO_2 via inhalation or intratracheal instillations caused malignant lung tumors (IARC, 2010). The titanium compound, titanocene, when suspended in trioctanoin and injected intramuscularly, is carcinogenic in rats and mice. According to IARC, there is inadequate evidence in humans and sufficient evidence in animals for the carcinogenicity of TiO_2 (IARC, 2010).

Titanium compounds and related metallocenes have recently shown chemotherapeutic activity toward gastrointestinal, breast, lung, and skin cancer (Desoize, 2004; Cini et al., 2017). The mechanism of action of titanium compounds appears different from platinum compounds, and nephrotoxicity and myelotoxicity are not prominent. TiO_2 nanoparticles have photocatalytic activity raising concerns over their use in sunscreens. While some studies show TiO_2 particles cause oxidative damage to DNA (IARC, 2010), much about the safety or toxicity of these nanoparticles is still unknown making their use in sunscreens somewhat controversial; although generally considered to be safe (Warheit and Donner, 2015; Grande and Tucci, 2016; McSweeney, 2016).

Uranium

Uranium (U) is an actinide metal with a long history of human use. It was discovered by the German chemist Martin Kloproth in 1789 in a mineral called pitchblende, and was named after Uranus, the planet, which had been discovered 8 years earlier. Uranium naturally occurs in three radioisotopes: ^{234}U, ^{235}U, and ^{238}U. The isotope ^{235}U is of particular interest in nuclear weapons and nuclear reactions. Thus, uranium ore is chemically enriched to increase ^{235}U content from 0.72% to 2% to 4%. The by-product of this process is called depleted uranium, which has decreased ^{235}U and has 40% less radioactivity than natural uranium (ATSDR, 2013b; Asic et al., 2017). Depleted uranium has been used in military applications as warheads and tank armor. Nonmilitary uses include counterweights in airplanes and shields against radiation in hospitals (Asic et al., 2017). The chemical toxicity of uranium compounds is a health concern, rather than their radiation. Depleted uranium has the same chemical toxicity potential as natural uranium (ATSDR, 2013b; Asic et al., 2017).

Uranium has five oxidation states but only the 4+ and 6+ forms are stable enough to be of practical importance. The 6+ oxidation state forms the uranyl ion (UO^{2+}), which further forms water-soluble compounds and is an important species of uranium in body fluids (Bjorklund et al., 2017). The uranyl ion is also the most prevalent form in the environment. The ecotoxicity of uranium and uranyl carbonate complexes to plants, aquatic life, and birds has been recently reviewed (Sheppard et al., 2005).

Absorption of uranium compounds is low by all routes of exposure (ATSDR, 2013b; Bjorklund et al., 2017). Absorption of inhaled uranium compounds occurs in respiratory tract via transfer across cell membranes, and is dependent on the particle size and solubility. Absorption from the gastrointestinal tract can vary from 0.1% to 6%, depending on the specific uranium compound. Once in the blood, uranium is distributed to the organs of the body. Uranium in body fluids generally exists as uranyl ion complexed with citrate and bicarbonate. Uranium preferentially distributes to the bone (66%), liver (16%), kidney (8%), and then other tissues (10%). Two-thirds of the uranium in the blood is excreted in urine over the first 24 hours, but the bone deposits of uranium last for about 1.5 years (ATSDR, 2013b; Bjorklund et al., 2017).

Toxicity The kidney is the most sensitive organ for uranium toxicity (ATSDR, 2013b). Occupational cohort studies do not provide evidence of increased risk of kidney-related mortality among uranium-exposed workers (Arzuaga et al., 2010). However, occupational and community-based studies of populations chronically exposed to elevated drinking-water concentrations of uranium provide some evidence of adverse renal effects, as assessed by biomarkers of proximal tubule damage such as urinary levels of glucose, calcium, and various low-molecular-weight proteins (Arzuaga et al., 2010). Indications of proximal tubule effects, as evidenced by increased urinary β2-microglobulin and retinol binding protein levels, were also seen in the most recent follow-up surveillance study of Gulf War veterans exposed to depleted uranium (Arzuaga et al., 2010). Humans appear to be less sensitive to the renal effects of uranium than rodents or other mammals, which has complicated understanding to the underlying mechanisms of disease promotion (Asic et al., 2017; Bjorklund et al., 2017). Pathological and functional changes occur within days after acute exposure and are manifested by injury to renal tubular epithelial cells (Asic et al., 2017). Overt renal effects are observed with peak kidney uranium concentrations above 2 μg U/g, but mild renal tubular dysfunction from chronic exposure may occur at even lower renal concentrations. There appears to be a trend toward increased severity of renal toxicity with increase in length exposure and urinary uranium levels (Arzuaga et al., 2010).

Challenges remain for establishing any causal relationship between human uranium exposure and birth defects and/or gonadal endocrine dysfunction, because these studies are confounded by co-exposure to other toxicants and inadequate exposure assessments (ATSDR, 2013b; Bjorklund et al., 2017). However, uranium and depleted uranium can be developmental toxicants when given orally or subcutaneously to mice. Decreased fertility, embryo/fetal toxicity, teratogenicity, and reduced growth of the offspring have been

observed in rodents after uranium exposure during different periods of gestation. Bone is a major site of uranium accumulation, and chronic uranium intoxication may result in diminished bone growth and osteoporosis. There is also increasing concern of potential neurotoxicity produced by uranium exposure (Jiang and Aschner, 2006).

A higher incidence of lung cancer has been found in uranium miners and is probably due to radon and its daughter products, but not to uranium itself (ATSDR, 2013b; Bjorklund et al., 2017). Gulf war veterans who were wounded subsequent to the explosion of armor-piercing shells containing depleted uranium often retain small fragments of the metal. This has created concern for the potential long-term effects of such embedded uranium fragments. Although there are no human data, studies in rats and mice indicate that embedded depleted uranium fragments can cause localized proliferative reactions and sarcomas in rats, as well as leukemia in mice (Asic et al., 2017). However, the studies are suspect due to the tendencies for sarcoma incidence of the rodent strains to imbedded particulates.

Vanadium

Vanadium (V) is a transition metal discovered in the early 1800s and is named after the goddess of beauty in Scandinavian mythology, *Vanadis*, because of its beautiful multicolored chemical compounds. It is an essential trace element for microorganisms and bacteria, but definitive evidence for essentiality in mammals is lacking and no specific biochemical function for vanadium has been identified. Vanadium has several oxidation states, the most common being 3+, 4+, and 5+. The metal can be found as halides, such as the tetrachloride, and oxides, such as vanadium pentoxide. Organometallic vanadium compounds are generally unstable. Vanadium is recovered from vanadium-containing ores and from fossil fuels. Vanadium compounds are used in the production of special steels and alloys, in the manufacture of pigments, in photography, and as catalysts (ATSDR, 2012d).

Food is the major source of vanadium exposure in the general population (ATSDR, 2012d). Significant amounts of vanadium are found in seafood, mushrooms, dill seed, milk, meat, cereals, and vegetables. Concentrations in the drinking water largely depend on geographic location. Concentrations in rural air were much lower than in urban air, especially along the eastern seacoast, largely due to oil-fired fuel combustion that has been phased out over the past decades (ATSDR, 2012d). The dietary daily intake is estimated in the range of 10 to 60 μg (ATSDR, 2012d).

The lungs absorb about 25% of soluble vanadium compounds, but the absorption of vanadium salts from the gastrointestinal tract is generally poor (ATSDR, 2012d). Some dermal absorption of soluble compounds is possible but probably represents a minor route for humans. Once absorbed, extracellular vanadium will be in the form of vanadate (5+) and most likely in the vanadyl (4+) form after entering cells. After experimental exposure by various routes in rodents, the highest amounts of vanadium are found in bone, kidney, liver, and spleen (ATSDR, 2012d). Brain levels are considerably lower than other tissues indicating limited transport across the blood–brain barrier. After oral exposure, vanadium shows a multicompartmental pattern with potential for accumulation and retention particularly in the bone (ATSDR, 2012d).

Toxicity The toxicity of vanadium compounds usually increases as the valence increases, the pentavalent compounds being the most toxic. After occupational exposure to airborne vanadium, its toxic actions are largely confined to irritation of the respiratory tract, eyes, and skin (ATSDR, 2012d). Interestingly, there is usually a latent period of 1 to 6 days before the adverse effects of vanadium appear, although the effects are usually reversible. Bronchitis and bronchopneumonia are more frequent in workers exposed to vanadium compounds. In industrial exposures to vanadium pentoxide dust, a characteristic greenish-black discoloration of the tongue occurs due to deposition of vanadium. There is some evidence that a sensitization reaction may occur with repeated exposures (ATSDR, 2012d). Gastrointestinal distress, nausea, vomiting, abdominal pain, cardiac palpitation, tremor, nervous depression, and kidney damage have also been linked with industrial vanadium exposure (ATSDR, 2012d; Fortoul et al., 2014).

There is clear evidence that inhaled vanadium pentoxide is carcinogenic in mice and rats producing benign and malignant lung tumors in a large NTP study (Ress et al., 2003). Vanadium can act as a tumor promoter in the mouse lung (Rondini et al., 2010). The major cellular mechanism for tumor promotion as well as a number of potential therapeutic effects may involve binding to a critical thiol in the active site of tyrosine phosphatases that suppress growth factor and insulin signaling. There are no data indicating a carcinogenic potential for vanadium compounds in humans, although vanadium pentoxide inhalation causes a variety of DNA damage in exposed workers (Fortoul et al., 2014).

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REFERENCES

Abukabda AB, Stapleton PA, Nurkiewicz TR. Metal nanomaterial toxicity variations within the vascular system. *Curr Environ Health Rep*. 2016;3:379–391.

Achary MS, Satpathy KK, Panigrahi S, et al. Concentration of heavy metals in the food chain components of the nearshore coastal waters of Kalpakkam, southeast coast of India. *Food Control*. 2017;72(pt B):232–243.

Aguirre JD, Culotta VC. Battles with iron: manganese in oxidative stress protection. *J Biol Chem*. 2012;287:13541–13548.

Ahmad F, Zhou Y. Pitfalls and challenges in nanotoxicology: a case of cobalt ferrite ($CoFe_2O_4$) nanocomposites. *Chem Res Toxicol*. 2017;30:492–507.

Ahmadibeni Y, Hanley M, White M, et al. Metal-binding properties of a dicysteine-containing motif in protein tyrosine kinases. *Chembiochem*. 2007;8:1592–1605.

Ahsan H, Chen Y, Kibriya MG, et al. Arsenic metabolism, genetic susceptibility, and risk of premalignant skin lesions in Bangladesh. *Cancer Epidemiol Biomarkers Prev*. 2007;16:1270–1278.

Alkim H, Koksal AR, Boga S, et al. Role of bismuth in the eradication of *Helicobacter pylori*. *Am J Ther*. 2017;24:e751–e757.

Allan AM, Hafez AK, Labrecque MT, et al. Sex-dependent effects of developmental arsenic exposure on methylation capacity and methylation regulation of the glucocorticoid receptor system in the embryonic mouse brain. *Toxicol Rep*. 2015;2:1376–1390.

Amicosante M, Berretta F, Rossman M, et al. Identification of HLA-DRPhebeta47 as the susceptibility marker of hypersensitivity to beryllium in individuals lacking the berylliosis-associated supratypic marker HLA-DPGlubeta69. *Respir Res*. 2005;6:94.

Andersen O, Aaseth J. A review of pitfalls and progress in chelation treatment of metal poisonings. *J Trace Elem Med Biol*. 2016;38:74–80.

Andrew AS, Karagas MR, Hamilton JW. Decreased DNA repair gene expression among individuals exposed to arsenic in United States drinking water. *Int J Cancer*. 2003;104:263–268.

Andrew AS, Mason RA, Memoli V, et al. Arsenic activates EGFR pathway signaling in the lung. *Toxicol Sci*. 2009;109:350–357.

Antonini JM, Taylor MD, Zimmer AT, et al. Pulmonary responses to welding fumes: role of metal constituents. *J Toxicol Environ Health A*. 2004;67:233–249.

Anttila A, Heikkila P, Pukkala E, et al. Excess lung cancer among workers exposed to lead. *Scand J Work Environ Health*. 1995;21:460–469.

Aposhian HV, Aposhian MM. Arsenic toxicology: five questions. *Chem Res Toxicol*. 2006;19:1–15.

Archer NP, Napier TS, Villanacci JF. Fluoride exposure in public drinking water and childhood and adolescent osteosarcoma in Texas. *Cancer Causes Control*. 2016;27:863–868.

Argos M, Rahman M, Parvez F, et al. Baseline comorbidities in a skin cancer prevention trial in Bangladesh. *Eur J Clin Invest*. 2013;43:579–588.

Arita A, Costa M. Epigenetics in metal carcinogenesis: nickel, arsenic, chromium and cadmium. *Metallomics*. 2009;1:222–228.

Arnold WR, Cotsifas JS, Smith DS, et al. A comparison of the copper sensitivity of two economically important saltwater mussel species and a review of previously reported copper toxicity data for mussels: important implications for determining future ambient copper saltwater criteria in the USA. *Environ Toxicol*. 2009;24:618–628.

Arzuaga X, Rieth SH, Bathija A, et al. Renal effects of exposure to natural and depleted uranium: a review of the epidemiologic and experimental data. *J Toxicol Environ Health B Crit Rev*. 2010;13:527–545.

Aschner M, Aschner JL. Cellular and molecular effects of trimethyltin and triethyltin: relevance to organotin neurotoxicity. *Neurosci Biobehav Rev*. 1992;16:427–435.

Asic A, Kurtovic-Kozaric A, Besic L, et al. Chemical toxicity and radioactivity of depleted uranium: the evidence from in vivo and in vitro studies. *Environ Res*. 2017;156:665–673.

ATSDR. *Toxicological Profile for Silver*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 1990. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=539&tid=97.

ATSDR. *Toxicological Profile for Thallium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 1992. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=309&tid=49.

ATSDR. *Toxicological Profile for Titanium Tetrachloride*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 1997. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=664&tid=122.

ATSDR. *Toxicological Profile for Fluorides, Hydrogen Fluoride, and Fluorine*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2003a. Available at: https://www.atsdr.cdc.gov/toxprofiles/TP.asp?id=212&tid=38.

ATSDR. *Toxicological Profile for Selenium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2003b. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=153&tid=28.

ATSDR. *Toxicological Profile for Cesium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2004a. Available at: https://www.atsdr.cdc.gov/toxprofiles/TP.asp?id=578&tid=107.

ATSDR. *Toxicological Profile for Cobalt*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2004b. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=373&tid=64.

ATSDR. *Toxicological Profile for Copper*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2004c. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=206&tid=37.

ATSDR. *Toxicological Profile for Nickel*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2005a. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp15.pdf.

ATSDR. *Toxicological Profile for Zinc*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2005b. Available at: https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=302&tid=54.

ATSDR. *Toxicological Profiles of Tin and Tin Compounds*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2005c. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=543&tid=98.

ATSDR. *Toxicological Profile for Arsenic*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2007a. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=22&tid=3.

ATSDR. *Toxicological Profile for Barium and Compounds*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2007b. Available at: https://www.atsdr.cdc.gov/toxprofiles/TP.asp?id=327&tid=57.

ATSDR. *Toxicological Profile for Lead*. Agency for Toxic Substances and Disease Registry; 2007c. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=96&tid=22.

ATSDR. *Toxicological Profile for Aluminum*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2008. Available at: https://www.atsdr.cdc.gov/toxprofiles/TP.asp?id=191&tid=34.

ATSDR. *Toxicological Profile for Cadmium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2012a. Available at: https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=48&tid=15.

ATSDR. *Toxicological Profile for Chromium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2012b. Available at: http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=62&tid=17.

ATSDR. *Toxicological Profile for Manganese*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2012c. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=102&tid=23.

ATSDR. *Toxicological Profiles for Vanadium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2012d. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=276&tid=50.

ATSDR. *Addendum to the Toxicological Profile for Mercury*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2013a.

ATSDR. *Toxicological Profile for Uranium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2013b. Available at: https://www.atsdr.cdc.gov/toxprofiles/TP.asp?id=440&tid=77.

ATSDR. *Addendum to the Toxicological Profile for Titanium Tetrachloride*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2014. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=664&tid=122.

ATSDR. *Addendum to the Toxicological Profile for Beryllium*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2015a. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=1441&tid=33.

ATSDR. *ATSDR's Substance Priority List*. Atlanta, GA: Agency for Toxic Substances and Disease Registery; 2015b. Available at: https://www.atsdr.cdc.gov/spl/index.html.

ATSDR. *Addendum to the Toxicological Profile for Arsenic*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2016. Available at: https://www.atsdr.cdc.gov/toxprofiles/Arsenic_addendum.pdf.

ATSDR. *Toxicological Profile for Antimony and Compounds*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2017a. Available at: https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=332&tid=58.

ATSDR. *Toxicological Profile for Molybdenum*. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2017b. Available at: https://www.atsdr.cdc.gov/ToxProfiles/tp.asp?id=1482&tid=289.

Bailey CH. Improved meta-analytic methods show no effect of chromium supplements on fasting glucose. *Biol Trace Elem Res*. 2014;157:1–8.

Bailey KA, Smith AH, Tokar EJ, et al. Mechanisms underlying latent disease risk associated with early-life arsenic exposure: current research trends and scientific gaps. *Environ Health Perspect*. 2016;124:170–175.

Bakir F, Damluji SF, Amin-Zaki L, et al. Methylmercury poisoning in Iraq. *Science*. 1973;181:230–241.

Baltaci AK, Yuce K, Mogulkoc R. Zinc metabolism and metallothioneins. *Biol Trace Elem Res*. 2018;183:22–31.

Barbosa F Jr, Tanus-Santos JE, Gerlach RF, et al. A critical review of biomarkers used for monitoring human exposure to lead: advantages, limitations, and future needs. *Environ Health Perspect*. 2005;113:1669–1674.

Barchowsky A, Dudek EJ, Treadwell MD, et al. Arsenic induces oxidant stress and NF-kappa B activation in cultured aortic endothelial cells. *Free Radic Biol Med*. 1996;21:783–790.

Barchowsky A, O'Hara KA. Metal-induced cell signaling and gene activation in lung diseases. *Free Radic Biol Med*. 2003;34:1130–1135.

Barr RD, Rees PH, Cordy PE, et al. Nephrotic syndrome in adult Africans in Nairobi. *Br Med J*. 1972;2:131–134.

Beckman JS, Estevez AG, Crow JP, et al. Superoxide dismutase and the death of motoneurons in ALS. *Trends Neurosci*. 2001;24:S15–S20.

Begum M, Horowitz J, Hossain MI. Low-dose risk assessment for arsenic: a meta-analysis approach. *Asia Pac J Public Health*. 2015;27(2):NP20–NP35.

Bellinger DC. The protean toxicities of lead: new chapters in a familiar story. *Int J Environ Res Public Health*. 2011;8:2593–2628.

Bellinger DC. Childhood lead exposure and adult outcomes. *JAMA*. 2017;317:1219–1220.

Bellinger DC, Burger J, Cade TJ, et al. Health risks from lead-based ammunition in the environment. *Environ Health Perspect*. 2013;121:A178–A179.

Berdoukas V, Coates TD, Cabantchik ZI. Iron and oxidative stress in cardiomyopathy in thalassemia. *Free Radic Biol Med*. 2015;88:3–9.

Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Arch Toxicol*. 2008;82:493–512.

Bjorklund G, Albert Christophersen O, Chirumbolo S, et al. Recent aspects of uranium toxicology in medical geology. *Environ Res*. 2017;156:526–533.

Blanusa M, Varnai VM, Piasek M, et al. Chelators as antidotes of metal toxicity: therapeutic and experimental aspects. *Curr Med Chem*. 2005;12:2771–2794.

Blayney MB. The need for empirically derived permeation data for personal protective equipment: the death of Dr. Karen E. Wetterhahn. *Appl Occup Environ Hyg*. 2001;16:233–236.

Blunden S, Wallace T. Tin in canned food: a review and understanding of occurrence and effect. *Food Chem Toxicol*. 2003;41:1651–1662.

Bolt HM. Arsenic: an ancient toxicant of continuous public health impact, from Iceman Otzi until now. *Arch Toxicol*. 2012;86:825–830.

Bommarito PA, Martin E, Fry RC. Effects of prenatal exposure to endocrine disruptors and toxic metals on the fetal epigenome. *Epigenomics*. 2017;9:333–350.

Bondy SC. The neurotoxicity of environmental aluminum is still an issue. *Neurotoxicology*. 2010;31:575–581.

Boshuizen M, van der Ploeg K, von Bonsdorff L, et al. Therapeutic use of transferrin to modulate anemia and conditions of iron toxicity. *Blood Rev*. 2017;31:400–405.

Bradberry SM, Wilkinson JM, Ferner RE. Systemic toxicity related to metal hip prostheses. *Clin Toxicol (Phila)*. 2014;52:837–847.

Brandao K, Deason-Towne F, Perraud AL, et al. The role of Mg^{2+} in immune cells. *Immunol Res*. 2013;55:261–269.

Brewer GJ, Askari FK. Wilson's disease: clinical management and therapy. *J Hepatol.* 2005;42(suppl):S13–S21.

Briasoulis E, Pavlidis N. Noncardiogenic pulmonary edema: an unusual and serious complication of anticancer therapy. *Oncologist*. 2001;6:153–161.

Bridges CC, Zalups RK. Molecular and ionic mimicry and the transport of toxic metals. *Toxicol Appl Pharmacol*. 2005;204:274–308.

Bridges CC, Zalups RK. The aging kidney and the nephrotoxic effects of mercury. *J Toxicol Environ Health B Crit Rev*. 2017a;20:55–80.

Bridges CC, Zalups RK. Mechanisms involved in the transport of mercuric ions in target tissues. *Arch Toxicol*. 2017b;91:63–81.

Bright P, Burge PS, OHickey SP, et al. Occupational asthma due to chrome and nickel electroplating. *Thorax*. 1997;52:28–32.

Brown MJ, Margolis S. Lead in drinking water and human blood lead levels in the United States. *MMWR Suppl*. 2012;61:1–9.

Browne D, Whelton H, O'Mullane D. Fluoride metabolism and fluorosis. *J Dent*. 2005;33:177–186.

Bucher JR, Hailey JR, Roycroft JR, et al. Inhalation toxicity and carcinogenicity studies of cobalt sulfate. *Toxicol Sci*. 1999;49:56–67.

Budtz-Jorgensen E, Bellinger D, Lanphear B, et al. An international pooled analysis for obtaining a benchmark dose for environmental lead exposure in children. *Risk Anal*. 2013;33:450–461.

Burris HH, Baccarelli AA, Wright RO, et al. Epigenetics: linking social and environmental exposures to preterm birth. *Pediatr Res*. 2016;79:136–140.

Cai L, Li XK, Song Y, et al. Essentiality, toxicology and chelation therapy of zinc and copper. *Curr Med Chem*. 2005;12:2753–2763.

Caito S, Aschner M. Neurotoxicity of metals. *Handb Clin Neurol*. 2015;131:169–189.

Calderon RL, Hudgens EE, Carty C, et al. Biological and behavioral factors modify biomarkers of arsenic exposure in a U.S. population. *Environ Res*. 2013;126:134–144.

Camacho LH, Soignet SL, Chanel S, et al. Leukocytosis and the retinoic acid syndrome in patients with acute promyelocytic leukemia treated with arsenic trioxide. *J Clin Oncol*. 2000;18:2620–2625.

Cambier S, Benard G, Mesmer-Dudons N, et al. At environmental doses, dietary methylmercury inhibits mitochondrial energy metabolism in skeletal muscles of the zebra fish (*Danio rerio*). *Int J Biochem Cell Biol*. 2009;41:791–799.

Campbell JR, Rosier RN, Novotny L, et al. The association between environmental lead exposure and bone density in children. *Environ Health Perspect*. 2004;112:1200–1203.

Carey AM, Lombi E, Donner E, et al. A review of recent developments in the speciation and location of arsenic and selenium in rice grain. *Anal Bioanal Chem*. 2012;402:3275–3286.

Carlier MF, Didry D, Valentin-Ranc C. Interaction between chromium GTP and tubulin. Stereochemistry of GTP binding, GTP hydrolysis, and microtubule stabilization. *J Biol Chem*. 1991;266:12361–12368.

Carlin DJ, Naujokas MF, Bradham KD, et al. Arsenic and environmental health: state of the science and future research opportunities. *Environ Health Perspect*. 2016;124:890–899.

Carmouche JJ, Puzas JE, Zhang X, et al. Lead exposure inhibits fracture healing and is associated with increased chondrogenesis, delay in cartilage mineralization, and a decrease in osteoprogenitor frequency. *Environ Health Perspect*. 2005;113:749–755.

CDC. *Low Level Lead Exposure Harms Children: A Renewed Call for Primary Prevention*. Atlanta, GA: Centers for Disease Control and Prevention; 2012. Available at: https://www.cdc.gov/nceh/lead/acclpp/final_document_030712.pdf.

Cervantes C, Campos-Garcia J, Devars S, et al. Interactions of chromium with microorganisms and plants. *FEMS Microbiol Rev*. 2001;25:335–347.

Chambers A, Krewski D, Birkett N, et al. An exposure-response curve for copper excess and deficiency. *J Toxicol Environ Health B Crit Rev*. 2010;13:546–578.

Chan HM, Zhu LF, Zhong R, et al. Nephrotoxicity in rats following liver transplantation from cadmium-exposed rats. *Toxicol Appl Pharmacol*. 1993;123:89–96.

Chen B, Arnold LL, Cohen SM, et al. Mouse arsenic (+3 oxidation state) methyltransferase genotype affects metabolism and tissue dosimetry of arsenicals after arsenite administration in drinking water. *Toxicol Sci*. 2011a;124:320–326.

Chen C, Xun P, Nishijo M, et al. Cadmium exposure and risk of prostate cancer: a meta-analysis of cohort and case-control studies among the general and occupational populations. *Sci Rep*. 2016a;6:25814.

Chen CY, Driscoll CT, Lambert KF, et al. Connecting mercury science to policy: from sources to seafood. *Rev Environ Health*. 2016b;31:17–20.

Chen F, Shi X. Intracellular signal transduction of cells in response to carcinogenic metals. *Crit Rev Oncol Hematol*. 2002;42:105–121.

Chen J, Liu G, Kang Y, et al. Coal utilization in China: environmental impacts and human health. *Environ Geochem Health*. 2014;36:735–753.

Chen JK, Lampel HP. Gold contact allergy: clues and controversies. *Dermatitis*. 2015;26:69–77.

Chen L, Jin T, Huang B, et al. Critical exposure level of cadmium for elevated urinary metallothionein—an occupational population study in China. *Toxicol Appl Pharmacol*. 2006;215:93–99.

Chen SJ, Zhou GB, Zhang XW, et al. From an old remedy to a magic bullet: molecular mechanisms underlying the therapeutic effects of arsenic in fighting leukemia. *Blood.* 2011b;117:6425–6437.

Chen Y, Graziano JH, Parvez F, et al. Arsenic exposure from drinking water and mortality from cardiovascular disease in Bangladesh: prospective cohort study. *BMJ*. 2011c;342:d2431.

Chen Y, Parvez F, Gamble M, et al. Arsenic exposure at low-to-moderate levels and skin lesions, arsenic metabolism, neurological functions, and biomarkers for respiratory and cardiovascular diseases: review of recent findings from the Health Effects of Arsenic Longitudinal Study (HEALS) in Bangladesh. *Toxicol Appl Pharmacol*. 2009;239(2):184–192.

Chen Y, Wu F, Parvez F, et al. Arsenic exposure from drinking water and QT-interval prolongation: results from the health effects of arsenic longitudinal study. *Environ Health Perspect*. 2013;121:427–432, 432e1–7.

Chervona Y, Costa M. The control of histone methylation and gene expression by oxidative stress, hypoxia, and metals. *Free Radic Biol Med*. 2012;53:1041–1047.

Chitambar CR. Medical applications and toxicities of gallium compounds. *Int J Environ Res Public Health*. 2010;7:2337–2361.

Chitambar CR. Gallium-containing anticancer compounds. *Future Med Chem*. 2012;4:1257–1272.
Chitambar CR. Gallium and its competing roles with iron in biological systems. *Biochim Biophys Acta*. 2016;1863:2044–2053.
Chitambar CR. The therapeutic potential of iron-targeting gallium compounds in human disease: from basic research to clinical application. *Pharmacol Res*. 2017;115:56–64.
Cini M, Bradshaw TD, Woodward S. Using titanium complexes to defeat cancer: the view from the shoulders of titans. *Chem Soc Rev*. 2017;46:1040–1051.
Clark DC, Shulman JD, Maupome G, et al. Changes in dental fluorosis following the cessation of water fluoridation. *Community Dent Oral Epidemiol*. 2006;34:197–204.
Clarkson TW. The three modern faces of mercury. *Environ Health Perspect*. 2002;110(suppl 1):11–23.
Clarkson TW, Vyas JB, Ballatori N. Mechanisms of mercury disposition in the body. *Am J Ind Med*. 2007;50:757–764.
Clemens F, Verma R, Ramnath J, et al. Amplification of the Ect2 proto-oncogene and over-expression of Ect2 mRNA and protein in nickel compound and methylcholanthrene-transformed 10T1/2 mouse fibroblast cell lines. *Toxicol Appl Pharmacol*. 2005;206:138–149.
Clyne N, Hofman-Bang C, Haga Y, et al. Chronic cobalt exposure affects antioxidants and ATP production in rat myocardium. *Scand J Clin Lab Invest*. 2001;61:609–614.
Cocco P, Dosemeci M, Heineman EF. Brain cancer and occupational exposure to lead. *J Occup Environ Med*. 1998;40:937–942.
Coffey R, Ganz T. Iron homeostasis: an anthropocentric perspective. *J Biol Chem*. 2017;292:12727–12734.
Cohen SM, Arnold LL, Beck BD, et al. Evaluation of the carcinogenicity of inorganic arsenic. *Crit Rev Toxicol*. 2013;43:711–752.
Cohen SM, Lippard SJ. Cisplatin: from DNA damage to cancer chemotherapy. *Prog Nucleic Acid Res Mol Biol*. 2001;67:93–130.
Cosselman KE, Navas-Acien A, Kaufman JD. Environmental factors in cardiovascular disease. *Nat Rev Cardiol*. 2015;12:627–642.
Costa M, Davidson TL, Chen H, et al. Nickel carcinogenesis: epigenetics and hypoxia signaling. *Mutat Res*. 2005;592:79–88.
Counter SA, Buchanan LH. Mercury exposure in children: a review. *Toxicol Appl Pharmacol*. 2004;198:209–230.
Cousins RJ. Gastrointestinal factors influencing zinc absorption and homeostasis. *Int J Vitam Nutr Res*. 2010;80:243–248.
Cousins RJ, Liuzzi JP, Lichten LA. Mammalian zinc transport, trafficking, and signals. *J Biol Chem*. 2006;281:24085–24089.
Cristaudo A, Sera F, Severino V, et al. Occupational hypersensitivity to metal salts, including platinum, in the secondary industry. *Allergy*. 2005;60:159–164.
Cubeddu LX. Drug-induced inhibition and trafficking disruption of ion channels: pathogenesis of QT abnormalities and drug-induced fatal arrhythmias. *Curr Cardiol Rev*. 2016;12:141–154.
D'Ippoliti D, Santelli E, De Sario M, et al. Arsenic in drinking water and mortality for cancer and chronic diseases in Central Italy, 1990–2010. *PLoS One*. 2015;10:e0138182.
Davidson PW, Myers GJ, Cox C, et al. Methylmercury and neurodevelopment: longitudinal analysis of the Seychelles child development cohort. *Neurotoxicol Teratol*. 2006;28:529–535.
Davis CD, Uthus EO, Finley JW. Dietary selenium and arsenic affect DNA methylation in vitro in Caco-2 cells and in vivo in rat liver and colon. *J Nutr*. 2000;130:2903–2909.
De Boeck M, Kirsch-Volders M, Lison D. Cobalt and antimony: genotoxicity and carcinogenicity. *Mutat Res*. 2003;533:135–152.
De Flora S. Threshold mechanisms and site specificity in chromium(VI) carcinogenesis. *Carcinogenesis*. 2000;21:533–541.
De Francisco N, Ruiz Troya JD, Aguera EI. Lead and lead toxicity in domestic and free living birds. *Avian Pathol*. 2003;32:3–13.
Degrandi TH, de Oliveira IM, d'Almeida GS, et al. Evaluation of the cytotoxicity, genotoxicity and mutagenicity of diphenyl ditelluride in several biological models. *Mutagenesis*. 2010;25:257–269.
Denkhaus E, Salnikow K. Nickel essentiality, toxicity, and carcinogenicity. *Crit Rev Oncol Hematol*. 2002;42:35–56.
DeRouen TA, Martin MD, Leroux BG, et al. Neurobehavioral effects of dental amalgam in children: a randomized clinical trial. *JAMA*. 2006;295:1784–1792.
Desoize B. Metals and metal compounds in cancer treatment. *Anticancer Res*. 2004;24:1529–1544.
Deubner DC, Lowney YW, Paustenbach DJ, et al. Contribution of incidental exposure pathways to total beryllium exposures. *Appl Occup Environ Hyg*. 2001;16:568–578.
DeVoto E, Yokel RA. The biological speciation and toxicokinetics of aluminum. *Environ Health Perspect*. 1994;102:940–951.
Dietert RR. Developmental immunotoxicology (DIT): windows of vulnerability, immune dysfunction and safety assessment. *J Immunotoxicol*. 2008;5:401–412.
Dietert RR, Lee JE, Hussain I, et al. Developmental immunotoxicology of lead. *Toxicol Appl Pharmacol*. 2004;198:86–94.
Diez S. Human health effects of methylmercury exposure. *Rev Environ Contam Toxicol*. 2009;198:111–132.
Dilruba S, Kalayda GV. Platinum-based drugs: past, present and future. *Cancer Chemother Pharmacol*. 2016;77:1103–1124.
Diwan BA, Kasprzak KS, Rice JM. Transplacental carcinogenic effects of nickel(II) acetate in the renal cortex, renal pelvis and adenohypophysis in F344/NCr rats. *Carcinogenesis*. 1992;13:1351–1357.
Doherty MJ, Healy M, Richardson SG, et al. Total body iron overload in welder's siderosis. *Occup Environ Med*. 2004;61:82–85.
Dopp E, Hartmann LM, Florea AM, et al. Environmental distribution, analysis, and toxicity of organometal(loid) compounds. *Crit Rev Toxicol*. 2004;34:301–333.
Drace K, Kiefer AM, Veiga MM. Cyanidation of mercury-contaminated tailings: potential health effects and environmental justice. *Curr Environ Health Rep*. 2016;3:443–449.
Drake PL, Hazelwood KJ. Exposure-related health effects of silver and silver compounds: a review. *Ann Occup Hyg*. 2005;49:575–585.
Drobna Z, Naranmandura H, Kubachka KM, et al. Disruption of the arsenic (+3 oxidation state) methyltransferase gene in the mouse alters the phenotype for methylation of arsenic and affects distribution and retention of orally administered arsenate. *Chem Res Toxicol*. 2009;22:1713–1720.
Drobna Z, Walton FS, Harmon AW, et al. Interspecies differences in metabolism of arsenic by cultured primary hepatocytes. *Toxicol Appl Pharmacol*. 2010;245:47–56.
Drouin E, Russo P, Tuchweber B, et al. North American Indian cirrhosis in children: a review of 30 cases. *J Pediatr Gastroenterol Nutr*. 2000;31:395–404.
Dugbartey GJ, Peppone LJ, de Graaf IA. An integrative view of cisplatin-induced renal and cardiac toxicities: molecular mechanisms, current treatment challenges and potential protective measures. *Toxicology*. 2016;371:58–66.
Dumas A, Couvreur P. Palladium: a future key player in the nanomedical field? *Chem Sci*. 6:2015;2153–2157.
Dunnick JK, Elwell MR, Radovsky AE, et al. Comparative carcinogenic effects of nickel subsulfide, nickel oxide, or nickel sulfate hexahydrate chronic exposures in the lung. *Cancer Res*. 1995;55:5251–5256.
Eisler R. Health risks of gold miners: a synoptic review. *Environ Geochem Health*. 2003;25:325–345.
Eizaga Rebollar R, Garcia Palacios MV, Morales Guerrero J, et al. Magnesium sulfate in pediatric anesthesia: the Super Adjuvant. *Paediatr Anaesth*. 2017;27:480–489.
Ekino S, Susa M, Ninomiya T, et al. Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning. *J Neurol Sci*. 2007;262:131–144.
Engstrom A, Skerving S, Lidfeldt J, et al. Cadmium-induced bone effect is not mediated via low serum 1,25-dihydroxy vitamin D. *Environ Res*. 2009;109:188–192.
Engstrom KS, Hossain MB, Lauss M, et al. Efficient arsenic metabolism—the AS3MT haplotype is associated with DNA methylation and expression of multiple genes around AS3MT. *PLoS One*. 2013;8:e53732.
Enterline PE, Day R, Marsh GM. Cancers related to exposure to arsenic at a copper smelter. *Occup Environ Med*. 1995;52:28–32.
Evans M, Elinder CG. Chronic renal failure from lead: myth or evidence-based fact? *Kidney Int*. 2011;79:272–279.
Faa G, Gerosa C, Fanni D, et al. Gold - old drug with new potentials. *Curr Med Chem*. 2018;25:75–84.
Fairweather-Tait SJ, Bao Y, Broadley MR, et al. Selenium in human health and disease. *Antioxid Redox Signal*. 2011;14:1337–1383.

Fang L, Xu C, Li J, et al. The importance of environmental factors and matrices in the adsorption, desorption, and toxicity of butyltins: a review. *Environ Sci Pollut Res Int*. 2017;24:9159–9173.

Farina M, Avila DS, da Rocha JB, et al. Metals, oxidative stress and neurodegeneration: a focus on iron, manganese and mercury. *Neurochem Int*. 2013;62:575–594.

Farzan SF, Li Z, Korrick SA, et al. Infant infections and respiratory symptoms in relation to in utero arsenic exposure in a U.S. cohort. *Environ Health Perspect*. 2016;124:840–847.

Faurschou A, Menne T, Johansen JD, et al. Metal allergen of the 21st century—a review on exposure, epidemiology and clinical manifestations of palladium allergy. *Contact Dermatitis*. 2011;64:185–195.

Ferrario D, Gribaldo L, Hartung T. Arsenic exposure and immunotoxicity: a review including the possible influence of age and sex. *Curr Environ Health Rep*. 2016;3:1–12.

Ferraro L, Tomasini MC, Tanganelli S, et al. Developmental exposure to methylmercury elicits early cell death in the cerebral cortex and long-term memory deficits in the rat. *Int J Dev Neurosci*. 2009;27:165–174.

Ferreccio C, Yuan Y, Calle J, et al. Arsenic, tobacco smoke, and occupation: associations of multiple agents with lung and bladder cancer. *Epidemiology*. 2013;24:898–905.

Ficker E, Kuryshev YA, Dennis AT, et al. Mechanisms of arsenic-induced prolongation of cardiac repolarization. *Mol Pharmacol*. 2004;66:33–44.

Finney J, Moon HJ, Ronnebaum T, et al. Human copper-dependent amine oxidases. *Arch Biochem Biophys*. 2014;546:19–32.

Fleming DE, Chettle DR, Webber CE, et al. The O'Flaherty model of lead kinetics: an evaluation using data from a lead smelter population. *Toxicol Appl Pharmacol*. 1999;161:100–109.

Fontenot AP, Falta MT, Kappler JW, et al. Beryllium-induced hypersensitivity: genetic susceptibility and neoantigen generation. *J Immunol*. 2016;196:22–27.

Forte G, Petrucci F, Bocca B. Metal allergens of growing significance: epidemiology, immunotoxicology, strategies for testing and prevention. *Inflamm Allergy Drug Targets*. 2008;7:145–162.

Fortoul TI, Rojas-Lemus M, Rodriguez-Lara V, et al. Overview of environmental and occupational vanadium exposure and associated health outcomes: an article based on a presentation at the 8th International Symposium on Vanadium Chemistry, Biological Chemistry, and Toxicology, Washington DC, August 15–18, 2012. *J Immunotoxicol*. 2014;11:13–18.

Fowler BA, Conner EA, Yamauchi H. Metabolomic and proteomic biomarkers for III-V semiconductors: chemical-specific porphyrinurias and proteinurias. *Toxicol Appl Pharmacol*. 2005;206:121–130.

Fox KA, Phillips TM, Yanta JH, et al. Fatal cobalt toxicity after total hip arthroplasty revision for fractured ceramic components. *Clin Toxicol (Phila)*. 2016;54:874–877.

Franco JL, Posser T, Dunkley PR, et al. Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. *Free Radic Biol Med*. 2009;47:449–457.

Freitas AJ, Rocha JB, Wolosker H, et al. Effects of Hg^{2+} and CH_3Hg^+ on Ca^{2+} fluxes in rat brain microsomes. *Brain Res*. 1996;738:257–264.

Galaris D, Pantopoulos K. Oxidative stress and iron homeostasis: mechanistic and health aspects. *Crit Rev Clin Lab Sci*. 2008;45:1–23.

Gamble MV, Liu X, Ahsan H, et al. Folate and arsenic metabolism: a double-blind, placebo-controlled folic acid-supplementation trial in Bangladesh. *Am J Clin Nutr*. 2006;84:1093–1101.

Gammella E, Buratti P, Cairo G, Recalcati S. The transferrin receptor: the cellular iron gate. *Metallomics*. 2017;9:1367–1375.

Garciafigueroa DY, Klei LR, Ambrosio F, et al. Arsenic-stimulated lipolysis and adipose remodeling is mediated by G-protein-coupled receptors. *Toxicol Sci*. 2013;134:335–344.

Gerhardsson L. Tellurium. In: Norberg GF, Fowler B. A., Norberg M, eds. *Handbook on the Toxicology of Metals: Edition 4*. London, UK: Elsevier; 2015:1217–1228.

Germolec DR, Spalding J, Yu HS, et al. Arsenic enhancement of skin neoplasia by chronic stimulation of growth factors. *Am J Pathol*. 1998;153:1775–1785.

Gibb HJ, Lees PS, Pinsky PF, et al. Clinical findings of irritation among chromium chemical production workers. *Am J Ind Med*. 2000a;38:127–131.

Gibb HJ, Lees PS, Pinsky PF, et al. Lung cancer among workers in chromium chemical production. *Am J Ind Med*. 2000b;38:115–126.

Gochfeld M. Factors influencing susceptibility to metals. *Environ Health Perspect*. 1997;105(suppl 4):817–822.

Gonzalez-Raymat H, Liu G, Liriano C, et al. Elemental mercury: its unique properties affect its behavior and fate in the environment. *Environ Pollut*. 2017;229:69–86.

Gonzalez C, Rosas-Hernandez H, Ramirez-Lee MA, et al. Role of silver nanoparticles (AgNPs) on the cardiovascular system. *Arch Toxicol*. 2016;90:493–511.

Goodman BP, Mistry DH, Pasha SF, et al. Copper deficiency myeloneuropathy due to occult celiac disease. *Neurologist*. 2009;15:355–356.

Goodman JE, Prueitt RL, Thakali S, et al. The nickel ion bioavailability model of the carcinogenic potential of nickel-containing substances in the lung. *Crit Rev Toxicol*. 2011;41:142–174.

Gordon T, Bowser D. Beryllium: genotoxicity and carcinogenicity. *Mutat Res*. 2003;533:99–105.

Goulle JP, Saussereau E, Mahieu L, et al. Application of inductively coupled plasma mass spectrometry multielement analysis in fingernail and toenail as a biomarker of metal exposure. *J Anal Toxicol*. 2009;33:92–98.

Goyer RA. Lead toxicity: current concerns. *Environ Health Perspect*. 1993;100:177–187.

Grande F, Tucci P. Titanium dioxide nanoparticles: a risk for human health? *Mini Rev Med Chem*. 2016;16:762–769.

Grashow R, Zhang J, Fang SC, et al. Toenail metal concentration as a biomarker of occupational welding fume exposure. *J Occup Environ Hyg*. 2014;11:397–405.

Greenberg MI, Vearrier D. Metal fume fever and polymer fume fever. *Clin Toxicol (Phila)*. 2015;53:195–203.

Greenough MA, Camakaris J, Bush AI. Metal dyshomeostasis and oxidative stress in Alzheimer's disease. *Neurochem Int*. 2013;62:540–555.

Grimsrud TK, Andersen A. Evidence of carcinogenicity in humans of water-soluble nickel salts. *J Occup Med Toxicol*. 2010;5:7.

Grimsrud TK, Andersen A. Unrecognized risks of nickel-related respiratory cancer among Canadian electrolysis workers. *Scand J Work Environ Health*. 2012;38:503–515.

Grober U, Schmidt J, Kisters K. Magnesium in prevention and therapy. *Nutrients*. 2015;7:8199–8226.

Gulson BL, Mizon KJ, Korsch MJ, et al. Mobilization of lead from human bone tissue during pregnancy and lactation—a summary of long-term research. *Sci Total Environ*. 2003;303:79–104.

Guo L, Harari E, Virmani R, et al. Linking hemorrhage, angiogenesis, macrophages, and iron metabolism in atherosclerotic vascular diseases. *Arterioscler Thromb Vasc Biol*. 2017;37:e33–e39.

Haase H, Engelhardt G, Hebel S, et al. Mercuric ions inhibit mitogen-activated protein kinase dephosphorylation by inducing reactive oxygen species. *Toxicol Appl Pharmacol*. 2011;250:78–86.

Hadrup N, Lam HR. Oral toxicity of silver ions, silver nanoparticles and colloidal silver—a review. *Regul Toxicol Pharmacol*. 2014;68:1–7.

Hallab NJ, Mikecz K, Vermes C, et al. Orthopaedic implant related metal toxicity in terms of human lymphocyte reactivity to metal-protein complexes produced from cobalt-base and titanium-base implant alloy degradation. *Mol Cell Biochem*. 2001;222:127–136.

Hamza I, Gitlin JD. Copper chaperones for cytochrome c oxidase and human disease. *J Bioenerg Biomembr*. 2002;34:381–388.

Handy RD. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? *Comp Biochem Physiol A Mol Integr Physiol*. 2003;135:25–38.

Hanzel CE, Verstraeten SV. Tl(I) and Tl(III) activate both mitochondrial and extrinsic pathways of apoptosis in rat pheochromocytoma (PC12) cells. *Toxicol Appl Pharmacol*. 2009;236:59–70.

Harlan WR. The relationship of blood lead levels to blood pressure in the U.S. population. *Environ Health Perspect*. 1988;78:9–13.

Hart BA, Potts RJ, Watkin RD. Cadmium adaptation in the lung—a double-edged sword? *Toxicology*. 2001;160:65–70,

Hartwig A. Metal interaction with redox regulation: an integrating concept in metal carcinogenesis? *Free Radic Biol Med*. 2013;55:63–72.

Hartwig A, Asmuss M, Blessing H, et al. Interference by toxic metal ions with zinc-dependent proteins involved in maintaining genomic stability. *Food Chem Toxicol*. 2002;40:1179–1184.

Haynes EN, Sucharew H, Kuhnell P, et al. Manganese exposure and neurocognitive outcomes in rural school-age children: the communities actively researching exposure study (Ohio, USA). *Environ Health Perspect*. 2015;123:1066–1071.

Hays AM, Lantz RC, Rodgers LS, et al. Arsenic-induced decreases in the vascular matrix. *Toxicol Pathol*. 2008;36:805–817.

Hedera P, Peltier A, Fink JK, et al. Myelopolyneuropathy and pancytopenia due to copper deficiency and high zinc levels of unknown origin II. The denture cream is a primary source of excessive zinc. *Neurotoxicology*. 2009;30:996–999.

Herroeder S, Schonherr ME, De Hert SG, et al. Magnesium—essentials for anesthesiologists. *Anesthesiology*. 2011;114:971–993.

Hoffman RS. Thallium poisoning during pregnancy: a case report and comprehensive literature review. *J Toxicol Clin Toxicol*. 2000;38:767–775.

Hoffman RS. Thallium toxicity and the role of Prussian blue in therapy. *Toxicol Rev*. 2003;22:29–40.

Hojyo S, Fukada T. Zinc transporters and signaling in physiology and pathogenesis. *Arch Biochem Biophys*. 2016;611:43–50.

Hopenhayn C, Huang B, Christian J, et al. Profile of urinary arsenic metabolites during pregnancy. *Environ Health Perspect*. 2003;111:1888–1891.

Horev-Azaria L, Baldi G, Beno D, et al. Predictive toxicology of cobalt ferrite nanoparticles: comparative in-vitro study of different cellular models using methods of knowledge discovery from data. *Part Fibre Toxicol*. 2013;10:32.

Hornos Carneiro MF, Barbosa F Jr. Gold nanoparticles: a critical review of therapeutic applications and toxicological aspects. *J Toxicol Environ Health B Crit Rev*. 2016;19:129–148.

Hosseini Koupaie E, Eskicioglu C. Health risk assessment of heavy metals through the consumption of food crops fertilized by biosolids: a probabilistic-based analysis. *J Hazard Mater*. 2015;300:855–865.

Hostynek JJ. Factors determining percutaneous metal absorption. *Food Chem Toxicol*. 2003;41:327–345.

Howe CG, Gamble MV. Influence of arsenic on global levels of histone posttranslational modifications: a review of the literature and challenges in the field. *Curr Environ Health Rep*. 2016;3:225–237.

Huggins FE, Huffman GP, Linak WP, et al. Quantifying hazardous species in particulate matter derived from fossil-fuel combustion. *Environ Sci. Technol*. 2004;38:1836–1842.

Hughes MF, Beck BD, Chen Y, et al. Arsenic exposure and toxicology: a historical perspective. *Toxicol Sci*. 2011;123:305–332.

Hughes MF, Devesa V, Adair BM, et al. Tissue dosimetry, metabolism and excretion of pentavalent and trivalent monomethylated arsenic in mice after oral administration. *Toxicol Appl Pharmacol*. 2005;208:186–197.

Hultman P, Pollard M. *Immunotoxicology of Metals: Handbook on the Toxicology of Metals*. London: Elsevier; 2015.

Hwang GW. Role of intracellular defense factors against methylmercury toxicity. *Biol Pharm Bull*. 2012;35:1881–1884.

IARC. *IARC Monographs on the Evaluation of Carcingenic Risks to Humans: Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide, and Vanadium Pentoxide*. Lyon, France: World Health Orginization; 2006.

IARC. *IARC Monograph on the Evaluation of Carcinogenic Risks to Humans: Carbon Black, Titanium Dioxide, and Talc*. Lyon, France: International Agency for Research on Cancer; 2010. Available at: http://monographs.iarc.fr/ENG/Monographs/vol93/mono93.pdf.

IARC. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Arsenic, Metals, Fibres, Dusts*. Lyon, France: International Agency for Research on Cancer; 2012. Available at: http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C.pdf.

IARC. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Arsenic, Metals, Fibres, Dusts: Nickel and Nickel Compounds*. Lyon, France: International Agency for Research on Cancer; 2017. Available at: http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C-10.pdf.

IOM. *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*. Washington, DC: National Academies Press; 2000a. Available at: http://doi.org/10.17226/9810.

IOM. *Institute of Medicine: Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B 6, Folate, Vitamin B 12, Pantothenic Acid, Biotin and Choline*. Washington, DC: National Academies Press; 2000b.

IOM. *Institute of Medicine, Food and Nutrition Board: Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington, DC: National Academies Press; 2001. Available at: https://doi.org/10.17226/10026.

IOM. *Institute of Medicine and National Research Council Dietary Supplements: A Framework for Evaluating Safety*. Washington, DC: National Academies Press.; 2005. Available at: https://doi.org/10.17226/10882.

Irgolic KJ, Woolson EA, Stockton RA, et al. Characterization of arsenic compounds formed by *Daphnia magna* and *Tetraselmis chuii* from inorganic arsenate. *Environ Health Perspect*. 1977;19:61–66.

Isani G, Carpene E. Metallothioneins, unconventional proteins from unconventional animals: a long journey from nematodes to mammals. *Biomolecules*. 2014;4:435–457.

Ishihara N, Matsushiro T. Biliary and urinary excretion of metals in humans. *Arch Environ Health*. 1986;41:324–330.

Ito K, Johnson S, Kheirbek I, et al. Intraurban variation of fine particle elemental concentrations in New York City. *Environ Sci Technol*. 2016;50:7517–7526.

Jacob P, Fesenko S, Bogdevitch I, et al. Rural areas affected by the Chernobyl accident: radiation exposure and remediation strategies. *Sci Total Environ*. 2009;408:14–25.

Jaishankar M, Tseten T, Anbalagan N, et al. Toxicity, mechanism and health effects of some heavy metals. *Interdiscip Toxicol*. 2014;7:60–72.

James KA, Byers T, Hokanson JE, et al. Association between lifetime exposure to inorganic arsenic in drinking water and coronary heart disease in Colorado residents. *Environ Health Perspect*. 2015;123:128–134.

Jarup L, Akesson A. Current status of cadmium as an environmental health problem. *Toxicol Appl Pharmacol*. 2009;238:201–208.

Jaw S, Jeffery EH. Role of metallothionein in biliary metal excretion. *J Toxicol Environ Health*. 1989;28:39–51.

Jha SK, Mishra VK, Sharma DK, et al. Fluoride in the environment and its metabolism in humans. *Rev Environ Contam Toxicol*. 2011;211:121–142.

Jiang GC, Aschner M. Neurotoxicity of depleted uranium: reasons for increased concern. *Biol Trace Elem Res*. 2006;110:1–17.

Jiang Y, Zheng W. Cardiovascular toxicities upon manganese exposure. *Cardiovasc Toxicol*. 2005;5:345–354.

Jin YH, Dunlap PE, McBride SJ, et al. Global transcriptome and deletome profiles of yeast exposed to transition metals. *PLoS Genet*. 2008;4:e1000053.

Johansson C, Castoldi AF, Onishchenko N, et al. Neurobehavioural and molecular changes induced by methylmercury exposure during development. *Neurotox Res*. 2007;11:241–260.

Julin B, Bergkvist C, Wolk A, et al. Cadmium in diet and risk of cardiovascular disease in women. *Epidemiology*. 2013;24:880–885.

Jurowska A, Jurowski K, Szklarzewicz J, et al. Molybdenum metallopharmaceuticals candidate compounds: the "Renaissance" of molybdenum metallodrugs? *Curr Med Chem*. 2016;23:3322–3342.

Kakinuma Y, Miyauchi T, Yuki K, et al. Novel molecular mechanism of increased myocardial endothelin-1 expression in the failing heart involving the transcriptional factor hypoxia-inducible factor-1alpha induced for impaired myocardial energy metabolism. *Circulation*. 2001;103:2387–2394.

Kaler SG. Inborn errors of copper metabolism. *Handb Clin Neurol*. 2013;113:1745–1754.

Kambe T, Takeda TA, Nishito Y. Activation of zinc-requiring ectoenzymes by ZnT transporters during the secretory process: biochemical and molecular aspects. *Arch Biochem Biophys*. 2016;611:37–42.

Kandimalla R, Vallamkondu J, Corgiat EB, et al. Understanding aspects of aluminum exposure in Alzheimer's disease development. *Brain Pathol*. 2016;26:139–154.

Kasprzak KS. Oxidative DNA and protein damage in metal-induced toxicity and carcinogenesis. *Free Radic Biol Med*. 2002;32:958–967.

Kasprzak KS, Sunderman FW Jr, Salnikow K. Nickel carcinogenesis. *Mutat Res*. 2003;533:67–97.

Kawata K, Yokoo H, Shimazaki R, et al. Classification of heavy-metal toxicity by human DNA microarray analysis. *Environ Sci Technol*. 2007;41:3769–3774.

Kazantzis G. Cadmium, osteoporosis and calcium metabolism. *Biometals*. 2004;17:493–498.

Kenyon EM, Del Razo LM, Hughes MF. Tissue distribution and urinary excretion of inorganic arsenic and its methylated metabolites in mice following acute oral administration of arsenate. *Toxicol Sci*. 2005;85:468–475.

Keogan DM, Griffith DM. Current and potential applications of bismuth-based drugs. *Molecules*. 2014;19:15258–15297.

Kim SH, Johnson VJ, Sharma RP. Mercury inhibits nitric oxide production but activates proinflammatory cytokine expression in murine macrophage: differential modulation of NF-kappaB and p38 MAPK signaling pathways. *Nitric Oxide*. 2002;7:67–74.

Kim SH, Sharma RP. Mercury alters endotoxin-induced inflammatory cytokine expression in liver: differential roles of p38 and extracellular signal-regulated mitogen-activated protein kinases. *Immunopharmacol Immunotoxicol*. 2005;27:123–135.

Kim Y, Lobdell DT, Wright CW, et al. Blood metal concentrations of manganese, lead, and cadmium in relation to serum ferritin levels in Ohio residents. *Biol Trace Elem Res*. 2015;165:1–9.

Kippler M, Engstrom K, Mlakar SJ, et al. Sex-specific effects of early life cadmium exposure on DNA methylation and implications for birth weight. *Epigenetics*. 2013;8:494–503.

Kitchin KT, Wallace K. The role of protein binding of trivalent arsenicals in arsenic carcinogenesis and toxicity. *J Inorg Biochem*. 2008;102:532–539.

Klaassen CD, Liu J, Choudhuri S. Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annu Rev Pharmacol Toxicol*. 1999;39:267–294.

Klei LR, Barchowsky A. Positive signaling interactions between arsenic and ethanol for angiogenic gene induction in human microvascular endothelial cells. *Toxicol Sci*. 2008;102:319–327.

Klei LR, Garciafigueroa DY, Barchowsky A. Arsenic activates endothelin-1 Gi protein–coupled receptor signaling to inhibit stem cell differentiation in adipogenesis. *Toxicol Sci*. 2013;131:512–520.

Klimisch HJ. Lung deposition, lung clearance and renal accumulation of inhaled cadmium chloride and cadmium sulphide in rats. *Toxicology*. 1993;84:103–124.

Kodama H, Fujisawa C, Bhadhprasit W. Pathology, clinical features and treatments of congenital copper metabolic disorders—focus on neurologic aspects. *Brain Dev*. 2011;33:243–251.

Kondo Y, Himeno S, Satoh M, et al. Citrate enhances the protective effect of orally administered bismuth subnitrate against the nephrotoxicity of *cis*-diamminedichloroplatinum. *Cancer Chemother Pharmacol*. 2004;53:33–38.

Kono S. Aceruloplasminemia: an update. *Int Rev Neurobiol*. 2013;110:125–151.

Kontoghiorghe CN, Kontoghiorghes GJ. Efficacy and safety of iron-chelation therapy with deferoxamine, deferiprone, and deferasirox for the treatment of iron-loaded patients with non-transfusion-dependent thalassemia syndromes. *Drug Des Devel Ther*. 2016;10:465–481.

Kopp SJ, Glonek T, Perry HM Jr, et al. Cardiovascular actions of cadmium at environmental exposure levels. *Science*. 1982;217:837–839.

Kosnett MJ. Heavy metal intoxication & chelators. In: Katzung BG, Trevor AJ, eds. *Basic & Clinical Pharmacology*. 13th ed. New York: McGraw-Hill Education; 2015.

Kozul CD, Ely KH, Enelow RI, et al. Low-dose arsenic compromises the immune response to influenza A infection in vivo. *Environ Health Perspect*. 2009a;117:1441–1447.

Kozul CD, Hampton TH, Davey JC, et al. Chronic exposure to arsenic in the drinking water alters the expression of immune response genes in mouse lung. *Environ Health Perspect*. 2009b;117:1108–1115.

Krapivinsky G, Krapivinsky L, Manasian Y, et al. The TRPM7 chanzyme is cleaved to release a chromatin-modifying kinase. *Cell*. 2014;157:1061–1072.

Kuo CC, Howard BV, Umans JG, et al. Arsenic exposure, arsenic metabolism, and incident diabetes in the strong heart study. *Diabetes Care*. 2015;38:620–627.

Lagerkvist BE, Linderholm H, Nordberg GF. Arsenic and Raynaud's phenomenon. Vasospastic tendency and excretion of arsenic in smelter workers before and after the summer vacation. *Int Arch Occup Environ Health*. 1988;60:361–364.

Laity JH, Andrews GK. Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). *Arch Biochem Biophys*. 2007;463:201–210.

Lamas GA, Navas-Acien A, Mark DB, et al. Heavy metals, cardiovascular disease, and the unexpected benefits of chelation therapy. *J Am Coll Cardiol*. 2016;67:2411–2418.

Lantz RC, Chau B, Sarihan P, et al. In utero and postnatal exposure to arsenic alters pulmonary structure and function. *Toxicol Appl Pharmacol*. 2009;235:105–113.

Laohaudomchok W, Lin X, Herrick RF, et al. Toenail, blood, and urine as biomarkers of manganese exposure. *J Occup Environ Med*. 2011;53:506–510.

Laraque D, Trasande L. Lead poisoning: successes and 21st century challenges. *Pediatr Rev*. 2005;26:435–443.

Larsen A, Stoltenberg M, Sondergaard C, et al. In vivo distribution of bismuth in the mouse brain: influence of long-term survival and intracranial placement on the uptake and transport of bismuth in neuronal tissue. *Basic Clin Pharmacol Toxicol*. 2005;97:188–196.

Larsson SC, Wolk A. Urinary cadmium and mortality from all causes, cancer and cardiovascular disease in the general population: systematic review and meta-analysis of cohort studies. *Int J Epidemiol*. 2015;45:782–791.

Lau A, Whitman SA, Jaramillo MC, et al. Arsenic-mediated activation of the Nrf2-Keap1 antioxidant pathway. *J Biochem Mol Toxicol*. 2013;27:99–105.

Laulicht F, Brocato J, Ke Q, Costa M. Genotoxicity of metal compounds. In: Norberg GF, Fowler BA, Norberg M, eds. *Handbook on the Toxicology of Metals*. 4th ed. Oxford, UK: Elsevier; 2015.

Leggett RW, Williams LR, Melo DR, et al. A physiologically based biokinetic model for cesium in the human body. *Sci Total Environ*. 2003;317:235–255.

Leifer CA, Dietert RR. Early life environment and developmental immunotoxicity in inflammatory dysfunction and disease. *Toxicol Environ Chem*. 2011;93:1463–1485.

Leikauf GD. Hazardous air pollutants and asthma. *Environ Health Perspect*. 2002;110(suppl 4):505–526.

Lemaire M, Lemarie CA, Flores Molina M, et al. Genetic deletion of LXRalpha prevents arsenic-enhanced atherosclerosis, but not arsenic-altered plaque composition. *Toxicol Sci*. 2014;142:477–488.

Lemaire M, Lemarie CA, Molina MF, et al. Exposure to moderate arsenic concentrations increases atherosclerosis in ApoE(–/–) mouse model. *Toxicol Sci*. 2011;122:211–221.

Lemaire M, Negro Silva LF, Lemarie CA, et al. Arsenic exposure increases monocyte adhesion to the vascular endothelium, a pro-atherogenic mechanism. *PLoS One*. 2015;10:e0136592.

Leslie EM, Liu J, Klaassen CD, et al. Acquired cadmium resistance in metallothionein-I/II(–/–) knockout cells: role of the T-type calcium channel Cacnalpha1G in cadmium uptake. *Mol Pharmacol*. 2006;69:629–639.

Leussink BT, Nagelkerke JF, van de Water B, et al. Pathways of proximal tubular cell death in bismuth nephrotoxicity. *Toxicol Appl Pharmacol*. 2002;180:100–109.

Levin R, Brown MJ, Kashtock ME, et al. Lead exposures in U.S. Children, 2008: implications for prevention. *Environ Health Perspect*. 2008;116:1285–1293.

Levy M, Leclerc BS. Fluoride in drinking water and osteosarcoma incidence rates in the continental United States among children and adolescents. *Cancer Epidemiol*. 2012;36:e83–e88.

Leyssens L, Vinck B, Van Der Straeten C, et al. Cobalt toxicity in humans: a review of the potential sources and systemic health effects. *Toxicology*. 2017;387:43–56.

Li J, Packianathan C, Rossman TG, et al. Nonsynonymous polymorphisms in the human AS3MT arsenic methylation gene: implications for arsenic toxicity. *Chem Res Toxicol*. 2017;30:1481–1491.

Liang X, Dempski RE, Burdette SC. Zn(2+) at a cellular crossroads. *Curr Opin Chem Biol*. 2016;31:120–125.

Liu J, Chen H, Miller DS, et al. Overexpression of glutathione *S*-transferase II and multidrug resistance transport proteins is associated with acquired tolerance to inorganic arsenic. *Mol Pharmacol*. 2001;60:302–309.

Liu J, Habeebu SS, Liu Y, et al. Acute CdMT injection is not a good model to study chronic Cd nephropathy: comparison of chronic CdCl2 and CdMT exposure with acute CdMT injection in rats. *Toxicol Appl Pharmacol*. 1998;153:48–58.

Liu J, Lu Y, Wu Q, et al. Mineral arsenicals in traditional medicines: orpiment, realgar, and arsenolite. *J Pharmacol Exp Ther*. 2008;326:363–368.

Liu J, Waalkes MP. Liver is a target of arsenic carcinogenesis. *Toxicol Sci*. 2008;105:24–32.
Liu J, Xie Y, Ducharme DM, et al. Global gene expression associated with hepatocarcinogenesis in adult male mice induced by in utero arsenic exposure. *Environ Health Perspect*. 2006;114:404–411.
Liu J, Zheng B, Aposhian HV, et al. Chronic arsenic poisoning from burning high-arsenic-containing coal in Guizhou, China. *Environ Health Perspect*. 2002;110:119–122.
Liu Z. Roles of vertebrate aquaglyceroporins in arsenic transport and detoxification. In: Jahn TP, Bienert GP, eds. *MIPs and Their Role in the Exchange of Metalloids*. New York: Springer; 2010:71–81.
Lomer MC, Hutchinson C, Volkert S, et al. Dietary sources of inorganic microparticles and their intake in healthy subjects and patients with Crohn's disease. *Br J Nutr*. 2004;92:947–955.
Loza-Rosas SA, Saxena M, Delgado Y, et al. A ubiquitous metal, difficult to track: towards an understanding of the regulation of titanium(iv) in humans. *Metallomics*. 2017;9:346–356.
Luebke RW, Chen DH, Dietert R, et al. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J Toxicol Environ Health B Crit Rev*. 2006;9:1–26.
Macfie A, Hagan E, Zhitkovich A. Mechanism of DNA-protein cross-linking by chromium. *Chem Res Toxicol*. 2010;23:341–347.
Madl AK, Liong M, Kovochich M, et al. Toxicology of wear particles of cobalt-chromium alloy metal-on-metal hip implants Part I: physicochemical properties in patient and simulator studies. *Nanomedicine*. 2015;11:1201–1215.
Magos L, Clarkson TW. Overview of the clinical toxicity of mercury. *Ann Clin Biochem*. 2006;43:257–268.
Makrilia N, Syrigou E, Kaklamanos I, et al. Hypersensitivity reactions associated with platinum antineoplastic agents: a systematic review. *Met Based Drugs*. 2010;2010:pii: 207084.
Malluche HH, Mawad H, Monier-Faugere MC. Effects of treatment of renal osteodystrophy on bone histology. *Clin J Am Soc Nephrol*. 2008;3(suppl 3):S157–S163.
Mantha M, Yeary E, Trent J, et al. Estimating inorganic arsenic exposure from U.S. rice and total water intakes. *Environ Health Perspect*. 2017;125:057005.
Manton WI, Angle CR, Krogstrand KL. Origin of lead in the United States diet. *Environ Sci Technol*. 2005;39:8995–9000.
Marchetti C. Molecular targets of lead in brain neurotoxicity. *Neurotox Res*. 2003;5:221–236.
Marchetti C. Interaction of metal ions with neurotransmitter receptors and potential role in neurodiseases. *Biometals*. 2014;27:1097–1113.
Maret W. Zinc in pancreatic islet biology, insulin sensitivity, and diabetes. *Prev Nutr Food Sci*. 2017;22:1–8.
Maret W, Sandstead HH. Zinc requirements and the risks and benefits of zinc supplementation. *J Trace Elem Med Biol*. 2006;20:3–18.
Markowitz GS, Radhakrishnan J, Kambham N, et al. Lithium nephrotoxicity: a progressive combined glomerular and tubulointerstitial nephropathy. *J Am Soc Nephrol*. 2000;11:1439–1448.
Martinez-Finley EJ, Aschner M. Recent advances in mercury research. *Curr Environ Health Rep*. 2014;1:163–171.
Martinez-Zamudio R, Ha HC. Environmental epigenetics in metal exposure. *Epigenetics*. 2011;6:820–827.
Matson Dzebo M, Arioz C, Wittung-Stafshede P. Extended functional repertoire for human copper chaperones. *Biomol Concepts*. 2016;7:29–39.
Mayser P, Schulz S. Precipitation of free fatty acids generated by Malassezia: a possible explanation for the positive effects of lithium succinate in seborrhoeic dermatitis. *J Eur Acad Dermatol Venereol*. 2016;30:1384–1389.
Mazumder DG, Dasgupta UB. Chronic arsenic toxicity: studies in West Bengal, India. *Kaohsiung J Med Sci*. 2011;27:360–370.
Mazumder DN. Effect of chronic intake of arsenic-contaminated water on liver. *Toxicol Appl Pharmacol*. 2005;206:169–175.
Mazur A, Maier JA, Rock E, et al. Magnesium and the inflammatory response: potential physiopathological implications. *Arch Biochem Biophys*. 2007;458:48–56.
McCormick NH, Hennigar SR, Kiselyov K, et al. The biology of zinc transport in mammary epithelial cells: implications for mammary gland development, lactation, and involution. *J Mammary Gland Biol Neoplasia*. 2014;19:59–71.
McKee AS, Fontenot AP. Interplay of innate and adaptive immunity in metal-induced hypersensitivity. *Curr Opin Immunol*. 2016;42:25–30.
McSweeney PC. The safety of nanoparticles in sunscreens: an update for general practice. *Aust Fam Physician*. 2016;45:397–399.
Meenakshi, Maheshwari RC. Fluoride in drinking water and its removal. *J Hazard Mater*. 2006;137:456–463.
Meharg AA, Williams PN, Adomako E, et al. Geographical variation in total and inorganic arsenic content of polished (white) rice. *Environ Sci Technol*. 2009;43:1612–1617.
Melnikov P, Zanoni LZ. Clinical effects of cesium intake. *Biol Trace Elem Res*. 2010;135:1–9.
Mercer JF. The molecular basis of copper-transport diseases. *Trends Mol Med*. 2001;7:64–69.
Merget R, Pham N, Schmidtke M, et al. Medical surveillance and long-term prognosis of occupational allergy due to platinum salts. *Int Arch Occup Environ Health*. 2017;90:73–81.
Mickle MH. Structure, use, and validation of the IEUBK model. *Environ Health Perspect*. 1998;106(suppl 6):1531–1534.
Montenegro MF, Barbosa F Jr, Sandrim VC, et al. A polymorphism in the delta-aminolevulinic acid dehydratase gene modifies plasma/whole blood lead ratio. *Arch Toxicol*. 2006;80:394–398.
Moon K, Guallar E, Navas-Acien A. Arsenic exposure and cardiovascular disease: an updated systematic review. *Curr Atheroscler Rep*. 2012;14:542–555.
Moon KA, Guallar E, Umans JG, et al. Association between exposure to low to moderate arsenic levels and incident cardiovascular disease. A prospective cohort study. *Ann Intern Med*. 2013;159:649–659.
Morgenstern A, Bruchertseifer F, Apostolidis C. Targeted alpha therapy with 213Bi. *Curr Radiopharm*. 2011;4:295–305.
Mosior M, Krawczak-Sadowska T, Wrobel A, et al. The effect of arsenate and vanadate ions on the critical cell volume of bovine erythrocytes. *Gen Physiol Biophys*. 1992;11:337–343.
Mukherjee SC, Saha KC, Pati S, et al. Murshidabad—one of the nine groundwater arsenic-affected districts of West Bengal, India. Part II: dermatological, neurological, and obstetric findings. *Clin Toxicol.(Phila)*. 2005;43:835–848.
Munger MA, Radwanski P, Hadlock GC, et al. In vivo human time-exposure study of orally dosed commercial silver nanoparticles. *Nanomedicine*. 2014;10:1–9.
Navas-Acien A, Guallar E, Silbergeld EK, et al. Lead exposure and cardiovascular disease—a systematic review. *Environ Health Perspect*. 2007;115:472–482.
Navas-Acien A, Selvin E, Sharrett AR, et al. Lead, cadmium, smoking, and increased risk of peripheral arterial disease. *Circulation*. 2004;109:3196–3201.
Navas-Acien A, Silbergeld EK, Streeter RA, et al. Arsenic exposure and type 2 diabetes: a systematic review of the experimental and epidemiological evidence. *Environ Health Perspect*. 2006;114:641–648.
Nayak NC, Chitale AR. Indian childhood cirrhosis (ICC) & ICC-like diseases: the changing scenario of facts versus notions. *Indian J Med Res*. 2013;137:1029–1042.
Ndagi U, Mhlongo N, Soliman ME. Metal complexes in cancer therapy—an update from drug design perspective. *Drug Des Devel Ther*. 2017;11:599–616.
Needleman H. Lead poisoning. *Annu Rev Med*. 2004;55:209–222.
Nemec AA, Barchowsky A. Signal transducer and activator of transcription 1 (STAT1) is essential for chromium silencing of gene induction in human airway epithelial cells. *Toxicol Sci*. 2009;110:212–223.
Nemec AA, Zubritsky LM, Barchowsky A. Chromium(VI) stimulates Fyn to initiate innate immune gene induction in human airway epithelial cells. *Chem Res Toxicol*. 2010;23:396–404.
Nesci S, Trombetti F, Pirini M, et al. Mercury and protein thiols: stimulation of mitochondrial F1FO-ATPase and inhibition of respiration. *Chem Biol Interact*. 2016;260:42–49.
Newman LS, Mroz MM, Balkissoon R, et al. Beryllium sensitization progresses to chronic beryllium disease: a longitudinal study of disease risk. *Am J Respir Crit Care Med*. 2005;171:54–60.
Ng JC, Wang JP, Zheng B, et al. Urinary porphyrins as biomarkers for arsenic exposure among susceptible populations in Guizhou province, China. *Toxicol Appl Pharmacol*. 2005;206:176–184.

Nierenberg DW, Nordgren RE, Chang MB, et al. Delayed cerebellar disease and death after accidental exposure to dimethylmercury. *N Engl J Med*. 1998;338:1672–1676.

Nigra AE, Ruiz-Hernandez A, Redon J, et al. Environmental metals and cardiovascular disease in adults: a systematic review beyond lead and cadmium. *Curr Environ Health Rep*. 2016;3:416–433.

Niu HX, Li SH, Li HY, et al. Clinicopathological features, diagnosis, and treatment of IgA nephropathy with minimal change disease related to exposure to mercury-containing cosmetics: a case report. *Clin Nephrol*. 2017;87:196–201.

Nordberg GF. Cadmium and health in the 21st century—historical remarks and trends for the future. *Biometals*. 2004;17:485–489.

Nordberg GF. Historical perspectives on cadmium toxicology. *Toxicol Appl Pharmacol*. 2009;238:192–200.

NRC. *Toxicological Effects of Methylmercury*. Washington, DC: National Academies Press; 2000. Available at: https://doi.org/10.17226/9899.

NRC. *Acute Exposure Guideline Levels for Selected Airborne Chemicals: Volume 6*. Washington, DC: National Academies Press.; 2008.

NRC. *Critical Aspects of EPA's IRIS Assessment of Inorganic Arsenic: Interim Report*. Washington, DC: Press TNA; 2014.

NTP. *NTP Monograph on Health Effects of Low-level Lead*. Research Triangle Park, NC: National Institute of Environmental Health Sciences; 2012. Available at: https://ntp.niehs.nih.gov/pubhealth/hat/noms/lead/index.html.

NTP. *Draft Report on Carcinogens Concept: Antimony Trioxide*. Research Triangle Park, NC: National Toxicology Program, National Institute of Environmental Health Sciences; 2016a. Available at: https://ntp.niehs.nih.gov/ntp/about_ntp/bsc/2016/december/meetingmaterials/draftantimonytrioxide_508.pdf.

NTP. *Natinal Toxicology Program Report on Carcinogens, Fourteenth Edition*. Research Triangle Park, NC: U.S. Department of Health and Human Services; 2016b. Available at: http://ntp.niehs.nih.gov/go/roc14.

Nuttall JR, Oteiza PI. Zinc and the aging brain. *Genes Nutr*. 2014;9:379.

O'Brien TJ, Ceryak S, Patierno SR. Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms. *Mutat Res*. 2003;533:3–36.

O'Hara KA, Klei LR, Barchowsky A. Selective activation of Src family kinases and JNK by low levels of chromium(VI). *Toxicol Appl Pharmacol*. 2003;190:214–223.

O'Hara KA, Nemec AA, Alam J, et al. Chromium (VI) inhibits heme oxygenase-1 expression in vivo and in arsenic-exposed human airway epithelial cells. *J Cell Physiol*. 2006;209:113–121.

Ogawa Y, Kawamura T, Shimada S. Zinc and skin biology. *Arch Biochem Biophys*. 2016;611:113–119.

Okoro HK, Fatoki OS, Adekola FA, et al. Human exposure, biomarkers, and fate of organotins in the environment. *Rev Environ Contam Toxicol*. 2011;213:27–54.

Oller AR, Costa M, Oberdorster G. Carcinogenicity assessment of selected nickel compounds. *Toxicol Appl Pharmacol*. 1997;143:152–166.

Oruch R, Elderbi MA, Khattab HA, et al. Lithium: a review of pharmacology, clinical uses, and toxicity. *Eur J Pharmacol*. 2014;740:464–473.

Packer M. Cobalt cardiomyopathy: a critical reappraisal in light of a recent resurgence. *Circ Heart Fail*. 2016;9(12):pii: e003604.

Pan Z, Choi S, Ouadid-Ahidouch H, et al. Zinc transporters and dysregulated channels in cancers. *Front Biosci (Landmark Ed)*. 2017;22:623–643.

Papanikolaou G, Pantopoulos K. Iron metabolism and toxicity. *Toxicol Appl Pharmacol*. 2005;202:199–211.

Park RM, Bena JF, Stayner LT, et al. Hexavalent chromium and lung cancer in the chromate industry: a quantitative risk assessment. *Risk Anal*. 2004;24:1099–1108.

Parvez F, Chen Y, Brandt-Rauf PW, et al. A prospective study of respiratory symptoms associated with chronic arsenic exposure in Bangladesh: findings from the Health Effects of Arsenic Longitudinal Study (HEALS). *Thorax*. 2010;65:528–533.

Parvez F, Chen Y, Yunus M, et al. Arsenic exposure and impaired lung function. Findings from a large population-based prospective cohort study. *Am J Respir Crit Care Med*. 2013;188:813–819.

Parvez F, Wasserman GA, Factor-Litvak P, et al. Arsenic exposure and motor function among children in Bangladesh. *Environ Health Perspect*. 2011;119:1665–1670.

Patel LM, Lambert PJ, Gagna CE, et al. Cutaneous signs of systemic disease. *Clin Dermatol*. 2011;29:511–522.

Patel PP, Patel PA, Zulf MM, et al. Association of dental and skeletal fluorosis with calcium intake and serum vitamin D concentration in adolescents from a region endemic for fluorosis. *Indian J Endocrinol Metab*. 2017;21:190–195.

Patra S, Vij M, Kancherala R, et al. Is Indian childhood cirrhosis an extinct disease now?—an observational study. *Indian J Pediatr*. 2013;80:651–654.

Patrick L. Lead toxicity, a review of the literature. Part 1: Exposure, evaluation, and treatment. *Altern Med Rev*. 2006;11:2–22.

Peres TV, Schettinger MR, Chen P, et al. Manganese-induced neurotoxicity: a review of its behavioral consequences and neuroprotective strategies. *BMC Pharmacol Toxicol*. 2016;17:57.

Perez-Ramirez C, Canadas-Garre M, Molina MA, et al. Contribution of genetic factors to platinum-based chemotherapy sensitivity and prognosis of non-small cell lung cancer. *Mutat Res*. 2017;771:32–58.

Perez AL, Tang WH. Contribution of environmental toxins in the pathogenesis of idiopathic cardiomyopathies. *Curr Treat Options Cardiovasc Med*. 2015;17:381.

Peter AL, Viraraghavan T. Thallium: a review of public health and environmental concerns. *Environ Int*. 2005;31:493–501.

Petrick JS, Blachere FM, Selmin O, et al. Inorganic arsenic as a developmental toxicant: in utero exposure and alterations in the developing rat lungs. *Mol Nutr Food Res*. 2009;53:583–591.

Pi J, He Y, Bortner C, et al. Low level, long-term inorganic arsenite exposure causes generalized resistance to apoptosis in cultured human keratinocytes: potential role in skin co-carcinogenesis. *Int J Cancer*. 2005;116:20–26.

Pollack AZ, Ranasinghe S, Sjaarda LA, et al. Cadmium and reproductive health in women: a systematic review of the epidemiologic evidence. *Curr Environ Health Rep*. 2014;1:172–184.

Prasad AS. Zinc deficiency: its characterization and treatment. *Met Ions Biol Syst*. 2004;41:103–137.

Prasad AV, Mohan N, Chandrasekar B, et al. Activation of nuclear factor kappa B in human lymphoblastoid cells by low-dose ionizing radiation. *Radiat Res*. 1994;138:367–372.

Prozialeck WC, Edwards JR. Early biomarkers of cadmium exposure and nephrotoxicity. *Biometals*. 2010;23:793–809.

Prozialeck WC, Edwards JR, Nebert DW, et al. The vascular system as a target of metal toxicity. *Toxicol Sci*. 2008;102:207–218.

Pullen-James S, Woods SE. Occupational arsine gas exposure. *J Natl Med Assoc*. 2006;98:1998–2001.

Rahman A, Vahter M, Ekstrom EC, et al. Arsenic exposure in pregnancy increases the risk of lower respiratory tract infection and diarrhea during infancy in Bangladesh. *Environ Health Perspect*. 2011;119:719–724.

Rahman MM, Chowdhury UK, Mukherjee SC, et al. Chronic arsenic toxicity in Bangladesh and West Bengal, India—a review and commentary. *J Toxicol Clin Toxicol*. 2001;39:683–700.

Raisbeck MF, Siemion RS, Smith MA. Modest copper supplementation blocks molybdenosis in cattle. *J Vet Diagn Invest*. 2006;18:566–572.

Ramsey KA, Bosco A, McKenna KL, et al. In utero exposure to arsenic alters lung development and genes related to immune and mucociliary function in mice. *Environ Health Perspect*. 2013a;121:244–250.

Ramsey KA, Larcombe AN, Sly PD, et al. In utero exposure to low dose arsenic via drinking water impairs early life lung mechanics in mice. *BMC Pharmacol Toxicol*. 2013b;14:13.

Ress NB, Chou BJ, Renne RA, et al. Carcinogenicity of inhaled vanadium pentoxide in F344/N rats and B6C3F1 mice. *Toxicol Sci*. 2003;74:287–296.

Reuben A, Caspi A, Belsky DW, et al. Association of childhood blood lead levels with cognitive function and socioeconomic status at age 38 years and with IQ change and socioeconomic mobility between childhood and adulthood. *JAMA*. 2017;317:1244–1251.

Reynolds M, Armknecht S, Johnston T, et al. Undetectable role of oxidative DNA damage in cell cycle, cytotoxic and clastogenic effects of Cr(VI) in human lung cells with restored ascorbate levels. *Mutagenesis*. 2012;27:437–443.

Riihimaki V, Aitio A. Occupational exposure to aluminum and its biomonitoring in perspective. *Crit Rev Toxicol*. 2012;42:827–853.

Ring G, O'Mullane J, O'Riordan A, et al. Trace metal determination as it relates to metallosis of orthopaedic implants: evolution and current status. *Clin Biochem*. 2016;49:617–635.

Risher JF, Amler SN. Mercury exposure: evaluation and intervention the inappropriate use of chelating agents in the diagnosis and treatment of putative mercury poisoning. *Neurotoxicology*. 2005;26:691–699.

Roberts EA, Socha P. Wilson disease in children. *Handb Clin Neurol*. 2017;142:141–156.

Rodriguez-Rubio L, Nava E, Del Pozo JSG, et al. Influence of the perioperative administration of magnesium sulfate on the total dose of anesthetics during general anesthesia. A systematic review and meta-analysis. *J Clin Anesth*. 2017;39:129–138.

Rohl C, Grell M, Maser E. The organotin compounds trimethyltin (TMT) and triethyltin (TET) but not tributyltin (TBT) induce activation of microglia co-cultivated with astrocytes. *Toxicol In Vitro*. 2009;23:1541–1547.

Rondini EA, Walters DM, Bauer AK. Vanadium pentoxide induces pulmonary inflammation and tumor promotion in a strain-dependent manner. *Part Fibre Toxicol*. 2010;7:9.

Rosen BP. Biochemistry of arsenic detoxification. *FEBS Lett*. 2002;529:86–92.

Rossman TG, Klein CB. Genetic and epigenetic effects of environmental arsenicals. *Metallomics*. 2011;3(11):1135–1141.

Rossman TG, Uddin AN, Burns FJ, et al. Arsenite cocarcinogenesis: an animal model derived from genetic toxicology studies. *Environ Health Perspect*. 2002;110(suppl 5):749–752.

Rossman TG, Uddin AN, Burns FJ. Evidence that arsenite acts as a cocarcinogen in skin cancer. *Toxicol Appl Pharmacol*. 2004;198:394–404.

Rowe CL. Bioaccumulation and effects of metals and trace elements from aquatic disposal of coal combustion residues: recent advances and recommendations for further study. *Sci Total Environ*. 2014;485–486, 490–496.

Roy S, Hardej D. Tellurium tetrachloride and diphenyl ditelluride cause cytotoxicity in rat hippocampal astrocytes. *Food Chem Toxicol*. 2011;49:2564–2574.

Rubio C, Rubio-Osornio M, Retana-Marquez S, et al. In vivo experimental models of epilepsy. *Cent Nerv Syst Agents Med Chem*. 2010;10:298–309.

Rudel H. Case study: bioavailability of tin and tin compounds. *Ecotoxicol Environ Saf*. 2003;56:180–189.

Ryu DY, Lee SJ, Park DW, et al. Dietary iron regulates intestinal cadmium absorption through iron transporters in rats. *Toxicol Lett*. 2004;152:19–25.

Ryu HW, Lee DH, Won HR, et al. Influence of toxicologically relevant metals on human epigenetic regulation. *Toxicol Res*. 2015;31:1–9.

Sabin BR, Grammer LC. Chapter 17: Occupational immunologic lung disease. *Allergy Asthma Proc*. 2012;33(suppl 1):S58–S60.

Saito M, Arakaki R, Yamada A, et al. Molecular mechanisms of nickel allergy. *Int J Mol Sci*. 2016;17(2):pii: E202.

Salnikow K, Zhitkovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem Res Toxicol*. 2008;21:28–44.

Salvador JA, Figueiredo SA, Pinto RM, et al. Bismuth compounds in medicinal chemistry. *Future Med Chem*. 2012;4:1495–1523.

Samikkannu T, Chen CH, Yih LH, et al. Reactive oxygen species are involved in arsenic trioxide inhibition of pyruvate dehydrogenase activity. *Chem Res Toxicol*. 2003;16:409–414.

Santos DB, Schiar VP, Paixao MW, et al. Hemolytic and genotoxic evaluation of organochalcogens in human blood cells in vitro. *Toxicol In Vitro*. 2009;23:1195–1204.

Satarug S, Garrett SH, Sens MA, et al. Cadmium, environmental exposure, and health outcomes. *Environ Health Perspect*. 2010;118:182–190.

Satarug S, Moore MR. Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. *Environ Health Perspect*. 2004;112:1099–1103.

Sawyer RT, Maier LA. Chronic beryllium disease: an updated model interaction between innate and acquired immunity. *Biometals*. 2011; 24:1–17.

Sayes CM, Reed KL, Warheit DB. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. *Toxicol Sci*. 2007;97:163–180.

Schiff MH, Whelton A. Renal toxicity associated with disease-modifying antirheumatic drugs used for the treatment of rheumatoid arthritis. *Semin Arthritis Rheum*. 2000;30:196–208.

Schnaas L, Rothenberg SJ, Flores MF, et al. Reduced intellectual development in children with prenatal lead exposure. *Environ Health Perspect*. 2006;114:791–797.

Schwarz G. Molybdenum cofactor and human disease. *Curr Opin Chem Biol*. 2016;31:179–187.

Schweizer U, Fradejas-Villar N. Why 21? The significance of selenoproteins for human health revealed by inborn errors of metabolism. *FASEB J*. 2016;30:3669–3681.

Sears ME, Kerr KJ, Bray RI. Arsenic, cadmium, lead, and mercury in sweat: a systematic review. *J Environ Public Health*. 2012;2012:184745.

Sedman RM, Beaumont J, McDonald TA, et al. Review of the evidence regarding the carcinogenicity of hexavalent chromium in drinking water. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2006;24:155–182.

Serefko A, Szopa A, Poleszak E. Magnesium and depression. *Magnes Res*. 2016;29:112–119.

Shelnutt SR, Goad P, Belsito DV. Dermatological toxicity of hexavalent chromium. *Crit Rev Toxicol*. 2007;37, 375–387.

Sheppard SC, Sheppard MI, Gallerand MO, et al. Derivation of ecotoxicity thresholds for uranium. *J Environ Radioact*. 2005;79:55–83.

Sherwood CL, Lantz RC, Boitano S. Chronic arsenic exposure in nanomolar concentrations compromises wound response and intercellular signaling in airway epithelial cells. *Toxicol Sci*. 2013;132:222–234.

Shim H, Harris ZL. Genetic defects in copper metabolism. *J Nutr*. 2003;133:1527s–1531s.

Shoults-Wilson WA, Reinsch BC, Tsyusko OV, et al. Effect of silver nanoparticle surface coating on bioaccumulation and reproductive toxicity in earthworms (*Eisenia fetida*). *Nanotoxicology*. 2011;5:432–444.

Siedlikowski M, Bradley M, Kubow S, et al. Bioaccessibility and bioavailability of methylmercury from seafood commonly consumed in North America: in vitro and epidemiological studies. *Environ Res*. 2016a;149:266–273.

Silbergeld EK. Facilitative mechanisms of lead as a carcinogen. *Mutat Res*. 2003;533:121–133.

Silbergeld EK, Patrick TE. Environmental exposures, toxicologic mechanisms, and adverse pregnancy outcomes. *Am J Obstet Gynecol*. 2005;192:S11–S21.

Silveira LJ, McCanlies EC, Fingerlin TE, et al. Chronic beryllium disease, HLA-DPB1, and the DP peptide binding groove. *J Immunol*. 2012;189:4014–4023.

Simeonova PP, Luster MI. Arsenic carcinogenicity: relevance of *c*-Src activation. *Mol Cell Biochem*. 2002;234–235, 277–282.

Simeonova PP, Wang S, Hulderman T, et al. *c*-Src-dependent activation of the epidermal growth factor receptor and mitogen-activated protein kinase pathway by arsenic. Role in carcinogenesis. *J Biol Chem*. 2002;277:2945–2950.

Sinczuk-Walczak H, Szymczak M, Halatek T. Effects of occupational exposure to arsenic on the nervous system: clinical and neurophysiological studies. *Int J Occup Med Environ Health*. 2010;23:347–355.

Singh R, Gautam N, Mishra A, et al. Heavy metals and living systems: an overview. *Indian J Pharmacol*. 2011;43, 246–253.

Sinha D, Biswas J, Bishayee A. Nrf2-mediated redox signaling in arsenic carcinogenesis: a review. *Arch Toxicol*. 2013;87:383–396.

Sivulka DJ. Assessment of respiratory carcinogenicity associated with exposure to metallic nickel: a review. *Regul Toxicol Pharmacol*. 2005;43:117–133.

Slikkerveer A, de Wolff FA. Pharmacokinetics and toxicity of bismuth compounds. *Med Toxicol Adverse Drug Exp*. 1989;4:303–323.

Smith AH, Ercumen A, Yuan Y, et al. Increased lung cancer risks are similar whether arsenic is ingested or inhaled. *J Expo Sci Environ Epidemiol*. 2009;19:343–348.

Smith AH, Marshall G, Yuan Y, et al. Increased mortality from lung cancer and bronchiectasis in young adults after exposure to arsenic in utero and in early childhood. *Environ Health Perspect*. 2006;114:1293–1296.

Smith AH, Marshall G, Yuan Y, et al. Evidence from Chile that arsenic in drinking water may increase mortality from pulmonary tuberculosis. *Am J Epidemiol*. 2011;173:414–420.

Smith AH, Steinmaus CM. Health effects of arsenic and chromium in drinking water: recent human findings. *Annu Rev Public Health*. 2009;30:107–122.

Smith D, Strupp BJ. The scientific basis for chelation: animal studies and lead chelation. *J Med Toxicol*. 2013;9:326–338.

Solenkova NV, Newman JD, Berger JS, et al. Metal pollutants and cardiovascular disease: mechanisms and consequences of exposure. *Am Heart J*. 2014;168:812–822.

Soliman AT, De Sanctis V, Yassin M, et al. Iron deficiency anemia and glucose metabolism. *Acta Biomed*. 2017;88:112–118.

Soucy NV, Ihnat MA, Kamat CD, et al. Arsenic stimulates angiogenesis and tumorigenesis in vivo. *Toxicol Sci*. 2003;76:271–279.

Soucy NV, Mayka D, Klei LR, et al. Neovascularization and angiogenic gene expression following chronic arsenic exposure in mice. *Cardiovasc Toxicol*. 2005;5:29–41.

Standeven AM, Wetterhahn KE. Chromium(VI) toxicity: uptake, reduction, and DNA damage. *J Am Coll Toxicol*. 1989;8:1275–1283.

States JC, Barchowsky A, Cartwright IL, et al. Arsenic toxicology: translating between experimental models and human pathology. *Environ Health Perspect*. 2011;119:1356–1363.

Steenland K, Boffetta P. Lead and cancer in humans: where are we now? *Am J Ind Med*. 2000;38, 295–299.

Steinmaus C, Ferreccio C, Acevedo J, et al. High risks of lung disease associated with early-life and moderate lifetime arsenic exposure in northern Chile. *Toxicol Appl Pharmacol*. 2016;313:10–15.

Stern BR. Essentiality and toxicity in copper health risk assessment: overview, update and regulatory considerations. *J Toxicol Environ Health A*. 2010;73:114–127.

Straif K, Benbrahim-Tallaa L, Baan R, et al. A review of human carcinogens—Part C: metals, arsenic, dusts, and fibres. *Lancet Oncol*. 2009;10:453–454.

Straub AC, Clark KA, Ross MA, et al. Arsenic-stimulated liver sinusoidal capillarization in mice requires NADPH oxidase-generated superoxide. *J Clin Invest*. 2008;118:3980–3989.

Straub AC, Klei LR, Stolz DB, et al. Arsenic requires sphingosine-1-phosphate type 1 receptors to induce angiogenic genes and endothelial cell remodeling. *Am J Pathol*. 2009;174:1949–1958.

Stringari J, Nunes AK, Franco JL, et al. Prenatal methylmercury exposure hampers glutathione antioxidant system ontogenesis and causes long-lasting oxidative stress in the mouse brain. *Toxicol Appl Pharmacol*. 2008;227:147–154.

Strupp C. Beryllium metal II. A review of the available toxicity data. *Ann Occup Hyg*. 2011;55:43–56.

Sun H, Brocato J, Costa M. Oral chromium exposure and toxicity. *Curr Environ Health Rep*. 2015;2:295–303.

Sun H, Shamy M, Costa M. Nickel and epigenetic gene silencing. *Genes (Basel)*. 2013;4:583–595.

Sundar S, Chakravarty J. Antimony toxicity. *Int J Environ Res Public Health*. 2010;7:4267–4277.

Tanaka A. Toxicity of indium arsenide, gallium arsenide, and aluminium gallium arsenide. *Toxicol Appl Pharmacol*. 2004;198:405–411.

Tchounwou PB, Yedjou CG, Patlolla AK, et al. Heavy metal toxicity and the environment. *EXS*. 2012;101:133–164.

Thevenod F, Lee WK. Cadmium and cellular signaling cascades: interactions between cell death and survival pathways. *Arch Toxicol*. 2013;87:1743–1786.

Thompson DF, Callen ED. Soluble or insoluble prussian blue for radiocesium and thallium poisoning? *Ann Pharmacother*. 2004;38:1509–1514.

Thyssen JP, Menne T. Metal allergy—a review on exposures, penetration, genetics, prevalence, and clinical implications. *Chem Res Toxicol*. 2010;23:309–318.

Tillman LA, Drake FM, Dixon JS, et al. Review article: safety of bismuth in the treatment of gastrointestinal diseases. *Aliment Pharmacol Ther*. 1996;10:459–467.

Tokar EJ, Benbrahim-Tallaa L, Waalkes MP. Metal ions in human cancer development. *Met Ions Life Sci*. 2011a;8:375–401.

Tokar EJ, Diwan BA, Waalkes MP. Early life inorganic lead exposure induces testicular teratoma and renal and urinary bladder preneoplasia in adult metallothionein-knockout mice but not in wild type mice. *Toxicology*. 2010;276:5–10.

Tokar EJ, Diwan BA, Ward JM, et al. Carcinogenic effects of "whole-life" exposure to inorganic arsenic in CD1 mice. *Toxicol Sci*. 2011b;119:73–83.

Tokar EJ, Qu W, Waalkes MP. Arsenic, stem cells, and the developmental basis of adult cancer. *Toxicol Sci*. 2011c;120(suppl 1):S192–S203.

Tong D, Ortega J, Kim C, et al. Arsenic inhibits DNA mismatch repair by promoting EGFR expression and PCNA phosphorylation. *J Biol Chem*. 2015;290:14536–14541.

Trepiccione F, Christensen BM. Lithium-induced nephrogenic diabetes insipidus: new clinical and experimental findings. *J Nephrol*. 2010;23(suppl 16):S43–S48.

Trumbo P, Yates AA, Schlicker S, et al. Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *J Am Diet Assoc*. 2001;101:294–301.

Tseng CH. Cardiovascular disease in arsenic-exposed subjects living in the arseniasis-hyperendemic areas in Taiwan. *Atherosclerosis*. 2008;199:12–18.

Tully DB, Collins BJ, Overstreet JD, et al. Effects of arsenic, cadmium, chromium, and lead on gene expression regulated by a battery of 13 different promoters in recombinant HepG2 cells. *Toxicol Appl Pharmacol*. 2000;168:79–90.

Tyler CR, Allan AM. The effects of arsenic exposure on neurological and cognitive dysfunction in human and rodent studies: a review. *Curr Environ Health Rep*. 2014;1:132–147.

Ueno S, Kashimoto T, Susa N, et al. Reduction in peripheral lymphocytes and thymus atrophy induced by organotin compounds in vivo. *J Vet Med Sci*. 2009;71:1041–1048.

Ueshima K. Magnesium and ischemic heart disease: a review of epidemiological, experimental, and clinical evidences. *Magnes Res*. 2005;18:275–284.

Valko M, Morris H, Cronin MT.Metals, toxicity and oxidative stress. *Curr Med Chem*. 2005;12:1161–1208.

Valko M, Rhodes CJ, Moncol J, et al. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact*. 2006;160:1–40.

van Bemmel DM, Li Y, McLean J, et al. Blood lead levels, ALAD gene polymorphisms, and mortality. *Epidemiology*. 2011;22:273–278.

Vilahur N, Vahter M, Broberg K. The epigenetic effects of prenatal cadmium exposure. *Curr Environ Health Rep*. 2015;2:195–203.

Vincent JB. Chromium: is it essential, pharmacologically relevant, or toxic? *Met Ions Life Sci*. 2013;13:171–198.

Vollet K, Haynes EN, Dietrich KN. Manganese exposure and cognition across the lifespan: contemporary review and argument for biphasic dose–response health effects. *Curr Environ Health Rep*. 2016;3:392–404.

von Ehrenstein OS, Mazumder DN, Yuan Y, et al. Decrements in lung function related to arsenic in drinking water in West Bengal, India. *Am J Epidemiol*. 2005;162:533–541.

von Lindern I, Spalinger S, Stifelman ML, et al. Estimating children's soil/dust ingestion rates through retrospective analyses of blood lead biomonitoring from the Bunker Hill Superfund Site in Idaho. *Environ Health Perspect*. 2016;124:1462–1470.

von Lindern IH, Spalinger SM, Bero BN, et al. The influence of soil remediation on lead in house dust. *Sci Total Environ*. 2003;303:59–78.

Vouk VB. General chemistry of metals. In: Friberg L, Norberg GF, Vouk VB, eds. *Handbook on the Toxicology of Metals*. New York: Elsevier; 1986:14–35.

Waalkes MP. Cadmium carcinogenesis. *Mutat Res*. 2003;533:107–120.

Waalkes MP, Diwan BA, Ward JM, et al. Renal tubular tumors and atypical hyperplasias in B6C3F1 mice exposed to lead acetate during gestation and lactation occur with minimal chronic nephropathy. *Cancer Res*. 1995;55:5265–5271.

Waalkes MP, Liu J, Goyer RA, et al. Metallothionein-I/II double knockout mice are hypersensitive to lead-induced kidney carcinogenesis: role of inclusion body formation. *Cancer Res*. 2004;64:7766–7772.

Waalkes MP, Liu J, Kasprzak KS, et al. Hypersusceptibility to cisplatin carcinogenicity in metallothionein-I/II double knockout mice: production of hepatocellular carcinoma at clinically relevant doses. *Int J Cancer*. 2006;119:28–32.

Waisberg M, Joseph P, Hale B, et al. Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology*. 2003;192:95–117.

Walton FS, Waters SB, Jolley SL, et al. Selenium compounds modulate the activity of recombinant rat AsIII-methyltransferase and the

methylation of arsenite by rat and human hepatocytes. *Chem Res Toxicol*. 2003;16:261–265.
Wang CH, Hsiao CK, Chen CL, et al. A review of the epidemiologic literature on the role of environmental arsenic exposure and cardiovascular diseases. *Toxicol Appl Pharmacol*. 2007;222:315–326.
Wang L, Son YO, Ding S, et al. Ethanol enhances tumor angiogenesis in vitro induced by low-dose arsenic in colon cancer cells through hypoxia-inducible factor 1 alpha pathway. *Toxicol Sci*. 2012;130:269–280.
Wang Z, Xia T, Liu S. Mechanisms of nanosilver-induced toxicological effects: more attention should be paid to its sublethal effects. *Nanoscale*. 2015;7:7470–7481.
Warheit DB, Donner EM. Risk assessment strategies for nanoscale and fine-sized titanium dioxide particles: recognizing hazard and exposure issues. *Food Chem Toxicol*. 2015;85:138–147.
Warniment C, Tsang K, Galazka SS. Lead poisoning in children. *Am Fam Physician*. 2010;81:751–757.
Wasserman GA, Liu X, Loiacono NJ, et al. A cross-sectional study of well water arsenic and child IQ in Maine schoolchildren. *Environ Health*. 2014;13:23.
Wasserman GA, Liu X, Parvez F, et al. Arsenic and manganese exposure and children's intellectual function. *Neurotoxicology*. 2011;32:450–457,
Weber KT. A Quebec quencher. *Cardiovasc Res*. 1998;40:423–425.
Weinberg ED. The hazards of iron loading. *Metallomics*. 2010;2:732–740.
Weiss KH, Stremmel W. Clinical considerations for an effective medical therapy in Wilson's disease. *Ann N Y Acad Sci*. 2014;1315:81–85.
Wester PO, Brune D, Nordberg G. Arsenic and selenium in lung, liver, and kidney tissue from dead smelter workers. *Br J Ind Med*. 1981;38(2):179–184.
White SJ, Shine JP. Exposure potential and health impacts of indium and gallium, metals critical to emerging electronics and energy technologies. *Curr Environ Health Rep*. 2016;3:459–467.
Whitehead MW, Thompson RP, Powell JJ. Regulation of metal absorption in the gastrointestinal tract. *Gut*. 1996;39:625–628.
WHO. *IPCS Environmental Health Criteria*. *Thallium*. Geneva: World Health Organization; 1996.
WHO. *IPCS Environmental Criteria. Aluminum*. Geneva: World Health Organization; 1997.
WHO. *IPCS Environmental Health Criteria. Palladium*. Geneva: World Health Organization; 2002. Available at: http://www.inchem.org/documents/ehc/ehc/ehc226.htm.
WHO. *Silver: Water Disinfection and Toxicity*. Geneva: World Health Orginization/Centre for Research into Environment and Health; 2014.
Willhite CC, Karyakina NA, Yokel RA, et al. Systematic review of potential health risks posed by pharmaceutical, occupational and consumer exposures to metallic and nanoscale aluminum, aluminum oxides, aluminum hydroxide and its soluble salts. *Crit Rev Toxicol*. 2014;44(suppl 4):1–80.
Williams PL, James RC, Roberts SM. *Principles of Toxicology : Environmental and Industrial Applications*. New York: Wiley; 2000.
Wright RO, Baccarelli A. Metals and neurotoxicology. *J Nutr*. 2007;137:2809–2813.
Wrobel JK, Power R, Toborek M. Biological activity of selenium: revisited. *IUBMB Life*. 2016;68:97–105.
Wu Y, Yang X, Ge J, et al. Blood lead level and its relationship to certain essential elements in the children aged 0 to 14 years from Beijing, China. *Sci Total Environ*. 2011;409:3016–3020.
Xiao T, Yang F, Li S, et al. Thallium pollution in China: a geo-environmental perspective. *Sci Total Environ*. 2012;421–422:51–58.
Yokohira M, Arnold LL, Pennington KL, et al. Effect of sodium arsenite dose administered in the drinking water on the urinary bladder epithelium of female arsenic (+3 oxidation state) methyltransferase knockout mice. *Toxicol Sci*. 2011;121, 257–266.
Zalups RK, Ahmad S. Molecular handling of cadmium in transporting epithelia. *Toxicol Appl Pharmacol*. 2003;186:163–188.
Zare B, Nami M, Shahverdi AR. Tracing tellurium and its nanostructures in biology. *Biol Trace Elem Res*. 2017;180:171–181.
Zhang C, Ferrari R, Beezhold K, et al. Arsenic promotes NF-kappaB-mediated fibroblast dysfunction and matrix remodeling to impair muscle stem cell function. *Stem Cells*. 2016;34:732–742.
Zheng W. Toxicology of choroid plexus: special reference to metal-induced neurotoxicities. *Microsc Res Tech*. 2001;52:89–103.
Zhitkovich A. Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI). *Chem Res Toxicol*. 2005;18:3–11.
Zhitkovich A, Song Y, Quievryn G, et al. Non-oxidative mechanisms are responsible for the induction of mutagenesis by reduction of Cr(VI) with cysteine: role of ternary DNA adducts in Cr(III)-dependent mutagenesis. *Biochemistry*. 2001;40:549–560.
Zhou X, Sun X, Cooper KL, et al. Arsenite interacts selectively with zinc finger proteins containing C3H1 or C4 motifs. *J Biol Chem*. 2011;286(26):22855–22863.
Zhou X, Sun X, Mobarak C, et al. Differential binding of monomethylarsonous acid compared to arsenite and arsenic trioxide with zinc finger peptides and proteins. *Chem Res Toxicol*. 2014;27:690–698.
Zwolak I, Zaporowska H. Selenium interactions and toxicity: a review. Selenium interactions and toxicity. *Cell Biol Toxicol*. 2012;28:31–46.

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chapter 24

Toxic Effects of Solvents and Vapors

James V. Bruckner, S. Satheesh Anand, and D. Alan Warren

Introduction

Is There a Solvent-Induced Chronic Encephalopathy?

Solvent Abuse

Environmental Contamination

Toxicokinetics
- Absorption
- Transport and Distribution
- Elimination
- Metabolism
- Physiological Modeling

Potentially Sensitive Subpopulations
- Endogenous Factors
  - *Children*
  - *Elderly*
  - *Gender*
  - *Genetics*
- Exogenous Factors
  - *P450 Inducers*
  - *P450 Inhibitors*
  - *Lifestyle*
  - *Solvent Mixtures*
  - *Diseases*

Chlorinated Hydrocarbons
- Trichloroethylene
  - *Metabolism*
  - *Modes of Carcinogenic Action in Target Tissues*
  - *Liver Cancer*
  - *Lung Cancer*
  - *Kidney Cancer*
  - *Cancer Epidemiology*
  - *Risk Assessment*
- Tetrachloroethylene
  - *Metabolism*
  - *Modes of Cytotoxicity/Carcinogenicity*
  - *Hepatorenal Toxicity*
  - *Cancer Bioassays in Rodents*
  - *Cancer Epidemiology Studies*
  - *Risk Assessment*
- 1,1,1-Trichloroethane
  - *Toxicokinetics (TK) and Metabolism*
  - *Toxicity*
  - *Potential Carcinogenicity*
- Methylene Chloride
  - *Metabolism*
  - *Modes of Toxicity/Carcinogenicity*
  - *Cancer Bioassays in Rodents*
  - *Cancer Epidemiology Studies*
  - *Risk Assessment*
- Carbon Tetrachloride
- Chloroform

Aromatic Hydrocarbons
- Benzene
- Toluene
- Xylenes and Ethylbenzene
- Styrene

Alcohols
- Ethanol
- Methanol

Glycols
- Ethylene Glycol
- Diethylene Glycol
- Propylene Glycol

Glycol Ethers
- Reproductive Toxicity
- Developmental Toxicity
- Hematotoxicity
- Immunotoxicity/Carcinogenicity

Fuels and Fuel Additives
- Automotive Gasoline
- Methyl Tertiary-Butyl Ether
- Jet Fuel
  - *Pulmonary Effects*
  - *Immune Effects*
  - *Dermal Effects*

Carbon Disulfide

Acknowledgments

References

INTRODUCTION

The term *solvent* refers to a class of organic chemicals of variable lipophilicity and volatility. These properties, coupled with small molecular size and lack of charge, make inhalation the major route of exposure and provide for ready absorption across membranes of the lung, gastrointestinal (GI) tract, and skin. In general, the lipophilicity of solvents increases with increasing numbers of carbon and/or halogen atoms, while volatility decreases. Organic solvents are frequently used to dissolve, dilute, or disperse materials that are insoluble in water. As such they are widely employed as degreasers and as constituents of paints, varnishes, lacquers, inks, aerosol spray products, dyes, and adhesives. Other uses are as intermediates in chemical synthesis, and as fuels and fuel additives. Most organic solvents are refined from petroleum. Many solvents such as naphthas and gasoline are complex mixtures, often consisting of hundreds of compounds. Early in the twentieth century, there were perhaps a dozen or so known and commonly used solvents. By 1981, this number had climbed to approximately 350 (OSHA, 2006).

Solvents are classified largely according to molecular structure or functional group. Classes of solvents include aliphatic hydrocarbons, many of which are halogenated (i.e., halocarbons);

aromatic hydrocarbons; alcohols; ethers; esters/acetates; amides/amines; aldehydes; ketones; and complex mixtures that defy classification. The main determinants of a solvent's inherent toxicity are: (1) its number of carbon atoms; (2) whether it is saturated or has double or triple bonds between adjacent carbon atoms; (3) its configuration (i.e., straight chain, branched chain, or cyclic); (4) whether it is halogenated; and (5) the presence of functional groups. Some class-wise generalizations regarding toxicity can be made. For example, the more lipophilic a hydrocarbon, the more potent a central nervous system (CNS) depressant it is; amides/amines tend to be potent sensitizers; aldehydes are particularly irritating; hydrocarbons that are extensively metabolized tend to be more cytotoxic/mutagenic; and many unsaturated, short-chain halocarbons are animal carcinogens. The toxicity of solvents within the same class can vary dramatically. For example, 1,1,1-trichloroethane (TRI) and 1,1,2-trichloroethylene (TCE) are both halocarbons with three chlorine atoms, yet unsaturated TCE is carcinogenic in the rat and mouse, but TRI is not. Similar results have been reported for 2,4- and 2,6-diaminotoluene in rodents, as only the 2,4-isomer is capable of inducing significant hepatocyte proliferation and liver tumors. Slight structural differences in solvent metabolites are also of toxicological consequence. The peripheral neuropathy induced by *n*-hexane and 2-hexanone is dependent on the production of the γ-diketone metabolite 2,5-hexanedione. Diketones lacking the gamma structure are not neurotoxic. Thus, subtle differences in chemical structure can translate into dramatic differences in toxicity.

Nearly everyone is exposed to solvents during their normal activities. Consider, for example, a person who works in an aircraft factory as a metal degreaser (TCE exposure) drives to the neighborhood bar after work and has a few drinks (ethanol exposure) and cigarettes (benzene and styrene exposure); stops on the way home at a self-service filling station for gasoline (benzene, toluene, 1,3-butadiene, etc. exposure) and the dry cleaner's for laundry (tetrachloroethylene [PERC] exposure); and after dinner enjoys his hobby of model shipbuilding that requires the use of glue (toluene exposure). Cigarette smoking is the primary source of benzene, toluene, and styrene exposure, as well as an important source of ethylbenzene and xylenes (Chambers et al., 2011). While everyone may not identify with the above scenarios, detailed surveys of indoor and outdoor air, such as the EPA's Total Exposure Assessment Methodology (TEAM) and National Human Exposure Assessment Survey (NHEXAS) studies, indicate that airborne solvent exposure is unavoidable. Drinking water is also a common source of solvent exposure due to discharge of solvents into surface water and groundwater and the presence of disinfection by-products, including the animal carcinogen chloroform ($CHCl_3$). Trichloroacetic acid (TCA) and dichloroacetic acid (DCA), metabolites of TCE and PERC, are also common drinking water disinfection by-products.

Environmental exposures to solvents in air and groundwater are frequent subjects of toxic tort litigation, despite concentrations that are typically in the low parts per billion (ppb) or parts per trillion (ppt) ranges. Multiple exposure pathways frequently exist (Fig. 24-1). Although not represented in Fig. 24-1, household use of solvent-contaminated water may result in solvent intake from inhalation and dermal absorption as well as ingestion (Weisel and Jo, 1996; Nuckols et al., 2005). In many cases, risk assessment guidelines stipulate that risks be determined for physiologically diverse individuals who are exposed to several solvents by multiple exposure pathways. As an aid to the risk assessment process, the U.S. Environmental Protection Agency (EPA) has derived toxicity factors for many of the most common/toxic solvents. These toxicity factors are referred to as reference concentrations (RfCs), reference doses (RfDs), and cancer slope factors (CSFs). Values for a number of these are available from the EPA's online Integrated Risk Information System (IRIS). Additional sources of exposure guidelines for noncancer endpoints are found in the *Toxicological Profiles* of the U.S. Agency for Toxic Substances and Disease Registry (ATSDR). These profiles often contain minimal risk levels (MRLs) that are derived in a similar manner to EPA's RfCs and RfDs, but are frequently based on different critical studies or derived with different uncertainty factors.

Occupational solvent exposures involve situations ranging from a secretary using typewriter correction fluid to the loading and off-loading of tanker trucks with thousands of gallons of gasoline. The greatest industrial use of solvents is as metal degreasers. This work environment is typically where the highest exposures occur, mainly via inhalation and secondarily via dermal contact. An estimated 10 million people are potentially exposed to organic solvents in the workplace (OSHA, 2006). Many of the most severe exposures to solvents have occurred as a result of their use in confined spaces with inadequate ventilation. While the U.S. Occupational Safety and Health Administration (OSHA) has established legally enforceable permissible exposure limits (PELs) for over 100 solvents, most PELs are outdated. The majority of existing PELs were adopted from a list of threshold limit values (TLVs) previously published by the American Conference of Governmental Industrial Hygienists (ACGIH). Many current TLVs are more stringent than PELs but do not carry the weight of law. Whereas the ACGIH's TLVs for an 8-hour workday, 40-hour workweek are designed to be protective for a working lifetime, its short-term exposure limits (STELs) and ceiling values are designed to protect against the acute effects of high-level, short-term solvent exposure. If warranted, ACGIH will assign a skin notation to a solvent, indicating that a significant contribution to overall exposure is possible by the dermal route, either by contact with vapors or direct skin contact with the liquid. ACGIH also categorizes chemicals for carcinogenicity in animals and humans. Biological monitoring in the workplace should find increasing use as technologic advances are made, because it often provides a better measure of exposure than classic industrial hygiene monitoring. The ACGIH has published over 50 Biological Exposure Indices (BEIs), on which bases the safety of internal measures of exposure can be judged (ACGIH, 2011).

Most solvent exposures involve a mixture of chemicals, rather than a single compound (see the subsection "Solvent Mixtures" in the sub section "Exogenous Factors"). Our knowledge of the toxicity of solvent mixtures is rudimentary relative to the toxicology of individual solvents. While the assumption is frequently made that the toxic effects of solvents are additive, the chemicals may also interact synergistically or antagonistically. For example, repetitive alcohol consumption induces certain cytochrome P450s (CYPs), and may therefore enhance the metabolic activation of other solvents to cytotoxic metabolites. Ethanol intake near the time of exposure to such solvents, in contrast, may competitively inhibit their metabolism and be protective. Another well-characterized example of solvent antagonism is the competitive metabolic interaction between benzene and toluene (Medinsky et al., 1994). Coexposure to these chemicals results in diminished benzene metabolism, genotoxicity, and erythropoietic toxicity relative to that which follows benzene exposure alone. It is now recognized that significant data gaps exist in the area of mixtures toxicology, and that these can be significant sources of uncertainty in risk assessments. Physiologically based toxicokinetic (PBTK) models are being developed by different research groups to predict the impact of metabolic induction and inhibition on the kinetics of individual components of specific

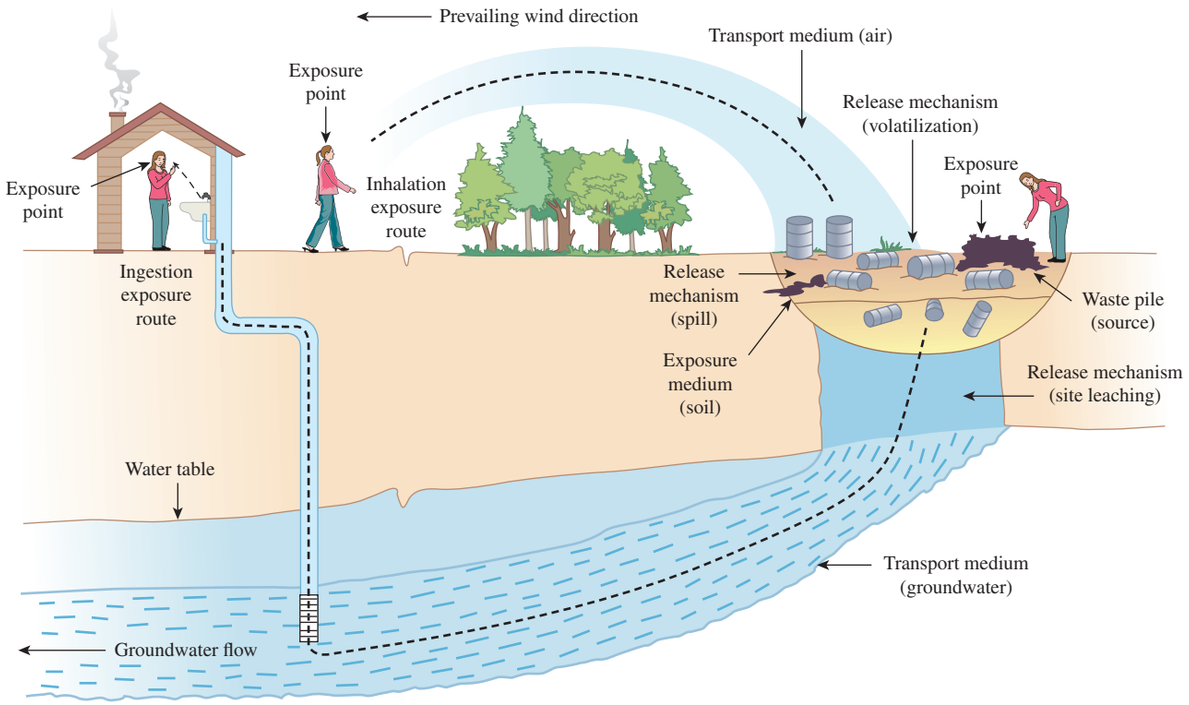

Figure 24-1. *Solvent exposure pathways and media.* (Adapted from EPA Risk Assessment Guidance for Superfund. *Human Health Evaluation Manual Part A, Interim Final*. Washington, DC: Office of Emergency and Remedial Response, 1989.)

mixtures (see the subsection "Physiological Modeling" in the section "Toxicokinetics").

Although some solvents are much less hazardous than others, virtually all can cause adverse effects. Provided that the dose or concentration is sufficient, most have the potential to induce some level of narcosis and cause respiratory and mucous membrane irritation. A number of solvents are animal carcinogens, but only a handful have been classified as known human carcinogens. Herein lies a major challenge for toxicology—determining the human relevance of tumors observed in chronic, high-dose rodent studies. As with other chemicals, whether adverse health effects occur from solvent exposure is dependent on several factors: (1) toxicity/carcinogenicity of the solvent; (2) exposure route; (3) amount or rate of exposure; (4) duration of exposure; (5) individual susceptibility; and (6) interactions with other chemicals. Adverse health effects may occur acutely and be readily discernible, or they may result from chronic exposure and have insidious onset. Numerous epidemiologic studies of environmentally and occupationally exposed populations have been conducted for some solvents, but most human risk assessments remain heavily reliant upon extrapolation from high-dose animal studies. One must bear in mind that the toxic effects and their underlying mechanisms discussed herein may be operative only in certain animal species or strains and under certain exposure conditions. Care must therefore be taken in generalizing beyond the experimental conditions under which data are collected. While a relatively small number of commercially available solvents is discussed in this chapter, those selected for discussion are thought to best demonstrate principles of solvent toxicology, are of particular commercial importance, and/or are currently garnering significant attention from the toxicological and regulatory communities. A book chapter that examines solvents from an organ systems standpoint, in contrast to discussion of individual solvents, is that of Gerr and Letz (1998).

IS THERE A SOLVENT-INDUCED CHRONIC ENCEPHALOPATHY?

The CNS-depressant effects of acute, high-level exposures and the potential for permanent neurologic damage in chronic solvent abusers are not a matter of debate. It is also clear that chronic, moderate-to-high-level exposure to a few solvents such as *n*-hexane and carbon disulfide can cause specific degenerative changes in the CNS or the peripheral nervous system (PNS). Far less clear is whether chronic, low-level exposure to virtually any solvent or solvent mixture can produce a pattern of neurologic dysfunction referred to as *painters' syndrome, organic solvent syndrome, psychoorganic syndrome, and chronic solvent encephalopathy* (CSE). CSE is characterized by nonspecific symptoms (e.g., headache, fatigue, mood disturbances, and sleep disorders) with or without changes in neuropsychological function. There is a reversible form of CSE referred to as *neurasthenic syndrome* that consists of symptoms only, and both "mild" and "severe" forms accompanied by objective signs of neuropsychological dysfunction that may or may not be fully reversible. Regardless of form, there is universal agreement that CSE is a nonprogressive disease in which no severe deterioration of functioning occurs after diagnosis, provided exposure ceases. An elevated prevalence of psychiatric disorders such as major depressive disorder, panic disorders, agoraphobia, and generalized anxiety disorder has been reported among CSE patients (Visser et al., 2011). The syndrome that is CSE was first described in the Scandinavian occupational literature in the late 1970s, in solvent-exposed painters (Axelson et al., 1976; Arlien-Soborg et al.,

1979). Since that time, numerous studies from Scandinavia have been published purporting that solvents as a class have chronic neurotoxic properties. These countries, as well as a few others in Europe (i.e., Germany, Austria, and Belgium) have passed legislation recognizing CSE as a compensable occupational disability (Triebig and Hallermann, 2001). Scientists outside of Scandinavia, including many in the United States, have generally been less willing to recognize CSE as a legitimate disease state and have published studies to the contrary (Triebig et al., 1988; Bleecker et al., 1991; Spurgeon et al., 1994).

In response to the numerous reports of CSE, two conferences were convened in 1985. The first was held in Copenhagen by the Nordic Council of the World Health Organization (WHO, 1985). The second, in Raleigh, NC, was attended by an international group of scientists from academia, industry, and government (Cranmer, 1986). The categorization scheme that resulted from the Raleigh meeting is presented in Table 24-1. The WHO scheme is similar. Among those who utilize the categorization scheme, it is generally believed that the most severe CSE category, type 3, results from repeated, severe intoxications like those experienced by solvent abusers. CSE types 1 and 2, in contrast, are thought to be associated with prolonged, low-to-moderate-level exposure common to work environments. A major criticism of the categorization scheme is the lack of consideration of inhaled solvent concentration and exposure duration. However, most CSE proponents believe that solvent exposure must occur for some 10 years before clinical symptoms are manifest. Citing growing acceptance of CSE outside of Scandinavia, some scientists and physicians have advocated for refinement of existing diagnostic criteria and a unified categorization scheme (van der Hoek et al., 2000, 2001). To this end, a European working group has published a consensus document updating the neuropsychological characteristics of CSE and providing guidelines for neuropsychological assessment during diagnosis of patients suspected of CSE (van Valen et al., 2012). Similarly, Verberk et al. (2012) published a set of "decision rules" to promote standardization in the diagnostic assessment of CSE, but found that application of 25 cognitive tests on patients suspected of CSE failed to show a characteristic profile of impairment and gave divergent results on tests of related mental functions.

CSE researchers typically rely upon self-reported symptoms and a clinical neuropsychological evaluation (Table 24-2), and to a much lesser extent on diagnostic tests such as electroencephalography and computerized brain tomography (Osterberg et al., 2000; Carter et al., 2002; Visser et al., 2012). Based upon reanalysis of patients diagnosed with CSE 20 years earlier, Bast-Pettersen (2009) concluded that symptoms were (and still are) not very useful in diagnosing CSE, and objective neuropsychological tests are necessary. However, it has been argued that many neuropsychological tests are of questionable validity, sensitivity, specificity, and predictive value. It has also been noted that many investigations of CSE are fraught with methodologic flaws. For example, CSE investigators frequently fail to measure premorbid function or intellect; employ a reference population; control adequately for the potential confounders of age, alcohol use, other CNS diseases, and other chemicals; corroborate functional deficits with objective evidence of brain disease; and/or examine exposure–response relationships. The importance of doing so is best exemplified by the reanalysis of individuals originally reported in the seminal study by Arlien-Soborg et al. (1979) to have "painters' syndrome." When the influences of age, education, and intelligence were considered, the previously reported reduction in neuropsychological test scores disappeared (Gade et al., 1988). Another example is that of Cherry et al. (1985), who demonstrated the importance of matching solvent-exposed and control groups for preexposure intellect before making a diagnosis of CSE. Subsequently, Albers et al. (1999, 2000) reportedly found no objective evidence of toxic encephalopathy or polyneuropathy among railroad workers with long-term solvent exposure and

Table 24-1

Proposed Categories of Solvent-Induced Encephalopathy

CATEGORY	CLINICAL MANIFESTATIONS
Type 1	*Symptoms only*: The patient complains of nonspecific symptoms such as fatigability, memory impairment, difficulty in concentration, and loss of initiative. These symptoms are reversible if exposure is discontinued, and there is no objective evidence of neuropsychiatric dysfunction.
Type 2A	*Sustained personality or mood change*: There is a marked and sustained change in personality involving fatigue, emotional lability, impulse control, and general mood and motivation.
Type 2B	*Impairment in intellectual function*: There is difficulty in concentration, impairment of memory, and a decrease in learning capacity. These symptoms are accompanied by objective evidence of impairment. There may also be minor neurologic signs. The complete reversibility of type 2B is questionable.
Type 3	*Dementia*: In this condition, marked global deterioration in intellect and memory is often accompanied by neurologic signs and/or neuroradiologic findings. This condition is, at best, poorly reversible, but is generally nonprogressive once exposure has ceased.

SOURCE: Reproduced from Cranmer (1986), with permission from Elsevier.

Table 24-2

Functions That May Be Assessed in a Neuropsychological Evaluation

Psychomotor functions
Reaction time
Motor speed and dexterity
Eye–hand coordination
Sustained attention/concentration and perceptual speed
Verbal and nonverbal memory
Immediate memory
Delayed memory
Learning
Visual constructive ability
Conceptual ability
Evaluation of personality and affect

SOURCE: Reproduced from Cranmer (1986), with permission from Elsevier.

diagnosis of CSE. However, these workers were found to have statistically significantly prolongated blink reflex latencies (a potential sensitive indicator of subclinical solvent neurotoxicity) compared with historical controls, even though blink reflex latencies of all workers remained within the range of normal (Albers et al., 2001). As to this result, the authors conclude *"… that the small group differences … are of no diagnostic importance and of uncertain physiologic importance, and they may reflect unrecognized confounders and technical factors."*

It is evident that resolution of the controversial issue of CSE will come only through the conduct of well-designed and controlled clinical and epidemiological studies, especially considering the absence of an appropriate animal model. Examples of such studies continue to originate largely from Europe and Scandinavia, including those examining CSE risk in relation to genetic polymorphisms in bioactivating and detoxifying enzymes and dopamine receptors (Kezic et al., 2006; Godderis et al., 2010). Another such study tested the hypothesis that disturbances within the frontostriatothalamic circuitry explained the symptomology (impaired psychomotor speed and attention) frequently observed in CSE patients (Visser et al., 2008). Confirmation of the hypothesis came in the form of reductions among CSE patients in striatal dopamine receptor binding and neurometabolite concentrations in frontal gray matter, as well as changes in fractional anisotropy (a measure of the uniformity of water movement within and along axons, where reductions are indicative of axonal injury) in the thalamus and caudate nucleus. While identification of such brain abnormalities brings some focus to the nonspecific clinical picture that is CSE, similar findings might be anticipated in normal aging or neurological disorders such as Parkinson's, for which solvents have been considered as risk factors (Lock et al., 2013). Though brief and somewhat dated, insightful reviews of CSE by Rosenberg (1995) and Schaumburg and Spencer (2000) have been published. These reviews conclude that the available literature, including the "landmark" North American study of 187 paint-manufacturing workers (Bleecker et al., 1991), does not support chronic low-level solvent exposure as a cause of *symptomatic* CNS or PNS dysfunction. This does not preclude, however, the possibility that such exposure can be associated with *subclinical* cognitive dysfunction in the form of slight psychomotor and attentional deficit disorders. For the viewpoint of CSE proponents, readers are directed to texts by Arlien-Soborg (1992) and Kilburn (1998), as well as a systematic review of all evidence on the course and prognostic factors of CSE through 2008 (van Valen et al., 2009).

A number of scientists have examined potential relationships between solvent exposure and motor and psychotic disorders. Causes of Parkinson disease (PD) are poorly understood, although case reports have associated PD with exposures to various solvents, notably trichloroethylene (TCE). Studies of different solvents, including TCE, have revealed adverse effects on the substantia nigra, a brain region in which dopamine deficiency due to loss of dopanergic neurons is implicated in typical motor symptoms of the disease in humans. There is no consistent evidence from such toxicology studies or epidemiological investigations that a specific solvent or class of solvents is a cause of Parkinson disease (Lock et al., 2013). Landtblom (1997) described a number of epidemiological studies of associations of occupational organic solvent exposure with multiple sclerosis (MS). Relative risks or odds ratio greater than 1 were reported in some instances. In a subsequent publication, Landtblom et al. (2003) examined the relationship between glutathione *S*-transferase M1 and CYP2 polymorphisms in leukocyte DNA and solvent exposure in MS patients. These null genotypes have been associated with decreased metabolic clearance of organic solvents. No differences in genetic predisposition were found between MS patients exposed and unexposed to solvents. Prolonged occupational exposure to organic solvents has also been linked to schizophrenia (Stein et al., 2010). Solvents are often implicated in idiopathic neurological disorders, although sufficient evidence for a causal association is lacking.

SOLVENT ABUSE

Inhalants are volatile substances that can be inhaled to induce a psychoactive or mind-altering effect. Their abuse became a major drug problem worldwide, particularly in disadvantaged populations and among adolescents (Williams and Storck, 2007). The epidemiology of inhalant abuse in the United States is receiving considerable attention (Spiller, 2004; Wu et al., 2004), with almost one in five 8th graders claiming to have used inhalants according to a National Institute on Drug Abuse (NIDA)-sponsored survey (Johnston et al., 2008). Solvents are among the most popular classes of drugs of abuse, given their presence in a multitude of inexpensive, readily available products that are legal to buy and possess. These products are used for common household and industrial purposes and include paint thinners and removers, dry-cleaning fluids, degreasers, gasoline, glues, typewriter fluid, nail polish remover, felt-tip marker fluids, and aerosols such as fabric protector sprays and spray paints. Solvents are often among the first drugs used by children and adolescents. Early use of inhalants is often a precursor to abuse of multiple illegal substances (Wu et al., 2004). Research suggests that adverse socioeconomic conditions, a history of child abuse, poor grades, and dropping out of school are all factors contributing to inhalant abuse (NIDA, 2005).

Solvent abuse is a unique exposure situation, in that participants repeatedly subject themselves to vapor concentrations high enough to produce effects that resemble alcohol intoxication. Solvents can be breathed in through the nose or the mouth by "sniffing" or "snorting" vapors from containers; spraying aerosols directly into the nose or mouth; "bagging" by inhaling vapors from a plastic or paper bag; or "huffing" from a solvent-soaked rag stuffed into the mouth (NIDA, 2005). Although dependent on the pattern of inhalation, blood levels of solvents typically peak a few minutes after inhalation begins, and the abuser can begin to experience effects after a matter of seconds. While intoxication may last only a few minutes, abusers frequently seek to prolong the "high" by inhaling repeatedly over the course of several hours. In extreme circumstances, death may be a consequence of cardiac arrhythmias, asphyxiation, and/or cachexia.

Relatively little is known about the neuropharmacology of abused solvents, although they appear to have much in common with the classical CNS-depressant drugs like ethanol and barbiturates, including the potential for tolerance and dependence (Balster, 1998). Commonly used solvents have been shown to alter the function of a variety of ligand-gated ion channels, including those activated by glycine, gamma-aminobutyric acid (GABA) and *N*-methyl-D-aspartate (NMDA) (Beckstead et al., 2000; Cruz et al., 2000). Recent evidence links high-level toluene exposure in rats to increased dopaminergic neurotransmission within the mesolimbic reward pathway, an effect thought to underlie the abuse potential of numerous drugs (Reigel and French, 2002; Reigel et al., 2004). Toluene, as well as TCE and TRI, has been shown to enhance serotonin-3 receptor function, which has also been implicated in the reinforcing properties of abused drugs (Lopreato et al., 2003).

Whereas the intoxicating effects achieved through acute solvent abuse are reversible, abuse may be continued for years and result in residual organ damage. For example, chronic abuse of products containing *n*-hexane and methyl-*n*-butyl ketone can cause

peripheral neuropathies. Blood dyscrasias, liver damage, kidney injury, and hearing impairment are seen in patients who have abused solvents injurious to these organs. It has been known for some time that the brain is not spared residual damage, with long-term neurologic and psychological sequelae (Caldemeyer et al., 1996; Lubman et al., 2008). Rosenberg et al. (2002) reported increased incidences of neurologic and neuropsychological effects in chronic solvent abusers compared to a control group of chronic drug abusers. Solvent abusers did significantly worse on tests of working memory and executive cognitive function, and a much higher percentage of the patients (44.0% vs. 25.5%) had structural abnormalities in subcortical regions of the brain (i.e., basal ganglia, cerebellum, pons, and thalamus) as visualized by MRI (Fig. 24-2). Solvent abusers also showed moderate-to-severe diffuse abnormality of the cerebral white matter, consistent with that seen in earlier studies of neuropsychologically impaired toluene abusers (Rosenberg et al., 1988a, 1988b). This condition was termed "white matter dementia," as myelinated neurons are white in appearance. It was characterized primarily by diffuse cerebral, cerebellar, and brainstem atrophy, and ventricular enlargement (Fig. 24-2). Dementia in toluene abusers has been referred to as "toluene leukoencephalopathy" (Filley et al., 2004). Leukoencephalopathy, also known as multifocal demyelinating disease, involves structural alteration of cerebral white matter, in which the myelin sheaths that cover nerve fibers are destroyed but axons are largely spared. Thus, toluene is among a long list of white matter toxins identified to date.

The Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV) listed 292.82 "inhalant-induced persisting dementia" and 292.84 "inhalant-induced mood disorder." The International Classification of Diseases-10 (ICD-10) included F18.7 "mental and behavior disorders due to use of volatile solvents; residual and late-onset psychiatric disorder." DSM-5 lists Inhalant Use

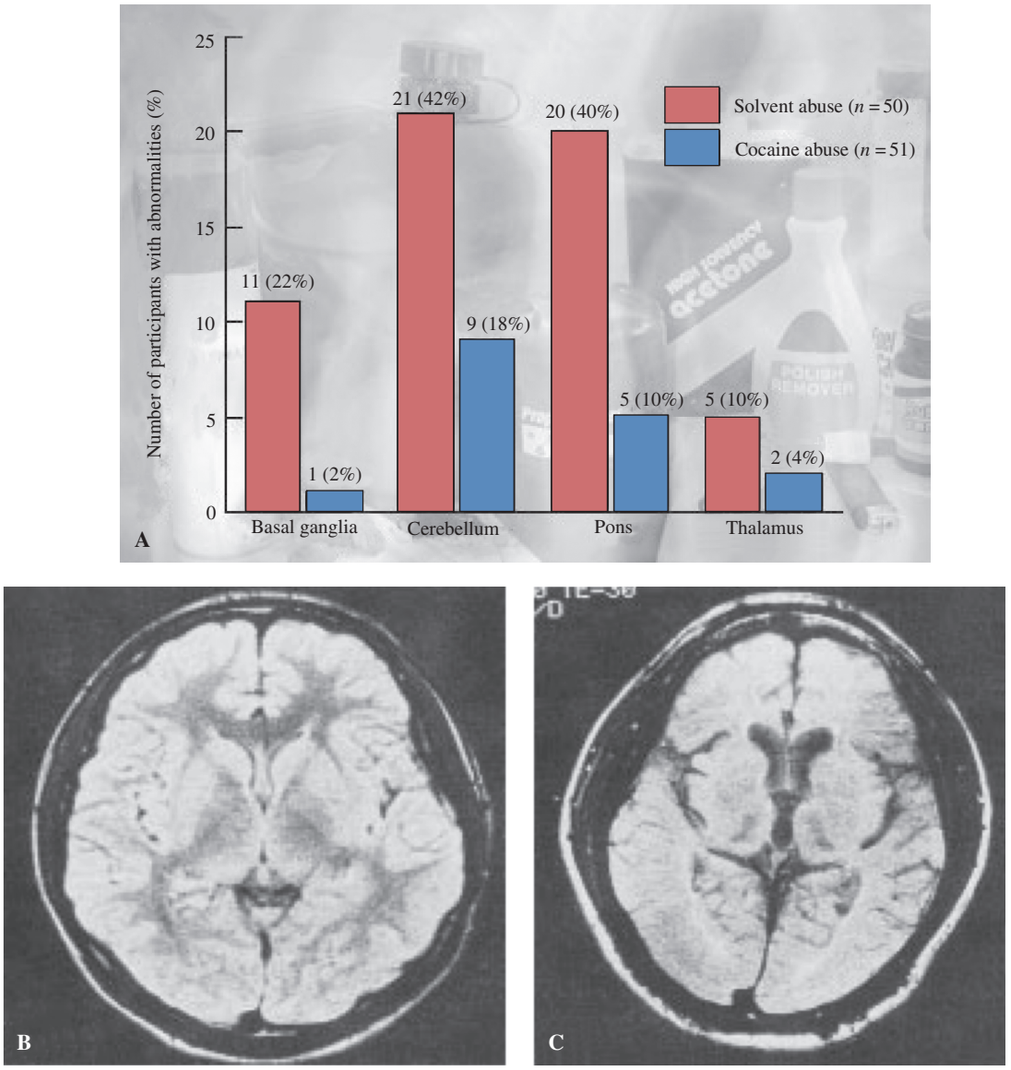

Figure 24-2. *(**A**) Inhalant and cocaine abusers with subcortical abnormalities, by brain region affected. (**B–C**) Brain damage in a toluene abuser.* (A) Magnetic resonance imaging (MRI) scans of chronic inhalant abusers and chronic cocaine abusers showed more frequent occurrence of abnormalities in the basal ganglia, cerebellum, pons, and thalamus for those who abused solvents. (Available at http://www.drugabuse.gov/NIDA notes/NNVol17N4/Chronic.html: from NIDA Notes, 2002.) MRI shows marked atrophy (shrinkage) of brain tissue in a toluene abuser (C) compared to a nonabusing individual (B). Note the smaller size and the larger empty (dark) space within the toluene abuser's brain. The white outer circle in each image is the skull. (Available at http://www.drugabuse.gov/PDF/RRInhalants.pdf.) (Courtesy of Neil Rosenberg, MD, as published in NIDA Research Report Series, March 2005.)

Disorder 304.60 (F18.20), which is defined as "a pattern of inhaling hydrocarbon-based fumes, such as those found in solvents or paints, for the purpose of altering the mental state and leading to significant impairment." The repeated practice is classified as a pattern of substance abuse.

ENVIRONMENTAL CONTAMINATION

Widespread use of solvents has resulted in their dissemination throughout the environment (Fig. 24-1). Everyone is exposed daily to solvents, as reflected by their common occurrence in blood of nonoccupationally exposed populations (Churchill et al., 2001; Sexton et al., 2005; Blount et al., 2006; Jia et al., 2012). Because solvents as a chemical class are volatile, the preponderance of solvents entering the environment do so by evaporation. The majority of the more volatile organic chemicals (VOCs) volatilize when products containing them (e.g., aerosol propellants, paint thinners, cleaners, and soil fumigants) are used as intended. Solvent loss into the atmosphere also occurs during production, processing, storage, and transport activities, resulting in elevated concentrations in air in the proximity of point sources. Winds dilute and disperse solvent vapors across the world. Atmospheric concentrations of most VOCs are usually extremely low (i.e., nondetectable to nanograms or a few micrograms per cubic meter (m^3) of air). Relatively high atmospheric concentrations of certain solvents (e.g., 10 to 520 μg/m^3, or 3 to 163 ppb of benzene) have been measured in urban areas, around petrochemical plants, and in the immediate vicinity of hazardous waste sites. Motor vehicle exhaust is a major contributor to hydrocarbon emissions (Mohamed et al., 2002). Fraser et al. (1998) measured very high levels of benzene, toluene, ethylbenzene, and xylenes in a Los Angeles roadway tunnel.

Solvent contamination of drinking water supplies is a major health concern. Although the majority of a solvent spilled onto the ground evaporates, some may permeate the soil and migrate through it until reaching groundwater or an impermeable material. In years past, the more lipophilic solvents were generally regarded as water insoluble. It is now recognized that all solvents are soluble in water to some extent. Some (e.g., alcohols, ketones, glycols, and glycol ethers) are freely water soluble. Maximum solubilities of some common hydrocarbon solvents range from 10 mg/L (ppm) for *n*-hexane to 24,000 ppm for bromochloromethane. High concentrations of VOCs are sometimes found in the effluent of facilities of rubber producers, chemical companies, petrochemical plants, and paper mills. Concentrations diminish rapidly after VOCs enter bodies of water, primarily due to dilution and evaporation. VOCs in waters rise to the surface or sink to the bottom, according to their density. VOCs on the surface will largely evaporate. VOCs on the bottom must depend on solubilization in water or mixing by current or wave action to reach the surface. VOCs in groundwater tend to remain trapped until the water reaches the surface, although some are subject to microbial modification. Concentrations in well water are rarely high enough for acute or subacute toxicity to be of concern. The very low levels of some solvents typically found in water have, however, caused a great deal of concern and debate about their toxic and carcinogenic potential.

Potential health effects of solvent contaminants of water have received considerable attention over the past 40+ years. A report by Mason and McKay (1975), of an increased incidence of cancer in persons who drank water from the Mississippi River, prompted the EPA to analyze the water supply of New Orleans. The finding of some 76 synthetic organic chemicals, many of which were solvents, prompted passage of the Safe Drinking Water Act in 1974. Chloroform ($CHCl_3$) is the most frequently found VOC in finished drinking water supplies in the United States (ATSDR, 1997a). It and certain other trihalomethanes are formed by reaction of the chlorine added as a disinfectant with natural organic compounds present in the water. Levels of solvents found in drinking water in the United States are typically in the nanogram per liter (ppt) to microgram per liter (ppb) range, though concentrations in the low milligram per liter (ppm) range are found in water from wells situated in solvent plumes from hazardous waste sites and other point discharges. Of the thousands of chemicals found at hazardous waste sites, 6 of the 10 most commonly present in groundwater are solvents (Pohl et al., 2008).

People are subjected to solvents in environmental media by inhalation, ingestion, and skin contact. Considerable effort was devoted by the EPA, from 1979 to 1985, to assess personal exposure to solvents in different locales of the United States. TRI, PERC, benzene, xylenes, and ethylbenzene were most frequently found in highest concentrations (about 1 to 32 μg/m^3) in air (Wallace et al., 1987). Exhaled breath levels of some chemicals (e.g., $CHCl_3$, ethylbenzene) corresponded to indoor air levels. Personal activities (e.g., smoking, visiting a dry cleaner or service station) and occupational exposures were believed to be largely responsible for exposures to other VOCs (e.g., benzene, toluene, xylenes, and PERC) (Ashley et al., 1994). Subsequent studies, accounting for all pertinent exposure pathways, were conducted by the EPA in the mid-1990s (Gordon et al., 1999). Elliott et al. (2006) more recently reported that indoor exposures to VOCs did not have adverse effects on the respiratory function of 953 adults surveyed, with the exception of 1,4-dichlorobenzene, a component of air fresheners, toilet bowl deodorants, and mothballs. It should be recognized that $CHCl_3$ and other VOCs volatilize to some degree during home water usage, particularly when the water is heated. Thus, a significant proportion of one's total exposure to VOCs in tap water can occur via inhalation (Weisel and Jo, 1996), though the contribution of dermal exposure is relatively modest (Thrall et al., 2002).

TOXICOKINETICS

Toxicokinetic (TK) studies are playing an increasingly important role in reducing uncertainties in risk assessments of solvents and other chemicals (Andersen, 2003; Clewell et al., 2004a; Creton et al., 2009). The fundamental goal of TK studies is to delineate the uptake and disposition of chemicals and certain of their metabolites in the body. It is now recognized that toxicity is a dynamic process, in which the degree and duration of injury of a target tissue are dependent on the net effect of toxicodynamic (TD) and TK processes including systemic absorption, tissue deposition, metabolism, interaction with cellular components, elimination, and tissue repair. Estimation of risk of toxicity from TK data is based on the fundamental concept that the intensity of response from an administered dose is dependent on the amount(s) of biologically active chemical moiety(ies) present in a target tissue (i.e., the tissue dose). A related concept is that the tissue dose in a given target organ in one species will have the same degree of effect as an equivalent target organ dose in a second species. This concept of tissue dose equivalence appears to be applicable in many cases to solvents, although TD differences are possible. Gaining an understanding of how the processes that govern solvent kinetics vary with dose, route of exposure, species, and even different individuals greatly reduces the number of assumptions that have to be made in assessment of health risks from exposure and effect data.

Volatility and lipophilicity are two of the most important properties of solvents that govern their absorption and disposition in the body. Most solvents are volatile under normal usage conditions,

though volatility varies from compound to compound. Lipophilicity can also vary substantially, from quite water soluble (e.g., glycols, esters, and alcohols) to quite lipid soluble (e.g., halocarbons and aromatic hydrocarbons). Many solvents of particular concern at present are relatively lipid soluble and volatile, hence their designation as VOCs. These compounds have relatively low molecular weight and are uncharged. Thus, they pass freely through membranes from areas of high-to-low concentration by passive diffusion.

Absorption

The majority of systemic absorption of inhaled VOCs occurs in the alveoli, although limited absorption has been demonstrated to occur in the upper respiratory tract (Stott and McKenna, 1984). Gases in the alveoli are thought to equilibrate almost instantaneously with blood in the pulmonary capillaries. Blood:air partition coefficients (PCs) of VOCs are important determinants of the extent of their uptake. A PC is defined by Gargas et al. (1989) as the ratio of concentration of VOC achieved between two different media at equilibrium. As blood is largely aqueous, the more hydrophilic solvents have relatively high blood:air PCs, which favor extensive uptake. Gargas et al. (1989) determined in vitro PCs for 55 VOCs in F344 rats. Human blood:air values were measured for 36 of the compounds. Because VOCs diffuse from areas of high-to-low concentration, increases in respiratory rate (to maintain a high alveolar concentration) and in cardiac output/pulmonary blood flow (to maintain a large concentration gradient by removing capillary blood containing the VOC) enhance systemic absorption.

Systemic uptake of solvents during ongoing inhalation exposures is dependent on tissue loading and metabolism, in addition to the factors noted above. Percent uptake is initially high, but progressively declines as the chemical accumulates in tissues, and the level of chemical in venous blood returning in the pulmonary circulation increases. A near steady state or equilibrium will be reached upon inhalation of a fixed concentration of lipophilic solvents. The approach to equilibrium is asymptotic. Despite continued inhalation of lipophilic solvents, levels in the blood and tissues (other than fat) generally only increase modestly. Percent uptake remains relatively constant for the duration of exposure, with metabolism and accumulation in adipose tissue largely responsible for the continuing absorption. Hydrophilic solvents take considerably longer to reach steady state, due to the extended time required for equilibration of chemical in the inspired air with that in total body water.

Solvents are well absorbed from the GI tract. Peak blood levels are observed within a few minutes of dosing fasted subjects, although the presence of fatty food in the GI tract can significantly delay absorption of lipophilic solvents. It is frequently assumed that 100% of an oral dose of most solvents is absorbed systemically. The vehicle or diluent in which a solvent is ingested can affect its absorption and TK. Kim et al. (1990a, 1990b), for example, found that a corn oil vehicle served as a reservoir in the gut to delay GI absorption of carbon tetrachloride (CCl_4) in rats. Although bioavailability of CCl_4 given in corn oil and in an aqueous emulsion was the same, peak blood CCl_4 levels and acute hepatotoxicity were much lower in the corn oil group. Other factors that can influence the oral absorption of certain classes of chemicals have relatively little influence on most lipophilic solvents, due to their rapid passive diffusion in the entire GI tract.

Dermal absorption of solvents can result in both local and systemic effects (Semple, 2004). Skin contact with vapors and concentrated solutions of solvents is a common occurrence in the workplace. Dermal contact with solvent contaminants of water can also occur in the home and in recreational settings (Weisel and Jo, 1996; Gordon et al., 2006). Lipophilic solvents penetrate the stratum corneum, the skin's barrier to absorption, by passive diffusion. Important determinants of the rate of dermal absorption of solvents include the chemical concentration, surface area exposed, exposure duration, integrity and thickness of the stratum corneum, and lipophilicity and molecular weight of the solvent (EPA, 1998a). Skin penetration can be quantified in laboratory animals and humans by a variety of in vitro and in vivo techniques (Morgan et al., 1991; Nakai et al., 1999). Although absorption rates measured by these methods may vary numerically, there is often good agreement in their relative ranking of chemicals' ability to penetrate the skin. Dermal permeability constants are typically two to four times lower for human than for rodent skin (McDougal et al., 1990). The extent of dermal absorption in occupational and environmental exposure settings should be taken into account when conducting risk assessments of solvents.

Transport and Distribution

Chemicals and nutrients absorbed into portal venous blood from the GI tract can be removed from the bloodstream by first-pass, or presystemic elimination. As VOCs are rapidly absorbed and human intestinal epithelium apparently does not contain cytochrome P450 2E1 (CYP2E1) (Paine et al., 2006), the primary CYP isoform that metabolizes low doses of many VOCs, their first-pass GI metabolism should be negligible. Blood in the portal venous circulation passes through the liver before entering the arterial circulation. Solvents are also subject to exhalation by the lungs during their first pass through the pulmonary circulation. Therefore, solvents that are well metabolized and quite volatile are most efficiently eliminated before they enter the arterial blood. The efficiency of the hepatic first-pass elimination is thus dependent on the chemical, as well as the rate at which it arrives in the liver. Pulmonary first-pass elimination, in contrast, is believed to be a zero-order process, as a fixed percentage of the chemical is thought to exit the pulmonary blood at each pass through the pulmonary circulation. Indeed, Lee et al. (1996) demonstrated in rats that pulmonary first-pass elimination of TCE was relatively constant over a range of doses, but that hepatic elimination was inversely related to dose. Thirty years ago Andersen (1981) theorized that the liver was capable of removing "virtually all" of an orally administered VOC, if the amount in the portal blood was not great enough to saturate metabolism. Liu et al. (2009) could not detect TCE in the arterial blood of rats gavaged with 0.1 μg TCE/kg in an aqueous emulsion, but did find TCE at bolus doses more than or equal to 1 μg/kg. Interestingly, bioavailability was consistently about 12% in the dosage range of 100 to 5000 μg TCE/kg. These findings indicate that first-pass elimination affords extrahepatic organs substantial, albeit not complete, protection from environmentally-encountered levels of well-metabolized VOCs.

Solvents are transported by the arterial blood and taken up by tissues according to their blood flow and mass and tissue:blood PCs (Astrand, 1983). Relatively hydrophilic solvents solubilize in plasma. Nevertheless, as much as 50% of such chemicals may still be carried by erythrocytes (Lam et al., 1990). These researchers found that lipophilic solvents do not bind appreciably to plasma proteins or hemoglobin, but partition into hydrophobic sites in these molecules. Lipophilic solvents also partition into phospholipids, lipoproteins, and cholesterol present in the blood. The brain is an example of a rapidly perfused tissue with a relatively high lipid content. Lipophilic solvents therefore quickly accumulate in the brain after the initiation of exposures (Warren et al., 2000). Inhalation of high concentrations of halothane, TCE, and $CHCl_3$ can produce

CNS effects as profound as surgical anesthesia within as little as 1 to 2 minutes. Redistribution to poorly perfused, lipoidal tissues subsequently occurs. Adipose tissue gradually accumulates relatively large amounts of VOCs, and slowly releases them back into the blood, due to high fat:blood PCs and low blood perfusion rate.

Route of exposure can significantly influence target organ deposition and toxicity of solvents. Direct route-to-route extrapolation does not take physiological differences in absorption pathways into account. All of the cardiac output passes through the pulmonary circulation versus about 20% for the GI tract. Also, the alveolar surface area for absorption is approximately 20 times that of the entire GI tract in humans. VOCs absorbed in the lungs directly enter the arterial circulation and are transported throughout the body. In contrast, VOCs absorbed from the GI tract largely enter the portal circulation and are subject to first-pass elimination by both the liver and the lungs. The GI epithelium contains certain monooxygenases, notably Cyp3a in rats (Mitschke et al., 2008) and CYP3A4 in humans (Ding and Kaminsky, 2003), though these isozymes do not participate to a significant extent in metabolism of most solvents. The liver, however, takes up and metabolizes a portion of many VOCs following their ingestion. It is not surprising then that extrahepatic organs receive a relatively high dose upon inhalation of VOCs. Inhalation of 1,1-dichloroethylene (1-1-DCE) produced substantially greater renal damage in rats than did gastric infusion of an equivalent dose over the same time-frame (Bruckner et al., 2010).

The pattern of ingestion of solvents can significantly influence their TK and health effects. For convenience, test chemicals are typically given daily to animals as a single bolus by gavage in short- and long-term oral toxicity and carcinogenicity studies. Actual human exposures to solvents in drinking water are quite different, in that people typically ingest water in divided doses. High, daily gavage doses of $CHCl_3$ and other halocarbons have produced hepatocellular carcinoma in B6C3F1 mice. No evidence of hepatic tumorigenesis was seen, however, when these mice were given the same doses of the chemical in their drinking water (Jorgenson et al., 1985; Klaunig et al., 1986). Female B6C3F1 mice exhibited hepatocellular necrosis and regenerative hyperplasia when gavaged with $CHCl_3$. Consumptions of the same doses in water were without ill effect (Larson et al., 1994). Similarly, the maximum blood level of CCl_4 was found to be much higher and hepatotoxicity more severe in rats dosed with CCl_4 by gavage, than in those given the same dose over 2 hours by gastric infusion (Sanzgiri et al., 1995, 1997). Similarly, 1,1-DCE was much more hepatotoxic to rats when given as an oral bolus than when infused for 2 hours into the stomach (Bruckner et al., 2010). Oral bolus doses of solvents can also cause damage of extrahepatic organs by exceeding the capacity of hepatic and pulmonary first-pass elimination, as well as protection and repair processes of hepatocytes.

Elimination

The rate of systemic elimination of different solvents varies considerably. The two major means of systemic elimination of VOCs are metabolism and exhalation. The rate and extent of metabolic clearance are dose- and compound-dependent. Exhalation is determined largely by the rate of pulmonary blood flow, the chemical's blood:air PC, and the alveolar ventilation rate. The more volatile, lipophilic VOCs are exhaled the most readily, because they have the lower blood:air PCs (Gargas et al., 1989). A good "case in point" is the comparison of toluene with acetone. The extent of CNS depression caused by each chemical is dependent on the concentration of the parent compound present in the brain (Bruckner and Peterson, 1981). As can be seen in Fig. 24-3, recovery of rats from toluene

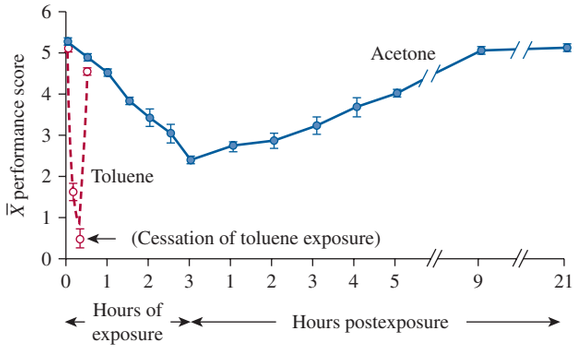

Figure 24-3. *Comparison of the induction of and recovery from toluene and acetone narcosis.* Rats inhaled 45 mg/L of toluene or acetone for 20 minutes or 3 hours, respectively. Animal performance/reflexes were monitored periodically as measures of the degree of CNS depression. (Reproduced from Bruckner and Peterson, 1981, with permission from Elsevier.)

anesthesia occurs within minutes of exposure cessation. This can be attributed to redistribution of toluene from the brain to body fat and other tissues, as well as to relatively rapid metabolism and exhalation. In contrast, recovery from acetone narcosis does not occur for at least 9 hours postexposure. As acetone is water soluble, limited amounts are deposited in the brain. It is instead distributed in the considerable volume of the blood and other body water. Clearance of acetone is slow due to its large volume of distribution and its relatively slow metabolism and exhalation. Acetone's water solubility is responsible for its relatively high blood:air PC and retention in the blood. Thus, acetone is available for diffusion into the brain and induction of a modest degree of CNS depression for a prolonged time.

Body fat plays an important role in the elimination of lipophilic solvents. Blood levels of such solvents drop very rapidly during the initial elimination phase following cessation of exposure. This so-called redistribution phase is characterized by rapid diffusion of solvents from the blood into tissues. Equilibration of blood and adipose tissue is prolonged due to the small fraction of cardiac output (about 3%) supplying fat depots. Body fat increases the volume of distribution and total body burden of lipophilic solvents. Deequilibration from adipose tissue during the terminal elimination phase is prolonged, due to low blood flow and high fat:blood PCs. Sato et al. (1975) demonstrated slower elimination of benzene from female than from male rats and humans, due to the females' higher body fat content. Rats with excess body fat experienced leukopenia during chronic benzene exposure, whereas their thinner counterparts did not.

Metabolism

Biotransformation plays a key role in modulating the toxicities of many solvents. Organic solvents are poorly soluble in water. Certain cellular enzymes can convert them to relatively water-soluble derivatives, which may be more readily eliminated in the largely aqueous urine and/or bile. Conversion of a bioactive parent compound to a less bioactive or inactive metabolite(s) that (is/are) efficiently eliminated is termed *metabolic inactivation*, or *detoxification*. Toluene, for example, accumulates in neuronal membranes and inhibits their functions. Toluene is metabolized to hydroxyl and carboxyl metabolites, which are too polar to accumulate or remain

in substantial quantities in neuronal membranes. Thus, metabolism serves to detoxify and accelerate the elimination of toluene. Metabolism of other solvents can produce reactive metabolites that are cytotoxic and/or mutagenic. This phenomenon is known as *metabolic activation*, or *bioactivation*. Benzene, for example, is oxidized to a variety of epoxides, quinones, and semiquinones that can produce hematopoietic toxicities and leukemia (Snyder, 2004). Benzene and many other VOCs are converted via multiple metabolic pathways to products of varying toxicity. Some of these competing pathways are considered bioactivation, others detoxification pathways. A variety of factors can influence the prominence of the different pathways and hence alter toxicity outcomes.

The initial step in biotransformation of relatively lipophilic solvents is catalyzed primarily by microsomal cytochrome P450s (CYPs). They are a "superfamily" of hemoproteins that act as terminal oxidases of the mixed-function oxidase (MFO) system. Their mode of action is described in Chap. 6. CYPs can catalyze a number of oxidative reactions, as well as certain reductive reactions (Omiecinski et al., 1999). Some 40 different human CYP isoforms have been identified to date, but six (CYPs 3A4, 2D6, 2C9, 2C19, 2E1, and 1A2) account for the majority of xenobiotic metabolism (Guengerich, 2006, Zanger and Schwab, 2013). CYPs are generally thought to have broad, overlapping substrate specificities, as demonstrated by Kobayashi et al. (2002) in rats. Xenobiotic metabolism under physiological conditions may favor one or two isoforms as the primary catalysts for a given chemical. Different isoforms can predominate at different concentrations of a chemical.

The outcome of exposure to a potentially toxic solvent can depend on the relative abundance of CYP isoforms. Expression of CYPs can differ considerably as a result of genetic polymorphisms and exposure to chemicals that induce or inhibit specific isoforms. A number of factors have been found to be sources of individual variability in CYP induction (Tang et al., 2005). Inheritable gene alterations, such as base changes and deletions, can result not only in functionally deficient enzymes, but also in the absence of certain P450s (Ma et al., 2004). As CYPs have different modes of regulatory control, their expression in response to various inducers and inhibitors varies. It should also be recognized that frequently there are interspecies differences in the presence, regulation, and catalytic activities of CYPs (Lewis et al., 1998; Guengerich, 2006).

There has been a focus on identification of CYPs that participate in the metabolism of individual solvents. Nakajima et al. (1992a) found that low concentrations of TCE in rat liver were metabolized primarily by CYP2E1, a constitutive high-affinity, low-capacity isoform. CYP2B1/2, a low-affinity, high-capacity form predominated under high-dose (substrate) conditions. CYP2E1 was observed to be a major contributor to the metabolism of TCE and benzene, but not toluene. CYP1A1 catalyzed the formation of *o*-cresol, but not benzyl alcohol from toluene. CYP1A1 oxidized TCE in mice but not in rats. Thus, CYP isoforms exhibit species selectivity, substrate selectivity, and regioselectivity for solvents (Nakajima, 1997).

CYP2E1 is a major catalyst of the oxidation of a variety of low-molecular-weight xenobiotics, including many solvents. CYP2E1 is found predominantly in liver, with lower levels present in kidneys, lungs, brain, testes, and other extrahepatic tissues of rodents and humans. Guengerich et al. (1991) reported that human CYP2E1 is primarily responsible for oxidation of some 16 halogenated and aromatic hydrocarbons, including benzene, styrene, $CHCl_3$, TCE, and vinyl chloride. These compounds are oxidized to electrophilic metabolites, capable of causing cytotoxicity and/or mutagenicity (Raucy et al., 1993). The enzyme is also responsible for reduction of CCl_4 to free radicals (see the discussion on CCl_4 in this chapter). CYP2E1 activity is associated with the pathogenesis of alcoholic cirrhosis, through formation of highly reactive oxygen radicals and acetaldehyde from ethanol (Gonzalez, 2005; Lu and Cederbaum, 2008). CYP2E1 is inducible by ethanol, acetone, pyridazine, chlorzoxazone, isoniazid, and other of its substrates. Activity of the enzyme varies from species to species and from human to human (Snawder and Lipscomb, 2000). Overnight fasting is widely recognized to induce CYP2E1, due in part to release of acetone during lipolysis (Bruckner et al., 2002). Starving rats, however, apparently must engage in coprophagy for induction to occur (Chung et al., 2001). Such differences in CYP2E1 activity/levels may play a major role in susceptibility to the toxicity and carcinogenicity of ethanol and a number of other solvents.

A variety of environmental factors can predispose people to harmful effects of solvents by altering P450s (Lof and Johanson, 1998). Ethanol is an effective CYP2E1 inducer when ingested repeatedly in substantial amounts (see the sub section "Exogenous Factors"). The person who drinks in such a manner may be subject to potentiation of solvent toxicity, due to increased solvent metabolic activation (Manno et al., 1996). Conversely, ethanol consumed at about the same time as a solvent exposure can be protective by competitively inhibiting metabolic activation of the solvent. Thus, the timing of exposures is important. Folland et al. (1976) reported severe CCl_4-induced hepatorenal injury in workers at an isopropyl alcohol bottling plant. These subjects' preexposure to the alcohol, which is metabolized to acetone, had markedly induced CYP2E1. Their induced condition resulted in a substantial increase in the metabolic activation and cytotoxicity of CCl_4, upon inhalation of normally nontoxic concentrations of the halocarbon.

The metabolic basis of solvent interactions receives considerable attention. The consequence of CYP2E1 induction by one solvent or drug depends on the nature of the second solvent. Kaneko et al. (1994) reported that ethanol pretreatment of rats has a more profound effect on the TK of TRI (poorly metabolized) than TCE (well metabolized). Similarly, ethanol pretreatment of rats results in a greater increase in metabolism and hepatotoxicity of CCl_4 (relatively poorly metabolized) than $CHCl_3$ (well metabolized) (Wang et al., 1997). As capacity-limited (i.e., metabolic capacity of the liver) metabolism prevails for poorly metabolized solvents, irrespective of dose, induction of their metabolism could be toxicologically significant in high (occupational) or low (environmental) exposure settings. In contrast, alterations of the bioactivation of low doses of well-metabolized solvents should be of little consequence, as their biotransformation is perfusion (i.e., hepatic blood flow) limited. Lipscomb et al. (2003), for example, utilized a PBTK model to calculate that a sixfold interindividual difference in TCE metabolism resulted in less than 2% change in the total amount of TCE oxidized by persons inhaling only 50 ppm for 8 hours or drinking 2 L of water containing 5 ppb TCE. Under such circumstances, interindividual metabolic differences should have little influence on cancer risks posed by trace levels of such solvents.

Physiological Modeling

Physiologically based toxicokinetic (PBTK) models have been developed to provide predictions of chemical concentrations in blood and tissues as a function of time (Dixit et al., 2003). PBTK models are thus used to relate the administered, or external dose to the blood and/or tissue (internal) dose of parent compound and/or bioactive moiety. If there is sufficient knowledge of the physiology of the test animal/tissue and interactions of the bioactive moiety with cellular components and ensuing responses, physiologically based toxicodynamic (PBTD) models can be developed (Conolly,

2002). Relatively few PBTD models have been published to date for solvents. Tan et al. (2003) linked an existing PBTK model for $CHCl_3$ with data for hepatocellular death and regenerative hyperplasia to predict liver tumor response in mice. Luke et al. (2010) expanded upon the work of Tan et al. (2003) by developing biologically based dose–response (BBDR) models for $CHCl_3$ and CCl_4, based upon key events of cytotoxicity and proliferation, to forecast liver tumor incidence in female B6C3F1 mice. The BBDR models consisted of a chemical-specific PBPK model for each chemical linked to a PBPD model of cytotoxicity and cellular proliferation, linked in turn to a clonal growth model to predict tumor incidence. Tan et al. (2011) have utilized models to evaluate the likelihood of TK and TD interactions of different chemical mixtures.

Development of PBTK models for anesthetic gases was initially undertaken in the 1920s and 1930s. Modeling was used in the design of experiments and dosage regimens for drugs, but the practice was not extended to industrial chemicals until the 1970s and 1980s. A "benchmark" paper by Ramsey and Andersen (1984) described the development of a PBTK model for styrene. By 1995, PBTK models had been developed for about 50 chemical environmental contaminants (Leung and Paustenbach, 1995). Some 28 of these 50 chemicals were solvents. Andersen (2003) more recently identified some 700 papers that dealt with PBTK models and/or their applications to toxicology and risk assessment. Thus, physiological models are being developed and used with increasing frequency to incorporate dosimetry and mechanistic data into non-cancer and cancer risk assessments (Krishnan and Johanson, 2005; Thompson et al., 2008), as well as pediatric drug development (Barrett et al., 2012).

Physiological modeling accounts for species- and dose-dependent shifts from linear to nonlinear kinetics, which impact tissue doses of bioactive moiety or moieties. The models are well suited for use with species-to-species extrapolations, because human physiological and metabolic parameter values can be inputted into animal models, and simulations of target tissue doses and effects in humans generated. Thus, solvent exposures necessary to produce the same target organ dose in humans, as those found experimentally to cause unacceptable cancer or noncancer risks in test animals, can be determined for some chemicals with reasonable certainty. In cases where there may be species differences in tissue sensitivity, PBTD models must be used to forecast toxicologically effective target organ doses.

The influence of interindividual variability in model parameters has received considerable attention (Price et al., 2003; Dorne, 2004). Jonsson and Johanson have performed model fitting in a Bayesian framework using Markov chain Monte Carlo simulation to reduce parameter uncertainty for methylene chloride (MC) (Jonsson and Johanson, 2001) and styrene (Jonsson and Johanson, 2002). A Bayesian analysis of a PBPK model for TCE yielded improved agreement of simulated and empirical TK data (Hack et al., 2006). Covington et al. (2007) utilized this methodology to estimate that only about 2% of low inhaled doses of PERC is metabolized by humans to TCA. Qui et al. (2010) subsequently reported a similar finding when they used the approach in an expanded and more detailed assessment of PERC kinetics and TCA production. The percentage is far lower than that has been assumed by regulatory agencies in the absence of data. Hierarchical analyses are proving quite useful in characterization of model parameter uncertainty and variability, and thereby in providing improved predictions of internal dosimetry for risk assessments.

PBTK modeling is finding increasing use in estimating exposure levels that result in concentrations of solvents measured in blood, exhaled breath, or urine (i.e., biomarkers) in humans. Different human populations may have highly variable exposures, activity, physiology, and kinetics. A procedure termed *reverse dosimetry* has been employed to reconstruct population exposures from limited biomarker data (Clewell et al., 2008). Reverse dosimetry, integrating PBPK modeling with exposure pattern characterization, Monte Carlo analysis, and other statistical tools, has been successfully employed to estimate distributions of exposures consistent with concentrations of TCE (Sohn et al., 2004), $CHCl_3$ (Tan et al., 2007; Lyons et al., 2008), and other trihalomethanes (Tan et al., 2007) measured in human blood or exhaled breath. These exposure distributions can be compared to occupational and environmental exposure guidelines, in order to evaluate potential health risks in different situations.

POTENTIALLY SENSITIVE SUBPOPULATIONS

There is considerable variability in responses of humans to solvents and other xenobiotics. Although information on interindividual differences in the kinetics and toxicity of solvents is usually limited, some significant differences are reported and should be taken into account in risk assessments (Grassman et al., 1998; Hattis et al., 1999). Children within specific age groups appear to be even more variable than adults (Hattis et al., 2003). Current EPA cancer and noncancer risk assessment practices are generally considered to be quite protective of potentially vulnerable subgroups, due to the use of multiple uncertainty factors and conservative default assumptions.

Endogenous Factors

Children Currently, there is considerable interest in regulatory and scientific issues related to protection of children's health (Hines et al., 2010; Landrigan and Goldman, 2011; Felter et al., 2015). The potential sensitivity of infants and children to pesticides and other chemicals was brought to the public's attention by a National Academy of Sciences' report (NAS, 1993). Ensuing Presidential Executive Order 13045 in 1997, directed all federal agencies to make protection of children a high priority and to consider risks to children when making policies and setting standards. The resulting Food Quality and Protection Act of 1996 dictated that an additional 10-fold safety/uncertainty factor be used in pesticide risk assessments when pediatric toxicity and TK data are unavailable. This is almost always the case for insecticides, solvents, and other chemicals. Application of the 10-fold children's safety factor to non-pesticides has been considered. The Pediatric Research Equity Act of 2003 requires pharmaceutical companies seeking FDA's approval of new drugs to assess a compound's safety and efficacy at different doses in pediatric patients or surrogates.

Very little information is available on the toxic potential of solvents and most other chemicals in infants and children. There are several well-known examples of compounds (e.g., lead, thalidomide, chloromycetin, and diazepam) to which fetuses or neonates are particularly susceptible. Results of experiments with chlorpyrifos in immature rats raised enough concern about possible neurodevelopmental disorders in children that use of a number of organophosphates was severely restricted, and most have been removed from the home-use market (Colborn, 2006). Compilations of maximally tolerated doses (MTDs) of chemotherapeutic drugs are among our most definitive data on age-dependent toxicity in humans. Clinical trials of diverse groups of antineoplastic drugs have revealed that their toxic effects are often similar qualitatively, and usually differ quantitatively by a factor of less than or equal to 2 (NAS, 1993). MTDs for anticancer drugs in children are

frequently higher than those for adults (Bruckner, 2000). Ginsberg et al. (2002) assembled a database with which TK parameters for adults and children can be compared for 45 drugs. These developmental metabolic profiles are relevant to some solvents and other environmental chemicals.

Increasing numbers of toxicology studies are being carried out in immature laboratory animals. Compilations of LD_{50} values and other data reveal that neonatal rodents are usually more sensitive to chemicals than adults (Hasegawa et al., 2007). Variances are usually no more than two- to threefold, but can be as much as 10-fold (Goldenthal, 1971). The younger and more immature a subject, the more different its response from that of the adult. The newborn mouse and rat more nearly resemble the human fetus during the third trimester than during the human neonate's initial weeks and months of life. Thus, it is not surprising that newborn rodents would be much more sensitive to many toxicants than adult rodents or human newborns. Growth and maturation are, of course, much more rapid in rodents. A few days growth of mice and rats can result in substantial changes in metabolism, disposition, and toxicity (Ginsberg et al., 2004). It is generally accepted that there are *windows of vulnerability*, or periods during the development of the nervous, audiovisual, immune, reproductive, endocrine, and other organ systems when the intricate, fragile maturational processes may be particularly sensitive to xenobiotics (Scheuplein et al., 2002; Makri et al., 2004). These windows occur at different ages and last for different periods of time in different species. This makes the choice of an appropriate animal model for children complex and cross-species comparisons more difficult than usual with adult risk assessments (Ginsberg et al., 2004; Morford et al., 2004).

Despite a paucity of data, some logical assumptions can be made about age-dependent changes in the TK and toxicity of solvents. GI absorption of solvents would not be expected to vary with age, as most solvents are absorbed rapidly and completely by passive diffusion from the entire GI tract. Systemic absorption of inhaled solvents may be greater in infants and children. Their cardiac output and respiratory rates are relatively high, although their alveolar surface area is lower than that of adults (Sarangapani et al., 2003). Reduced plasma protein binding in neonates and infants may be of consequence for certain solvent metabolites. Low-binding capacity for TCA, for example, could result in more of the mouse hepatocarcinogen being available for hepatic uptake, as well as for renal clearance. Extracellular water, expressed as percent of body weight, is highest in newborns and diminishes through childhood. The larger volume of distribution for water-soluble solvents results in their slower clearance and longer duration of action. Body fat is high from about 6 months to 3 years of age, and then steadily decreases until adolescence, when it increases again in females. As lipophilic solvents accumulate in adipose tissue, more body fat would result in higher body burdens, slower clearance, and longer duration of action.

Changes in xenobiotic biotransformation during maturation may have the greatest impact on susceptibility to chemical toxicity and carcinogenicity. Poisonings of premature and term human newborns by benzyl alcohol, hexachlorophene, diazepam, chloramphenicol, and certain other compounds are primarily attributable to deficits in hepatic phase II metabolic (i.e., conjugative) capacity. Maturational studies of human liver reveal that CYP isoforms are expressed asynchronously (Hines, 2008; 2013). There have been comprehensive efforts to characterize the ontogeny of human hepatic phase I enzymes from gestation through adolescence. Levels of CYP2E1, the primary catalyst of oxidation of a variety of solvents, are very low in fetal liver, increase steadily for the first year, and remain relatively constant through adolescence (Johnsrud et al., 2003). Blanco et al. (2000) failed to find an age-related difference in the maximal in vitro activity of CYP2E1 or other CYP isoforms in liver microsomes from donors from infancy to 93 years old. More rapid systemic clearance of many compounds by young children than by adults (Ginsberg et al., 2002) is generally believed to result from the child's liver's larger volume and more rapid perfusion rate (Murry et al., 1995; Sarangapani et al., 2003). Glomerular filtration and renal tubular secretion are quite low at birth, but increase substantially during the first 6 months to 1 year (Scheuplein et al., 2002). The half-life of creatinine, a substance eliminated entirely by glomerular filtration, is about half the adult value at 1 year. The net result of multiple age-related TK changes, some with offsetting effects, is difficult to predict.

A number of authorities have advocated the use of PBTK/TD models to collectively account for the unique and changing characteristics of children (Clewell et al., 2004a; Daston et al., 2004). A few PBTK models have been published for predicting the internal dosimetry of chemicals in immature rats. Timchalk et al. (2007) developed a BBDR model for chlorpyrifos (CPF), a widely used organophosphate insecticide. More pronounced depression of erythrocyte and brain acetylcholinesterase (AChE) activities, apparently resulting from higher CPF-oxon blood and brain levels, were forecast in preweanling than in adult rats. These age-dependent differences in AChE depression were not manifest at low CPF doses (Marty et al., 2012). A human PBTK model for the chemical did not predict differences between 3-year-olds and adults in inhibition of brain or erythrocyte AChE activity (Hinderliter et al., 2011).

PBTK modeling has been used to forecast the TK of six inhaled VOCs (TCE, PERC, benzene, $CHCl_3$, methylene chloride [MC], and methyl ethyl ketone) in 10-day-old and adult rats (Rodriguez et al., 2007). VOC blood levels were forecast to be up to 3.8-fold higher in the preweanlings, due primarily to their lower metabolism and faster respiration. Many of the input parameters were estimated or scaled, however, rather than measured. Empirical data were not provided to assess the accuracy of the simulations. Tornero-Velez et al. (2010) developed a PBTK model that clarified why preweanling rats are more susceptible than adults to the acute toxicity of high doses of deltamethrin (DLM), a pyrethroid insecticide. These researchers were among the first to rely primarily on age-specific input parameters and to demonstrate concordance between measured and simulated time-courses of a bioactive moiety (i.e., the neurotoxic parent compound). Blood and brain levels of DLM were inversely related to age in 10-, 21-, 40-, and 90-day-old rats. Inefficient metabolic detoxification was shown to be a major contributor to preweanling rats' susceptibility to DLM (Anand et al., 2006a), though immaturities in other kinetic processes may also be factors (Mortuza etl al., 2018).

Effort is being devoted to development of PBTK models for VOCs in human infants and children. Clewell et al. (2004a) constructed what they termed a life-stage model to simulate blood levels of four solvents (isopropanol, vinyl chloride, MC, and PERC) and their primary metabolites in different life stages. This was intended to be a preliminary model, in view of the lack of validated models for the younger groups and the required number of simplifying assumptions in the face of uncertainties about many age-specific parameters. In general, dose metrics for the chemicals across life stages were within a factor of 2, except during the early postnatal period when the largest TK differences occurred. Gentry et al. (2003) took a similar approach to forecast internal dosimetry of vinyl chloride, isopropanol, MC, and PERC in human offspring during pregnancy and lactation. In general, blood concentrations of the VOCs were lower in neonates during lactation than in the fetus during gestation. Fetal/neonatal VOC exposures

were generally several orders of magnitude lower than maternal exposures. Sarangapani et al. (2003) developed a PBTK model that integrated key age-specific respiratory tract parameters, such that the disposition of four VOCs (styrene, vinyl chloride, isopropanol, and PERC) could be forecast in children and adults. Differences in blood levels of parent compounds in infants and adults were comparable or varied less than twofold during the first year of life. Nong et al. (2006) incorporated measured age-specific liver volumes and CYP2E1 content into a PBPK model for toluene to predict internal doses (blood levels) of the parent compound in adults and in children of different age groups. CYP2E1 content was the key model input parameter for neonates, whereas liver blood-flow rate was predominant after a few months of age. Combined interindividual and interage variability in the area under blood level versus time curves (AUCs) was within a factor of 2, except for the neonates, which had somewhat higher AUCs.

The aforementioned findings suggest that differences in dosimetry of parent solvents across life stages are usually well within the 3.3 TK component of the classical 10-fold interspecies uncertainty factor. Relative little work has been done, however, to model solvent metabolite dosimetry in different age groups. Potential PD differences should also be taken into account in children's risk assessments.

Elderly Age-related changes in effects of xenobiotics have primarily been of interest in the field of drug therapy (Bressler and Bahl, 2003; McLean and LeCouteur, 2004; Reeve et al., 2015). More attention is now being paid to exposures and vulnerabilities of the elderly to other chemicals (Geller and Zenick, 2005). The EPA encourages evaluation of toxicity across the entire lifespan, with a focus on lifestages with their own unique sensitivity (Brown et al., 2008). Data are sorely lacking, however, on the relative susceptibility of geriatric populations to solvents and many other industrial chemicals. The aging CNS undergoes changes (e.g., neuronal loss, altered neurotransmitter and receptor levels, and reduced adaptability to effects of toxicants) (Ginsberg et al., 2005), that may predispose to more pronounced neurologic effects by solvents. Older people do appear to be more sensitive to the CNS-depressant effects of ethanol, due in part to lower gastric alcohol dehydrogenase (ADH) activity (Seitz et al., 1993). Kiesswetter et al. (1997) observed the most pronounced neurobehavioral effects in older workers with the highest single or mixed organic solvent exposures. Memory, attention, visual perception, and motor skills diminish with aging, even in the absence of chemical exposure. Thus, these findings could be interpreted as merely additive (i.e., aging + solvents effects), or as exacerbation during a vulnerable life stage. Paradoxically, a subsequent investigation by Kiesswetter et al. (2000), a study of toluene-exposed rotogravure printers, revealed fewer symptoms and better psychometric performance in older workers with higher exposure levels. A limited number of studies of this phenomenon by other investigators are no more definitive.

Investigations of adverse effects of solvents on old animals have been quite limited. Attention has been primarily focused on age-related susceptibility of rodents to liver damage. Susceptibility varies dramatically from one chemical to another. Rikans and Hornbrook (1997) reviewed studies showing increased susceptibility of geriatric F344 rats to allyl alcohol, but there was no apparent age dependency with bromobenzene. Results varied with species, strain, and gender. CCl_4 hepatotoxicity was less severe in 28-month-old female F344 rats than in young adults. Wauthier et al. (2004) saw no changes in CYP2E1 protein or mRNA in 18-month-old male Wistar rats, but did observe decreased CYP2E1 activity. The investigators attributed this phenomenon to oxidative stress on the isoform. More severe CCl_4-induced DNA damage in hepatocytes from older mice was shown to be due to preexisting oxidative DNA damage and a higher incidence of DNA strand breaks in the older animals (Lopez-Diazguerrero et al., 2005). Thus, it appears normal aging processes may be accentuated by chemical stressors.

There are few pertinent geriatric animal or human toxicology studies, but age-specific factors that govern the TK of solvents have received considerable attention. Clewell et al. (2002), Sarangapani et al. (2003), and Thompson et al. (2009) compiled comprehensive reviews and evaluations of age-specific TK parameters, though data for many age groups are inadequate. Splanchnic blood flow decreases with age more rapidly than cardiac output. This is unlikely to significantly affect the GI absorption of solvents, as they are readily absorbed by passive diffusion. Dermal absorption could be influenced by loss of integrity of the stratum corneum, reduction in skin surface lipids and atrophy of the dermal capillary network (Roskos et al., 1989). Total lung capacity does not change with age, but vital capacity (mobile volume) diminishes progressively due to loss of lung elasticity and strength of the respiratory muscles. Residual capacity (fixed volume) increases with progressive narrowing and closure of small airways (Ritschel, 1988). Other alterations that may contribute to lower pulmonary absorption include decreases in alveolar surface area, membrane permeability and capillary blood volume, and thicker alveolar cell membranes (Clewell et al., 2002). Nevertheless, inhalation PBTK model predictions of steady-state blood concentrations of isopropanol, styrene, vinyl chloride, and PERC differed very little among 10-, 15-, 25-, 50-, and 75-year-old humans (Sarangapani et al., 2003).

Age influences the distribution of xenobiotics in the body, as well as their metabolism and elimination (Herrlinger and Klotz, 2001; Mangoni and Jackson, 2003). With aging, body fat content usually increases substantially at the expense of the lean mass (i.e., skeletal muscle) and body water. Thus, polar solvents tend to reach higher blood levels during exposures. Relatively lipid-soluble solvents accumulate in adipose tissue and are released slowly to sites of action, metabolism, and elimination. Ginsberg et al. (2005) assembled clearance and half-life data for 46 drugs in 4500 subjects. Clearance was typically slower and half-life longer after the age of 60 years, particularly in those 80 to 84 years old. Cardiac output diminishes 30% to 40% between the ages of 25 and 65 years, as do renal and hepatic blood flows (McLean and Le Couteur, 2004). This research group and Clewell et al. (2002) concluded that reduced hepatic clearance of flow- and capacity-limited xenobiotics in the elderly was due primarily to reductions in liver blood flow and liver size. McLean and Le Couteur (2004) presented evidence that aging of the sinusoidal endothelium reduced oxygen delivery and drug transfer from blood to hepatocytes. Schmucker (2005) and Klotz (2009) did not find significant relationships between aging and CYP activities in human liver. Cotreau et al. (2005) did describe some reports of age-related decreases in CYP3A4, an isoform important in metabolism of a number of drugs in humans, but of limited relevance to solvent metabolism.

Recent findings indicate that toxic compounds, both xenobiotics and certain products of intermediary metabolism, are major contributors to macromolecular damage and dysregulation involved in aging (Zimniak, 2008). Important endogenous toxicants include reactive carbonyl compounds and electrophiles formed by peroxidation of polyunsaturated lipids. Enzymes capable of reducing carbonyl groups and glutathione *S*-transferases (GSTs) are key protection mechanisms. Lee et al. (2008) characterized age-related changed in the expression of a substantial number of xenobiotic metabolizing enzymes in the liver of aged male F344 and Brown Norway rats. GST expression diminished with age. Most data,

however, indicate that phase II (conjugation) pathways in humans are not altered by aging (Mitchell et al., 2011). Maurya and Rizvi (2010) reported an increase in plasma GST activity and antioxidant potential with age in a study of 80 healthy male and female human subjects 18 to 85 years old. It was hypothesized that this was in response to increased production of reactive oxygen moieties with advancing age.

Information is available on certain age-related aspects of xenobiotic clearance other than metabolism. Plasma protein binding of drugs generally remains unaltered or diminishes modestly with age (Grandison and Boudinot, 2000). Hepatic and/or renal dysfunction more often account for altered protein binding. Little information apparently exists on effects of aging on most transporters (Kinirons and O'Mahony, 2004), though Lee et al. (2008) did see diminished expression of efflux transporters in the livers of aging male rats. Renal clearance diminishes with advancing age, due to parallel annual decreases of about 0.65% in glomerular filtration and maximal tubular secretory capacity beyond the age of 30 years. The foregoing TK factors may significantly influence the internal dosimetry of polar solvent metabolites, and in turn certain responses of geriatric populations.

PBTK models can be useful for integrating age-related physiological and metabolic changes into a quantitative framework to forecast their net effect on blood and tissue time courses of parent compounds and metabolites (Schlender et al., 2016). Very few such "geriatric" models, however, have been published. McMahon et al. (1994) employed a PBTK model to demonstrate that reduced urinary elimination of benzene metabolites by geriatric mice was due to decreased renal blood flow rather than altered formation of metabolites. Corley et al. (2005c) incorporated a number of age-, gender-, and species-specific physiological and biochemical parameters into a preexisting PBTK model for 2-butoxyethanol (BE), in order to predict the kinetics of BE and butoxyacetic acid, BE's major metabolite, in male and female mice and rats of different ages. The life-stage PBTK model of Clewell and colleagues was used to simulate human blood concentrations of several VOCs and their major metabolite (or metabolic rate) from infancy through the age of 75 years (Sarangapani et al., 2003). Blood levels of the parent compounds remained relatively constant during adulthood, but metabolite levels or amounts metabolized frequently varied during the pediatric and geriatric years. In a subsequent publication, blood levels of PERC and its major metabolite, TCA, were predicted to progressively rise during adulthood and old age by a PBTK model. Clewell et al. (2004b) attributed this phenomenon to relatively low pulmonary and metabolic clearance, coupled with accumulation of the lipophilic solvent in ample amounts of adipose tissue. Much work remains to be done to refine these generic PBTK models and to integrate them with significant geriatric PD changes (Burton et al., 2005).

Gender Some of the physiological and biochemical differences between men and women have the potential to alter tissue dosimetry and health effects of certain chemicals (Arbuckle, 2006; Vahter et al., 2007). Clewell et al. (2002) have provided one of the most comprehensive reviews of the potential impact of gender differences on solvents' TK. There was no convincing evidence of sex differences in GI absorption rate constants. The investigators located few data on gender-dependent dermal or pulmonary absorption of solvents or other chemicals. Sarangapani et al. (2003) published tables of a number of factors (e.g., cardiac output, pulmonary ventilation rate, and pulmonary surface area) that dictate pulmonary absorption of VOCs. These rates and values were generally higher for males, but a PBPK model's predictions of steady-state blood concentrations for inhaled isopropanol, styrene, vinyl chloride, and PERC were largely sex-independent (Sarangapani et al., 2003).

Distribution of water- and lipid-soluble solvents can vary substantially between men and women (Schwartz, 2003). Most men have more lean body mass and larger body size. Women typically have smaller volumes of distribution for polar solvents, but larger volumes of distribution for lipophilic solvents (Gandhi et al., 2004). Lean body mass decreases from 76% to 52%, and body fat increases from 33% to 49% in females between the ages of 25 years and 65 to 70 years. Nomiyama and Nomiyama (1974) found that women retain less inhaled acetone and ethyl acetate than similarly exposed men. Levels of toluene and TCE in expired air of females were lower, reflecting greater fat deposition and retention of these lipophilic chemicals. Similarly, women exhibited significantly higher respiratory uptake of 1,3-butadiene, another lipophilic VOC (Lin et al., 2001b). Clewell et al. (2004b) utilized their life-stage PBTK model to predict blood levels of PERC and TCA, its major metabolite, in men and women over a lifetime of daily ingestion of 1 μg PERC/kg. The women were predicted to attain significantly higher blood PERC and TCA concentrations, ostensibly due to PERC's greater storage in fat and its relatively low pulmonary and metabolic clearance.

The major sex differences in P450-mediated hepatic metabolism in rats are not seen in humans or most other mammals. Rinn et al. (2004) did see significant differences in gene expression in the liver, kidney, and reproductive tissues of male and female mice. Some of the differentially expressed genes involve drug and steroid metabolism, but the biological significance of these variances is unknown. No marked gender differences in P450-catalyzed oxidation reactions have been identified in humans (Mugford and Kedderis, 1998). Other investigators have described modestly higher levels and activities of certain liver P450s in males, including CYP1A2, CYP2D6, and CYP2E1 (Schwartz, 2003). Many CYP3A substrates do not exhibit sex-dependent differences in clearance, though some researchers report differences for certain substrates (Cotreau et al., 2005). Gender-specific metabolism data for most hydrocarbon solvents are lacking. Men exhibit somewhat lower blood ethanol levels than women upon ingestion of equal doses, due in part to more extensive ADH-catalyzed metabolism of the alcohol by the male gastric mucosa (Frezza et al., 1990). UDP-glucuronyl transferase activity appears to be somewhat higher in males. Women seem to have modestly lower glomerular filtration rates, though information on gender-dependent renal tubular secretion and reabsorption processes is lacking.

A gender-specific PBTK model was developed to assess the relative risks of benzene exposures (Brown et al., 1998). Women were found to have a higher blood:air partition coefficient, a higher percent of body fat and higher maximum rate of benzene metabolism than men. Women exhibited higher blood benzene levels and a 23% to 26% increase in benzene metabolism, potentially placing them at greater risk than men with equivalent exposures.

Relatively little is known about potential influences of contraceptives, hormone replacement therapy, or pregnancy on the metabolism and disposition of xenobiotics (Gleiter and Gundert-Remy, 1996). The menstrual cycle with its hormone changes may influence the metabolism of some xenobiotics (Fletcher et al., 1994). Watanabe et al. (1997) found that estradiol changes during the estrous cycle of rats affected both UDP-glucuronyl transferase and NADPH-cytochrome *c* reductase activities. Experiments by Nakajima et al. (1992b) demonstrated that pregnancy of rats resulted in significant reductions in hepatic CYP content, as well as the metabolism of both toluene and TCE. Pregnancy results in a 40% increase in tidal volume, which may increase the absorption of inhaled VOCs. Plasma volume increases about 50% in pregnant women, resulting in a decrease in albumin concentration and plasma protein binding of many drugs (Fletcher et al., 1994).

Cardiac output increases about 50%, due to increases in stroke volume and heart rate (Silvaggio and Mattison, 1994). Uterine blood flow, renal plasma flow, and glomerular filtration rise substantially, though no information on hepatic blood flow is apparently available. These TK factors need to be considered when assessing risks and setting occupational exposure limits for these subpopulations.

The effects of pregnancy on peroxisome-proliferator-activated receptors (PPARs) are being investigated. PPARs are transcription factors that belong to the nuclear hormone receptor superfamily. PPARs regulate genes involved in cell differentiation, development, and metabolism. The three identified and described PPAR isoforms are PPARα, PPARβ/δ, and PPARγ. Among the isoforms, PPARα has the greatest influence on cellular homeostasis and carcinogenicity. However, all three PPAR isoforms play essential roles in physiologic change and development in the fetoplacental unit. Abnormalities in PPAR-regulated pathways may be implicated in reproductive and gestational disease (Toth et al., 2007; Borel et al., 2008). Two TCE and PERC metabolites, TCA and DCA, can induce modest PPARα activation in humans. The combined effects of pregnancy and low-level PPAR activation, if any, are unknown.

Gentry et al. (2003) described an approach for use of PBTK modeling to compare maternal and fetal/neonatal blood and tissue dosimetry during pregnancy and lactation. In general, blood levels of isopropanol, vinyl chloride, MC, and PERC were predicted to be lower in the human neonate during lactation than in the fetus during gestation. Simulated vinyl chloride levels, for example, were four orders of magnitude lower in the neonates. Fetal/neonatal isopropanol levels were forecast to be orders of magnitude lower than maternal levels. Corley et al. (2003) reviewed PBTK/TD modeling of the developing embryo, fetus, and neonate.

Genetics A variety of genetic polymorphisms for biotransformation have been found to occur at different frequencies in different ethnic groups. Polymorphisms for xenobiotic-metabolizing enzymes may affect the quantity and quality of enzymes and the outcomes of exposures to solvents and other chemicals (Wormhoudt et al., 1999; Johansson and Ingelman-Sundberg, 2011). It is important to note that culturally linked environmental factors also contribute to ethnic differences in metabolism and disposition of solvents and other chemicals. It is often difficult to disentangle the influences of genetic traits from those of different lifestyles, socioeconomic status, and geographic settings.

Ethnic differences in regulating the expression of CYP isozymes and other enzymes are associated with some variations in xenobiotic metabolism and susceptibility to certain cancers (Raunio et al., 1995; Rodriguez-Antona and Ingelman-Sundberg, 2006). Shimada et al. (1994) found that Caucasians had higher total hepatic CYP levels than Japanese. Caucasians exhibited higher CYP2E1 activities, as reflected by aniline *p*-hydroxylation and 7-ethoxycoumarin *O*-deethylation. Individuals with two linked polymorphisms in the transcription regulatory region of the CYP2E1 gene exhibited greater expression of the isoform. Reported frequencies of these rare alleles were 2% in Caucasians, 2% to 5% in African Americans, and 24% to 27% in Japanese (Kato et al., 1992). Kawamoto et al. (1995) found that a CYP2E1 polymorphism in Japanese workers did not affect metabolism of toluene to benzoic acid, but that CYP1A1 and aldehyde dehydrogenase (ALDH2) polymorphisms did. ALDH2 deficiency can result in elevated CNS levels of toluene and benzaldehyde (Wilkins-Haug, 1997) in toluene-exposed individuals. About half of the Japanese population lacks ALDH2, due to a structural point mutation in the ALDH2 gene. Pronounced interethnic differences in rates of ethanol metabolism have been associated with a number of ADH and ALDH polymorphisms (Pastino et al., 2000). Results of investigations of relationships between occurrence of defective alleles for CYP2E1 and cancer incidence have been contradictory. Studies with more statistical power of selected ethnic groups are needed. Molecular markers clearly indicate variances in CYP genes, but biologically plausible mechanisms linking specific genotypes with specific outcomes need to be established.

Ethnic differences have been demonstrated for some phase II biotransformation reactions. Glutathione *S*-transferases (GSTs) are a family of enzymes, some of which promote the detoxification of electrophilic metabolites by catalyzing their conjugation with reduced glutathione (GSH). In contrast, GST theta-mediated conjugation with GSH is the first step in metabolic activation of methylene chloride (MC) to formaldehyde, a putative carcinogen (see MC subsection). Individuals with a null/null genotype for GST theta are thus at a lower risk of developing cancer from MC (El-Masri et al., 1999; Haber et al. 2002). The prevalence of this genotype ranges from 10% in Mexican Americans to 60% to 65% in Chinese and Koreans (Nelson et al., 1995). GST theta also catalyzes dehalogenation of DCA to glyoxylic acid. DCA, a minor product of TCE and PERC oxidation, is a hepatocarcinogen in high doses in mice and rats. DCA is cleared more slowly from the systemic circulation of persons with a GST theta polymorphism, possibly resulting in increased risk of liver cancer upon TCE or PERC exposure (Shroads et al., 2011). GSTs participate in conjugating relatively small percentages of doses of TCE and PERC, but these pathways can lead to production of cytotoxic, mutagenic metabolites in the kidney. Contrary to findings in a more limited study, Wiesenhutter et al. (2007) saw no association between deletion polymorphisms of GST-T1 and GST-M1 and incidence of renal cell cancer (RCC) in 134 RCC cases (20 with TCE exposure) and 401 matched controls. More recently Moore et al. (2010) studied gene variants in 1097 RCC cases and 1476 controls. A significant association was found between TCE exposure and RCC in individuals with at least one intact GST-T1 allele, but not in individuals with two deleted alleles.

Genetic polymorphisms, variable transporter activities, genetic variants of receptors and regulatory proteins, and environmental factors can play roles in individual variability in CYP induction (Tang et al., 2005). There are substantial differences in activity and/or content of CYP2E1 within the U.S. population. Lipscomb (2004) incorporated extremes in CYP2E1-mediated metabolism into a PBTK model for TCE and found there was very little impact on TCA dosimetry. Intersubject differences in hepatic blood flow did have a pronounced effect on TCE biotransformation, as TCE is an extensively metabolized (blood flow limited) compound. Wenker et al. (2001) similarly reported that interindividual genetic polymorphisms in CYP2E1 and several other CYPs did not affect the metabolism of styrene, another flow-limited compound. Kedderis (1997) utilized a PBTK-modeling approach to confirm this phenomenon, and found the opposite to be true for poorly metabolized (capacity limited) VOCs such as PERC and CCl_4.

Exogenous Factors

P450 Inducers Considerable effort has been devoted to investigation of effects of different enzyme inducers on xenobiotic metabolism and toxicity (Hukkanen, 2012). Preexposure to drugs and other compounds, which induce CYP isoforms that metabolically activate chemicals, can markedly potentiate the chemicals' toxicity/carcinogenicity (Ioannides and Lewis, 2004; Dekant, 2009). As described in the subsection "Metabolism," CYP2E1 is a high-affinity, low-capacity isoform responsible for activation of a wide variety of low-molecular-weight hydrocarbon and halocarbon solvents to cytotoxins and mutagens (Guengerich et al., 1991).

A number of CYP2E1 substrates (e.g., ethanol and other alcohols, acetone and other ketones, pyridine and pyridazine, isoniazid, acetaminophen, chlorzoxazone) can act as inducers of the isoform. The minimal hepatocytotoxicity caused by a low dose of CCl_4 alone (Panel B, Fig. 24-4) can be contrasted with the centrilobular necrosis (Panel C, Fig. 24-4) in rats pretreated with 2-butanol before the same CCl_4 dose (Traiger and Bruckner, 1976). CYP2E1 is present in many tissues, as are other CYPs that metabolize solvents. Although levels/activities are highest in liver, induction in extrahepatic tissues can result in increased metabolic activation in situ that may be toxicologically significant. It is worthy of note that many chemicals that induce CYPs also induce detoxifying enzymes (e.g., epoxide hydrolase, glucuronyl- and sulfotransferases). Thus, the net outcome of preexposure to an inducing agent on solvent toxicity should be addressed on a case by case basis. A number of naturally occurring organosulfur compounds in allium vegetables have been demonstrated to induce phase II enzymes in the liver, kidney, intestines, and lungs of Wistar rats (Guyonnet et al., 1999).

It should be recognized that timing of exposures to two or more CYP substrates is important. Sato et al. (1991) demonstrated that ethanol competitively inhibited the biotransformation of TCE when the exposures were concurrent. Alternatively, preexposure to the alcohol induced TCE metabolism. As described previously in the "Metabolism" subsection, increased metabolic capacity in an individual is of little consequence for environmentally encountered levels of blood flow-limited (i.e., extensively metabolized) solvents (e.g., TCE, vinyl chloride, MC, and $CHCl_3$). Thus, genetically predisposed or induced individuals should be at no greater cancer risk from oxidative metabolites of low doses of these solvents than the average person.

P450 Inhibitors An increasing number of drugs and other chemicals, dietary supplements, fruit juices, and vegetable constituents are being identified as inhibitors and/or inducers of CYPs, phase II enzymes, and efflux transporters (Lin and Lu, 2001; Huang and Lesko, 2004). It should be recalled that mechanisms of P450 inhibition can be categorized as reversible, quasi-reversible, and irreversible. Reversible inhibition is transient, with the normal function of the CYP continuing after elimination of the inhibitor. Both quasi- and irreversible inhibitions result from the enzyme's formation of a reactive metabolite. With quasi-reversible inhibition, the metabolite and enzyme form a complex that is so stable that the intact enzyme may or may not be available for further metabolism. With mechanism based on suicide inhibition, the reactive metabolite irreversibly inhibits the enzyme by binding to its active site. Experiments by Lilly et al. (1998) indicate that this is the mechanism by which epoxide metabolites of *trans*- and *cis*-1,2-dichloroethylene inhibit Cyp2e1. Fisher et al. (2004) subsequently reported that metabolites generated from a single oral dose of CCl_4 as low as 1 mg/kg are suicide inhibitors of Cyp2e1 and Cyp2b2 in B6C3F1 mice. The net result of this action by CCl_4 was reduced formation of TCA from coadministered PERC. Blobaum (2006) described reversible inactivation of Cyp2e and Cyp2b isoforms during their mechanism-based inactivation by small *tert*-butyl terminal alkynes.

Flavonoids in grapefruit juice were one of the first classes of naturally occurring compounds to be documented to produce clinically significant increases in levels of therapeutic agents metabolized by CYP3A4 (Chan et al., 1998). Flavonoids in red wine exerted a similar effect on intestinal CYP3A4, but white wine had little influence. Bergamottin, a furanocoumarin in grapefruit and lime juice, was subsequently found to inhibit human CYP3A4 activity (Bailey et al., 2003) as well as intestinal P-glycoprotein efflux transporters (Dresser and Bailey, 2003). St. John's wort, a

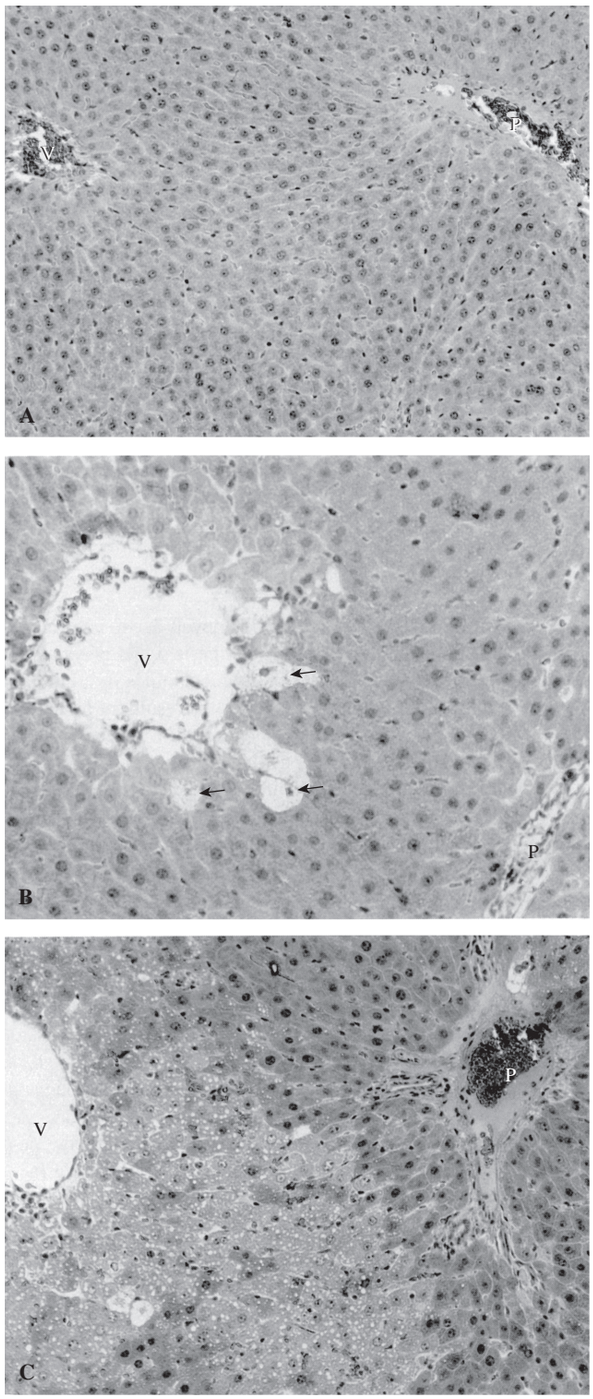

Figure 24-4. *Potentiation of CCl_4 hepatotoxicity by 2-butanol.* Rats were (A) untreated; (B) given 0.1 mL/kg CCl4/kg IP; or (C) pretreated with 2-butanol (2.2 mL/kg PO) 16 hours before 0.1 mL/kg CCl_4 IP. Central veins and portal triads are designated V and P, respectively. Occasional hepatocytes adjacent to the central vein are vacuolated in (B). Note the demarcation between vacuolated/necrotic centrilobular and midzonal cells, and normally appearing periportal cells in (C). Hematoxylin and eosin stain. (A) and (C) × 315; (B) × 480. (Reproduced from Traiger and Bruckner, 1976, with permission from ASPET.)

top-selling dietary supplement taken for depression, selectively induced CYP3A4 in the small intestine (Huang and Lesko, 2004). Echinacea inhibited human intestinal CYP3A, but induced hepatic CYP3A. Ginseno-sides exhibited no or weak inhibition of several human CYPs, but some of the compounds' major intestinal metabolites inhibited a wide range of CYPs (Liu et al., 2006). Certain components of ginko biloba, frequently used for memory impairment, were potent inhibitors of human recombinant CYP1B1, CYP1A1, and CYP1A2 (Chang et al., 2006). Identification of isoform-selective, nontoxic inhibitors may eventually lead to modulation of human xenobiotic metabolism for therapeutic purposes. There are large interindividual responses to CYP enzyme inducers and inhibitors (Lin and Lu, 2001).

Lifestyle Exercise can significantly affect the kinetics of xenobiotics, but is often not considered in occupational risk assessments of solvents. Exercise increases two of the major determinants of VOC uptake, alveolar ventilation rate and cardiac output/pulmonary blood flow. Polar solvents with relatively high blood:air PCs (e.g., acetone, ethanol, and ethylene glycol) are very rapidly absorbed into the pulmonary circulation. Alveolar ventilation is rate limiting for these chemicals. In contrast, pulmonary blood flow and metabolism are rate limiting for uptake of the more lipophilic solvents (Johanson and Filser, 1992). Heavy exercise can increase pulmonary uptake of relatively polar solvents as much as fivefold in human subjects (Astrand, 1983). Light exercise doubles uptake of relatively lipid-soluble solvents, but no further increases occur at higher workloads. Blood flow to the liver and kidneys diminishes with exercise, so biotransformation of well-metabolized solvents and urinary elimination of polar metabolites may be diminished (Lof and Johanson, 1998). Dankovic and Bailer (1994) modified the human PBTK model of Reitz et al. (1989) for methylene chloride (MC) to reflect light work conditions. The modified model predicted that light exercise would result in a twofold increase in hepatic MC deposition and in metabolite formation via CYP- and GSH-dependent pathways. Jonsson et al. (2001) developed a population PBTK model for humans inhaling MC during moderate-to-heavy exercise. Increased workload was predicted by PBTK modeling to produce an 87% increase in internal exposure to components of a mixture of toluene, ethylbenzene, and xylene (Dennison et al., 2005).

Dietary habits can influence the absorption, metabolism, and toxicity of solvents in several ways. Factors that influence gastric emptying would not be expected to influence systemic absorption of VOCs, as they are so rapidly absorbed by passive diffusion throughout the GI tract. The mere bulk of food in the GI lumen, however, can inhibit absorption by preventing contact of the chemical with the GI epithelium. VOCs in the GI tract partition into dietary lipids, largely remaining there until the lipids are emulsified and absorbed. This serves to substantially delay uptake of lipophilic solvents such as CCl_4, resulting in lower, but prolonged blood CCl_4 levels and diminished hepatotoxicity in rats (Kim et al., 1990a). Food intake increases splanchnic blood flow, which favors GI absorption. The elevation in liver blood flow would be expected to enhance the biotransformation of low doses of well-metabolized solvents, but to have relatively little effect on metabolism of relatively high (i.e., saturating) doses. Consumption of a high-fat diet results in a selective increase in hepatic CYP2E1 activity and protein levels in male Sprague–Dawley rats (Raucy et al., 1991). Elevation of plasma ketone bodies appears to be implicated in this process, because acetone is a potent CYP2E1 inducer. Carbohydrate deficiency has been recognized for many years to enhance the metabolism of a series of VOCs in rats, but not to alter their conjugation (Sato and Nakajima, 1985). Psoralens (found in parsley, parsnip, and celery) reduce CYP1A2 activity, whereas food cooked over charcoal enhances it. Effects of other foods, fruit juices, and food supplements are addressed in the preceding subsections on P450 inducers and inhibitors.

Fasting for 1 to 3 days can significantly alter the hepatotoxicity of medium to high doses of VOCs that undergo metabolic activation. Fasting results in decreased hepatic concentrations of reduced glutathione (GSH), due to the cessation of intake of amino acids required for its synthesis. GSH plays a key role in detoxifying electrophilic metabolites of a number of VOCs. Conversely, conjugation of GSH with TCE or PERC can lead to formation of limited quantities of cytotoxic, mutagenic metabolites (see subsections on TCE and PERC). Short-term fasting (about 8 to 24 hours) results in induction of liver Cyp2e1 and increase in metabolism of a number of aromatic hydrocarbons and halocarbons (Nakajima et al., 1982). The hepatotoxicity of CCl_4, which requires Cyp2e1-catalyzed metabolic activation, is more severe in fasted animals (Bruckner et al., 2002). In contrast, long-term food deprivation (i.e., starvation) results in decreased synthesis and activity of Cyps. In a more recent study, male Wistar rats were dietarily restricted for 1 day, after which hepatic Cyp2e1 content was significantly increased and GSH depleted compared to conventionally fed rats. AST and ALT were elevated after oral CCl_4 and $CHCl_3$ in dietarily restricted rats compared to those conventionally fed (Qin et al., 2007).

Many physiological and biochemical processes that impact solvent TK exhibit circadian, or diurnal rhythms. A series of investigations has been conducted to delineate molecular control mechanisms of diurnal oscillation in mammals. The phenomenon appears to be controlled by a "master pacemaker" in the suprachiasmatic nuclei (SCN) of the hypothalamus of mice (Teboul et al., 2009). Genetic and genomic analyses of the SCN and liver indicate that a relatively small number of output genes are directly regulated by core oscillator components. Rate-limiting steps in major cellular pathways/processes are being identified as key sites of circadian regulation. Experiments reveal that transcriptional feedback is required for maintenance of circadian rhythmicity (Sato et al., 2006).

Several research groups have discovered circadian rhythms in susceptibility of rats to liver damage by various VOCs, including CCl_4, $CHCl_3$, DCE, ethanol, and styrene. In each instance, the chemicals were most toxic when given during the initial part of the rodents' dark/active cycle. Low hepatic GSH levels at this time were an important contributing factor to the increased liver injury by DCE and $CHCl_3$. Restricted food intake during sleep resulted in lipolysis and formation of acetone, which contributed to the diurnal induction of Cyp2e1 and the ensuing increases in hepatic metabolic activation and cytotoxicity of CCl_4 in male rats during the early part of the animals' active cycle (Bruckner et al., 2002). Skrzypinska-Gawrysiak et al. (2000) also observed that male mice were most susceptible to CCl_4 hepatotoxicity when dosed at a similar time. Zhang et al. (2009) observed the highest expression of mRNAs of Cyp2e1 and a number of other Cyps in liver microsomes of male CD-1 mice during their active/dark cycle. In-phase expression of certain hepatic transcription factors suggested that the factors are important in coupling the clock with expression of drug metabolism genes. Clearance rates for a number of well-metabolized compounds, including ethanol (Sturtevant et al., 1978) and nicotine (Gries et al., 1996), are maximal in human subjects during the first part of their active/wake cycle. Hepatic blood flow and acetone levels in expired air of humans peak before breakfast. Thus, humans may also be more susceptible during this time to the toxicity of solvents that undergo metabolic activation.

Ethanol is an effective CYP2E1 inducer when ingested repeatedly in substantial amounts. There are numerous reports of marked potentiation of hepatic or renal damage by ethanol or other alcohols in persons occupationally exposed to potent hepatorenal toxicants, such as CCl_4 (Folland et al., 1976; Manno et al., 1996). CCl_4 is not extensively metabolized, but its free radical metabolites are extremely cytotoxic, as described in the subsection on the chemical. A group of moderate drinkers exposed to 1,1,1-trichlorethane (TRI) vapor at 175 ppm showed a significant increase in metabolism and metabolic clearance of the chemical (Johns et al., 2006). TRI is a relatively nontoxic, poorly metabolized solvent. Kaneko et al. (1994) exposed ethanol-pretreated rats by inhalation to TCE or TRI at 50 to 1000 ppm. TRI metabolism was enhanced at all vapor concentrations, but TCE metabolism was enhanced by ethanol only at the highest vapor concentration (1000 ppm), as its biotransformation is perfusion-limited (limited by hepatic blood flow). Low levels of TCE entering the liver are extensively metabolized, even in nondrinkers who still have substantial CYP2E1 activity. Kedderis (1997) predicted that a 10-fold increase in CYP2E1 activity in humans inhaling TCE at 10 ppm would result in only a 2% increase in TCE metabolism by the liver. Thus, increased bioactivation capacity due to ethanol or other factors should not increase risks of toxicity or cancer from low-level exposures to TCE, DCE, MC, vinyl chloride, benzene, or other extensively metabolized VOCs. In contrast, Kedderis (1997) predicted a 10-fold increase in CYP2E1 activity would result in a 3.8-fold increase in PERC metabolites in the liver of persons inhaling 10 ppm PERC. Increased bioactivation of less extensively metabolized VOCs could thus enhance risks of adverse effect of low-level exposure to such chemicals.

Timing of ethanol consumption and VOC exposure is important. Prior repeated exposure to ethanol is necessary for substantial CYP2E1 synthesis to occur. Concurrent exposure to ethanol and a VOC, however, may often be protective from both well-metabolized and poorly metabolized solvents. If a VOC and ethanol are both metabolized by CYP2E1, the xenobiotics compete for available isozyme. Muller et al. (1975) observed that concurrent intake of ethanol and inhalation of TCE at 50 ppm by human subjects resulted in a marked decrease in urinary excretion of TCE's major metabolites, TCA, and trichloroethanol (TCOH). In this instance, ethanol would afford protection against these oxidative metabolites. Metabolism of ethanol produces an excess of NAD, a cofactor that favors formation of TCOH from chloral hydrate, at the expense of TCA. Reduced formation of TCE would be protective against TCA-induced hepatic tumors. Larson and Bull (1989), however, observed this interaction in rats only with very high doses of TCE and ethanol.

Medications and drugs of abuse that induce or inhibit CYP2E1 and other enzymes involved in the metabolism of VOCs can potentially alter the chemicals' toxicity or carcinogenicity. Nakajima et al. (1992a) showed that pretreatment of rats with phenobarbital, ethanol, or 3-methylcholanthrene significantly increased TCE oxidation. The same would be expected to occur in humans at high TCE doses. Some drugs (such as cycloheximide, disulfiram, and chloramphenicol) and the aforementioned natural constituents of plants inhibit CYP2E1. Those compounds, in sufficient doses, would be protective against high doses of TCE and other VOCs that are bioactivated by CYP2E1.

Tobacco smoke contains a number of compounds that are strong CYP inducers. Polycyclic hydrocarbons, such as 3-methylcholanthrene, are potent-inducing agents. The polycyclic hydrocarbons primarily stimulate synthesis of CYP1A1 and CYP1A2, isozymes that play a modest role in catalyzing the biotransformation of TCE (Nakajima et al., 1992a). Nicotine, however, is a strong Cyp2e1 inducer in rats (Micu et al., 2003). Cigarette smoke is known to induce Cyp2e1 in both rodents and humans. Yue et al. (2009) recently observed that chronic nicotine treatment also increased voluntary ethanol intake by rats, thereby further enhancing hepatic Cyp2e1 and Cyp2b1/2 levels.

Solvent Mixtures Many occupational and environmental exposures to VOCs involve multiple chemicals. That is particularly true of contaminated environmental media, in that widespread use of solvents leads to their volatilization and entry into surface water and groundwater. VOCs spilled onto the ground largely evaporate. VOC levels in air in the breathing zone of the general population are typically in the ppb range. Lin et al. (2008) examined correlations between VOC concentrations in air and blood. Spilled VOCs also leach through porous soils into groundwater and remain trapped there. The groundwater at about 90% of 1608 hazardous-waste sites on the U.S. National Priorities List contains VOCs. TCE is the most frequently found of all chemicals, followed by lead, PCE, and benzene (Pohl et al., 2008). The most common four-component VOC mixture is TCE, PCE, TRI, and 1,1-dichloroethane. ATSDR (2004) published a toxicologic profile addressing potential health risks posed by this four-component mixture. Many U.S. cities' drinking-water supplies contain complex mixtures of VOCs. Total concentrations generally range from parts per trillion (ppt) to parts per billion (ppb) (Moran et al., 2007; Rowe et al., 2007). Trace amounts of a variety of VOCs are also present in the blood of many nonoccupationally exposed members of the general population (Churchill et al., 2001; Blount et al., 2006).

Most studies have involved experiments with binary or ternary mixtures. One chemical may have no effect on, potentiate (enhance), or antagonize (inhibit) adverse actions of a second or third chemical (Tan et al., 2011). Knowledge of mechanisms of VOC interactions involves largely the influence of one VOC on the metabolic activation or inactivation of another. Koizumi et al. (1982) published the results of one of the first such studies. They found that coexposure of rats to PERC and TRI resulted in significant suppression of TRI metabolism. Workers exposed to TCE and PERC were found to have lower urinary concentrations of TCE metabolites than workers exposed to TCE alone (Seiji et al., 1989). The interaction resulted from competitive metabolic inhibition, wherein the amounts of the combined chemicals exceeded the metabolic capacity of the study subjects. Such an interaction is protective against cytotoxicity and carcinogenicity, in that the bioactivation of both TCE and PERC is reduced. Conversely, systemic concentrations of parent VOCs would be increased, and this might enhance neurologic effects of sufficiently high doses (Pohl and Scinicariello, 2011).

PBTK modeling has been used by several research groups to predict the metabolic and toxicologic consequences of exposure to VOC mixtures. Competitive metabolic inhibition was evident in a PBTK-model approach to studying TCE and 1,1-DCE (El-Masri et al., 1996) and TCE and vinyl chloride (Barton et al., 1995). Later PBTK modeling efforts predicted interaction thresholds below which competitive metabolic inhibition would not occur. Dobrev et al. (2001), for example, reported that the thresholds for interaction of TCE with PERC and TRI vapor in rats were 25 and 135 ppm, respectively, when the TCE concentration was 50 ppm. Those findings imply that protection from adverse effects caused by oxidative metabolites would occur in occupational settings when vapor concentrations were relatively high. An increase in blood TCE concentrations under these exposure conditions, however, was predicted to result in a disproportionate increase in formation of nephrotoxic GSH conjugation products in humans (Dobrev et al., 2002). Competitive metabolic inhibition, with potentiation or protection

from adverse effects of VOCs, would not occur upon exposure to environmentally relevant concentrations.

Additivity of toxic effects of chemicals that act by similar mechanisms is typically assumed in the absence of experimental evidence to the contrary. Stacey (1989) studied the joint action of TCE and PERC on the liver and kidneys of rats. Combined administration of near-toxic-threshold doses of the two solvents produced modest hepatorenal toxicity. Jonker et al. (1996) provided evidence that TCE and PERC in combination with two other similarly acting solvents affected kidney weight in rats given subtoxic doses of each chemical by gavage for 32 days. Competitive metabolic inhibition, as described above, would result in less than additive adverse effects when metabolites are the bioactive moieties (Pohl and Scinicariello, 2011). Goldsworthy and Popp (1987) found that the joint effect of TCE and PERC on peroxisome proliferation in the liver and kidneys of mice and rats was less than additive. Competitive metabolic inhibition at relatively high exposure levels of toluene, ethylbenzene, and xylene has been predicted by PBTK modeling to result in higher internal exposures (and CNS depressant effects) than would occur with simple additivity (Dennison et al., 2005).

Few toxicity or carcinogenicity studies of complex chemical mixtures including VOCs have been conducted. The National Toxicology Program (NTP, 1993a) supplied F344 rats and B6C3F1 mice with drinking water containing 24 contaminants for up to 26 weeks. The mixture contained TCE, PERC, MC, TRI, DCE, 1,1-dichloroacetic acid, other solvents, heavy metals, polychlorinated biphenyls, and a phthalate. The total no-observed-adverse-effect level (NOAEL) for histologic changes in the liver was 11 ppm of the complex mixture in rats. Suppression of immune function occurred in female mice that consumed 756 ppm of the mixture for 2 weeks or 378 ppm for 13 weeks. A follow-up study in chemically tumor-initiated rats showed that the contaminant mixture did not promote preneoplastic foci in the liver (Benjamin et al., 1999). Wang et al. (2002a) supplied ICR mice with water containing $CHCl_3$, 1,1-dichloroacetic acid, DCE, TRI, TCE, and PERC for 16 and 18 months. There was a trend of increasing frequency of hepatocellular neoplasms in the male mice and an increasing incidence of mammary adenocarcinomas in the high-dose female mice. The total concentration of VOCs in the drinking water of females was about 1555 ppb. Most of the mixture was TCE (471 ppb) and PERC (606 ppb). These concentrations are far lower than have previously been reported to produce tumors. The results must be regarded as preliminary, in that the study design had a number of limitations, and the results have not been replicated. In addition, male B6C3F1 mice are particularly susceptible to hepatic tumors (Haseman et al., 1998), and mice metabolically activate a substantially greater proportion of VOC doses than do humans.

Diseases Illness can be a major source of variability in response to solvents. Impaired drug metabolism and clearance are commonly seen in patients with cirrhosis and hepatitis (Tan and Schiano, 2012). Reduced metabolism of solvents may result from decrease in hepatic parenchymal mass, diminished enzymatic activity, and/or decreased portal blood flow (Morgan and McLean, 1995). Lower levels of CYP2E1, CYP1A2, and GSH are seen in livers of patients with cirrhosis (Murray, 1992). Reduced P450-catalyzed metabolic activation of certain solvents can be protective, but diminished capacity to conjugate electrophilic metabolites with GSH would have the opposite effect. Plasma levels of albumin and a_1-acid glycoprotein fall in cirrhotic patients. Thus, plasma protein binding of many xenobiotic metabolites decreases and their rate of elimination increases (Morgan and McLean, 1995). Definitive information is lacking, however, on the net effect of common liver diseases on solvents.

Chronic kidney disease is becoming increasingly prevalent in the United States over the past decade (Coresch et al., 2007). Progressive loss of kidney function leads to impaired renal excretion of numerous chemicals and metabolites that may be toxic or pharmacologically active. Kidney disease can affect uptake and efflux transporters and metabolic enzymes in the liver and the GI tract (Nolin et al., 2008). Chronic renal failure can result in downregulation of the expression of some liver CYPs, but have no effect on others (Leblond et al., 2001). Effects on hepatic clearance of therapeutic chemicals can thus vary widely. The mechanisms of these effects on gene expression are unclear, as is their applicability to solvents. The plasma protein binding of many xenobiotics is reduced in patients with compromised renal function, apparently due to retention of substances that compete for protein-binding sites, as well as reduced albumin synthesis. Clearance of highly metabolized xenobiotics thus appears to depend on potentially offsetting influences of altered metabolism, decreased plasma protein binding, and decreased renal excretion (Yuan and Venitz, 2000). Impairment of renal metabolic activation via oxidation or β-lyase-mediated conversion of GSH metabolic intermediates of TCE and PERC (see subsections on these chemicals for details) to reactive, mutagenic metabolites would be protective.

Diabetes mellitus is a metabolic disease characterized by hyperglycemia as a result of insulin deficiency (type 1) or insulin resistance (type 2). Type 2 accounts for 90% of cases. Approximately 30 million people suffer from diabetes in the United States. A prominent effect of chemically induced type 1 diabetes in rats is induction of Cyp2e1. Elevation of acetone, but apparently not the other ketone bodies is responsible for the Cyp2e1 increase. Hypoinsulinemia may play a role, in that insulin downregulates Cyp2e1 mRNA in rat hepatoma cells and primary cultures of rat hepatocytes (Cheng and Morgan, 2001). In contrast, CYP2E1 is not affected in type 2 diabetic animals (Sawant et al., 2004). Neither type 1 nor type 2 diabetes influences CYP2E1 activity in humans (Lucas et al., 1998; Cheng and Morgan, 2001). Nevertheless, animal studies show that type 2 diabetes increases susceptibility to hepatotoxicants including allyl alcohol and CCl_4 (Sawant et al., 2004), apparently due to inhibition of tissue repair. Devi and Mehendale (2005, 2006) subsequently demonstrated inhibition of expression of genes involved in cell division and protease inhibitors, as well as enhanced gene expression of proteases in type 1 diabetic rats. These events may be important in delayed tissue repair following chemical cytotoxicity in diabetics.

Persons with bacterial infections may be more sensitive to cytotoxic actions of solvents. Endotoxin, which includes a lipopolysaccharide, is released from the cell wall of gram-negative organisms. The lipopolysaccharide causes the release of inflammatory mediators, which alter cell membranes, intercellular signaling, and gene expression (Roth et al., 1997). These effects may render cells more susceptible to damage by solvents and other chemicals. Exposure of animals to small amounts of endotoxin potentiates liver injury by CCl_4, halothane, allyl alcohol, ethanol, and other solvents (Roth et al., 1997). Endotoxin apparently activates Kupffer cells to release inflammatory mediators and cytotoxic moieties to hepatocytes (Thurman, 1998).

CHLORINATED HYDROCARBONS

Trichloroethylene

TCE was widely used as a solvent and metal degreasing agent in the United States from the early 1900s to the 1980s (Bakke et al., 2007). Workers were initially subjected routinely to quite high exposures.

NIOSH (1989) estimated that 401,000 persons at 23,225 plants in the United States were potentially exposed to TCE. TCE has been identified at over one-half of the nearly 1300 hazardous waste sites that make up the EPA's National Priorities List (ATSDR, 2006a; Pohl et al., 2008). TCE is released into the atmosphere from vapor degreasing operations; however, direct discharges to surface water and groundwater from disposal operations were frequent occurrences. As a result, TCE can be released to indoor air by vapor intrusion through underground walls and floors and by volatilization from the water supply. TCE has recently received a great deal of attention from the scientific and regulatory communities, due in large part to the EPA's effort to update the chemical's two-decade-old risk assessment (EPA, 2011).

Moderate-to-high doses of TCE, as with other halocarbons, are associated with a number of noncancer toxicities (Barton and Das, 1996; ATSDR, 1997d). TCE has been implicated in the development of autoimmune disorders and immune system dysfunction (Cooper et al., 2009; Lan et al., 2010; Gilbert, 2014a, 2014b), and has been investigated for its potential as a male reproductive toxicant (Forkert et al., 2003; Xu et al., 2004). The effect of gestational exposure to TCE or its oxidative metabolites on cardiac development is a subject that has invoked considerable debate, as conflicting results have been published (Fisher et al., 2001; Johnson et al., 2003; Watson et al., 2006). The issue of TCE's potential effect on ocular development has also reemerged (Warren et al., 2006). Nevertheless, cancer remains the dominant issue for TCE (Jollow et al., 2009; Lash et al., 2014). TCE and its potential health risks have been reviewed by NAS (2006, 2009), EPA (2011), ATSDR (2014), and IARC (2014).

Metabolism The adverse effects of TCE, other than CNS depression, are generally believed to be associated with TCE's metabolites. CNS depression is due to the parent compound and a major metabolite, trichloroethanol. Knowledge of TCE's metabolism in different species and under different exposure conditions is thus a prerequisite to understanding its mechanisms of action and assessing human health risks. A great deal of experimentation at many biological levels has been expended over the last five decades to these ends.

TCE is rapidly and extensively absorbed into the systemic circulation via the oral and inhalation routes. Dermal absorption is considerably slower and less extensive. The majority of TCE is oxidized in the liver, while a small amount is conjugated in the liver with glutathione (GSH) by GSH S-transferases (GSTs). The oxidative pathway is shown on the left of Fig. 24-5, while the GSH pathway is shown on the right. This diagram is a simplification of a more complex metabolic scheme described in detail by Lash et al. (2000a, 2014).

The initial step in the oxidative pathway is catalyzed by microsomal cytochrome P-450s (CYPs). CYP2E1, as described previously under the section on Metabolism under the section on Toxicokinetics, is the primary isozyme responsible for oxidation of low concentrations of TCE (Lipscomb et al., 1997; Kim and Ghanayem, 2006a; Ramdhan et al., 2008). CYP-catalyzed oxidation of TCE in rodents and humans, in decreasing order of magnitude, is as follows: mice>rats>humans (Lash et al., 2000a, 2014). Whether or not TCE is initially converted to TCE oxide is controversial. Cai and Guengerich (2001) were able to detect formation of trace amounts of the epoxide by phenobarbital-induced rat liver CYPs, but not by human liver CYPs. The majority of TCE is apparently converted to an oxygenated TCE-CYP intermediate, which rearranges to form chloral, a major metabolic intermediate. Chloral is oxidized to chloral hydrate (CH), a sedative and hypnotic still widely used in medical and dental procedures for infants and children (Buck, 2005; Heistein et al., 2006; Wilson et al., 2014). CH is both oxidized to TCA and reduced to TCOH. Much TCOH is conjugated with glucuronic acid (GLU) and excreted in the urine. TCOH-GLU that is excreted in the bile is hydrolyzed in the intestine, reabsorbed, and oxidized in part to TCA (Stenner et al., 1997). Chiu et al. (2007) observed that concentrations of TCA were significantly lower than TCOH and TCOH-GLU concentrations in the blood of humans who inhaled TCE at 1 ppm for 6 hours. Modest amounts of DCA apparently are produced from TCA and TCOH in mice, but relatively little DCA is formed in rats or humans. Trace amounts of DCA were detected in one study of TCE-exposed humans (Fisher et al., 1998) but not in other studies (Lash et al., 2000b; Bloeman et al., 2001). Very high doses of both TCA and DCA have been shown to be hepatic carcinogens in mice (Bull, 2000). It is generally accepted that TCA is a non-genotoxic liver carcinogen in B6C3F1 mice, although its ability to cause liver cancer in humans has been discounted by findings in numerous laboratory investigations (Bull, 2000). The possible role of DCA in human liver cancer is even more controversial (Walgren et al., 2005; Caldwell and Keshava, 2006; Klaunig et al., 2007).

The GSH conjugation pathway is quite similar qualitatively, but not quantitatively, in rats and humans. The initial step in this second, minor pathway involves conjugation of TCE with GSH to form *S*-(1,2-dichlorovinyl)GSH (DCVG). DCVG formation occurs primarily in the liver at a rate about 10 times greater in rats than in humans (Green et al., 1997a). Much of the DCVG is excreted via the bile into the intestines and converted to *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). That metabolite is reabsorbed and taken up by the liver, where a portion is detoxified by *N*-acetylation. Bernauer et al. (1996) exposed rats and humans to TCE vapor at up to 160 ppm for 6 hours. The rats excreted eight times more *N*-acetyl-DCVC in their urine than did the human volunteers at each exposure level. Some DCVC is taken up by the kidneys and further metabolized there by the enzyme β-lyase to *S*-(1,2-dichlorovinyl)thiol (DCVSH). DCVSH is then converted to highly reactive products, including DCVC sulfoxide, chlorothioketene, and thionoacylchloride (Lash et al., 2014). Metabolic activation of DCVC to chlorothioketene was shown to occur 11 times more rapidly in rats than in humans (Green et al., 1997a). Lash et al. (2001) also demonstrated that cultured rat renal cells were more sensitive to DCVC than human renal cells. Chlorothioketene and similarly unstable congeners are capable of covalently binding to renal cellular proteins and DNA. This results in genotoxicity and cytotoxicity, with ensuing regenerative hyperplasia and potentially renal cell carcinoma.

Modes of Carcinogenic Action in Target Tissues Both metabolic pathways are implicated in the carcinogenicity of TCE: reactive metabolite(s) of the GSH pathway in kidney tumors in rats, and oxidative metabolites in liver and lung tumors in mice. That tumor formation in many cases is species-, strain-, sex-, and route of exposure–dependent has provided clues as to TCE's modes of carcinogenic action. Whereas substantial progress has been made on the mechanistic front, the reader should not infer from the text that follows that all modes of action are known with certainty.

Liver Cancer It is well established that TCE, when given chronically in very high doses by gavage, can produce an increased incidence of hepatocellular carcinoma in B6C3F1 mice, but not in other strains of mice or in rats. This differential susceptibility can be explained in part by the greater capacity of the mouse to bioactivate relatively large quantities of TCE via the oxidative pathway. The B6C3F1 mouse produces a substantially larger amount of TCA after TCE exposures than do unresponsive strains of mouse, rats,

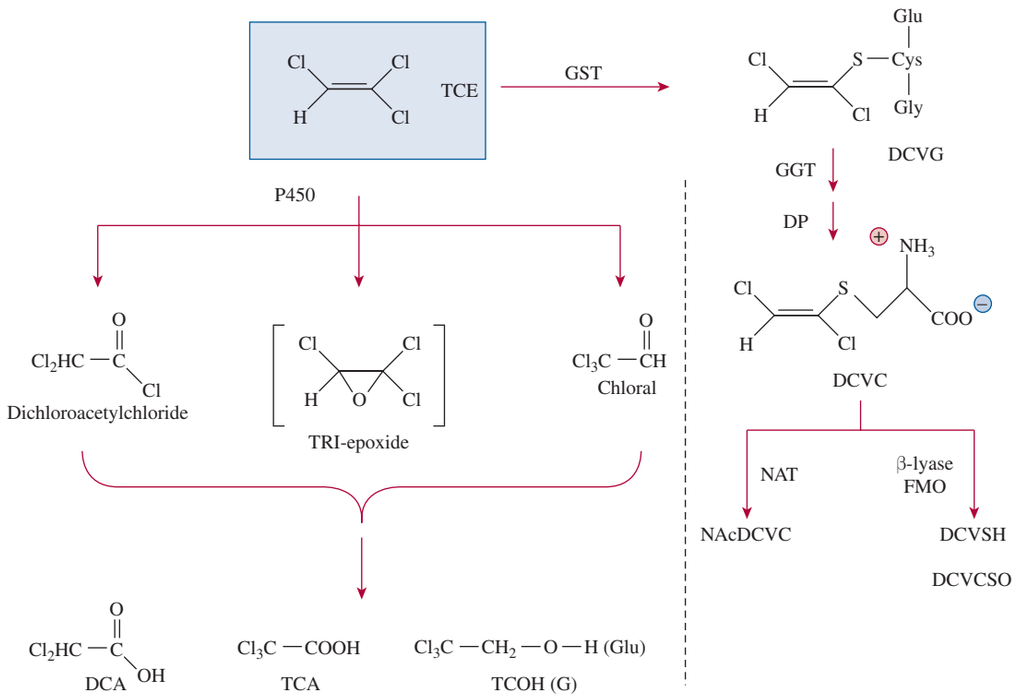

Figure 24-5. *General scheme of metabolism for TCE.* β-lyase, cysteine conjugate β-lyase; DCA, dichloroacetate; DCVC,S-(1,2-dichlorovinyl)-L-cysteine; DCVSH, 1,2-dichlorovinylthiol; DCVCSO, DCVC sulfoxide; DCVG, S-(1,2-dichlorovinyl)glutathione; DP, dipeptidase; FMO, flavin-containing monooxygenase; GGT, γ-glutamyltransferase; GST, GSH S-transferase; P450, cytochrome P450; TCA, trichloroacetate; TCOH, trichloroethanol; TCOG, TCOH glucuronide. (Reproduced from Lash et al., 2005, with permission from Elsevier.)

or humans. The susceptibility of the B6C3F1 mouse is also likely related to the high (42.2%) incidence of liver adenoma/carcinoma in male controls (Haseman et al., 1998). This phenomenon may be due to an abnormally high population of spontaneously initiated cells in these animals' liver. Mice express very low levels of epoxide hydrolase (Lorenz et al., 1984), the enzyme that catalyzes the hydrolytic degradation/detoxification of reactive epoxide metabolites of TCE and other VOCs.

Chloral hydrate (CH) induces hepatic tumors in male B6C3F1 mice, but not in F344 rats. Female B6C3F1 mice gavaged with up to 100 mg CH/kg/day for 104 weeks showed no increase in liver tumors, but the male mice did exhibit increased incidences of hepatoma and/or hepatocellular carcinoma. CH is rapidly converted to TCA and TCOH in rodents and humans. Merdink et al. (2008) detected only trace amounts of DCA in blood and urine of male human subjects dosed with 500 or 1500 mg of CH. An epidemiology study of the possible association of short-term clinical administration of CH as a sedative-hypnotic and cancer risk in 2290 patients was conducted by Haselkorn et al. (2006). The authors concluded there was no persuasive evidence of a causal relationship between CH and cancer in humans.

TCA and DCA are the most likely candidates for proximate hepatocarcinogens produced by TCE's oxidative pathway. TCA is a species-specific carcinogen that induces peroxisome proliferation and hepatocellular carcinoma in male and female B6C3F1 mice when administered in very high doses in drinking water or by gavage. TCA does not produce liver tumors in any strain of rats tested under these conditions (NAS, 2006, 2009). Very high doses of DCA, however, produce hepatic tumors in both B6C3F1 mice and F344 rats. Large, repeated doses of DCA and TCA initially stimulate, and then depress the growth of normal hepatocytes (Bull, 2000). This may confer a growth advantage to initiated cells, and is referred to as *negative selection*. At high tumorigenic doses, DCA (but not TCA) is thought to stimulate cell replication within liver tumors. If indeed both DCA and TCA contribute to tumorigenesis, findings by Bull et al. (2002) indicate they do so by distinct mechanisms. DCA-promoted liver tumors differed phenotypically from those promoted by TCA. DCA- and TCA-induced tumors also differ as to whether their depression of cytosine methylation is reversible upon cessation of treatment (Tao et al., 1998). This is particularly important as DNA hypomethylation, including that of the proto-oncogenes c-jun and c-myc, may be an epigenetic mechanism for the tumorigenicity of DCA and TCA (Tao et al., 2004). It appears that hypomethylation due to TCA and DCA induces DNA replication and prevents the methylation of newly synthesized strands of DNA (Ge et al., 2001). Tao et al. (2000) have reported that DCA and TCA do so by virtue of their depletion of *S*-adenosylmethionine, which normally supplies the methyl group for the methylation process. It should be recognized that these are not genotoxic mechanisms. The effects disappear as soon as these metabolites are eliminated.

A primary mode of action of TCA, and to a smaller extent DCA, is activation of peroxisome proliferator-activated receptor alpha (PPARα). As peroxisomes contain a variety of oxidative enzymes, PPARα activation produces oxidative stress, which can be manifest as lipid peroxidation, oxidative DNA damage, and transcription factor activation (O'Brien et al., 2005). Stimulation of PPARα can enhance DNA replication, resulting in expansion of some clones of hepatocytes and suppression of apoptosis, so initiated and precancerous cells will be spared. Male wild-type mice

dosed orally with TCE exhibit hepatocyte proliferation and changes in expression of genes involved in cell growth (Laughter et al., 2004). PPARα-null mice are refractory to those effects, which are associated with carcinogenesis. Mice expressing human PPARα fail to show increases in markers of cell proliferation and are resistant to liver cancer if treated with PPARα agonists (Morimura et al., 2006; Yang et al., 2008). The concentration of PPARα in human cells is about 10% of that in the livers of rodents (Palmer et al., 1998; Klaunig et al., 2003). Many toxicologists have judged that the mode of action for hepatic carcinogenesis observed in mice after administration of peroxisome-proliferation-inducing drugs and other chemicals, such as TCA, makes it unlikely that such chemicals pose a hepatic cancer risk in humans (Cattley et al., 1998; Clewell and Andersen, 2004; Klaunig et al., 2007; Gonzalez and Shah, 2008). It was concluded by an expert review panel that the PPARα mode of action for liver cancer in mice is not relevant to humans (NAS, 2006). However, others have raised questions about the interpretation of PPARα actions and whether it is the only relevant mode of action for such chemicals (Keshava and Caldwell, 2006). This has continued to be a subject of debate (NAS, 2008).

It is important to recognize that stimulation or inhibition of cell growth through PPARα activation ceases when the metabolites are eliminated (Miller et al., 2000). Thus, such alteration of cell signaling is not a genotoxic mechanism of action. Very high concentrations of DCA and CH have a weak genotoxic action in vitro. Bull (2000) and Moore and Harrington-Brock (2000), however, conclude that it is unlikely that those metabolites would cause tumors in any organ through genotoxicity or mutagenicity at exposure concentrations relevant to humans.

Lung Cancer As discussed in the review paper by Green (2000), inhaled TCE is carcinogenic to the mouse lung but not to that of the rat. Oral TCE is not carcinogenic to the lung, probably due to first-pass hepatic metabolism that limits the amount of TCE reaching the lungs. The primary target of TCE in the mouse lung is the nonciliated Clara cell. Cytotoxicity is characterized by vacuolization and increased replication of these cells in the bronchiolar epithelium. A dose-dependent reduction in the CYP activity in Clara cells is observed as well. This loss of metabolic activation capacity can be thought of as an adaptive response. Clara cells recover morphologically during repeated daily inhalation exposures to TCE.

Chloral is the putative toxicant responsible for pulmonary tumor formation. Clara cells of the mouse efficiently metabolize TCE to chloral. Chloral accumulates, due to its efficient production and low activity of ADH, the enzyme responsible for its reduction to TCOH. The Clara cells' lack of glucuronosyltransferase, the enzyme that normally catalyzes the formation of TCOH-GLU, has also been implicated in chloral accumulation. Species differences in susceptibility of the lung to TCE are due in part to mouse lung Clara cells having a much higher level of Cyp2e1 than those of the rat, and thus a much higher capacity to metabolize TCE to chloral. Also, Clara cells in mice are much more numerous than in rats. Clara cells are rare in human lungs. A critical role for chloral is supported by the finding that its administration to mice, but not that of TCA or TCOH, causes Clara cell toxicity identical to that of TCE. Chloral does appear to have some genotoxic potential, especially in regard to inducing aneuploidy. However, the fact that tumors are not seen in species where cytotoxicity does not occur strongly implicates cytotoxicity and reparative proliferation in tumor formation. In an effort to test the hypothesis that bronchiolar damage by TCE is associated with bioactivation within Clara cells, Forkert et al. (2006) administered TCE i.p. to CD-1 mice. The result was dose-dependent production of dichloroacetyl lysine adducts in Clara cells (used as an in vivo marker of TCE metabolism) that correlated with bronchiolar damage. The work also suggested that CYP2F2 may play a more important role than CYP2E1 in TCE metabolism and cytotoxicity within the mouse lung (Forkert et al., 2005).

Kidney Cancer TCE was given in corn oil to F344/N rats and B6C3F1 mice of both sexes by gavage five times weekly at doses up to 1000 mg/kg in rats and 6000 mg/kg in mice in a 13-week study, as well as up to 1000 mg/kg to both species and sexes in a 103-week study (NTP, 1990). A low, but statistically significant increase in renal tumor incidence was observed only in the male rats given TCE at 1000 mg/kg for 2 years. Two-year gavage studies of TCE, in four additional rat strains, were also conducted (NTP, 1988). In all strains of rats tested, cytomegaly and karyomegaly of tubular cells in the renal corticomedullary region were seen. Frank toxic nephropathy was observed with higher frequency in male rats beginning at 52 weeks of exposure. Renal adenomas or adenocarcinomas were occasionally seen in male rats of different strains after 2 years of the repetitive, high-dose oral exposure regimen.

Adverse effects of TCE on the kidneys are due largely to metabolites formed via the GSH conjugation pathway (Lash et al., 2000a, 2014). As described previously, conjugation of TCE with GSH to form *S*-(1,2-dichlorovinyl)GSH (DCVG) occurs primarily in the liver, which is secreted into bile and blood. DCVG in the bile is converted in the gut to *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), which is reabsorbed into the bloodstream. As described previously, humans have a lower capacity than rats to metabolize TCE by the GSH pathway. Lash et al. (1999) were able to detect DCVG in the blood of humans who inhaled TCE at 50 or 100 ppm for 4 hours, but Bloeman et al. (2001) could not find DCVG or DCVC in the urine of similarly exposed subjects. DCVG in the blood is taken up by the kidneys and metabolized to DCVC by γ-glutamyltransferase and a dipeptidase. Lash et al. (2001) observed the following decreasing order of toxic potency in freshly isolated rat cortical cells: DCVC > DCVG >> TCE. DCVC can be detoxified by acetylation or activated further by two pathways: (1) cleavage by renal cytosolic and mitochondrial β-lyases to dichlorothioketene, which in turn can lose a chloride ion to yield chlorothioketene or tautomerize to form chlorothionacyl chloride (the latter two moieties are very reactive and acylate proteins and DNA); and (2) oxidation by renal CYPs or flavin-containing monoxygenases to DCVC sulfoxide (DCVCS), a reactive epoxide. Lash et al. (1994) reported that DCVCS was a more potent nephrotoxicant than DCVC in vitro and in vivo in rats. Apoptosis was observed after as little as 1 hour of incubation of cultured human renal proximal tubular (RPT) cells with DCVC and DCVCS (Lash et al., 2003, 2005). Cellular proliferation, accompanied by increased expression of proteins associated with cellular growth, differentiation, stress, and apoptosis, was also an early response to low doses. Necrosis, however, was a late, high-dose phenomenon. Exposure of human RPT cells to DCVC at lower concentrations for 10 days also resulted in expression of genes associated with cell proliferation, apoptosis, and stress (Lash et al., 2005), as well as repair and DCVC metabolism (Lash et al., 2006).

Animal studies typically provide insight into mode of action, but in the case of TCE-induced renal cell carcinoma (RCC), human studies have been of significant value. Bruning et al. (1997a) analyzed tumor tissues from 23 RCC patients with occupational histories of long-term, high-level TCE exposure. Tumor cell DNA was isolated and analyzed for somatic mutations of the von-Hippel Lindau (VHL) tumor suppressor gene. Compared to VHL gene mutation rates of 33% to 55% in unexposed RCC patients, all 23 TCE-exposed RCC patients exhibited aberrations of the VHL gene.

In a follow-up study, Brauch et al. (1999) sought to determine whether TCE produced a specific mutation of the VHL gene. These investigators analyzed VHL gene sequences in DNA isolated from RCC tissues from patients exposed to high levels of TCE in metal-processing factories. Renal cell tumors of TCE-exposed patients showed somatic VHL mutations in 33 of 44 cases (75%). Of the 33 cases with VHL mutations, a specific mutational hot spot at VHL nucleotide 454 was observed in 13 cases. The nucleotide 454 mutation was not found in any of the 107 RCC patients without TCE exposure or among 97 healthy subjects, 47 of whom had a history of TCE exposure. These data suggest that the VHL gene may be a specific and susceptible target of reactive GSH pathway metabolites, a concept strengthened by a more recent study reporting VHL nucleotide 454 mutation among TCE-exposed but not in nonexposed RCC patients (Brauch et al., 2004). More recently, however, Charbotel et al. (2007) found no associations between the number and type of VHL mutations in TCE-exposed and unexposed RCC patients. The subject awaits further clarification (Chow and Devesa, 2008).

The aforementioned reactive products of DCVC are both cytotoxic and mutagenic. Adverse effects on proximal tubule cells include alkylation of cytosolic and mitochondrial structural and enzymatic proteins, oxidative stress, marked ATP depletion, and perturbations of calcium homeostasis. Tubular necrosis ensues, with subsequent proliferation that can alter gene expression, which in term may modify cell growth and differentiation. Genes associated with stress, apoptosis, repair, and proliferation were upregulated almost twofold in cultured human renal tubular cells exposed to subtoxic doses of DCVC for 10 days (Lock et al., 2006). Mechanisms of noncarcinogenic and carcinogenic action are discussed in detail by Lash et al. (2014) and Lock and Reed (2006).

Bruning and Bolt (2000) opined that reactive metabolite(s) of the GSH pathway may have a genotoxic effect on the proximal tubule of the human kidney, but that full development of a malignant tumor requires a promotional effect such as cell proliferation in response to tubular damage. If this is true, RCC secondary to TCE exposure would be a threshold response. The question of whether chronic tubular damage is a prerequisite to renal tumor formation is quite important. Evidence has come from the use of electrophoresis to examine protein excretion patterns in the urine of RCC patients with and without a history of chronic, high-level TCE exposure (Bruning et al., 1996). Protein excretion patterns indicative of tubular damage were identified in all their 17 TCE-exposed cases, but in only about one-half of 35 controls. Bruning et al. (1999) subsequently published the results of a larger study supportive of this concept. Some 93% of 41 RCC patients with high TCE exposure exhibited elevated urinary α_1-microglobulin excretion versus 46% of 50 RCC patients without a history of TCE exposure. Similar findings were reported in an updated study by Bolt et al. (2004), and in an investigation by Green et al. (2004) of 70 electronics workers who inhaled an average of 32 ppm TCE for 4 years.

Results of an investigation by Mally et al. (2006) provide additional insight into the TCE renal carcinogenesis threshold premise. A strain of rats (Eker) uniquely susceptible to renal carcinogens was administered 100, 250, 500, and 1000 mg TCE/kg by gavage 5 days/week for 13 weeks. The Eker rat is a unique animal model for RCC, carrying a germ-line alteration of the *Tsc*-2 tumor-suppressor gene. Results showed a significant increase in cell proliferation in renal tubular cells but no increase in preneoplastic renal lesions or tumor incidence. In vitro studies were conducted on primary Eker rat renal epithelial cells by exposing them to the TCE metabolite DCVC dissolved in water at 10 to 50 μM for 8, 24, and 72 hours. Concentrations of DCVC that reduced rat renal cell survival to 50% also resulted in cell transformation. No carcinogen-specific mutations were identified in the *VHL* or *Tsc-2* tumor-suppressor genes in the transformed cells. Renal cell carcinomas in the Eker rat have substantial similarities to human RCC. It is not entirely clear that this or any contemporary experimental animal model adequately mirrors humans with regard to the effects of TCE-induced mutations in the *VHL* gene, but the authors firmly suggest that TCE-mediated renal carcinogenicity may occur only secondarily to nephrotoxicity and sustained regenerative cell proliferation.

Metabolism of TCE by the GSH pathway is similar qualitatively, but not quantitatively in rats and humans. *N*-acetyl-DCVC, the major detoxification product of DCVC, was found in the urine of humans and rats after 6 hours of inhalation of up to 160 ppm TCE (Bernauer et al., 1996). Cumulative excretion of the *N*-acetyl derivative was seven- to eightfold greater in the rats. Bruning et al. (1997b) originally reported an increased likelihood of RCC patients with high TCE exposure having a functional GSH S-transferase isozyme GSTT1 or GSTM1 genotype. This isozyme GSTT1 is thought to be primarily involved in TCE metabolism, while GSTM1 is believed to detoxify epoxides. A reassessment by the investigators of a larger population, however, revealed no such relationships (Wiesenhutter et al., 2007). Moore et al. (2010), however, completed an assessment of an even larger population. There was an increased risk of RCC in workers with at least one intact GSTT1 allele, but not in persons with two deleted alleles (i.e., null genotype).

It is also worthy of note that male rats and humans are apparently at greater risk of TCE-induced kidney cancer than their female counterparts. Blood DCVG levels were 3.4-fold higher in male than in female volunteers inhaling 50 or 100 ppm TCE (Lash et al., 1999). Male rats display higher GSH conjugation, γ-glutamyl transpeptidase activity, and cysteine conjugate β-lyase activity than female rats. Taken together, results of the cited studies indicate that male humans and male rats both possess GSH conjugation capacity and can produce the critical TCE metabolite DCVC. Renal carcinoma has been observed in male rats and male workers when both have been exposed to very high TCE concentrations for prolonged periods of time. These observations show data congruence, indicating that the conjugation pathway plays a central role in induction of renal carcinoma in males of both species. As described previously, rats have a significantly greater capacity to metabolically activate TCE to DCVC by this pathway than do humans. Rat renal cortical cells, in turn, are more susceptible to injury by DCVC than their human counterparts.

Cancer Epidemiology There have been many published studies of cancer incidence and mortality in TCE-exposed populations. Most of the epidemiology studies in the United States prior to 2000 involved workers in the aircraft maintenance and manufacturing industries. There were also investigations of Swedish, Finnish, German, and Danish worker cohorts. Results of these assessments have been mixed, ranging from no association to limited evidence. The major studies with some exposure data constituted what Wartenberg et al. (2000) referred to as Tier I studies, which received a greater weighting when making causal inferences than lower tier cohort, case–control, and community-based investigations. Among the Tier I studies, evidence for an excess incidence of cancer was strongest for kidney (rate ratios [RR] = 1.7, 95% confidence interval [CI] = 1.1 to 2.7), liver (RR = 1.9, CI = 1.0 to 3.4), and non-Hodgkin's lymphoma (NHL) (RR = 1.5, CI = 0.9 to 2.3) (Wartenberg et al., 2000).

Occupational exposure studies have provided the strongest evidence to date for an association between TCE and RCC. Henschler et al. (1995) described a cohort of male cardboard factory workers who were exposed to moderate to extremely high concentrations of

TCE vapor. Five of the 169 exposed workers had been diagnosed with kidney cancer versus none of the 190 controls. This resulted in standardized incidence ratios (SIRs) of 7.97 (CI = 2.59 to 18.59) and 9.66 (CI = 3.14 to 22.55), using Danish and German Cancer Registry data for comparison, respectively. German researchers also conducted a hospital-based case–control study with 58 RCC patients and 84 patients from accident wards who served as controls. Of the 58 RCC patients, 19 had histories of occupational TCE exposure of at least 2 years, compared to only 5 of the controls. After adjustment for potential confounders, an association between RCC and long-term exposure to TCE was reported (odds ratio [OR] = 10.80, CI = 3.36 to 34.75) (Vamvakas et al., 1998). In an expanded German case–control study of 134 RCC cases and 401 controls, excess risks for those working longest with TCE (OR = 1.8, CI = 1.01 to 3.20) and those reporting narcotic symptoms (OR = 3.71, CI = 1.80 to 7.54) were reported (Bruning et al., 2003). Narcosis was thought to be associated with peak exposures. These workers reported frequent dizziness, requiring them to seek fresh air several times daily. More recent investigations have generally involved surveys of populations with lower exposures. The results of these investigations have generally been indicative of an association between TCE and RCC, although some findings were negative or did not reach statistical significance (Raaschou-Nielsen et al., 2003; Wong, 2004; Chang et al., 2005; Alexander et al., 2006; Boice et al., 2006, Mandel et al., 2006; Moore et al., 2010). A number of these assessments involved a limited number of subjects. Charbotel et al. (2006) studied 86 RCC patients and 316 matched controls from an area in France with high TCE exposures in local industries. There was a 64% increase in RCC risk with TCE exposure, with the risk doubling in persons with a high cumulative dose, increasing more (OR = 2.74, CI = 1.06 to 7.07) when peak exposures were also taken into account. The OR was still high, but not statistically significant after adjusting for exposure to cutting fluids, because of the study's lack of power. A meta-analysis based on existing studies was conducted, because risk estimates were modest. Statistically significant meta-relative risks of 1.26 to 1.32 were reported by Karami et al. (2012). Cichocki et al. (2016) recently reviewed findings of epidemiological studies of TCE exposures and cancer risks.

Epidemiological evidence of NHL or cancer of the liver, lung, or other organs has been weaker than that for the kidney. There appeared to be a dose-dependent increase in the standardized incidence ratio (SIR) for NHL, with increased duration of TCE exposure in Danish workers (Raaschou-Nielsen et al., 2003). These researchers also reported an SIR of 1.7 for esophageal adenocarcinoma. Seidler et al. (2007) described the association between TCE exposure of more than 35 ppm/year and malignant lymphoma (OR = 2.1, CI = 1.0 to 4.8) as being of borderline statistical significance. Lan et al. (2010) recently observed a dose-dependent decline in major types of lymphocytes in TCE-exposed workers in China. Little or no association with NHL incidence was seen by Boice et al. (2006) or Zhao et al. (2005) in aerospace employees. Evidence of lung cancer in persons occupationally exposed to TCE is limited to nonexistent (Hansen et al., 2001; Raaschou-Nielsen et al., 2003; Zhao et al., 2005; Boice et al., 2006). The latter group of scientists' estimates of SIRs for lung cancer, for example, were 1.4 for men and 1.9 for women. Most estimates of liver cancer risk in TCE-exposed workers have also been low. SIRs of 2.6 (Hansen et al., 2001) and 2.8 (Raaschou-Nielsen et al., 2003), resulting from a small number of cases, were among the highest reported.

Risk Assessment Attempts to understand the mechanistic underpinnings of TCE's carcinogenicity in rodent models and their relevance to humans have resulted in a *massive* body of published data. TCE provides a stellar example of how experimental data from laboratory and epidemiological studies may or may not impact cancer risk assessment in a regulatory context. The EPA began in the 1990s to revise its guidelines for cancer risk assessment. The final EPA *Guidelines for Carcinogen Risk Assessment* were released in March 2005. TCE was utilized as a pilot chemical for evaluation and implementation of this guidance document. The guidelines emphasize a scientific "weight of evidence" approach that includes characterization of dose–response relationships, modes of action, and metabolic/TK processes. Where adequate data are available to support reversible binding of the carcinogenic moiety to biological molecules as the initiating event, a nonlinear (i.e., threshold) risk assessment/approach is to be used. Otherwise, the default assumption of a linear (i.e., no-threshold) model/approach is to be used to estimate cancer risk. As described above, there is considerable evidence that TCE's oxidative metabolites act via non-genotoxic modes of action. PPARα activation and induction of mouse liver tumors were judged to be irrelevant to humans (NAS, 2006). Despite a substantial increase in information in the last decade, Caldwell and Keshava (2006) contended that knowledge of mechanisms and human relevance was still insufficient to depart from the default assumption. This is exemplified by the critical response of EPA scientists (Caldwell et al., 2006) to the effort by Clewell and Andersen (2004, 2006) to apply a margin of exposure approach (nonlinear dose–response extrapolation) in their TCE risk assessment. In contrast to liver cancer, kidney cancer is widely accepted to be qualitatively similar in rats and humans, though rats form greater quantities of reactive metabolites via the GSH pathway. Despite genotoxic events, kidney tumor formation in humans may require promotion resulting from frank cytotoxicity (Bruning and Bolt, 2000). Caldwell and Keshava (2006), however, opine that there may be other modes of action of multiple metabolites operative at low doses. This logic has been retained in the EPA's *IRIS Toxicological Review of TCE* (2011).

The EPA (2001a) draft "TCE Risk Assessment of TCE" elicited considerable debate, which prompted the EPA and other federal agencies to request a scientific review of the document by a NAS expert panel. Their report was released in 2006. It concluded there was concordance between rat and human studies of renal carcinogenicity. The preponderance of evidence indicated that humans would be much less susceptible than mice to liver and lung carcinogenesis. It was recommended, among other things, that a new meta-analysis of epidemiological data be conducted. Kelsh and his co-workers recently completed meta-analyses of occupational study data. Studies were classified as group I or II, depending on the quality of their design and exposure assessment. The summary relative risk estimate (SRRE) across all such studies was 1.42 (CI = 1.17 to 1.77) for kidney cancer (Kelsh et al., 2010). The same research group performed meta-analyses for occupational TCE exposures and liver cancer (Alexander et al., 2007) and NHL (Mandel et al., 2006). The highest SSREs calculated for liver cancer and NHL in any study groupings were 1.30 and 1.59, respectively. The authors noted that the results of many studies were inconsistent, information on TCE exposure was often quite limited, and recognized confounding factors were not always taken into account. More recently NAS (2009) evaluated published occupational epidemiology studies, and concluded that evidence was inadequate/insufficient to determine whether there was as association with NHL or cancer of the liver, lung, or any other organ. TCE is now classified as a known human carcinogen by the EPA (2011), carcinogenic to humans (Group I) by IARC (2014), and known to be a human carcinogen by NTP (2015). All three agencies based their determination on the strength of epidemiologic evidence for kidney cancer. The IARC Group I

classification was also supported by strong mechanistic evidence of renal carcinogenicity (Rusyn et al., 2014). The relative consistency of findings across studies, positive exposure–response relationships, and results of meta-analyses argue against chance for a causal association (Cichocki et al., 2016).

Tetrachloroethylene

Tetrachloroethylene (perchloroethylene, PERC) is commonly used as a dry cleaner, fabric finisher, degreaser, rug and upholstery cleaner, paint and stain remover, solvent, and chemical intermediate. The highest exposures usually occur in occupational settings via inhalation. Much attention is being focused on adverse health effects that may be experienced by dry cleaners and other persons living in the proximity of such facilities (Garetano and Gochfeld, 2000). Echeverria et al. (1995), for example, reported adverse effects on visuospatial functions of dry cleaners. PERC is frequently detected in the low ppt range in the breath and blood of the general populace (Ashley et al., 1994; Churchill et al., 2001; Blount et al., 2006). Although releases are primarily to the atmosphere, PERC enters surface water and groundwater by accidental and intentional discharges (ATSDR, 1997c). Levels in the ppb range were reported in municipal water in areas of New England, where PERC was used in a process to treat plastic water pipe (Paulu et al., 1999). Pohl et al. (2008) reported that PERC was the third most frequently found chemical contaminant in groundwater at hazardous waste sites in the United States. A dry cleaner adjacent to Camp Lejeune, in North Carolina, was the source of PERC contamination of some wells that supplied drinking water to the marine base. This contamination prompted assessment of potential health effects in employees and persons stationed at the base (NAS, 2009).

Metabolism The systemic disposition and metabolism of PERC and TCE are similar in many respects, though PERC is much less extensively metabolized (ATSDR, 1997c; Chiu et al., 2007, Cichocki et al., 2016). Both chemicals are well absorbed from the lungs and GI tract, distributed to tissues according to their blood flow and lipid content, partially exhaled unchanged, and metabolized. PERC, like TCE, is metabolized by cytochrome P450 (CYP)-catalyzed oxidation and glutathione (GSH) conjugation. CYP2E1, however, is not thought to play a major role, in that PERC is believed to be oxidized primarily by the Cyp2b family in the rat (Hanioka et al., 1995). In humans, CYP2B6 is the primary isoform responsible for PERC metabolism, and there are minor contributions by CYP1A1 and CYP2C8 (White et al., 2001). The initial metabolite is the epoxide PERC oxide. This metabolic intermediate can be biotransformed to several products (Lash and Parker, 2001). The primary one is trichloroacetyl chloride, which reacts with water to form TCA, the predominant PERC metabolite found in the urine of rodents and humans (Birner et al., 1996; Volkel et al., 1998). Some TCA is converted to DCA.

A small proportion of absorbed PERC undergoes conjugation with GSH to form *S*-(1,2,2-trichlorovinyl)GSH (TCVG). This initial metabolic step is catalyzed by GSH S-transferases and occurs primarily in the liver. TCVG is converted to *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC). TCVC, like the DCVC formed from TCE, is both detoxified in the liver by *N*-acetylation and metabolically activated by β-lyases in the kidneys. 2,2-Dichlorothioketene can decompose to DCA. Hence, DCA is derived from both GSH- and CYP-dependent biotransformation of PERC. PERC is conjugated with GSH more extensively by rats (1% to 2%) (Dekant et al., 1986) than is TCE (less than 0.005%) (Green et al., 1997a). The extent of GSH conjugation of PERC increases when the oxidative pathway begins to become saturated at high exposure levels. Metabolic products of GSH conjugates of TCE and PERC are primary contributors to the halocarbons' nephrotoxicity (Lash et al., 2007; Cichocki et al., 2016).

Modes of Cytotoxicity/Carcinogenicity PERC-induced hepatic injury is believed to be a consequence of its oxidative metabolism (Lash and Parker, 2001; Guyton et al., 2014). Although PERC is more poorly metabolized by CYPs than TCE, two additional intermediate metabolites of PERC appear to contribute to its hepatocytotoxicity: the initial oxidation product, PERC oxide; and one of its convertants, trichloroacetyl chloride. TCA is primarily responsible for activation of the nuclear receptor PPARα, which stimulates peroxisomal enzymes and selected CYPs involved in lipid metabolism. This results in peroxisome proliferation, which generates reactive oxygen moieties that can cause lipid peroxidation, cellular injury, and altered expression of cell-signaling proteins (Bull, 2000). Lash et al. (2007) more recently demonstrated that CYP inhibition resulted in reduced injury of hepatocytes isolated from male F344 rats and exposed to PERC. GSH depletion increased cellular injury, apparently because of a shift from the GSH to the oxidative pathway.

The metabolism and mode of nephrotoxicity of PERC and TCE appear to be quite similar, although PERC and its metabolites are somewhat more potent. Renal effects of both halocarbons are due primarily to metabolites formed via the GSH pathway (Lash and Parker, 2001). The sites, enzymes, and products associated with PERC biotransformation are almost identical to those associated with TCE. The primary difference is that TCVG and TCVC are produced from PERC, and DCVG and DCVC are formed from TCE. TCVC can be detoxified by acetylation or cleaved by renal cytosolic and mitochondrial β-lyases to trichlorothioketene, which loses a chloride ion to form dichlorothioketene. The latter is a very reactive moiety that binds to cellular proteins and DNA. TCVC, as noted above, can be enzymatically oxidized to form the very reactive TCVC sulfoxide (TCVCS) (Krause et al., 2003). TCVCS was shown to be more nephrotoxic than TCVC in male rats on i.p. injection (Elfarra and Krause, 2007). TCVC caused more pronounced necrosis of renal proximal tubular cells in male rats than did DCVC after i.v. injection (Birner et al., 1997). Lash et al. (2002) similarly found that PERC and TCVG were more toxic than TCE and DCVG in vivo to renal cortical cells of F344 rats. Cells from male rats were more sensitive than cells from females to PERC- and TCVG-induced mitochondrial state 3 respiratory inhibition and cytotoxicity. Isolated rat hepatocytes and their mitochondria, however, were unaffected by PCE and TCVC. Elevated GSH levels enhanced TCE-induced and PERC-induced cytotoxicity in suspensions of rat renal cortical cells but not hepatocytes (Lash et al., 2007). Thus, PERC's GSH metabolites are both sex- and organ-specific.

Hepatorenal Toxicity PERC, like TCE, has quite limited ability to adversely affect the liver or kidneys of rodents. Near-lethal i.p. doses of PERC were required to cause acute liver injury in mice (Klaassen and Plaa, 1966). Buben and O'Flaherty (1985) saw manifestations of modest hepatocellular damage in male mice given 500 to 2000 mg PERC/kg daily for 6 weeks by corn oil gavage. A lack of dose-dependence reflected saturation of metabolic activation in this dosage range. Philip et al. (2007) reported that male mice given 150 to 1000 mg PERC/kg by aqueous gavage initially exhibited a dose-dependent increase in serum alanine aminotransferase (ALT) activity, but levels of the enzyme regressed substantially over a 30-day dosing period. Hayes et al. (1986) found no consistent dose-related changes in any clinical chemistry parameter in male or female rats that consumed up to 1440 mg PERC/kg in their

drinking water for 90 days. Liver injury was not seen in B6C3F1 mice or Osborne–Mendel rats gavaged with up to about 1000 mg/kg daily for 78 weeks (NCI, 1977a). Dose-dependent karyomegaly was observed in the renal proximal tubular epithelium of mice and rats inhaling PERC for 103 weeks (NTP, 1986). This change was most prominent in the male rats. Tinston (1995) reported mild, progressive glomerulonephropathy and increased pleomorphism of renal tubular nuclei in male but not female rats that inhaled 1000 ppm PERC for up to 19 weeks. Green et al. (1990) found increases in protein droplets and cell proliferation in the proximal tubular epithelium of male F344 rats gavaged with 1500 mg PERC/kg daily for up to 42 days. These alterations were accompanied by an increase in α_{2u}-globulin, a male rat-specific protein (Fig. 24-6). Goldsworthy et al. (1988) reported similar findings in male, but not female F344 rats gavaged for 10 days with 1000 mg PERC/kg/day. The accumulation of α_{2u}-globulin is cytotoxic, causing cellular necrosis and compensatory cellular proliferation in the P2 segment of renal proximal tubules (Borghoff et al., 1990).

Humans should be less susceptible to hepatorenal injury by PERC than rodents, due to lower target-organ doses of the parent compound and its bioactive metabolites. Rats achieve a substantially higher internal dose of VOCs than do humans upon inhalation exposures. Volkel et al. (1998) subjected rats and people to identical PERC inhalation regimens. Blood TCA concentrations were 3 to 10 times higher in the rats. DCA was not detectable in human urine, but substantial amounts were found in the rat urine. A study of the urinary excretion of total trichloro-metabolites by PERC-exposed workers led Ohtsuki et al. (1983) to conclude that the capacity of humans to metabolize PERC was rather low. Lash and Parker (2001) noted that saturation of PERC metabolism occurred at lower doses

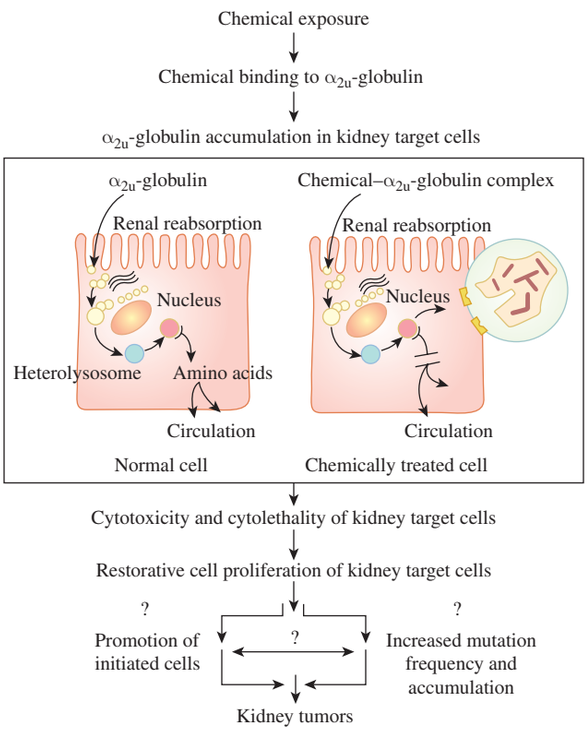

Figure 24-6. *Proposed mechanism of solvent-induced kidney cancer in male rats involving α_{2u}-globulin.* (Used with permission of Borghoff et al. CIIT Activities 1996;16(10):1–8.)

in humans than in rodents. This indicates that humans have lower capacity to form biologically active metabolites from moderate-to-high PERC doses. The difference is reflected in the finding of much lower concentrations of protein adducts in the blood of humans than in the blood of rats subjected to equivalent PERC inhalation exposures (Pahler et al., 1999).

PERC has limited ability to damage the liver of humans. Stewart et al. (1977) found no evidence of hepatotoxicity in six male and six female volunteers exposed randomly to PERC at 0, 25, or 100 ppm 5.5 hours/day, 5 days/week for 11 weeks. Serum ALT activity was not increased in 22 dry cleaners examined by Lauwerys et al. (1983). A research group in Italy studied 141 employees exposed to PCE in small laundries and dry cleaning shops (Gennari et al., 1992). No worker exhibited clinical signs of hepatic dysfunction or abnormal serum enzyme concentrations, although there did appear to be an increase in one isozyme of γ-glutamyltransferse, which was said to be associated with hepatobiliary impairment. Another investigation of dry cleaners failed to reveal increases in serum enzymes, but did show mild-to-moderate changes in hepatic parenchyma revealed by ultrasonography (Brodkin et al., 1995). Thus, considerable experience in occupational settings demonstrates that humans may develop mild but reversible liver injury upon chronic exposure to high concentrations of PERC (ATSDR, 1997c).

Occupational exposures to PERC vapor have led to several reports of mild renal tubular damage (ATSDR, 1997c). Employees of dry cleaning shops have been the subjects of a number of studies of potential kidney effects. Increased concentrations of lysosomal or β-glucuronidase activity were described in the urine of dry cleaners exposed to 10 (Franchini et al., 1983) and 23 ppm PERC (Vyskocil et al., 1990) for 9 to 14 years. In a more comprehensive study, a number of urinary indices indicative of early glomerular and tubular changes were increased in 50 dry cleaners who inhaled about 15 ppm PERC for 10 years (Mutti et al., 1992). Verplanke et al. (1999) monitored several indexes of tubular and glomerular function in Dutch dry cleaning workers, but found an increase only in retinol-binding protein in their urine. Other investigators have failed to find evidence of renal effects in such populations. A laboratory study of 10 male and 10 female adults who inhaled PERC at up to 150 ppm for as long as 7.5 hours/day for 5 days did not show changes from pre-exposure baseline BUN levels (Stewart et al., 1981). Hake and Stewart (1977) described a dry cleaner worker who was found unconscious in a pool of PERC, where he had been for an estimated 12 hours. Laboratory tests revealed hematuria and proteinuria that lasted for 10 and 20 days, respectively. Mild hepatic damage was revealed by transient increases in serum enzymes. On the basis of the foregoing occupational experiences, PERC has shown limited ability to cause diffuse changes along the nephron, although extremely high exposures can lead to pronounced changes in humans.

Cancer Bioassays in Rodents High, chronic doses of PERC have been demonstrated to produce species- and in certain cases strain- and gender-specific tumors in some organs of mice and rats. Male and female B6C3F1 mice gavaged with very high doses of PERC (stabilized with epichlorohydrin) for up to 78 weeks, exhibited an increase in hepatocellular carcinoma (NCI, 1977a). No increase in tumor incidence was seen in Osborne–Mendel rats. A significant increase in hepatocellular neoplasms (adenomas and carcinomas combined) was seen in B6C3F1 mice inhaling 100 or 200 ppm PERC for 2 years (NTP, 1986). There was also a low incidence of renal adenomas and carcinomas in male but not female F344 rats exposed for 2 years to 200 or 400 ppm PERC vapor. The incidence of renal neoplasms in the males was 1 of 49 controls, 3 of 49 exposed at 200 ppm, and 4 of 49 exposed at 400 ppm (NTP,

1986). Even though the changes were not statistically significant, it was noted that these particular tumors are *rare* in F344/N male rats, so they were thought to have been caused by PERC exposure. There was also a significant increase over controls in mononuclear-cell leukemia (MNL) in the male and female rats, but it was not clearly dose-dependent. The incidence of this form of leukemia can exceed 70% in F344 controls (Caldwell, 1999; Ishmael and Dugard, 2006). MNL in this rat strain apparently arises from large granular lymphocytes. This leukemic origin is very uncommon in humans (Caldwell, 1999). Gliomas were found in two female and four male F344/N rats exposed to PERC at 400 ppm and in one control male (NTP, 1986). The increase in incidence of this tumor was not statistically significant. Thus, the brain tumors were not considered by NTP (1986) to have been induced by exposure to PERC. The overall incidence of Leydig cell (testicular) tumors was 70%, 80%, and 82% in the 0-, 200-, and 400-ppm groups, respectively. Haseman et al. (1998) reported that NTP control F344 rats have an extremely high spontaneous incidence (89.1%) of Leydig cell tumors. Therefore, these tumors are believed to be irrelevant to men (Ishmael and Dugard, 2006). No increases in lung proliferative lesions were seen in B6C3F1 mice of either sex after inhalation of PERC at 100 or 200 ppm for 103 weeks, nor were lung neoplasms seen in male or female F344/N rats exposed at 200 or 400 ppm for 103 weeks (NTP, 1986).

Cancer Epidemiology Studies There have been many epidemiology studies of cancer incidence and mortality in groups of dry cleaners and other persons occupationally exposed to PERC (ATSDR, 1997c; NAS, 2009; EPA, 2008; Guyton et al., 2014). Some researchers have reported findings of excess incidences of different cancers, while others have not. Frequently, there was not a determination of the degree or duration of PERC exposure, or consideration of major confounding factors (e.g., exposure to other solvents/chemicals, smoking, alcohol consumption, socioeconomic status). Nevertheless, there was sufficient information for Weiss (1995) to conclude that cigarette smoking and alcohol consumption could only partially account for an increased rate of esophageal cancer in dry cleaners. In this instance kidney cancer incidence did not appear to be elevated. Blair et al. (2003) conducted and updated a mortality assessment of a cohort of dry cleaners and found an increased risk of death from esophageal cancer (Standardized Mortality Ratio [SMR] = 2.2, 95% confidence interval [CI] = 1.5 to 3.3). No exposure-response pattern was seen. Chang et al. (2005) did not find any cases of esophageal cancer in a Taiwanese cohort of electronics workers, while Lynge et al. (2006) observed a decreased incidence in dry cleaners in Nordic countries. Nevertheless, NAS (2009) concluded there was limited/suggestive evidence of an association between chronic PERC exposure and esophageal cancer.

There has been little evidence of an association between occupational PERC exposure and liver cancer. Increased incidences or mortality were not seen in cohort studies (Blair et al., 2003; Chang et al., 2005) or in a case–control study (Lynge et al., 2006). A rate ratio (RR) of just 0.76 (CI = 0.38 to 1.52) was estimated in the latter investigation of Nordic dry cleaning workers. A mortality odds ratio (MOR) of 2.57 (CI = 1.21 to 5.46) was reported by Lee et al. (2003) in another case–control study, but the exposure assessment was weak. The NAS (2009) concluded there was insufficient/inadequate evidence to determine whether an association exists between chronic PERC exposure and hepatobiliary cancer.

Epidemiology studies of populations exposed to PERC generally have shown no increase in risk of lung cancer. Blair et al. (2003), however, in an updated mortality analysis of dry cleaners, reported a SMR of 1.5 (CI = 1.2 to 1.9) for workers with medium to high exposures. This study adequately characterized exposures and included a relatively large number of subjects. Thus, the NAS (2009) concluded there is limited/suggestive evidence of an association between chronic PERC exposure and lung cancer.

Investigations of kidney cancer risk in PERC-exposed populations have yielded mixed results. A number of studies have not revealed an increased risk (Boice et al., 1999; Ruder et al., 2001; Blair et al., 2003; Mundt et al., 2003; Lynge et al., 2006). However, some of the assessments lacked power due to a limited number of study subjects. Although Blair et al. (2003) reported a very low SMR for kidney cancer deaths in dry cleaners with little or no PERC exposures; the SMR was 1.5 for those with medium or high exposure. Increased incidences have also been reported by Mandel et al. (1995), Pesch et al. (2000), and McCredie and Stewart (1993). The NAS (2009) concluded there is limited/suggestive evidence of an association between chronic PERC exposure and kidney cancer in humans. There are two higher NAS risk categories that require stronger evidence than "limited/suggestive."

There is limited evidence of associations between occupational PCE exposure and bladder cancer, non-Hodgkin lymphoma, and multiple myeloma (Guyton et al., 2014). In several surveys of dry cleaning workers, increasing risks were seen with increasing duration or intensity of exposure. Vlaanderen et al. (2014) concluded that their meta-analysis demonstrated an increased risk of bladder cancer in dry cleaners reported in both case–control and cohort studies, as well as some evidence of exposure-response relationships.

Risk Assessment A great deal of research has been conducted to characterize PERC's dose–response relationships, modes of action, and metabolism/toxicokinetics in rodents and humans. It is apparent from the previous discussion there is a considerable body of information available from human experience/exposures to PERC, as well as extensive data sets from studies in different species of laboratory animals. Both types of data are important in assessing risks to human health. Knowledge of the relevance of the animal data to humans is essential in order to meaningfully extrapolate from one species to another.

Humans appear to be less susceptible than rodents to the toxic or carcinogenic actions of PERC, as they are to those of TCE. Humans absorb less inhaled PERC and TCE, attain lower target organ doses of the parent compounds, have lower oxidative and GSH conjugation capacity, and inactivate epoxide intermediates more efficiently. Upon equivalent inhalation exposures of Wistar rats and humans to PERC, the rats excreted substantially larger amounts of TCA, DCA, and acetylated TCVC (Volkel et al., 1998). These observations were consistent with in vitro findings of greater conversion of PERC to TCVC by rat liver cytosol and of 10-fold higher β-lyase–mediated metabolism of TCVC by rat than by human kidney (Green et al., 1990). These data indicate that the human kidney has limited capacity to generate reactive metabolites from PERC by the GSH and β-lyase pathways. Biotransformation of PERC by the GSH conjugation pathway appears to be quite similar qualitatively, but not quantitatively in male rats and humans.

PERC-induced accumulation of α2μ-globulin in the P2 segment of renal proximal tubules and the accompanying cytotoxic and regenerative changes are *unique* to the male rat (Borghoff et al., 1990). α2μ-Globulin is synthesized under androgenic control in the liver of male rats, reabsorbed by the P2 segment, and undergoes hydrolytic digestion. Accumulation of α2μ in cytoplasmic droplets elicits tubular cell necrosis and compensatory cell proliferation (Goldsworthy et al., 1988). Sustained cell proliferation can promote clonal expansion of spontaneously or chemically initiated cells in proximal tubules to form preneoplastic and neoplastic lesions

(Swenberg, 1993). The increase in cell proliferation is reversible, as is the binding of halocarbon metabolites to α2μ-globulin. Thus, this mode of action is not genotoxic and is considered by IARC and the EPA to be irrelevant to humans. Melnick and Kohn, (1999), however, argue that some data are inconsistent with this conclusion, and that alternative mechanisms may exist. Doi et al. (2007) find some contribution of α2μ-globulin nephropathy to renal tumors produced by several structurally diverse chemicals in rats, but conclude that other critical processes are probably involved.

Several comprehensive reviews of PERC's toxicity and carcinogenicity have been published. Lash and Parker (2001) and Cichocki et al. (2016) compiled reviews of the metabolism, toxicity, and modes of toxicity of the chemical in laboratory animals and humans. The NAS (2009) recently published an evaluation of risks of use of TCE- and PERC-contaminated drinking water. It contained an extensive review of the metabolism, toxicity, and carcinogenicity of the two VOCs. The EPA (2008) released a draft document in support of its IRIS risk assessment, entitled *Toxicological Review of Tetrachloroethylene (Perchloroethylene)*. Quite recently, the NAS (2010) published a review of the EPA document. Questions were raised that called into question the soundness and reliability of EPA's proposed toxicity and cancer risk estimates. Stated weaknesses included a lack of critical analysis of data, preference given to studies (e.g., genotoxicity, epidemiological) showing positive results, and lack of an integrated consideration of the weight of evidence. The EPA subsequently published its final IRIS document (EPA, 2012). EPA classifies PER as "likely to be carcinogenic to humans." IARC (2014) classifies it as Group 2A (probably carcinogenic to humans), while NTP (2015) terms PCE "reasonably anticipated to be a human carcinogen."

1,1,1-Trichloroethane

1,1,1-Trichloroethane (methyl chloroform, TRI) is a widely used organic solvent. Its popularity as a metal degreaser, general purpose solvent, spot cleaner, and component of aerosols and a variety of household products increased substantially with the decline in manufacture of other halocarbons found to be high-dose rodent carcinogens and potential human carcinogens (ATSDR, 2006b). Utilization of TRI diminished during the 1990s, however, due to its ozone-depleting properties (Doherty, 2000). The volatile organic chemical (VOC) was to be phased out under the Montreal Protocol by 2002, but is still manufactured in the United States and utilized as a precursor for hydrofluorocarbons. TRI is also still present in some cleaning products. TRI was found in groundwater at 21% of hazardous waste sites surveyed in the United States (Pohl et al., 2008). TRI and other VOCs in groundwater can remain trapped for years and serve as a source of low-level exposure. The highest exposures to TRI occurred via inhalation in occupational settings, but people encounter the VOC at home by use of commercial products and tap water containing it. Ashley et al. (1994) reported TRI and other VOCs in the blood of 75% of 600+ nonoccupationally exposed individuals. TRI and TCE were also frequently detected in a subset of 982 adults examined in the NHANES III survey (Churchill et al., 2001), and in 951 members of the general population (Blount et al., 2006).

Toxicokinetics (TK) and Metabolism The TK of TRI has been characterized in rodents and in humans. TRI is rapidly and extensively absorbed from the lungs (Schumann et al., 1982) and GI tract. Systemic uptake and blood levels are elevated in humans inhaling TRI and other VOCs when they exercise. Dallas et al. (1989) observed that uptake of inhaled TRI diminished from 80+ % to 50% during 2 hours of exposure, due to its systemic accumulation as a result of slow metabolism. Monster (1979) similarly noted in humans that the capacity of the body to absorb TRI vapor was less than that for TCE, due to TRI's relatively poor metabolism. The bioavailability of comparable oral doses of TRI and TCE in rats is about 85% and 45%, respectively. TRI, like other VOCs, is distributed throughout the body, with fat achieving the highest concentrations (Schumann et al., 1982). As TRI biotransformation is quite limited, the VOC is cleared primarily by exhalation by rodents (Schumann et al., 1982; Reitz et al., 1988) and humans (Nolan et al., 1984). Prior exposure to CYP2E1 inducers such as ethanol can enhance the metabolic clearance of capacity-limited chemicals like TRI (Johns et al., 2006).

A number of physiologically based toxicokinetic (PBTK) models for TRI in rodents and in humans have been published. Lu et al. (2008) evaluated the suitability of each of these for use in the EPA IRIS database. The model of Reitz et al. (1988) proved the most satisfactory, in that it accurately predicted the time course of TRI in mouse, rat, and human blood for different inhalation scenarios. No empirical data were available for assessing the accuracy of simulations of brain TRI levels. Warren et al. (1998), however, demonstrated a high degree of correlation between brain and blood levels in TRI-exposed mice and rats, as well as reasonable correlation between brain levels and CNS effects of the VOC. TK data from human and rodent studies were used by Lu et al. (2008) to simulate internal dose metrics for appropriate human exposure scenarios for derivation of IRIS reference values.

Toxicity The primary pharmacological manifestation of acute or chronic inhalation of TRI is CNS depression, ranging in severity from slight headache or dizziness to anesthesia and death (ATSDR, 2006b). The current threshold limit value (TLV) of 350 ppm was established to prevent decrements in workers' mental and physical functions. Volunteers who inhaled 450 ppm TRI for 4 hours showed no effects on psychophysiological functions (Salvini et al., 1971). Two of 11 other subjects inhaling 500 ppm TRI 6.5 to 7 hours daily for 5 days exhibited difficulty balancing on one foot (Stewart et al., 1969). Muttray et al. (2000) found no psychophysiological effects in volunteers breathing 200 ppm TRI for 4 hours, but did report EEG changes and slight tiredness in their subjects. Warren et al. (2000) observed rapid, parallel increases in blood and brain TRI concentrations in mice inhaling the VOC. Mice breathing high vapor levels initially exhibited increased locomotor activity, followed by decreased activity. Mattsson et al. (1993) saw large evoked potential and EEG changes in F344 rats during inhalation of 2000 ppm TRI, but found no evidence of neurotoxicity (e.g., residual neurological functional or morphological changes) after 13 weeks of exposure of ≤2000 ppm 6 hours daily, 5 days per week. Most studies of long-term occupational exposures have not revealed residual neurological effects, although one assessment of workers subject to near-anesthetic vapor levels did reveal deficits in memory and balance (ATSDR, 2006b). It is worthy of note that very high inhaled concentrations of TRI, particularly when accompanied by hypoxia and stress, can sensitize the myocardium to catecholamines, producing cardiac arrhythmias (Reinhardt et al., 1973).

TRI has a very limited cytotoxic potential, ostensibly due to its limited biotransformation to relatively nontoxic metabolites. A near-lethal acute i.p. dose (3350 mg TRI/kg) was required to significantly enhance serum ALT activity in mice (Klaassen and Plaa, 1966). Male rats given a single oral dose of about 2500 mg/kg exhibited a transient increase in serum aspartate aminotransferase activity, but no ALT increase (Tyson et al., 1983). These enzymes are released from damaged or necrotic hepatocytes into the bloodstream. Male rats

gavaged five times weekly for as long as 12 days with up to 5 g TRI/kg died from effects of repeated, protracted CNS depression, but exhibited only slight hepatotoxicity (Bruckner et al., 2001). Quast et al. (1988) saw only minimal histological changes in the liver of F344 rats inhaling 1500 ppm TRI 6 hours daily, five times/week for up to 2 years. Rats gavaged daily for 21 days with a high dose of TRI showed increased urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity, but no microscopic evidence of injury indicative of renal toxicity (NTP, 1996). Liver and/or kidney injury are usually absent or quite modest in occupationally exposed populations, even in fatal cases (ATSDR, 2006b). A report by Hodgson et al. (1989) is an exception. They described four TRI-exposed workers who exhibited elevated serum ALT activity and fatty vacuolation of hepatocytes. In addition, Brogen et al. (1986) reported that 10% of a group of metal workers exposed to TRI, TCE, and freon 113 had elevated NAG activity in their urine.

Potential Carcinogenicity The NCI (1977b) conducted a 78-week study in which B6C3F1 mice and F344 rats of both sexes received high doses of TRI daily by gavage. There was no increase in cancers attributable to TRI. In a screening study, Maltoni et al. (1986) observed an apparent increase in leukemias in male and female Sprague–Dawley rats gavaged with 500 mg TRI/kg/day for 104 weeks. Statistical analyses were not presented, and the authors stated that definite conclusions could not be drawn from their work due to limitations in the design and size of the experiment. A two-year inhalation study in B6C3F1 mice and F344 rats of both sexes revealed no evidence of tumorigenicity due to TRI (Quast et al., 1988). Few epidemiological studies of TRI-exposed populations have been conducted. Infante-Rivard et al. (2005) did report a high risk (odds ratio [OR] = 7.55, 95% CI = 0.92 to 61.97) of childhood leukemia in offspring of women exposed to TRI from 2 years before pregnancy up to birth. Gold et al. (2011) reported an OR of 1.8 (1.1 to 2.9) for multiple myeloma in persons exposed to TRI. It should be recognized that workers are commonly exposed to multiple solvents in the workplace. TRI is currently designated as having inadequate information for assessment of human carcinogenic potential by the EPA (2007a).

Methylene Chloride

Methylene chloride (dichloromethane, MC) has enjoyed widespread use as a solvent in industrial processes, manufacture of drugs, degreasing agents, aerosol propellants, agriculture, and food preparation. MC was commonly used to decaffeinate coffee and tea. Thus, large numbers of people have been exposed occupationally and in the home. The primary route of exposure to this very volatile solvent is inhalation. The preponderance of MC escaping into the environment does so by volatilization (ATSDR, 2000a). The VOC is also frequently found in wastewater discharges and in air and water at hazardous waste sites (Pohl et al., 2008). In 2012, a total of 3.4 million pounds of MC was released into the environment in the United States (EPA, 2015).

Metabolism The TK of MC has been well characterized in humans and rodents. MC metabolism occurs primarily in the liver but also in the lung. MC is rapidly absorbed and distributed throughout the body (Angelo et al., 1986). Inhaled MC reached a near steady state in the blood of human subjects with 1 to 2 hours of continuous exposure (DiVincenzo and Kaplan, 1981). Less than 5% of the absorbed dose was exhaled unchanged. Some 25% to 34% was exhaled as carbon monoxide (CO), the major end-metabolite of MC. Exposure of the volunteers to 50, 100, 150, and 200 ppm for 7.5 hours produced peak blood carboxyhemoglobin saturations of 1.9%, 3.4%, 5.3%, and 6.8%, respectively. MC was very rapidly eliminated from the body and did not accumulate over a five-day exposure regimen. As shown in Fig. 24-7, metabolism of MC in humans and rodents is thought to occur via three pathways (Andersen et al., 1987). One entails cytochrome P450 2E1 (CYP2E1)-catalyzed oxidation to CO via formyl chloride, a reactive intermediate. The second, a glutathione (GSH)-mediated pathway, involves the theta-class GSH S-transferase (GST), GST-T1. The GST-catalyzed pathway results in the formation of a GSH conjugate that is eventually metabolized to carbon dioxide. The conjugation of MC with GSH results in formation of two reactive intermediates that have been proposed to be involved in MC toxicity, *S*-(chloromethyl) GSH and formaldehyde. Oxidation is a high-affinity, low-capacity pathway that predominates at the relatively low MC concentrations present in occupational and environmental settings. The GST conjugation is a low-affinity, high-capacity pathway operative at the high exposure levels used in cancer bioassays (Green, 1997b). With the third and minor pathway, it is postulated that CO_2 is also formed via the oxidative pathway by reaction of formyl chloride with a nucleophile such as GSH (Watanabe and Guengerich, 2006). The abilities of different species to metabolize MC in the liver by the GST pathway are as follows: mouse>>rat>human high conjugators>hamster>human non-conjugators (Reitz et al., 1989; Thier et al., 1998). Interindividual variation in the ability to biotransform MC via GST-T1 is associated with genetic polymorphisms in humans (Haber et al., 2002).

Physiologically based TK (PBTK) models for MC have been developed to assess the relative importance of the oxidative and GST pathways in MC's toxicity and carcinogenicity. A PBTK model by Andersen et al. (1987) was validated by comparing simulations of blood MC time-course data with data from experiments with mice, rats, and humans. Tumor incidences in mice in chronic bioassays by NTP (Mennear et al., 1988) and Serota et al. (1986a, 1986b) were consistent with model predictions of liver and lung doses of GSH metabolites, but not oxidative metabolites. After extensive review, the EPA adopted this model as a reasonable means of extrapolating NTP bioassay results in mice to humans. This was the first use of PBTK modeling by EPA in a cancer risk assessment. Other investigators such as Reitz et al. (1988, 1989) published deterministic models for MC, which also provided internal dosimetry point estimates of GST pathway metabolites. Forecasts of relatively low tissue doses of GST metabolites in humans resulted in part from the need to saturate the oxidative pathway before appreciable GSH metabolites could be formed.

Recently, probabilistic PBTK models for MC have been developed, which allow for inclusion of intra- and interspecies variability in model predictions, as well as quantitative assessment of model uncertainty. Values for model input parameters and TK data from rodent studies from a variety of sources were subjected to Markov Chain Monte Carlo (MCMC) analysis, a Bayesian optimization technique. With this approach, the prior input information can be combined to obtain posterior distributions of key model parameters. El-Masri et al. (1999) and Jonsson and Johanson (2001) used Bayesian analysis solely to evaluate the influence of GST-T1 polymorphism on human cancer risk. Marino et al. (2006) used MCMC analysis to develop a probabilistic PBTK model for MC in mice. The resulting dose metrics (mg MC metabolized by GST/L tissue/day) were 3- to 4-fold higher than contemporary EPA estimates. David et al. (2006) applied this modeling approach to humans. Inclusion of GSH nonconjugators resulted in a unit cancer risk estimation 500-fold lower than the EPA unit risk at that time. The EPA (2010a) concluded in an extensive assessment that this was the best available PBTK model, despite some uncertainties.

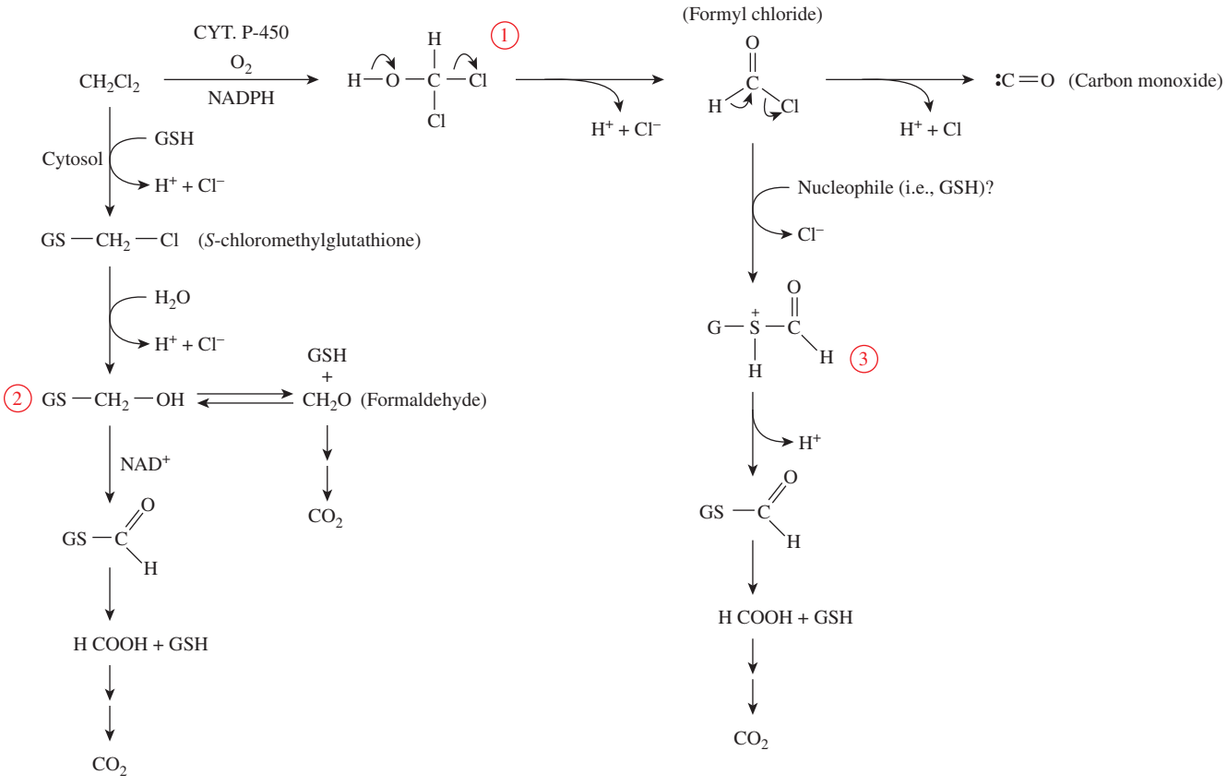

Figure 24-7. *Proposed pathways for methylene chloride (CH_2Cl_2) metabolism.* (1) Mixed function oxidase pathway; (2) glutathione transferase pathway; and (3) nucleotide pathway. (Modified from Andersen et al., 1987, with permission from Elsevier.)

Modes of Toxicity/Carcinogenicity MC has a quite limited cytotoxicity potential. Male and female B6C3F1 mice consuming up to about 2000 mg MC/kg/day in their drinking water for 90 days showed no adverse effects (Kirschmann et al., 1986). Similarly dosed male and female F344 rats exhibited mild-to-moderate hepatocellular lipid vacuolation and elevated serum enzyme activities at daily dosage levels as low as 166 to 209 mg/kg/day (Kirschmann et al., 1986). Hepatic centrilobular vacuolation and focal necrosis occurred in the liver of rats inhaling 500 to 4000 ppm MC 6 hours/day, 5 days/week for 2 years (Burek et al., 1984; Mennear et al., 1988). Manifestations of kidney damage have been rare in laboratory animals, but have occasionally been reported in persons subjected to high vapor levels (ATSDR, 2000a; EPA, 2010a). There is little information on the identity of MC metabolites that adversely affect the liver or kidney. As described previously, the CO formed by oxidation of MC binds to hemoglobin to produce dose-dependent increases in carboxyhemoglobin. Offspring of pregnant rats inhaling low concentrations of CO have been reported to exhibit permanent learning and memory impairment (De Salvia et al., 1995). It is generally accepted that tissue hypoxia can contribute to CNS-depressant effects of MC. There are few reports of residual neurological dysfunction in MC-exposed workers (Lash et al., 1991; ATSDR, 2000a).

There has been a great deal of research to define mechanisms of MC carcinogenicity, in order to more clearly understand the relevance of the murine tumors to humans (Green, 1997b). Liver and lung tumors in mice do not seem to be associated with overt cytotoxicity or increased replicative DNA synthesis (Maronpot et al., 1995). Induction of the tumors in mice is generally considered to be due to a reactive intermediate generated via the GST pathway (Andersen et al., 1987). GST-T1 in liver and lung catalyzes conversion of MC to S-(chloromethyl)glutathione ($GSCH_2Cl$), which apparently breaks down rapidly to GSH and formaldehyde. Both $GSCH_2Cl$ and formaldehyde are reactive with DNA. MC is usually mutagenic in bacterial assays containing GSH/GST activity. MC produces DNA single-strand breaks (SSBs) in vitro in mouse hepatocytes and lung Clara cells, in which GST is localized in the nucleus (Mainwaring et al., 1996). DNA SSBs were induced in the mouse hepatocytes by a 60-fold lower MC concentration than in rat hepatocytes. Rat liver does not show preferential nuclear localization of GST-T1. Some human hepatocytes apparently exhibit nuclear localization, others cytoplasmic (Sherratt et al., 2002). Negative results have been seen in a variety of genotoxicity assays with rat or hamster cell lines with little or no GST activity (EPA, 2010a). Positive results for sister chromatid exchanges, chromosomal aberrations, and the micronucleus test have been obtained in experiments with human cell lines and isolated cells. Negative results were seen in unscheduled DNA synthesis, DNA SSBs, and DNA-protein cross-links (DPX). There is limited evidence of formation of $GSCH_2Cl$ DNA adducts in some hybrid in vitro systems, but the adducts' instability presents considerable technical challenges to their study. They have yet to be isolated in vivo. Formaldehyde produces both DPX and SSBs, indicative of its prominent role in MC's carcinogenicity (Graves and Green, 1996).

Cancer Bioassays in Rodents High, chronic exposures to MC have been found to produce species- and gender-specific tumors in some organs of mice and rats. Serota et al. (1986a, 1986b)

administered a series of doses of MC to F344 rats and B6C3F1 mice in their drinking water for 2 years. The male mice showed a trend for an increase in hepatocellular adenomas or carcinomas, but the modest response was not dose-dependent. There was a statistically significant increase in neoplastic nodules or hepatocellular carcinoma in some groups of female F344 rats. Burek et al. (1984) saw a significant increase in salivary gland sarcomas in male Sprague–Dawley (S-D) rats that inhaled 3500 ppm 6 hours/day, 5 days/week for 2 years. The number of benign mammary tumors per tumor-bearing female S-D rat increased with increasing exposure level, though the number of tumor-bearing rats was not significantly elevated over controls. Similarly exposed hamsters were unaffected. In a two-year follow-up study (Nitschke et al., 1988), female S-D rats inhaling 500 ppm MC also only exhibited an increased number of benign mammary tumors per tumor bearing rat. No increase in malignant tumors was manifest in male rats. NTP conducted an inhalation study, in which F344/N rats and B6C3F1 mice inhaled up to 4000 ppm MC 6 hours/daily, five times weekly for 2 years (Mennear et al., 1988). There were weak trends for neoplastic nodules or hepatocellular carcinoma, as well as benign mammary tumors in female rats. Male and female mice inhaling 2000 or 4000 ppm MC exhibited statistically significant, dose-dependent increases in hepatocellular adenoma and carcinoma, as well as bronchoalveolar adenoma and carcinoma. There were similar findings in a follow-up study of female B6C3F1 mice inhaling 2000 ppm MC for 2 years (Maronpot et al., 1995). The incidences of these hepatic and lung tumors in control B6C3F1 mice are quite high (Haseman et al., 1998).

Cancer Epidemiology Studies Despite a substantial number of epidemiology studies of MC-exposed workers, evidence of associations between MC and specific tumors is not strong. There have been four cohort mortality studies of employees at facilities where MC was used as a solvent for cellulose acetate. There were no increased risks of cancer mortality for all tissues or for lung or breast. In just one assessment was there an elevated risk (standardized mortality ratio [SMR] = 2.98 [95% CI = 0.81 to 7.63]) of death from liver and biliary tract cancer (Lanes et al., 1993). This was apparently the sole report of increased hepatic cancer mortality in an occupational population. No investigations provide evidence of an association between MC and lung or kidney cancer (ATSDR, 2000a; EPA, 2010a). Cantor et al. (1995) conducted a case–control study of 33,509 occupationally exposed women, but found little association between MC exposure probability and breast cancer mortality. Blair et al. (1998), however, reported a rate ratio (RR) of 3.0 (1.0 to 8.8) for breast cancer in 3605 women employed at Hill Air Force Base. Blair et al. (1998) also estimated RRs of 3.0 (0.9 to 10.0) and 3.4 (0.9 to 13.2) for non-Hodgkin's lymphoma and multiple myeloma, respectively, in MC-exposed aircraft maintenance employees of both sexes. Occasionally, excess risks of other cancers have been found in highly exposed groups. Gibbs et al. (1996), for example, computed a statistically significant SMR of 2.08 for prostate cancer death of cellulose acetate workers with a latency period of at least 20 years since their first exposure to 350 to 700 ppm MC. In a study of association between MC and astrocytic brain cancer, Heineman et al. (1994) calculated an odds ratio (OR) of 2.4 for males with a high probability of MC exposure and for intense exposure versus unexposed controls. Lastly, Infante-Rivard et al. (2005) reported an increased risk of childhood leukemia (OR = 3.22 [0.88 to 11.7]) in offspring of mothers with probable or definite occupational MC exposure. Nevertheless, most investigations have revealed weak or no apparent associations between relatively high MC inhalation exposures in industry and cancers (Dell et al., 1999; Starr et al., 2006).

Risk Assessment A considerable body of scientific information supports the following conclusion: Should MC be a carcinogen in humans, it is much less potent than in rodents, notably mice. DPX were detected in hepatocytes that had been isolated from B6C3F1 mice and incubated for 2 hours with MC. DPX were not found in hepatocytes of F344 rats, Syrian golden hamsters, or three human subjects (Casanova et al., 1996, 1997). RNA-formaldehyde cross-links, however, were found in hepatocytes of all species. These links were 4-, 7-, and 14-fold higher in cells from mice than in cells from rats, humans, and hamsters, respectively. Metabolism of MC via the GSH pathway is an order of magnitude greater in mouse than in rat liver. Metabolic rates in hamster and human liver are even lower (Reitz et al. 1989; Thier et al., 1998; Sherratt et al., 2002). High GST-T1 activity was quantified in the nuclei of mouse centrilobular hepatocytes. Mice may be unique, in that the extensive metabolic activation of MC to an unstable intermediate occurs in the proximity of the DNA. It would be useful in future PBTK models to include subcompartments for cytosolic and nuclear GST activities (Starr et al., 2006). GST-T1 was also detected in relatively high levels in mouse lung Clara cells and ciliated cells at alveolar/bronchiolar junctions (Mainwaring et al., 1996). Clara cells are present in much lower numbers in rats, and are rare in human lungs. $GSCH_2Cl$ apparently causes SSB in vivo and in vitro in DNA of mouse liver and lung (Graves et al., 1995). No DNA breaks were detected in hamster or human hepatocytes in vitro.

The EPA (2010a) has recently concluded that MC is likely to be carcinogenic in humans and appears to act via a mutagenic mode of action. The designation of "likely to be carcinogenic in humans" was based largely upon the NTP (Mennear et al., 1988) findings of cancer at two sites (liver and lung) in male and female B6C3F1 mice. More limited findings of certain tumors in MC-exposed rats were considered to be supporting evidence. Epidemiological studies were said to provide some evidence of an association between occupational MC exposure and brain and liver cancer. As described in the previous paragraph, bioactivation of MC is qualitatively, but not quantitatively similar in mice and humans. Due to the similarity, the EPA (2010a) reasons that the apparent mode of action (mutagenicity of GST pathway metabolites) is biologically plausible in humans.

Carbon Tetrachloride

Carbon tetrachloride (CCl_4) previously enjoyed widespread use as a solvent, cleaning agent, fire extinguisher, synthetic intermediate, grain fumigant, and human anthelmintic. Its use has steadily declined since the 1970s, due to its hepatorenal toxicity, carcinogenicity, and contribution to atmospheric ozone depletion (ATSDR, 2005). Nevertheless, CCl_4 appears to be ubiquitous in ambient air in the United States, and it is still found in some water wells and waste sites. CCl_4 is a classic hepatotoxicant, but kidney injury is often more severe in humans.

The time course of CCl_4-induced acute liver injury has been well characterized (ATSDR, 2005). Early signs of hepatocellular injury in rats include dissociation of polysomes and ribosomes from rough endoplasmic reticulum, disarray of smooth endoplasmic reticulum, inhibition of protein synthesis, and triglyceride accumulation. Hypomethylation of RNA is thought to contribute to inhibition of lipoprotein synthesis, thereby playing a role in steatosis (Clawson et al., 1987). Ingested CCl_4 reaches the liver, undergoes metabolic activation, produces lipid peroxidation, covalently binds, and inhibits microsomal ATPase activity within minutes in rats. Single cell necrosis, evident 5 to 6 hours postdosing, progresses to

maximal centrilobular necrosis within 24 to 48 hours. Most microsomal enzyme activities are significantly depressed (Recknagel et al., 1989). A variety of cytoplasmic enzymes are released from dead and dying hepatocytes into the bloodstream. The activity of these enzymes in serum generally parallels the extent of necrosis in the liver. Cellular regeneration, manifest by increased DNA synthesis and cell cycle progression, is maximal 36 to 48 hours postdosing (Rao et al., 1997).

The bioactivation of CCl_4 is required for its conversion to a variety of cytotoxic metabolites. It is widely recognized that CCl_4 is bioactivated by cytochromes P450 via reductive dehalogenation to trichloromethyl radicals (CCl_3•), which can react in turn with oxygen to form trichloromethyl peroxy free radicals (CCl_300•). Both unstable radicals bind covalently to a variety of cellular components including enzymatic and structural proteins and polyunsaturated fatty acids in membranes. This results in lipoperoxidation, loss of intracellular and cellular membrane integrity, and leakage of enzymes (Plaa, 2000; Weber et al., 2003). By-products of lipid peroxidation include reactive aldehydes, which can form adducts with proteins and DNA, contributing to cytotoxicity and carcinogenicity, respectively (Manibusan et al., 2007). Liu et al. (1995) have proposed that CCl_4 oxidative stress in the liver enhances nuclear factor kappa B activity, which in turn promotes expression of pro-inflammatory cytotoxic cytokines. Shi et al. (1998) proposed apoptosis as an additional/alternate mechanism of CCl_4-induced cell death.

Perturbation of intracellular calcium (Ca^{2+}) homeostasis appears to be an integral part of CCl_4 cytotoxicity (Stoyanovsky and Cederbaum, 1996). Increased cytosolic Ca^{2+} levels may result from influx of extracellular Ca^{2+} due to plasma membrane damage and from decreased intracellular Ca^{2+} sequestration. Elevation of intracellular Ca^{2+} in hepatocytes can cause activation of phospholipase A_2 and exacerbation of membrane damage (Glende and Recknagel, 1992). Elevated Ca^{2+} may also be involved in alterations in calmodulin and phosphorylase activity, as well as changes in nuclear protein kinase C activity (Omura et al., 1999). High intracellular Ca^{2+} levels activate a number of catabolic enzymes including proteases, endonucleases, and phospholipases, which kill cells via apoptosis or necrosis (Weber et al., 2003). The hydrolytic enzyme calpain mediates progression of acute CCl_4-induced liver injury by leaking from dying hepatocytes and attacking neighboring cells (Limaye et al., 2003). Ca^{2+} may stimulate the release of cytokines and eicosanoids from Kupffer cells. Edwards et al. (1993) demonstrated that destruction of Kupffer cells prior to CCl_4 dosing of rats resulted in significant reductions in neutrophil infiltration and hepatocellular injury. Macrophages are known to release a number of inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), that are cytotoxic (Morio et al., 2001).

Development of cellular resistance and tissue repair are important in limiting CCl_4 hepatotoxicity, and in recovery (Mehendale, 2005). Alterations in transmembrane carrier proteins have been discovered in hepatocytes of CCl_4-treated mice. CCl_4 results in reduced expression of genes associated with extraction of bile acids and organic ions from sinusoidal blood, as well as upregulation of certain detoxification genes (Aleksunes et al., 2005). CCl_4 also produces differential upregulation of multidrug resistance proteins that are involved in export of oxidative stress products and metabolites. Hepatocellular regeneration has been shown to begin within 6 hours of a small dose of CCl_4, just as centrilobular necrosis is becoming evident (Lockard et al., 1983). This early-phase regeneration (arrested G_2 hepatocytes activated to proceed through mitosis) is followed at about 24 hours by the secondary phase of regeneration (hepatocytes mobilized from G_o/G_1 to proceed through mitosis; Bell et al., 1988). CCl_4 hepatotoxicity is obviously a complex, multifactorial process that is likely to continue to receive attention from researchers.

CCl_4 has frequently been used as a model hepatotoxic compound with which to examine the influence of various factors that alter P450s. CYP2E1 is primarily responsible for catalyzing the bioactivation of low doses of CCl_4 in humans. CYP3A contributes to the metabolism of higher doses (Zangar et al., 2000). The preeminent role of Cyp2e1 in animals is clearly demonstrated by the protection afforded CCl_4-treated rodents by Cyp2e1 antibody (Castillo et al., 1992), the Cyp2e1 inhibitor 3-amino-1,2,4-triazole (Padron et al., 1996), and the absence of CYP2E1 expression (Wong et al., 1998). As discussed previously in the subsection on P450 inducers, a variety of conditions that induce CYP2E1 potentiate CCl_4 hepatotoxicity in test animals and humans. Sufficient doses of CYP2E1 inhibitors, including natural constituents of foods (described in the subsection on P450 inhibitors), can inhibit CCl_4 toxicity (Lieber, 1997). Taieb et al. (2005) discovered protein 8, a transcription factor that regulates the expression of genes that protect cells from stress, rapidly triggered Cyp2e1 downregulation in CCl_4-dosed mice, thereby minimizing CCl_4 bioactivation.

CCl_4 has been found to be a hepatocarcinogen in rodents, but there is relatively little experimental evidence of genotoxicity or carcinogenicity in humans (ATSDR, 2005; EPA, 2010b). There have been extensive studies of its potential genotoxic and mutagenic effects, but the results are largely negative in bacterial and in mammalian systems. Araki et al. (2004) did report that CCl_4 was mutagenic in a strain of *E. Coli* that is particularly sensitive to oxidative damage. As early as the 1940s, the National Cancer Institute conducted a series of chronic bioassays in which very high oral bolus doses of CCl_4 were given to mice and rats. Large increases over controls in incidences of liver tumors were found in male and female mice (ATSDR, 2005). Relatively little was seen in rats, but hamsters were susceptible to CCl_4-induced liver cancer. Nagano et al. (2007) published the results of a study in which F344 rats and BDF1 mice of both sexes were exposed 6 hours daily, five times weekly for 104 weeks to 0, 5, 25, or 125 ppm CCl_4 vapor. There was a significant increase in hepatocellular adenomas and carcinomas in the male and female rats at the highest exposure level. Significant increases in these tumors and in adrenal pheochromocytomas were manifest in the 25- and 125-ppm male and female mice. There was a statistically significant, but more modest elevation in hepatocellular adenomas, but not carcinomas in the 5-ppm female mice. Degenerative and necrotic hepatic changes were seen in the liver of all groups of animals with liver tumors except the 5-ppm female mice. There has been limited evidence of associations between occupational CCl_4 exposure and certain cancers described in some epidemiology studies, but the data were not conclusive. Exposures to CCl_4 were poorly characterized and confounded by the concurrent exposures of most subjects to other chemicals in the workplace. There were no reported associations with liver cancer.

The weight of scientific evidence indicates that CCl_4 is more likely an indirect than a direct acting mutagen/carcinogen (EPA, 2010b). Manibusan et al. (2007) concluded that sustained cell death, regeneration and proliferation enhance the likelihood of unrepaired spontaneous-lipid peroxidation and endonuclease-induced mutations that may lead to hepatocarcinogenesis. Jiang et al. (2004) observed changes in expression of genes involved in cell death, proliferation, DNA damage, and fibrogenesis in livers of mice given a high CCl_4 dose daily for 4 weeks. Four weeks after cessation of this treatment, most gene expression profiles returned to control levels, except fibrogenesis. Bioactivated CCl_4 can apparently exert modest genotoxic effects, such as DNA breakage and related sequelae only under highly cytotoxic conditions. ACGIH (2011) assigned

CCl_4 the designation of A2 (suspected human carcinogen), in light of its threshold mode of action and its very weak or absent genotoxicity. Germany placed CCl_4 into its category 4, indicating that genotoxicity plays no, or at most a minor role in its mode of action (MAK, 2011). IARC (1999) classified CCl_4 as Group B2 "possibly carcinogenic to humans." The EPA (2010b), however, categorized the chemical as "likely to be carcinogenic to humans." Despite acknowledging the correlation between hepatocellular cytotoxicity, regenerative hyperplasia, and induction of liver tumors in rodents, concern about the reactivity of direct and indirect products of CCl_4 metabolism and about limited knowledge of key events led the agency to conclude that "the mode of action was unknown." This led to use of a linear model to estimate human cancer risks from oral and inhalation exposures. PBPK models were used to estimate mouse internal doses and human equivalent doses as part of this risk assessment.

Chloroform

The primary use of chloroform ($CHCl_3$, trichloromethane) is in the production of the refrigerant chlorodifluoromethane (Freon 22), but this use is diminishing as chlorine-containing fluorocarbons are phased out under the Montreal Protocol. $CHCl_3$ was among the first inhalation anesthetics, but it was replaced by safer compounds after about 1940. $CHCl_3$ is a by-product of drinking water chlorination and has been quantified in municipal drinking water supplies in concentrations as high as several hundred ppb, although concentrations are usually less than 25 ppb (ATSDR, 1997a). $CHCl_3$ has also been found in ppb concentrations in swimming pool water and surrounding air (Aggazzotti et al., 1995). Like many other halocarbons, $CHCl_3$ can invoke CNS symptoms at subanesthetic concentrations similar to those of alcohol intoxication and can sensitize the myocardium to catecholamines, possibly resulting in cardiac arrhythmias. A recent 28-day drinking water study in female B6C3F1 mice at levels up to 250 ppm failed to show any biologically relevant changes in immune parameters (Auttachoat et al., 2009).

The reproductive and developmental toxicities of $CHCl_3$ are rather unremarkable. Schwetz et al. (1974) found that inhalation of 100 to 300 ppm $CHCl_3$ by pregnant rats caused a high incidence of fetal resorption, retardation of fetal development, and a low incidence of fetal anomalies. Murray et al. (1979) reported that gestational exposure to 100 ppm $CHCl_3$ resulted in the decreased ability of mice to maintain pregnancy, as well as cleft palate, decreased fetal weight and length, and decreased ossification in pups. Very high $CHCl_3$ concentrations retarded development and induced diffuse cell death in cultured rat embryos (Brown-Woodman et al., 1998). These studies support $CHCl_3$ as a weak teratogen, but negative studies employing maternally toxic doses argue against this characterization (Thompson et al., 1974; NTP, 1997a). The EPA's 2001 IRIS profile for $CHCl_3$ notes that in reproductive/developmental studies, maternal toxicity and fetal effects occurred at doses higher than those that produced liver toxicity.

Hepatotoxicity serves as the basis for EPA's benchmark dose-based RfD (EPA, 2001b). Under certain conditions $CHCl_3$ is hepatotoxic and nephrotoxic. These toxicities are potentiated by aliphatic alcohols, ketones, DCA, and TCA (Davis, 1992). Albeit at low doses, numerous disinfection by-products such as the rodent carcinogens TCA and DCA are routinely consumed with $CHCl_3$ in finished drinking water. For this reason, mixture studies are particularly relevant. Consider, for example, the studies of Pereira et al. (2001) in which *N*-methyl-*N*-nitrosourea-initiated B6C3F1 mice were exposed to DCA with or without $CHCl_3$. $CHCl_3$ prevented hypomethylation, which would have resulted in increased mRNA expression of the proto-oncogene c-myc and promotion of liver tumors by DCA. Conversely, $CHCl_3$ increased DCA-induced DNA hypomethylation and enhanced the DCA promotion of kidney tumors. Thus, concurrent exposure to two rodent carcinogens, $CHCl_3$ and DCA, resulted in less than additive activity in one organ and synergism in another (Pereira et al., 2001; Tao et al., 2005). This exemplifies the difficulty in assessing the risks posed by solvent mixtures.

Tolerance or adaptation to $CHCl_3$'s hepatorenal toxicity and carcinogenicity has been observed in some mouse strains after repeated exposure. This phenomenon was first investigated by Pereira and Grothaus (1997) who reported preexposure of mice to low doses of $CHCl_3$ in drinking water induced resistance to hepatotoxicity and cell proliferation following a higher gavage dose. This was presumed at the time to result from suicidal inhibition of the cytochrome P450s (CYPs) responsible for $CHCl_3$'s activation. More recently, mice exposed daily for 7, 14, and 30 days were found to have a robust regenerative response in target tissues, which prevented the progression of injury (Anand et al., 2006b). Blood and tissue levels of $CHCl_3$ after repeated exposure were substantially lower than those following a single exposure, owing to increased elimination of $CHCl_3$ via exhalation. These same researchers also reported that priming mice with $CHCl_3$ prior to a lethal dose stimulated compensatory hepatogenic and nephrogenic repair, limiting the progression of injury and resulting in 100% survival. Relative to unprimed mice, there was no difference in hepatic or renal Cyp2e1 activity, though glutathione (GSH) and GSH reductase activity were upregulated in the kidney (but not in the liver), with a consequent decrease in renal covalent binding. The area under the blood $CHCl_3$ versus time curve (AUC) was 40% lower in primed versus unprimed mice, but increased elimination via exhalation was not responsible for the reduction in internal exposure in this particular case (Philip et al., 2006). Taken together, these studies suggest that TK and TD factors contribute to the tolerance observed to $CHCl_3$ toxicity. Mehendele and colleagues have conducted a series of other studies with $CHCl_3$, alone or in combination with other hepatotoxicants, to further discern the role of tissue repair in toxicant-induced injury. These studies have provided valuable insight into the importance of both the timing of the tissue repair response and its magnitude as pivotal determinants of the outcome of toxicant-induced injury, emphasizing the need to consider repair processes in predictive toxicology (Anand et al., 2003, 2005; Mehendale, 2005).

The status of $CHCl_3$ as a rodent carcinogen is indisputable. It causes liver and kidney tumors that are species-, strain-, sex-, and route of exposure–dependent. $CHCl_3$-induced liver tumors in mice and their dependence on ongoing liver necrosis were reported near the end of World War II (Eschenbrenner and Miller, 1945). These same authors observed that male but not female mice suffered kidney necrosis. This observation was supported by the report of Roe et al. (1979) that $CHCl_3$ ingested in a toothpaste base resulted in renal tumors in male but not female mice. This sex difference is thought to be attributable to testosterone-mediated differences in renal Cyp activity (Smith et al., 1984). The NCI (1976) cancer bioassay demonstrated renal tumors in male rats and an extremely high incidence of liver tumors in both sexes of B6C3F1 mice gavaged with $CHCl_3$ in corn oil. In 1985, Jorgenson and colleagues reported that daily doses of $CHCl_3$ in drinking water comparable to those in the NCI gavage assay also produced renal tumors in rats, but failed to cause liver tumors in B6C3F1 mice. This finding provided evidence that the dose rate of $CHCl_3$ was a determinant of liver tumor formation, supporting the existence of a threshold mechanism. Hard et al. (2000) have reevaluated the kidneys from the Jorgenson et al. (1985) study and have confirmed the presence

of chronic renal tubule injury, indicative of renal tumor formation via a non-genotoxic mechanism. Furthermore, in vivo studies using CYP450 reductase knockout mouse models have provided evidence that renal CYP450 enzymes, as opposed to those of hepatic origin, play an essential role in $CHCl_3$ renal toxicity (Fang et al., 2008; Liu et al., 2013). In what may be a landmark study, Larson et al. (1994) compared cytotoxicity and cell proliferation in female B6C3F1 mice given $CHCl_3$ by gavage in corn oil versus ad libitum ingestion in drinking water. As seen in Fig. 24-8, the hepatocyte-labeling index, a measure of the proliferative response, differed between the two exposure regimens at comparable doses. Pereira (1994) reported essentially the same observation. This suggests that ingestion of $CHCl_3$ in small increments, similar to drinking water patterns of humans, fails to produce a sufficient amount of cytotoxic metabolite(s) per unit time to overwhelm detoxification and other protective mechanisms.

Potentiation of $CHCl_3$'s toxicity by CYP inducers and protection by GSH and P450 inhibitors suggest that a metabolite, presumably phosgene, is responsible for $CHCl_3$'s hepatorenal toxicity. Both target organs metabolize $CHCl_3$ to phosgene. There is evidence that CYP2E1 and CYP2B1/2 metabolically activate $CHCl_3$. The former isoform is thought to catalyze $CHCl_3$ metabolism at a lower substrate concentration than the latter (Nakajima et al., 1995). By using an irreversible CYP2E1 inhibitor in Cyp2e1-knockout mice, Constan et al. (1999) have demonstrated that metabolism of $CHCl_3$ by Cyp2e1 is required for liver and kidney necrosis and cell proliferation. The electrophilic intermediate generated by $CHCl_3$'s metabolism (i.e., phosgene) is initially detoxified by covalently binding to cytosolic GSH, though suggestive evidence of an antioxidative role for metallothionein also exists (Abbassi et al., 2010). Once GSH is depleted, phosgene is free to covalently bind to hepatic and renal proteins and lipids. Such binding damages membranes and other intracellular structures, leading to necrosis and subsequent reparative cellular proliferation. Sustained proliferation with repeated exposures promotes tumor formation in rodents by irreversibly "fixing" spontaneously altered DNA and clonally expanding initiated cells. The expression of certain genes, including *myc* and *fos*, is altered during regenerative cell proliferation in response to $CHCl_3$-induced cytotoxicity (Sprankle et al., 1996; Kegelmeyer et al., 1997). While the identity of phosgene's intracellular targets is largely unknown, Guastadisegni et al. (1999) have reported that phosgene reacts with phosphatidylethanolamine (PE). The adduct formed appears to consist of two PE moieties cross-linked at the amino head groups by the carbonyl moiety of phosgene. $CHCl_3$-modified PE preferentially accumulates on inner mitochondrial membranes, inducing ultrastructural modifications and inhibiting functions of the organelle. These researchers observed the induction of hepatic apoptosis and necrosis in $CHCl_3$-treated rats and pointed out that apoptosis may be initiated by the release of regulatory factors normally sequestered in mitochondria, in particular Ca^{2+}. Evidence that Ca^{2+} perturbation plays a role in $CHCl_3$ toxicity comes from a report of Ca^{2+} mobilization in Madin–Darby canine kidney cells using Fura-2 as a Ca^{2+} probe. $CHCl_3$, albeit in millimolar concentrations, increased the cytosolic Ca^{2+} levels by releasing Ca^{2+} from multiple sites within the cell (Jan et al., 2000). Burke et al. (2007) described $CHCl_3$-induced hepatocyte death as occurring in two phases: a metabolic phase characterized by GSH depletion, and an oxidative phase characterized by mitochondrial permeability transition and protein nitration.

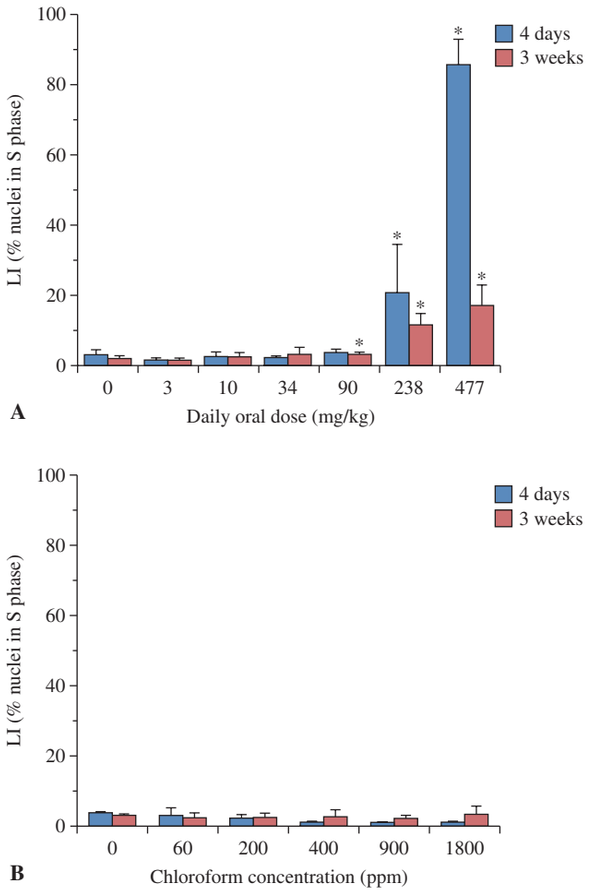

Figure 24-8. *Hepatocyte liver LI in female B6C3F1 mice given $CHCl_3$ orally in corn oil (**A**) or drinking water (**B**) for 4 days or 3 weeks.*

There is no evidence of covalent binding of $CHCl_3$ metabolites to nucleic acids. There is binding to nuclear histone, which plays a key role in controlling DNA expression and might be a mechanism of $CHCl_3$'s carcinogenicity (Diaz and Castro, 1980; Fabrizi et al., 2003). It has been hypothesized that the induction of oxidative stress and depletion of GSH by $CHCl_3$ may lead to indirect genotoxicity that could contribute to carcinogenicity. This hypothesis is supported by the small dose-dependent increase in M(1) dG adducts (malondialdehyde reacts with DNA to form adducts to deoxyguanosine), DNA strand breakage, and lipid peroxidation in $CHCl_3$-treated rat hepatocytes in the absence of any increase in DNA oxidation (Beddowes et al., 2003). Such a mechanism would still be threshold dependent, given its reliance on the initial depletion of antioxidants.

The EPA (2001b) classifies $CHCl_3$ as a probable human carcinogen (group B2), meaning there is sufficient evidence for carcinogenicity in animals and inadequate or no evidence in humans. Experimental evidence and the prevailing opinion that $CHCl_3$ is non-genotoxic indicate that the relationship between $CHCl_3$ dose and tumor formation is nonlinear. The EPA has, as is called for in its *Guidelines for Carcinogen Risk Assessment* (EPA, 2005), considered the mode of action in the determination of $CHCl_3$'s cancer risk and relied on a nonlinear dose–response approach and the use of margin-of-exposure analysis. In doing so, the Agency concluded that the RfD for noncancer effects, based on the dog study of Heywood et al. (1979), was adequately protective for cancer by the oral route on the basis of cancer and noncancer effects having a common link through cytotoxicity.

The wealth of mechanistic data available continues to inform the risk assessment for $CHCl_3$. For example, Constan et al. (2002)

exercised a PBTK dosimetry model to compare hepatic responses in mice and humans to inhaled $CHCl_3$. They concluded that no safety factor was needed to account for interspecies differences in inhalation cancer risk. Additionally, Tan et al. (2003) published a PBTK/TD model for $CHCl_3$ to describe the plausible mechanism linking the hepatic metabolism of $CHCl_3$ to hepatocellular killing and regenerative proliferation, thereby creating a predictive model that most accurately reflects the current science. This model, originally calibrated with data from female B6C3F1 mice, was subsequently modified, scaled to humans, and exercised to derive RfCs and RfDs based on liver and kidney dose metrics (Liao et al., 2007). More recently, a PBTK model was used to estimate 24-hour amounts of $CHCl_3$ metabolized in the kidneys of rats and mice. These estimates were used as internal doses with which to construct dose–response curves for multiple endpoints of kidney toxicity. Application of BMD methodology then revealed similarities in $CHCl_3$ response across species and exposure routes not previously seen when inhaled concentrations or oral doses were used in dose–response construction. Such similarities ultimately increase confidence in the use of the $CHCl_3$ database for human health risk assessment (Sasso et al., 2013). The database also includes a two-year bioassay in male F344 rats that was positive for kidney cancer when $CHCl_3$ exposure was by both the inhalation and oral routes, but negative when by either route alone (Nagano et al., 2006). This study, therefore, serves as a reminder that the assumption of additivity for multiple exposure routes may underestimate actual risk.

AROMATIC HYDROCARBONS

Benzene

Benzene is a volatile aromatic hydrocarbon solvent, a predominant air pollutant and an important industrial chemical. Benzene produced commercially in the United States is derived primarily from petroleum. Benzene has been utilized as a general purpose solvent, but it is now used principally in the synthesis of other chemicals (ATSDR, 2006c). The percentage by volume of benzene in gasoline is 1% to 2%. Benzene plays an important role in unleaded gasoline due to its antiknock properties. Inhalation is the primary route of exposure in industrial and in everyday settings. Benzene is present in the atmosphere both from natural sources such as forest fires and oil seeps and from industrial uses including automobile exhaust and gasoline vapor emission. Cigarette smoke is the major source of benzene in the home (Wallace, 1996). Smokers have benzene body burdens that are six to ten times greater than those of nonsmokers. Passive smoke can be a significant source of benzene exposure to nonsmokers. Because of the health risks of benzene exposure, the use and environmental release of this chemical has significantly diminished in the last two decades.

There is strong evidence from epidemiological studies that high-level benzene exposures result in an increased risk of acute myelogenous leukemia (AML) in humans (Bergsagel et al., 1999; ATSDR, 2006a). Evidence of increased risks of other cancers in such populations is less compelling. Only AML incidence was significantly elevated in the largest cohort study to date, in which about 75,000 benzene-exposed workers in 12 cities in China were evaluated (Yin et al., 1996). Types of cancer other than AML have been tentatively attributed to benzene. Marginal, nonsignificant increases were seen for lung cancer and chronic myelogenous leukemia. Some investigations of persons exposed to engine exhausts have reported a significant association with multiple myeloma. Bezabeth et al. (1996) and Bergsagel et al. (1999) have concluded, however, that there is no scientific evidence to support a causal relationship between benzene exposure and multiple myeloma or non-Hodgkin's lymphoma. Increased incidences of malignant lymphomas and a variety of solid tumors were found in male and female B6C3F1 mice dosed orally with high doses of benzene for up to 103 weeks (Huff et al., 1989). Male and female F344 rats in this bioassay exhibited excesses of Zymbal gland, skin, and oral cavity carcinomas. Benzene exposure via inhalation, dermal, and i.p. routes also produced a variety of tumors in rodents. Thus, benzene is clearly an animal and human carcinogen, but major species differences exist.

The most important adverse effect of benzene is hematopoietic toxicity, which precedes leukemia. Chronic, high benzene exposure can lead to bone marrow damage, which may be manifest initially as anemia, leukopenia, thrombocytopenia, or a combination of these. Bone marrow depression appears to be dose-dependent in both laboratory animals and humans. Continued exposure may result in marrow aplasia and pancytopenia, an often fatal outcome. Survivors of aplastic anemia frequently exhibit a preneoplastic state, termed myelodysplasia, which may progress to myelogenous leukemia (Snyder, 2002; Bird et al., 2005). Decreased lymphocyte counts are shown to be the early and consistent finding in animals and humans following benzene exposure (Goldstein, 1988). Hematological effects in workers with prolonged benzene exposure in the range of 1 to 30 ppm and above have been well documented (Ward et al., 1996). However, there is conflicting evidence for effects at levels less than 1 ppm, the Permissible Exposure Level (PEL) recommended by U.S. Occupational Safety and Health Administration. Three large epidemiological studies (two in the United States and one in the Netherlands) have not revealed any difference in blood parameters between exposed (less than 1 ppm) and nonexposed workers (Collins et al., 1997; Tsai et al., 2004; Swaen et al., 2010). However, Lan et al. (2004) have reported dose-dependent hematotoxicity in some 250 Chinese shoe workers who inhaled low (≤ 1 to ≥ 10 ppm) levels of benzene. Exposures of 100 of these employees to less than or equal to 1 ppm reduced mature white cell and platelet counts, as well as myeloid progenitor cell colony formation. The progenitors were more sensitive to benzene than mature white cells. Polymorphisms in myeloperoxidase (an enzyme that metabolizes benzene to toxic quinones and free radicals) and NAD(P)H:quinone oxidoreductase (an enzyme that protects against these moieties) conferred increased susceptibility to white cell decreases. Turtletaub and Mani (2003) found that formation of DNA and protein adducts in mouse liver and bone marrow was dose-dependent over a range of benzene doses, the lowest of which was 5 μg/kg.

Adverse health outcomes of chronic benzene exposure have been known for a long time, but the mechanisms by which benzene causes effects remain incompletely understood. The toxicity of benzene is dependent upon its metabolites. Thus, it is essential to understand the metabolism of benzene in order to address its mechanisms of toxicity. The initial metabolic step (Fig. 24-9), oxidation of benzene to an epoxide (i.e., benzene oxide), is catalyzed primarily by hepatic CYP2E1. Valentine et al. (1996) demonstrated that benzene-treated Cyp2e1 knockout mice had relatively low levels of all benzene metabolites in their urine. A five-day, 200-ppm benzene inhalation regimen produced severe genotoxicity and cytotoxicity in wild-type B6C3F1 mice, but no adverse effects in the knockout mice. Benzene oxide, which is in equilibrium with its oxepin form, is further metabolized by three pathways: (1) conjugation with GSH to form a premercapturic acid, which is converted to phenyl-mercapturic acid; (2) rearrangement nonenzymatically to form phenol; and (3) hydration by epoxide hydrolase to benzene dihydrodiol, which in turn can be oxidized by dihydrodiol dehydrogenase to catechol. If phenol is hydroxylated in the *ortho* position, more catechol will be formed. Catechol can be converted to *o*-benzoquinone. If benzene

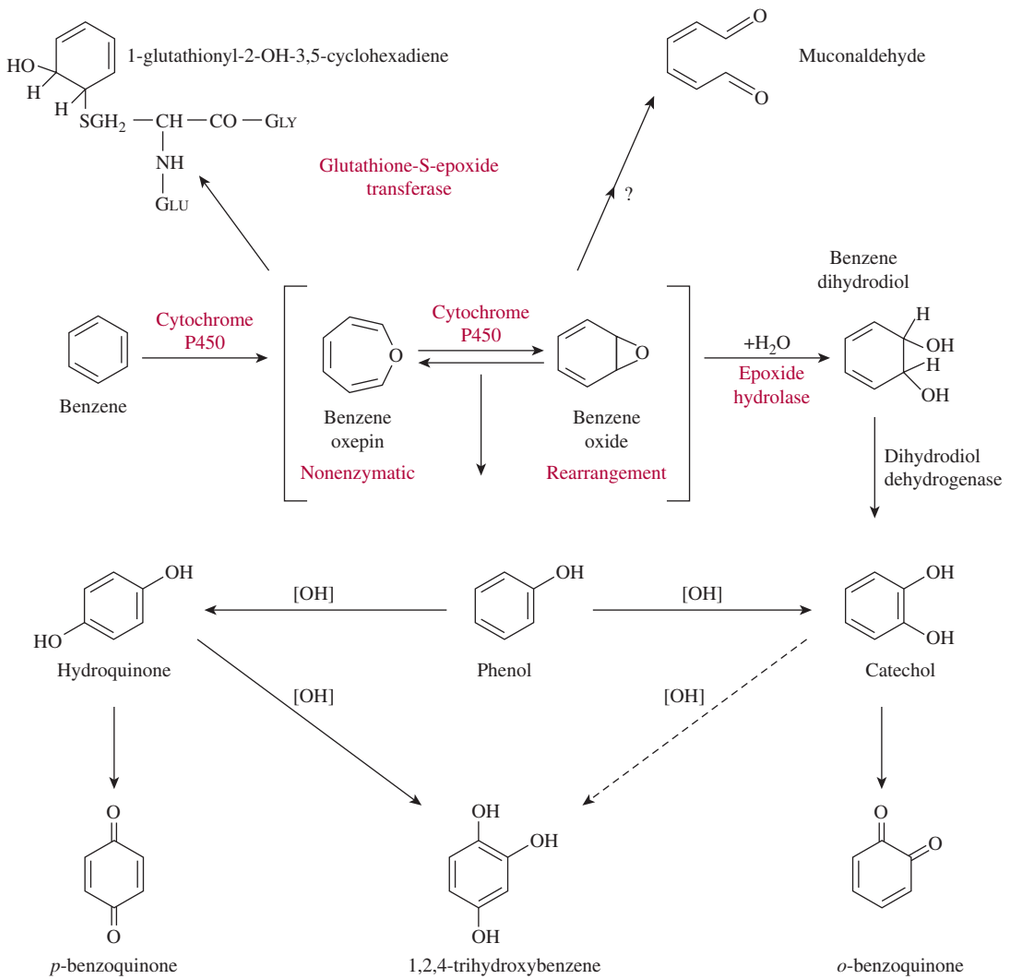

Figure 24-9. *Biotransformation of benzene.* A question mark leads from the oxepin-oxide compartment to muconaldehyde because the substrate for the ring opening has yet to be identified. The dotted line leading to 1,2,4-trihydroxybenzene (1,2,4-T) indicates that it is not clear what the relative contributions of hydroquinone and catechol are to 1,2,4-T. (Modified and used with permission of Rangan and Snyder, 1997.)

is hydroxylated in the *para* position, *p*-hydroquinone is formed. It can be oxidized to *p*-benzoquinone. The *o*- and *p*-benzoquinones are believed to be among the ultimate toxic metabolites of benzene. Another potentially toxic metabolite, muconaldehyde, may arise from ring opening of oxepin. Muconaldehyde undergoes a series of reactions that ultimately lead to *t,t*-muconic acid, an end metabolite found in the urine (Golding and Watson, 1999; Snyder, 2004). While hydroquinone, phenol, catechol, benzoquinone, and muconaldehyde are apparently involved in benzene toxicity, the glutathione conjugate of mercapturic acid, and the glucuronide and sulphate conjugates of hydroquinone, catechol, phenol, and benzoquinone are the results of detoxifying processes (McHale et al., 2012).

Liver is the primary metabolic organ, while bone marrow is the target organ for benzene. Transport of primary metabolites and further metabolism in bone marrow is thought by many authorities to play the key role in myelotoxicity. Levels of DNA and protein adducts were significantly higher in bone marrow than liver of benzene-dosed mice (Turtletaub and Mani, 2003). Bernauer et al. (1999) measured activity and levels of Cyp2e1 in the liver and bone marrow of five strains of mice of varying sensitivity to benzene. Cyp2e1 amounts and activities were considerably greater in the liver than the marrow, but no interstrain differences were seen in either tissue. It is likely that non-CYP enzymes (e.g., peroxidase, myeloperoxidase, and cyclooxygenase) play an important role in generation of semiquinones and quinones in bone marrow (Ross et al., 1996). Lindstrom et al. (1997) have reported that benzene oxide has an estimated half-life of 7.9 minutes in rat blood, and thus may be able to travel from the liver to the bone marrow. The quinones are thought to be too reactive to be transported in the bloodstream. Reactivity of muconaldehyde in the presence of GSH suggests that this metabolite would not reach bone marrow; however, metabolites of muconaldehyde, which are less reactive, could be transported to bone marrow and reoxidized. It has been generally accepted that phenolic conjugates are formed in the liver and transported via the blood to the bone marrow, where they are hydrolyzed and oxidized to quinones. Researchers have been unable to reproduce benzene toxicity by giving individual phenolic metabolites to animals, but coadministration of metabolites, phenol and muconaldehyde with hydroquinone, reproduced the myelotoxic effects of benzene (Witz et al., 1996; Monks et al., 2010). Snyder (2004) concluded that

benzene hematopoietic toxicity and leukemogenesis are primarily a function of the bone marrow, a site remote from the liver where substantial benzene metabolism occurs. *t,t*-Muconaldehyde and 1,4-benzoquinone are the most potent in inhibiting erythropoiesis, followed by hydroquinone and catechol, coadministration of phenol with hydroquinone and catechol, and certain glutathione adducts of hydroquinone (Snyder and Hedli, 1996).

Benzene's metabolic pathways appear to be qualitatively, though not quantitatively similar in species studied to date (Henderson, 1996). Mice have a greater overall capacity to metabolize benzene than do rats or primates. Mani et al. (1999) reported good correlation between protein–DNA adduct levels in bone marrow and susceptibility to benzene genotoxicity and carcinogenesis in F344 rats and three strains of mice. The B6C3F1 mouse shows the highest adduct levels and is the most sensitive of the animals tested. Powley and Carlson (1999) reported similar findings upon quantification of benzene metabolism in mouse, rat, rabbit, and human lung and liver microsomes. A paucity of information is available on the ability of human bone marrow to metabolically activate benzene and/or its metabolites. Rappaport et al. (2009; 2010) suggested that humans metabolize benzene to phenol and muconic acid by two enzymes, a high-affinity enzyme that is active primarily below 1 ppm, leading to muconic acid and a low-affinity enzyme active primarily above 1 ppm, leading to phenol. The authors also suggested that CYP2E1 was active primarily at higher concentrations and CYP2E1 and CYP2A13 could be contributing at lower concentrations.

Factors that alter the metabolism of benzene have the potential to influence the hematopoietic toxicity and carcinogenicity of the VOC. Pretreatment of rats with ethanol, a Cyp2e1 inducer, enhanced metabolism of benzene and potentiated its acute and subacute myelotoxicity in rats (Nakajima et al., 1985) and mice (Marrubini et al., 2003). Phenobarbital had a negligible effect. Pretreatment of male B6C3F1 mice with acetone, another Cyp2e1 inducer, increased benzene oxidation by about fivefold (Kenyon et al., 1996). Pretreatment with diethyldithiocarbamate, a Cyp2e1 inhibitor, completely abolished benzene oxidation. Coexposure of F344 rats to gasoline and benzene resulted in competitive metabolic inhibition (Travis et al., 1992). Metabolism of benzene by hepatic microsomes from 10 human donors was directly proportional to CYP2E1 activity in the samples (Seaton et al., 1994). Thus, it would be anticipated that CYP2E1 polymorphisms may influence susceptibility to benzene toxicity. Male mice have consistently been found to be more sensitive than females to genotoxic effects of benzene. PBTK analysis of data from gas uptake experiments with B6C3F1 mice of both sexes revealed that the optimized maximum rate of benzene metabolism was twice as high in males (Kenyon et al., 1996). It is not known whether there is a sex-dependent difference in benzene metabolism in humans. Sato et al. (1975) saw a more rapid rate of pulmonary elimination of benzene in men than in women following inhalation of 25 ppm for 2 hours. Benzene was retained longer in the females due to their higher body fat content. Unfortunately, most epidemiology studies of benzene-exposed workers have not provided a gender comparison. Li et al. (1994) did not see a statistically significant difference in cancer mortality between male and female workers exposed to benzene, though risks tended to be somewhat higher for males. Polymorphisms in enzymes involved in benzene metabolism such as CYP2E1, epoxide hydrolase, myeloperoxidase, glutathione S-transferases, and quinone reductases will also influence the toxicity of benzene. Increased susceptibility to hematotoxicity, as evidenced by lower WBC counts, was found in workers with null-GSTTI, null-GSTMl, and CYP2El (rs2031920 and rs3813867) genotypes (Ye et al., 2015).

There are a number of cell populations in the bone marrow that may serve as targets for benzene metabolites. Benzene exposure in vivo results in inhibited growth and development of pluripotential bone marrow stem cells. More mature precursors, such as stromal cells and erythroid and myeloid colony-forming units, are also affected. Trush et al. (1996) point out that hydroquinone-induced inhibition of interleukin (IL)-1 synthesis by stromal macrophages results in altered differentiation of myeloid and lymphoid cells that are normally active in immune responses. These investigators also note that killing of stromal macrophages and fibroblasts could result in such a pronounced reduction of cytokines and growth factors that immature and committed hematopoietic progenitors would die from apoptosis. The erythroid series is more susceptible than the myeloid series to benzene-induced cytotoxicity. Immature myeloid cells can proliferate when the development of erythroid cells is restricted and acquire neoplastic characteristics upon dedifferentiation. The end result is AML (Golding and Watson, 1999).

Investigations of benzene toxicity/leukemogenesis have uncovered a variety of potential mechanisms (Golding and Watson, 1999; Snyder, 2002; Bird et al., 2005). As mentioned before, experimental evidence indicates that the complementary actions of benzene and several of its metabolites are required for myelotoxicity. It has been recognized for 20 years that a number of benzene metabolites can bind covalently to GSH, proteins, DNA, and RNA. This can result in disruption of the functional hematopoietic microenvironment by inhibition of enzymes, destruction of certain cell populations, and alteration of the growth of other cell types. Covalent binding of hydroquinones to spindle-fiber proteins will inhibit cell replication (Smith, 1996). In vitro studies have established that reactive benzene metabolites bind covalently to DNA of several tissues of different species. Through the use of accelerator mass spectrophotometry, Creek et al. (1997) have demonstrated DNA binding in mice exposed to extremely low ^{14}C-benzene levels. The binding was dose-dependent over a wide range of doses. DNA adduct levels were so low, however, that this mechanism alone may be insufficient to fully account for leukemogenic effects. Altered gene expression related to DNA methylation in workers exposed to benzene (Yang et al., 2014) indicates a role for a non-genotoxic mechanism in benzene toxicity. However, further investigations are required to fully elucidate the role of non-genotoxic mechanisms. The role of aryl hydrocarbon receptor (AhR) in benzene-induced hematoxicity is emerging. AhR-knockout mice were completely resistant to hematotoxicity of benzene, suggesting a central role of AhR in benzene toxicity (Yoon et al., 2002), but the mechanisms for this receptor are still under investigation. A review of literature by Gasiewicz et al. (2010) indicated that AhR regulates the expression of enzymes involved in benzene toxicity and plays a major role in the regulation of hematopoietic stem cells. In order to identify critical data gaps and prioritize research, Meek and Klaunig (2010) proposed five key events in the mode of action for benzene-induced leukemia: (1) benzene metabolism via cytochrome P450; (2) interaction of benzene metabolites with target cells in the bone marrow; (3) formation of initiated, mutated target cells; (4) selective proliferation of the mutated cells; and (5) production of leukemia.

It appears likely that oxidative stress contributes to benzene toxicity. As the bone marrow is rich in peroxidase activity, phenolic metabolites of benzene can be activated there to reactive quinone derivatives. Ross et al. (1996) discovered that myeloperoxidase present in murine and human progenitor cells could bioactivate hydroquinone to *p*-benzoquinone. Electron spin resonance experiments by Hiraku and Kawanishi (1996) revealed the formation of a semiquinone radical in *p*-benzoquinone-treated HL-60 cells (a human myeloid cell line). This suggested that reactive oxygen

moieties (e.g., O_2^- and H_2O_2) are produced via the formation of the semiquinone radicals. These active oxygen species can cause DNA strand breaks or fragmentation, leading to cell mutation or apoptosis, respectively. Ross et al. (1996), however, pointed out that quinones can also inhibit proteases involved in induction of apoptosis. These authors noted that modulation of apoptosis may lead to aberrant hematopoiesis and neoplastic progression. Badham et al. (2010) recently demonstrated that in utero exposure to benzene in mice increased oxidative stress in fetal tissue. The increase in oxidative stress was also associated with changes in redox-sensitive signaling pathways involved in normal hematopoiesis.

A number of biomarkers of exposure to benzene have been developed and carefully evaluated. Concentrations of the parent compound in exhaled breath parallel blood concentrations. Clayton et al. (1999) reported a high correlation between the extent of smoking-related activities and levels of benzene in exhaled breath of humans. Urinary excretion of a variety of benzene metabolites (i.e., phenol, catechol, hydroquinone, 1,2,4-trihydroxybenzene, *S*-phenylmercapturic acid, and *t,t*-muconic acid) have been shown to be correlated with benzene exposure in occupational settings. Phenol, catechol, and hydroquinone, however, are neither sensitive nor specific biomarkers, because relatively high levels are found in nonexposed individuals (Medeiros et al., 1997). Similarly, *t,t*-muconic acid is not specific, because it is a metabolite of sorbic acid, a common food additive. Boogaard and van Sittert (1996) conclude that *S*-phenylmercapturic acid, an end-product of the conjugation of benzene oxide and GSH, is a suitable urinary biomarker for low-level benzene exposure because of its specificity and relatively long half-life. Adducts to hemoglobin and cysteine groups of proteins have been demonstrated in rodents, but not in humans. DNA damage has been detected in benzene-exposed workers (Liu et al., 1996; Andreoli et al., 1997), though such measures have not yet found widespread acceptance as biomarkers of effect. Altered gene expression in benzene-exposed workers could serve as potential biomarkers for leukemia. McHale et al. (2011) reported an increase in the expression of 16 genes that are involved in the immune response, inflammatory response, cell adhesion, cell-matrix adhesion, and blood coagulation. Forrest et al. (2005) reported altered expressions of CXCL16, ZNF331, JUN, and PF4 by microarray analyses along with real-time PCR confirmation. Li et al. (2014) reported increased copy-number variations in several chromosomes and expression of NOTCH1 and BSG genes. However, further investigations to characterize/validate gene expression are necessary to identify consistent biomarkers that can predict benzene exposure and/or toxicity.

A number of PBTK models have been developed to aid in predicting risks of myelotoxicity and leukemia posed to humans by benzene. Medinsky et al. (1989) published one of the first such models for benzene. They assumed that benzene metabolism followed Michaelis–Menten kinetics and occurred via benzene oxide. B6C3F1 mice, which are more sensitive than F344 rats to benzene myelotoxicity, were predicted to metabolize two to three times more inhaled benzene. This and other PBTK models do not accurately simulate some laboratory data sets. Modeling benzene metabolism and disposition is difficult because of its inherent complexity and variability. In light of intricate dose–response relationships, Medinsky et al. (1996) emphasized the importance of considering competitive metabolic interactions between benzene and its metabolites, as well as the balance between enzymatic activation and inactivation processes. Cole et al. (2001) employed in vitro metabolic parameters for several pathways to construct a PBTK model that simulated most tissue dosimetry data sets for benzene-exposed mice quite well. Yokley et al. (2006) described a PBTK model that simulated tissue doses of benzene, benzene oxide, phenol, and hydroquinone in humans exposed orally and by inhalation. A Markov Chain Monte Carlo statistical technique was used to assess dissimilarities in population distributions of key model parameters. Variability in metabolic parameters and certain physiological parameters (e.g., organ weight) had to be inputted to accurately predict the range of human values.

Benzene is concluded to be a known human carcinogen by regulatory agencies around the world. The International Agency for Research on Cancer (IARC) classifies benzene as a Group 1 carcinogen (carcinogenic to humans). Benzene is characterized as a known human carcinogen for all routes of exposures based on strong evidence in human and laboratory animals (EPA, 2007). Maximum contaminant levels (MCLs) of 0.005 and 0.01 mg/L have been established for benzene in drinking water (EPA, 2002; WHO, 2004). For the protection of workers, OSHA has established a TLV of 0.5 ppm as an 8-hour TWA. ACGIH has also established a Biological Exposure Index (BEI) for benzene of 25 μg of *S*-phenylmercapturic acid and 500 μg of *t,t*-muconic acid per g of creatinine for a urine sample collected at the end of a workshift. Readers are encouraged to review the recent NTP's 12th edition of Report on Carcinogens and ATSDR's Toxicological Profile for Benzene (2006c) for other regulatory levels for benzene exposure.

Toluene

Toluene occurs naturally in crude oil and in the tolu tree and is present in paints, lacquers, thinners, cleaning agents, glues, and many other products. Toluene is also used in the production of other chemicals. Gasoline, which contains 5% to 7% toluene by weight, is the largest source of atmospheric emissions and exposure of the general populace. In the United States, concentrations of toluene in outdoor air range from 1 to 35 ppb by volume (ATSDR, 2000b). Higher indoor levels of toluene are reported where products such as paint thinners, solvents, or tobacco products are used. Inhalation is the primary route of exposure, though skin contact occurs frequently. Toluene is a favorite of solvent abusers, who intentionally inhale high concentrations to achieve a euphoric effect (Filley et al., 2004). Large amounts of toluene enter the environment each year by volatilization. Relatively small amounts are released in industrial wastewater. Toluene is frequently found in water, soil, and air at hazardous waste sites (Pohl et al., 2008).

Toluene TK has been thoroughly characterized in humans and laboratory animals. Toluene is well absorbed from the lungs and GI tract. It rapidly accumulates in and can affect the brain, due to that organ's high rate of blood perfusion and relatively high lipid content. Toluene subsequently is deposited in other tissues according to their lipid content, with adipose tissue attaining the highest levels.

Toluene is well metabolized, but a portion is exhaled unchanged. Cytochrome P450s (CYPs) catalyze metabolism of toluene primarily to benzyl alcohol and lesser amounts of cresols (ATSDR, 2000b). Benzyl alcohol is converted by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) to benzoic acid, which is primarily conjugated with glycine and eliminated in the urine as hippuric acid. Nakajima et al. (1992a) reported that Cyp2e1 and Cyp2c11 are primarily responsible for catalyzing the initial hydroxylation step in rat liver at low and high toluene levels, respectively. CYP2E1 is most active at low doses in humans (Nakajima et al., 1997). A minor CYP-related pathway involves a transient epoxidation of the aromatic ring to form cresols, which undergo a variety of conjugation reactions, forming sulfates and glucuronides. Glutathione conjugation may also occur resulting in *S*-benzylglutathione and *S*-benzylmercapturic acid by

conjugation to benzyl alcohol, as well as *S*-*p*-toluyl glutathione and *S*-*p*-toluylmercaptic acid by conjugation of the epoxidated ring. Benignus et al. (1998) attempted to use a PBTK model to relate toluene blood levels to behavioral effects in rats and humans. It appeared that humans were more sensitive to increases in blood levels, but more rat data and much more human data are needed for model validation. Blood toluene and urine hippuric and cresol levels are commonly used as biomarkers for occupational exposures to toluene. Cosnier et al. (2014) reported mercapturic acid as an alternate to the traditional biomarkers, as it was not impacted by coexposure to other chemicals that impact toluene metabolism.

The CNS is the primary target organ of toluene and other alkylbenzenes. Cardiac, renal, and hepatic toxicities as well as fetal alcohol-like syndrome have occasionally been reported. Increased incidence of spontaneous abortion was linked to toluene exposure at workplaces, but has not been supported by the results of animal testing. Manifestations of acute exposure range from slight dizziness and headache to unconsciousness, respiratory depression, and death. Occupational inhalation exposure guidelines are established to prevent significant decrements in psychomotor functions. Acute encephalopathic effects are rapidly reversible upon cessation of exposure (Fig. 24-3), and are not associated with neuroimaging changes (Filley et al., 2004). Subtle neurologic effects have been described in some groups of occupationally exposed individuals. Exposure to about 100ppm toluene for years may result in subclinical effects, as evidenced by altered brainstem auditory-evoked potentials (Abbate et al., 1993) and changes in visual-evoked potentials (Vrca et al., 1995). Foo et al. (1990) reported a good correlation between toluene exposure and poor scores on neurobehavioral tests of 30 female rotogravure workers.

Toluene is the most commonly misused solvent due to its alcohol-like effects of euphoria and excitation. Motor incoordination, dizziness, relaxation, and lightheadedness are additional characteristic of toluene intoxication, as well as illusions and hallucinations. Even acute inhalation can lead to life threatening conditions when poor oxygenation leads to cardiac arrhythmias and other complications associated with hypokalemia (Bowen, 2011). "Sudden sniffing death" has been documented since the 1970s (see "Solvent Abuse" section). Severe neurotoxicity is often diagnosed in persons who have abused toluene for a prolonged period. A relatively specific neurobehavioral profile is manifest, including inattention, apathy, memory dysfunction, diminished visuospatial skills, frontal lobe dysfunction, and psychiatric status (Filley et al., 2004). Magnetic resonance imaging reveals ventricular enlargement, cerebral atrophy, and white matter hyperintensity, a characteristic profile termed toluene leukoencephalopathy. Such changes represent severe, chronic myelotoxicity. More advanced neuroimaging techniques may be able to reveal mild, subtle changes associated with early alterations in myelin of workers as well as toluene abusers (Caldemeyer et al., 1996; Filley et al., 2004). Rat pups subjected to high doses of toluene on days 6 to 19 of gestation exhibited a significant reduction in myelination per forebrain cell when they were 21 days old (Gospe and Zhou, 1998).

There is limited information on mechanisms by which toluene and similar solvents produce acute or residual CNS effects (Balster, 1998). The Meyer–Overton theory of partitioning of the lipophilic parent compounds into membrane lipids has been widely accepted for a century. It has been proposed that the presence of solvent molecules in cholesterol-filled interstices between phospholipids and sphingolipids changes membrane fluidity, thereby altering intercellular communication and normal ion movements (Engelke et al., 1996). Such a process is reversible. An alternate hypothesis is that toluene partitions into hydrophobic regions of proteins and interacts with them, thereby altering membrane-bound enzyme activity and/or receptor specificity in a reversible manner (Balster, 1998). Other evidence suggests that toluene and other VOCs may act acutely by enhancing $GABA_A$ receptor function (Mihic et al., 1994), attenuating NMDA receptor-stimulated calcium flux (Cruz et al., 1998), attenuating nicotinic acetylcholine receptors (Bale et al., 2002), enhancing glycine receptors (Bale et al., 2005), and/or activating dopamingeric systems (von Euler, 1994). Using an in vivo microdialysis study and a schedule-controlled operant behavioral task, it was shown that glutamate levels were decreased in mice after acute toluene exposure (Win-Shwe et al., 2009). In addition to neurotransmitter systems, immune and hypothalamic neurohormonal systems also are proposed to play a role in toluene-induced neurotoxicity (Win-Shwe and Fujimaki, 2010). Hester et al. (2011) demonstrated altered expression of genes associated with synaptic transmission and plasticity following acute toluene exposure. The mechanism of chronic toluene neurotoxicity is unknown. Toluene is known to be deposited in brain areas with the highest myelin content. Astrocytosis frequently accompanies myelin disruption (Gotohda et al., 2000; Filley et al., 2004).

As toluene is metabolized by CYPs, ADH, and ALDH, the chemical can interact with other xenobiotics metabolized by these enzymes. Concurrent exposure to solvents metabolized by the same CYP isoforms can result in competitive metabolic inhibition. Inoue et al. (1988) observed that benzene and toluene suppressed one another's metabolism in humans. Thus, the risk of leukopenia in workers exposed to benzene and toluene should be less than that in workers exposed to benzene alone. Pryor and Rebert (1992) found that toluene greatly reduced manifestations of peripheral neuropathy caused by *n*-hexane in rats. Although no interaction between toluene and xylenes was seen in humans inhaling low levels of each, simultaneous exposure to higher levels results in mutual metabolic suppression (Tardif et al., 1991). Prior exposure to P450 inducers can result in increased rates of toluene metabolism/elimination and more rapid recovery from toluene-induced CNS depression. Nakajima and Wang (1994) observed that high concentrations of inhaled toluene moderately induced four of the six CYP isoforms that metabolize it, but inhibited the other two in rat liver.

High-level prenatal toluene exposures have produced growth and skeletal retardations in offspring of rodents and humans. The term "fetal solvent syndrome" was used to describe toluene-abusing women's children who exhibit microcephaly and cranial facial features similar to those with fetal alcohol syndrome (FAS) (Wilkins-Haug, 1997). Exposure to a pattern of binge toluene abuse caused growth retardation, gross morphological anomalies, skeletal abnormalities, and soft tissue anomalies in a dose-related fashion (Bowen et al., 2009). Growth and skeletal retardation were the common findings in the developmental toxicity studies in animals. The reproductive and developmental toxic potential of toluene was evaluated in a two-generation study of Sprague–Dawley rats (Roberts et al., 2003). Both sexes of each generation were exposed to 0, 100, 500, or 2000 ppm toluene vapor 6 hours/day, 7 days/week for 80 days premating and for 15 days of mating. Pregnant animals were also exposed from gestation days 1 to 20 and lactation days 5 to 21. No adverse effects were seen at any dose on fertility, reproductive performance, or maternal or pup behavior. Reduced fetal body weight and skeletal anomalies associated with growth retardation were manifest in the offspring of both generations subjected to 2000 ppm, but not to lower vapor levels (Roberts et al., 2003).

Existing studies suggest that toluene is not carcinogenic or mutagenic. IARC classified toluene as not classifiable as a human carcinogen. The U.S. EPA determined that there is inadequate information to assess the carcinogenic potential of toluene. ACGIH

(2011) has established a TLV of 20 ppm as an 8-hour TWA to protect workers from subclinical changes in blue-yellow color vision and the potential for spontaneous abortion. The current ACGIH Biological Exposure Index I (BEI) for toluene is 0.02 mg of toluene/L blood, 0.03 mg toluene/L urine, and 0.3 mg *o*-cresol/g of creatinine for urine collected at the end of a workshift.

Xylenes and Ethylbenzene

Large numbers of people are exposed to xylenes and ethylbenzene occupationally and environmentally (Wallace et al., 1987). Xylenes (ATSDR, 2006d) and ethylbenzene (ATSDR, 2010a), like benzene and toluene, are major components of gasoline and fuel oil. Xylenes are also used in the production of ethylbenzene. The primary uses of xylenes and ethylbenzene industrially are as solvents and synthetic intermediates. Most of these aromatics that are released into the environment evaporate into the atmosphere. They may also enter groundwater from oil and gasoline spills, leakage of storage tanks, and migration from waste sites.

The TK and acute toxicity of toluene, xylenes, and other aromatic solvents are quite similar. Xylenes and the others are well absorbed from the lungs and GI tract, distributed to well-perfused and lipophilic tissues such as liver, fat, brain and skin, exhaled unchanged to some extent, well metabolized by hepatic CYP P450s, and largely excreted as urinary metabolites (Lof and Johanson, 1998). In humans, hepatic microsomal CYP2E1 is the primary enzyme involved in the metabolism of xylenes to methylbenzylalcohols, leading to the formation of methylhippuric acid isomers. Phase II metabolism occurs primarily by conjugation with glycine, which is rate limiting in humans. Conjugation with glucuronic acid and aromatic hydroxylation to form xylenols also occur to some extent. Ethylbenzene is also metabolized primarily by CYP-mediated oxidation followed by conjugation. The major metabolites are benzoic acids, glycine conjugates (e.g., hippuric acid), 1-phenylethanol, mandelic acid, and phenylglyoxylic acid. Nielsen and Alarie (1982) state that the potency of benzene and a series of alkylbenzenes, as sensory irritants of the upper respiratory tract of mice, increases with increasing lipophilicity. Acute lethality of hydrocarbons (i.e., CNS-depressant potency) also varies directly with lipophilicity (Swann et al., 1974). Some of the alkylbenzenes including xylenes and ethylbenzene are shown to be ototoxic in rats. There is limited evidence that chronic occupational exposure to xylenes is associated with residual neurologic effects (ATSDR, 2006d). Limited human data suggest that systemic effects associated with ethylbenzene exposure are respiratory tract and ocular irritation, possible ototoxicity, and hematological alterations (ATSDR, 2010a). Exposure conditions, including the presence of other solvents, have usually not been characterized in such reports. Uchida et al. (1993), for example, reported increased subjective CNS symptoms in workers exposed for about 7 years to a mean of 21 ppm xylenes. The prevalence of symptoms was not dose-dependent, nor were other solvent exposures characterized. This vapor concentration is significantly lower than current occupational exposure standards in the United States, which are established on the basis of acute irritancy or CNS effects. Savolainen et al. (1981) observed improved performance on a series of psychophysiological tests by humans inhaling 200 ppm *m*-xylene for 4 hours. An excitatory stage is often initially manifest in subjects, followed by functional inhibition with higher vapor concentrations and/or longer exposure periods. The current permissible exposure limit in the United States for xylenes and ethylbenzene is 100 ppm.

Xylenes and ethylbenzene appear to have very limited capacity to adversely affect organs other than the CNS. Mild, transient liver and/or kidney toxicity have/has occasionally been reported in humans exposed to high vapor concentrations of xylenes. Little evidence of hepatorenal injury is typically manifest in laboratory animals (ATSDR, 2006c). Xylenes and ethylbenzene increase liver weight and moderately induce liver CYP P450s in laboratory animals (ATSDR, 2006c, 2010a). The *o, p*, and *m* isomers of xylene vary somewhat in their capacity to induce different CYP P450 isoforms in different organs of rats (Backes et al., 1993). Concurrent exposure to an alkylbenzene and another compound metabolized by CYP P450s generally results in their competitive metabolic inhibition. Competitive metabolic interaction between xylene and ethylbenzene following inhalation exposure has been reported (Campbell and Fisher, 2007). Preexposure to an alkylbenzene, conversely, can result in increased metabolism of the other chemical.

Xylene levels in the blood and levels of its metabolite, methylhippuric acid, in urine are the primary biomarkers used to detect exposure to xylene. Ethylbenzene levels in blood and mandelic acid and phenylglyoxylic acid in urine are used as biomarkers of ethylbenzene exposure. However, mandelic acid and phenylglyoxylic acid are also metabolites of styrene (ATSDR, 2006d, 2000a).

Xylene's odor threshold is about 1 ppm, which is 100 times lower than the OSHA PEL. IARC has determined that xylene is not classifiable as to its carcinogenicity to humans. Ethylbenzene caused kidney injury and an increased incidence of renal adenoma and carcinoma (combined) in male F344 rats exposed to 750 ppm for up to 2 years (NTP, 1997b). However, no association has been found between the occurrence of tumors and exposure to ethylbenzene in humans. A battery of in vivo and in vitro genetic toxicity assays has shown that ethylbenzene is not genotoxic. IARC classified ethylbenzene as a possible human carcinogen.

Styrene

Styrene is primarily used in the manufacture of polystyrene items and in copolymers with acrylonitrile or 1,3-butadiene to produce synthetic rubber, latex, and reinforced plastics (Gibbs and Mulligan, 1997). Worker exposures in the rubber industry are of greatest concern toxicologically. Styrene is also often detected in the blood of nonoccupationally exposed populations (Churchill et al., 2001; Blount et al., 2006). Sources include tobacco smoke, auto exhaust, and emissions from building materials. Discharges from industry are the major source of environmental pollution (ATSDR, 2010b). An estimated 90,000 U.S. workers are potentially exposed to styrene (OSHA, 2016).

Central nervous system effects and mucous membrane and respiratory irritation are the most commonly reported effects in humans following styrene exposure. Workers exposed to about 25 ppm exhibit signs of mild hepatic injury and cholestasis (Brodkin et al., 2001). Styrene is damaging to the nasal epithelium of rats and mice, and hepatotoxic and pneumotoxic in mice. Species differences in styrene toxicity can be explained by metabolic differences. Styrene is metabolized principally by Cyp2e1 in liver and Cyp2f2 in lung to styrene-7,8-oxide (SO) (Linhart et al., 2000; Carlson, 2003). SO, ultimate toxicant, can bind covalently to proteins and nucleic acids. This epoxide is detoxified by the actions of epoxide hydrolase and glutathione S-transferase (GST) (Chung et al., 2006; Carlson, 2010). SO is detoxified much more efficiently by epoxide hydrolase in rats and humans than in mice. Among the three species, mouse liver and lungs have the greatest capacity to form SO from styrene (ATSDR, 2010b). Many of the enzymes involved in styrene metabolism are known to be polymorphic, so polymorphism may play a role in its toxicity.

There is considerable debate as to whether styrene is a human carcinogen. It is carcinogenic to the forestomach of orally dosed

mice and rats (Roe, 1994). Results of in vitro and in vivo mutagenicity and genotoxicity studies of styrene have been mixed (ATSDR, 2010b). Chromosomal aberrations, micronuclei, and/or sister chromatid exchange have been reported in employees in some high-exposure occupational settings, but not in others. Increased rates of different cancers have been reported in workers exposed to 1,3-butadiene and styrene in the synthetic rubber industry. The excesses of cancers have largely been attributed to 1,3-butadiene, though styrene may modify the actions of 1,3-butadiene and/or be implicated itself (Matonoski et al., 1997). Occasional findings of small increases in incidences of lymphatic and hematopoietic neoplasms in epidemiological studies of workers exposed primarily to styrene are not robust (IARC, 2002; ATSDR, 2010b). Tumors of the esophagus, pancreas, and kidney were also reported in reinforced plastic industry workers. No increases in any tumors were seen in male or female Sprague–Dawley rats exposed 6 hours daily, 5 days weekly for 104 weeks to as high as 1000 ppm styrene vapor (Cruzan et al., 1998). A similar inhalation cancer bioassay, however, revealed increases in pulmonary adenomas and/or carcinomas in male and female CD-1 mice (Cruzan et al., 2001). Gavage studies have yielded variable results (ATSDR, 2010b). In an NCI (1979) study, dose-related increases in the combined incidence of benign and malignant lung tumors were noted in mice following chronic gavage dosing. In an oral study in which pregnant dams were given a single dose of styrene on gestation day 17, and offspring were exposed to high oral doses weekly for 16 weeks after weaning, a significant increase in lung tumors was observed in these offspring following a 100-week exposure period (Ponomarkov and Tomatis, 1978). The same authors reported no significant tumor incidences in a second study in which a different strain of mice was exposed to much lower doses of styrene on gestation day 17 followed by once a week exposure of offspring for 120 weeks. Lung tumor response observed in mice is most likely due to cytotoxicity caused by the in situ formation of SO, followed by cell proliferation (ATSDR, 2010b). The relevance of mouse lung tumors to humans has been questioned due to species differences in lung metabolism of styrene (Carlson, 2008). The absence of preneoplastic lesions and lung tumors in Cyp2f2-null and humanized CD-1 mice exposed to 120 ppm of styrene vapor for 104 weeks indicates that styrene is a rodent, not a human carcinogen (Cruzan et al., 2017). Three modes of action for styrene-induced tumors have been proposed by NTP 2011: genotoxicity, cytotoxic effects, and immunosuppression. Genotoxicity was identified as relevant to all cancer sites, and cytotoxicity and immunosuppression were identified as site-specific.

Noncancer effects reported include mucous membrane irritation, changes in color vision, hearing loss, and neurocognitive impairment (ATSDR, 2010b). A recent review of available case reports, cross-sectional studies, and mortality studies suggested occupational exposure to styrene is a potential risk factor for nonmalignant respiratory disease, and related morbidity and mortality (Nett et al., 2017). The European Commission listed styrene as an endocrine disruptor, based primarily on reports of increased prolactin levels in workers exposed to the chemical. However, acute workplace-related stress is proposed as an alternative mode of action for elevated prolactin levels in workers (Gelbke et al., 2015).

Workers in the reinforced plastics industry are exposed to styrene and SO. Tornero-Velez and Rappaport (2001) used a modified PBTK model to predict the relative contributions of inhaled styrene and SO to systemic levels of SO in humans. Inhaled SO was forecast to present the greater cytogenetic hazard. Sarangapani et al. (2003) created a PBTK model with a multicompartment representation of the respiratory tract that included species-specific quantitative information on physiology, cellular composition, and metabolic capacity. The model-based analysis indicated that humans would be 100-fold less susceptible than mice to pulmonary tumors, based on predictions of SO concentrations in terminal bronchioles. Wenker et al. (2001) attempted to correlate key P450 metabolic capacities of 20 male volunteers with styrene clearance. Lack of correlation was attributed to the low inhaled vapor concentrations and styrene's blood flow-limited metabolism.

Based on the limited evidence in humans and evidence in mice, NTP (2011) classified styrene as "reasonably anticipated to be a human carcinogen," and IARC classified it as "possibly carcinogenic to humans." For workers' protection, OSHA (2006) established a permissible exposure limit (PEL) of 100 ppm, and ACGIH established a TLV of 20 ppm as an 8-hour TWA (ACGIH, 2011). The FDA has determined that the styrene concentration in bottled drinking water should not exceed 0.1 ppm.

ALCOHOLS

Ethanol

Many humans experience greater exposure to ethanol (ethyl alcohol, alcohol) than to any other solvent. Not only is ethyl alcohol used as an additive in gasoline, as a solvent in industry, in many household products and in pharmaceuticals, but it is also heavily consumed in intoxicating beverages. Frank toxic effects are less important occupationally than injuries resulting from psychomotor impairment. Driving under the influence of alcohol is, of course, the major cause of fatal auto accidents. In many states in the United States, a blood alcohol level of 80 mg/100 mL blood (80 mg%) is prima facie evidence of "driving while intoxicated." One's blood alcohol level and the time necessary to achieve it are controlled largely by the rapidity and extent of consumption of the chemical. Ethanol is distributed in body water and to some degree in adipose tissue. Alcohol is eliminated primarily by metabolism (90% to 95%), with the remainder exiting via urinary excretion and exhalation. The blood level in an average adult decreases by approximately equal to 16 mg% per hour. Thus, a person with a blood alcohol level of 120 mg% would require approximately equal to 8 hours to reach negligible levels.

Ethanol is metabolized to acetaldehyde by three enzymes: The major pathway involves ADH-catalyzed oxidation to acetaldehyde. ADH is present in various tissues including stomach, lung, eye, and liver, but the highest concentration is found in the cytoplasm of hepatocytes (Yin et al., 1999). While hepatic ADH largely contributes to ethanol oxidation in humans (Lee et al., 2006), a PBTK model indicates both hepatic and gastric ADH contribute to first-pass metabolism of ethanol in rats, with gastric ADH playing a greater role at lower ethanol doses (Pastino and Conolly, 2000).

The oxidation ADH catalyzes is reversible, but the acetaldehyde that is formed is rapidly oxidized by acetaldehyde dehydrogenase (ALDH) to acetate. Liver mitochondrial ALDH is the major enzyme responsible for acetaldehyde clearance. However, a recent PBTK model predicted the reversible conversion of acetaldehyde to alcohol by ADH to be an essential step in the systemic clearance of acetaldehyde (Umulis et al., 2005). A second enzyme, catalase, utilizes H_2O_2 supplied by the actions of NADPH oxidase and xanthine oxidase. There is usually little H_2O_2 available in hepatocytes to support the reaction, so it is unlikely that catalase will normally account for more than 10% of ethyl alcohol metabolism. The third enzyme, CYP2E1, is the principal isoform of the hepatic microsomal ethanol oxidizing system.

Ethanol interacts with other solvents that are also metabolized by ADH and CYP2E1. Ethanol can be an effective antidote for

poisoning by methanol, ethylene glycol, and diethylene glycol. As ethyl alcohol has a relatively high affinity for ADH, it competitively inhibits the metabolic activation of other alcohols and glycols. Preexposure to a single high dose or multiple doses of ethanol can induce CYP2E1, thereby enhancing the metabolic activation and potentiating the toxicity of a considerable number of other solvents and drugs such as acetaminophen (Lieber, 1997; Klotz and Ammon, 1998). Manno et al. (1996), for example, describe heavy drinkers who developed severe hepatorenal toxicity from CCl_4 exposures, which caused no ill effects in nondrinkers. Other alcohols, such as 2-butanol (Fig. 24-4), can have analogous effects (Traiger and Bruckner, 1976). Chronic heavy drinkers may develop more severe hepatotoxicity associated with acetaminophen overdosing than nonalcoholics, due to increased formation and reduced detoxification of toxic metabolites (Riordan and Williams, 2002; Rumack, 2004). Such interactions are also described under the "Metabolism" and "Exogenous Factors" subsections in this chapter.

A variety of factors that modulate ADH, CYP2E1, and ALDH can influence adverse effects experienced by drinkers. Relatively low ADH and CYP2E1 activities increase systemic alcohol levels and prolong its effects, while low ALDH results in elevated acetaldehyde levels. Long-term ingestion of high alcohol doses can lead to alcohol dependency and cirrhosis, whereas high acetaldehyde levels may result in acute toxicity due to covalent binding to proteins and other macromolecules (Niemela, 1999). Ethanol-metabolizing enzymes exist in multiple molecular forms that are genetically controlled. Genetic polymorphisms of these enzymes contribute to different disease outcomes in different ethnic and racial populations (Crabb et al., 2004; Russo et al., 2004). Asians have higher frequencies of an ADH1*2 variant, which encodes for a rapidly metabolizing form of ADH, preventing alcoholism. White and black populations in the United States predominantly have an ADH1*1 allele (Russo et al., 2004), which encodes for a relatively inactive ADH (Lee et al., 2006), leading to increased risk of alcohol dependency. As for ALDH, some 50% of Asians have high levels of the inactive allele, ALDH2*2. Hence, this population experiences acetaldehyde-induced flushing, tachycardia, nausea, vomiting, and hyperventilation upon alcohol consumption. Whereas this syndrome offers protection against developing alcoholism, it increases the risk of acetaldehyde-related cancers, including esophageal, stomach, colon, lung, head, and neck tumors (Vasiliou et al., 2004). The proposed mechanism for these cancers is that constant interaction of acetaldehyde with DNA leads to mutations in procarcinogenic or antiapoptotic genes (Israel et al., 2011). While higher frequencies ADHI*2 and ALDH2*2 result in increased internal exposure of acetaldehyde, the incidences of esophageal, stomach, colon, lung, head, and neck tumors in individuals who carry ADH1*2 is markedly less. A possible explanation for this difference is that increase in acetaldehyde levels in ADH1*2 individuals is brief due to further metabolism of acetaldehyde, whereas ALDH2*2 individuals have sustained exposure to acetaldehyde. Hispanics, in contrast, have very low levels of ADH1*2 and ALDH2*2, predisposing them to alcoholism and their offspring to fetal alcohol syndrome (FAS). Disulfiram, an ALDH inhibitor, is used to treat alcoholism, by enhancing acetaldehyde levels when alcohol is consumed. Whereas CYP2E1 polymorphism is reported in humans (Zintzaras et al., 2006), its role in the pathogenicity of alcoholism is still unclear.

Gender differences in responses to ethanol are well recognized. Women are more sensitive to alcohol, and exhibit higher mortality at lower levels of consumption than men (Sato et al., 2001; Brienza and Stein, 2002). Women exhibit somewhat higher blood levels than men following ingestion of equivalent doses of ethanol (Pikaar et al., 1988). This phenomenon appears to be due in part to more extensive ADH-catalyzed metabolism of ethanol by the male gastric mucosa (Frezza et al., 1990; Seitz et al., 1993). Kinetic data indicate that hepatic ADH does not play a role in the sex difference in first-pass elimination of ethanol, though Chrostek et al. (2003) did report higher activities of ADH isoforms that efficiently oxidize ethanol in the liver of male subjects. Sex differences in ethanol metabolism in the remainder of the body appear to be small or nonexistent. A second factor contributing to the higher blood ethanol levels and greater CNS effects in women is their smaller volume of distribution for relatively polar solvents such as alcohols. It is well known that women are more susceptible to alcohol-induced hepatitis and cirrhosis (Thurman, 2000). A postulated mechanism for this sex difference is described below.

Alcohol now is recognized as the leading preventable cause of birth defects and developmental disorders (Review in Warren et al., 2011). The severity of birth defects resulting from exposure of the developing embryo or fetus to alcohol is determined by multiple factors, including genetic background, timing and level of alcohol exposure, pattern of drinking, and nutritional status. Peak maternal blood alcohol concentration is apparently the most important determinant of the likelihood of alcohol-related developmental effects. Overconsumption during all three trimesters of pregnancy can result in certain manifestations, dependent on the period of gestation during which ingestion occurs. The most serious adverse consequence of prenatal alcohol exposure is fetal alcohol syndrome (FAS), which has an estimated prevalence that ranges from 0.5 to 7.0 cases per 1000 births in the United States. Diagnostic criteria for FAS include: (1) heavy maternal alcohol consumption during gestation; (2) pre- and postnatal growth retardation; (3) craniofacial malformations including microcephaly; and (4) mental retardation. However, there are other alcohol-related brain and behavioral abnormalities that are not diagnosed as FAS because of the lack of abnormal facial features. This range of deficits now is referred to as fetal alcohol spectrum disorders (FASD) and is estimated to occur in 1% of births. A recent study revealed that 86.5% of children and adolescents (4 to 18 years old) with FASD had never been previously diagnosed or had been misdiagnosed (Chasnoff et al., 2015). Thus it is important to identify FASD as early in the life as possible to treat and prevent complications. Lecuyer et al. (2017), based on the results of studies in an animal model and in humans, suggested that measurement of placental growth factor in the placenta or fetal blood could serve as a predictive marker for FASD.

Despite an intensive research effort for three decades, the mechanisms underlying FAS remain unclear. Exposure of embryonic tissue to ethanol adversely affects many cellular functions critical to development, including protein and DNA synthesis, uptake of critical nutrients such as glucose and amino acids, and changes in several kinase-mediated signal transduction pathways (Shibley and Pennington, 1997). Alcohol may also harm the fetus indirectly as a result of the mother's malnutrition. Numerous mechanisms have been suggested as contributing to alcohol-induced fetal damage, although none has been established with certainty (Goodlett and Horn, 2001). Some studies have suggested that oxidative stress on fetal tissues is responsible (Henderson et al., 1999), while others have implicated ethanol's effects on neurotransmitter-gated ion channels, particularly the NMDA receptor (Costa et al., 2000), and its ability to trigger cell death via necrosis and apoptosis (Ikonomidou et al., 2000; Goodlett and Horn, 2001). Others have reported that ethanol produces a long-lasting reduction in synaptic efficacy (Bellinger et al., 1999) and alters the fetal expression of developmentally important genes such as msx2 and insulin-like growth factors (Singh et al., 1996; Rifas et al., 1997). Other studies suggest that NMDA and GABA receptor-mediated apoptosis

play critical roles in learning impairment (Ikonomidou et al., 2000; Olney et al., 2002; Toso et al., 2006). Acetaldehyde may also contribute to the development of FAS, as it is shown to accumulate in the fetal brain after prenatal alcohol exposure (Hamby-Mason et al., 1997) and to be cytotoxic to cultured embryonic brain cells (Lee et al., 2005). Ethanol may also cause brain damage through endoplasmic reticulum (ER) stress in neurons (Yang and Luo, 2015). The potential mechanisms for ER stress include production of acetaldehyde adducts, oxidative stress, and disruption of intracellular calcium homeostasis. Animal models of FASD indicate that ethanol induces neuroimmune activation in the developing brain, resulting in microglial activation and production of pro-inflammatory molecules that are postulated to alter neuron survival and function and lead to long-term neuropathological and cognitive defects (Drew and Kane, 2014). Other mechanisms of FASD include: (1) alterations in the regulation of gene expression (e.g., reduced retinoic acid signaling, altered DNA methylation); (2) interference with mitogenic and growth factor responses involved in neural stem cell proliferation, migration, and differentiation; (3) disturbances in molecules that mediate cell-cell interactions (L1, NCAM, loss of trophic support); and (4) derangements of glial proliferation, differentiation, and function (Guerri et al., 2009; Ramsay, 2010).

Genetic variability in metabolic enzymes is another proposed mechanism for FAS. A role for CYP2E1 in the induction of FAS has been hypothesized, given its expression in human cephalic tissues during embryogenesis (Boutelet-Bochan et al., 1997). Results of in vitro experiments indicate that human CYP2E1 is effective in production of reactive oxygen intermediates (e.g., hydroxy radicals, superoxide anion, and H_2O_2) and in causation of lipid peroxidation (Dai et al., 1993). Membrane lipids and a variety of enzymes are targets for free radical attack. Albano et al. (1999) have demonstrated a marked increase in covalent binding of hydroxyethyl radicals to hepatic microsomal proteins from rats following chronic ethanol administration. There is not yet direct evidence that hydroxyethyl radicals contribute to lipid peroxidation, but they do readily react with α-tocopherol, GSH, and ascorbic acid, thereby potentially lowering liver antioxidant levels in vivo. Albano et al. (1999) also describe evidence that hydroxyethyl radical-protein adducts in hepatocytes induce immune responses, which may contribute to chronic ethanol hepatotoxicity (i.e., alcoholic liver disease [ALD]).

ALD is one of the most prevalent and fatal conditions of alcohol abuse. It is the 12th leading cause of death in the United States and accounts for approximately 4% of global mortality. The progression of ALD follows a characteristic pattern marked by appearance of fatty liver, hepatocyte necrosis, inflammation, regeneration nodules, fibrosis, and cirrhosis. Development of steatosis, characterized by accumulation of triglyceride droplets in the liver, is the initial stage of ALD. This stage is symptomless and reversible, if alcohol consumption is reduced. Despite extensive research, the mechanisms underlying ALD are still unclear. It is postulated that chronic alcohol consumption increases the gut permeability to lipopolysaccharide (LPS), an endotoxin that is a major constituent of the outer membrane of gram negative bacteria. LPS binds to receptors in Kupffer cells to produce reactive oxygen species (ROS), which activate NF-kB, leading to TNF- α and other inflammatory mediators that are cytotoxic to hepatocytes and chemoattractants for neutrophils (Bautista, 2000; Diehl, 2000). These mediators include interleukins, prostaglandins, free radicals, and eicosanoids. TNF-α signaling also increases mitochondrial ROS, which trigger apoptotic cell death in liver. Apoptotic cells in turn result in increased inflammatory response. Pro-inflammatory cytokines and oxidative stress stimulate collagen synthesis by hepatic stellate cells, leading to alcoholic fibrosis (Lieber, 2004). Chronic alcohol consumption also inhibits components of innate immunity, such as natural killer (NK) cells. The inhibition of NK cells decreases NK cell-mediated killing of stellate cells, contributing to fibrosis (Mandrekar and Szabo, 2009; Miller et al., 2011). Alcohol-induced oxidative stress is thought to cause liver injury by promoting the development of both humoral and cellular immune responses (Vidali et al., 2008). CYP2E1 can generate ROS during its catalytic cycle. Because CYP2E1 metabolizes ethanol and increases during ethanol exposure, it is suggested to play a significant role in ethanol-induced liver toxicity. Lu et al. (2010) recently reported that wild-type mice and humanized CYP2E1 (knock-in) mice had significant ethanol-induced oxidative stress and fatty liver, but CYP2E1 knock-out mice did not have these changes. Another mode of cell death, necroptosis (necrosis through apoptosis-regulated pathway), has been reported in mice after chronic ethanol exposure. Necroptosis is activated by receptor-interacting protein kinase 3, which is dependent on CYP2E1 expression (Roychowdhury et al., 2013). Necroptosis also exacerbates chronic inflammation by pro-inflammatory pathways.

Acetaldehyde is capable of upregulating collagen synthesis either directly or indirectly by inducing TGF-β1. Hence, polymorphisms of ALDH can increase the risk of fibrosis (Purohit and Brenner, 2006). Kupffer cells of female rats are more sensitive to endotoxin than those of males. Estrogen increases the sensitivity of Kupffer cells of rats to endotoxin, leading to increased production of TNF-α and death of hepatocytes (Thurman, 2000; Yin et al., 2000; Colantoni et al., 2003). This phenomenon is believed to account in part for the more severe hepatitis and cirrhosis commonly seen in female alcoholics (Thurman, 2000).

Alcohol-induced damage of the liver and other tissues is believed to result in part from nutritional disturbances, as well as toxic effects (Lieber, 2004; DiCecco and Francisco-Ziller, 2006). Lack of money, poor judgment, prolonged inebriation, and appetite loss contribute to poor nutrition and weight loss in alcoholics. A high percentage of calories in the alcoholic's diet is furnished by alcohol. Malabsorption of thiamine, diminished enterohepatic circulation of folate, degradation of pyridoxal phosphate, and disturbances in the metabolism of vitamins A and D can occur (Mezey, 1985). Recent research showed fungal overgrowth in the intestine of mice from chronic alcohol exposure exacerbated ALD (Yang et al., 2017).

Prostaglandins released from endotoxin-activated Kupffer cells may be responsible for a hypermetabolic state in the liver. With the increase in oxygen demand, the viability of centrilobular hepatocytes would be most compromised, due to their relatively poor oxygen supply (Thurman, 1998). Metabolism of ethanol via ADH and ALDH results in a shift in the redox state of the cell. The metabolites and the more reduced state can result in hyperlactacidemia, hyperlipidemia, hyperuricemia, and hyperglycemia, leading to increased steatosis and collagen synthesis (Lieber, 2004).

Alcoholism can result in damage of extrahepatic tissues. Alcoholic myopathy is one of the more common consequences, occurring in 50% of alcohol abusers. The condition is characterized by reductions in skeletal muscle mass and strength (Adachi et al., 2003; Preedy et al., 2003). Alcoholic cardiomyopathy is a complex process that occurs in 20% to 25% of alcoholics. It results from decreased synthesis of cardiac contractile proteins, attack of oxygen radicals, increases in endoplasmic reticulum Ca^{2+}-ATPase and an antibody response to acetaldehyde–protein adducts (Richardson et al., 1998; Preedy et al., 2003). Interestingly, light-to-moderate drinking is reported to protect against atherosclerosis in the carotid artery, a major cause of ischemic stroke (Hillbom, 1999). Alcohol increases high-density lipoprotein (HDL) cholesterol by inducing the constituents, apo I and II, by increasing catabolism of very low-density lipoproteins and chylomicrons, and by delaying HDL

catabolism (Kolovou et al., 2006). It is also hypothesized that ethanol metabolism in the vascular wall may inhibit oxidation of low-density lipoproteins (LDL), a requisite for atherogenesis. Phenolic antioxidants in wines may also inhibit LDL oxidation as well as reduce platelet aggregation. Conversely, heavy drinking appears to deplete antioxidants and have the opposite effects (Hillbom, 1999; Kolovou et al., 2006). Recent heavy drinking increases the risk of both hemorrhagic and ischemic strokes. Increased oxidative stress and suppressed immunity caused by chronic alcohol exposure increases the risk for acute lung injury, pneumonia, acute respiratory distress syndrome, and chronic obstructive pulmonary disease (Traphagen et al., 2015). Other organ systems can also be adversely affected in alcoholics including the brain and GI tract (Kril et al., 1997; Rajendram and Preedy, 2005).

There is concern about the role of ethyl alcohol in carcinogenesis, due to the frequent consumption of alcoholic beverages by millions of people. IARC (1988) concluded that there was "sufficient evidence" for causation of tumors of the oral cavity, pharynx and larynx, esophagus, and liver of humans. In a 2007 meeting, IARC added breast and colorectal cancers to the list of cancers causally linked with alcohol consumption (Baan et al., 2007). Genotoxic effects by acetaldehyde and elevated estrogen levels are likely mechanisms for breast cancer (Boeffetta and Hashibe, 2006). According to the World Health Organization Global Burden of Disease Project, drinking accounts for about 3.2% of all deaths and 3.6% of all cancers (Rehm et al., 2004). The original associations between alcohol and cancers came primarily from epidemiological case–control and cohort studies. One such cohort study of 276,000 American men showed increase in total cancer risk with increasing ethanol consumption (Boeffetta and Garfinkel, 1990). In support of epidemiological studies, a rat study showed that ethanol induces tumors in various organs including oral cavity, tongue, and lips (Soffritti et al., 2002). While mechanisms for alcohol-related carcinogenesis are not fully understood, metabolism plays a major role (Seitz and Stickel, 2007). Ethanol is shown to induce CYP2E1, which is known to generate ROS that damage DNA, lipids, and proteins. Additionally, acetaldehyde, which is carcinogenic to humans, reacts with DNA, forming adducts. Evidence suggests that the risk of alcohol-induced cancer in humans is modulated by genetic polymorphisms in enzymes responsible for alcohol metabolism, folate metabolism, and DNA repair (Boeffetta and Hashibe, 2006). Evidence also suggests that epigenetic alterations, specifically DNA methylation and histone acetylation and methylation, by ethanol lead to cancer development (French, 2013; Varela-Rey et al., 2013). Several mechanisms may mediate the effects of ethanol on DNA methylation, including reduced folate levels, inhibition of DNA methyltransferase and inhibition of enzymes that lead to lower levels of the methyl donor, S-adenosylmethionine (SAMe). Chronic ethanol feeding alters the methylation and acetylation of histones in the liver, leading to upregulation of p21, causing cell cycle arrest and DNA damage. This, in turn, results in a decrease in DNA methylation. Ethanol may also stimulate carcinogenesis by interfering with retinoic acid metabolism. Ethanol and smoking act synergistically to cause oral, pharyngeal, and laryngeal cancers. Age, sex, and ethnicity have also been reported to be factors. It is generally accepted that alcohol induces liver cancer by causing cirrhosis or other chronic liver damage and/or by enhancing the bioactivation of other carcinogens (Table 24-3).

Therapeutic intervention in ALD is a topic of great public health interest. In rat models, treatment with betaine and SAMe has decreased blood alcohol levels and liver pathology. Betaine was more effective than SAMe. The proposed mechanism for its decrease in ethanol toxicity is increased metabolism of ethanol by higher NAD+ generation, through conversion of norepinephrine to epinephrine. However, SAMe treatment did not decrease liver pathology in a 6-month clinical trial. Betaine is also proposed to protect leaky gut by regulating crucial methylation reactions required for maintaining gut barrier integrity. Based on the findings in animal studies, it is strongly recommended that betaine be added to alcoholic beverages to prevent ALD (Neuman et al., 2015).

Table 24-3

Possible Mechanisms of Ethanol Carcinogenicity

Congeners: additives and contaminants in alcoholic beverages influence carcinogenicity
CYP2E1 induction by ethanol increases metabolic activation of procarcinogens
Ethanol acts as a solvent for carcinogens, enhancing their absorption into tissues of the upper GI tract. Ethanol affects the actions of certain hormones on hormone-sensitive tissues
Immune function is suppressed by alcohol
Absorption and bioavailability of nutrients are reduced by alcohol

SOURCE: Adapted with permission from Ahmed (1995).

An international symposium (Purohit et al., 2005), sponsored by the National Institute on Alcohol Abuse and Alcoholism in 2004, concluded that chronic ethanol consumption may promote carcinogenesis by: (1) production of acetaldehyde, a weak mutagen and carcinogen; (2) induction of CYP2E1 and its associated oxidative stressors and conversion of procarcinogens to carcinogens; (3) depletion of SAM and, consequently, induction of global DNA hypomethylation; (4) increased production of inhibitory guanine nucleotide regulatory proteins and components of extracellular signal-regulated kinase-mitogen-activated protein kinase signaling; (5) accumulation of iron and associated oxidative stress; (6) inactivation of the tumor suppressor gene *BRCA1* and increased estrogen responsiveness (primarily in the breast); and (7) impairment of retinoic acid metabolism. Jeannot et al. (2011) recently reported that ethanol may act by promoting spontaneously initiated cells, especially *H-ras* mutated cells.

Methanol

Methanol (methyl alcohol, wood alcohol, and CH_3OH) is primarily used as a starting material for the synthesis of chemicals such as formaldehyde, acetic acid, methacrylates, ethylene glycol, and methyl tertiary-butyl ether (MTBE). CH_3OH is found in windshield washer fluid, carburetor cleaners, antifreeze, and copy machine toner, and serves as fuel for Sterno™ heaters, model airplanes, and Indianapolis 500 racecars. It also functions as a denaturant for some ethyl and isopropyl alcohols, rendering them unfit for consumption. It is used to a limited extent as an alternative fuel for fleet vehicles (usually in a mixture of 85% CH_3OH and 15% gasoline) and is being explored as a gasoline additive and hydrogen source for fuel cell vehicles. Exposure of the general population also occurs via the consumption of fruits, fruit juices, vegetables, and alcoholic beverages that contain free CH_3OH or CH_3OH precursors. Indirect exposure occurs via the hydrolysis of the artificial sweetener, aspartame, and subsequent CH_3OH absorption from the gut. Very low-level exposures may occur via ambient air and drinking water. Of all chemicals reported, CH_3OH was ranked No. 1 for fugitive air

emissions, No. 3 for point source air emissions, and No. 2 for surface water discharges according to the 2004 Toxic Release Inventory (EPA, 2006a). Nevertheless, persistence and bioaccumulation in the environment are not expected. The consumption of adulterated "bootleg" whiskey is a major cause of CH_3OH poisoning.

Serious CH_3OH toxicity is most commonly associated with ingestion. Following ingestion, the parent alcohol can cause CNS depression, ataxia, difficult breathing, acute gastritis or pancreatitis, anorexia, intense abdominal pain, vomiting, and diarrhea (Tephly, 1991). Left untreated, acute CH_3OH poisoning in humans is characterized by an asymptomatic latent period of 12 to 24 hours followed by formic acidemia, ocular toxicity, coma, and in extreme cases death (Lanigan, 2001). These effects are caused by its metabolites. Respiratory failure or sudden respiratory arrest is the most common cause of death in methanol poisoning. Visual disturbances generally develop between 18 and 48 hours after ingestion and range from mild photophobia and misty or blurred vision to markedly reduced visual acuity and complete blindness (Eells et al., 1996). Although there is considerable variability among individuals in susceptibility to CH_3OH, a frequently cited lethal oral dosage is 1 mL/kg. Blindness and death have been reported with dosages as low as 0.1 mL/kg (ATSDR, 1993). CH_3OH's target within the eye is the retina, specifically the optic disk and optic nerve. Optic disk edema and hyperemia are seen, along with morphological alterations in the optic nerve head and the intraorbital portion of the optic nerve. Both axons and glial cells exhibit altered morphologies (Kavet and Nauss, 1990). Rods and cones, the photoreceptors of the retina, are also altered functionally and structurally (Seme et al., 1999). Evidence is accumulating that Müller cells, neuroglia that function in the maintenance of retinal structure and in intra- and intercellular transport, are early targets of CH_3OH (Garner et al., 1995a). There are indications of mitochondrial disruption in Müller and photoreceptor cells, which is consistent with the long-held view that formate inhibits the energy-generating mitochondrial enzyme, cytochrome *c* oxidase, which is critical for the proper functioning of highly oxidative organs like the retina. This mechanism might explain, at least in part, CH_3OH's selective toxicity to photoreceptors and other highly metabolically active cells (Eells et al., 1996). Interestingly, exposure of rats to monochromatic red radiation from light-emitting diode arrays can aid in the recovery of rod and cone function in CH_3OH-treated rats and protect the retina from histopathological changes characteristic of formate toxicity (Eells et al., 2003). This "photobiomodulation" is thought to be mediated by the ability of monochromatic red to near-IR light to improve mitochondrial respiratory chain function (i.e., increase cytochrome *c* oxidase activity), thereby initiating a signaling cascade that promotes cellular proliferation and cytoprotection.

Significant species differences exist in the susceptibility of CH_3OH toxicity. Acute ocular toxicity, metabolic acidosis, CNS depression, and death in humans are not seen with rodents (Tephly, 1991). Conversely, rodents can be susceptible to delayed adverse effects of methanol such as developmental toxicity. Therefore, elucidation of the mechanism of CH_3OH's acute toxicity was hampered for years by the lack of appropriate animal models. Largely based on the work of Gilger and Potts (1955), it became apparent that only nonhuman primates respond to CH_3OH similarly to humans. Humans and nonhuman primates metabolize CH_3OH to formaldehyde (HCOH) by ADH, whereas rodents utilize catalase. In all mammalian species, HCOH is very rapidly converted via formaldehyde dehydrogenase (FLDH) to formate, which is further metabolized to CO_2. The conversion of formate to CO_2 occurs via a two-step, tetrahydrofolate (THF)-dependent pathway. First, formate is converted to 10-formyl-THF by formyl-THF synthetase, after which 10-formyl-THF is oxidized to CO_2 by formyl-THF dehydrogenase (F-THF-DH). Because rodents have much higher hepatic THF levels than primates, formate does not accumulate as it does in humans and monkeys (Medinsky and Dorman, 1995; Martinasevic et al., 1996). Another possible explanation is the lower F-THF-DH activity in primate liver (Johlin et al., 1989). It is further speculated that primates are more susceptible to CH_3OH and formate toxicity due to their relative inability to excrete excess formate via the kidneys (Smith and Taylor, 1982). Irregardless, susceptibility to CH_3OH-induced ocular toxicity is dependent on the relative rate of formate clearance. Dietary and chemical depletion of endogenous folate cofactors in rats have been shown to increase formate accumulation following CH_3OH, resulting in the development of metabolic acidosis and ocular toxicity similar to that observed in humans (Eells et al., 1996, 2000). Sweeting et al. (2010) reported that rabbits, similar to humans and monkeys, metabolize CH_3OH by ADH and exhibit slower CH_3OH clearance and increased formate accumulation, suggesting that rabbits resemble primates and humans more closely than rodents. A simplified scheme of CH_3OH metabolism is presented in Fig. 24-10. CH_3OH is primarily metabolized by ADH. The alcohol is also metabolized by the inducible hepatic microsomal ethanol oxidizing system. The polymorphism of these enzymes, as well as enzymes involved in folate metabolism, impact methanol toxicity.

For years there was considerable debate whether HCOH or formate was responsible for CH_3OH's ocular toxicity. The finding that HCOH does not accumulate following CH_3OH treatment, even in folate-deficient monkeys that are profoundly sensitive to CH_3OH, argues against a role for HCOH. Also, species of differing susceptibility exhibit comparable blood HCOH half-lives. In contrast, formate has been shown to accumulate in the human and monkey following CH_3OH treatment. CH_3OH-derived formate has also been quantified in the vitreous humor, retina, and to a lesser extent the optic nerve (Eells et al., 1996). Moreover, CH_3OH-induced retinal dysfunction, as indicated by diminution of the amplitude of electroretinogram (ERG) *a* and *b* waves, is negatively correlated in a linear fashion with blood formate concentrations (Eells et al., 1996). Others have reported similar relationships between blood formate and ERG responses indicative of photoreceptor dysfunction (Seme et al., 1999). Formate has also been shown to induce ocular toxicity in monkeys and formate oxidation-inhibited rats in the absence of metabolic acidosis or a reduction in blood pH (Martin-Amat et al., 1978; Eells et al., 1996). Direct incubation of formate with cultured ocular cells caused ATP depletion and cytotoxicity (Treichel et al., 2003). Thus, formate appears to act

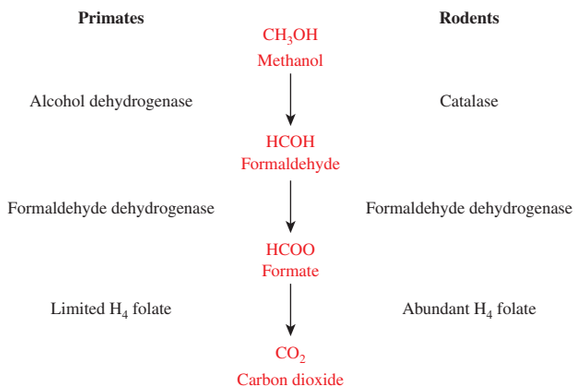

Figure 24-10. *Scheme for the metabolism of methanol.* Major enzymes are listed for primates (left) and rodents (right). Conversion of formate to CO_2 is rapid in rodents, but relatively slow in primates. (Used with permission of Dorman DC, Welsch F: CIIT Activities. 1996;16:1–7.)

as a direct ocular toxin and not indirectly through the induction of an acidotic state, though acidosis may potentiate formate toxicity (since the inhibition of cytochrome oxidase increases as pH decreases and acidosis allows for greater diffusion of formic acid into cells).

The question has been raised whether ocular toxicity is simply a function of circulating formate reaching the visual tract, or whether metabolism in retinal or optic nerve tissues generates toxic metabolites locally. This is a legitimate question considering that metabolism of CH_3OH to HCOH via peroxisomal enzymes (catalase) has been demonstrated in rat retina in vitro (Garner et al., 1995a), and the presence of cytoplasmic FLDH activity has been demonstrated in several regions of the rat and mouse eye, including the retina (Messiha and Price, 1983; McCaffery et al., 1991). By use of a folate-deficient rat model, Garner et al. (1995b) showed that a level of blood formate generated by i.v. infusion of pH-buffered formate did not diminish the ERG *b*-wave amplitude generated by Müller cells of the retina, as did a comparable blood level of formate derived from CH_3OH. This suggests that the intraretinal metabolism of CH_3OH is necessary for the initiation of retinal toxicity by formate. Not only are the enzymes necessary to produce formate present in the retina, but so too are folate and F-THF-DH. The later was found to be localized in the mitochondria of Müller cells, prompting the suggestion that F-THF-DH may serve a dual role, one protective of the Müller cell and the other toxic. Protection would come in the form of formate oxidation; toxicity from the overconsumption or depletion of ATP required for formate metabolism via the folate pathway (Martinasevic et al., 1996).

The aforementioned findings raise concerns about the safety of CH_3OH exposure. Chamber studies of human volunteers exposed to 200 ppm CH_3OH for 4 or 6 hours showed no blood formate accumulation above background (Lee et al., 1992; d'Alessandro et al., 1994). Whereas this might be considered evidence that exposure at the 2011 ACGIH TLV and OSHA PEL of 200 ppm poses no risk of ocular toxicity, such an interpretation may not be valid, given that ocular toxicity may be a function of intraretinal CH_3OH metabolism rather than circulating formate levels.

The effects of acute, high CH_3OH exposures are well characterized compared to those of chronic, low-level exposures. Few reproductive/developmental studies and even fewer cancer bioassays have been conducted, due to the fact that rodents are not an ideal animal model for CH_3OH. Nonetheless, Soffritti et al. (2002) conducted a bioassay in which Sprague–Dawley rats received CH_3OH in their drinking water (0, 500, 5000, or 20,000 ppm *ad libitum* for 104 weeks). The investigators concluded that CH_3OH was a multipotent carcinogen. However, they referenced two Japanese bioassays that found no evidence of carcinogenicity in B6C3F1 mice or F344 rats exposed 20 hours/day to 10, 100, or 1000 ppm CH_3OH by inhalation for 18 and 24 months, respectively (NEDO, 1987; Katoh, 1989). A recent review of carcinogenicity and genetic toxicity studies concluded that CH_3OH is not likely to be carcinogenic in humans (Cruzan, 2009).

The NTP Center for the Evaluation of Risks to Human Reproduction released a monograph on CH_3OH's reproductive/developmental toxicity, largely based on an expert panel report (NTP, 2003, 2004b). Among the conclusions was that CH_3OH is a potential developmental toxicant in humans, provided a high enough blood concentration of the parent compound (assumed to be the proximate teratogen) is achieved. As this conclusion was largely based on data in rodents that metabolize CH_3OH much differently than humans, its validity has been called into question (Clary, 2003). As such, the few reproductive/developmental studies in nonhuman primates are particularly informative. These studies reported that maternal exposure of monkeys to inhaled CH_3OH (200, 600, or 1800 ppm, 2.5 hours/day, 7 days/week prior to breeding and throughout pregnancy) was not associated with maternal toxicity, reproductive loss, or congenital malformations in offspring, but was associated with a 6- to 8-day reduction in the mean length of pregnancy (Burbacher et al., 2004a, 2004b). Studies in rodents and rodent whole embryo cultures have implicated dysregulated cell death in the pathogenesis of CH_3OH-induced malformations, examined GSH status in the developing conceptus as it relates to vulnerability, and explored the basis for the increased sensitivity of the mouse embryo compared to the rat (Harris et al., 2003, 2004; Degitz et al., 2004; Hansen et al., 2005). Because the deposition of CH_3OH and formic acid in rabbits is shown to be similar to humans, Sweeting et al. (2011) considered rabbits as an appropriate model for assessing the risk of human developmental toxicity. Three strains of mice and New Zealand rabbits were given a single dose of CH_3OH during gestation. Two strains of mice were susceptible to CH_3OH teratogenicity. One strain of mice and the rabbits were resistant. Despite similar CH_3OH and formic acid disposition in three strains of mice, the finding that one strain did not exhibit teratogenic effects suggests mechanisms other than pharmacokinetics could be involved. A role for reactive oxygen species is suspected. The study also raised concerns about using rodents as a surrogate to predict human developmental toxicity of CH_3OH.

Whereas there is still much to be learned about the mechanisms of CH_3OH toxicity, what is known allows for effective therapies, if they are applied in a timely manner. The American Academy of Clinical Toxicology has published practice guidelines for the treatment of CH_3OH poisoning (Barceloux et al., 2002). In cases of severe CH_3OH poisoning, there is a direct correlation between the formic acid concentration and increased morbidity and mortality. Sodium bicarbonate is usually given i.v. to correct severe acidosis, and case reports suggest that it may enhance renal formate excretion. Metabolic blockade is usually achieved with ethanol or 4-methylpyrazole (fomepizole), both acting as effective competitive inhibitors of ADH. Fomepizole is the primary antidote for methanol poisoning due to its efficacy and lower toxicity profile. ADH-inhibition therapy is most effective when administered at the initial stages of poisoning. Folate or folinic acid (activated folate) therapy is also indicated to increase the efficiency of formate oxidation. Hemodialysis is generally indicated when acidemia, high CH_3OH concentrations, or visual symptoms are present, although there is conflicting data on whether it appreciably shortens the elimination half-life of formate (Kerns et al., 2002; Hantson et al., 2005). Lastly, the treatment threshold of 20 mg CH_3OH/dL of blood in a nonacidotic patient arriving early for care has been questioned as being overly conservative. This stems from a comprehensive review of worldwide CH_3OH poisonings that identified 126 mg/dL as the lowest early blood CH_3OH level ever clearly associated with acidosis (Kostic and Dart, 2003). CH_3OH exemplifies the benefits of knowing a chemical's mode of action when treating the poisoned patient. This knowledge also aids in identifying potentially sensitive subpopulations, such as those suffering from dietary folate deficiency. Research suggests, however, that even in a state of folate deficiency, the body probably contains sufficient folate stores to effectively detoxify small doses of CH_3OH-derived formate from exogenous sources (Medinsky et al., 1997).

GLYCOLS

Ethylene Glycol

Ethylene glycol (1,2-dihydroxyethane, EG) is a constituent of antifreeze, deicers, hydraulic fluids, drying agents, and inks, and is used to make plastics and polyester fibers. Workers may be exposed

dermally or by inhalation when solutions containing EG are heated or sprayed. The most important exposure route is ingestion, as EG may be accidentally swallowed, taken deliberately in suicide attempts, or used as a cheap substitute for ethanol. "Antifreeze" poisoning occurs frequently in cats and dogs that find its taste appealing. Using the National Poison Data System, a retrospective analysis found 45,097 reported cases of EG exposure from January 2006 to December 2013, resulting in 154 deaths (Jobson et al., 2015). EG enters the environment as a result of disposal of industrial and consumer products containing the chemical. It partitions into surface water and groundwater, but does not persist in any environmental medium and is practically nontoxic to aquatic organisms (Staples et al., 2001).

The TK profile of EG is well characterized. All laboratory mammals and humans metabolize EG similarly. A series of papers have been published describing the TK of EG in Sprague–Dawley rats and CD-1 mice after administration of a single dose by the i.v., oral, or percutaneous routes (Frantz et al., 1996a–c). Absorption from the GI tract of rodents and humans is very rapid and virtually complete, whereas cutaneous and pulmonary absorption are relatively slow and less extensive. Once absorbed, EG is distributed throughout the total body water. As illustrated in Fig. 24-11, EG is metabolized by NAD^+-dependent ADH to glycolaldehyde and on to glycolic acid (GA). GA is oxidized to glyoxylic acid by GA oxidase and lactic dehydrogenase. Glyoxylic acid may be converted to formate and CO_2, or oxidized by glyoxylic acid oxidase to oxalic acid (OA) (Wiener and Richardson, 1988; Frantz et al., 1996b). The rate-limiting step in the metabolism of EG is the conversion of GA to glyoxylic acid. EG has a half-life in humans of 3 to 8.6 hours (Leth and Gregersen, 2005). Under conditions of repetitive, low-dose exposure, EG is not expected to bioaccumulate, given its rapid metabolism and elimination. Pregnancy status has no bearing on the TK of EG, GA, or OA, as demonstrated by comparisons of pregnant and nonpregnant Sprague–Dawley rats (Pottenger et al., 2001).

The minimum acute lethal dose of EG in humans is estimated at about 1.4 mL/kg, which equates to 100 mL for a 70-kg adult (LaKind et al., 1999; Hess et al., 2004). Acute poisoning entails three clinical stages after an asymptomatic period, during which EG is metabolized: (1) a period of inebriation, the duration and degree depending on dose; (2) the cardiopulmonary stage 12 to 24 hours after exposure, characterized by tachycardia and tachypnea, which may progress to cardiac failure and pulmonary edema; and (3) the renal toxicity stage 24 to 72 hours postexposure. Metabolic acidosis, due largely to GA accumulation, can develop and become progressively more severe during stages 2 and 3 (Jacobsen et al., 1984; Moreau et al., 1998; Egbert and Abraham, 1999). Hypocalcemia can result from Ca^{2+} chelation by OA to form Ca^{2+} oxalate monohydrate (COM) crystals. Deposition of these crystals in kidney tubules is associated with organ damage and potentially with acute renal failure. Nephrotoxicity appears to be an acute, high-dose phenomenon, as no demonstrable kidney damage has been reported in occupational studies of groups such as airport deicing workers or Finnish auto mechanics (Laitinen et al., 1995; Gerin et al., 1997). COM crystal deposition has been reported in the walls of CNS blood vessels, with associated inflammation, edema, and sometimes neuropathy (Froberg et al., 2006).

Based on high-dose rodent studies, including two lifetime dietary studies, EG has very limited chronic toxicity potential,

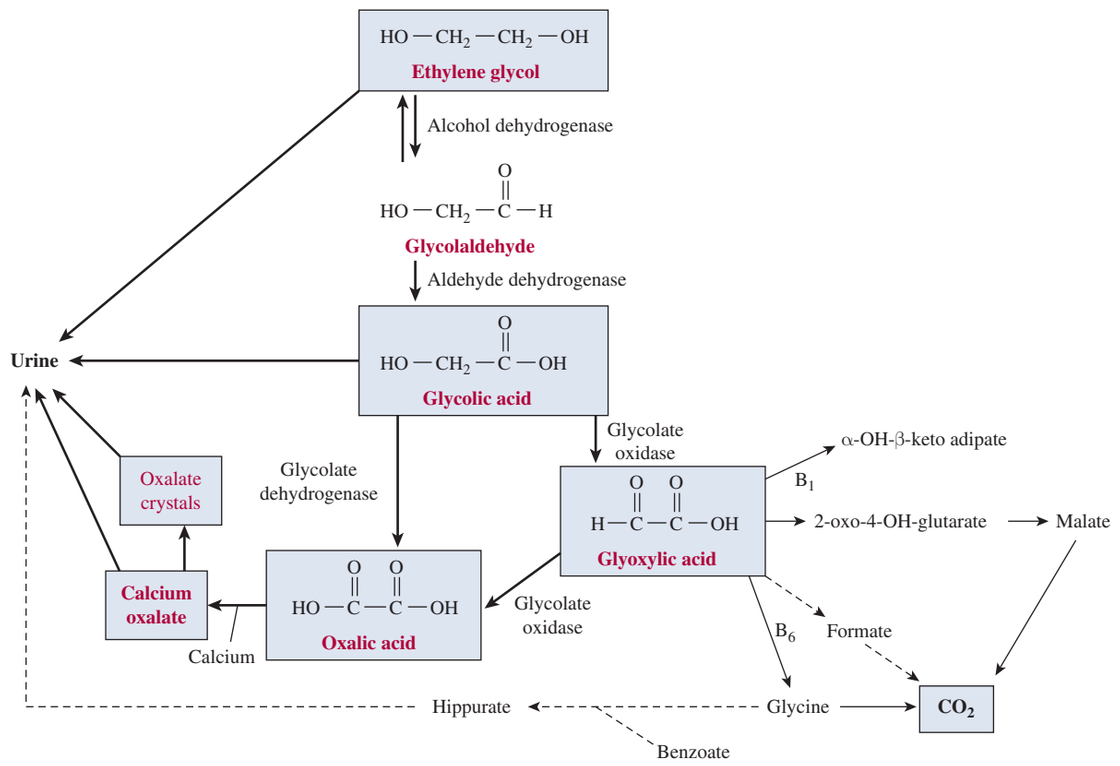

Figure 24-11. *Metabolic scheme for ethylene glycol in animals.* Key metabolites that have been observed in vivo are highlighted in boxes. Dashed lines are theoretical pathways that have not been verified in vivo or in vitro. (Used with permission of Corley et al., 2005a.)

exhibits no evidence of carcinogenicity, and does not appear to be a reproductive toxicant (DePass et al., 1986; NTP, 1993b). However, it can cause adverse developmental effects such as skeletal and soft tissue malformations and delayed ossification when administered during gestation at high doses (more than or equal to 500 mg/kg/day) (ATSDR, 1997b). GA appears to be the proximate developmental toxicant, with metabolic acidosis playing an exacerbating, but not obligatory role (Carney et al., 1996, 1999). Pottenger et al. (2001) have demonstrated that at teratogenic doses of EG, blood levels of GA are disproportionately high relative to the EG dose, further supporting GA as the proximate teratogen and implying a role for metabolic saturation. Corley et al. (2005d) have detailed the key events in EG-induced developmental toxicity and suggest the disruption of *hox* gene expression, fluid imbalance, and dysregulation of cell death in the embryo as mechanistic possibilities. The mechanism of GA's teratogenic action, however, remains largely unknown, as does the relevance of defects in rodents given the absence of reported developmental effects in humans. The NTP Center for the Evaluation of Risks to Human Reproduction has released a monograph on EG's reproductive/developmental toxicity largely based on an expert panel report (NTP, 2004d, 2004f). The overall conclusion was that EG exposures below the level of metabolic saturation should not result in developmental toxicity in humans. Further, because environmental and occupational exposures to humans are 2 to 3 orders of magnitude lower than those expected to result in metabolic saturation, such exposures are of negligible concern. Corley et al. (2005a) have developed a PBTK model of EG and GA and compared internal dose surrogates in rats and humans. Based on the comparisons, they also concluded that occupationally or environmentally exposed humans are unlikely to achieve blood levels of GA that have been associated with developmental toxicity in rats. This PBTK model has now been extended to include glyoxylic acid, OA, and the precipitation of calcium oxalate in the kidney (Corley et al., 2011). Carney et al. (2008) have demonstrated a role for toxicokinetic factors, including the rate of maternal metabolism of EG to GA, in the insensitivity of rabbits to teratogenicity compared to the rat. These authors point out that based on toxicokinetics, the rabbit is the more relevant model of human EG exposure. Furthermore, Carney et al. (2011) have experimentally confirmed that a dose rate of EG sufficient to saturate GA's metabolism to glyoxylic acid is critical for developmental toxicity, supporting the C_{max} rather than AUC value of embryonic GA as the key metric related to risk.

The chief concern about EG is renal toxicity after high dose, acute exposure. Although EG must be metabolized to toxic intermediates to induce kidney damage, the specific metabolite(s) and underlying mechanism(s) responsible remain to be fully elucidated. There is agreement, however, that the process involves a toxicant-induced proximal tubular necrosis leading to loss of renal function. Poldelski et al. (2001) exposed isolated mouse proximal tubule segments and human proximal tubular epithelial cells (HK-2) to GA, glycoaldehyde, glyoxylate, or OA for 15 to 60 minutes, on which basis they concluded glycoaldehyde and glyoxylate are the principal metabolites responsible for EG nephrotoxicity. This is contrary to the prevailing opinion that renal toxicity is due to the terminal metabolite, OA, which precipitates in the kidney in the form of COM crystals (Guo and McMartin, 2005; Guo et al., 2007; McMartin, 2009). Corley et al. (2005d) have listed the key mechanistic events in the renal toxicity of EG: (1) metabolism of EG to OA via GA; (2) concentration of OA in tubular urine → precipitation of OA with Ca^{2+} → buildup of COM crystals in renal tubular epithelium → adherence of COM crystals to the plasma membrane of proximal tubular cells → subsequent intracellular uptake of COM crystals by endocytosis; and (3) physical trauma by COM crystals and/or production of free radicals and lipid peroxidation leading to cell necrosis, apoptosis, and renal tubular degeneration. Clearly, the weight of evidence implicates OA is critical in the induction of renal damage, but a possible role for less frequently observed hippuric acid crystals and direct cytotoxicity by other metabolites cannot be ruled out. In vitro assays have indicated that both oxalate and COM crystals are injurious to renal epithelial cells (Thamilselvan and Khan, 1998). Also, renal damage has been observed occasionally following exposure to EG, without documentation of crystals in the kidney. Nonetheless, EG has been used as a model crystal-forming renal toxicant to determine the basis for COM crystal retention in the kidney. Studies with EG suggest that crystal retention in the kidney may depend on the expression of the proteins hyaluronan, osteopontin, and their mutual cell surface receptor CD44, all of which are upregulated in response to renal injury/inflammation, and under normal circumstances play a role in reestablishment of epithelial barrier integrity and restoration of renal function. However, their upregulation could turn a noncrystal-binding epithelium into a crystal-binding one, thereby setting the stage for crystal retention and renal injury (Asselman et al., 2003; Verhulst et al., 2003).

The intracellular target(s) of COM crystals is(are) not known with certainty, but it is well established that mitochondrial damage is a major mechanism for chemically mediated renal tubular necrosis. As such, COM crystals in the cytoplasm could directly affect mitochondria or be "dissolved" within the cell to release oxalate and Ca^{2+} ions that could do so. McMartin and Wallace (2005) hypothesized as much and demonstrated that COM produced a dose-dependent decrease in State 3 respiration in isolated rat kidney mitochondria, which they believe may be responsible for induction of a phenomenon known as mitochondrial permeability transition (MPT). MPT is characterized by an otherwise impenetrable inner mitochondrial membrane undergoing transformation, whereby it becomes penetrable to solutes or large molecules. As a result of MPT induction, mitochondria undergo a rapid and progressive osmotic swelling, depolarization of mitochondrial membrane potential, and inhibition of oxidative phosphorylation/ATP synthesis, all of which can lead to either oncotic/ischemic cell death or apoptotic cell death. Additional insight into the pathogenesis of EG-induced renal damage has been provided by Chen et al. (2004), who dosed male rats with 0.75% EG in their drinking water for 2, 4, and 8 weeks, after which the kidneys were processed for RNA isolation and microarray analysis using a rat-based chip. Changes in the expression of genes associated with tubular structure and function, oxidative damage, and inflammation were common. Increased expression of mitochondrial uncoupling protein was also observed, providing additional evidence of a mitochondrial target. Alternatively, the study of Hovda et al. (2010) raised the possibility of a lysosomal target and subsequent enzymatic destruction based on electron micrographs of human and rat proximal tubule cells that had internalized COM crystals.

In cases of severe EG poisoning, early diagnosis and aggressive therapeutic intervention are essential for a favorable clinical outcome. A plasma EG level of 20 mg/dL is considered the threshold for kidney toxicity, if therapeutic strategy is based on EG concentration alone (Hess et al., 2004). However, no signs of renal injury have developed in patients at initial plasma GA concentrations of up to 10.1 mM or 76.7 mg/dL (Brent et al., 1999; Hess et al., 2004). In any case, treatment of EG poisoning involves three primary goals: (1) correction of the patient's metabolic acidosis; (2) inhibition of EG metabolism to its toxic metabolites; and (3) removal of EG and its toxic metabolites by hemodialysis, if necessary. As Guo and McMartin (2005) demonstrated that the renal

cytotoxicity of COM crystals is potentiated by severe acidosis, its correction with bicarbonate should help ameliorate the development of renal toxicity. As with CH_3OH, ethanol and 4-methylpyrazole (fomepizole) are frequently given as antidotes for EG poisoning and can prevent renal injury if given early in the course of intoxication. These ADH inhibitors block EG's metabolic bioactivation, thus minimizing the formation of toxic metabolites and allowing EG to be eliminated unchanged by the kidneys. There is increasing evidence that i.v. fomepizole may be more efficacious than ethanol as an antidote and obviate the need for hemodialysis in most EG-poisoned patients with normal renal function (Moreau et al., 1998; Brent et al., 1999; Sivilotti et al., 2000; Scalley et al., 2002; Buchanan et al., 2010). Watson (2000) has even stated that the results of fomepizole therapy support the recommendation that ethanol's use should be limited to settings where fomepizole is not available or contraindicated. The same editorial by Watson also supports the use of hemodialysis when renal insufficiency or metabolic acidosis are present rather than the traditional criterion of serum EG concentrations more than 50 mg/dL. Interestingly, Corley and McMartin (2005) have refined a previously published PBTK model for EG and its GA metabolite in rats and humans to include hemodialysis, ethanol, and fomepizole as therapeutic interventions. This enabled the model to describe data from several human case reports of EG poisoning and demonstrated that fomepizole, if administered early enough, can indeed be more effective than ethanol or hemodialysis in inhibiting EG metabolism. McQuade et al. (2014) have reviewed the diagnostic challenges associated with EG poisoning and recommend starting antidotal therapy based on patient history and indirect tests rather than waiting for chromatographic confirmation. Evidence-based consensus guidelines for the out-of-hospital management of those suffering unintentional EG exposure have been published (Caravati et al., 2005).

From a regulatory standpoint, EPA has established an RfD of 2 mg/kg/day based on a NOAEL for kidney toxicity in the chronic rat feeding study by DePass et al. (1986) (EPA, 2006b). Using International Programme for Chemical Safety guidelines, Palmer and Brent (2005) have derived a "chemical-specific adjustment factor" of 43.7 mg/kg/day based on a NOAEL for acute renal toxicity in humans. Although considerably higher than EPA's RfD, it is comparable to the Benchmark Dose 0.5 ($BMD_{0.5}$) for EG-induced nephrotoxicity of 49 mg/kg/day derived by Health Canada (2000). More recently, Snellings et al. (2013) derived an alternative RfD based on an oxalate crystal-induced nephropathy mode of action. They applied Benchmark Dose methods to dose–response data consisting of severity and incidence measures of kidney lesions in male Wistar rats (Corley et al., 2008) as a function of PBTK-derived maximum concentrations of OA in the kidney. The resulting RfD of 15 mg/kg/day reflects a reduction in overall uncertainty, as male Wistar rats are considerably more sensitive to EG than their Fischer-344 counterparts used by DePass and colleagues. The nature of this strain difference in sensitivity has been experimentally examined (Li and McMartin, 2009; Li et al., 2010). The refined RfD is apt to be somewhat conservative, because humans are thought to be less sensitive than Wistar rats based on comparisons of COM-induced cell death of cultured proximal tubule cells in the two species (Guo and McMartin, 2005).

Diethylene Glycol

Diethylene glycol (DEG) is similar in physicochemical properties to EG, but has a higher boiling point, viscosity, and specific gravity. DEG serves as a chemical intermediate in the production of polyester resins and polyurethanes, and as a solvent for shellacs and printing ink. It is hygroscopic, which leads to applications as a drying agent for natural and industrial gases, a humectant for cork and paper, and an additive in cosmetics. Inhalation exposure is likely to occur by aerosols rather than vapor because of its low vapor pressure, and would be limited to industrial operations in which DEG is heated or where fogs or mists are generated. DEG's solvent properties make it useful in a variety of consumer products such as brake fluid and lubricants, making it accessible for intentional and mistaken ingestion. Unlike for EG, an RfD has not been established for DEG. Neither OSHA nor ACGIH has established occupational exposure limits, though the Occupational Alliance for Risk Science reaffirmed in 2016 a Workplace Environmental Exposure Level (WEEL) for the respirable fraction of DEG of 10 mg/m^3 as an 8-hour TWA (TERA, 2017).

DEG's use as an excipient in a liquid sulfanilamide preparation resulted in 105 deaths in the United States in 1937. This mass poisoning incident prompted passage of the Food, Drug and Cosmetic Act of 1938 (Wax, 1995). Use of DEG-contaminated propylene glycol or glycerin in various pharmaceuticals has caused multiple fatalities from renal failure in Nigeria, Bangladesh, India, and Haiti. In the Haitian incident, 109 cases of acute renal failure (with 88 deaths) were identified in children who received a locally manufactured acetaminophen syrup containing DEG-contaminated glycerin (O'Brien et al., 1998). The median lethal dose of DEG was estimated at 1.34 mL/kg. Renal failure was the "hallmark" finding in these cases, but hepatitis, pancreatitis, and severe neurologic manifestations (e.g., encephalopathy, optic neuritis with retinal edema, and unilateral facial paralysis) were frequently seen. Alfred et al. (2005) have presented seven cases of DEG poisoning characterized by metabolic acidosis, renal failure, and in three patients, neurotoxicity. In one of the more bizarre case reports, Marraffa et al. (2008) detailed multisystem organ failure following the intentional ingestion of 16 ounces of wallpaper stripper containing 26% DEG. Schep et al. (2009) have summarized the main aspects of DEG poisoning including epidemiology, toxicokinetics, toxicity and its mechanisms, clinical features, diagnosis, and management. These authors, like others, describe a final phase of DEG poisoning consisting of encephalopathy and multiple cranial and peripheral neuropathies with fatal and near-fatal outcomes. Though mechanism(s) remain unclear, delayed neurological sequelae are typically seen only in those patients with severe metabolic acidosis and renal failure (Reddy et al., 2010; Imam et al., 2014; Gupta et al., 2017).

Compared to EG, toxicological data for DEG in animals are quite limited. Consistent with observations in human poisonings, Fitzhugh and Nelson (1946) reported dose-dependent hepatorenal injury in rats that consumed diets containing DEG. Kraul et al. (1991) reported oliguria or polyuria, proteinuria, and other manifestations indicative of renal tubule injury in rats given a single i.p. dose of DEG. Similar to EG, renal tubular necrosis and COM crystal deposition in tubules have been observed following acute exposure of male rats (Hebert et al., 1978). As for reproductive/developmental effects, high-dose gavage and dietary studies during gestation in mice, rats, and rabbits have largely been negative for embryotoxicity and teratogenicity. One study in mice employing a continuous breeding protocol has revealed diminished reproductive performance and fertility, as well as limited data on craniofacial malformations in live-born and dead pups at drinking water exposures equivalent to 6.1 g/kg/day (Williams et al., 1990). Citing this lone positive result and the developmental toxicity profile of EG, Ballantyne and Snellings (2005) gavaged CD-1 mice and CD rats with a range of DEG doses (559 to 11,180 mg/kg/day) on gestation days 6 to 15. They reported no embryotoxic or teratogenic effects at any dose in either species, except for delayed ossification consistent with reduced fetal body weight in rats.

Like EG, DEG is well absorbed from the GI tract, distributed throughout total body water and organs on the basis of blood flow, and initially metabolized by ADH and subsequently by ALDH (Heilmair et al., 1993). The ether linkage of DEG is not cleaved and no appreciable amounts of EG or EG metabolites are formed from DEG, although small amounts of OA have occasionally been reported (Hebert et al., 1978; Winek et al., 1978). Based on studies in rats and dogs, unchanged DEG recovered in urine constitutes the majority of oral doses, with 2-hydroxyethoxyacetic acid (HEAA) and diglycolic acid (DGA) accounting for most of the remainder as major and minor urinary metabolites, respectively (Lenk et al., 1989; Wiener and Richardson, 1989; Mathews et al., 1991). Differences in the toxicity/potency of DEG and EG can thus be explained by differences in their TK profiles.

DEG's nephrotoxic moiety has only recently been elucidated, largely owing to the efforts of a single research group. Besenhofer et al. (2010) first demonstrated the essentiality of metabolism by showing that fomepizole not only blocked the formation of HEAA and DGA in DEG-treated Wistar rats, but also prevented metabolic acidosis and kidney toxicity. Next, it was shown that despite HEAA's dominance over DGA in the blood, renal concentrations were similar, owing to a 100-fold concentrative mechanism for DGA (Besenhofer et al., 2011). This mechanism was speculated to be DGA's ability to act as a substrate for a sodium dicarboxylate transporter that normally enables renal tubule cells to accumulate Krebs-cycle intermediates, to which DGA has structural similarity. Support for this mechanism came from in vitro studies showing DGA, but not HEAA or DEG, produced a dose-dependent necrosis in cultured human proximal tubule (HPT) cells that was significantly diminished when HPT cells were incubated with a sodium dicarboxylate transporter inhibitor (Landry et al., 2011). Thus, DGA was identified as the metabolite responsible for DEG's nephrotoxicity, whereas these same studies support HEAA as the metabolite responsible for the metabolic acidosis characteristic of severe DEG poisoning. Subsequently, Wistar and Fischer-344 rats were shown to react similarly to orally administered DEG, with both showing kidney necrosis and markedly elevated kidney DGA at a 10 g/kg dose, but showing neither at doses of 2 and 5 g/kg (Landry et al., 2015). As might be expected, Wistar rats receiving DGA by oral gavage responded similarly, once again showing a steep, threshold-dependent dose–response where renal DGA accumulation and renal toxicity were strongly associated (Robinson et al., 2017). As noted by the authors, the steep, threshold-dependent dose response suggests toxicity is secondary to an as-yet-unidentified-saturable process that could involve the downstream metabolism of DGA or saturation of transpoter-mediated efflux of DGA from renal tubule cells. As for the process by which necrotic renal tubule cell death occurs, evidence suggests that DGA causes mitochondrial dysfunction via multiple mechanisms, including the chelation of free Ca^{2+} that regulates the organelle's metabolism (Conrad et al., 2016). Interestingly, DEG's hepatotoxicity also appears to be dependent upon the accumulation of DGA in the liver. As with EG, ADH inhibitors can be effective antidotes for DEG, as demonstrated by the use of fomepizole and hemodialysis to successfully treat a 17-month-old girl who ingested DEG (Brophy et al., 2000). Owing to its hygroscopic nature, DEG is used to dehydrate natural gas in off-shore extraction plants and to prevent formation of gas hydrates. DEG's ecological toxicity is thus at issue, as it may be released into the sea accidentally or in discharged produced waters. Recently published studies detail the application of bioassays with marine/estuarine species to the ecotoxicity risk assessment of DEG (Tornambe et al., 2012; Manfra et al., 2015).

Propylene Glycol

Propylene glycol (PG) is used as an intermediate in the synthesis of polyester fibers and resins, as a component of automotive antifreeze/coolants, and as a deicing fluid for aircraft. As PG is "generally recognized as safe" by the FDA, it is a constituent of many cosmetics, processed foods, and tobacco products, and serves as a diluent for oral, dermal, and i.v. drug preparations (ATSDR, 1997b). The most important routes of exposure in the general population are ingestion and dermal contact with products containing the compound. Workers involved in manufacturing or use of products containing PG may be exposed to concentrations higher than the general population, with the likelihood of inhalation exposure increasing when materials are heated or sprayed. Neither OSHA nor ACGIH has a workplace exposure limit for PG, but the American Industrial Hygiene Association (AIHA) has established a workplace environmental exposure limit of 10 mg/m^3 (3.2 ppm) as an 8-hour TWA. The inhalation route of exposure is of growing relevance for the general population, as PG is the predominant carrier for nicotine in e-cigarettes (Burstyn, 2014; Pisinger and Dossing, 2014). As there are no controlled long-term studies of the effects of inhaling heated aerosolized PG in humans, the toxicological implications of e-cigarette use are uncertain. However, Kienhuis et al. (2015) have examined the risk of inhaling vapor from the e-cigarette variant known as a shish-pen, and concluded that PG concentrations after one puff are sufficiently high to potentially cause irritation of the airways. Others have questioned the applicability of the margin of exposure analysis used to arrive at this conclusion (Farsalinos and Baeyens, 2016). The use and disposal of deicing solutions is the major means by which PG is released to the environment. PG has a high mobility in soil and the potential to leach into groundwater, but is neither persistent nor bioaccumulative. Its soil and water half-lives are a few days under aerobic or anaerobic conditions. The environmental distribution, fate, and effects of PG and its dimer, trimer, and tetramer forms have been recently reviewed (West et al., 2014).

PG has a very low order of acute and chronic toxicity (ATSDR, 1997b; LaKind et al., 1999), targets no specific organ system, and there have been no accounts of human fatalities. However, large i.v. doses given over short periods of time can be toxic, manifesting as hyperosmolality, increased anion gap metabolic acidosis, acute kidney injury, and sepsis-like syndrome. Clinical studies and case reports speak of individuals with reactions to PG-containing drug preparations, particularly where preexisting conditions exist. For example, a patient with renal insufficiency secondary to chronic cocaine use developed metabolic acidosis after receiving lorazepam for sedation where PG was a component of the i.v. formulation (Cawley, 2001). Wilson et al. (2000, 2005) reported on a case of nearly fatal PG toxicity after i.v. diazepam for alcohol withdrawal, and later on a case series detailing the risk of PG toxicity from i.v. benzodiazepine therapy. Several studies have reported on the accumulation of PG in critically ill adults receiving continuous i.v. lorazepam infusion (Nelson et al., 2008; Horinek et al., 2009), while others have focused on PG exposure to neonatal and pediatric patients (Shehab et al., 2009; Hansen et al., 2015). Less conventional PG exposures have also been documented. Take the case of metabolic acidosis attributable to the ingestion of a large quantity of a popular cinnamon-flavored whiskey in which PG is an ingredient (Cunningham et al., 2015). Also, Glover and Reed (1996) reported a case in which a 2-year-old child experienced CNS depression and anion-gap acidosis after ingesting a hair gel containing PG. Brief, but insightful reviews of PG's clinical toxicity have been published, including one oriented to children (Zar et al., 2007; Lim et al., 2014). Practitioners should exercise caution when treating patients with PG-containing medications in large

doses or over a prolonged period, especially patients with compromised hepatic and renal elimination capacities due to age or disease. Toxicity surveillance is typically carried out by monitoring serum osmolar and anion gaps as well as serum levels of PG.

Toxicity studies of PG in lab animals can be found in the literature, but findings of adverse effects are rare. Christopher et al. (1990) noted increases in anion-gap acidosis, CNS depression, and ataxia in cats ingesting high doses of PG, consistent with progressively elevated plasma lactate levels. The susceptibility of cats to PG may be unique, owing to their lack of major UDP-glucuronosyltransferase enzymes necessary for glucuronidation and normal renal clearance (Court, 2013). PG has been overwhelmingly negative in tests for mutagenic or genotoxic activity and was negative for carcinogenicity in a chronic feeding study in rats (Gaunt et al., 1972), a chronic inhalation study in monkeys (Robertson et al., 1947), and a chronic initiation-promotion dermal study in mice (Stenback and Shubik, 1974). As for PG's reproductive/developmental toxicity, the NTP Center for the Evaluation of Risks to Human Reproduction released a monograph on the subject largely based on an expert panel report (NTP, 2004e, 2004g). The overall conclusion was that there is negligible concern for adverse developmental/reproductive toxicity from PG exposures in humans, because animal studies have shown no such effect even at the highest doses tested. Recent reviews of the toxicology of propylene glycols, including PG, have been published affirming their very low risk to human health (Fiume et al., 2012; Fowles et al., 2013).

PG's TK profile explains its relative lack of toxicity. As with other glycols, PG is readily absorbed from the GI tract and distributed throughout total body water and to organs on the basis of blood flow. Approximately 55% of PG is metabolized by ADH to lactaldehyde, while a significant percentage (about 45%) is excreted unchanged by the kidneys (Morshed et al., 1988). PG has a mean serum half-life of 2 to 4 hours in humans. Whereas excessive lactic acid from lactaldehyde metabolism is primarily responsible for metabolic acidosis observed in extreme exposures, lactate is a good substrate for gluconeogenesis, an efficient detoxification mechanism (NTP, 2004e, 2004g). This detoxification mechanism typically does not allow lactic acid to accumulate to toxic levels, even under saturable metabolic conditions. The rate-limiting step in PG metabolism is its conversion to lactaldehyde. Saturation of this metabolic step in humans occurs at doses 8- to 10-fold lower than observed in laboratory animals. This is protective, because PG has a lower general toxicity than its metabolites (NTP, 2004e, 2004g). As for EG and DEG, ADH inhibitors may competitively inhibit PG metabolism and thus be beneficial in the PG-poisoned patient. This is exemplified by a case of co-ingestion of ethanol and PG-containing antifreeze by a 61-year-old man absent of significant acid–base disturbance and minimal lactate elevation (Brooks and Wallace, 2002).

Two structural analogues of PG also have low hazard profiles. DPG (dipropylene glycol) and tripropylene glycol (TPG) are both widely used in personal care products such as perfumes, facial makeup, stick deodorants, and shaving and skin-care preparations. Their low hazard profiles are predictable, given that DPG is rapidly converted to PG, and TPG is rapidly hydrolyzed to DPG, which is further hydrolyzed to PG. The toxicity of these compounds has been summarized (UNEP, 1994, 2001). A negative DPG cancer bioassay was conducted in F344 rats and B6C3F1 mice receiving the chemical via drinking water (Hooth et al., 2004; NTP, 2004c).

GLYCOL ETHERS

If one alcohol residue of ethylene glycol ($HO—CH_2—CH_2—OH$) is replaced by an ether, the resulting compound is a monoalkyl glycol ether such as ethylene glycol monomethyl ether (EGME), also called 2-methoxyethanol (2-ME; $CH_3—O—CH_2—CH_2—OH$). If both alcohols are replaced by ethers, the result is a di-alkyl glycol ether such as ethylene glycol dimethyl ether ($CH_3—O—CH_2—CH_2—O—CH_3$). The alkyl group at the end of the ether linkage may be a straight or branched short-chain moiety (e.g., methyl, ethyl, *n*-propyl, isopropyl, or butyl). The butyl moiety results in one of the most widely used glycol ethers, 2-butoxyethanol (2-BE; $CH_3—CH_2—CH_2—CH_2—O—CH_2—CH_2—OH$). Acetates of monoalkyl ethers such as 2-ME acetate ($CH_3—CO—O—CH_2—CH_2—O—CH_3$) are also common solvents that undergo rapid ester hydrolysis to their parent glycol ethers in vivo, and thus tend to exhibit the same toxicity profiles as unesterified glycols. Glycol ethers exhibit properties of both alcohols and ethers and are thus soluble in water and most organic solvents. This dual solubility and a favorable evaporation rate make glycol ethers very popular solvents for surface coatings such as varnishes and latex paints. Glycol ethers also find use as solvents in paint thinners and strippers, inks, metal cleaning products, liquid soaps, and household cleaners, and are used as jet fuel anti-icing additives and in semiconductor fabrication. Human exposure occurs mainly via inhalation, but also by dermal absorption.

Although glycol ether metabolism varies with chemical structure, some generalizations are possible (Fig. 24-12). For ethylene glycol monoalkyl ethers, the major metabolic pathway is oxidation via ADH and ALDH to alkoxyacetic acids. For example, 2-ME and 2-BE are metabolized to methoxyacetic acid (MAA; $CH_3—CO—O—CH_2—O—CH_3$) and butoxyacetic acid (BAA; $CH_3—CO—O—CH_2—O—CH_2—CH_2—CH_2—CH_3$), respectively. The competing *O*-dealkylase pathway results in the cleavage of the ether linkage to form ethylene glycol and an alkyl aldehyde. Medinsky et al. (1990) reported that the relative contribution of the oxidative pathway to metabolism of ethylene glycol ethers increases with increasing alkyl chain length, whereas that of the *O*-dealkylase pathway decreases. In contrast, the propylene series of glycol ethers (e.g., propylene glycol monomethyl ether [PGME]) is predominantly biotransformed to PG via *O*-dealkylation. Glycol ethers may also be conjugated with glucuronide or sulfate, but this is thought to occur mainly after saturation of other metabolic pathways. Based on studies with rat skin, glycol ethers can undergo cutaneous metabolism via ADH and ALDH. However, their rapid penetration of the skin is thought to significantly reduce the potential for first-pass dermal metabolism (Lockley et al., 2005). Like that of many other solvents, the toxicokinetics of glycol ethers may be influenced by age. This was shown in inhalation chamber studies with PGME where aging human volunteers (more than 58 years) excreted higher concentrations of free PGME compared to conjugated PGME than their 20- to 25-year-old counterparts (Hopf et al., 2012).

In vitro and in vivo toxicity studies demonstrate that some glycol ethers and their oxidative metabolites are reproductive, developmental, hematologic, and immunologic toxicants by all exposure routes. A few have also tested positive in rodent cancer bioassays. Two glycol ethers, 2-BE and 2-phenoxyethanol (PhE), have even been shown to induce apoptosis and disturb glucose metabolism in the brains of rats subcutaneously dosed over 4 weeks (Pomierny et al., 2016), raising concerns for glycol ether exposure as a risk factor for neurodegenerative disease. The metabolism of glycol ethers is considered a prerequisite to their toxicity, as the alkoxyacetic acids are usually regarded as the ultimate toxicants. Their acetaldehyde precursors have also been implicated on occasion. A critical role for metabolism is supported by the differential toxicities of glycol ethers metabolized via the oxidative and *O*-dealkylase pathways (Miller et al., 1984; Ghanayem et al., 1987). This differential toxicity has resulted in a dramatic shift away from the production and use of certain low-molecular-weight ethylene glycol ethers and their acetate

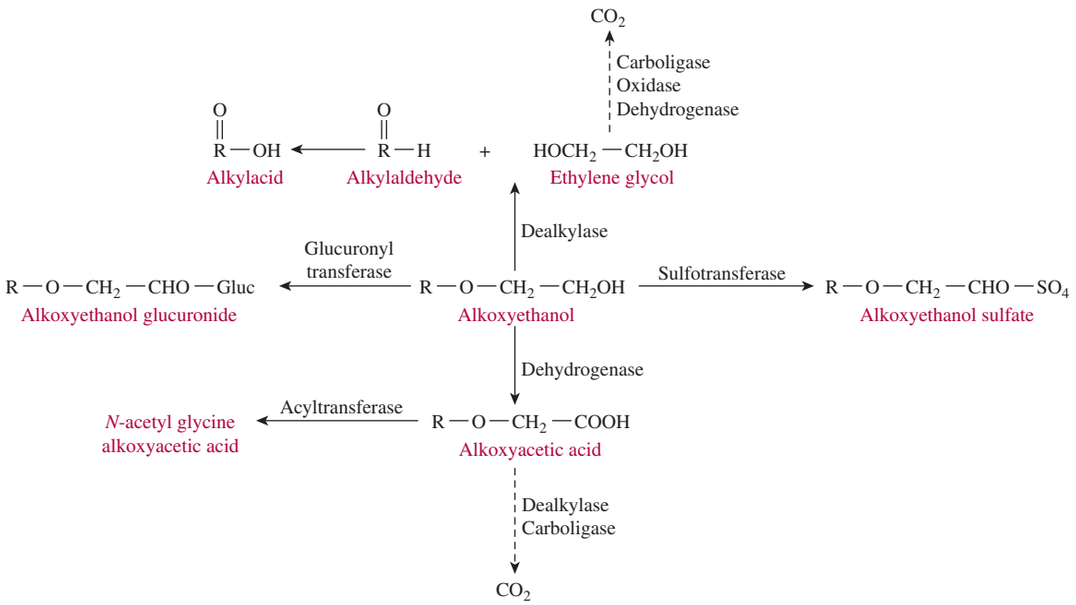

Figure 24-12. *Metabolism of glycol ethers.* R denotes alkyl group of —CH3,—CH2—CH3, or—CH2—CH2—CH2—CH3 for methoxy-, ethoxy-, or butoxyethanol, respectively. The formation of alkoxy glucuronide or sulfate conjugates has been identified only for butoxyethanol. (Reproduced from Medinsky et al., 1990, with permission from Elsevier.)

esters (e.g., 2-ME and 2-ethylene glycol monoethyl ether or 2-EE) toward those with more favorable toxicity profiles such as ethylene, diethylene, and triethylene glycol butyl ethers and those in the low-molecular-weight propylene series like PGME (de Ketttenis, 2005; Spencer, 2005; Robinson et al., 2009). Like several glycol ethers of the ethylene series, PBTK models have been developed for PGME and its acetate in rats and humans and exercised for risk assessment, including derivation of an RfC and an RfD (Corley et al., 2005b; Kirman et al., 2005; Lemazurier et al., 2005). Another PBTK modeling effort with glycol ethers was undertaken to demonstrate the utility of using an in vitro alternative to live animals (i.e., embryonic stem cells) to generate toxicity data as model input for the derivation of dose–response curves for in vivo developmental toxicity (Louisse et al., 2010b). One of the latest modeling efforts was applied to PhE and its 2-phenoxyacetic acid metabolite (PhAA). Model predictions based on systemic AUCs of PhE and PhAA as dose metrics resulted in favorable margins of exposure for use of PhE in personal care products (Troutman et al., 2015). Structure–activity relationships have been discerned in studies of glycol ethers with various chemical substitution patterns (Hardin et al., 1984; Rawlings et al., 1985; Hardin and Eisenmann, 1987; Ghanayem et al., 1989). The reproductive and developmental toxicities of the monoalkyl glycol ethers, for example, tend to decrease with increasing alkyl chain length, whereas hematotoxicity increases. Thus, structure–activity relationships may not be universally applicable across multiple toxicological endpoints. The utility of a "category approach" to predicting hemolysis and testicular toxicity for glycol ethers and chemicals related by structure, active metabolites, and/or mode of action has been recently demonstrated (Yamada et al., 2012, 2014).

Reproductive Toxicity

Epidemiological studies have reported associations between glycol ether exposure and increased risk for spontaneous abortion, menstrual disturbances, and subfertility among women employed in the semiconductor industry (Schenker et al., 1995; Correa et al., 1996; Chen et al., 2002; Hsieh et al., 2005). These associations appear biologically plausible given that the reproductive toxicity of 2-ME and its active metabolite, MAA, is manifested as ovarian luteal cell hypertrophy and increased progesterone production in the female rat. Furthermore, MAA increased progesterone production in cultured human luteal cells at the same concentration it did in rat luteal cells, implying that it has the potential to alter ovarian luteal function in women (Almekinder et al., 1997; Davis et al., 1997; Dodo et al., 2009; Taketa et al., 2011). Another monoalkyl glycol ether, 2-EE, was shown capable of rendering female CD-1 mice infertile when examined using the Fertility Assessment by Continuous Breeding protocol and high concentrations in drinking water (Lamb et al., 1984). Additional support for an effect on female reproduction comes from the study of 519 French women in whom urinary PhAA was significantly associated with longer time to pregnancy (Garlantezec et al., 2013). By contrast, diethylene glycol monobutyl ether did not disrupt the estrous cycle or impair fertility in rats (Sitarek et al., 2012). Reproductive effects, primarily reversible spermatotoxicity, have also been described for men exposed to glycol ethers, in some cases at concentrations well below current OSHA PELs for 2-ME (25 ppm) and 2-EE (200 ppm). For instance, Welch et al. (1988) found that painters exhibited oligospermia and azoospermia following average exposure to 2-EE and 2-ME at 2.7 and 0.8 ppm, respectively. In addition, men exposed to a mean level of 6.6 ppm 2-EE in a foundry had decreased numbers of sperm per ejaculate (Ratcliffe et al., 1989). Futhermore, a large case–control study of male fertility clinic patients revealed a highly significant association between a diagnosis of impaired fertility and the detection of ethoxyacetic acid in urine (Veulemans et al., 1993). Yet another case–control study of fertility clinic patients has reported significant associations between low motile sperm count and moderate or high glycol ether exposures in the preceding 3 months (Cherry et al., 2008).

Testicular effects in men are supported by experimental observations in animals including seminiferous tubule atrophy, abnormal sperm head morphology, necrotic spermatocytes, decreased sperm motility and count, and infertility (Lamb et al., 1984; Foote et al., 1995; Watanabe et al., 2000; Wang et al., 2006). Spermatocytes are among the first cells to be visibly affected following glycol ether exposure, and their death involves apoptosis (Chapin et al., 1984; Brinkworth et al., 1995; Ku et al., 1995). Creasy and Foster (1984) noted a consistent order of spermatocyte susceptibility following oral administration of 2-ME or 2-EE to rats: dividing spermatocytes > early-pachytene spermatocytes > late-pachytene spermatocytes > mid-pachytene spermatocytes. Spermatocytes in the leptotene/zygotene stages of cell division, late-stage spermatids, and spermatogonia can be affected if the dose is increased and exposure prolonged.

As for mechanism of action, studies suggest several possibilities. Beattie et al. (1984) reported that rates of lactate accumulation in cultured rat Sertoli cells were significantly decreased by MAA. Lactate is the preferred metabolic substrate of spermatocytes. Also, 2-EE has been shown to increase oxygen consumption and decrease ATP levels in pachytene spermatocytes in a manner consistent with an uncoupled oxidative state (Oudiz and Zenick, 1986). Mebus et al. (1989) have, in addition, demonstrated that serine, acetate, sarcosine, and glycine attenuated the spermatotoxicity of 2-ME in the rat, suggesting MAA may interfere with the availability of one-carbon units for incorporation into purine and pyrimidine bases necessary for nucleic acid synthesis in pachytene spermatocytes. A mechanistic role for Ca^{2+} has been hypothesized and investigated in a series of studies by Chapin and colleagues. Ghanayem and Chapin (1990) observed that a Ca^{2+} channel blocker afforded protection against 2-ME-induced pachytene spermatocyte cell death. These authors reasoned that 2-ME perturbed Ca^{2+} homeostasis, which is consistent with observations of spermatocyte mitochondrial disruption. Involvement of Ca^{2+} was further suggested by observations that 2-ME activates a Ca^{2+}-dependent nuclease, cyclophilin A, found in pachytene spermatocytes and associated with spermatocyte apoptosis (Wine et al., 1997). Whereas an increase in intracellular Ca^{2+} is thought to trigger endonuclease activation, the protection afforded by Ca^{2+} channel blockers against MAA-induced spermatocyte apoptosis is apparently not mediated by preventing a rise in intracellular free Ca^{2+} (Li et al., 1997). Rather, because an intact relationship between Sertoli and germ cells is necessary for the morphological expression of MAA-induced spermatocyte apoptosis, it was reasoned that spermatocyte apoptosis is mediated by Sertoli cell-generated factor(s). This hypothesis proposes that transfer of this factor(s) from Sertoli cells into germ cells (or initiation of spermatocyte apoptosis by 2-ME-damaged Sertoli cells through direct Sertoli cell to germ cell communication) can be inhibited by Ca^{2+} channel blockers through their membrane-stabilizing effects and/or interaction with protein kinase C and/or calmodulin, both of which have demonstrated roles in apoptosis. The inhibition of protein kinase C and calmodulin has been shown to block MAA-induced spermatocyte cell death (Li et al., 1997).

Jindo et al. (2001) advanced the research of their predecessors by using cultured seminiferous tubules of juvenile rats to demonstrate that MAA-induced spermatocyte apoptosis could be blocked with protein kinase inhibitors. Several kinases (e.g., Src) increased immediately *around* dying spermatocytes in the immediate proximity of Sertoli cells. An increase was also noted in the phosphorylation of the endoplasmic reticulum chaperone glucose-regulated protein 94, known also as endoplasmin, that was located *inside* dying spermatocytes. This work implicates a role for kinase activity in the pathogenesis of MAA-induced spermatocyte apoptosis and suggests the involvement of Sertoli cells. Yet another investigation examined the role of tyrosine kinase pp60 (rat testicular Src), a tyrosine kinase encoded by the Src gene and involved in an array of cell signaling pathways, for its involvement in 2-ME-induced spermatocyte apoptosis. Sertoli cell cytoplasm was observed to be the principal site of Src immunoreactivity in control testis, while 2-ME treatment significantly induced Src expression in dying spermatocytes. In addition, MAA-induced apoptosis was blocked using Src inhibitors, further supporting a role for rat testicular Src in Sertoli-germ cell communication and spermatocyte toxicity of 2-ME (Wang et al., 2000). Furthermore, a suppression subtractive hybridization technique using whole testes from 2-ME-treated mice was employed to create mouse testis cDNA libraries enriched for gene populations either upregulated or downregulated by 2-ME (Wang and Chapin, 2000). A total of 70 clones were screened, and six of them were shown to be differentially expressed in the 2-ME lesion, three with increased expression, and three were suppressed. Interestingly, predicted peptide sequences of the six genes revealed several conserved motifs such as phosphorylation sites for protein kinase C and tyrosine kinase. Importantly, these gene changes were apparent at multiple germ cell stages and were localized in multiple germ cell types (Sertoli, interstitial, and peritubular cells). This further suggests the involvement of cell types other than the dying spermatocyte in the pathogenesis of 2-ME-induced spermatocyte death and helps explain the requirement for *intact* seminiferous tubules for in vitro replication of the pathology observed in vivo. Additional insights into the testicular toxicity of glycol ethers continue to come from gene/protein expression and metabolic profiling studies of 2-ME-treated rats and monkeys and MAA-treated cultured mouse Leydig cells, including those profiling post-transcriptional regulatory microRNAs (Yamamoto et al., 2007; Bagchi and Waxman, 2008; Wade et al., 2008; Bagchi et al., 2010; Takei et al., 2010; Fukushima et al., 2011; Sakurai et al., 2015). Such studies have raised several possible mechanisms, including oxidative stress as a precursor to spermatocyte apoptosis, altered androgen receptor signaling, inhibition of the flavoprotein dehydrogenase enzyme family, and increased acetylation of core histones in several testis germ cell populations.

Developmental Toxicity

Exposure to certain glycol ethers during organogenesis (e.g., 2-ME and 2-EE) is toxic to the developing embryo, with effects seen in several animal models including nonhuman primates (Hardin et al., 1986; Scott et al., 1989). Others such as ethylene glycol butyl, propyl, and monohexyl ethers, and most PG ethers have either not induced fetal malformations or have a lower potential for developmental toxicity (Tyl et al., 1989; Spencer, 2005). Structural anomalies in rodents have included a variety of minor skeletal variations, hydrocephalus, exencephaly, cardiovascular malformations, dilatation of the renal pelvis, craniofacial anomalies, and digit malformations. In the absence of structural defects, electrocardiograms of fetal rats from dams treated with 2-ME during gestation showed persistent, aberrant QRS waves, suggestive of an intraventricular conduction delay (Toraason and Breitenstein, 1988). Neurobehavioral changes and regional brain alterations of several neurotransmitters in offspring of rats treated with 2-ME or 2-EE have been reported (Nelson and Brightwell, 1984).

There remains considerable uncertainty surrounding the mechanism(s) by which glycol ethers exert their developmental effects. 2-ME has served as a model toxicant to investigate the disposition of weak acids in the maternal–fetal unit and the hypothesis that weak acids such as MAA exert their effects by altering

embryonic pH at critical stages of organogenesis (Nelson et al., 1989; Clarke et al., 1992; O'Flaherty et al., 1995; Terry et al., 1995; Louisse et al., 2010a). Ambroso et al. (1998) have applied confocal laser scanning microscopy, classical histopathology, and in situ immunohistochemistry to demonstrate that a dose-dependent increase and expansion of apoptosis in gestation day 8 mouse embryos could underlie 2-ME-induced neural tube defects. Similarly, associations have been made in mice between a maternal dose of MAA on gestation day 11, apoptosis in embryonic forelimb buds, and digit malformations (Haryono et al., 2011). In vitro studies have also been informative, such as that of Scofield et al. (2006) that utilized MAA-exposed chick forelimb bud micromass cultures to show mitochondrial-mediated apoptosis during chondrogenesis. An apoptotic mechanism has also been hypothesized for malformations induced by several prototypical teratogens like retinoic acid and ethanol. Further mechanistic insights have come from exposure of cultured, murine embryonic forelimbs to 2-ME and MAA. Dayan and Hales (2014) have reported that MAA was considerably more potent than 2-ME in inducing limb abnormalities, and unlike 2-ME, induced the acetylation of p53 with resultant increases in *p*21 mRNA expression and cleaved caspase-3 protein levels, markers for cell cycle arrest and apoptosis, respectively. Thus, the induction of acetylation via an increase in histone acetyltransferase activity and/or decrease in histone deacetylase activity, surfaces as a viable explanation for MAA's testicular and developmental toxicities alike.

Few epidemiological studies have addressed developmental effects of glycol ethers. Saavedra et al. (1997) described facial malformations and varying degrees of mental retardation in 44 offspring of mothers who were exposed occupationally to 2-ME and ethylene glycol at a factory producing capacitors in Mexico. Two other reports on what appears to be a subset of the same population describe these dysmorphic features and mental retardation, along with telomere shortening and chromosomal rearrangements in the in utero–exposed offspring (El-Zein et al., 2002, 2007). There are a few reports published from a multicenter case–control study in Europe designed to investigate the role of maternal exposures at work and congenital malformations (Ha et al., 1996; Cordier et al., 1997; Lorente et al., 2000). Preliminary results (Ha et al., 1996) of evaluation of offspring of mothers who were exposed to glycol ethers at work during pregnancy found excesses of oral clefts (OR = 2.0; 95% CI = 1.1 to 4.1) and CNS malformations (OR = 1.8; 95% CI = 1.1 to 3.3). In a study of 984 cases of major congenital malformations, Cordier et al. (1997) reported an overall OR of congenital malformations associated with glycol ether exposure of 1.44 (95% CI = 1.10 to 1.90), with significant associations for glycol ether exposure with cleft lip, multiple anomalies, and neural tube defects. Lorente et al. (2000) studied 100 mothers of babies with oral clefts and 751 mothers of healthy babies and reported a nonsignificant OR of 1.7 (95% CI = 0.9 to 3.3) for maternal occupational exposure to glycol ethers and cleft lip, with or without cleft palate. Maldonado et al. (2003) have reviewed the epidemiological evidence and determined that it is insufficient to determine whether occupational exposure to glycol ethers causes human congenital malformations.

Out of concern for the potential of ethylene glycol monoalkyl ethers as developmental toxicants, several PBTK models have been developed. Hays et al. (2000) developed a PBTK model for 2-ME and MAA in the pregnant rat that was capable of predicting embryonic concentrations. Gargas et al. (2000a, 2000b) applied a PBTK model to estimate inhaled concentrations of 2-EE, its acetate ester, and 2-ME in humans that would result in blood levels equivalent to those observed at the rat NOAELs and LOAELs for developmental effects. Sweeney et al. (2001) applied Monte Carlo simulations to the models of Gargas and co-workers to account for the variability in TK and TD factors among humans and laboratory animals and derived occupational exposure limits to protect workers from developmental effects of 2-ME and 2-EE that were 1 to 2 orders of magnitude lower than current OSHA PELs. It is worthy of note that 2-ME has been largely removed from commerce due to its teratogenic potency.

Hematotoxicity

Some glycol ethers are hemolytic to red blood cells (RBCs). Typically, the osmotic balance of cells is disrupted, they imbibe water and swell, their ATP concentration decreases, and hemolysis occurs (Ghanayem, 1989). Nyska et al. (1999) reported that subchronic exposure to 2-BE causes disseminated thrombosis and bone infarctions in female, but not male rats, likely due to impedance of blood flow by intravascular hemolysis. It is thought that females might be susceptible because they are less efficient in eliminating BAA, the hemolytic metabolite of 2-BE, and exhibit higher peak blood BAA levels. Young adult rats are more resistant to the hematologic effects of 2-BE than older rats, an observation attributed to depressed degradation and renal clearance of BAA in the older rats.

Species differ dramatically in their sensitivities to glycol ether-induced RBC deformity and hemolysis. Humans are less susceptible than rodents. This lower susceptibility even applies to RBCs from potentially sensitive subpopulations, such as the elderly and persons with hereditary blood disorders (Udden, 1994; Udden and Patton, 1994). A good example of using PBTK models in human risk assessment has been published by Corley et al. (1994). Based on comparisons of model output with data collected by Udden and colleagues on levels of 2-BE required to affect osmotic fragility of human RBCs, Corley and co-workers concluded that humans are unlikely to achieve hemolytic blood levels of BAA unless very large volumes of 2-BE are intentionally ingested. Udden (2005) more recently reported on the hemolytic effects of diethylene glycol butyl ether (DGBE) and its principal metabolite, butoxyethoxyacetic acid (BEAA), using rat and human RBCs in vitro. BEAA had weak hemolytic activity on rat erythrocytes, which is consistent with the finding of mild hemolysis when DGBE is administered to rats by gavage. However, such effects were absent in human RBCs exposed to DGBE or BEAA, indicating that it is unlikely hemolysis will occur in humans exposed to DGBE. Johnson et al. (2005) confirmed DGBE's low order of hematotoxicity in a 13-week drinking water study in F344 rats that identified a NOAEL of 250 mg/kg/day, with minimal but statistically significant decreases in RBC count, hemoglobin, and hematocrit at 1000 mg/kg/day.

Hoflack et al. (1997) have shown 2-BE capable of inducing apoptosis in a human leukemia cell line and hypothesized that the hematopoietic toxicity of 2-BE may be the result of its ability to induce apoptotic cell death. However, once inside the cell it is not entirely clear how hemolysis is accomplished, although the RBC membrane has long been the suspected target. Udden and Patton (2005) have utilized BAA to examine the mechanism of glycol ether hemolysis in rat RBCs in vitro. They concluded that the mode of action of BAA is to cause a colloid osmotic lysis of the RBC and speculated the following scenario: BAA causes Na^+ and Ca^{2+} to enter the cell. Ca^{2+} initially has a protective effect via the Ca^{2+}-activated potassium channel, which facilitates the loss of potassium, thereby compensating for the osmotic effect of increased cell Na^+. Ca^{2+} subsequently has deleterious effects through activation of proteases and the loss of the normal asymmetric distribution of phospholipids (e.g., phosphatidylserine) in the membrane bilayer. These authors noted that preliminary studies in their laboratory have shown the movement of phosphatidylserine from the inner to the

outer leaflet of the lipid bilayer of rat RBCs incubated with BAA. This "externalization" of phospatidylserine is associated with adhesion of RBCs to endothelial cells and the generation of thrombin, which is most interesting given reports of disseminated thrombosis and infarction in 2-BE-treated rats (Nyska et al., 1999; Ghanayem et al., 2001).

Immunotoxicity/Carcinogenicity

Based on changes in thymus and splenic weights/cellularities and a variety of in vitro and in vivo immune function assays, the immune system is a potential target for the oxidative metabolites of some glycol ethers. 2-ME and MAA have been employed almost exclusively in immunotoxicity investigations of glycol ethers. Not only have adult animals proven susceptible, but 2-ME exposure of pregnant mice induces fetal thymic atrophy/hypocellularity and a reduction in fetal liver prolymphocytes with potential implications for fetal immunity (Holladay et al., 1994). Using B6C3F1 mice and gavage exposure, House et al. (1985) were among the first to report that 2-ME and MAA reduced thymus weight. Kayama et al. (1991) subsequently reported that 2-ME selectively depleted immature thymocytes in mice. Exon et al. (1991) not only reported thymic atrophy in rats exposed to 2-ME in drinking water, but also decreased antibody production, decreased splenocyte production of interferon-γ, and a reduction in spleen cellularity. Around the same time, the first in a lengthy series of studies by Smialowicz and colleagues was published (Smialowicz et al., 1991a, 1991b, 1992, 1994; Williams et al., 1995; Kim and Smialowicz, 1997). This series has reported decreased thymus weights, reduced lymphoproliferative responses to mitogens, and reduced IL-2 production in splenocytes of F344 rats exposed to 2-ME by gavage. It has also generated data indicating that not all glycol ethers are immunosuppressive, that mice are relatively insensitive to glycol ether immunosuppression compared to rats, and that rats of various strains show differential sensitivities. The Smialowicz series has further demonstrated that the relative insensitivity of mice is not a function of their more rapid clearance of MAA; that 2-ME is immunotoxic when applied dermally to F344 rats; and that questions remain as to 2-ME's proximate immunotoxicant, as 2-methoxyacetaldehyde is more immunotoxic than MAA based on the ability to suppress IgM and IgG production by lymphocytes in F344 rats.

As for cancer, only a few chronic bioassays have been conducted with glycol ethers. Two-year inhalation bioassays of 2-BE in F344 rats and B6C3F1 mice revealed some evidence of carcinogenicity in male mice, based on increased incidences of hemangiosarcoma of the liver, as well as some evidence of carcinogenic activity in female mice, based on increased incidences of forestomach squamous cell papilloma or carcinoma (mainly papilloma) (NTP, 2000). In June, 2004, an IARC working group evaluated the cancer risk of 2-BE and concluded that it is not classifiable as to its human carcinogenicity (Cogliano et al., 2005). Likewise, EPA's IRIS profile for 2-BE currently indicates that the human carcinogenic potential of 2-BE cannot be determined at this time (EPA, 2006c). Since NTP's bioassays were completed, numerous studies have shed light on 2-BE's possible modes of action related to liver hemangiosarcomas and forestomach tumors and their implications for risk assessment (Park et al., 2002; Siesky et al., 2002; Poet et al., 2003; Boatman et al., 2004; Klaunig and Kamendulis, 2005; Corthals et al., 2006). As discussed by Gift (2005), these studies suggest the following scenario: 2-BE consumed while grooming is metabolized to irritant metabolites in the forestomach and/or irritant metabolites are formed in the upper respiratory tract and swallowed → chronic irritation → inflammation → hyperplastic effects → forestomach tumors. As for liver hemangiosarcomas, the following is suggested: 2-BE is metabolized to BAA → BAA causes hemolysis of RBCs → hemosiderin (iron) derived from released hemoglobin is taken up by and stored in phagocytic cells (e.g., Kupffer cells) of the spleen and liver → oxidative damage and increased synthesis of endothelial DNA are initiated by reactive oxygen species from excess iron or Kupffer cells, producing cytokines/growth factors that suppress apoptosis and promote cell proliferation → endothelial DNA mutations → potentiation and promotion of hepatic neoplastic cell populations. As further discussed by Gift (2005), the evidence suggests nonlinear modes of action in both cases and questionable human relevance of both tumor types. Several PBTK models have been developed and subsequently refined for 2-BE and BAA to aid in risk assessments (Corley et al., 1994, 2005c; Lee et al., 1998; Franks et al., 2006).

Spencer et al. (2002) have reported a two-year inhalation bioassay of PGME in F344 rats and B6C3F1 mice that did not result in increases in neoplasia in either species except for kidney adenomas in male rats related to α_{2u}-globulin nephropathy. In contrast, a two-year inhalation bioassay with propylene glycol mono-*t*-butyl ether (PGMBE) in F344 rats and B6C3F1 mice resulted in α_{2u}-globulin nephropathy in male rats, but also liver tumors in male and female B6C3F1 mice at the highest concentration tested (1200 ppm) (NTP, 2004a; Doi et al., 2004). Dill et al. (2004) have published information on PGMBE TK in rats and mice that demonstrate saturation of PGMBE metabolism/elimination at this tumor-producing concentration. The genotoxicity of some glycol ethers and their metabolites has been evaluated, with most exhibiting a lack of genotoxic potential and others yielding weakly positive responses in certain tests. Therefore, the role of genetic toxicology in the toxicities discussed above cannot be summarily dismissed, but is of unknown significance (Elliot and Ashby, 1997; NTP, 2000; Ballantyne and Vergnes, 2001).

FUELS AND FUEL ADDITIVES

Automotive Gasoline

Automotive gasoline is a complex mixture of hundreds of hydrocarbons predominantly in the C_4 to C_{12} range. The sheer number of people exposed in the manufacture, distribution, and use of gasoline make characterization of its acute and chronic toxicities important. Generalizations regarding gasoline toxicity must be made with care, because its composition varies with the crude oil from which it is refined, the refining process, and the use of specific additives. Experiments conducted with fully vaporized gasoline may not be predictive of actual risk, because humans are exposed primarily to the more volatile components in the range of C_4 to C_5. These hydrocarbons are generally regarded as less toxic than their higher-molecular-weight counterparts. Concern about gasoline exposure is fueled in part by the toxicities of certain components, some of which are classified by EPA as known or probable human carcinogens (e.g., benzene and 1,3-butadiene). The ACGIH has established a TLV for gasoline of 300 ppm to prevent ocular and upper respiratory tract irritation and a STEL of 500 ppm to avoid acute CNS depression.

Inhalation exposure to gasoline has been measured for service station attendants, self-service customers, truck drivers, distribution workers, and workmen removing leaking underground storage tanks (Kearney and Dunham, 1986; Shamsky and Samimi, 1987). In one survey, short-term exposures of self-service customers averaged about 6 ppm. The TLV is rarely exceeded in occupationally exposed individuals, due in part to the use of vapor scavenging systems. Brief exposures in excess of the STEL have, however,

been documented for workers engaged in bulk handling operations (Phillips and Jones, 1978). The most extreme exposures occur to those intentionally sniffing gasoline for its euphoric effects. Several case reports of acute and chronic encephalopathies are testament to the dangers of this habit (Valpey et al., 1978; Fortenberry, 1985). In these cases, the identity of the offending agent(s) is often unclear. Gasoline is one of the most popular and lethal inhalants (Spiller, 2004; Wu et al., 2004), with deaths reported even among Aboriginal people in South Australia (Byard et al., 2003). An all too common occurrence is the ingestion of gasoline during siphoning events, something that may occur at epidemic rates following adverse climatic events that result in gasoline shortages (Kim et al., 2013). This is typically followed by a burning sensation in the mouth and pharynx, as well as nausea, vomiting, and diarrhea resulting from GI irritation. If aspirated into the lungs, gasoline may produce pulmonary epithelial damage, edema, and pneumonitis. Thus, emetic therapy for gasoline ingestion is usually contraindicated.

Between 1986 and 2004, EPA identified 447,233 releases from underground petroleum storage tanks, many of which threaten groundwater that serves as the primary drinking water source for nearly one-half the U.S. population. Despite the number of releases, few community health studies have been conducted, and those that have are typically driven by concerns over leukemia risk owing to gasoline's benzene content. Consider for example the retrospective cohort study of the "Tranguch Gasoline Spill" in northeastern Pennsylvania (Patel et al., 2004). The standard incidence ratio for leukemia of all types was significantly elevated (4.40; 95% CI = 1.09 to 10.24), consistent with that reported by the Pennsylvania Department of Health. However, the excess was based on only four cases, two of which had a history of smoking, a potential confounder. In addition, exposure was not well characterized, and only two of the subjects had acute myelogenous leukemia (AML), the leukemia type most strongly associated with benzene. A follow-up study reported significantly elevated SIRs for total leukemia (7.69: 95% CI = 1.58 to 22.46) and AML (11.54: 95% CI = 2.38 to 33.69) during the period 4 to 10 years subsequent to the spill, with only three of six total leukemia cases being AML (Talbott et al., 2011). While such results raise concern for low-level benzene exposures among some, others point to the pitfalls of drawing causal inferences from environmentally exposed populations based on such limited data. Several meta-analyses have examined populations occupationally exposed to gasoline for hematolymphopoietic cancers, with little to no evidence of increased risk (Dement et al., 1997; Kane and Newton, 2010; Keenan et al., 2013). Still other studies have examined residences next to gasoline stations as a risk factor for childhood leukemia (Brosselin et al., 2009; Steinmaus and Smith, 2017) and genotoxicity and oxidative stress in gasoline station attendants (Hallare et al., 2009; Uzma et al., 2010; Moro et al., 2013), some with positive results.

Reese and Kimbrough (1993) and Caprino and Togna (1998) have reviewed the acute toxicity of gasoline and its additives. Like some other solvents, gasoline can sensitize the heart to catecholamines, defat the skin upon repeated contact, and induce hepatic P450s and UDP-glucuronyltransferase activities (Poon et al., 1995). There is a paucity of data on the reproductive toxicity of gasoline, but reports of enhanced estrogen metabolism and uterine atrophy among unleaded gasoline (UG)-treated mice suggest that this endpoint warrants investigation (Standeven et al., 1994a). Although dated, the study of Lykke and Stewart (1978) is of interest, because rats exposed to leaded gasoline at one-third the ACGIH TLV (i.e., 100 ppm) for 40 hours/week for 6 to 12 weeks were observed to have a progressive interstitial fibrosis of the lungs associated with irregular alveolar collapse. The question of whether there is a "fetal gasoline syndrome" has been raised, although case reports are confounded by tetraethyl lead, alcohol abuse, and the possibility that an aberrant gene is distributed within the small Amerindian population where the cases reside (Hunter et al., 1979). The question of developmental exposure was recently answered, in part, by a series of three studies employing the same exposure regimen. Pregnant rats were exposed to vapors of UG containing no ethanol, 15% ethanol or 85% ethanol for 6.5 hours/day on GD9 to GD20. One study not only examined gestationally exposed offspring using tests of motor activity and a functional observational battery, but also reported on cell-mediated and humoral immunity, systolic blood pressure, and glucose homeostasis (Bushnell et al., 2015). In another, offspring underwent cognitive testing as adults using trace fear conditioning, Morris water maze, and operant procedures (Oshiro et al., 2015). In the third, male offspring underwent neurophysiological assessment of auditory, peripheral nerve, somatosensory, and visual system function as adults (Herr et al., 2016). As for results, the few adverse effects were minor, not consistently related to the ethanol content of vapors, and occurred at vapor concentrations orders of magnitude higher than those anticipated to the general population. The above series of developmental toxicology studies was conducted in response to a rule issued by EPA in 1994 under the Clean Air Act, requiring testing of motor vehicle fuels and fuel additives. Another series of rodent studies designed to meet these same legislative requirements examined the toxicology of inhaled vapor condensates of gasoline or gasoline blended with various fuel oxygenates to assess the influence of additives on the hazard potential of evaporative emissions. The series was published as supplementary volume 70, issue 2 of *Regulatory Toxicology and Pharmacology*. It consists of studies evaluating developmental toxicity (Roberts et al., 2014a, 2014b), neurotoxicity (O'Callaghan et al., 2014), subchronic toxicity (Clark et al., 2014), genotoxicity using the micronucleus and sister chromatid exchange tests (Schreiner et al., 2014), reproductive toxicity (Gray et al., 2014), and immunotoxicity (White et al., 2014). Overall, the studies' results suggest that evaporative emissions pose a low hazard potential for the general public, as wide margins of safety between real-world exposure scenarios and NOAELs were evident.

Prior to the identification of α_{2u}-globulin as the principal accumulating protein in the syndrome referred to as α_{2u}-globulin nephropathy, Kuna and Ulrich (1984) reported regenerative epithelium and dilated tubules in the kidneys of male rats exposed to 1552 ppm UG for 90 days. At about the same time, a chronic inhalation study revealed not only nephropathy, but also increased renal tumors in male rats (MacFarland et al., 1984). Subsequent studies by Halder et al. (1986) and Aranyi et al. (1986) showed that such nephropathy could not be produced by exposure of rats to a mixture of the butane and pentane components of gasoline or the 0°F to 145°F gasoline distillation fraction. These are thought to be more representative of human occupational exposures than wholly vaporized gasoline. In addition, the authors of a gavage screening study of 15 pure hydrocarbons and gasoline fractions concluded that branched aliphatic alkane components were primarily responsible for the nephropathy (Halder et al., 1985).

Investigations of mechanisms of the nephropathy and renal tumors included an assessment of unscheduled (a measure of genotoxicity) and replicative DNA synthesis (a measure of cell proliferation) in rat kidney cells exposed in vitro and in vivo to UG. No unscheduled DNA synthesis occurred, even at a tumorigenic dose, while a five- to eightfold increase in cell proliferation was observed (Loury et al., 1987). In a publication the same year by Olson et al. (1987), UG was reported to result in an increase in hyaline droplets harboring large accumulations of α_{2u}-globulin within

proximal convoluted tubule epithelial cells. It was hypothesized that α_{2u}-globulin accumulated secondary to a defect in renal lysosomal degradation of the protein (Fig. 24-6). Supportive evidence for this hypothesis came from the demonstration that inhibition of the lysosomal peptidase, cathepsin B, caused a rapid accumulation of phagolysosomes and α_{2u}-globulin in the kidney similar to that of UG (Olson et al., 1988).

Further progress in elucidating the mechanism of α_{2u}-globulin nephropathy came from the demonstration that the UG component, 2,2,4-trimethylpentane (TMP), itself an inducer of α_{2u}-globulin nephropathy, was metabolized to 2,4,4-trimethyl-2-pentanol (TMPOH), which was selectively retained by the kidney of male rats. Subsequently, it was demonstrated that the sex-specific retention of TMPOH in the kidney was due to reversible binding with α_{2u}-globulin. This binding rendered the protein less digestible by lysosomal enzymes, which accounted for its accumulation (Charbonneau and Swenberg, 1988). This accumulation, in turn, led to cellular degeneration and necrosis, primarily in the P_2 segment of the proximal tubule. In response, regenerative proliferation occurs and promotes formation of renal cell tumors by irreversibly "fixing" spontaneously altered DNA and clonally expanding initiated cells. The promotional effects of gasoline and TMP on atypical cell foci and renal cell tumors have been demonstrated in male rats following initiation with *N*-ethyl-*N*-hydroxyethylnitrosamine (Short et al., 1989). NCI–Black–Reiter male rats, the only rat strain not to synthesize α_{2u}-globulin, are resistant to gasoline- and TMP-induced nephropathy (Dietrich and Swenberg, 1991). Thus, gasoline and TMP have been of great value in elucidating the mechanism of α_{2u}-globulin nephropathy and shedding light on its implications for renal tumorigenesis. Most toxicologists, and indeed the EPA, have concluded that renal tumors secondary to α_{2u}-globulin nephropathy are of little relevance, because humans do not synthesize α_{2u}-globulin.

Chronic inhalation of gasoline at high concentrations has also resulted in increased hepatocellular adenomas and carcinomas in female B6C3F1 mice, possibly due to the promotion of spontaneously initiated cells that occur with unusually high frequency in this mouse strain (MacFarland et al., 1984). This possibility is supported by reports that UG is a CYP inducer, mitogen, and liver tumor promoter in *N*-nitrosodiethylamine (DEN)-initiated female B6C3F1 mice (Standeven and Goldsworthy, 1993; Standeven et al., 1995; Moser et al., 1996a). CYP induction by UG has been attributed to "heavy UG" (components with boiling points more than 100°C), whereas mitogenic activity is highly concentrated in UG components boiling from 100 to 132°C, for which the 2,2,3-, 2,2,4-, and 2,3,4-trimethylpentane isomers appear at least partially responsible (Standeven and Goldsworthy, 1994). It has been hypothesized that the liver tumor-promoting activity of UG is secondary to its estrogen antagonism, given that (1) UG is not a hepatocarcinogen in male mice; (2) estrogen inhibits liver tumor development initiated in mice but potentiates liver tumor promotion by UG; and (3) UG induces hepatic estrogen metabolism (Standeven et al., 1994b). The hypothesis that liver tumor promotion by UG depends on its interaction with estrogen is supported by the demonstration that tumor-promoting activity of UG was greatly attenuated in ovariectomized mice relative to intact mice (Moser et al., 1997). Further, the addition of estrogen to DEN-treated mice substantially reduces the percentage of hepatic foci with decreased levels of TGF-β1 compared to DEN-treated control mice or DEN + UG-treated mice, suggesting a promotional mechanism involving estrogen and the dysregulation of tumor growth factor(s) (Moser et al., 1996c). Whereas much attention has been given to its promotional potential, UG may also damage DNA, as it reportedly induces unscheduled DNA synthesis in hepatocytes from male and female mice treated in vivo and in cultured mouse, rat, and human hepatocytes (IARC, 1989b). The antioxidant vitamins A and E confer protection against the hepatotoxic effects of gasoline vapors in male and female rats, consistent with gasoline's ability to induce hepatic oxidative stress (Uboh et al., 2009). The epidemiological evidence for an association between gasoline exposure and cancer in humans is inconclusive. Raabe (1993) has reviewed the carcinogenic potential of gasoline. IARC (1989b) classifies it as possibly carcinogenic to humans (Group 2B) primarily due to its benzene content. A comprehensive review of gasoline toxicity, though somewhat dated, is provided in ATSDR's (1995) *Toxicological Profile for Gasoline*.

Vehicle emissions from gasoline combustion are a major contributor to urban air pollution, which is at unhealthy levels in numerous cities. In response, the Clean Air Act Amendments of 1990 require the use of oxygenated gasoline in such areas. Oxygenated gasoline contains additives that add oxygen to gasoline, thereby boosting its octane quality, enhancing combustion, and reducing exhaust emissions. Methyl tertiary butyl ether (MTBE) and ethanol are the two most common oxygenates, although use of the former is being rapidly phased out due to widespread groundwater contamination and health concerns. As a result, the demand for ethanol–gasoline blends is increasing dramatically, raising concerns about how the two components might interact toxicologically. There is a dearth of information on this issue, but a 4-week inhalation study of an ethanol–gasoline mixture (6130 ppm ethanol and 500 ppm gasoline) in rats concluded that coexposure showed additive and possibly some synergistic effects on growth, neurochemistry, and histopathology of the adrenal gland and respiratory tract. Effects were described as generally mild and adaptive in nature, and returned to normal after exposure cessation (Chu et al., 2005). The risks and benefits of ethanol as an oxygenate are discussed in detail by Williams et al. (2003), who point out several reasons why ethanol in gasoline can increase groundwater plume lengths and persistence of gasoline constituents in groundwater if ethanol blends are released into the environment. The most obvious concern is that longer or more persistent gasoline plumes could lead to a higher probability of gasoline constituents affecting public water wells. Meanwhile, another fuel additive, methylcyclopentadienyl manganese tricarbonyl (MMT), is receiving attention due to concerns that it could increase manganese inhalation exposures and pose a risk for neurotoxicity. The combustion of MMT gasoline results in the emission of fine Mn particulates mainly as Mn sulfate and Mn phosphate and smaller amounts of oxides. Several studies characterizing vehicular exhaust using MMT gasoline and describing the neurobehavioral toxicology of Mn have been published (Normandin et al., 2004; Pfeifer et al., 2004; Reaney et al., 2006; Tapin et al., 2006; Carmona et al., 2014; Su et al., 2016). Of particular note is the series of studies mandated under the Clean Air Act's Alternative Tier 2 test program that generated TK data on Mn in animals and enabled the development of PBPK models for Mn of potential value in human health risk assessment (Dorman et al., 2012; Taylor et al., 2012). Although largely beyond the scope of this chapter, gasoline engine exhaust has been classified as possibly carcinogenic to humans (Group 2B), based largely on sufficient evidence in animals when condensates and exhaust extracts are tested (IARC, 1989a).

Methyl Tertiary-Butyl Ether

MTBE is made by reacting methanol with isobutylene. While its high octane rating made it a logical replacement for tetraethyl lead as an octane booster for gasoline, its popularity as a gasoline oxygenator drove massive increases in U.S. production. As an oxygenator,

MTBE makes fuel combustion more complete, thereby reducing pollutant emissions from automobile exhaust as mandated by the Clean Air Act Amendments of 1990. By 1997, MTBE was being used at the rate of 10 million gal/day, with more than one-third of the usage in California (Williams et al., 2000a). While routine, low-level exposure of customers occurred at self-service stations, heightened concern for MTBE resulted primarily from its contamination of groundwater by leaking underground gasoline tanks. It is highly water soluble, travels faster and farther in water than other gasoline components, and is resistant to degradation. Because of the widespread occurrence of MTBE in water supplies, in 1998 the EPA formed the *Blue Ribbon Panel on Oxygenates in Gasoline* that recommended a substantial reduction in MTBE use and the eventual removal of the oxygenate requirement for reformulated gasoline. As a result, MTBE has not been used in significant quantities in reformulated gasoline since 2005. However, it finds occasional use as an FDA-approved drug for the dissolution of gallstones in the event surgical or endoscopic options are unavailable (Kim et al., 2015). It is also being investigated for its potential as an anti-angiogenic treatment for solid tumors (Kozlosky et al., 2013).

MTBE is well absorbed following oral, inhalation, and dermal exposure of humans and rats (Dekant et al., 2001; Prah et al., 2004; McGregor, 2006). The majority of absorbed MTBE is exhaled unchanged. Some MTBE is oxidized to *tert*-butyl alcohol (TBA) and HCOH. Whereas this oxidation is primarily CYP2A6-mediated in humans (Hong et al., 1999), it is largely Cyp2b1-mediated in rats (Turini et al., 1998). TBA is relatively water soluble, so it tends to remain in the blood and extracellular fluid, and is slowly exhaled. TBA is further metabolized, first to 2-methyl-1,2-propanediol and then to 2-hydroxyisobutyrate, the major urinary metabolites of MTBE. In addition, glucuronide and sulfate conjugates of TBA are found in trace amounts in urine. Although HCOH is one of the oxidative metabolites of MTBE, it is undetectable after MTBE exposure in humans and rats, presumably due to its rapid metabolism. PBTK models have been developed to describe the dosimetry of MTBE and TBA following inhalation and oral exposures in rats and humans (Borghoff et al., 1996; Licata et al., 2001). These early PBTK models were subsequently expanded to account for MTBE and TBA binding of α_{2u}-globulin in kidney (Leavens and Borghoff, 2009; Borghoff et al., 2010).

Concern for MTBE has led to numerous toxicity studies in humans and rodents, and a number of reviews of MTBE's toxicity are available (ATSDR, 1996b; EPA, 1997; Borak et al., 1998; WHO, 1998; McGregor, 2006). The review of Borak et al. (1998) focuses upon the acute human health effects of MTBE. It concludes, based on 19 reports of inhalation exposure to MTBE alone or in gasoline and 12 reports of parenteral MTBE administration to dissolve cholesterol gallstones, that no significant association exists between MTBE exposure and the acute symptoms commonly attributed to it. These symptoms include headache, eye, nose and throat irritation, cough, nausea, dizziness, and disorientation. The more recent review of McGregor (2006) is supportive of this conclusion. In 1988, the EPA and industry developed a Testing Consent Order for MTBE under the Toxic Substances Control Act that precipitated investigations of MTBE's potential two-generation reproductive toxicity, developmental toxicity, in vivo mutagenicity, subchronic inhalation toxicity, oncogenicity, and neurotoxicity. Results of these studies, published in a supplement to the *Journal of Applied Toxicology*, are a major addition to the toxicity literature on MTBE and define several NOAELs (Bevan et al., 1997a, 1997b; Bird et al., 1997; Daughtrey et al., 1997; Lington et al., 1997; McKee et al., 1997). Nearly two decades later, another series of studies was published in a supplement to *Regulatory Toxicology and Pharmacology*, the purpose of which was to determine whether the addition of oxygenates to gasoline increased the hazard potential of evaporative emissions (Schreiner et al., 2011; Clark et al., 2014; Gray et al., 2014; O'Callaghan et al., 2014; Roberts et al., 2014a, 2014b; White et al., 2014). This latest series not only examined MTBE as a gasoline additive, but also several other ether and alcohol oxygenates.

The publication of Bird et al. (1997) in the first series mentioned above is actually a recapitulation of reports by Chun et al. (1992) and Burleigh-Flayer et al. (1992), two of only three MTBE inhalation cancer bioassays. These bioassays exposed male and female F344 rats and CD-1 mice to MTBE vapor, whereas a third inhalation study exposed male and female F344 rats to gasoline vapor condensate containing MTBE (Benson et al., 2011). The results of two oral chronic bioassays have been published, the first being that of Belpoggi et al. (1995, 1997) who dosed male and female Sprague–Dawley rats with MTBE in an olive oil vehicle by gavage. The other oral study is that of Dodd et al. (2013) in which Wistar rats of both sexes were exposed to MTBE in drinking water for 2 years. The results of the five pure- and mixed-exposure MTBE animal cancer bioassays are presented in Table 24-4. In addition, Cirvello et al. (1995) and NTP (1995) have reported some evidence for the carcinogenicity of TBA in the kidney and thyroid after long-term drinking water exposure. Taken at face value, one might interpret these cancer bioassay findings as ample evidence of carcinogenicity in animals and suggestive of a cancer risk for humans. The relevance of these findings to humans, however, has been a source of debate among toxicologists. Critics have questioned these studies on the basis of (1) the appropriateness of a combined incidence category for leukemias and lymphomas;

Table 24-4

Summary Results of MTBE Cancer Bioassays

AUTHORS	ANIMAL STRAIN/ SPECIES	EXPOSURE ROUTE	POSITIVE RESULTS
Chun et al. (1992)	Fischer 344 rats	Inhalation	Kidney and testicular tumors (males)
Burleigh-Flayer et al. (1992)	CD-1 mice	Inhalation	Liver adenomas (females)
Belpoggi et al. (1995)	Sprague–Dawley rats	Oral gavage	Testicular tumors (males) Leukemia + lymphoma (females)
Dodd et al. (2013)	Wistar rats	Drinking Water	None
Benson et al. (2011)	Fischer 344 rats	Inhalation*	None**

** Exposure was to gasoline vapor condensate with a relative concentration of MTBE of 21.3%*

*** MTBE failed to clearly enhance the production of any proliferative lesion type*

(2) the possibility that renal tumors were secondary to male rat-specific α_{2u}-globulin nephropathy; (3) the possibility that Leydig cell tumors were a function of abnormally low testicular tumor rates in control animals or increased survival time of treated rats; (4) the questionable relevance of testicular tumors in rats to humans, given the species' differential responses of Leydig cells to proliferative stimuli; (5) the possibility that liver and kidney tumors are the result of high-dose-induced chronic cytotoxicity, cell death, and reparative cell proliferation; (6) the questionable relevance of inhalation bioassays to prediction of drinking water risks; (7) the use of an oil rather than water dosing vehicle, which could unduly influence MTBE's oral TK; (8) the incidence of brain astrocytomas falling within historical control ranges for Wistar rats; and (9) relative to humans, the increased susceptibility of the rodent to thyroid gland neoplasia when secondary to thyroid dysfunction. Mennear (1997) and McGregor (2006) have discussed a number of these issues. In addition, a review of MTBE's mode of action for cancer endpoints in rodents led Cruzan et al. (2007) to conclude that the weight of the evidence supported a non-genotoxic mode of action and comparatively less risk to humans, if any, for tumorigenic responses. In contrast, Mehlman (2002) has argued that NTP's failure to list MTBE in its *9th Report on Carcinogens* and in so doing, acknowledge it as a probable human carcinogen, "… did a disservice to the general public." More recently, Bogen and Heilman (2015) have reassessed MTBE's cancer potency in light of new data on MTBE and metabolite tumorigenicity, genotoxicity, and cytotoxicity. They note that of 53 genotoxicity tests performed for MTBE, there is not a single unequivocally positive test result. Accordingly, they have concluded that MTBE is unlikely to increase cancer risk unless chronic exposures are sufficient to induce target tissue toxicity. They nonetheless derived a cancer potency estimate of 1.8×10^{-5} $(mg/kg/day)^{-1}$ based on a hypothetical linear risk and genotoxic mode of action, on which basis they concluded that if MTBE is carcinogenic to humans, it is among the weakest 10% of chemical carcinogens evaluated by EPA.

As an outgrowth of the uncertainties surrounding MTBE's human carcinogenicity risk, several mechanistic studies have been published. For example, after only 10 days of MTBE inhalation exposure, a strong positive linear relationship between renal α_{2u}-globulin concentration and cell proliferation was seen in the male F344 rat (Prescott-Matthews et al., 1997). This study, unlike the chronic bioassay of Chun et al. (1992), definitively identified the accumulating protein as α_{2u}-globulin. Williams and Borghoff (2001) have shown that TBA interacts with α_{2u}-globulin, which explains its accumulation in the male rat kidney following MTBE or TBA exposure. MTBE has been shown to be a hepatic mitogen in the female mouse, but not a promoter of tumor formation in DEN-initiated female mouse liver (Moser et al., 1996b). It has been suggested in light of these findings that MTBE may promote the growth of spontaneously initiated cell populations having genetic lesions different from those produced by DEN. Casanova and Heck (1997) have reported a lack of concentration-, species-, and sex-dependence in the formation of HCOH-induced DNA–protein cross-links and RNA–HCOH adducts in isolated female CD-1 mouse hepatocytes incubated with MTBE. As the cancer bioassay data suggest that hepatocarcinogenicity varies with all of these factors, these results do not support a role for HCOH in MTBE-induced liver tumor formation. Studies also raise the possibility that MTBE-induced tumor formation might be hormonally mediated, as MTBE can cause endocrine dysregulation in rodents at high doses (Moser et al., 1998; Williams et al., 2000b). However, MTBE and TBA do not appear to directly impact the steroidogenic pathways involved in estrogen and androgen production, nor does MTBE interact with the hypothalamic–pituitary–gonadal axis of zebrafish or fathead minnows used in endocrine screening tests (de Peyster et al., 2014; Mihaich et al., 2015). Changes in triiodothyronine, luteinizing hormone, testosterone, and estradiol levels have been discussed as possible mechanisms of MTBE-induced Leydig cell cancer (Williams and Borghoff, 2000; Williams et al., 2000b; de Peyster et al., 2003).

While several mechanistic issues surrounding MTBE's toxicity have yet to be resolved, there is no evidence that MTBE-oxygenated gasoline ever posed an imminent threat to public health. EPA's Office of Water has concluded that available data are inadequate to estimate potential health risks of MTBE at low exposure levels in drinking water, and the Agency's IRIS Database remains devoid of an RfD or cancer potency estimate. Fortunately, water containing MTBE has an unpleasant taste and odor, which may alert consumers to the fact that their water is contaminated. Based on the results of studies of taste and odor thresholds for humans, an advisory guidance range of 20 to 40 μg/L has been set by the EPA to assure consumer acceptance and provide a large margin of safety from toxicity and carcinogenicity. While no national standard exists, MTBE is a regulated drinking water contaminant in California that adopted a primary MCL of 13 μg/L corresponding to the de minimis cancer risk derived from animal studies. Additionally, California established a secondary MCL of 5 μg/L to address taste and odor concerns. Whether the suspension of MTBE's use in reformulated gasoline was a prudent public health measure depends in part on the risks associated with increased auto emissions and ozone formation. Two articles that discuss this subject in considerable detail are those by Spitzer (1997) and Erdal et al. (1997). In the United States, there was a time when 85% of reformulated gasoline contained MTBE—now gasoline blended with 10% ethanol accounts for 95% of the fuel consumed in motor vehicles with gasoline engines. Other ether and alcohol oxygenates are used more sparingly, with ethyl *tertiary*-butyl ether (ETBE) and to a lesser extent, *tertiary*-amyl methyl ether (TAME), receiving the most scrutiny from a toxicological standpoint. The carcinogenic potential of ETBE has been examined in two bioassays in F344 rats, with no carcinogenic effect when given in drinking water, but hepatocellular adenomas in males upon inhalation (Suzuki et al., 2012; Saito et al., 2013). Like MTBE, ETBE does not appear to be genotoxic and experimental evidence supports a mechanism involving hepatocyte oxidative stress and subsequent regenerative cell proliferation (Kakehashi et al., 2013). In addition, ETBE delivered by gavage to F344 rats has shown the ability for multi-organ tumor promotion (Hagiwara et al., 2011). As he did for MTBE, McGregor (2007) has reviewed the toxicity of ETBE.

Jet Fuel

Jet A, jet propellant-5 (JP-5), and JP-8 are the predominant jet fuels in use today. All are kerosene-like mixtures of hundreds of aliphatic and aromatic hydrocarbons. Jet A is commercial aviation fuel, JP-5 is used primarily for Navy shipboard aircraft, and JP-8 is the battlefield fuel for all NATO forces and is used not only for aircraft, but also for ground vehicles and other equipment such as generators, cooking stoves, and tent heaters (NAS, 2003). These jet fuels differ slightly in hydrocarbon composition and/or additives with such differences intended to impart added safety, enhance combat aircraft survivability, and simplify battlefield logistics. Currently, some military units within the contiguous United States are converting from JP-8 to JAA (i.e., Jet A with an additive package) in an effort to reduce fuel cost and simplify the logistics of obtaining

fuel. There is also a move afoot to alleviate dependence on petroleum-based jet fuel by using synthetic versions produced from coal or natural gas or a blend of synthetic and petroleum-based fuels.

JP-8 is thought to be the largest toxicant exposure for the U.S. Armed Forces and commercial aviation fuel among the most common sources of nonmilitary occupational chemical exposure. As such, the Department of Defense (DoD) has a vested interest in jet fuel toxicology, for which it has sponsored research for decades. These research efforts have been summarized in book form (Witten et al., 2011). Civilian and military personnel are exposed to jet fuel by inhalation and dermal contact. Exposure can occur to liquid, vapor, or aerosol, each phase having a distinct composition and toxicity profile. Exposure is prevalent in aircraft refueling and maintenance operations and ground crews positioned behind jet aircraft during "cold starts" can become "drenched" in aerosol emissions. Jet fuel can be released into the environment by in-flight jettisoning and spills or leaks to soil or water during use, storage, or transportation. In many cases, DoD is responsible for the cost of remediating contaminated military sites and contractor facilities, not to mention its responsibility to safeguard the health of military personnel. For this purpose, the U.S. military has adopted an 8-hour TWA PEL and 15-min STEL of 200 and 1000 mg/m^3, respectively, applicable to JP-5 and JP-8. Additionally, the National Advisory Committee on Acute Exposure Guideline Levels (AEGLs) has established AEGL1 and AEGL2 values for emergency response purposes in the event of an accidental or intentional release of JP-5 or JP-8 (NRC, 2011). Given the difficulty in measuring jet fuel exposure via the inhalation and dermal routes, considerable effort has gone into identifying biomarkers of JP-8 exposure using U.S. Air Force personnel as study subjects. Candidate exposure surrogates include urinary 1- and 2-naphthol and 2-hydroxyfluorene; blood concentrations of *o*-xylene and *m/p*-xylene; urinary (2-methoxyethoxy) acetic acid, a metabolite of the anti-icing additive diethylene glycol monomethyl ether; and naphthyl-keratin adducts (i.e., reactive naphthalene metabolites bound to skin keratins) that are indicative of dermal exposure only (Kang-Sickel et al., 2011; B'Hymer et al., 2012; Smith et al., 2012; Rodrigues et al., 2014; Maule et al., 2016).

The complexity of jet fuel mixtures presents multiple challenges to toxicologists and risk assessors alike. One such challenge is to generate experimental exposures to jet fuel that accurately reflect those occurring in humans, a task made more difficult by the need to sometimes distinguish aerosol droplets from their surrounding vapors. The NAS Subcommittee on JP8 has reviewed the methods used to generate exposure atmospheres in several published studies using aerosol/vapor mixtures and suspects JP-8 concentrations may have been grossly underreported, particularly in studies by Witten and colleagues (NAS, 2003). Because of concerns surrounding the quantitative accuracy of the exposure atmospheres, the NAS Subcommittee recommended an examination of the methods used for their characterization. To this end, Dietzel et al. (2005) have developed and validated a GC/MS method for JP-8, which was subsequently used to characterize the aerosol and vapor fractions of aerosolized fuel in one of the University of Arizona-based inhalation chambers previously utilized by Witten and colleagues. Subsequent publications have detailed the development and validation of new methods to generate and evaluate jet fuel vapor and aerosol fractions for experimentation (Gregg et al., 2006; Martin et al., 2010; Tremblay et al., 2010). One application of these new methods came in the form of a detailed assessment of jet fuel pharmacokinetics and use of the data to develop a PBPK model for JP-8 and synthetic jet fuel in the rat (Martin et al., 2012).

Jet A, JP-5, and JP-8 have similar toxicity profiles, which suggest their toxicities are largely a function of hydrocarbon content rather than additives. Toxicity data on jet fuels have been well summarized (ATSDR, 1998; NAS, 2003; Ritchie et al., 2003; Mattie and Sterner, 2011; NRC, 2011), with most studies focusing on the two main portals of entry (i.e., lung and skin) and the immune system. In subchronic and chronic rodent inhalation studies of jet fuel vapor, the chief findings have been renal toxicity and neoplasia in male rats consistent with α_{2u}-globulin nephropathy (Mattie et al., 1991; Bruner et al., 1993). Despite the liver, kidneys, and testes in humans not being considered particularly sensitive targets of jet fuels, proteomic analyses of these tissues have been conducted in male rats subchronically exposed to JP-8 vapor (Witzmann et al., 2000a, 2000b, 2003). Similarly, gene expression in the whole brain of rats repeatedly exposed to JP-8 vapor has been examined (Lin et al., 2001a, 2004), driven largely by reports of cognitive and motor deficits and neurochemical changes in jet fuel–exposed workers and experimental animals (Baldwin et al., 2001, 2007; Ritchie et al., 2001; Rossi et al., 2001; Bell et al., 2005). Among U.S. Air Force personnel enrolled in the Occupational JP8 Exposure Neuroepidemiology Study (OJENES), repeated workday exposures to JP-8 did not result in performance deviations from reference ranges on most neuropsychological tasks (Proctor et al., 2011). Experimental studies do not support jet fuel as a teratogen or strong reproductive toxicant, and genotoxicity studies have been contradictory. Like several aromatic hydrocarbon solvents, JP-8 exposure enhances noise-induced hearing loss, apparently by causing the death of outer hair cells in the cochlea and suppressing central auditory function (Kaufman et al., 2005; Fechter et al., 2007, 2010, 2012; Guthrie et al., 2014, 2015). Perhaps most intriguing, however, are reports that JP-8 exposure in the gestating rat is capable of inducing the epigenetic transgenerational inheritance of adult-onset diseases, including obesity and reproductive disorders in both sexes (Manikkam et al., 2012; Tracey et al., 2013).

Pulmonary Effects The pulmonary effects of an aerosol/vapor mixture of JP-8 were initially investigated in rats with nose-only exposures designed to simulate military flightline exposures (Hays et al., 1995; Pfaff et al., 1995, 1996). Functional changes in the form of increases in pulmonary resistance and alveolar permeability were accompanied by a decrease in the concentration of the tachykinin Substance P (SP) in bronchoalveolar lavage fluid. Pathological changes were observed in lower pulmonary structures including inflammation of the terminal bronchioles, degeneration of alveolar type II epithelial (AIIE) cells, and disruption of terminal bronchial airway epithelium. Most interestingly, the activity of neutral endopeptidase (NEP), an enzyme responsible for the metabolism of SP in the lung, was increased by exposure and a significant inverse relationship between SP and NEP activity demonstrated. Thus, JP-8 appears to exhibit a rather novel mechanism of lung injury that involves the reduction or depletion of SP due to its enhanced metabolism by NEP.

Because SP participates in the maintenance of airway epithelial cell competency, the effect of JP-8 and *n*-tetradecane (C_{14}), a primary constituent of JP-8, on epithelial barrier integrity was examined in vitro using paracellular mannitol flux in BEAS-2B human bronchial epithelial cells (Robledo et al., 1999). Noncytotoxic concentrations of JP-8 and C_{14} produced dose-dependent increases in transepithelial mannitol flux that spontaneously reversed to control values over a 48-hour recovery period. This suggests that JP-8 and C_{14} compromise the integrity of intercellular tight junctions that may precede and initiate the pathological alterations observed in whole animal studies. Evidence was also collected that SP's protective effect on the lung is largely mediated through the plasma membrane-bound neurokinin receptor, NK_1, present on airway epithelium from the trachea to the respiratory bronchioles (Robledo and Witten, 1999).

Among the most affected alveolar cells in rodent studies are AIIE cells. In support of the hypothesis that apoptotic cell death is responsible at least partially for JP-8's cytotoxicity in the lung, Stoica et al. (2001) reported that JP-8 results in morphological and biochemical changes characteristic of apoptosis in the rat AIIE cell line, RLE-6TN. Further, Boulares et al. (2002) have collected data that strongly suggest JP-8 triggers apoptosis in rat lung epithelial cells by inducing the generation of reactive oxygen species, depleting/reducing intracellular GSH, and markedly decreasing mitochondrial membrane potential, thereby initiating the apoptotic cascade (i.e., caspase-3 activation and DNA fragmentation).

A characteristic feature of the lung inflammatory response to JP-8 in rodents is vacuolization of AIIE cells and accumulation of pulmonary alveolar macrophages (PAM). The findings of Wang et al. (2002b) suggest that JP-8 causes pro-inflammatory cytokine secretion not only by PAM, but also AIIE cells. The prolonged production of pro-inflammatory cytokines, together with the proteases produced by activated macrophages and neutrophils, is capable of producing a sustained immune response with increased risk for lung damage. Moreover, cocultures of AIIE cells and primary PAM indicate that the balance of cytokines released in response to JP-8 could possibly be regulated in vivo by cross-communication between the two cell types. Espinoza et al. (2005) showed that the JP-8-induced expression of pro-inflammatory cytokine genes in AIIE cells was mediated by the activation of PARP-1 (an enzyme coactivator of NF-κβ) and NF-κβ (a transcription factor that controls the expression of a variety of genes involved in inflammatory responses). The release of cytokines in the lung in response to JP-8 is similar to cytokine release from epidermal cells, which is thought to mediate, at least in part, the dermal and immune toxicities of JP-8 (see below). Further insight into the pulmonary effects of jet fuel has come from examinations of aerosolized JP-8's effect on gene and protein expression in lung cytosol and lung tissue of rats and mice (Witzmann et al., 1999; Drake et al., 2003; Espinoza et al., 2005). Like its petroleum-based counterpart, the pulmonary toxicity of synthetic jet fuel has been examined in nose-only exposures to male mice and in cultured AIIE cells of rats (Wong et al., 2009; Robb et al., 2010).

Immune Effects The immune system appears to be as susceptible to jet fuel as the lung, if not more so. Detrimental effects on the immune system of mice have been reported for aerosolized Jet A, JP-8, and JP-8+100 (Harris et al., 2001a). Some effects were apparent just 1 hour after a single inhalation exposure with continued deterioration with each successive exposure (Harris et al., 2002). As first reported by Harris et al. (1997a), mice exposed nose-only to a JP-8 aerosol/vapor mix exhibited decreased spleen and thymus weights and cellularities and an altered number of viable immune cells in lymph nodes, bone marrow, and peripheral blood. Depending on the immune tissue examined, different immune cell sub-populations were lost, including T and B cells and macrophages. In addition, JP-8 affected immune function as demonstrated by a concentration-dependent suppression of T-cell proliferation upon stimulation with the mitogen concanavalin A. In a short-term exposure study, Harris et al. (1997b) determined that JP-8-induced immunotoxicity persisted for at least 1 month after insult. These same authors later expanded the number of immune parameters examined and reported that aerosolized JP-8 exposure of mice nearly completely ablated natural killer cell function, suppressed the generation of lymphokine-activated killer cell activity, suppressed the generation of cytotoxic T lymphocytes from precursor T cells, and inhibited helper T-cell activity (Harris et al., 2000). As even a 1-hour exposure to aerosolized JP-8 elevated serum levels of interleukin-10 (IL-10) and prostaglandin E2 (PGE2), these two immunosuppressive cytokines should be suspected as means by which jet fuel can modulate immune function (Harris et al., 2007a).

Ullrich (1999) has shown that like inhalation, dermal application of JP-8 to mice can induce immune suppression. He found IL-10 in the serum of JP-8 dermally treated mice and interpreted this as suggestive of an immune suppressive mechanism involving the upregulation of cytokine release. A follow-up study suggested that the immunosuppressive effect of dermally applied JP-8 was specific to cell-mediated immune reactions (i.e., T-helper cell-driven cell-mediated immunity), as JP-8 had no effect on antibody production in immunized mice (Ullrich and Lyons, 2000). Ramos et al. (2002) have demonstrated that the dermal application of JP-8 and Jet A suppresses delayed-type hypersensitivity and immunologic memory upon rechallenge with a fungal pathogen, raising the possibility that jet fuel exposure is capable of depressing the protective effect of prior vaccination. He and his colleagues used platelet-activating factor (PAF) receptor antagonists to show that the PAF receptor, a signaling phospholipid that activates the transcription of genes for PGE2 and IL-10 in keratinocytes, plays a critical role in jet fuel–induced immune suppression (Ramos et al., 2004). This, coupled with the demonstration that jet fuel–induced immunosuppression can be reversed by inhibiting prostaglandin secretion with a selective COX-2 inhibitor or injecting an anti-IL-10 antibody (Ullrich and Lyons, 2000), strongly supports an essential role for PGE2 and IL-10 in the sequence of events culminating in immunosuppression. Using synthetic jet fuel's absence of aromatic hydrocarbons to their experimental advantage, Ramos et al. (2007) demonstrated that the aromatic hydrocarbons in JP-8 are the active agents that induce immune suppression. This is seemingly due to the aromatic fraction's ability to produce reactive oxygen species (ROS) in the skin that activates NF-κβ, which in turn activates COX-2, thereby inducing the secretion of PGE2 (Ramos et al., 2009). Such a mode of action is consistent with the demonstration that functional mast cells and their PGE2 are essential to jet fuel immunosuppression, as is mast cell migration from the skin to draining lymph nodes that enables transmission of the immunosuppressive signal from skin to the immune system (Limon-Flores et al., 2009). Similar to that seen for pulmonary toxicity, aerosolized SP can both prevent and reverse some facets of JP-8-induced immunotoxicity, suggesting a key mechanistic role for the neuropeptide (Harris et al., 2001b, 2007c). Also, as reported in AIIE cells, JP-8 induces apoptosis in primary mouse thymocytes (Stoica et al., 2001). Exposure of mice to JP-8 in utero is reportedly immunosuppressive, whether dams are exposed by gavage or aerosol inhalation (Keil et al., 2003; Harris et al., 2007b). Lastly, immunosuppression secondary to jet fuel exposure has been shown to potentiate the growth and metastasis of tumors in experimental animal models (Harris et al., 2007c) and increase the severity of viral infection and related mortality in mice (Harris et al., 2008).

Dermal Effects Owing to reports of severe contact dermatitis among military personnel, the dermal toxicity of jet fuel has been the subject of intensive investigation. A recent examination of dermal exposure in 124 U.S. Air Force fuel-cell maintenance workers, using a noninvasive tape-strip technique and naphthalene as a surrogate, confirmed that the skin provides a significant exposure route for JP-8 (Chao et al., 2005). Dermal absorption and penetration of JP-8 and its component hydrocarbons have been examined in vitro using dermatomed rat skin and static diffusion cells and in vivo in weanling pigs and humans (McDougal et al., 2000; Singh et al., 2003; Kim et al., 2006a). Their cutaneous toxicity has been explored in pigs, rats, rabbits, and in vitro using human epidermal

keratinocytes (HEK) (Kabbur et al., 2001; Monteiro-Riviere et al., 2001; Chou et al., 2003; Singh and Singh, 2004). Dermal exposure to jet fuel can lead to skin irritation and sensitization and the disruption of skin barrier function. Research implicates cytokine release, oxidative stress, and DNA damage/fragmentation as mechanistic underpinnings (Allen et al., 2000; Rogers et al., 2001; Gallucci et al., 2004). In stark contrast to traditional histopathology techniques, Sharma and Locke (2010) used noninvasive magnetic resonance microimaging as a means of skin damage assessment. They noted exfoliated epidermis, dermis thickening, and hair follicle atrophy in nude rats sprayed with heated JP-8 vapor for 1 minute and examined 24 hours later. Chronic dermal application of middle distillate fuels such as jet fuel can be weakly carcinogenic, though it has been effectively argued that such tumorigenicity is secondary to chronic irritation (Nessel, 1999; Nessel et al., 1999).

The ultrastructural analysis of pig skin exposed to cotton fabric saturated with jet fuel suggests that the primary effect of exposure is damage of the stratum corneum barrier (Monteiro-Riviere et al., 2004). This same publication reported that IL-8 release from HEK after JP-8 exposure was decreased by SP, which is an agonist for the NK_1 receptor present in keratinocytes and mechanistically linked to IL release. The attenuation of IL release in keratinocytes by SP and the protection SP affords against pulmonary and immunotoxicity suggests that there may be a common mechanistic linkage to these toxicities. However, studies suggest that jet fuel–induced cell death in skin is via necrosis, not apoptosis as observed in some other cell types such as AIIE cells and T lymphocytes (Stoica et al., 2001). This was also demonstrated by Rosenthal et al. (2001), who observed JP-8-induced necrotic rather than apoptotic cell death in mouse skin fibroblasts and HEK in culture or grafted onto nude mice. These authors used immunoblot analysis to determine that necrosis of HEK appeared to be associated with the perturbation of the ratio between anti- and pro-survival members of the Bcl-2 family of proteins (i.e., the toxic, higher level of JP-8 decreased levels of the pro-survival proteins Bcl-2 and Bcl-xL, while simultaneously elevating levels of the anti-survival proteins Bad and Bak). This led to the suggestion that high intrinsic levels of Bcl-2 and Bcl-xL may prevent apoptotic death of keratinocytes at low concentrations of JP-8, whereas modulation of Bcl-2 family members by high doses may lead to necrotic cell death.

As the skin allows for the selective absorption and penetration of various jet fuel components, one cannot assume that the internal or target tissue dose of chemical is qualitatively or quantitatively the same as that of the external dose (McDougal and Robinson, 2002). This has led to efforts to identify the offending components of jet fuel mixtures. Using static diffusion cells, McDougal et al. (2000) identified 12 components of JP-8 that fully penetrated rat skin and six components, all aliphatic hydrocarbons, which were partially absorbed or retained by the skin. This led these researchers to speculate that the aliphatic components of jet fuel may be the cause of skin irritation. Allen et al. (2001) exposed HEK to micromolar concentrations of four aliphatic hydrocarbon components of jet fuel and found that they all induced IL-8 release at subtoxic doses, further implicating aliphatic components. However, Chou et al. (2002) exposed HEK to 10 aliphatic jet fuel hydrocarbons (C6–C16) and found that the higher cytotoxicity of the shorter chain aliphatics did not correlate with their ability to induce IL-8 release, which peaked at mid-chain lengths (i.e., C9–C13). The toxicological interactions of jet fuel's aromatic and aliphatic components on HEK cells have also been explored (Yang et al., 2006). The cytotoxicity and IL-8 release from these "mixed" hydrocarbon exposures were not always predictable based on the cytotoxic and IL-8 profiles of individual components. Muhammad et al. (2005) exposed pigs topically to cotton fabric soaked with one of eight aliphatic hydrocarbons or one of six aromatic hydrocarbons and monitored skin irritation. Based on these data, coupled with data on IL-8 release, in vitro absorption and cytotoxicity, they postulate that tridecane and tetradecane may be the two most important hydrocarbons responsible for jet fuel–induced skin irritation. Jet fuel exemplifies the difficulty of accurately predicting the dermal risk posed by a complex mixture based on limited knowledge of a few individual components. Aside from the skin as a target tissue, dermal exposure to JP-8 may result in systemic levels of hydrocarbons, the prediction of which may aid in risk assessment. Toward this end, Kim et al. (2006b) published a dermatotoxicokinetic model of the skin that quantitatively characterized the TK of three aromatic and three aliphatic jet fuel components following application of a single dose of JP-8 to the forearm of human volunteers (Kim et al., 2006a). This model was subsequently expanded to include an inhalation component and exercised to describe the relative contribution of dermal and inhalation exposures to the end-exhaled air concentrations of naphthalene among JP-8 exposed U.S. Air Force personnel. The median relative contribution of dermal exposure to the end-exhaled breath concentration of naphthalene was 4% (10th percentile 1% and 90th percentile 11%) (Kim et al., 2007). As in lung tissue and lung cytosol of rodents, the capacity of JP-8 to alter gene or protein expression in HEK and rat epidermis has been examined (Espinoza et al., 2004; Witzmann et al., 2005; Chou et al., 2006; McDougal et al., 2007).

CARBON DISULFIDE

The toxicity of carbon disulfide (CS_2) was first recognized during the nineteenth century, when it was widely employed as a solvent to soften rubber. CS_2 is among the current 187 chemicals regulated under the Clean Air Act because of reported high emissions and the potential for human exposure and serious health effects. The major uses of CS_2 are in the production of rayon fiber, cellophane, and CCl_4 and as a solubilizer for waxes and oils (ATSDR, 1996a). Historically, exposures were particularly high during the early period of rayon production, and studies of these workers have been very informative as to CS_2's toxicity. Human exposure is predominantly occupational, although CS_2 has been identified in at least 200 current or former EPA National Priority List hazardous waste sites (ATSDR, 1996a). Most industrial releases are to the atmosphere. The general public may be subjected to low vapor levels as demonstrated by detection of CS_2 in samples of breath and indoor and outdoor air surveyed in and around New York City (Phillips, 1992). Exposure to dithiocarbamate pesticides and drugs (e.g., the alcohol aversion drug, disulfiram) can result in indirect exposure to CS_2, as it is a product of their metabolism. However, there is evidence that the metabolic production of CS_2 is not a unifying explanation for the neuropathies frequently associated with dithiocarbamate exposure (Tonkin et al., 2000; Mulkey, 2001).

The relative contributions of parent compound and metabolites to most CS_2-induced toxicities are unknown. Two distinct metabolic pathways for CS_2 exist: (1) the direct interaction of CS_2 with free amine and sulfhydryl groups of amino acids and polypeptides to form dithiocarbamates and trithiocarbonates; and (2) microsomal metabolism of CS_2 to reactive sulfur intermediates capable of covalently binding tissue macromolecules (Graham et al., 1995) (Fig. 24-13). The conjugation of CS_2 with sulfhydryls of cysteine or glutathione (GSH) results in the formation of 2-thiothiazolidine-4-carboxylic acid (TTCA), which is excreted in urine and has been frequently used as a biomarker of CS_2 exposure, especially among viscose rayon workers (Riihimaki et al., 1992; Lee et al., 1995). Several limitations of TTCA as a biomarker have been noted, and

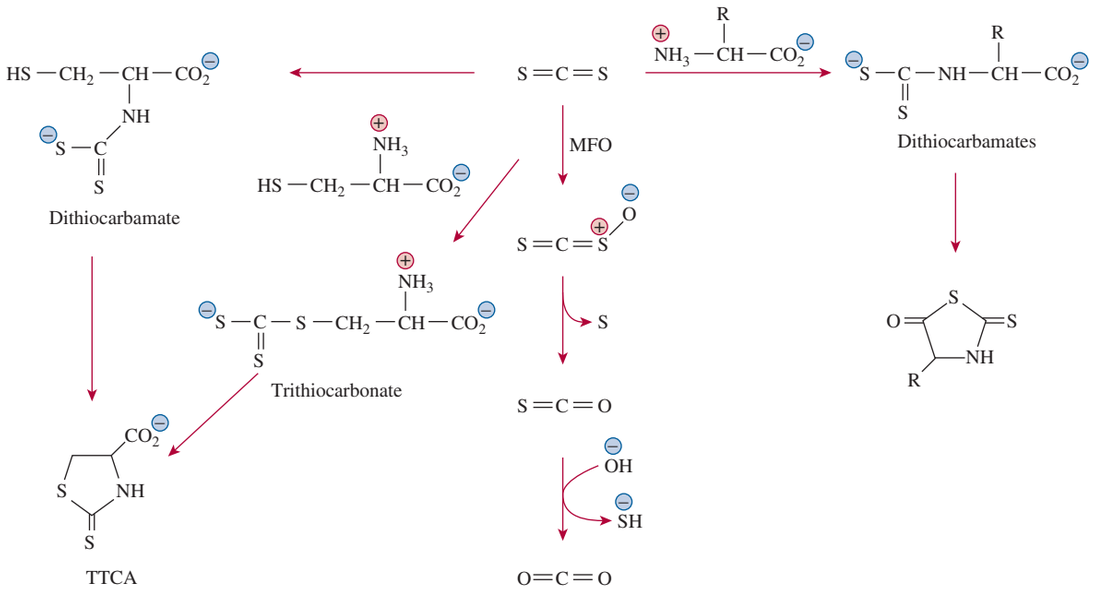

Figure 24-13. *Metabolism of carbon disulfide (CS_2).* CS_2 is metabolized by the mixed-function oxidase (MFO) system to carbonyl sulfide, atomic sulfur, and HS–. Reaction of CS2 with sulfhydryls of cysteine or GSH yields trithiocarbonates, which can cyclize to form thiazolidine-2-thione-4-carboxylic acid (TTCA). Reaction of CS2 with amino groups of amino acids results in dithiocarbamate derivatives, which can cyclize to yield 2-thio-5-thiazolidinones; reaction of cysteine amine could also produce TTCA. CS2 is also metabolized in the liver by P450s to an unstable oxygen intermediate, which spontaneously generates atomic sulfur, carbonyl sulfide (COS), and CO2. (Reproduced with permission of Graham et al., 1995.)

covalently cross-linked erythrocyte spectrin and hemoglobin have been discussed as potential alternatives (Valentine et al., 1993, 1998). Nonetheless, the current ACGIH Biological Exposure Index (BEI) for CS_2 is 0.5 mg of TTCA per g of creatinine for a urine sample collected at the end of a workshift (ACGIH, 2011).

A few comprehensive reviews of CS_2's toxicity have been published (Beauchamp et al., 1983; ATSDR, 1996a; WHO, 2002; Gelbke et al., 2009). CS_2 is capable of targeting multiple organ systems including the cardiovascular system, central and peripheral nervous systems, male and female reproductive systems, eyes (retinal angiopathy and impairment of color vision), and ears (hearing loss). CS_2 toxicity requires frequent and prolonged exposures in occupational settings. The nervous and cardiovascular systems have garnered the most attention. The most common neurotoxic effect is a distal sensorimotor neuropathy that preferentially affects long axons in the PNS and CNS (particularly the ascending and descending tracks of the spinal cord and the visual pathways). Encephalopathy with motor and cognitive impairment has also been reported following chronic, low-level exposure to CS_2 (Graham et al., 1995). Several MRI studies report diffuse white matter lesions in chronically exposed workers similar to that described for "toluene leukoencephalopathy" among solvent abusers (Cho et al., 2002; Ku et al., 2003). For those particularly interested in the nervous system, the classic paper by Richter (1945) detailing his observations of chronic CS_2 poisoning in monkeys is recommended. Rosenberg (1995) described the following clinical syndromes associated with CS_2: (1) acute and chronic encephalopathy (often with prominent psychiatric manifestations); (2) polyneuropathy (both peripheral and cranial); (3) Parkinsonism; and (4) asymptomatic CNS and PNS dysfunction. Pathologic changes occur in both the CNS and PNS. CNS pathology consists of neuronal degeneration throughout the cerebral hemispheres, with maximal diffuse involvement in the frontal regions. Cell loss is also noted in the globus pallidus, putamen and cerebellar cortex, with loss of Purkinje cells. Vascular abnormalities with endothelial proliferation of arterioles may be seen, sometimes associated with focal necrosis or demyelination. PNS changes consist primarily of myelin swelling and fragmentation and large focal axonal swellings, characteristic of distal axonopathy.

Significant contributions to the understanding of CS_2's neurotoxicity have come from collaborative research by the NIEHS, EPA, and several universities (Harry et al., 1998; Sills et al., 2005). Studies were conducted with F344 rats subchronically exposed to a range of CS_2 concentrations, in order to define the onset and temporal progression of neurotoxicity as manifest by multiple endpoints. These specific endpoints included toxicokinetic (TK) changes in blood CS_2 levels and urinary TTCA, covalent cross-linking of blood and spinal cord proteins, alterations in axon/Schwann cell interactions as indicated by nerve growth factor mRNA expression, morphology of distal axonopathy, nerve conduction velocity and action potential, and behavioral assessment using a Functional Observational Battery. This research showed sensitive endpoints at the cellular level that progressed to alterations in hindlimb and forelimb function, followed by electrophysiological and morphological changes. The TK data provided useful information about internal exposure to CS_2, but its use in the prediction of biological effects was limited (Moorman et al., 1998). The collaborative efforts support the theory that the axonal degeneration that underlies CS_2's central–peripheral neuropathy results from the reaction of CS_2 (and perhaps carbonyl sulfide [COS]) with protein amino groups to yield initial adducts (dithiocarbamate derivatives). The adducts decompose to an electrophile (isothiocyanate for CS_2 and isocyanate for COS), which in turn reacts with protein nucleophiles on neurofilaments to cause covalent protein cross-linking. Progressive cross-linking of neurofilaments occurs during neurofilament transport along the axon, and covalently cross-linked masses of

neurofilaments are thought to occlude axonal transport at the nodes of Ranvier, ultimately resulting in axonal swelling and degeneration (similar to that seen with 2,5-hexanedione). It should be noted that several other mechanisms for the disruption of neurofilament transport that underlie CS_2's axonopathy have been proposed including: impaired energy metabolism, metal ion chelation by CS_2's dithiocarbamate derivatives, induction of vitamin deficiency, disruption of cytoskeletal protein association by the increased phosphorylation of neurofilaments, changes in neurofilament content due to an imbalance of neurofilament synthesis and degradation by activated calpains, and production of reactive oxygen species and enhanced lipid peroxidation (Wilmarth et al., 1993; Graham et al., 1995; Song et al., 2009; Sun et al., 2009).

One of the potentially more important outcomes of the collaborative research effort described above was the identification of thiourea cross-linking structures on erythrocyte spectrin and hemoglobin. This cross-linking exhibited a linear dose–response over the range of inhaled CS_2 concentrations examined, was detectable at subneurotoxic exposure levels, preceded axonal structural damage, and was positively correlated with neurofilament cross-linking (i.e., spectrin and hemoglobin cross-linking reflect neurofilament cross-linking). These findings suggest the utility of spectrin and hemoglobin cross-linking as sensitive biomarkers of exposure and effect, and potential alternatives or supplements to TTCA that was found to lack dose proportionality in the same studies (Valentine et al., 1997, 1998; Moorman et al., 1998). More recently, Chinese researchers collected serum from CS_2-exposed workers with damaged nerve fibers and surveyed it for aberrantly expressed microRNAs (miRNAs) compared to control serum (first reported in mammalian systems in 2001, miRNAs are noncoding RNAs that usually act as gene silencers and influence a wide range of biological processes). Two miRNAs were identified, miR-150 and miR-30a, both of which were upregulated and capable of discerning damaged nerve fiber cases from controls. These and other miRNAs appear to hold promise as noninvasive biomarkers for a damaged nervous system (Guo et al., 2015).

Numerous worker studies support an association between CS_2 exposure and cardiovascular disease and related mortality. Elevated mortality from cardiovascular disease has been reported among viscose rayon workers in Finland, the United Kingdom, the United States, Scandinavia, and Poland (WHO, 2002). Viscose rayon worker studies reporting excess cardiovascular morbidity expand the range to Germany (Drexler et al., 1996) and Japan (Takebayashi et al., 2004). Further support for a CS_2–cardiovascular disease link comes from examination of cardiovascular disease risk factors in workers, and to a lesser extent experimental rodent studies. In workers, CS_2 exposure has been associated with elevations in blood pressure, total and LDL cholesterol, triglycerides, apolipoproteins, and lipid peroxidation in plasma, as well as reductions in HDL and antioxidant status (Vanhoorne et al., 1992; Wronska-Nofer et al., 2002; Luo et al., 2003). Wronska-Nofer (1979) conducted studies in rats supporting a role for CS_2 in the elevation of blood cholesterol, whereas Lewis et al. (1999) found that exposure to as little as 50 ppm CS_2 significantly enhanced the rate of arterial fat deposition in mice placed on a Western style, high-fat diet. Sulsky et al. (2002) have reviewed 37 studies addressing the CS_2–cardiovascular disease association and concluded that epidemiological evidence for an association was "mixed," with an effect on total and/or LDL cholesterol being the most consistent finding but of limited magnitude and uncertain clinical significance. Tan et al. (2002) conducted a meta-analysis of 11 cohort studies on CS_2's cardiovascular effects that showed a small, but significant, correlation between CS_2 exposure and cardiovascular disease prevalence (pooled RR = 1.56; 95% CI = 1.12 to 2.1). More recently, a cross-sectional study of 290 exposed and 137 nonexposed German workers in a rayon manufacturing plant reported increased intima-media thickness (an established marker of atherosclerosis and a reasonable surrogate marker of cardiovascular risk) among those with long-term exposures to high CS_2 levels. However, it was noted that no significant changes in intima-media thickness were found in the group of workers whose exposure never exceeded 10 ppm (Schramm et al., 2016). This is in agreement with another study of German viscose workers that examined an array of biomarkers of cardiovascular risk where average CS_2 exposure was less than 5 ppm (Domergue et al., 2016). No associations between long-term or recent CS_2 exposure and cardiovascular risk parameters were detected. Taken together, studies suggest that CS_2 has the ability to accelerate atherosclerosis, provided an exposure threshold is exceeded. Further, some have speculated that like neurotoxicity, protein cross-linking may also be involved in CS_2's promotion of the atherosclerotic process (Lewis et al., 1999).

Price et al. (1997) pointed out that the cardiovascular mortality excesses seen in most published studies were among workers chronically exposed to high concentrations that no longer are observed in the workplace. These authors reviewed historical exposure and mortality data in the viscose rayon industry and estimated that chronic exposures of 15 to 20 ppm would not be associated with an increased risk of mortality due to ischemic heart disease. They were also instrumental in applying the benchmark dose method toward the establishment of EPA's RfC for CS_2 of 0.7 mg/m^3 (about 0.22 ppm), that is based on reduced maximum motor conduction velocity in the peroneal nerves of the NIOSH cohort of viscose rayon workers reported by Johnson et al., 1983; Price et al., 1996; EPA, 2006d. For the protection of workers, OSHA has established a permissible exposure limit (PEL) of 20 ppm as an 8-hour TWA, a 30 ppm ceiling for 30 minutes, and a 100 ppm peak. The ACGIH TLV, as an 8-hour TWA, was reduced from 10 to 1 ppm in 2006, based on neurologic endpoints. As a result, the ACGIH also reduced CS_2's BEI from 5 to 0.5 mg TTCA/g creatinine in 2009. Gelbke et al. (2009) reviewed the health effects of CS_2 in the viscose industry and concluded that while some uncertainties exist, available data generally support an occupational exposure limit of 10 ppm.

One of the uncertainties surrounding Gelbke et al.'s (2009) general support of an occupationoral exposure limit of 10 ppm involved the reproductive capacity of female workers. Menstrual disorders, spontaneous abortion, premature birth, and extended time to pregnancy have been reported among workers, with recently published mouse studies shedding light on potential mechanisms. For example, CS_2 administered to gestating mice resulted in DNA damage and apoptosis of endometrial cells (with upregulation of Bax and caspase-3 genes and downregulation of the Bcl-2 gene in uterine tissue), dramatic elevations in uterine tissue of 8-OH-dG (a biomarker of oxidative DNA damage), and a reduction in the number of implanted blastocysts (Zhang et al., 2013; Yang et al., 2014). In addition, gestational exposure to CS_2 was shown to disrupt murine embryo implantation by decreasing the uterine expression of the cell adhesion molecule known as integrin β3, a biomarker of uterine receptivity in humans as well (Sun et al., 2013). These same researchers later reported the ability of CS_2 to decrease the sex steroid hormones, progesterone and estrogen, and in turn downregulate the cell adhesion protein and mRNA of leukemia inhibitory factor (LIF) in maternal uterine tissue (Wang et al., 2015). Not only do progesterone and estrogen stimulate LIF synthesis from endometrial epithelial cells, but LIF serves as an upstream regulatory protein of integrin β3, potentially explaining integrin β3's loss and embryo implantation failure. The possibility also exists that CS_2's reproductive toxicity may be male-mediated in some cases,

as suggested by the health histories of 100 male viscose rayon workers employed 10 years prior to study, all of whom were asked about occurrences of spontaneous abortion and fetal deaths (Patel et al., 2004). This "epidemiological" study reported an incidence of miscarriage of 5.71% among workers exposed to 1.695 ppm CS_2 on average compared to 18.91% among those exposed to an average concentration of 12.28 ppm. CS_2 has been reported to result in testicular atrophy and reduced sperm count, with two recent rat studies elucidating possible mechanisms. Both exposed male rats to CS_2 via inhalation, with the first implicating reduced nitric oxide (a second messenger in the reproductive system) levels in serum and tissues of the hypothalamic–pituitary–gonadal axis in decreases in sex hormones and sperm quantity and quality (Huang et al., 2012). The second study reported CS_2-induced apoptosis in testicular germ cells and a role for the mitochondrial membrane permeability transition in the cell death (Guo et al., 2014).

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REFERENCES

Abbassi R, Chamkhia N, Sakly M. Chloroform-induced oxidative stress in rat liver: implication of metallothionein. *Toxicol Ind Health*. 2010;26:487–496.

Abbate C, Giorgianni C, Munao F, Brecciaroli R. Neurotoxicity induced by exposure to toluene. An electropyhysiologic study. *Int Arch Occup Environ Health*. 1993;64:389–392.

ACGIH (American Conference of Governmental Industrial Hygienists). *Threshold Limit Values (TLVs) and Biological Exposure Indices (BEIs)*. Cincinnati, OH, 2011. Available at: www.acgih.org.

Adachi J, Asano M, Ueno Y, et al. Alcoholic muscle disease and biomembrane perturbations. *Nutr Biochem*. 2003;14:616–625.

Aggazzotti G, Fantuzzi G, Righi E, Predieri G. Environmental and biological monitoring of chloroform in indoor swimming pools. *J Chromatogr A*. 1995;710:181–190.

Ahmed FE. Toxicological effects of ethanol on human health. *Crit Rev Toxicol*. 1995;25:347–367.

Albano E, French SW, Ingelman-Sundberg M. Hydroxyethyl radicals in ethanol hepatotoxicity. *Front Biosci*. 1999;4:533–540.

Albers JW, Wald JJ, Garabrant DH, et al. Neurologic evaluation of workers previously diagnosed with solvent-induced toxic encephalopathy. *JOEM*. 2000;42:410–423.

Albers JW, Wald JJ, Trask CL, et al. Evaluation of blink reflex results obtained from workers previously diagnosed with solvent-induced toxic encephalopathy. *JOEM*. 2001;43:713–722.

Albers JW, Wald JJ, Werner RA, et al. Absence of polyneuropathy among workers previously diagnosed with solvent-induced toxic encephalopathy. *JOEM*. 1999;41:500–509.

Aleksunes LM, Slitt AM, Cherrington NJ, et al. Differential expression of mouse hepatic transporter genes in response to acetaminophen and carbon tetrachloride. *Toxicol Sci*. 2005;83:44–52.

Alexander DD, Kelsh MA, Mink PJ. A meta-analysis of occupational trichloroethylene exposure and liver cancer. *Int Arch Occup Environ Health*. 2007;81: 127–143.

Alexander DD, Mink PJ, Mandel JH, Kelsh MA. A meta-analysis of occupational trichloroethylene exposure and multiple myeloma or leukemia. *Occup Med*. 2006;56:485–493.

Alfred S, Coleman P, Harris D, et al. Delayed neurologic sequelae resulting from epidemic diethylene glycol poisoning. *Clin Toxicol*. 2005;43:155–159.

Allen DG, Riviere JE, Monteiro-Riviere NA. Identification of early biomarkers of inflammation produced by keratinocytes exposed to jet fuels jet A, JP-8, and JP-8 (100). *J Biochem Mol Toxicol*. 2000;14:231–237.

Allen DG, Riviere JE, Monteiro-Riviere NA. Analysis of interleukin-8 release from normal human epidermal keratinocytes exposed to aliphatic hydrocarbons: delivery of hydrocarbons to cell cultures via complexation with α-cyclodextrin. *Toxicol in Vitro*. 2001;15:663–669.

Almekinder JL, Lennard DE, Walmer DK, et al. Toxicity of methoxyacetic acid in cultured human luteal cells. *Fundam Appl Toxicol*. 1997;38:191–194.

Ambroso JL, Stedman DB, Elswick BA, Welsch F. Characterization of cell death induced by 2-methoxyethanol in CD-1 mouse embryos on gestation day 8. *Teratology*. 1998;58:231–240.

Anand SS, Kim K-B, Padilla S, et al. Ontogeny of hepatic and plasma metabolism of deltamethrin in vitro: Role in age-dependent acute neurotoxicity. *Drug Metab Dispos*. 2006a;34:389–397.

Anand SS, Mumtaz MM, Mehendale HM: Dose-dependent liver regeneration in chloroform, trichloroethylene and allyl alcohol ternary mixture hepatotoxicity in rats. *Arch Toxicol*. 2005;79:671–682.

Anand SS, Philip BK, Palkar PS, et al. Adaptive tolerance in mice upon subchronic exposure to chloroform: Increased exhalation and target tissue regeneration. *Toxicol Appl Pharmacol*. 2006b;213:267–281.

Anand SS, Soni MG, Vaidya VS, et al. Extent and timeliness of tissue repair determines the dose-related hepatotoxicity of chloroform. *Int J Toxicol*. 2003;22:25–33.

Andersen ME. A physiologically based toxicokinetic description of the metabolism of inhaled gases and vapors: analysis at steady state. *Toxicol Appl Pharmacol*. 1981;60:509–526.

Andersen ME. Toxicokinetic modeling and its applications in chemical risk assessment. *Toxicol Lett*. 2003;138:9–27.

Andersen ME, Clewell HJ III, Gargas ML, et al. Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol Appl Pharmacol*. 1987;87:185–205.

Andreoli C, Leopardi P, Crebelli R. Detection of DNA damage in human lymphocytes by alkaline single cell gel electrophoresis after exposure to benzene or benzene metabolites. *Mutat Res*. 1997;377:95–104.

Angelo MJ, Pritchard AB, Hawkins DR, et al. The pharmacokinetics of dichloromethane. II. Disposition in Fischer 344 rats following intravenous and oral administration. *Food Chem Toxicol*. 1986;24:975–980.

Araki A, Kamigaito N, Sasaki T, Matsushima T. Mutagenicity of carbon tetrachloride and chloroform in *Salmonella typhimurium.* TA 98, TA 100, TA 1535, and TA 1537, and *Escherichia coli* WP2uvrA/pKM101 and WP2/pKM101, using a gas exposure method. *Environ Mol Mutagen*. 2004;43: 128–133.

Aranyi C, O'Shea WJ, Halder CA, et al. Absence of hydrocarbon-induced nephropathy in rats exposed subchronically to volatile hydrocarbon mixtures pertinent to gasoline. *Toxicol Ind Health*. 1986;2:85–98.

Arbuckle TE. Are there sex and gender differences in acute exposure to chemicals in the same setting? *Environ Res*. 2006;101:195–204.

Arlien-Soborg P. *Solvent Neurotoxicity*. Boca Raton, FL: CRC Press; 1992.

Arlien-Soborg P, Bruhn P, Gyldensted P, Melgaard B. Chronic painters' syndrome: chronic toxic encephalopathy in house painters. *Acta Neurol Scand*. 1979;60:149–156.

Ashley DL, Bonin MA, Cardinali FL, et al. Blood concentrations of volatile organic compounds in a nonoccupationally exposed US population and in groups with suspected exposure. *Clin Chem*. 1994;40:1401–1404.

Asselman M, Verhulst A, de Broe ME, Verkoelen CF. Calcium oxalate crystal adherence to hyaluronan-, osteopontin-, and CD44-expressing injured/regenerating tubular epithelial cells in rat kidneys. *J Am Soc Nephrol*. 2003;14:3155–3166.

Astrand I. Effect of physical exercise on uptake, distribution and elimination of vapors in man. In: Fiserova-Bergerova V, ed. *Modeling of Inhalation Exposure to Vapors: Uptake, Distribution, and Elimination*. Vol 2. Boca Raton, FL: CRC Press; 1983:107–130.

ATSDR (Agency for Toxic Substances and Disease Registry). Methanol toxicity. *Am Fam Physician*. 1993;47:163–171.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Gasoline*. Atlanta, GA: Public Health Service; 1995.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Carbon Disulfide*. Atlanta, GA: Public Health Service; 1996a.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile For Methyl T-Butyl Ether*. Atlanta, GA: Public Health Service; 1996b.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Chloroform*. Atlanta, GA: Public Health Service; 1997a.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Ethylene Glycol and Propylene Glycol*. Atlanta, GA: Public Health Service; 1997b.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Tetrachloroethylene*. Atlanta, GA: Public Health Service; 1997c.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Trichloroethylene*. Atlanta, GA: Public Health Service; 1997d.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Jet Fuels (JP-5 and JP-8)*. Atlanta, GA: Public Health Service; 1998.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Methylene Chloride*. Atlanta, GA: Public Health Service; 2000a.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Toluene*. Atlanta, GA: Public Health Service; 2000b.

ATSDR (Agency for Toxic Substances and Disease Registry). *Interaction Profile for 1,1,1-Trichloroethane, 1,1-Dichloroethane, Trichloroethylene and Tetrachloroethylene*. Atlanta, GA: Public Health Service; 2004.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Carbon Tetrachloride*. Atlanta, GA: Public Health Service; 2005.

ATSDR (Agency for Toxic Substances and Disease Registry). *ToxFAQs for Trichloroethylene (TCE)*. Atlanta, GA: Public Health Service; 2006a. http://www.atsdr.ege.gov/tfacts19.html.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for 1,1,1-Trichloroethane (Update)*. Atlanta, GA: Public Health Service; 2006b.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Benzene*. Atlanta, GA: Public Health Service; 2006c.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Xylenes*. Atlanta, GA: Public Health Service; 2006d.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Ethylbenzene*. Atlanta, GA: Public Health Service; 2010a.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Styrene*. Atlanta, GA: Public Health Service; 2010b.

ATSDR (Agency for Toxic Substances and Disease Registry). *Draft Toxicological Profile for Trichloroethylene*. Atlanta, GA: Public Health Service; 2014.

Auttachoat W, Germolec DR, Collins BJ, et al. Immunotoxicological profile of chloroform in female B6C3F1 mice when administered in drinking water. *Drug Chem Toxicol*. 2009;32:77–87.

Axelson O, Hane M, Hogstedt C. A case-referent study of neuropsychiatric disorders among workers exposed to solvents. *Scand J Work Environ Health*. 1976;2:14–20.

Baan R, Straif K, Grosse Y, et al. Carcinogenicity of alcoholic beverages. WHO International Agency for Research on Cancer Monograph Working Group. *Lancet Oncol*. 2007;8: 292–293.

Backes WL, Sequeira DJ, Cawley GF, Eyer CS. Relationship between hydrocarbon structure and induction of P450: effects on protein levels and enzyme activities. *Xenobiotica*. 1993;23:1353–1366.

Badham HJ, Renaud SJ, Wan J, Linn LM. Benzene-induced oxidative stress: effects on embryonic signaling pathways. *Chem-Biol Interact*. 2010;184:218–221.

Bagchi G, Waxman DJ. Toxicity of ethylene glycol monomethyl ether: impact of testicular gene expression. *Int J Androl*. 2008;31:269–274.

Bagchi G, Zhang Y, Waxman DJ. Impact of methoxyacetic acid on mouse Leydig cell gene expression. *Reprod Biol Endocrinol*. 2010;8:65.

Bailey DG, Dresser GK, Bend JR. Bergamottin, lime juice, and red wine as inhibitors of cytochrome P450 3A4 activity: comparison with grapefruit juice. *Clin Pharmacol Therap*. 2003;73:529–537.

Bakke B, Stewart PA, Waters MA. Use of and exposure to trichloroethylene in U.S. industry: a systematic literature review. *J Occup Environ Hyg*. 2007;4:375–390.

Baldwin CM, Figueredo AJ, Wright LS, et al. Repeated aerosol-vapor JP-8 jet fuel exposure affects neurobehavior and neurotransmitter levels in a rat model. *J Toxicol Environ Health Part A*. 2007;70:1203–1213.

Baldwin CM, Houston FP, Podgornik MN, et al. Effects of aerosol-vapor JP-8 jet fuel on the functional observational battery, and learning and memory in the rat. *Arch Environ Health*. 2001;56:216–226.

Bale AS, Smothers CT, Woodward JJ. Inhibition of neuronal nicotine acetylcholine receptors by the abused solvent, toluene. *Br J Pharmacol*. 2002;137:375–383.

Bale AS, Tu Y, Carpenter-Hyland EP, et al. Alterations in glutamatergic and gabaergic ion channel activity in hippocampal neurons following exposure to the abused solvent toluene. *Neuroscience*. 2005;30: 197–206.

Ballantyne B, Snellings WM. Developmental toxicity study with diethylene glycol dosed by gavage to CD rats and CD-1 mice. *Food Chem Toxicol*. 2005;43:1637–1646.

Ballantyne B, Vergnes JS. In vitro and in vivo genetic toxicology studies with diethylene glycol monohexyl ether. *J Appl Toxicol*. 2001;21:449–460.

Balster RL. Neural basis of inhalant abuse. *Drug Alcohol Dep*. 1998;512:207–214.

Barceloux DG, Bond GR, Krenzelok EP, et al. American Academy of Clinical Toxicology practice guidelines on the treatment of methanol poisoning. *J Toxicol Clin Toxicol*. 2002;40:415–446.

Barrett JS, Alberighi OCD, Läer S, and Meibohm B. Physiologically Based Pharmacokinetic (PBPK) Modeling in Children. *Clin Pharmacol Therap*. 2012;92:40–49.

Barton HA, Creech JR, Godin CS, et al. Chloroethylene mixtures: pharmacokinetic modeling and in vitro metabolism of vinyl chloride, trichloroethylene, and trans-1,2-dichloroethylene in rat. *Toxicol Appl Pharmacol*. 1995;130:237–247.

Barton HA, Das S. Alternative for a risk assessment on chronic non-cancer effects from oral exposure to trichloroethylene. *Regul Toxicol Pharmacol*. 1996;24:269–285.

Bast-Pettersen R. The neuropsychological diagnosis of chronic solvent induced encephalopathy (CSE)—a reanalysis of neuropsychological test results in a group of CSE patients diagnosed 20 years ago, based on comparison with matched controls. *Neurotoxicology*. 2009;30: 1195–1201.

Bautista AP. Impact of alcohol on the ability of Kupffer cells to produce chemokines and its role in alcoholic liver disease. *J Gastroenterol Hepatol*. 2000;15:349–356.

Beattie PJ, Welsh MJ, Brabec MJ. The effect of 2-methoxyethanol and methoxyacetic acid on Sertoli cell lactate production and protein synthesis in vitro. *Toxicol Appl Pharmacol*. 1984;76:56–61.

Beauchamp RO, Bus JS, Popp JA, et al. A critical review of the literature on carbon disulfide toxicity. *Crit Rev Toxicol*. 1983;11:169–278.

Beckstead MJ, Weiner JL, Eger EI, et al. Glycine and gamma-aminobutyric acid (A) receptor function is enhanced by inhaled drugs of abuse. *Mol Pharmacol*. 2000;57:1199–1205.

Beddowes EJ, Faux SP, Chipman JK. Chloroform, carbon tetrachloride and glutathione depletion induce secondary genotoxicity in liver cells via oxidative stress. *Toxicology*. 2003;187:101–115.

Bell AN, Young RA, Lockard VG, Mehendale HM. Protection of chlordecone-potentiated carbon tetrachloride hepatotoxicity and lethality by partial hepatectomy. *Arch Toxicol*. 1988;61:392–405.

Bell IR, Brooks AJ, Baldwin CM, et al. JP-8 jet fuel exposure and divided attention test performance in 1991 Gulf War veterans. *Aviat Space Environ Med*. 2005;76:1136–1144.

Bellinger FP, Bedi KS, Wilson P, Wilce PA. Ethanol exposure during the third trimester equivalent results in long-lasting decreased synaptic efficacy but not plasticity in the CA1 region of the rat hippocampus. *Synapse*. 1999;31:51–58.

Belpoggi F, Soffritti M, Filippini F, Maltoni C. Results of long-term experimental studies on the carcinogenicity of methyl *tert* -butyl ether. *Ann NY Acad Sci*. 1997;837:77–95.

Belpoggi F, Soffritti M, Maltoni C. Methyl-tertiary-butyl ether (MTBE)-a gasoline additive-causes testicular and lymphophaematopoietic cancers in rats. *Toxicol Ind Health*. 1995;11:119–149.

Benignus VA, Boyes WK, Bushnell PG. A dosimetric analysis of behavioral effects of acute toluene exposure in rats and humans. *Toxicol Sci*. 1998;43:186–195.

Benjamin SA, Yang RS, Tessari JD, et al. Lack of promotional effects of groundwater contaminant mixtures on the induction of preneoplastic foci in rat liver. *Toxicology*. 1999;137:137–149.

Benson JM, Gigliotti AP, March TH, et al. Chronic carcinogenicity study of gasoline vapor condensate (GVC) and GVC containing methyl tertiary-butyl ether in F344 rats. *J Toxicol Environ Health, Part A*. 2011;74:638–657.

Bergsagel DE, Wong O, Bergsagel PL, et al. Benzene and multiple myeloma: appraisal of the scientific evidence. *Blood*. 1999;94:1174–1182.

Bernauer U, Birner G, Dekant W, Henschler D. Biotransformation of trichloroethene: dose-dependent excretion of 2,2,2-trichloro-metabolites and mercapturic acids in rats and humans after inhalation. *Arch Toxicol*. 1996;70: 338–346.

Bernauer U, Vieth B, Ellrich R, et al. CYP2E1-dependent benzene toxicity: The role of extrahepatic benzene metabolism. *Arch Toxicol*. 1999;73:189–196.

Besenhofer LM, Adegobyega PA, Bartels M, et al. Inhibition of metabolism of diethylene glycol prevents target organ toxicity in rats. *Toxicol Sci*. 2010;117:25–35.

Besenhofer LM, McLaren MC, Latimer B, et al. Role of tissue metabolite accumulation in the renal toxicity of diethylene glycol. *Toxicol Sci*. 2011;123:374–383.

Bevan C, Neeper-Bradley TL, Tyl RW, et al. Two-generation reproductive toxicity study of methyl tertiary-butyl ether (MTBE) in rats. *J Appl Toxicol*. 1997a;17(S1):S13–S19.

Bevan C, Tyl RW, Neeper-Bradley TL, et al. Developmental toxicity evaluation of methyl tertiary-butyl ether (MTBE) by inhalation in mice and rabbits. *J Appl Toxicol*. 1997b;17(S1):S21–S29.

Bezabeth S, Engel A, Morris CB, Lamm SH. Does benzene cause multiple myeloma? An analysis of the published case–control literature. *Environ Health Perspect*. 1996;104(suppl 6):1393–1398.

B'Hymer C, Mathias P, Krieg E, et al. (2-Methoxyethoxy)acetic acid: a urinary biomarker of exposure for jet fuel JP-8. *Int Arch Occup Environ Health*. 2012;85:413–420.

Bird MG, Burleigh-Flayer HD, Chun JS, et al. Oncogenicity studies of inhaled methyl tertiary-butyl ether (MTBE) in CD-1 mice and F-344 rats. *J Appl Toxicol*. 1997;17:S45–S55.

Bird MG, Greim H, Snyder R, Rice JM. International symposium: recent advances in benzene toxicity. *Chem-Biol Interact*. 2005;153–154:1–5.

Birner G, Bernauer U, Werner M, Dekant W.Biotransformation, excretion, and nephrotoxicity of haloalkane-derived cysteine S-conjugates. *Arch Toxicol*. 1997;72:1–8.

Birner G, Rutkowska A, Dekant W. N-acetyl-S-(1,2,2-trichlorovinyl)-L-cysteine and 2,2,2-trichloroethanol: Two novel metabolites of tetrachloroethylene in humans after occupational exposure. *Drug Metab Dispos*. 1996;14:41–48.

Blair A, Hartge P, Stewart PA, et al. Mortality and cancer incidence of aircraft maintenance workers exposed to trichloroethylene and other organic solvents and chemicals: extended follow-up. *Occup Environ Med*. 1998;55:161–171.

Blair A, Petralia SA, Stewart PA. Extended mortality following of a cohort of dry cleaners. *Ann Epidemiol*. 2003;13:50–56.

Blanco JG, Harrison PL, Evans WE, Relling MV. Human cytochrome P450 maximal activities in pediatric versus adult liver. *Drug Metab Dispos*. 2000;28:379–382.

Bleecker ML, Bolla KI, Agnew J, et al. Dose-related subclinical neurobehavioral effects of chronic exposure to low levels of organic solvents. *Am J Ind Med*. 1991;19:715–728.

Blobaum AL. Mechanism-based inactivation and reversibility: Is there a new trend in the inactivation of cytochrome P450 enzymes? *Drug Metab Dispos*. 2006;34:1–7.

Bloemen LJ, Monster AC, Kezic S, et al. Study on the cytochrome P-450- and glutathione-dependent biotransformation of trichloroethylene in humans. *Int Arch Occup Environ Health*. 2001;74:102–108.

Blount BC, Kobelski RJ, McElprang DO, et al. Quantification of 31 volatile organic compounds in whole blood using solid-phase microextraction and gas chromatography-mass spectrometry. *J Chromatogr B*. 2006;832:292–301.

Boatman R, Corley R, Green T, et al. Review of studies concerning the tumorigenicity of 2-butoxyethanol in B6C3F1 mice and its relevance for human risk assessment. *J Toxicol Environ Health B Crit Rev*. 2004;7:385–398.

Bogen KT, Heilman JM. Reassessment of MTBE cancer potency considering modes of action for MTBE and its metabolites. *Crit Rev Toxicol*. 2015;45:1–56.

Boeffetta P, Garfinkel L. Alcohol drinking and mortality among men enrolled in an American Cancer Society prospective study. *Epidemiology*. 1990;1:342–348.

Boeffetta P, Hashibe M. Alcohol and cancer. *Lancet Oncol*. 2006;7:149–156.

Boice JD Jr, Marano DE, Fryzek JP, et al. Mortality among aircraft manufacturing workers. *Occup Environ Med*. 1999;56:581–597.

Boice JD, Marano DE, Cohen SS, et al. Mortality among Rocketdyne workers who tested rocket engines, 1948-1999. *J Occup Environ Med*. 2006;48:1070–1092.

Bois FY. Applications of population approaches in toxicology. *Toxicol Lett*. 2001;120:385–394.

Bolt HM, Lammert M, Selinski S, Bruning T. Urinary alpha 1-microglobulin excretion as a biomarker of renal toxicity in trichloroethylene-exposed persons. *Int Arch Occup Environ Health*. 2004;77:186–190.

Boogaard PJ, van Sittert NJ. Suitability of *S*-phenylmercapturic acid and *trans-trans*-muconic acid as biomarkers for exposure to low concentrations of benzene. *Environ Health Perspect*. 1996;104(suppl 6):1151–1157.

Borak J, Pastides H, Van Ert M, et al. Exposure to MTBE and acute human health effects: A critical literature review. *Hum Ecol Assess*. 1998;4:177–200.

Borel V, Gallot D, Marceau G, et al. Placental implications of peroxisome proliferator-activated receptors in gestation and parturition. *PPAR Res*. 2008;758562.

Borghoff SJ, Murphy JE, Medinsky MA. Development of physiologically based pharmacokinetic model for methyl tertiary-butyl ether and tertiary-butanol in male Fischer-344 rats. *Fundam Appl Toxicol*. 1996;30:264–275.

Borghoff SJ, Parkinson H, Leavens TL. Physiologically based pharmacokinetic rat model for methyl *tertiary*-butyl ether; comparison of selected dose metrics following various MTBE exposure scenarios used for toxicity and carcinogenicity evaluation. *Toxicology*. 2010;275:79–91.

Borghoff SJ, Short BG, Swenberg JA. Biochemical mechanisms and pathobiology of α_{2u}-globulin nephropathy. *Annu Rev Pharmacol Toxicol*. 1990;30:349–367.

Boulares AH, Contreras FJ, Espinoza LA, Smulson ME. Roles of oxidative stress and glutathione depletion in JP-8 jet fuel-induced apoptosis in rat lung epithelial cells. *Toxicol Appl Pharmacol*. 2002;180:92–99.

Boutelet-Bochan H, Huang Y, Juchau MR. Expression of CYP2E1 during embryogenesis and fetogenesis in human cephalic tissues: Implications for the fetal alcohol syndrome. *Biochem Biophys Res Commun*. 1997;238:443–447.

Bowen SE. Two-serious and challenging medical complications associated with volatile substance misuse: sudden sniffing death and fetal solvent syndrome. *Subst Use Misuse*. 2011;46:68–72.

Bowen SE, Irtenkauf S, Hannigan JH, Stefanski AL. Alterations in rat fetal morphology following abuse patterns of toluene exposure. *Reprod Toxicol*. 2009;27:161–169.

Brauch H, Weirich G, Hornauer MA, et al. Trichloroethylene exposure and specific somatic mutations in patients with renal cell carcinoma. *J Natl Cancer Inst*. 1999;91:854–861.

Brauch H, Weirich G, Klein B, et al. VHL mutations in renal cell cancer: Does occupational exposure to trichloroethylene make a difference? *Toxicol Lett.* 2004;151:301–310.

Brent J, McMartin K, Phillips S, et al. Fomepizole for the treatment of ethylene glycol poisoning. Methylpyrazole for Toxic Alcohols Study Group. *N Engl J Med*. 1999;340:832–838.

Bressler R, Bahl JJ. Principles of drug therapy for the elderly patient. *Mayo Clin Proc*. 2003;78:1564–1577.

Brienza RS, Stein MD. Alcohol use disorders in primary care: do gender-specific differences exist? *J Gen Intern Med*. 2002;17:387–397.

Brinkworth MH, Weinbauer GF, Schlatt S, Nieschlag E. Identification of male germ cells undergoing apoptosis in adult rats. *J Reprod Fertil*. 1995;105:25–33.

Brodkin CA, Daniell W, Checkoway H, et al. Hepatic ultrasonic changes in workers exposed to perchloroethylene. *Occup Environ Med*. 1995;52:679–685.

Brodkin CA, Moon J-D, Camp J, et al. Serum hepatic biochemical activity in two populations of workers exposed to styrene. *Occup Environ Med*. 2001;58:95–102.

Brogen C-H, Christensen JM, Rasmussen K. Occupational exposure to chlorinated organic solvents and its effect on renal excretion of N-acetyl-Beta-D-glucosaminidase. *Arch Toxicol*. 1986;9(suppl): 460–464.

Brooks DE, Wallace KL. Acute propylene glycol ingestion. *J Toxicol Clin Toxicol*. 2002;40:513–516.

Brophy PD, Tenebein M, Gardner J, et al. Childhood diethylene glycol poisoning treated with alcohol dehydrogenase inhibitor Fomepizole and hemodialysis. *Am J Kidney Dis*. 2000;35:958–962.

Brosselin P, Rudant J, Orsi L, et al. Acute childhood leukaemia and residence next to petrol stations and automotive repair garages: the ESCALE study (SFCE). *Occup Environ Med*. 2009;66:598–606.

Brown EA, Shelley ML, Fisher JW. A pharmacokinetic study of occupational and environmental exposure with regard to gender. *Risk Anal*. 1998;18:205–213.

Brown RC, Barone S, Kimmel CA. Children's health risk assessment: incorporating a lifestage approach into the risk assessment process. *Birth Defects Res*. 2008;83: 511–521.

Brown-Woodman PDC, Hayes LC, Huq F, et al. In vitro assessment of the effect of halogenated hydrocarbons: Chloroform, dichloromethane, and dibromomethane on embryonic development of the rat. *Teratology*. 1998;57:321–333.

Bruckner JV. Differences in sensitivity of children and adults to chemical toxicity: the NAS panel report. *Regul Toxicol Pharmacol*. 2000;31:280–285.

Bruckner JV, Kyle GM, Luthra R, et al. Acute, short-term and subchronic oral toxicity of 1,1,1-trichloroethane in rats. *Toxicol Sci*. 2001;60:363–372.

Bruckner JV, Peterson RG. Evaluation of toluene and acetone inhalant abuse. I. Pharmacology and pharmacodynamics. *Toxicol Appl Pharmacol*. 1981;61:27–38.

Bruckner JV, Ramanathan R, Lee KM, Muralidhara S. Mechanisms of circadian rhythmicity of carbon tetrachloride hepatotoxicity. *J Pharmacol Exp Therap*. 2002;300:273–281.

Bruckner JV, White CA, Muralidhara S, Dallas CE. Effect of exposure route and dosage regimen on the toxicokinetics and target organ toxicity of 1,1-dichloroethylene. *J Pharmacol Exp Therap*. 2010;333:519–527.

Bruner RH, Kinkead ER, O'Neill TP, et al. The toxicologic and oncogenic potential of JP-4 jet fuel vapors in rats and mice: 12-Month intermittent inhalation exposures. *Fundam Appl Toxicol*. 1993;20:97–110.

Bruning T, Bolt HM. Renal toxicity and carcinogenicity of trichloroethylene: key results, mechanisms, and controversies. *Crit Rev Toxicol*. 2000;30:253–285.

Bruning T, Golka K, Makropoulos V, Bolt HM. Preexistence of chronic tubular damage in cases of renal cell cancer after long and high exposure to trichloroethylene. *Arch Toxicol*. 1996;70:259–260.

Bruning T, Weirich G, Hornauer MA, et al. Renal cell carcinomas in trichloroethene (TRI) exposed persons are associated with somatic mutations in the von Hippel–Lindau (VHL) tumour suppressor gene. *Arch Toxicol*. 1997a;71:332–335.

Bruning T, Lammert M, Kempkes M, et al. Influence of polymorphisms of GSTM1 and GSTT1 for risk of renal cell cancer in workers with long-term high occupational exposure to trichloroethene. *Arch Toxicol*. 1997b;71:596–599.

Bruning T, Mann H, Melzer H, et al. Pathological excretion patterns of urinary proteins in renal cell cancer patients exposed to trichloroethylene. *Occup Med*. 1999;49:299–305.

Bruning T, Pesch B, Wiesenhutter B, et al. Renal cell cancer risk and occupational exposure to trichloroethylene: Results of a consecutive case–control study in Arnsberg, Germany. *Am J Ind Med*. 2003;43:274–285.

Buben JA, O'Flaherty EJ. Delineation of the role of metabolism in the hepatotoxicity of trichloroethylene and perchloroethylene. A dose-effect study. *Toxicol Appl Pharmacol*. 1985;78:105–122.

Buchanan JA, Alhelail M, Cetaruk EW, et al. Massive ethylene glycol ingestion treated with fomepizole alone—a viable therapeutic option. *J Med Toxicol*. 2010;6:131–134.

Buck ML. The use of chloral hydrate in infants and children. *Ped Pharmacotherap*. 2005;11:1–4.

Bull RJ. Mode of action of liver tumor induction by trichloroethylene and its metabolites, trichloroacetate and dichloroacetate. *Environ Health Perspect*. 2000;108(suppl 2):241–259.

Bull RJ, Orner GA, Cheng RS, et al. Contribution of dichloroacetate and trichloroacetate to liver tumor induction in mice by trichloroethylene. *Toxicol Appl Pharmacol*. 2002;182:55–65.

Burbacher TM, Grant KS, Shen DD, et al. Chronic maternal methanol inhalation in nonhuman primates (*Macaca fascicularis*): reproductive performance and birth outcome. *Neurotoxicol Teratol*. 2004a;26: 639–650.

Burbacher TM, Shen DD, Lalovic B, et al. Chronic maternal methanol inhalation in nonhuman primates (*Macaca fascicularis*): exposure and toxicokinetics prior to and during pregnancy. *Neurotoxicol Teratol*. 2004b;26:201–221.

Burek JD, Nitschke KD, Bell TJ, et al. Methylene chloride: a two-year inhalation toxicity and oncogenicity study in rats and hamsters. *Fundam Appl Toxicol*. 1984;4:30–47.

Burke AS, Redeker K, Kurten RC, et al. Mechanisms of chloroform-induced hepatotoxicity: Oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. *J Toxicol Environ Health Part A*. 2007;70:1936–1945.

Burleigh-Flayer HD, Chun JS, Kintigh WJ. *Methyl Tertiary Butyl Ether: Vapor Inhalation Oncogenicity Study in CD Mice*. Export, PA: Bushy Run Research Center; 1992.

Burstyn I. Peering through the mist: Systematic review of what the chemistry of contaminants in electronic cigarettes tells us about health risks. *BMC Public Health*. 2014;14:18.

Burton DA, Allen MC, Bird JL, Faragher RA. Bridging the gap: ageing, pharmacokinetics and pharmacodynamics. *J Pharm Pharmacol*. 2005;57:671–679.

Bushnell PJ, Beasley TE, Evansky PA, et al. Toxicological assessments of rats exposed prenatally to inhaled vapors of gasoline and gasoline-ethanol blends. *Neurotoxicol Teratol*. 2015;49:19–30.

Byard RW, Chivell WC, Gilbert JD. Unusual facial markings and lethal mechanisms in a series of gasoline inhalation deaths. *Am J Foren Med Path*. 2003;24:298–302.

Cai H, Guengerich FP. Reaction of trichloroethylene and trichloroethylene oxide with cytochrome P450 enzymes: Inactivation and sites of modification. *Chem Res Toxicol*. 2001;14: 451–458.

Caldemeyer KS, Armstrong SW, George KK, et al. The spectrum of neuroimaging abnormalities in solvent abuse and their clinical correlation. *J Neuroimag*. 1996;6:167–173.

Caldwell DJ. Review of mononuclear cell leukemia in F-344 rat bioassays and its significance to human cancer risk: a case study using alkyl phthalates. *Regul Toxicol Pharmacol*. 1999;30:40–53.

Caldwell JC, Evans MV, Marcus AH, et al. Comments on article "Applying mode-of-action and pharmacokinetic considerations in contemporary cancer risk assessments: An example with trichloroethylene" by Clewell and Andersen. *Crit Rev Toxicol*. 2006;36:291–294.

Caldwell JC, Keshava N. Key issues in the modes of action and effects of trichloroethylene metabolites for liver and kidney tumorigenesis. *Environ Health Perspect*. 2006;114:1457–1463.

Campbell JL Jr, Fisher JW. A PBPK modeling assessment of the competitive metabolic interactions of JP-8 vapor with two constituents, m-xylene and ethylbenzene. *Inhal Toxicol*. 2007;19:265–273.

Cantor KP, Stewart PA, Brinton LA, Dosemaci M. Occupational exposures and female breast cancer mortality in the United States. *J Occup Environ Med*. 1995;37:336–348.

Caprino L, Togna GI. Potential health effects of gasoline and its constituents: a review of current literature (1990–1997) on toxicological data. *Environ Health Perspect*. 1998;106:115–125.

Caravati EM, Erdman AR, Christianson G, et al. Ethylene glycol exposure: an evidence-based consensus guideline for out-of-hospital management. *Clin Toxicol*. 2005;43:327–345.

Carlson GP. In vitro metabolism of styrene to styrene oxide in liver and lung of CYP2E1 knockout mice. *J Toxicol Environ Health A*. 2003;66:861–869.

Carlson GP. Critical appraisal of the expression of cytochrome P450 enzymes in human lung and evaluation of the possibility that such expression provides evidence of potential styrene tumorigenicity in humans. *Toxicology*. 2008;254:1–10.

Carlson GP. Metabolism and toxicity of styrene in microsomal epoxide hydrolase-deficient mice. *J Toxicol Environ Health A*. 2010;74: 1689–1699.

Carmona A, Roudeau S, Perrin L, et al. Environmental manganese compounds accumulate as Mn (II) within the Golgi apparatus of dopamine cells: Relationship between speciation, subcellular distribution and cytotoxicity. *Metallomics*. 2014;6:822–832.

Carney EW, Freshour NL, Dittenber DA, Dryzga MD. Ethylene glycol developmental toxicity: unraveling the roles of glycolic acid and metabolic acidosis. *Toxicol Sci*. 1999;50:117–126.

Carney EW, Liberacki AB, Bartels MJ, Breslin WJ. Identification of proximate toxicant for ethylene glycol developmental toxicity using rat whole embryo culture. *Teratology*. 1996;53:38–46.

Carney EW, Tornesi B, Liberacki AB, et al. The impact of dose rate on ethylene glycol developmental toxicity and pharmacokinetics in pregnant CD rats. *Toxicol Sci*. 2011;119:178–188.

Carney EW, Tornesi B, Markham DA, et al. Species-specificity of ethylene glycol-induced development toxicity: Toxicokinetic and whole embryo culture studies in the rabbit. *Birth Defects Res (Part B)*. 2008;83: 573–581.

Carter N, Iregren A, Soderman E, et al. EUROQUESST – A questionnaire for solvent related symptoms: Factor structure, item analysis and predictive validity. *Neurotoxicology*. 2002;23:711–717.

Casanova M, Bell DA, Heck H d'A. Dichloromethane metabolism to formaldehyde and reaction of formaldehyde with nucleic acids in hepatocytes of rodents and humans with and without glutathione *S*-transferase T1 and M1 genes. *Fundam Appl Toxicol*. 1997;37:168–180.

Casanova M, Conolly RB, Heck H d'A. DNA–protein cross-links (DPX) and cell proliferation in B6C3F1 mice but not Syrian golden hamsters exposed to dichloromethane: Pharmacokinetics and risk assessment with DPX as dosimeter. *Fundam Appl Toxicol*. 1996;31:103–116.

Casanova M, Heck H d'A. Lack of evidence for the involvement of formaldehyde in the hepatocarcinogenicity of methyl tertiary-butyl ether in CD-1 mice. *Chem-Biol Interact*. 1997;105:131–143.

Castillo T, Koop DR, Kamimura S, et al. Role of cytochrome P-450 2E1 in ethanol-, carbon tetrachloride- and iron-dependent microsomal lipid peroxidation. *Hepatology*. 1992;16:992–996.

Cattley RC, DeLuca J, Elcombe C, et al. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? *Regul Toxicol Pharmacol*. 1998;27:47–60.

Cawley MJ. Short-term lorazepam infusion and concern for propylene glycol toxicity: case report and review. *Pharmacotherapy*. 2001;21:1140–1144.

Chambers DM, Ocariz JM, McGuirk MF, Blount BC. Impact of cigarette smoking on volatile organic compound (VOC) blood levels in the U.S. population. *Environ Internat*. 2011;37:1321–1328.

Chan WK, Nguyen LT, Miller VP, Harris RZ. Mechanism-based inactivation of human cytochrome P450 3A4 by grapefruit juice and red wine. *Life Sci*. 1998;62:135–142.

Chang TKH, Chen J, Yeung EYH. Effect of *Ginkgo biloba* extract on procarcinogen-bioactivating human CYP enzymes: Identification of isorhamnetin, kaempferol, and quercetin as potent inhibitors of CYP1B1. *Toxicol Appl Pharmacol*. 2006;213:18–26.

Chang YM, Tai CF, Yang SC, et al. Cancer incidence among workers potentially exposed to chlorinated solvents in an electronics factory. *J Occup Health*. 2005;47:171–180.

Chao YC, Gibson RL, Nylander-French LA. Dermal exposure to jet fuel (JP-8) in US Air Force personnel. *Ann Occup Hyg*. 2005;49:639–645.

Chapin RE, Dutton SD, Ross MD, et al. The effects of ethylene glycol monomethyl ether on testicular histology in F344 rats. *J Androl*. 1984;5:369–380.

Charbonneau M, Swenberg JA. Studies on the biochemical mechanism of α_u-globulin nephropathy in rats. *CIIT Activities*. 1988;8:1–5.

Charbotel B, Fevotte J, Hours M, et al. Case–control study on renal cell cancer and occupational exposure to trichloroethylene. Part II: Epidemiological aspects. *Ann Occup Hyg*. 2006;50:777–787.

Charbotel B, Gad S, Caiola D, et al. Trichloroethylene exposure and somatic mutations of the VHL gene in patients with renal cell carcinoma. *J Occup Med Toxicol*. 2007;2:13–20.

Chasnoff IJ, Wells AM, King L. Misdiagnosis and missed diagnoses in foster and adopted children with prenatal alcohol exposure. *Pediatrics*. 2015;135:264–270.

Chen DH, Kaung HL, Miller CM, et al. Microarray analysis of changes in renal phenotype in the ethylene glycol rat model of urolithiasis: Potential and pitfalls. *BJU Int*. 2004;94:637–650.

Chen PC, Hsieh GY, Wang JD, Cheng TJ. Prolonged time to pregnancy in female workers exposed to ethylene glycol ethers in semiconductor manufacturing. *Epidemiology*. 2002;13:191–196.

Cheng P-Y, Morgan ET. Hepatic cytochrome P450 regulation in disease states. *Curr Drug Metab*. 2001;2:165–183.

Cherry NH, Hutchins H, Pace T, Waldron JA. Neurobehavioral effects of repeated occupational exposure to toluene and paint solvents. *Br J Ind Med*. 1985;42:291–300.

Cherry N, Moore H, McNamee R, et al. Occupation and male fertility: Glycol ethers and other exposures. *Occup Environ Med*. 2008;65:708–714.

Chiu WA, Micallef S, Monster AC, Bois FY. Toxicokinetics of inhaled trichloroethylene and tetrachloroethylene in humans at 1 ppm: empirical results and comparisons with previous studies. *Toxicol Sci*. 2007;95:23–26.

Cho SK, Kim RH, Yim SH, et al. Long-term neuropsychological effects and MRI findings in patients with CS_2 poisoning. *Acta Neurol Scand*. 2002;106:269–275.

Chou CC, Riviere JE, Monteiro-Riviere NA. Differential relationship between the carbon chain length of jet fuel aliphatic hydrocarbons and their ability to induce cytotoxicity vs. interleukin-8 release in human epidermal keratinocytes. *Toxicol Sci*. 2002;69:226–233.

Chou CC, Riviere JE, Monteiro-Riviere NA. The cytotoxicity of jet fuel aromatic hydrocarbons and dose-related interleukin-8 release from human epidermal keratinocytes. *Arch Toxicol*. 2003;77:384–391.

Chou CC, Yang JH, Chen SD, et al. Expression profiling of human epidermal keratinocyte response following 1-minute JP-8 exposure. *Cutan Ocul Toxicol*. 2006;25:141–153.

Chow W-H, Devesa SS. Contemporary epidemiology of renal cell cancer. *Cancer J*. 2008;14: 288–301.

Christopher MM, Eckfeldt JH, Eaton JW. Propylene glycol ingestion causes *D*-lactic acidosis. *Lab Invest*. 1990;62:114–118.

Chrostek L, Jelski W, Szmitkowski M, Puchalski Z. Gender-related differences in hepatic activity of alcohol dehydrogenase isoenzymes and aldehyde dehydrogenase in humans. *J Clin Lab Anal*. 2003;17:93–96.

Chu I, Poon R, Valli V, et al. Effects of an ethanol-gasoline mixture: Results of a 4-week inhalation study in rats. *J Appl Toxicol*. 2005;25:193–199.

Chun JS, Burleigh-Flayer HD, Kintigh WJ. *Methyl Tertiary Butyl Ether: Vapor Inhalation Oncogenicity Study in Fisher 344 Rats*. Export, PA: Bushy Run Research Center; 1992.

Chung HC, Sung SH, Kim JS, et al. Lack of cytochrome P450 2E1 (CYP2E1) induction in the rat liver by starvation without coprophagy. *Drug Metab Dispos*. 2001;29: 213–216.

Chung J-K, Yuan W, Liu G, Zheng J. Investigation of bioactivation and toxicity of styrene in CYP2E1 transgenic cells. *Toxicology*. 2006;226: 99–106.

Churchill JE, Ashley DL, Kaye WE. Recent chemical exposures and blood volatile organic compound levels in a large population-based sample. *Arch Environ Health*. 2001;56:157–166.

Cichocki JA, Guyton KZ, Guha N, et al. Target organ metabolism, toxicity, and mechanism of trichloroethylene and perchloroethylene: Key similarities, differences, and data gaps. *J Pharmacol Exp Therap*. 2016;359:110–123.

Cirvello JD, Radovsky A, Heath JE, et al. Toxicity and carcinogenicity of *t*-butyl alcohol in rats and mice following chronic exposure in drinking water. *Toxicol Ind Health*. 1995;11:151–165.

Clark CR, Schreiner CA, Parker CM, et al. Health assessment of gasoline and fuel oxygenate vapors: Subchronic inhalation toxicity. *Regul Toxicol Pharmacol*. 2014;70:S18–S28.

Clarke DO, Duignan JM, Welsch F. 2-Methoxyacetic acid dosimetryteratogenicity relationships in CD-1 mice exposed to 2-methoxyethanol. *Toxicol Appl Pharmacol*. 1992;114:77–87.

Clary JJ. Methanol, is it a developmental risk to humans? *Reg Toxicol Pharmacol*. 2003;37:83–91.

Clawson GA, MacDonald J, Woo C. Early hypomethylation of 2-0-ribose moieties in hepatocytes cytoplasmic ribosomal RNA underlies the protein synthesis defect produced by CCl_4. *J Cell Biol*. 1987;105:705–711.

Clayton CA, Pellizzari ED, Whitmore RW, et al. National Human Exposure Assessment Survey (NHEXAS): distributions and associations of lead, arsenic and volatile organic compounds in EPA Region 5. *J Expos Anal Environ Epidemiol*. 1999;9:381–392.

Clewell HJ, Andersen ME. Applying mode-of-action and pharmacokinetic considerations in contemporary cancer risk assessments: An example with trichloroethylene. *Crit Rev Toxicol*. 2004a;34:385–445.

Clewell HJ, Andersen ME. Response to comments by Caldwell et al. on article "Applying mode-of-action and pharmacokinetic considerations in contemporary cancer risk assessments: an example with trichloroethylene." *Crit Rev Toxicol*. 2006;36:295–298.

Clewell HJ, Gentry PR, Covington TR, et al. Evaluation of the potential impact of age- and gender-specific pharmacokinetic differences on tissue dosimetry. *Toxicol Sci*. 2004b;79:381–393.

Clewell HJ, Tan YM, Campbell JL, Andersen ME. Quantitative interpretation of human biomonitoring data. *Toxicol Appl Pharmacol*. 2008;231:122–133.

Clewell HJ, Teeguarden J, McDonald T, et al. Review and evaluation of the potential impact of age- and gender-specific pharmacokinetic differences on tissue dosimetry. *Crit Rev Toxicol*. 2002;32(5):329–389.

Cogliano VJ, Grosse Y, Baan RA, et al. Meeting report: Summary of IARC monographs on formaldehyde, 2-butoxyethanol, and 1-tert-butoxy-2-propanol. *Environ Health Perspect*. 2005;113:1205–1208.

Colantoni A, Idilman R, De Maria N, et al. Hepatic apoptosis and proliferation in male and female rats fed alcohol: role of cytokines. *Alcohol Clin Exp Res*. 2003;27:1184–1189.

Colborn T. A case for revisiting the safety of pesticides: a closer look at neurodevelopment. *Environ Health Perspect*. 2006;114:10–17.

Cole CE, Tran HT, Schlosser PM. Physiologically based pharmacokinetic modeling of benzene metabolism in mice through extrapolation from in vitro to in vivo. *J Toxicol Environ Health A*. 2001;62:439–465.

Collins JJ, Ireland BK, Easterday PA, et al. Evaluation of lymphopenia among workers with low-level benzene exposure and the utility of routine data collection. *J Occup Environ Med*. 1997;39:232–237.

Conolly R. The use of biologically based modeling in risk assessment. *Toxicology*. 2002;181-182:275–279.

Conrad T, Landry GM, Aw TY, et al. Diglycolic acid, the toxic metabolite of diethylene glycol, chelates calcium and produces renal mitochondrial dysfunction *in vitro*. *Clin Toxicol*. 2016;54:501–511.

Constan AA, Sprankle CS, Peters JM, et al. Metabolism of chloroform by cytochrome P4502E1 is required for induction of toxicity in the liver, kidney, and nose of male mice. *Toxicol Appl Pharmacol*. 1999;160: 120–126.

Constan AA, Wong BA, Everitt JI, Butterworth BE. Chloroform inhalation exposure conditions necessary to initiate liver toxicity in female B6C3F1 mice. *Toxicol Sci*. 2002;66:201–208.

Cooper GS, Markris SL, Nietert PG, Jinot J. Evidence of autoimmune-related effects of trichloroethylene exposure from studies in mice and humans. *Environ Health Perspect*. 2009;117:696–702.

Cordier S, Bergeret A, Goujard J, et al. Congenital malformations and maternal occupational exposure to glycol ethers. *Epidemiology*. 1997;8:355–363.

Coresch J, Selvin E, Stevens LA, et al. Prevalence of chronic kidney disease in the United States. *JAMA*. 2007;398: 2038–2047.

Corley RA, Bartels MJ, Carney EW, et al. Development of a physiologically based pharmacokinetic model for ethylene glycol and its metabolite, glycolic acid, in rats and humans. *Toxicol Sci*. 2005a;85:476–490.

Corley RA, Bormett GA, Ghanayem BI. Physiologically based pharmacokinetics of 2-butoxyethanol and its major metabolite, 2-butoxyacetic acid, in rats and humans. *Toxicol Appl Pharmacol*. 1994;129:61–79.

Corley RA, Gies RA, Wu H, Weitz KK. Development of a physiologically based pharmacokinetic model for propylene glycol monomethyl ether and its acetate in rats and humans. *Toxicol Lett*. 2005b;156:193–213.

Corley RA, Grant DM, Farris E, et al. Determination of age and gender differences in biochemical processes affecting the disposition of 2-butoxyethanol and its metabolites in mice and rats to improve PBPK modeling. *Toxicol Lett*. 2005c;156:127–161.

Corley RA, Mast TJ, Carney EW, et al. Evaluation of physiologically based models of pregnancy and lactation for their application in children's health risk assessments. *Crit Rev Toxicol*. 2003;33:137–211.

Corley RA, McMartin KE. Incorporation of therapeutic interventions in physiologically based pharmacokinetic modeling of human clinical case reports of accidental or intentional overdosing with ethylene glycol. *Toxicol Sci*. 2005;85:491–501.

Corley RA, Meek ME, Carney EW. Mode of action: Oxalate crystal-induced renal tubule degeneration and glycolic acid-induced dysmorphogenesis—renal and developmental effects of ethylene glycol. *Crit Rev Toxicol*. 2005d;35:691–702.

Corley RA, Saghir SA, Bartels MJ, et al. Extension of PBPK model for ethylene glycol and glycolic acid to include the competitive formation and clearance of metabolites associated with kidney toxicity in rats and humans. *Toxicol Appl Pharmacol*. 2011;250:229–244.

Corley RA, Wilson DM, Hard GC, et al. Dosimetry considerations in the enhanced sensitivity of male Wistar rats to chronic ethylene glycol-induced nephrotoxicity. *Toxicol Appl Pharmacol*. 2008;228:165–178.

Correa A, Gray R, Cohen R, et al. Ethylene glycol ethers and risks of spontaneous abortion and subfertility. *Am J Epidemiol*. 1996;143:707–717.

Corthals SM, Kamendulis LM, Klaunig JE. Mechanism of 2-butoxyethanol-induced hemangiosarcomas. *Toxicol Sci*. 2006;92:378–386.

Cosnier F, Nunge H, Brochard C, et al. Impact of coexposure on toluene biomarkers in rats. *Xenobiotica*. 2014;44:217–228.

Costa ET, Savage DD, Valenzuela CF. A review of the effects of prenatal or early postnatal ethanol exposure on brain ligand-gated ion channels. *Alcohol Clin Exp Res*. 2000;24:706–715.

Cotreau MM, von Moltke LL, Greenblatt DJ. The influence of age and sex on the clearance of cytochrome P450 3A substrates. *Clin Pharmacokinet*. 2005;44:33–60.

Court MH. Feline drug metabolism and disposition: Pharmacokinetic evidence for species differences and molecular mechanisms. *Vet Clin North Am Small Anim Pract*. 2013;43:1039–1054.

Covington TR, Gentry PR, Van Landingham CB, et al. The use of Markov chain Monte Carlo uncertainty analysis to support a public health goal for perchloroethylene. *Regul Toxicol Pharmacol*. 2007;47:1–18.

Crabb DW, Matsumoto M, Chang D, You M. Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. *Proc Nutr Soc*. 2004;63:49–63.

Cranmer JM. Proceedings of the workshop on neurobehavioral effects of solvents. *Neurotoxicology*. 1986;7:1–95.

Creasy DM, Foster PMD. The morphological development of glycol ether-induced testicular atrophy in the rat. *Exp Mol Pathol*. 1984;40:169–176.

Creek MR, Mani C, Vogel JS, Turteltaub KW. Tissue distribution and macro-molecular binding of extremely low doses of [^{14}C]-benzene in B6C3F1 mice. *Carcinogenesis*. 1997;18:2421–2427.

Creton S, Billington R, Davies W, et al. Application of pharmacokinetics to improve chemical risk assessment: Implications for the use of animals. *Regul Toxicol Pharmacol*. 2009;55:291–299.

Cruz SL, Balster RL, Woodward JJ. Effects of volatile solvents on recombinant *N* -methyl-d-aspartate receptors expressed in *Xenopus* oocytes. *Br J Pharmacol*. 2000;131:1303–1308.

Cruz SL, Mirshahi T, Thomas B, et al. Effects of the abused solvent toluene on recombinant *N* -methyl-d-aspartate and non-*N* -methyl-d-aspartate receptors expressed in Xenopus oocytes. *J Pharmacol Exp Therap*. 1998;286:334–340.

Cruzan G. Assessment of the cancer potential of methanol. *Crit Rev Toxicol*. 2009;39:347–363.

Cruzan G, Borghoff SJ, de Peyster A, et al. Methyl *tertiary*-butyl ether mode of action for cancer endpoints in rodents. *Regul Toxicol Pharmacol*. 2007;47:156–165.

Cruzan G, Bus JS, Banton MI, et al. Complete attenuation of mouse lung cell proliferation and tumorigenicity in CYP2F2 knockout and CYP2F1 humanized mice exposed to inhaled styrene for up to 2 years supports a lack of human relevance. *Toxicol Sci*. 2017;159:413–421.

Cruzan G, Cushman JR, Andrews LS, et al. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. *Toxicol Sci*. 1998;46:266–281.

Cruzan G, Cushman JR, Andrews LS, et al. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. *J Appl Toxicol*. 2001;21:185–198.

Cunningham CA, Ku K, Sue GR. Propylene glycol poisoning from excess whiskey ingestion: a case of high osmolal gap metabolic acidosis. *J Investig Med High Impact Case Rep*. 2015;3:1–2.

Dai Y, Rashba-Step J, Cederbaum AI. Stable expression of human cytochrome P4502E1 in HepG2 cells: characterization of catalytic activities and production of reactive oxygen intermediates. *Biochemistry*. 1993;32:6928–6937.

d'Alessandro A, Osterloh JD, Chuwers P, et al. Formate in serum and urine after controlled methanol exposure at the threshold limit value. *Environ Health Perspect*. 1994;102:178–181.

Dallas CE, Ramanathan R, Muralidhara S, et al. The uptake and elimination of 1,1,1-trichloroethane during and following inhalation exposures in rats. *Toxicol Appl Pharmacol*. 1989;98: 385–397.

Dankovic DA, Bailer AJ. The impact of exercise and intersubject variability on dose estimates for dichloromethane derived from a physiologically based pharmacokinetic model. *Fundam Appl Toxicol*. 1994;22:20–25.

Daston G, Faustman E, Ginsberg G, et al. A framework for assessing risks to children from exposure to environmental agents. *Environ Health Perspect*. 2004;112:238–256.

Daughtrey WC, Gill MW, Pritts IM, et al. Neurotoxicological evaluation of methyl tertiary-butyl ether in rats. *J Appl Toxicol*. 1997;17(S1):S57–S64.

David RM, Clewell HJ, Gentry PR, et al. Revised assessment of cancer risk to dichloromethane. II. Application of probabilistic methods to cancer risk determinations. *Regul Toxicol Pharmacol*. 2006;45:55–65.

Davis BJ, Almekinder JL, Flagler N, et al. Ovarian luteal cell toxicity of ethylene glycol monomethyl ether and methoxy acetic acid in vivo and in vitro. *Toxicol Appl Pharmacol*. 1997;142:328–337.

Davis ME. Dichloroacetic acid and trichloroacetic acid increase chloroform toxicity. *J Toxicol Environ Health*. 1992;37:139–148.

Dayan C, Hales BF. Effects of ethylene glycol monomethyl ether and its metabolite, 2-methoxyacetic acid, on onganogenesis stage mouse limbs *in vitro*. *Birth Defects Res Part B*. 2014;101:254–261.

de Ketttenis P. The historic and current use of glycol ethers: a picture of change. *Toxicol Lett*. 2005;156:5–11.

de Peyster A, MacLean KJ, Stephens BA, et al. Subchronic studies in Sprague–Dawley rats to investigate mechanisms of MTBE-induced Leydig cell cancer. *Toxicol Sci*. 2003;72:31–42.

De Salvia MA, Cagiano R, Carratu MR, et al. Irreversible impairment of active avoidance behavior in rats prenatally exposed to mild concentrations of carbon monoxide. *Psychopharmacology*. 1995;122:66–71.

Degitz SJ, Rogers JM, Zucker RM, Hunter ES III. Developmental toxicity of methanol: pathogenesis in CD-1 and C57BL/6J mice exposed in whole embryo culture. *Birth Defects Res A Clin Mol Teratol*. 2004;70:179–184.

Dekant W. The role of biotransformation and bioactivation in toxicity. In: Luch A, ed. *Molecular Clinical and Environmental Toxicology*. Vol 1. Switzerland: Birkhauser Verlag; 2009:57–86.

Dekant W, Bernauer U, Rosner E, Amberg A. Biotransformation of MTBE, ETBE, and TAME after inhalation or ingestion in rats and humans. *Health Effects Inst Res Report*. 2001;102:29–71.

Dekant W, Metzler M, Henschler D. Identification of S-(1,2,2-trichlorovinyl)-N-acetylcysteine as a urinary metabolite of tetrachloroethylene: Bioactivation through glutathione conjugation as a possible explanation for its nephrocarcinogenicity. *J Biochem Toxicol*. 1986;1:57–71.

Dell LD, Mundt KA, McDonald M, et al. Critical review of the epidemiology literature on the potential cancer risks of methylene chloride. *Int Arch Occup Environ Health*. 1999;72:429–442.

Dennison JE, Bigelow PL, Mumtaz MM, et al. Evaluation of potential toxicity from co-exposure to three CNS depressants (toluene, ethylbenzene, and xylene) under resting and working conditions using PBPK modeling. *J Occup Environ Hyg*. 2005;2:127–135.

DePass LR, Garman RH, Woodside MD, et al. Chronic toxicity and oncogenicity studies of ethylene glycol in rats and mice. *Fund Appl Toxicol*. 1986;7:547–565.

de Peyster A, Mihaich E, Kim DH, et al. Responses of the steroidogenic pathway from exposure to methyl-tert-butyl ether and tert-butanol. *Toxicology*. 2014;319:23–37.

Devi SS, Mehendale HM. Disrupted G1 to S phase clearance via cyclin signaling impairs liver tissue repair in thioacetamide-treated type I diabetic rats. *Toxicol Appl Pharmacol*. 2005;207:89–102.

Devi SS, Mehendale HM. Microarray analysis of thioacetamide-treated type I diabetic rats. *Toxicol Appl Pharmacol*. 2006;212:69–78.

DiVincenzo GD, Kaplan CJ. Effect of exercise or smoking on the uptake, metabolism and excretion of methylene chloride vapor. *Toxicol Appl Pharmacol*. 1981;59:141–148.

Diaz G, Castro JA. Covalent binding of chloroform metabolites to nuclear proteins—no evidence for binding to nucleic acids. *Cancer Lett*. 1980;9:213–218.

DiCecco SR, Francisco-Ziller N. Nutrition in alcoholic liver disease. *Nutr Clin Pract*. 2006;21:245–254.

Diehl AM. Cytokine regulation of liver injury and repair. *Immunol Rev*. 2000;174:160–171.

Dietrich DR, Swenberg JA. NCI–Black–Reiter (NBR) male rats fail to develop renal disease following exposure to agents that induce α-2u-globulin (α_{2u}) nephropathy. *Fundam Appl Toxicol*. 1991;16:749–762.

Dietzel KD, Campbell JL, Bartlett MG, et al. Validation of a gas chromatography/mass spectrometry method for quantification of aerosolized Jet Propellant 8. *J Chromatogr A*. 2005;1093:11–20.

Dill J, Fuciarelli A, Lee K, et al. Toxicokinetics of propylene glycol mono-*t*-butyl ether following intravenous or inhalation exposure in rats and mice. *Inhal Toxicol*. 2004;16:271–290.

Ding X, Kaminsky LS. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol*. 2003;43:149–173.

DiVincenzo GD, Kaplan CJ. Uptake, metabolism, and elimination of methylene chloride vapor by humans. *Toxicol Appl Pharmacol*. 1981;59:130–140.

Dixit R, Riviere J, Krishnan K, Andersen ME. Toxicokinetics and physiologically based toxicokinetics in toxicology and risk assessment. *J Toxicol Environ Health B*. 2003;6:1–40.

Dobrev ID, Andersen ME, Yang RS. In silico toxicology: stimulating interaction thresholds for human exposure to mixtures of trichloroethylene, tetrachloroethylene, and 1,1,1-trichloroethane. *Environ Health Perspect*. 2002;110:1031–1039.

Dobrev ID, Andersen ME, Yang RSH. Assessing interaction thresholds for trichloroethylene in combination with tetrachloroethylene and 1,1,1-trichloroethane using gas uptake studies and PBPK modeling. *Arch Toxicol*. 2001;75:134–144.

Dodd D, Willson G, Parkinson H, Bermudez E. Two-year drinking water carcinogenicity study of methyl tertiary-butyl ether (MTBE) in Wistar rats. *J Appl Toxicol*. 2013;33:593–606.

Dodo T, Taketa Y, Sugiyama M, et al. Collaborative work on evaluation of ovarian toxicity. 11) Two- or four-week repeated-dose studies and fertility study of ethylene glycol monomethyl ether in female rats. *J Toxicol Sci*. 2009;34(Special Issue I):SP121–SP128.

Doherty RE. A history of the production and use of carbon tetrachloride, tetrachloroethylene, trichloroethylene and 1,1,1-trichloroethane in the United States: Part 2-Trichloroethylene and 1,1,1-trichloroethane. *J Environ Forensics*. 2000;1:83–93.

Doi AM, Hill G, Seely J, et al. α2μ-Globulin in nephropathy and renal tumors in National Toxicology Program studies. *Toxicol Pathol*. 2007;35:533–540.

Doi AM, Roycroft JH, Herbert RA, et al. Inhalation toxicology and carcinogenesis studies of propylene glycol mono-t-butyl ether in rats and mice. *Toxicology*. 2004;199:1–22.

Domergue J, Lison D, Haufroid V. No evidence of cardiovascular toxicity in workers exposed below 5 ppm carbon disulfide. *Int Arch Occup Environ Health*. 2016;89:835–845.

Dorman DC, Andersen ME, Roper JM, Taylor MD. Update on a pharmacokinetic-centric alternative tier II program for MMT—Part I: Program implementation and lessons learned. *J Toxicol*. 2012;2012:1–10.

Dorne JLCM. Impact of inter-individual differences in drug metabolism and pharmacokinetics on safety evaluation. *Fund Clin Pharmacol*. 2004;18:609–620.

Drake MG, Witzmann FA, Hyde J, Witten ML. JP-8 jet fuel exposure alters protein expression in the lung. *Toxicology*. 2003;191:199–210.

Dresser GK, Bailey DG. The effects of fruit juices on drug disposition: a new model for drug interactions. *Eur J Clin Invest*. 2003;33(suppl 2):10–16.

Drew PD, Kane CJ. Fetal alcohol spectrum disorders and neuroimmune changes. *Int Rev Neurobiol*. 2014;118:41–80.

Drexler H, Ulm K, Hardt R, et al. Carbon disulphide: IV. Cardiovascular function in workers in the viscose industry. *Int Arch Occup Environ Health*. 1996;69:27–32.

Echeverria D, White RF, Sampaio C. A behavioral evaluation of PCE exposure in patients and dry cleaners: a possible relationship between clinical and preclinical effects. *J Occup Environ Med*. 1995;37:667–680.

Edwards MJ, Keller BJ, Kauffman FC, Thurman RG. The involvement of Kupffer cells in carbon tetrachloride toxicity. *Toxicol Appl Pharmacol*. 1993;119:275–279.

Eells JT, Henry MM, Lewandowski MF, et al. Development and characterization of a rodent model of methanol-induced retinal and optic nerve toxicity. *Neurotoxicology*. 2000;21:321–330.

Eells JT, Henry MM, Summerfelt P. Therapeutic photobiomodulation for methanol-induced retinal toxicity. *Proc Natl Acad Sci*. 2003;100:3439–3444.

Eells JT, Salzman MM, Lewandowski MR, Murray TG. Formate-induced alterations in retinal function in methanol-intoxicated rats. *Toxicol Appl Pharmacol*. 1996;140:58–69.

Egbert PA, Abraham K. Ethylene glycol intoxication: Pathophysiology, diagnosis, and emergency management. *ANNA J*. 1999;26:295–302.

Elfarra AA, Krause RJ. S-(1,2,2-trichlorovinyl)-L-cysteine sulfoxide, a reactive metabolite of S-(1,2,2-trichlorovinyl)-L-cysteine formed in rat liver and kidney microsomes, is a potent nephrotoxicant. *J Pharmacol Exp Therap*. 2007;321:1095–1101.

Elliot BM, Ashby J. Review of the genotoxicity of 2-butoxyethanol. *Mutat Res*. 1997;387:89–96.

Elliott L, Longnecker MP, Kissling GE, London SJ. Volatile organic compounds and pulmonary function in the Third National Examination Survey. *Environ Health Perspect*. 2006;114:1210–1214.

El-Masri HA, Bell DA, Portier CJ. Effects of glutathione transferase theta polymorphism on the risk estimates of dichloromethane to humans. *Toxicol Appl Pharmacol*. 1999;158:221–230.

El-Masri HA, Tessari JD, Yang RS. Extrapolation of an interaction threshold for the joint toxicity of trichloroethylene and 1,1-dichloroethylene: Utilization of a PBPK model. *Arch Toxicol*. 1996;70:527–539.

El-Zein R, Abdel-Rahman SZ, Morris DL, Legator MS. Exposure to ethylene glycol monomethyl ether: Clinical and cytogenetic findings. *Arch Environ Health*. 2002;57:371–376.

El-Zein R, Albrecht T, Knutson E, et al. Reduction in telomere length in individuals exposed in utero to glycol ether. *Arch Environ Occup Health*. 2007;62:161–163.

Engelke M, Tahti H, Vaalavirta L. Perturbation of artificial and biological membranes by organic compounds of aliphatic, alicyclic and aromatic structure. *Toxicol In Vitro*. 1996;10:111–115.

EPA (U.S. Environmental Protection Agency). *Drinking Water Advisory: Consumer Acceptability Advice and Health Effects Analysis on Methyl Tertiary-Butyl Ether (MtBE)*. Washington, DC: Office of Water; December 1997.

EPA (U.S. Environmental Protection Agency). *Risk Assessment Guidance for Superfund. Human Health Evaluation Manual Supplemental Guidance*, Dermal Risk Assessment, Interim Guidance, Vol I. Washington, DC: Office of Emergency and Remedial Response; 1998a.

EPA (Environmental Protection Agency). *On-line Integrated Risk Information System (IRIS) Substance File—1,1,1-Trichloroethane*. Washington, DC, 1998b. Available at: http://www.epa.gov/iris/subst/0020.htm.

EPA (U.S. Environmental Protection Agency). *Trichloroethylene Health Risk Assessment: Synthesis and Characterization*. External Review Draft. EPA/600/P-01/002A. National Center for Environmental Assessment, Washington, DC, 2001a.

EPA (U.S. Environmental Protection Agency). *On-line Integrated Risk Information System (Iris) Substance File—Chloroform*. Washington, DC, 2001b. Available at: http://www.epa.gov/iris/subst/0025.htm.

EPA (U.S. Environmental Protection Agency). *National Primary Drinking Water Regulations*. EPA/816/F-02/013, Office of Ground Water and Drinking Water, Washington, DC, 2002. Available at: http://www.epa.gov/safewater/mcl.html.

EPA (U.S. Environmental Protection Agency). *Guidelines for Carcinogen Risk Assessment*. Washington, DC, Risk Assessment Forum, EPA/630/P-03/001F, 2005.

EPA (U.S. Envioronmental Protection Agency). *EPA's Toxics Release Inventory*, 2006a. Available at: http://semanticommunity.info/EPA/Web.

EPA (U.S. Environmental Protection Agency). *On-line Integrated Risk Information System (Iris) Substance File—Ethylene Glycol*. Washington, DC, July 30, 2006b. Available at: http://www.epa.gov/iris/subst/0238.htm.

EPA (U.S. Environmental Protection Agency). On-line Integrated Risk Information System (Iris) Substance File—Ethylene Glycol mMonobutyl Ether (EGBE) (2-Butoxyethanol). Washington, DC, August 1, 2006c. Available at: http://www.epa.gov/iris/subst/0500.htm.

EPA (U.S. Environmental Protection Agency). On-line Integrated Risk Information System (Iris) Substance File—Carbon Disulfide. Washington, DC, July 17, 2006d. Available at: http://www.epa.gov/iris/subst/0217.htm.

EPA (U.S. Environmental Protection Agency). On-line Integrated Risk Information System (Iris) Substance File—Benzene. Washington, DC, 2007. Available at: http://www.epa.gov/iris/subst/index.html.

EPA (U.S. Environmental Protection Agency). *Toxicological Review of Tetrachloroethylene (Perchloroethylene). In Support of IRIS*. External Review Draft, EPA/635/R-08/011A, Washington, DC, 2008.

EPA (U.S. Environmental Protection Agency). *Toxicological Review of Dichloromethane (Methylene Chloride). In Support of IRIS*. External Review Draft, EPA/635/R-10/003A, Washington, DC, 2010a.

EPA (U.S.Environmental Protection Agency). On-line Integrated Risk Information System (Iris) Substance File—Carbon Tetrachloride. Washington, DC, 2010b. Available at: http://www.epa.gov/iris/subst/0020.htm.

EPA (U.S. Environmental Protection Agency). *Toxicological Review of Trichloroethylene. In Support of IRIS*. External Review Draft. EPA/635/R-09/011F, Washington, DC, 2011.

EPA (U.S. Environmental Protection Agency). TRI explorer (2012 Data update released March 2014), 2015.

Erdal S, Gong H Jr, Linn WS, Rykowski R. Projection of health benefits from ambient ozone reduction related to the use of methyl tertiary butyl ether (MTBE) in the reformulated gasoline program. *Risk Anal*. 1997;17:693–704.

Eschenbrenner AB, Miller E. Induction of hepatomas in mice by repeated oral administration of chloroform, with observations on sex differences. *J Natl Cancer Inst*. 1945;5:251–255.

Espinoza LA, Li P, Lee RY, et al. Evaluation of gene expression profile of keratinocytes in response to JP-8 jet fuel. *Toxicol Appl Pharmacol*. 2004;200:93–102.

Espinoza LA, Valikhani M, Cossio MJ, et al. Altered expression of γ-Synuclein and detoxification-related genes in lungs of rats exposed to JP-8. *Am J Resp Cell Mol*. 2005;32:192–200.

Exon JH, Mather GG, Bussiere JL. Effects of subchronic exposure of rats to 2-methoxyethanol or 2-butoxyethanol: Thymic atrophy and immunotoxicity. *Fundam Appl Toxicol*. 1991;16:830–840.

Fabrizi L, Taylor GW, Canas B, et al. Adduction of the chloroform metabolite phosgene to lysine residues of human histone H2B. *Chem Res Toxicol*. 2003;16:266–275.

Fang C, Behr M, Xie F, et al. Mechanism of chloroform-induced renal toxicity: non-involvement of hepatic cytochrome P450-dependent metabolism. *Toxicol Appl Pharmacol*. 2008;227:48–55.

Farsalinos KE, Baeyens F. Harmful effects from one puff of shisha-pen vapor: methodological and interpretational problems in the risk assessment analysis. *Tob Induc Dis*. 2016;14:22.

Fechter LD, Fisher JW, Chapman GD, et al. Subchronic JP-8 jet fuel exposure enhances vulnerability to noise-induced hearing loss in rats. *J Toxicol Environ Health Part A*. 2012;75:299–317.

Fechter LD, Gearhart C, Fulton S. Ototoxic potential of JP-8 and a Fischer-Tropsch synthetic jet fuel following subacute inhalation exposure in rats. *Toxicol Sci*. 2010;116:239–248.

Fechter LD, Gearhart C, Fulton S, et al. JP-8 fuel can promote auditory impairment resulting from subsequent noise exposure in rats. *Toxicol Sci*. 2007;98:510–524.

Filley CM, Halliday W, Kleinschmidt-DeMasters BK. The effects of toluene on the central nervous system. *J Neuropathol Exp Neurol*. 2004; 63:1–12.

Fisher J, Lumpkin M, Boyd J, et al. PBPK modeling of the interaction between carbon tetrachloride and tetrachloroethylene in mice. *Environ Toxicol Pharmacol*. 2004;16:93–105.

Fisher JW, Channel SR, Eggers JS, et al. Trichloroethylene, trichloroacetic acid, and dichloroacetic acid: do they affect fetal rat heart development? *Int J Toxicol*. 2001;20:257–267.

Fisher JW, Mahle D, Abbas R. A human physiologically based pharmacokinetic model for trichloroethylene and its metabolites, trichloroacetic acid and free trichloroethanol. *Toxicol Appl Pharmacol*. 1998;152:339–359.

Fitzhugh OG, Nelson AA. Comparison of the chronic toxicity of triethylene glycol with that of diethylene glycol. *J Ind Hyg Toxicol*. 1946;28:40–43.

Fiume MM, Bergfeld WF, Belsito DV, et al. Safety assessment of propylene glycol, tripropylene glycol, and PPGs as used in cosmetics. *Int J Toxicol*. 2012;31(suppl 2):245S–260S.

Fletcher CV, Acosta CP, Strykowski JM. Gender differences in human pharmacokinetics and pharmacodynamics. *J Adoles Health*. 1994;15:619–629.

Folland DS, Schaffner W, Ginn EH, et al. Carbon tetrachloride toxicity potentiated by isopropyl alcohol. *JAMA*. 1976;236:1853–1856.

Foo SC, Jeyaratnam J, Koh D. Chronic neurobehavioral effects of toluene. *Br J Ind Med*. 1990;47:480–484.

Foote RH, Farrell PB, Schlafer DH, et al. Ethylene glycol monomethyl ether effects on health and reproduction in male rabbits. *Reprod Toxicol*. 1995;9:527–539.

Forkert P-G, Baldwin RM, Millen B, et al. Pulmonary bioactivation of trichloroethylene to chloral hydrate: relative contributions of CYP2E1, CYP2F, and CYP2B1. *Drug Metab Dispos*. 2005;33:1429–1437.

Forkert P-G, Lash L, Tardif R, et al. Identification of trichloroethylene and its metabolites in human seminal fluid of workers exposed to trichloroethylene. *Drug Metab Dispos*. 2003;31:306–311.

Forkert P-G, Millen B, Lash LH, et al. Pulmonary bronchiolar cytotoxicity and formation of dichloroacetyl lysine protein adducts in mice treated with trichloroethylene. *J Pharmacol Exp Ther*. 2006;316:520–529.

Forrest MS, Lan Q, Hubbard AE, et al. Discovery of novel biomarkers by microarray analysis of peripheral blood mononuclear cell gene expression in benzene-exposed workers. *Environ Health Perspec*. 2005;113: 801–807.

Fortenberry JD. Gasoline sniffing. *Am J Med*. 1985;79:740–744.

Fowles JR, Banton MI, Pottenger LH. A toxicological review of the propylene glycols. *Crit Rev Toxicol*. 2013;43:363–390.

Foxenberg RJ, Ellison CA, Knaak JB, et al. Cytochrome P450-specific human PBPK/PD models for the organophosphorus pesticides: Chlorpyrifos and parathion. *Toxicology*. 2011;285:57–66.

Franchini I, Cavtorta A, Falzoi M, et al. Early indicators of renal damage in workers exposed to organic solvents. *Int Arch Occup Environ Health*. 1983;52:1–9.

Franks SJ, Spendiff MK, Cocker J, Loizou GD. Physiologically based pharmacokinetic modeling of human exposure to 2-butoxyethanol. *Toxicol Lett*. 2006;162:164–173.

Frantz SW, Beskitt JL, Grosse CM, et al. Pharmacokinetics of ethylene glycol. I. Plasma disposition after single intravenous, peroral, or percutaneous doses in female Sprague–Dawley rats and CD-1 mice. *Drug Metab Dispos*. 1996a;24:911–921.

Frantz SW, Beskitt JL, Grosse CM, et al. Pharmacokinetics of ethylene glycol II. Tissue distribution, dose-dependent elimination, and identification of urinary metabolites following single intravenous, peroral or percutaneous doses in female Sprague–Dawley rats and CD-1 mice. *Xenobiotica*. 1996b;26:1195–1220.

Frantz SW, Beskitt JL, Tallant MJ, et al. Pharmacokinetics of ethylene glycol. III. Plasma disposition and metabolic fate after single increasing intravenous, peroral, or percutaneous doses in the male Sprague–Dawley rat. *Xenobiotica*. 1996c;26:515–539.

Fraser MP, Cass GR, Simoneit BRT. Gas-phase and particle-phase organic compounds emitted from motor vehicle traffic in a Los Angeles roadway tunnel. *Environ Sci Technol*. 1998;32:2051–2060.

French SW. Epigenetic events in liver cancer resulting from alcoholic liver disease. *Alcohol Res*. 2013;35:57–67.

Frezza M, di Padova C, Pozzato G, et al. High blood alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. *N Engl J Med*. 1990;322:95–99.

Froberg K, Dorion RP, McMartin KE. The role of calcium oxalate crystal deposition in cerebral vessels during ethylene glycol poisoning. *Clin Toxicol*. 2006;44:315–318.

Fukushima T, Taki K, Ise R, et al. MicroRNAs expression in the ethylene glycol monomethyl ether-induced testicular lesion. *J Toxicol Sci*. 2011;36:601–611.

Gade A, Mortensen EL, Bruhn P. "Chronic painter's syndrome." A reanalysis of psychological test data in a group of diagnosed cases, based on comparisons with matched controls. *Acta Neurol Scand*. 1988;77: 293–306.

Gallucci RM, O'Dell SK, Rabe D, Fechter LD. JP-8 jet fuel exposure induces inflammatory cytokines in rat skin. *Int Immunopharmacol*. 2004;4:1159–1169.

Gandhi M, Aweeka F, Greenblatt RM, Blaschke TF. Sex differences in pharmacokinetics and pharmacodynamics. *Ann Rev Pharmacol Toxicol*. 2004;44:499–523.

Garetano G, Gochfeld M. Factors affecting tetrachloroethylene concentrations in residences above dry cleaning establishments. *Arch Environ Health*. 2000;55:59–68.

Gargas ML, Burgess RJ, Voisard DE, et al. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. *Toxicol Appl Pharmacol*. 1989;98:87–99.

Gargas ML, Tyler TR, Sweeney LM, et al. A toxicokinetic study of inhaled ethylene glycol monomethyl ether (2-ME) and validation of a physiologically based pharmacokinetic model for the pregnant rat and human. *Toxicol Appl Pharmacol*. 2000a;165:53–62.

Gargas ML, Tyler TR, Sweeney LM, et al. A toxicokinetic study of inhaled ethylene glycol ethyl ether acetate and validation of a physiologically based pharmacokinetic model for rat and human. *Toxicol Appl Pharmacol*. 2000b;165:63–73.

Garlantezec R, Warembourg C, Monfort C, et al. Urinary glycol ether metabolites in women and time to pregnancy: the PELAGIE Cohort. *Environ Health Perspect*. 2013;121:1167–1173.

Garner CD, Lee EW, Louis-Ferdinand RT. Muller cell involvement in methanol-induced retinal toxicity. *Toxicol Appl Pharmacol*. 1995a;130:101–107.

Garner CD, Lee EW, Terzo TS, Louis-Ferdinand RT. Role of retinal metabolism in methanol-induced retinal toxicity. *J Toxicol Environ Health*. 1995b;44:43–56.

Gasiewicz TA, Singh KP, Casado FL. The aryl hydrocarbon receptor has an important role in the regulation of hematopoiesis: Implications for benzene-induced hematopoietic toxicity. *Chem-Biol Interact*. 2010;184:246–251.

Gaunt IF, Carpanini FM, Grasso P, Lansdown AB. Long-term toxicity of propylene glycol in rats. *Food Cosmet Toxicol*. 1972;10:151–162.

Ge R, Yang S, Kramer PM, et al. The effect of dichloroacetic acid and trichloroacetic acid on DNA hypomethylation and cell proliferation in B6C3F1 mice. *J Biochem Mol Toxicol*. 2001;15:100–106.

Gelbke HP, Goen T, Maurer M, Sulsky SI. A review of health effects of carbon disulfide in viscose industry and a proposal for an occupational exposure limit. *Crit Rev Toxicol*. 2009;39(suppl 2):1–126.

Gelbke HP, Banton M, Leibold E, et al. A critical review finds styrene lacks direct endocrine disruptor activity. *Crit Rev Toxicol*. 2015;45:727–764.

Geller AM, Zenick H. Aging and the environment: a research framework. *Environ Health Perspect*. 2005;113:1257–1262.

Gennari P, Naldi M, Motta R, et al. Gamma-glutamyltransferase isozymes pattern in workers exposed to tetrachloroethylene. *Am J Ind Med*. 1992;21: 661–671.

Gentry PR, Covington TR, Clewell HJ III. Evaluation of the potential impact of pharmacokinetic differences on tissue dosimetry in offspring during pregnancy and lactation. *Regul Toxicol Pharmacol*. 2003;38:1–16.

Gerin M, Patrice S, Begin D, et al. A study of ethylene glycol exposure and kidney function of aircraft de-icing workers. *Int Arch Occup Environ Health*. 1997;69:255–265.

Gerr F, Letz R. Organic solvents. In: Rom WD, ed. *Environmental and Occupational Medicine*. 3rd ed. Philadelphia, PA: Lippincott-Raven; 1998:1091–1108.

Ghanayem BI. Metabolic and cellular basis of 2-butoxyethanol-induced hemolytic anemia in rats and assessment of human risk in vitro. *Biochem Pharmacol*. 1989;38:1679–1684.

Ghanayem BI, Burka LT, Matthews HB. Metabolic basis of ethylene glycol monobutyl ether (2-butoxyethanol) toxicity: Role of alcohol and aldehyde dehydrogenases. *J Pharmacol Exp Therap*. 1987;242:222–231.

Ghanayem BI, Burka LT, Matthews HB. Structure–activity relationships for the in vitro hematotoxicity of n-alkoxyacetic acids, toxic metabolites of glycol ethers. *Chem-Biol Interact*. 1989;70:339–352.

Ghanayem BI, Chapin RE. Calcium channel blockers protect against ethylene glycol monomethyl ether (2-methoxyethanol)-induced testicular toxicity. *Exp Mol Pathol*. 1990;52:279–290.

Ghanayem BI, Long PH, Ward SM, et al. Hemolytic anemia, thrombosis, and infarction in male and female F344 rats following gavage exposure to 2-butoxyethanol. *Exp Toxicol Pathol*. 2001;53:97–105.

Gibbs BF, Mulligan CN. Styrene toxicity: An ecotoxicological assessment. *Ecotoxicol Environ Safety*. 1997;38:181–194.

Gibbs GW, Amsel J, Soden K. A cohort mortality study of cellulose triacetate-fiber workers exposed to methylene chloride. *J Occup Environ Med*. 1996;38:693–697.

Gift JS. U.S. EPA's IRIS assessment of 2-butoxyethanol: The relationship of noncancer to cancer effects. *Toxicol Lett*. 2005;156:163–178.

Gilger AP, Potts AM. Studies on the visual toxicity of methanol: V. The role of acidosis in experimental methanol poisoning. *Am J Ophthalmol*. 1955;39:63–86.

Ginsberg G, Hattis D, Miller R, Sonawane B. Pediatric pharmacokinetic data: Implications for environmental risk assessment for children. *Pediatrics*. 2004;113:973–983.

Ginsberg G, Hattis D, Russ A, Sonawane B. Pharmacokinetic and pharmacodynamic factors that can affect sensitivity to neurotoxic sequelae in elderly individuals. *Environ Health Perspect*. 2005;113:1243–1249.

Ginsberg G, Hattis D, Sonawane B, et al. Evaluation of child/adult pharmacokinetic differences from a database derived from the therapeutic drug literature. *Toxicol Sci*. 2002;66:185–200.

Ginsberg G, Slikker W Jr, Bruckner JV, Sonawane B. Incorporating children's toxicokinetics into a risk framework. *Environ Health Perspect*. 2004;112:272–283.

Gleiter CH, Gundert-Remy U. Gender differences in pharmacokinetics. *Eur J Drug Metab Pharmacokinet*. 1996;21:123–128.

Glende EA Jr, Recknagel RO. Phospholipase A2 activation and cell injury in isolated rat hepatocytes exposed to bromotrichloromethane, chloroform and 1,1-dichloroethylene as compared to effects of carbon tetrachloride. *Toxicol Appl Pharmacol*. 1992;113:159–162.

Glover ML, Reed MD. Propylene glycol: the safe diluent that continues to cause harm. *Pharmacotherapy*. 1996;16:690–693.

Godderis L, Maertens N, de Gelder V, et al. Genetic susceptibility in solvent induced neurobehavioral effects. *Neurotox Res*. 2010;17:268–278.

Gold LS, Stewart PA, Milliken K, et al. The relationship between multiple myeloma and occupational exposure to six chlorinated solvents. *Occup Environ Med*. 2011;68: 391–399.

Goldenthal EI. A compilation of LD50 values in newborn and adult animals. *Toxicol Appl Pharmacol*. 1971;18:185–207.

Golding BT, Watson WP. Possible mechanisms of carcinogenesis after exposure to benzene. In: Singer B, Bartsch H, eds. *Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis*. Lyons, France: ARC; 1999:75–88, IARC Publ No. 150.

Goldstein BD. Benzene toxicity. *Occup Med*. 1988;33:541–554.

Goldsworthy TL, Lyght O, Bumett VL, Popp JA. Potential role of α-2μ-globulin, protein droplet accumulation, and cell replication in the renal carcinogenicity of rats exposed to trichloroethylene, perchloroethylene and pentachloroethane. *Toxicol Appl Pharmacol*. 1988;96:367–379.

Goldsworthy TL, Popp JA. Chlorinated hydrocarbon-induced peroxisomal enzyme activity in relation to species and organ carcinogenicity. *Toxicol Appl Pharmacol*. 1987;88:225–233.

Gonzalez FJ. Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. *Mutat Res*. 2005;569: 101–110.

Gonzalez FJ, Shah YM. PPARα: Mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. *Toxicology*. 2008;246:2–8.

Goodlett CR, Horn KH. Mechanisms of alcohol-induced damage to the developing nervous system. *Alcohol Res Health*. 2001;25:175–184.

Gordon SM, Brinkman MC, Ashley DL, et al. Changes in breath trihalomethane levels resulting from household water-use activities. *Environ Health Perspect*. 2006;114:514–521.

Gordon SM, Callahan PJ, Nishioka MG, et al. Residential environmental measurements in the National Human Exposure Assessment Survery (NHEXAS) pilot study in Arizona: Preliminary results for pesticides and VOCs. *J Expos Anal Environ Epidemiol*. 1999;9:456–470.

Gospe SM Jr, Zhou SS. Toluene abuse embryopathy: Longitudinal neurodevelopmental effects of prenatal exposure to toluene in rats. *Reprod Toxicol*. 1998;12:119–126.

Gotohda T, Takunaga I, Kubo S-i, et al. Effect of toluene inhalation on astrocytes and neutrotrophic factor in rat brain. *Forensic Sci Internat*. 2000;113:233–238.

Graham DG, Amarnath V, Valentine WM, et al. Pathogenetic studies of hexane and carbon disulfide neurotoxicity. *Crit Rev Toxicol*. 1995;25:91–112.

Grandison MK, Boudinot FD. Age-related changes in protein binding of drugs: implications for therapy. *Clin Pharmacokinet*. 2000;38:271–290.

Grassman JA, Kimmel CA, Neumann DA. Accounting for variability in responsiveness in human risk assessment. In: Neumann DA, Kimmel CA, eds. *Human Variability in Response to Chemical Exposures*. Washington, DC: International Life Sciences Institute; 1998:1–26.

Graves RJ, Coutts C, Green T. Methylene chloride-induced DNA damage: an interspecies comparison. *Carcinogenesis*. 1995;16:1919–1926.

Graves RJ, Green T. Mouse liver glutathione S-transferase mediated metabolism of methylene chloride to a mutagen in the CHO/HRPT assay. *Mutat Res*. 1996;367:143–150.

Gray TM, Steup D, Roberts LG, et al. Health assessment of gasoline and fuel oxygenate vapors: Reproductive toxicity assessment. *Regul Toxicol Pharmacol*. 2014;70:S48–S57.

Green T. Methylene chloride induced mouse liver and lung tumors: an overview of the role of mechanistic studies in human safety assessment. *Hum Exp Toxicol*. 1997b;16:3–13.

Green T. Pulmonary toxicity and carcinogenicity of trichloroethylene: Species differences and modes of action. *Environ Health Perspect*. 2000;108(suppl 2):261–264.

Green T, Dow J, Ellis MK, et al. The role of glutathione conjugation in the development of kidney tumors in rats exposed to trichloroethylene. *Chem-Biol Interact*. 1997a;105:99–117.

Green T, Dow J, Ong CN, et al. Biological monitoring of kidney function among workers occupationally-exposed to trichloroethylene. *Occup Environ Med*. 2004;61:312–317.

Green T, Odum J, Nash JA, et al. Perchloroethylene-induced rat kidney tumors: an investigation of the mechanisms involved and their relevance to humans. *Toxicol Appl Pharmacol*. 1990;103:77–89.

Gregg SD, Fisher JW, Bartlett MG. A review of analytical methods for the identification and quantification of hydrocarbons found in jet propellant 8 and related petroleum based fuels. *Biomed Chromatogr*. 2006;20:492–507.

Gries J-M, Benowitz N, Verotta D. Chronopharmacokinetics of nicotine. *Clin Pharmacol Ther*. 1996;60:385–395.

Guastadisegni C, Balduzzi M, Mancuso MT, Di Consiglio E. Liver mitochondria alterations in chloroform-treated Sprague–Dawley rats. *J Toxicol Environ Health*. 1999;57:415–429.

Guengerich FP. Cytochrome P450s and other enzymes in drug metabolism and toxicity. *AAPS J*. 2006;8:E101–E111.

Guengerich FP, Kim DH, Iwasaki M. Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol*. 1991;4:168–179.

Guerri C, Bazinet A, Riley EP. Foetal alcohol spectrum-disorders and alterations in brain and behavior. *Alcohol Alcohol*. 2009;44:108–114.

Guo C, McMartin KE. The cytotoxicity of oxalate, metabolite of ethylene glycol, is due to calcium oxalate monohydrate formation. *Toxicology*. 2005;208:347–355.

Guo C, Cenac TA, Li Y, McMartin KE. Calcium oxalate, and not other metabolies, is responsible for the renal toxicity of ethylene glycol. *Toxicol Lett*. 2007;173:8–16.
Guo L, Luo C, Fan J, et al. Serum miRNA profiling identifies miR-150/30a as potential biomarker for workers with damaged nerve fibers from carbon disulfide. *Ind Health*. 2015;53:38–47.
Guo Y, Wang W, Dong Y, et al. Carbon disulfide induces rat testicular injury via mitochondrial apoptotic pathway. *Chemosphere*. 2014;108:367–375.
Gupta A, Diaz FJ, Lal A, et al. Basal ganglion hemorrhage as delayed complication of diethylene glycol ingestion. *Am J Forensic Med Pathol*. 2017;38:39–42.
Guthrie OW, Wong BA, McInturf SM, et al. Inhalation of hydrocarbon jet fuel suppress central auditory nervous system function. *J Toxicol Environ Health Part A*. 2015;78:1154–1169.
Guthrie OW, Xu H, Wong BA, et al. Exposure to low levels of jet-propulsion fuel impairs brainstem encoding of stimulus intensity. *J Toxicol Environ Health Part A*. 2014;77:261–280.
Guyonnet D, Siess M-H, Le Bon A-M, Suschetet M. Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. *Toxicol Appl Pharmacol*. 1999;154:50–58.
Guyton KZ, Hogan KA, Scott CS, et al. Human health effects of tetrachloroethylene: key findings and scientific issues. *Environ health Perspect*. 2014;122:325–334.
Ha M-C, Cordier S, Dananche B, et al. Congenital malformations and occupational exposure to glycol ethers: A European collaborative case–control study. *Occup Hyg*. 1996;2:417–421.
Haber LT, Maier A, Gentry PR, et al. Genetic polymorphisms in assessing interindividual variability in delivered dose. *Regul Toxicol Pharmacol*. 2002;35:177–197.
Hack CE, Chiu WA, Zhao QJ, Clewell HJ. Bayesian population analysis of a harmonized physiologically based pharmacokinetic model of trichloroethylene and its metabolites. *Regul Toxicol Pharmacol*. 2006;46:63–83.
Hake CL, Stewart RD. Human exposure to tetrachloroethylene: inhalation and skin contact. *Environ Health Perspect*. 1977;21:231–238.
Hagiwara A, Doi Y, Imai N, et al. Medium-term multi-organ carcinogenesis bioassay of ethyl tertiary-butyl ether in rats. *Toxicology*. 2011;289:160–166.
Halder CA, Holdsworth CE, Cockrell BY, Piccirillo VJ. Hydrocarbon nephropathy in male rats: Identification of the nephrotoxic components of unleaded gasoline. *Toxicol Ind Health*. 1985;1:67–87.
Halder CA, Van Gorp GS, Hatoum NS, Warne TM. Gasoline vapor exposures. Part II. Evaluation of the nephrotoxicity of the major C4/C5 hydrocarbon components. *Am Ind Hyg Assoc J*. 1986;47:173–175.
Hallare AV, Gervasio MKR, Gervasio PLG, Acacio-Claro PJB. Monitoring genotoxicity among gasoline station attendants and traffic enforcers in the City of Manila using the micronucleus assay with exfoliated epithelial cells. *Environ Monit Assess*. 2009;156:331–341.
Hamby-Mason R, Chen JJ, Schenker S, et al. Catalase mediates acetaldehyde formation from ethanol in fetal and neonatal rat brain. *Alcoholism: Clin Exper Res*. 1997;21:1063–1072.
Hanioka N, Jinno H, Toyo'oka T, et al. Induction of rat liver drug metabolizing enzymes by tetrachloroethylene. *Arch Environ Contam Toxicol*. 1995;28:273–280.
Hansen J, Raaschou-Nielson O, Christensen JM, et al. Cancer incidence among Danish workers exposed to trichloroethylene. *J Occup Environ Med*. 2001;43:133–139.
Hansen JM, Contreras KM, Harris C. Methanol, formaldehyde, and sodium formate exposure in rat and mouse conceptuses: a potential role of the visceral yolk sac in embryotoxicity. *Birth Defects Res A Clin Mol Teratol*. 2005;73:72–82.
Hansen L, Lange R, Gupta S. Development and evaluation of a guideline for monitoring propylene glycol toxicity in pediatric intensive care unit receiving continuous infusion Lorazepam. *J Pediatr Pharmacol Ther*. 2015;20:367–372.
Hantson P, Haufroid V, Wallemacq P. Formate kinetics in methanol poisoning. *Human Exp Toxicol*. 2005;24:55–59.
Hard GC, Boorman GA, Wolf DC. Re-evaluation of the 2-year chloroform drinking water carcinogenicity bioassay in Osborne–Mendel rats supports chronic renal tubule injury as the mode of action underlying the renal tumor response. *Toxicol Sci*. 2000;53:237–244.
Hardin BD, Eisenmann CJ. Relative potency of four ethylene glycol ethers for induction of paw malformations in the CD-1 mouse. *Teratology*. 1987;35:321–328.
Hardin BD, Goad PT, Burg JR. Developmental toxicity of four glycol ethers applied cutaneously to rats. *Environ Health Perspect*. 1984;57:69–74.
Hardin BD, Goad PT, Burg JR. Developmental toxicity of diethylene glycol monomethyl ether (diEGME). *Fundam Appl Toxicol*. 1986;6:430–439.
Harris C, Dixon M, Hansen JM. Glutathione depletion modulates methanol, formaldehyde and formate toxicity in cultured rat conceptuses. *Cell Biol Toxicol*. 2004;20:133–145.
Harris C, Wang SW, Lauchu JJ, Hansen JM. Methanol metabolism and embryotoxicity in rat and mouse conceptuses: Comparisons of alcohol dehydrogenase (ADH1), formaldehyde dehydrogenase (ADH3), and catalase. *Reprod Toxicol*. 2003;17:349–357.
Harris DT, Sakiestewa D, Robledo RF, Witten M. Immunotoxicological effects of JP-8 jet fuel exposure. *Toxicol Ind Health*. 1997a;13:43–55.
Harris DT, Sakiestewa D, Robledo RF, Witten M. Short-term exposure to JP-8 jet fuel results in long-term immunotoxicity. *Toxicol Ind Health*. 1997b;13:559–570.
Harris DT, Sakiestewa D, Robledo RF, Witten M. Protection from JP-8 jet fuel induced immunotoxicity by administration of aerosolized substance P. *Toxicol Ind Health*. 1997c;13:571–588.
Harris DT, Sakiestewa D, Robledo RF, et al. Effects of short-term JP-8 jet fuel exposure on cell-mediated immunity. *Toxicol Ind Health*. 2000;16:78–84.
Harris DT, Sakiestewa D, Titone D, Witten M. JP-8 jet fuel exposure rapidly induces high levels of IL-10 and PGE2 secretion and is correlated with loss of immune function. *Toxicol Ind Health*. 2007a;23:223–230.
Harris DT, Sakiestewa D, He X, et al. Effects of in utero JP-8 jet fuel exposure on the immune systems of pregnant and newborn mice. *Toxicol Ind Health*. 2007b;23:545–552.
Harris DT, Sakiestewa D, He X, et al. JP-8 jet fuel exposure suppresses the immune response to viral infections. *Toxicol Ind Health*. 2008;24:209–216.
Harris DT, Sakiestewa D, Titone D, et al. JP-8 jet fuel exposure potentiates tumor development in two experimental model systems. *Toxicol Ind Health*. 2007c;23:617–623.
Harris DT, Sakiestewa D, Titone D, et al. Jet fuel-induced immunotoxicity. *Toxicol Ind Health*. 2001a;16:261–265.
Harris DT, Sakiestewa D, Titone D, et al. Substance P as prophylaxis for JP-8 jet fuel-induced immunotoxicity. *Toxicol Ind Health*. 2001b;16:253–259.
Harris DT, Sakiestewa D, Titone D, et al. JP-8 jet fuel exposure results in immediate immunotoxicity, which is cumulative over time. *Toxicol Ind Health*. 2002;18:77–83.
Harry GJ, Graham DG, Valentine WM, et al. Carbon disulfide neurotoxicity in rats: VIII. Summary. *Neurotoxicology*. 1998;19:159–162.
Haryono A, Surjono TW, Sutasurya LA, Sudarwati S. Methoxyacetic acid induced apoptosis on the forelimb bud of Swiss Webster mice. *Hayati J Biosci*. 2011;18:33–36.
Hasegawa R, Hirata-Koizumi M, Dourson M, et al. Pediatric susceptibility to 18 industrial chemicals: a comparative analysis of newborn with young animals. *Regul Toxicol Pharmacol*. 2007;47:296–307.
Haselkorn T, Whittemore AS, Udaltsova N, Friedman GD. Short-term chloral hydrate administration and cancer in humans. *Drug Safety*. 2006;29:67–77.
Haseman JK, Hailey JR, Morris RW. Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: a National Toxicology Program update. *Toxicol Pathol*. 1998;26:428–441.
Hattis D, Banati P, Goble R, Burmaster DE. Human interindividual variability in parameters related to health risks. *Risk Anal*. 1999;19:711–726.
Hattis D, Ginsberg G, Sonawane B, et al. Differences in pharmacokinetics between children and adults-II. Children's variability in drug elimination half-lives and in some parameters needed for physiologically based pharmacokinetic modeling. *Risk Anal*. 2003;23:117–142.

Hayes JR, Condie LW Jr, Borzelleca JF. The subchronic toxicity of tetrachloroethylene (perchloroethylene) administered in the drinking water of rats. *Fund Appl Toxicol*. 1986;7:119–125.

Hays AM, Parliman G, Pfaff JK, et al. Changes in lung permeability correlate with lung histology in a chronic exposure model. *Toxicol Ind Health*. 1995;11(3):325–36.

Hays SM, Elswick BA, Blumenthal GM, et al. Development of a physiologically based pharmacokinetic model of 2-methoxyethanol and 2-methoxyacetic acid disposition in pregnant rats. *Toxicol Appl Pharmacol*. 2000;163:67–74.

Health Canada. Priority substances list. *State-of-the-Science Report for Ethylene Glycol*. Health Canada and Environment Canada, 2000. http://www.ec.gc.ca/substances/ese/eng/psap/final/ethyleneglycol.cfm.

Hebert JL, Fabre M, Auzepy P, Paillas J. Acute experimental poisoning by diethylene glycol: Acid base balance and histological data in male rats. *Toxicol Eur Res*. 1978;1:2890–2894.

Heilmair R, Lenk W, Lohr D. Toxicokinetics of diethylene glycol (DEG) in the rat. *Arch Toxicol*. 1993;67:655–666.

Heineman EF, Cocco P, Gomez MR, et al. Occupational exposure to chlorinated aliphatic hydrocarbons and risk of astrocytic brain cancer. *Am J Ind Med*. 1994;26:155–169.

Heistein LC, Ramaciotti C, Scott WA, et al. Chloral hydrate sedation for pediatric echocardiography: Physiologic responses, adverse events, and risk factors. *Pediatrics.* 2006;117:e434–e441.

Henderson GI, Chen JJ, Schenker S. Ethanol, oxidative stress, reactive aldehydes, and the fetus. *Front Biosci*. 1999;4:D541–D550.

Henderson RF. Species differences in the metabolism of benzene. *Environ Health Perspect*. 1996;104(suppl 6):1173–1175.

Henschler D, Vamvakas S, Lammert M, et al. Increased incidence of renal cell tumors in a cohort of cardboard workers exposed to trichloroethene. *Arch Toxicol*. 1995;69:291–299.

Herr DW, Freeborn DL, Degn L, et al. Neurophysiological assessment of auditory, peripheral nerve, somatosensory, and visual system function after developmental exposure to gasoline, E15, and E85 vapors. *Neurotoxicol Teratol*. 2016;54:78–88.

Herrlinger C, Klotz U. Drug metabolism and drug interactions in the elderly. *Best Pract Res Clin Gastroenterol*. 2001;15:897–918.

Hess R, Bartels MJ, Pottenger LH. Ethylene glycol: an estimate of tolerable levels of exposure based on a review of animal and human data. *Arch Toxicol*. 2004;78:671–680.

Hester SD, Johnstone AF, Boyes WK, et al. Acute toluene exposure alters expression of genes in the central nervous system associated with synaptic structure and function. *Neurotoxicol Terotol*. 2011;33:521–529.

Heywood R, Sortwell RJ, Noel PRB, et al. Safety evaluation of toothpaste containing chloroform. III. Long-term study in beagle dogs. *J Environ Pathol Toxicol*. 1979;2:835–851.

Hillbom M. Oxidants, antioxidants, alcohol and stroke. *Front Biosci*. 1999;4:67–71.

Hinderliter PM, Price PS, Bartels MJ, et al. Development of a sources-to-outcome model for dietary exposures to insecticide residues: an example using chloripyrifos. *Regul Toxicol Pharmacol*. 2011;61:82–92.

Hines RN. The ontogeny of drug metabolism enzymes and implications for adverse drug events. *Pharmacol Therap*. 2008;118:250–267.

Hines RN. Developmental expression of drug metabolising enzymes: Impact on disposition in neonates young children. *Int J Pharm*. 2013;452:3–7.

Hines RN, Sargent D, Autrup H, et al. Approaches for assessing risks to sensitive populations: lessons learned from evaluating risks in the pediatric population. *Toxicol Sci*. 2010;113:4–26.

Hiraku Y, Kawanishi S. Oxidative DNA damage and apoptosis induced by benzene metabolites. *Cancer Res*. 1996;56:5172–5178.

Hodgson MJ, Heyl AE, Van Thiel DH. Liver disease associated with exposure to 1,1,1-trichloroethane. *Arch Intern Med*. 1989;149:1793–1798.

Hoflack J-C, Vasseur P, Poirier GG. Glycol ethers induce death and necrosis in human leukemia cells. *Biochem Cell Biol*. 1997;75:415–425.

Holladay SD, Comment CE, Kwon J, Luster MI. Fetal hematopoietic alterations after maternal exposure to ethylene glycol monomethyl ether: prolymphoid cell targeting. *Toxicol Appl Pharmacol*. 1994;129:53–60.

Hong J-Y, Wang Y-Y, Bondoc FY, et al. Metabolism of methyl *tert*- butyl ether and other gasoline ethers by human liver microsomes and heterologously expressed human cytochromes P450: identification of CYP2A6 as a major catalyst. *Toxicol Appl Pharmacol*. 1999;160:43–48.

Hooth MJ, Herbert RA, Haseman JK, et al. Toxicology and carcinogenesis studies of dipropylene glycol in rats and mice. *Toxicology*. 2004;204:123–140.

Hopf NB, VErnez D, Berthet A, et al. Effect of age on toxicokinetics among human volunteers exposed to propylene glycol methyl ether (PGME). *Toxicol Lett*. 2012;211:77–84.

Horinek EL, Kiser TH, Fish DN, MacLaren R. Propylene glycol accumulation in critically ill patients receiving continuous intravenous Lorazepam infusions. *Ann Pharmacother*. 2009;43:1964–1971.

House RV, Lauer LD, Murray MJ, et al. Immunological studies in B6C3F1 mice following exposure to ethylene glycol monomethyl ether and its principal metabolite methoxyacetic acid. *Toxicol Appl Pharmacol*. 1985;77:358–362.

Hovda KE, Guo C, Austin R, McMartin KE. Renal toxicity of ethylene glycol results from internalization of calcium oxalate crystals by proximal tubule cells. *Toxicol Lett*. 2010;192:365–372.

Hsieh GY, Wang JD, Cheng TJ, Chen PC. Prolonged menstrual cycles in female workers exposed to ethylene glycol ethers in the semiconductor manufacturing industry. *Occup Environ Med*. 2005;62:510–516.

Huang S-M, Lesko LJL. Drug–drug, drug–dietary supplement, and drug–citrus fruit and other food interactions: what have we learned? *J Clin Pharmacol*. 2004;44:559–569.

Huang X, Zhou Y, Ma J, et al. Nitric oxide mediated effects on reproductive toxicity caused by carbon disulfide in male rats. *Environ Toxicol Pharmacol*. 2012;34:679–687.

Huff JE, Haseman JK, DeMarini DM, et al. Multiple-site carcinogenicity of benzene in Fischer 344 rats and B6C3F1 mice. *Environ Health Perspect*. 1989;82:125–163.

Hukkanen J. Induction of cytochrome P450 enzymes: a view on human in vivo findings. *Expert Rev Clin Pharmacol*. 2012;5:569–585.

Hunter AGW, Thompson D, Evans JA. Is there a fetal gasoline syndrome? *Teratology*. 1979;20:75–80.

IARC (International Agency for Research on Cancer). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Alcohol Drinking*. Vol 44. Lyon, France: World Health Organization; 1988:251–259.

IARC (International Agency for Research on Cancer). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Diesel and Gasoline Engine Exhausts and Some Nitroarenes*. Vol 46. Lyon, France: World Health Organization; 1989a.

IARC (International Agency for Research on Cancer). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Occupational Exposures in Petroleum Refining: Crude Oil and Major Petroleum Fuels*. Vol 45. Lyon, France: World Health Organization; 1989b.

IARC (International Agency for Research on Cancer). *Re-evaluation of Some Organic Chemicals, Hydrazine, and Hydrogen Peroxide*. Vol 71. Lyon, France: World Health Organization; 1999:401–432.

IARC (International Agency for Research on Cancer). *Summaries and Evaluations—Styrene (Group 2B)*. Vol 82. Lyon, France: World Health Organization; 2002:437.

IARC (International Agency for Research on Cancer). *Trichloroethylene, Tetrachloroethylene, and Some Other Chlorinated Agents*. Vol 106. Lyon, France: World Health Organization; 2014.

Ikonomidou C, Bittigau P, Ishimaru MJ, et al. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science*. 2000;287:1056–1060.

Imam YZB, Kamran S, Karim H, et al. Neurological manifestation of recreational fatal and near-fatal diethylene glycol poisonings. *Medicine*. 2014;93:1–8.

Infante-Rivard C, Siemiatycki R, Nadon L. Maternal exposure to occupational solvents and childhood leukemia. *Environ Health Perspect*. 2005;113:787–792.

Inoue O, Seiji K, Watanabe T, et al. Mutual metabolic suppression between benzene and toluene in man. *Int Arch Occup Environ Health*. 1988;60:15–20.

Ioannides C, Lewis DFV. Cytochrome P450 in the bioactivation of chemicals. *Current Topics Med Chem*. 2004;5:1767–1788.

Ishmael J, Dugard PH. A review of perchloroethylene and rat mononuclear cell leukemia. *Regul Toxicol Pharmacol*. 2006;45:178–184.

Israel Y, Rivera-Meza M, Quintanilla ME, et al. Acetaldehyde burst protection of ADH1B*2 against alcoholism: an additional hormesis protection

against esophageal cancers following alcohol consumption? *Alcohol Clin Exp Res*. 2011;35:306–810.
Jacobsen D, Ovrebo S, Ostborg J, Sejersted OM. Glycolate causes the acidosis in ethylene glycol poisoning and is effectively removed by hemodialysis. *Acta Med Scand*. 1984;216:409–416.
Jan CR, Chen LW, Lin MW. $Ca^{(2+)}$ mobilization evoked by chloroform in Madin–Darby canine kidney cells. *J Pharmacol Exp Ther*. 2000;292:995–1001.
Jeannot E, Pogribny IP, Breland FA, Rusyn I. Chronic administration of ethanol leads to an increased incidence of hepatocellular adenoma by promoting H-ras-mutated cells. *Cancer Lett*. 2011;301:161–167.
Jia C, Yu X, Masik W. Blood/air distribution of volatile organic compounds (VOCs) in a nationally representative sample. *Sci Total Environ*. 2012;419:225–232.
Jiang Y, Liu J, Waalkes M, Kang YJ. Changes in gene expression associated with carbon tetrachloride-induced liver fibrosis persist after cessation of dosing in mice. *Toxicol Sci*. 2004;79: 404–410.
Jindo T, Wine RN, Li L-H, Chapin RE. Protein kinase activity is central to rat germ cell apoptosis induced by methoxyacetic acid. *Toxicol Pathol*. 2001;29:607–616.
Jobson MA, Hogan SL, Maxwell CS, et al. Clinical features of reported ethylene glycol exposures in the United States. *PLos One*. 2015;10:e0143044.
Johanson G, Filser JG. Experimental data from closed chamber gas uptake studies in rodents suggest lower uptake rate of chemical than calculated from literature values on alveolar ventilation. *Arch Toxicol*. 1992;66:291–295.
Johansson I, Ingelman-Sundberg M. Genetic polymorphism and toxicology—with emphasis on cytochrome P450. *Toxicol Sci*. 2011;120:1–13.
Johlin FC, Swain E, Smith C, Tephly TR. Studies on the mechanism of methanol poisoning: purification and comparison of rat and human liver 10-formyltetrahydrofolate dehydrogenase. *Mol Pharmacol*. 1989;35:745–750.
Johns DO, Daniell WE, Shen DD, et al. Ethanol induced increase in the metabolic clearance of 1,1,1-trichloroethane in human volunteers. *Toxicol Sci*. 2006;92: 61–70.
Johnson BL, Boyd J, Burg JR, et al. Effects on the peripheral nervous system of workers' exposure to carbon disulfide. *Neurotoxicology.* 1983;4:53–65.
Johnson KA, Baker PC, Kan HL, et al. Diethylene glycol monobutyl ether (DGBE): Two- and thirteen-week oral toxicity studies in Fischer 344 rats. *Food Chem Toxicol*. 2005;43:467–481.
Johnson PD, Goldberg SJ, Mays MZ, Dawson BV, et al. Threshold of trichloroethylene contamination in maternal drinking waters affecting fetal heart development in the rat. *Environ Health Perspect*. 2003;111:289–292.
Johnsrud EK, Koukouritaki SB, Divakaran K, et al. Human hepatic CYP2E1 expression during development. *J Pharmacol Exp Therap*. 2003;307:402–407.
Johnston LD, O'Malley PM, Bachman JG, Schulenberg JE. *Monitoring the Future National Results on Adolescent Drug Use: Overview of Key Findings*. Bethesda, MD: National Institute on Drug Abuse, 2008, NIH Publication No. 09-7401, pp. 1–67.
Jollow DJ, Bruckner JV, McMillan DC, et al. Trichloroethylene risk assessment: a review and commentary. *Crit Rev Toxicol*. 2009;39:782–797.
Jonker D, Woutersen RA, Fernon VJ. Toxicity of mixtures of nephrotoxicants with similar or dissimilar mode of action. *Food Chem Toxicol*. 1996;34:1075–1082.
Jonsson F, Bois F, Johanson G. Physiologically based pharmacokinetic modeling of inhalation exposure of humans to dichloromethane during moderate to heavy exercise. *Toxicol Sci*. 2001;89:209–218.
Jonsson F, Johanson G. A Bayesian analysis of the influence of GSTT1 polymorphism on the cancer risk estimate for dichloromethane. *Toxicol Appl Pharmacol*. 2001;174:99–112.
Jonsson F, Johanson G. Physiologically based modeling of the inhalation kinetics of styrene in humans using a Bayesian population approach. *Toxicol Appl Pharmacol*. 2002;179:35–49.
Jorgenson TA, Meierhenry EF, Rushbrook CJ, et al. Carcinogenicity of chloroform in drinking water to male Osborne–Mendel rats and female B6C3F1 mice. *Fundam Appl Toxicol*. 1985;5:760–769.
Kabbur MB, Rogers JV, Gunasekar PG, et al. Effect of JP-8 jet fuel on molecular and histological parameters related to acute skin irritation. *Toxicol Appl Pharmacol*. 2001;175:83–88.
Kakehashi A, Hagiwara A, Imai N, et al. Mode of action of ethyl *tertiary*-butyl ether hepatotumorigenicity in the rat: Evidence for a role of oxidative stress via activation of CAR, PXR and PPAR signaling pathways. *Toxicol Appl Pharmacol*. 2013;273:390–400.
Kane EV, Newton R. Occupational exposure to gasoline and the risk of non-Hodgkin lymphoma: A review and meta-analysis of the literature. *Cancer Epidemiol*. 2010;34:516–522.
Kaneko T, Wang P-Y, Sato A. Enzymes induced by ethanol differently affect the pharmacokinetics of trichloroethylene and 1,1,1-trichloroethane. *Occup Environ Med*. 1994;51:113–119.
Kang-Sickel J-CC, Butler MA, Frame L, et al. The utility of naphthylkeratin adducts as biomarkers for jet-fuel exposure. *Biomarkers*. 2011;16:590–599.
Karami S, Lan Q, Rothman N, et al. Occupational trichloroethylene exposure and kidney cancer risk: a meta-analysis. *Occup Environ Med*. 2012;69:858–867.
Kato S, Shields PG, Caparoso NE, et al. Cytochrome P450IIE1 genetic polymorphisms, racial variation, and lung cancer risk. *Cancer Res*. 1992;52:6712–6715.
Katoh M. New Energy Development Organization data. Presented at the Methanol Vapors and Health Effects Workshop: What we know and what we need to know—Summary Report. Washington, DC, ILSI Risk Science Institute/U.S. Environmental Protection Agency/Health Effects Institute/American Petroleum Institute, A-7, 1989.
Kaufman LR, LeMasters GK, Olsen DM, Succop P. Effects of concurrent noise and jet fuel exposure on hearing loss. *J Occup Environ Med*. 2005;47:212–218.
Kavet R, Nauss KM. The toxicity of inhaled methanol vapors. *Crit Rev Toxicol*. 1990;21:21–50.
Kawamoto T, Koga M, Murata K, et al. Effects of ALDH2, CYP1A1, and CYP2E1 genetic polymorphisms and smoking and drinking habits on toluene metabolism in humans. *Toxicol Appl Pharmacol*. 1995;133: 295–304.
Kayama F, Yamashita U, Kawamoto T, Kodama Y. Selective depletion of immature thymocytes by oral administration of ethylene glycol monomethyl ether. *Int J Immunopharmacol*. 1991;13:531–540.
Kearney CA, Dunham DB. Gasoline vapor exposures at a high volume service station. *Am Ind Hyg Assoc J*. 1986;47:535–539.
Kedderis GL. Extrapolation of in vitro enzyme induction data to humans in vivo. *Chem-Biol Interact*. 1997;107:109–121.
Keenan JJ, Gaffney S, Gross SA, et al. An evidence-based analysis of epidemiologic associations between lymphatic and hematopoietic cancers and occupational exposure to gasoline. *Hum Exp Toxicol*. 2013;32:1007–1027.
Kegelmeyer AE, Sprankle CS, Horesovsky GJ, Butterworth BE. Differential display identified changes in mRNA levels in regenerating livers from chloroform-treated mice. *Mol Carcinogen*. 1997;20: 288–297.
Keil DE, Warren DA, Jenny MJ, et al. Immunological function in mice exposed to JP-8 jet fuel in utero. *Toxicol Sci*. 2003;76:347–356.
Kelsh MA, Alexander DD, Mink PJ, Mandel JH. Occupational trichloroethylene exposure and kidney cancer: A meta-analysis. *Epidemiology*. 2010;21: 95–102.
Kenyon EM, Kraichely RE, Hudson KT, Medinsky MA. Differences in rates of benzene metabolism correlate with observed genotoxicity. *Toxicol Appl Pharmacol*. 1996;136:49–56.
Kerns W, Tomaszewski C, McMartin K, et al. Formate kinetics in methanol poisoning. *Clin Toxicol*. 2002;40:137–143.
Keshava N, Caldwell JC. Key issues in the role of peroxisome proliferator-activated receptor agonism and cell signaling in trichloroethylene toxicity. *Environ Health Perspect*. 2006;114: 1464–1470.
Kezic S, Calkoen F, Wenker M, et al. Genetic poylmorphisms of metabolic enzymes modifies the risk of chronic solvent-induced encephalopathy. *Toxicol Ind Health*. 2006;22:281–289.
Kienhuis AS, Soeteman-Hernandez LG, Gox PMJ, et al. Potential harmful health effects of inhaling nicotine-free shisha-pen vapor: A chemical risk assessment of the main components propylene glycol and glycerol. *Tob Induc Dis*. 2015;13:15.
Kiesswetter E, Sietmann B, Seeber A. Standardization of a questionnaire for neurotoxic symptoms. *Environ Res*. 1997;73:73–80.

Kiesswetter E, Sietmann B, Zupanic M, Seeber A. Neurobehavioral study on the interactive effects of age and solvent exposure. *Neurotoxicology*. 2000;21:685–696.

Kilburn KH. *Chemical Brain Injury*. New York: Van Nostrand Reinhold; 1998.

Kim B-S, Smialowicz RJ. The role of metabolism in 2-methoxyethanol induced suppression of in vitro polyclonal antibody responses by rat and mouse lymphocytes. *Toxicology*. 1997;123:227–239.

Kim D, Andersen ME, Chao Y-CE, et al. PBTK modeling demonstrates contribution of dermal and inhalation exposure components to end-exhaled breath concentrations of naphthalene. *Environ Health Perspect*. 2007;115:894–901.

Kim D, Andersen ME, Nylander-French LA. Dermal absorption and penetration of jet fuel components in humans. *Toxicol Lett*. 2006a;165: 11–21.

Kim D, Andersen ME, Nylander-French LA. A dermatotoxicokinetic model of human exposures to jet fuel. *Toxicol Sci*. 2006b;93:22–33.

Kim D, Ghanayem BI. Comparative metabolism and disposition of trichloroethylene in CYP2E1-/- and wild-type mice. *Drug Metab Dispos*. 2006a;34:2020–2027.

Kim G, Malayaman SN, Green MS. Use of methyl tert-butyl ether for the treatment of refractory intrahepatic biliary strictures and bile casts: a modern perspective. *Case Rep Surg*. 2015;2015:408175.

Kim HJ, Bruckner JV, Dallas CE, Gallo JM. Effect of dosing vehicles on the pharmacokinetics of orally administered carbon tetrachloride in rats. *Toxicol Appl Pharmacol*. 1990a;102:50–60.

Kim HJ, Oden'hal S, Bruckner JV. Effect of oral dosing vehicles on the acute hepatotoxicity of carbon tetrachloride in rats. *Toxicol Appl Pharmacol*. 1990b;102:34–49.

Kim HK, Takematsu M, Biary R, et al. Epidemic gasoline exposures following hurricane Sandy. *Prehosp Disaster Med*. 2013;28:586–591.

Kinirons MT, O'Mahony MS. Drug metabolism and aging. *Br J Clin Pharmacol*. 2004;57:540–544.

Kirman CR, Sweeney LM, Corley R, Gargas ML. Using physiologically based pharmacokinetic modeling to address nonlinear kinetics and changes in rodent physiology and metabolism due to aging and adaptation in deriving reference values for propylene glycol methyl ether and propylene glycol methyl ether acetate. *Risk Anal*. 2005;25:271–284.

Kirschmann JC, Brown NM, Coots RH, et al. Review of investigations of dichloromethane metabolism and subchronic oral toxicity as the basis for the design of chronic oral studies in rats and mice. *Food Chem Toxicol*. 1986;24:943–949.

Klaassen CD, Plaa GL. Relative effects of various chlorinated hydrocarbons on liver and kidney function in mice. *Toxicol Appl Pharmacol*. 1966;9:139–151.

Klaunig JE, Babich MA, Baetcke KP, et al. PPARalpha agonist-induced rodent tumors: Modes of action and human relevance. *Crit Rev Toxicol*. 2003;33:655–780.

Klaunig JE, Babich MA, Cook JC, et al. PPARα and effects of TCE. *Environ Health Perspect*. 2007;115:A14–A15.

Klaunig JE, Kamendulis LM. Mode of action of butoxyethanol-induced mouse liver hemangiosarcomas and hepatocellular carcinomas. *Toxicol Lett*. 2005;156:107–115.

Klaunig JE, Ruch RJ, Pereira MA. Carcinogenicity of chlorinated methane and ethane compounds administered in drinking water to mice. *Environ Health Perspect*. 1986;69:89–95.

Klotz U. Pharmacokinetics and drug metabolism in the elderly. *Drug Metab Rev*. 2009;41:67–76.

Klotz U, Ammon E. Clinical and toxicological consequences of the inductive potential of ethanol. *Eur J Clin Pharmacol*. 1998;54:7–12.

Kobayashi K, Urashima K, Shimada N, Chiba K. Substrate specificity for rat cytochrome P450 (CYP) isoforms: Screening with cDNA-expressed systems in rats. *Biochem Pharmacol*. 2002;63:889–896.

Koizumi A, Kumai M, Ikeda M. In vivo suppression of 1,1,1-trichloroethane metabolism by co-administered tetrachloroethylene: an inhalation study. *Bull Environ Contam Toxicol*. 1982;29: 196–199.

Kolovou GD, Salpea KD, Anagnostopoulou KK, Mikhailidis DP. Alcohol use, vascular disease, and lipid-lowering drugs. *J Pharmacol Exp Ther*. 2006;318:1–7.

Kostic MA, Dart RC. Rethinking the toxic methanol level. *J Toxicol Clin Toxicol*. 2003;41:793–800.

Kozlosky J, Bonventre J, Cooper K. Methyl tert butyl ether (MTBE) is anti-angiogenic in both *in vitro* and *in vivo* mammalian model systems. *J Appl Toxicol*. 2013;33:820–827.

Kraul H, Jahn F, Braunlich H. Nephrotoxic effects of diethylene glycol (DEG) in rats. *Exp Pathol*. 1991;42:27–32.

Krause RJ, Lash LH, Elfarra AA. Human kidney flavin-containing monooxygenases and their potential roles in cysteine S-conjugate metabolism and nephrotoxicity. *J Pharmacol Exp Therap*. 2003;304:185–191.

Kril JJ, Halliday GM, Svoboda MD, Cartwright H. The cerebral cortex is damaged in chronic alcoholics. *Neuroscience*. 1997;79:983–998.

Krishnan K, Johanson G. Physiologically-based pharmacokinetic and toxicokinetic models in cancer risk assessment. *J Environ Sci Health*. 2005;23:31–53.

Ku MC, Huang CC, Kuo HC, et al. Diffuse white matter lesions in carbon disulfide intoxication: Microangiopathy or demyelination. *Eur Neurol.* 2003;50:220–224.

Ku WW, Wine RN, Chae BY, et al. Spermatocyte toxicity of 2-methoxyethanol (ME) in rats and guinea pigs: Evidence for the induction of apoptosis. *Toxicol Appl Pharmacol*. 1995;134:100–110.

Kuna RA, Ulrich CE. Subchronic inhalation toxicity of two motor fuels. *J Am Col Toxicol*. 1984;3:217–229.

Laitinen J, Liesivuori J, Savolainen H. Exposure to glycols and their renal effects in motor servicing workers. *Occup Med*. 1995;45:259–262.

LaKind JS, McKenna EA, Hubner RP, Tardiff RG. A review of the comparative mammalian toxicity of ethylene glycol and propylene glycol. *Crit Rev Toxicol*. 1999;29:331–365.

Lam C-W, Galen TJ, Boyd JF, Pierson DL. Mechanism of transport and distribution of organic solvents in blood. *Toxicol Appl Pharmacol*. 1990;104:117–129.

Lamb JC IV, Gulati DK, Russell VS, et al. Reproductive toxicity of ethylene glycol monoethyl ether tested by continuous breeding of CD-1 mice. *Environ Health Perspect*. 1984;57:85–90.

Lan O, Zhang L, Li G, et al. Hematotoxicity in workers exposed to low levels of benzene. *Science*. 2004;306: 1774–1776.

Lan Q, Zyang L, Tang X, et al. Occupational exposure to trichloroethylene is associated with a decline in lymphocyte subsets and soluble CD27 and CD30 markers. *Carcinogenesis*. 2010;31:1592–1596.

Landrigan PJ, Goldman LR. Children's vulnerability to toxic chemicals: A challenge and opportunity to strengthen health and environmental policy. *Health Affairs*. 2011;30: 842–850.

Landry GM, Dunning CL, Abreo F, et al. Diethylene glycol-induced toxicities show marked threshold dose response in rats. *Toxicol Appl Pharmacol*. 2015;282:244–251.

Landry GM, Martin S, McMartin KE. Diglycolic acid is the nephrotoxic metabolite in diethylene glycol poisoning inducing necrosis in human proximal tubule cells *in vitro*. *Toxicol Sci*. 2011;124:35–44.

Landtblom A-M. Exposure to organic solvents and multiple sclerosis. *Neurology.* 1997;49:570–574.

Landtblom A-M, Wateson M, Ahmad A, Soderkvist P. Multiple sclerosis and exposure to organic solvents investigated by genetic polymorphisms of the GSTM1 and CYP2D6 enzyme systems. *Neurol Sci*. 2003;24:248–251.

Lanes SF, Rothman KJ, Dreyer NA, et al. Mortality update of cellulose fiber production workers. *Scand J Work Environ Health*. 1993;19:426–428.

Lanigan S. Final report on the safety assessment of methyl alcohol. *Int J Toxicol*. 2001;20(suppl 1):57–85.

Larson JL, Bull RJ. Effect of ethanol on the metabolism of trichloroethylene. *J Toxicol Environ Health*. 1989;28:395–406.

Larson JL, Wolf DC, Butterworth BE. Induced cytotoxicity and cell proliferation in the hepatocarcinogenicity of chloroform in female B6C3F1 mice: comparison of administration by gavage in corn oil vs ad libitum in drinking water. *Fundam Appl Toxicol*. 1994;22:90–102.

Lash AA, Becker CE, So Y, Shore M. Neurotoxic effects of methylene chloride: are they long lasting in humans? *Br J Ind Med*. 1991;48:418–426.

Lash LH, Chiu WA, Guyton KZ, Rusyn I. Trichloroethylene biotransformation and its role in mutagenicity, carcinogenicity and target organ toxicity. *Mut Res*. 2014;762:22–36.

Lash LH, Fisher JW, Lipscomb JC, Parker JC. Metabolism of trichloroethylene. *Environ Health Perspect*. 2000a;108(suppl 2):177–200.
Lash LH, Parker JC. Hepatic and renal toxicities associated with perchloroethylene. *Pharmacol Rev*. 2001;53:177–208.
Lash LH, Parker JC, Scott CS. Modes of action of trichloroethylene for kidney tumorigenesis. *Environ Health Perspect*. 2000b;108(suppl 2):225–240.
Lash LH, Putt DA, Brashear WT, et al. Identification of S-(1,2-dichlorovinyl) glutathione in the blood of human volunteers exposed to trichloroethylene. *J Toxicol Environ Health A*. 1999;56: 1–21.
Lash LH, Putt DA, Huang P, et al. Modulation of hepatic and renal metabolism and toxicity of trichloroethylene and perchloroethylene by alterations in status of cytochrome P450 and glutathione. *Toxicology*. 2007;235:11–26.
Lash LH, Putt DA, Hueni SE, et al. Roles of necrosis, apoptosis, and mitochondrial dysfunction in S-(1,2-dichlorovinyl)-L-cysteine sulfoxide-induced cytotoxicity in primary cultures of human renal proximal tubular cells. *J Pharmacol Exp Therap*. 2003;305:1163–1172.
Lash LH, Putt DA, Hueni SE, Horwitz BP. Molecular markers of trichloroethylene-induced toxicity in human kidney cells. *Toxicol Appl Pharmacol*. 2005;206:157–168.
Lash LH, Putt DA, Parker JC. Metabolism and tissue distribution of orally administered trichloroethylene in male and female rats: identification of glutathione- and cytochrome P-450-derived metabolites in liver, kidney, blood, and urine. *J Toxicol Environ Health A*. 2006;69: 1285–1309.
Lash LH, Qian W, Putt DA, et al. Renal and hepatic toxicity of trichloroethylene and its glutathione-derived metabolites in rats and mice: Sex-, species- and tissue-dependent differences. *J Pharmacol Exp Therap*. 2001;297:155–164.
Lash LH, Qian W, Putt DA, et al. Renal toxicity of perchloroethylene and S-(1,2,2-trichlorovinyl)glutathione in rats and mice: Sex- and species-dependent differences. *Toxicol Appl Pharmacol*. 2002;179:163–171.
Lash LH, Sausen PJ, Duescher RJ. Roles of cysteine β-lyase and S-oxidase in nephrotoxicity: studies with S-(1,2-dichlorovinyl)-4-cysteine and S-(1,2-dichlorovinyl)-L-cysteine sulfoxide. *J Pharmacol Exp Therap*. 1994;269:374–383.
Laughter AR, Dunn CS, Swanson CL, et al. Role of the peroxisome proliferators-activated receptor alpha (PPARalpha) in responses to trichloroethylene and metabolites, trichloroacetate and dichloroacetate in mouse liver. *Toxicology*. 2004;203:83–98.
Lauwerys R, Herbrand J, Buchet JP, et al. Health surveillance of workers exposed to tetrachloroethylene in dry cleaning shops. *Int Arch Occup Environ Health*. 1983;52: 69–77.
Leavens TL, Borghoff SJ. Physiologically based pharmacokinetic model of methyl tertiary butyl ether and tertiary butyl alcohol dosimetry in male rats based on binding to α2u-globulin. *Toxicol Sci*. 2009;109:321–335.
Leblond F, Guevin C, Demers C, et al. Downregulation of hepatic cytochrome P450 in chronic renal failure. *J Am Soc Nephrol*. 2001;12:326–332.
Lecuyer M, Laquerriere A, Bekri S, et al. PLGF, a placental marker of fetal brain defects alter in utero alcohol exposure. *Acta Neuropathol Commun*. 2017;6:44.
Lee BL, Yang XF, New AL, Ong CN. Liquid chromatographic determination of urinary 2-thiothiazlidine-4-carboxylic acid, a biomarker of carbon disulphide exposure. *J Chromatogr B*. 1995;668:265–272.
Lee EW, Terzo TS, D'Arcy JB, et al. Lack of blood formate accumulation in humans following exposure to methanol vapor at the current permissible exposure limit of 200 ppm. *Am Ind Hyg Assoc J*. 1992;53:99–104.
Lee JS, Ward WO, Wolf DC, et al. Coordinated changes in xenobiotic metabolizing enzyme gene expression in aging male rats. *Toxicol Sci*. 2008;106:263–283.
Lee KM, Bruckner JV, Muralidhara S, Gallo JM. Characterization of presystemic elimination of trichloroethylene and its nonlinear kinetics in rats. *Toxicol Appl Pharmacol*. 1996;139:262–271.
Lee KM, Dill JA, Chou BJ, Roycroft JH. Physiologically based pharmacokinetic model for chronic inhalation of 2-butoxyethanol. *Toxicol Appl Pharmacol*. 1998;153:211–226.
Lee LJ, Chung CW, Ma YC, et al. Increased mortality odds ratio of male liver cancer in a community contaminated by chlorinated hydrocarbons in groundwater. *Occup Environ Med*. 2003;60:364–369.
Lee RD, An SM, Kim SS, et al. Neurotoxic effects of alcohol and acetaldehyde during embryonic development. *J Toxicol Environ Health A*. 2005;68:2147–2162.
Lee SL, Chau GY, Yao CT, et al. Functional assessment of human alcohol dehydrogenase family in ethanol metabolism: Significance of first-pass metabolism. *Alcohol Clin Exp Res*. 2006;30:1132–1142.
Lemazurier E, Lecomte A, Robidel F, Bois FY. Propylene glycol monomethyl ether. A three-generation study of isomer beta effects on reproduction and developmental parameters in rats. *Toxicol Ind Health*. 2005;21:33–40.
Lenk W, Lohr D, Sonnenbichler J. Pharmacokinetics and biotransformation of diethylene glycol and ethylene glycol in the rat. *Xenobiotica*. 1989;19:961–979.
Leth PM, Gregersen M. Ethylene glycol poisoning. *Forensic Sci Int*. 2005;155:179–184.
Leung H-W, Paustenbach DJ. Physiologically based pharmacokinetic and pharmacodynamic modeling in health risk assessment and characterization of hazardous substances. *Toxicol Lett*. 1995;79:55–65.
Lewis DFV, Ioannides C, Parke DV. Cytochrome P450 and species differences in xenobiotic metabolism and activation of carcinogen. *Environ Health Perspect*. 1998;106:633–641.
Lewis JG, Graham DG, Valentine WM, et al. Exposure of C57BL/6 mice to carbon disulfide induces early lesions of atherosclerosis and enhances arterial fatty deposits induced by a high fat diet. *Toxicol Sci*. 1999;49:124–132.
Li G-L, Linet MS, Hayes RB, et al. Gender differences in hematopoietic and lymphoproliferative disorders and other cancer risks by major occupational group among workers exposed to benzene in China. *J Occup Med*. 1994;36:875–881.
Li K, Jing Y, Yang C, et al. Increased leukemia-associated gene expression in benzene-exposed workers. *Sci Rep*. 2014;4:5369.
Li L-H, Wine RN, Miller DS, et al. Protection against methoxyacetic-acid-induced spermatocyte apoptosis with calcium channel blockers in cultured rat seminiferous tubules: Possible mechanisms. *Toxicol Appl Pharmacol*. 1997;144:105–119.
Li Y, McLaren MC, McMartin KE. Involvement of urinary proteins in the rat strain differences in sensitivity to ethylene glycol-induced renal toxicity. *Am J Physiol Renal Physiol*. 2010;299:F605–F615.
Li Y, McMartin KE. Strain differences in urinary factors that promote calcium oxalate crystal formation in the kidneys of ethylene glycol-treated rats. *Am J Physiol Renal Physiol*. 2009;296:F1080–F1087.
Liao KH, Tan Y-M, Conolly RB, et al. Bayesian estimation of pharmacokinetic and pharmacodynamics parameters in a mode-of-action-based cancer risk assessment for chloroform. *Risk Anal*. 2007;27: 1535–1551.
Licata AC, Dekant W, Smith CE, Borghoff SJ. A physiologically based pharmacokinetic model for methyl *tert* -butyl ether in humans: Implementing sensitivity and variability analyses. *Toxicol Sci*. 2001;62:191–204.
Lieber CS. Cytochrome P4502E1: its physiological and pathological role. *Physiol Rev*. 1997;77:517–544.
Lieber CS. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. *Alcohol*. 2004;34:9–19.
Lilly PD, Thorton-Manning JR, Gargas ML, et al. Kinetic characterization of CYP2E1 inhibition in vivo and in vitro by the chloroethylenes. *Arch Toxicol*. 1998;72:609–621.
Lim TY, Poole RL, Pageler NM. Propylene glycol toxicity in children. *J Pediatr Pharmacol Ther*. 2014;19:277–282.
Limaye PB, Apte UM, Shankar K, et al. Calpain release from dying hepatocytes mediates progression of acute liver injury induced by model hepatotoxicants. *Toxicol Appl Pharmacol*. 2003;191:211–226.
Limon-Flores AY, Chacon-Salinas R, Ramos G, Ullrich SE. Mast cells mediate the immune suppression induced by dermal exposure to JP-8 jet fuel. *Toxicol Sci*. 2009;112:144–152.
Lin B, Ritchie GD, Rossi J III, Pancrazio JJ. Gene expression profiles in the rat central nervous system induced by JP-8 jet fuel vapor exposure. *Neurosci Lett*. 2004;363:233–238.
Lin JH, Lu AYH. Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *Ann Rev Pharmacol Toxicol*. 2001;41:535–567.

Lin B, Ritchie GD, Rossi J III, Pancrazio JJ. Identification of target genes responsive to JP-8 exposure in the rat central nervous system. *Toxicol Ind Health*. 2001a;17:262–269.

Lin Y, Egeghy PP, Rappaport SM. Relationships between levels of volatile organic compounds in air and blood from the general population. *J Exposure Sci Environ Epidemiol*. 2008;18:421–429.

Lin Y-S, Smith TJ, Kelsey KT, Wypij D. Human physiological factors in respiratory uptake of 1,3-butadiene. *Environ Health Perspect*. 2001b;109:921–926.

Lindstrom AB, Yeowell-O'Connell K, Waidyanatha S, et al. Measurement of benzene oxide in the blood of rats following administration of benzene. *Carcinogenesis*. 1997;18:1637–1641.

Lington AW, Dodd DE, Ridlon SA, et al. Evaluation of 13-week inhalation toxicity study on methyl *t* -butyl ether (MTBE) in Fischer 344 rats. *J Appl Toxicol*. 1997;17(S1):S37–S44.

Linhart I, Gut I, Smejkal J, Novak J. Biotransformation of styrene in mice. Stereochemical aspects. *Chem Res Toxicol*. 2000;13:36–44.

Lipscomb JC. Evaluating the relationship between variance in enzyme expression and toxicant concentration in health risk assessment. *Human Ecol Risk Assess*. 2004;10:39–55.

Lipscomb JC, Teuschler LK, Swartout J, et al. The impact of cytochrome P450 2E1-dependent metabolic variance on a risk-relevant pharmacokinetic outcome in humans. *Risk Anal*. 2003;23:1221–1238.

Liu L, Zhang Q, Feng J, et al. The study of DNA oxidative damage in benzene-exposed workers. *Mutation Res*. 1996;370:145–150.

Liu S-L, Esposti SD, Yao T, et al. Vitamin E therapy of acute CCl_4-induced hepatic injury in mice is associated with inhibition of nuclear factor kappa B binding. *Hepatology*. 1995;22:1474–1481.

Liu S, Yao Y, Lu S, et al. The role of renal proximal tubule P450 enzymes in chloroform-induced nephrotoxicity: utility of renal specific P450 reductase knockout mouse models. *Toxicol Appl Pharmacol*. 2013;272:230–237.

Liu Y, Bartlett MG, White CA, et al. Presystemic elimination of trichloroethylene in rats following environmentally relevant oral exposures. *Drug Metab Dispos*. 2009;37:1994–1998.

Liu Y, Zhang J-W, Li W, et al. Ginsenoside metabolites, rather than naturally occurring ginsenosides, lead to inhibition of human cytochrome P450 enzymes. *Toxicol Sci*. 2006;91:356–364.

Lock EA, Barth JL, Argraves SW, Schnellmann RG. Changes in gene expression in human renal proximal tubule cells exposed to low concentrations of S-(1,2-dichlorovinyl)-l-cysteine, a metabolite of trichloroethylene. *Toxicol Appl Pharmacol*. 2006;216:319–330.

Lock EA, Reed CJ. Trichloroethylene: Mechanisms of renal toxicity and renal cancer and relevance to risk assessment. *Toxicol Sci*. 2006;91:313–331.

Lock EA, Zhang J, Checkoway H. Solvents and Parkinson's disease: a systematic review of toxicological and epidemiological evidence. *Toxicol Appl Pharmacol*. 2013;266:345–355.

Lockard VG, Mehendale HM, O'Neal RM. Chlordecone-induced potentiation of carbon tetrachloride hepatotoxicity: a morphometric and biochemical study. *Exp Mol Pathol*. 1983;39:246–256.

Lockley DJ, Howes D, Williams FM. Cutaneous metabolism of glycol ethers. *Arch Toxicol*. 2005;79:160–168.

Lof A, Johanson G. Toxicokinetics of organic solvents: a review of modifying factors. *Crit Rev Toxicol*. 1998;28:571–650.

Lopez-Diazguerrero NE, Luna-Lopez A, Gutierrez-Ruiz MC, et al. Susceptibility of DNA to oxidative stressors in young and aging mice. *Life Sci*. 2005;77:2840–2854.

Lopreato GF, Phelan R, Borghese CM, et al. Inhaled drugs of abuse enhance serotonin-3 receptor function. *Drug Alcohol Depend*. 2003;70:11–15.

Lorente C, Cordier S, Bergeret A, et al. Maternal occupational risk factors for oral clefts. Occupational exposure and congenital malformation working group. *Scand J Work Environ Health*. 2000;26:137–145.

Lorenz J, Glatt HR, Fleishmann R, et al. Drug metabolism in man and its relationship to that in three rodent species: monooxygenase, epoxide hydrolase, and glutathione *S*-transferase activities in subcellular fractions of lung and liver. *Biochem Med*. 1984;32:43–56.

Louisse J, Bai Y, Verwei M, et al. Decrease of intracellular pH as possible mechanism of embryotoxicity of glycol ether alkoxyacetic acid metabolites. *Toxicol Appl Pharmacol*. 2010a;245:236–243.

Louisse J, de Jong E, van de Sandt JJM, et al. The use of *in vitro* toxicity data and physiologically based kinetic modeling to predict dose-response curves for *in vivo* developmental toxicity of glycol ethers in rat and man. *Toxicol Sci*. 2010b;118:470–484.

Loury DJ, Smith-Oliver T, Butterworth BE. Assessment of unscheduled DNA and replicative DNA synthesis in rat kidney cells exposed in vitro or in vivo to unleaded gasoline. *Toxicol Appl Pharmacol*. 1987;87:127–140.

Lu Y, Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. *Free Radic Biol Med*. 2008;44: 723–738.

Lu Y, Rieth S, Lohitnavy M, et al. Application of PBPK modeling in support of the derivation of toxicity reference values for 1,1,1-trichloroethane. *Regul Toxicol Pharmacol*. 2008;50:249–260.

Lu Y, Wu D, Wang X, et al. Chronic alcohol-induced liver injury and oxidant stress are decreased in cytochrome P4502E1 knockout mice and restored in humanized cytochrome P4502E1 knock-in mice. *Free Radic Biol Med*. 2010;49:1406–1416.

Lubman DI, Yucel M, Lawrence AJ. Inhalant abuse among adolescents: neurobiological considerations. *Br J Pharmacol*. 2008;154:316–326.

Lucas D, Farez C, Bardou LG, et al. Cytochrome P450 2E1 activity in diabetic and obese patients as assessed by chlorzoxazone hydroxylation. *Fundam Clin Pharmacol*. 1998;12:553–558.

Luke NS, Sams R, DeVito MJ, et al. Development of a quantitative model incorporating key events in a hepatotoxic mode of action to predict tumor incidence. *Toxicol Sci*. 2010;115:253–266.

Luo JC, Chang HY, Chang SJ, et al. Elevated triglyceride and decreased high density lipoprotein level in carbon disulfide workers in Taiwan. *J Occup Environ Med*. 2003;45:73–78.

Lykke AWJ, Stewart BW. Fibrosing alveolitis (pulmonary interstitial fibrosis) evoked by experimental inhalation of gasoline vapours. *Experientia*. 1978;34:498.

Lynge E, Andersen A, Rylander L, et al. Cancer in persons working in dry cleaning in the Nordic countries. *Environ Health Perspect*. 2006;114:213–219.

Lyons MA, Yang RSH, Mayeno AN, Reisfeld B. Computational toxicology of chloroform: reverse dosimetry using Bayesian inference, Markov chain Monte Carlo simulation, and human biomonitoring data. *Environ Health Perspect*. 2008;116:1040–1046.

Ma JD, Nafziger AN, Bertino JS Jr. Genetic polymorphisms of cytochrome P450 enzymes and the effect on interindividual, pharmacokinetic variability in extensive metabolizers. *J Clin Pharmacol*. 2004;44: 447–456.

MacFarland HN, Ulrich CE, Holdsworth CE, et al. A chronic inhalation study with unleaded gasoline vapor. *J Am Col Toxicol*. 1984;3:231–248.

Mainwaring GW, Williams SM, Foster JR, et al. The distribution of Theta-class glutathione S-transferase in the liver and lung of mouse, rat and human. *Biochem J*. 1996;318: 297–303.

MAK. List of MAK and BAT Values. Deutsche Forschungsgemeinschaft. Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area. Report No. 47, 2011. Available at: http://www.dfg.de.

Makri A, Goveia M, Balbus J, Parkin R. Children's susceptibility to chemicals: a review by developmental stage. *J Toxicol Environ Health Part B*. 2004;7:417–435.

Maldonado G, Delzell E, Tyl RW, Sever LE. Occupational exposure to glycol ethers and human congenital malformations. *Int Arch Occup Environ Health*. 2003;76:405–423.

Mally A, Walker CL, Everitt JI, et al. Analysis of renal cell transformation following exposure to trichloroethene in vivo and its metabolite *S*-(dichlorovinyl)-l-cysteine in vitro. *Toxicology*. 2006;224:108–118.

Maltoni C, Cotti G, Patella V. Results of long-term carcinogenicity bioassays on Sprague-Dawley rats of methyl chloroform, administered by ingestion. *Acta Oncol.* 1986;7:101–107.

Mandel JH, Kelsh MA, Mink PJ. Occupational trichloroethylene exposure and non-Hodgkin's lymphoma: a meta-analysis and review. *Occup Environ Med*. 2006;63:597–607.

Mandel JS, McLaughlin JK, Schlehofer B, et al. International renal-cell cancer study. IV. Occupation. *Int J Cancer*. 1995;61:601–605.

Mandrekar P, Szabo G. Signaling pathways in alcohol-induced liver inflammation. *J Hepatol*. 2009;50:1258–1266.

Manfra L, Tornambe A, Savorelli F, et al. Ecotoxicity of diethylene glycol and risk assessment for marine environment. *J Hazard Mater*. 2015;284:130–135.

Mangoni AA, Jackson SHD. Age-related changes in pharmacokinetics and pharmacodynamics: basic principles and practical applications. *Br J Clin Pharmacol*. 2003;57:6–14.

Mani C, Freeman S, Nelson DO, et al. Species and strain comparisons in the macromolecular binding of extremely low doses of [^{14}C] benzene in rodents using accelerator mass spectrophotometry. *Toxicol Appl Pharmacol*. 1999;159:83–90.

Manibusan MK, Odin M, Eastmond DA. Postulated carbon tetrachloride mode of action: a review. *J Environ Sci Health C*. 2007;25:185–209.

Manikkam M, Guerrero-Bosagna C, Tracey R, et al. Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PLos One*. 2012;7:e31901.

Manno M, Rezzadore M, Grossi M, Sbrana C. Potentiation of occupational carbon tetrachloride toxicity by ethanol abuse. *Hum Exp Toxicol*. 1996;15:294–300.

Marino DJ, Clewell HJ, Gentry PR, et al. Revised assessmenmt of cancer risk to dichloromethane. I. Bayesian PBPK and dose-response modeling in mice. *Regul Toxicol Pharmacol*. 2006;45:44–54.

Maronpot RR, Devereux TR, Hegi M, et al. Hepatic and pulmonary carcinogenicity of methylene chloride in mice: a search for mechanisms. *Toxicology*. 1995;102:73–81.

Marraffa JM, Holland MG, Stork CM, et al. Diethylene glycol: widely used solvent presents serious poisoning potential. *J Emerg Med*. 2008;35:401–406.

Marrubini G, Castoldi AF, Coccini T, Manzo L. Prolonged ethanol ingestion enhances benzene myelotoxicity and lowers urinary concentrations of benzene metabolite levels in CD-1 mice. *Toxicol Sci*. 2003;75:16–24.

Martin SA, Campbel JL, Tremblay RT, Fisher JW. Development of a physiologically based pharmacokinetic model for inhalation of jet fuels in the rat. *Inhal Toxicol*. 2012;24:1–26.

Martin SA, Tremblay RT, Brunson KF, et al. Characterization of a nose-only inhalation exposure system for hydrocarbon mixtures and jet fuels. *Inhal Toxicol*. 2010;22:382–393.

Martin-Amat G, McMartin KE, Hayreh SS, et al. Methanol poisoning: ocular toxicity produced by formate. *Toxicol Appl Pharmacol*. 1978;45:201–208.

Martinasevic K, Green MD, Baron J, Tephly TR. Folate and 10-formyltetrahydrofolate dehydrogenase in human and rat retina: Relation to methanol toxicity. *Toxicol Appl Pharmacol*. 1996;141:373–381.

Marty MS, Andrus AK, Bell MP, et al. Cholinesterase inhibition and toxicokinetics in immature and adult rats after acute or repeated exposures to chloropyrifos or chlorpyrifos-oxon. *Regul Toxicol Pharmacol*. 2012;63:209–224.

Mason TJ, McKay FW. U.S. Cancer Mortality by County:1950–1969. NIH Publ. No. 74-615. National Cancer Institute, Bethesda, MD, 1975.

Mathews JM, Parker MK, Matthews HB. Metabolism and disposition of diethylene glycol in rat and dog. *Drug Metab Dispos*. 1991;19:1066–1070.

Matonoski G, Elliott E, Tao X, et al. Lymphohematopoietic cancers and butadiene and styrene exposure in synthetic rubber manufacture. *Ann NY Acad Sci*. 1997;837:157–169.

Mattie DR, Alden CL, Newell TK, et al. A 90-day continuous vapor inhalation toxicity study of JP-8 jet fuel followed by 20–21 months of recovery in Fischer 344 rats and C57BL/6 mice. *Toxicol Pathol*. 1991;19:77–87.

Mattie DR, Sterner TR. Past, present and emerging toxicity issues for jet fuel. *Toxicol Appl Pharmacol*. 2011;254:127–132.

Mattsson JL, Albee RR, Lomax LG, et al. Neutotoxicologic examination of rats exposed to 1,1,1-trichloroethane vapor for 13 weeks. *Neurotoxicol Teratol*. 1993;15:313–326.

Maule AL, Proctor SP, Blount BC, et al. Volatile organic compounds in blood as biomarkers of exposure to JP-8 jet fuel among US Air Force personnel. *JOEM*. 2016;58:24–29.

Maurya PK, Rizvi SI. Age-dependent changes in glutathione-S-transferase: correlation with total plasma antioxidant potential and red cell intracellular glutathione. *Ind J Clin Biochem*. 2010;25:398–400.

McCaffery P, Tempst P, Lara G, Drager U. Aldehyde dehydrogenase as a positional marker in the retina. *Development*. 1991;112:693–702.

McCredie M, Stewart JH. Risk factors for kidney cancer in New South Wales, IV. Occupation. *Br J Ind Med*. 1993;50:349–354.

McDougal JN, Garrett CM, Amato CM, Berberich SJ. Effects of brief cutaneous JP-8 jet fuel exposures on time course of gene expression in the epidermis. *Toxicol Sci*. 2007;95:495–510.

McDougal JN, Jepson GW, Clewell HJ III, et al. Dermal absorption of organic chemical vapors in rats and humans. *Fundam Appl Toxicol*. 1990;14:299–308.

McDougal JN, Pollard DL, Weisman W, et al. Assessment of skin absorption and penetration of JP-8 jet fuel and its components. *Toxicol Sci*. 2000;55:247–255.

McDougal JN, Robinson PJ. Assessment of dermal absorption and penetration of components of a fuel mixture (JP-8). *Sci Total Environ*. 2002;288:23–30.

McGregor D. Methyl tertiary-butyl ether: studies for potential human health hazards. *Crit Rev Toxicol*. 2006;36:319–358.

McGregor D. Ethyl tertiary-butyl ether: a toxicological review. *Crit Rev Toxicol*. 2007;37:287–312.

McHale CM, Zhang I, Lan Q, et al. Global gene expression profiling of a population exposed to a range of benzene levels. *Environ Health Perspect*. 2011;119:628–634.

McHale CM, Zhang I, Smith MT. Current understanding of the mechanism of benzene-induced leukemia in humans: Implications for risk assessment. *Carcinogenesis*. 2012;33:240–252.

McKee RH, Vergnes JS, Galvin JB, et al. Assessment of the in vivo mutagenic potential of methyl tertiary-butyl ether. *J Appl Toxicol*. 1997;17(S1):S31–S367.

McLean AJ, Le Couteur DG. Aging biology and geriatric clinical pharmacology. *Pharmacol Rev*. 2004;56:163–184.

McMahon TF, Medinsky MA, Birnbaum LS. Age-related changes in benzene disposition in male C57BL/6N mice described by a physiologically based model. *Toxicol Lett*. 1994;74:241–253.

McMartin K. Are calcium oxalate crystals involved in the mechanism of acute renal failure in ethylene glycol poisoning? *Clin Toxicol*. 2009;47:859–869.

McMartin KE, Wallace KB. Calcium oxalate monohydrate, a metabolite of ethylene glycol, is toxic for rat renal mitochondrial function. *Toxicol Sci*. 2005;84:195–200.

McQuade DJ, Dargan PI, Wood DM. Challenges in the diagnosis of ethylene glycol poisoning. *Ann Clin Biochem*. 2014;51:167–178.

Mebus CA, Welsch F, Working PK. Attenuation of 2-methoxyethanol induced testicular toxicity in the rat by simple physiological compounds. *Toxicol Appl Pharmacol*. 1989;99:110–121.

Medeiros AM, Bird MG, Witz G. Potential biomarkers of benzene exposure. *J Toxicol Environ Health*. 1997;51:519–539.

Medinsky MA, Dorman DC. Recent developments in methanol toxicity. *Toxicol Lett* 1995;82/83:707–711.

Medinsky MA, Dorman DC, Bond JA, et al. Pharmacokinetics of methanol and formate in female cynomolgus monkeys exposed to methanol vapors. *Res Rep Health Effects Inst*. 1997;77:1–38.

Medinsky MA, Kenyon EM, Seaton MJ, Schlosser PM. Mechanistic considerations in benzene physiological model development. *Environ Health Perspect*. 1996;104(suppl 6):1399–1404.

Medinsky MA, Sabourin PJ, Lucier G, et al. A physiological model for simulation of benzene metabolism by rats and mice. *Toxicol Appl Pharmacol*. 1989;99:193–206.

Medinsky MA, Schlosser PM, Bond JA. Critical issues in benzene toxicity and metabolism: The effect of interactions with other organic chemicals on risk assessment. *Environ Health Perspect*. 1994;102(suppl 9):119–124.

Medinsky MA, Singh G, Bechtold WE, et al. Disposition of three glycol ethers administered in drinking water to male F344/N rats. *Toxicol Appl Pharmacol*. 1990;102:443–455.

Meek ME, Klaunig JE. Proposed mode of action of benzene-induced leukemia: interpreting available data and identifying critical data gaps for risk assessment. *Chem-Biol Interact*. 2010;184:279–285.

Mehendale HM. Tissue repair: an important determinant of final outcome of toxicant-induced injury. *Toxicol Pathol*. 2005;33:41–51.

Mehlman MA. Carcinogenicity of methyl-tertiary butyl ether in gasoline. *Ann NY Acad Sci*. 2002;982:149–159.

Melnick RL, Kohn MC. Possible mechanisms of induction of renal tubule cell neoplasms in rats associated with α_{2u}-globulin: Role of protein accumulation versus ligand delivery to the kidney. In: Cappen CC, Dybing E, Rice JM, Wilbourn JD, eds. *Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis*. Lyons, France, 1999, IARC Publ No 147:119–137.

Mennear JH. Carcinogenicity studies on MTBE: critical review and interpretation. *Risk Anal*. 1997;17:673–681.

Mennear JH, McConnell EE, Huff JE, et al. Inhalation and carcinogenesis studies of methylene chloride (dichloromethane) in F344/N rats and B6C3F1 mice. *Ann NY Acad Sci*. 1988;534:343–351.

Merdink JL, Robison LM, Stevens DK, et al. Kinetics of chloral hydrate and its metabolites in male human volunteers. *Toxicology*. 2008;245:130–140.

Messiha FS, Price J. Properties and regional distribution of ocular aldehyde dehydrogenase in the rat. *Neurobehav Toxicol Teratol*. 1983;5:251–254.

Mezey E. Metabolic effects of ethanol. *Fed Proc*. 1985;44:134–138.

Micu AL, Miksys S, Sellers DL, et al. Rat hepatic CYP2E1 is induced by very low nicotine doses: An investigation of induction, time course, dose response and mechanism. *J Pharmacol Exp Therap*. 2003;306:941–947.

Mihaich E, Erler S, Le Blanc G, Gallagher S. Short-term fish reproduction assays with methyl tertiary butyl ether with zebrafish and fathead minnow: implications for evaluation of potential for endocrine activity. *Environ Toxicol Chem*. 2015;34:2013–2022.

Mihic SJ, McQuilkin SJ, Eger EI, et al. Potentiation of γ-aminobutyric acid type A receptor-mediated chloride currents by novel halogenated compounds correlated with their abilities to induce general anesthesia. *Mol Pharmacol*. 1994;46:851–857.

Miller AM, Horiguchi N, Jeong W-I, et al. Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines. *Alcohol Clin Exp Res*. 2011;35:787–793.

Miller JH, Minard KR, Wind RA, et al. In vivo MRI measurements of tumor growth induced by dichloroacetate: implications for mode of action. *Toxicology*. 2000;145:115–125.

Miller RR, Hermann EA, Young JT, et al. Ethylene glycol monomethyl ether and propylene glycol monomethyl ether: Metabolism, disposition, and subchronic inhalation toxicity studies. *Environ Health Perspect*. 1984;57:233–239.

Mitchell SJ, Kane AE, Hilmer SN. Age-related changed in the hepatic pharmacology and toxicology of paracetamol. *Current Gerontol Geriatr Res*. 2011;2011:1–14.

Mitschke D, Reichel A, Fricker G, Moenning U. Characterization of cytochrome P450 expression along the entire length of the intestine of male and female rats. *Drug Metab Dispos*. 2008;36:1039–1045.

Mohamed MF, Kang D, Aneja VP. Volatile organic compounds in some urban locations in United States. *Chemosphere*. 2002;47:863–882.

Monks TJ, Butterworth M, Lau SS. The fate of benzene-oxide. *Chem-Biol Interact*. 2010;184: 201–206.

Monster AC. Difference in uptake, elimination, and metabolism in exposure to trichloroethylene, 1,1,1-trichloroethane and tetrachloroethylene. *Int Arch Occup Environ Health*. 1979;42:311–317.

Monteiro-Riviere N, Inman A, Riviere J. Effects of short-term high-dose and low-dose exposure to Jet A, JP-8 and JP-8 + 100 jet fuels. *J Appl Toxicol*. 2001;21:485–494.

Monteiro-Riviere NA, Inman AO, Riviere JE. Skin toxicity of jet fuels: ultra-structural studies and the effects of substance P. *Toxicol Appl Pharmacol*. 2004;195:339–347.

Moore LE, Boeffetta P, Karami S, et al. Occupational trichloroethylene exposure and renal carcinoma risk: Evidence of genetic susceptibility by reductive metabolism gene variants. *Cancer Res*. 2010;70: 6527–6536.

Moore MM, Harrington-Brock K. Mutagenicity of trichloroethylene and its metabolites: Implications for the risk assessment of trichloroethylene. *Environ Health Perspect*. 2000;108(suppl 2):215–223.

Moorman MP, Sills RC, Collins BJ, Morgan DL. Carbon disulfide neurotoxicity in rats: II. Toxicokinetics. *Neurotoxicology*. 1998;19:89–97.

Moran MJ, Zogorski JS, Squillace PJ. Chlorinated solvents in groundwater of the United States. *Environ Sci Technol*. 2007;41:74–81.

Moreau CL, Kerns W II, Tomaszewski CA, et al. Glycolate kinetics and hemodialysis clearance in ethylene glycol poisoning. META Study Group. *J Toxicol Clin Toxicol*. 1998;36:659–666.

Morford LL, Henck JW, Breslin WJ, DeSesso JM. Hazard identification and predictability of children's health risk from animal data. *Environ Health Perspect*. 2004;112: 266–271.

Morgan DL, Cooper SW, Carlock DL, et al. Dermal absorption of neat and aqueous volatile organic chemicals in the Fischer 344 rat. *Environ Res*. 1991;55:51–63.

Morgan DJ, McLean AJ. Clinical pharmacokinetic and pharmacodynamics considerations in patients with liver disease. An update. *Clin Pharmacokinet*. 1995;29:370–391.

Morimura K, Cheung C, Ward JM, et al. Differential susceptibility of mice humanized for peroxisome proliferator-activated receptor (alpha) to Wy-14,643-induced liver carcinogenesis. *Carcinogenesis*. 2006;27: 1074–1080.

Morio LA, Chiu H, Sprowles KA, et al. Distinct roles of tumor necrosis factor-α and nitric oxide in acute liver injury induced by carbon tetrachloride in mice. *Toxicol Appl Pharmacol*. 2001;172:44–51.

Moro AM, Charao MF, Brucker N, et al. Genotoxicity and oxidative stress in gasoline station attendants. *Mutat Res*. 2013;754:63–70.

Morshed KM, Nagpaul JP, Majumdar S, et al. Kinetics of propylene glycol elimination and metabolism in the rat. *Biochem Med Metab Biol*. 1988;39:90–97.

Mortuga T, Chen C, White CA, et al. Toxicokinetic of deltamethrin: Dosage dependency which effects and low-dose age equivalent dosimetry in rate. *Toxicol Sci*. 2018;162:327–336.

Moser GJ, Wolf DC, Sar M, et al. Methyl tertiary butyl ether-induced endocrine alterations in mice are not mediated through the estrogen receptor. *Toxicol Sci*. 1998;41:77–87.

Moser GJ, Wolf DC, Wong BA, Goldsworthy TL. Loss of tumor-promoting activity of unleaded gasoline in *N*-nitrosodiethylamine-initiated ovariectomized B6C3F1 mouse liver. *Carcinogenesis*. 1997;18:1075–1083.

Moser GJ, Wong BA, Wolf DC, et al. Comparative short-term effects of methyl tertiary butyl ether and unleaded gasoline vapor in B6C3F1 mice. *Fundam Appl Toxicol*. 1996a;31:173–183.

Moser GJ, Wong BA, Wolf DC, et al. Methyl tertiary butyl ether lacks tumor-promoting activity in *N*-nitrosodiethylamine-initiated B6C3F1 female mouse liver. *Carcinogenesis*. 1996b;17:2753–2761.

Moser GJ, Wolf DC, Harden R, et al. Cell proliferation and regulation of negative growth factors in mouse liver foci. *Carcinogenesis*. 1996c;17:1835–1840.

Mugford CA, Kedderis GL. Sex-dependent metabolism of xenobiotics. *Drug Metab Rev*. 1998;30:441–498.

Muhammad F, Monteiro-Riviere NA, Riviere JE. Comparative in vivo toxicity of topical JP-8 jet fuel and its individual hydrocarbon components: identification of tridecane and tetradecane as key constituents responsible for dermal irritation. *Toxicol Pathol*. 2005;33:258–266.

Mulkey ME. The determination of whether dithiocarbamate pesticides share a common mechanism of toxicity. Internal memorandum, U.S. EPA Office of Pesticide Programs, December 19, 2001:1–38. Available at: http://www.epa.gov/oppsrrdl/cumulative/dithiocarb.pdf.

Muller G, Spassowski M, Henschler D. Metabolism of trichloroethylene in man. III. Interaction of trichloroethylene and ethanol. *Arch Toxicol*. 1975;33:173–189.

Mundt KA, Birk T, Burch MT. Critical review of the epidemiological literature on occupational exposure to perchloroethylene and cancer. *Int Arch Occup Environ Health*. 2003;76:473–491.

Murray FJ, Schwetz BA, McBride JG, Staples RE. Toxicity of inhaled chloroform in pregnant mice and their offspring. *Toxicol Appl Pharmacol*. 1979;50:515–522.

Murray M. P450 enzymes. Inhibition mechanisms, genetic regulation and effects of liver disease. *Clin Pharmacokinet*. 1992;23:132–146.

Murry DJ, Crom WR, Reddick WE, et al. Liver volume as a determinant of drug clearance in children and adolescents. *Drug Metab Dispos*. 1995;23:1110–1116.

Mutti A, Alinovi R, Bergamaschi E, et al. Nephropathies and exposures to perchloroethylene in dry-cleaners. *Lancet*. 1992;340:189–183.

Muttray A, Kurten R, Jung D, et al. Acute effects of 200 ppm 1,1,1-trichloroethane on the human EEG. *Eur J Med Res*. 2000;5:375–384.

Nagano K, Kano H, Arito H, et al. Enhancement of renal carcinogenicity by combined inhalation and oral exposures to chloroform in male rats. *J Toxicol Environ Health, Part A*. 2006;69:1827–1842.

Nagano K, Sasaki T, Umeda Y, et al. Inhalation carcinogenicity and chronic toxicity of carbon tetrachloride in rats and mice. *Inhal Toxicol*. 2007;19: 1089–1103.

Nakai JS, Stathopulos PB, Campbell GL, et al. Penetration of chloroform, trichloroethylene, and tetrachloroethylene through human skin. *J Toxicol Environ Health*. 1999;58:157–170.

Nakajima T. Cytochrome P450 isoforms and the metabolism of volatile hydrocarbons of low relative molecular mass. *J Occup Health*. 1997;39:83–91.

Nakajima T, Elovaara E, Okino T, et al. Different contributions of cytochrome P450 2E1 and P450 2B1/2 to chloroform hepatotoxicity in rat. *Toxicol Appl Pharmacol*. 1995;133:215–222.

Nakajima T, Koyama Y, Sato A. Dietary modification of metabolism and toxicity of chemical substances—with special reference to carbohydrate. *Biochem Pharmacol*. 1982;31:1005–1011.

Nakajima T, Okuyama S, Yonekura I, Sato A. Effects of ethanol and phenobarbital administration on the metabolism and toxicity of benzene. *Chem-Biol Interact*. 1985;55:23–38.

Nakajima T, Wang R-S. Induction of cytochrome P450 by toluene. *Int J Biochem*. 1994;26:1333–1340.

Nakajima T, Wang R-S, Elovaara E, et al. A comparative study on the contribution of cytochrome P450 isozymes to metabolism of benzene, toluene and trichloroethylene in rat liver. *Biochem Pharmacol*. 1992a;43:251–257.

Nakajima T, Wang R-S, Elovaara E, et al. Toluene metabolism by cDNA-expressed human hepatic cytochrome P450. *Biochem Pharmacol*. 1997;53:271–277.

Nakajima T, Wang R-S, Katakura Y, et al. Sex-, age- and pregnancy-induced changes in the metabolism of toluene and trichloroethylene in rat liver in relation to the regulation of cytochrome P450IIE1 and P450IIC11 content. *J Pharmacol Exp Therap*. 1992b;261:869–874.

NAS (National Academy of Sciences). *Pesticides in the Diets of Infants and Children*. National Research Council. Washington, DC: National Academy Press; 1993:1–12.

NAS (National Academy of Sciences). *Toxicologic Assessment of Jet-Propulsion Fuel 8*. Washington, DC: National Academies Press; 2003.

NAS (National Academy of Sciences). *Assessing the Human Health Risks of Trichloroethylene: Key Scientific Issues*. Washington, DC: National Academy Press; 2006.

NAS (National Academy of Sciences). *Mouse Liver Tumors: Benefits and Constraints on Use in Human Health Risk Assessment, Qualitative and Quantitative Aspects*. Risk Analysis Issues and Reviews Newsletter No. 2, 2008.

NAS (National Academy of Sciences). *Contaminated Water Supplies at Camp Lejeune: Assessing Potential Health Effects*. Washington, DC: National Academy Press; 2009.

NAS (National Academy of Sciences). *Review of the Environmental Protection Agency's Draft IRIS Risk Assessment of Tetrachloroethylene*. Washington, DC: National Academy Press; 2010.

NCI (National Cancer Institute). *Carcinogenesis Bioassay of Chloroform*. National Technical Information Service No. PB264018/AS. Bethesda, MD: NCI, 1976.

NCI (National Cancer Institute). *Bioassay of Tetrachloroethylene for Possible Carcinogenicity*. NCI: DHEW Publ. No. NIH 77-813, Bethesda, MD: NCI, 1977a.

NCI (National Cancer Institute). *Bioassay of 1,1,1-Trichloroethane for Possible Carcinogenicity*. DHEW Pub. No. NIH 77-803, NCI-CG-TR-3, Bethesda, MD, 1977b.

NCI (National Cancer Institute). *Bioassay of Styrene for Possible Carcinogenicity.* Technical Report Series No. 185. NIH 79-1741, NCI-CG-TR-185, Bethesda, MD, 1979.

NEDO (New Energy Development Organization). Toxicological research of methanol as a fuel for power station: Summary report on test with monkeys, rats and mice. *Tokyo*. 1987;1–296.

Nelson BK, Brightwell WS. Behavioral teratology of ethylene glycol monomethyl and monoethyl ethers. *Environ Health Perspect*. 1984;57:43–46.

Nelson BK, Vorhees CV, Scott WJ, Hastings L. Effects of 2-methoxyethanol on fetal development, postnatal behavior, and embryonic intracellular pH of rats. *Neurotoxicol Teratol*. 1989;11:273–284.

Nelson HH, Wiencke JK, Christiani DC, et al. Ethnic differences in the prevalence of the homozygous deleted genotype of glutathione *S*-transferase theta. *Carcinogenesis*. 1995;16:1243–1245.

Nelson JL, Haas CE, Habtemariam B, et al. A prospective evaluation of propylene glycol clearance and accumulation during continuous-infusion Lorazepam in critically ill patients. *J Intensive Care Med*. 2008;23:184–194.

Nessel CS. A comprehensive evaluation of the carcinogenic potential of middle distillate fuels. *Drug Chem Toxicol*. 1999;22:165–180.

Nessel CS, Freeman JJ, Forgash RC, McKee RH. The role of dermal irritation in the skin tumor promoting activity of petroleum middle distillates. *Toxicol Sci*. 1999;49:48–55.

Nett RJ, Cox-Ganser JM, Hubbs AF, et al. Non-malignant respiratory disease among workers in industries using styrene—a review of the evidence. *Am J Ind Med*. 2017;60:163–180.

Neuman MG, Malnick S, Maor Y, et al. Alcoholic liver disease: Clinical and translational research. *Exp Mol Pathol*. 2015;99:596–610.

NIDA (National Institute on Drug Abuse). *Research Report Series: Inhalant Abuse*. (NIH Publication No. 05-3818). Bethesda, MD, 2005:1–8.

Nielsen GD, Alarie Y. Sensory irritation, pulmonary irritation, and respiratory stimulation by airborne benzene and alkylbenzenes: Prediction of safe industrial exposure levels and correlation with their thermodynamic properties. *Toxicol Appl Pharmacol*. 1982;65:459–477.

Niemela O. Aldehyde–protein adducts in the liver as a result of ethanol-induced oxidative stress. *Front Biosci*. 1999;4:506–513.

NIOSH (National Institute of Occupational Safety and Health). 1981–83 National Occupational Exposure Survey (NOAES), 1989.

Nitschke KD, Burek JD, Bell TJ, et al. Methylene chloride: a 2-year inhalation toxicity and oncogenicity study in rats. *Fundam Appl Toxicol*. 1988;11:48–59.

Nolan RJ, Freshour NL, Rick DL, et al. Kinetics and metabolism of inhaled methyl chloroform (1,1,1-trichloroethane) in male volunteers. *Fund Appl Toxicol*. 1984;4:654–662.

Nolin TD, Naud J, Leblond FA, Pichette V. Emerging evidence of the impact of kidney disease on drug metabolism and transport. *Clin Pharmacol Ther*. 2008;83:898–903.

Nomiyama K, Nomiyama H. Respiratory retention, uptake and excretion of organic solvents in man. *Int Arch Arbeitsmed*. 1974;32:75–83.

Nong A, McCarver DG, Hines RN, Krishnan K. Modeling interchild differences in pharmacokinetics on the basis of subject-specific data on physiology and hepatic CYP2E1 levels: A case study with toluene. *Toxicol Appl Pharmacol*. 2006;214:78–87.

Normandin L, Ann Beaupre L, Salehi F, et al. Manganese distribution in the brain and neurobehavioral changes following inhalation exposure of rats to three chemical forms of manganese. *Neurotoxicology*. 2004;25:433–441.

NRC (National Research Council). *Acute Exposure Guideline Levels for Selected Airborne Chemicals: Vol 10*. Washington, DC: The National Academies Press. Available at: https://doi.org/10.17226/13247, 2011.

NTP (National Toxicology Program). *Toxicology and Carcinogenesis Studies of Tetrachloroethylene (Perchloroethylene) in F344/N Rats and B6C3F1 Mice*. Research Triangle Park, NC, 1986.

NTP (National Toxicology Program). *Toxicology and Carcinogenesis Studies of Trichloroethylene in Four Strains of Rats (ACI, August, Marshall, Osborne-Mendel) (Gavage Studies)*. NIH Publ. No. 88-2529. Research Triangle Park, NC, 1988.

NTP (National Toxicology Program). *Carcinogenesis Studies of Trichloroethylene (Without Epichlorohydrin) in F344/N Rats and B6C3F1 Mice (Gavage Studies)*. NIH Pub. No. 90-1779. Research Triangle Park, NC, 1990.

NTP (National Toxicology Program). *Toxicity Studies of a Chemical Mixture of 25 Groundwater Contaminants Administered in Drinking Water to F344/N Rats and B6C3F1 Mice*. (Tech Rep. Ser. No. 35; NIH Publ. No. 93-3384). Research Triangle Park, NC, 1993a.

NTP (National Toxicology Program). *Toxicology and Carcinogenesis Studies of Ethylene Glycol in B6C3F1 Mice (Feed Studies)*. NIH Publ. No 93-3144. Research Triangle Park, NC, 1993b.

NTP (National Toxicology Program). *Toxicology and Carcinogenesis Studies of t-Butyl Alcohol in F344/N Rats and B6C3F1 Mice* (Tech. Rep. Ser. No. 436; NIH Publ. No. 95-3167). Research Triangle Park, NC, 1995.

NTP (National Toxicology Program). NTP Technical Report on Renal Toxicity Studies of Selected Halogenated Ethanes Administered by

Gavage to F344/N Rats. (Toxicity Report Series 45, NIH Publ No. 96-3935), Research Triangle Park, NC, 1996.
NTP (National Toxicology Program). Chloroform. NTP reproductive assessment by continuous breeding study. *Environ Health Perspect*. 1997a;105(suppl 1):285–286.
NTP (National Toxicology Program). *Toxicology and Carcinogenesis Studies of Ethylbenzene in F344/N Rats and B6C3F1 Mice. (Inhalation Studies)*. Research Triangle Park, NC, 1997b.
NTP (National Toxicology Program). NTP Toxicology and Carcinogenesis Studies 2-Butoxyethanol (CAS No. 111-76-2) in F344/N Rats and B6C3F1 Mice (Inhalation Studies). *Natl Toxicol Program Tech Rep Ser*. 2000;484:1–290.
NTP (National Toxicology Program). *NTP-CERHR Monograph on the Potential Human and Reproductive and Developmental Effects of Methanol*. NTP-Center for the Evaluation of Risks to Human Reproduction, U.S. DHHS, NIH Publication No. 03-4478, September 2003.
NTP (National Toxicology Program). NTP technical report on the toxicology and carcinogenesis studies of propylene glycol mono-t-butyl ether (CAS No. 57018-52-7) in F344/N rats and B6C3F1 mice and a toxicology study of propylene glycol mono-*t*-butyl ether in male NBR rats (inhalation studies). *Natl Toxicol Program Tech Rep Ser*. 2004a;515:1–306.
NTP (National Toxicology Program). NTP-CERHR expert panel on the reproductive and developmental toxicity of methanol. *Reprod Toxicol*. 2004b;18:303–390.
NTP (National Toxicology Program). NTP toxicology and carcinogenesis studies of dipropylene glycol (CAS No. 25265-71-8) in F344/N rats and B6C3F1 mice (drinking water studies). *Natl Toxicol Program Tech Rep Ser*. 2004c;511:6–260.
NTP (National Toxicology Program). NTP-CERHR expert panel report on the reproductive and developmental toxicity of ethylene glycol. *Repro Toxicol*. 2004d;18:457–532.
NTP (National Toxicology Program). NTP-CERHR expert panel report on the reproductive and developmental toxicity of propylene glycol. *Repro Toxicol*. 2004e;18:533–579.
NTP (National Toxicology Program). NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Ethylene Glycol. *NTP CERHR MON*. 2004f;11:1–III36.
NTP (National Toxicology Program). NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Propylene Glycol (PG). *NTP CERHR MON*. 2004g;12:i–III6.
NTP (National Toxicology Program). *12th Report on Carcinogens (ROC)*. Public Health Service, 2011. Available at: http://niehs.nih.gov/go/roc12.
NTP (National Toxicology Program). *14th Report on Carcinogens: Monograph on Trichloroethylene*. U.S. Department of Health and Human Services, Research Triangle Park, NC, 2015.
Nuckols JR, Ashley DL, Lyu C, et al. Influence of tap water quality and household water use activities on indoor air and internal dose levels of trihalomethanes. *Environ Health Perspect* 2005;113:863–870.
Nyska A, Maronpot RR, Long PH, et al. Disseminated thrombosis and bone infarction in female rats following inhalation exposure to 2-butoxyethanol. *Toxicol Pathol*. 1999;27:287–294.
O'Brien KL, Selanikio JD, Hecdivert C, et al. Epidemic of pediatric deaths from acute renal failure caused by diethylene glycol poisoning. *JAMA*. 1998;279:1175–1180.
O'Brien ML, Spear BT, Glauert HP. Role of oxidative stress in peroxisome proliferator mediated carcinogenesis. *Crit Rev Toxicol*. 2005;35:61–88.
O'Callaghan JP, Daughtrey WC, Clark CR, et al. Health assessment of gasoline and fuel oxygenate vapors: Neurotoxicity evaluation. *Regul Toxicol Pharmacol*. 2014;70:S35–S42.
O'Flaherty EJ, Nau H, McCandless D, et al. Physiologically based pharmacokinetics of methoxyacetic acid: Dose-effect considerations in C57BL/6 mice. *Teratology*. 1995;52:78–89.
Ohtsuki T, Sato K, Koizumi A, et al. Limited capacity of humans to metabolize tetrachloroethylene. *Int Arch Occup Environ Health*. 1983;51:381–390.
Olney JW, Wozniak DF, Farber NB, et al. The enigma of fetal alcohol neurotoxicity. *Ann Med*. 2002;34:109–119.
Olson MJ, Garg BD, Murty CVR, Roy AK. Accumulation of α_{2u}-globulin in the renal proximal tubules of male rats exposed to unleaded gasoline. *Toxicol Appl Pharmacol*. 1987;90:43–51.
Olson MJ, Mancini MA, Garg BD, Roy AK. Leupeptin-mediated alteration of renal phagolysosomes: Similarity to hyaline droplet nephropathy of male rats exposed to unleaded gasoline. *Toxicol Lett*. 1988;41:245–254.
Omiecinski CJ, Remmel RP, Hosagrahara VP. Concise review of the cytochrome P450s and their roles in toxicology. *Toxicol Sci*. 1999;4:151–156.
Omura M, Katsumata T, Misawa H, Yamaguchi M. Decreases in protein kinase and phosphatase activities in the liver nuclei of rats exposed to carbon tetrachloride. *Toxicol Appl Pharmacol*. 1999;160:192–197.
OSHA (Occupational Safety and Health Administration). *Solvents*. July 3, 2006. Available at: http://www.osha.gov/archive/oshinfo/priorities/solvents.html.
OSHA (Occupational Safety and Health Administration). Styrene. https://www.osha.gov/SLTC/styrene. 2016.
Oshiro WM, Beasley TE, McDaniel KL, et al. Prenatal exposure to vapors of gasoline-ethanol blends causes few cognitive deficits in adult rats. *Neurotoxicol Teratol*. 2015;49:59–73.
Osterberg K, Orbaek P, Karlson B, et al. A comparison of neuropsychological tests for the assessment of chronic toxic encephalopathy. *Am J Ind Med*. 2000;38:666–680.
Oudiz D, Zenick H. In vivo and in vitro evaluations of spermatotoxicity induced by 2-ethoxyethanol treatment. *Toxicol Appl Pharmacol*. 1986;84:576–583.
Padron AG, de Toranzo EGD, Castro JA. Depression of liver microsomal glucose 6-phosphatase activity in carbon tetrachloride-poisoned rats. Potential synergistic effects of lipid peroxidation and of covalent binding of haloalkane-derived free radicals to cellular components in the process. *Free Radic Biol Med*. 1996;21:81–87.
Pahler A, Parker J, Dekant W. Dose-dependent protein adduct formation in liver, kidney, and blood of rats and in human blood after perchloroethylene inhalation. *Toxicol Sci*. 1999;48:5–13.
Paine MF, Hart HL, Ludington SS, et al. The human intestinal cytochrome P450 "pie." *Drug Metab Dispos*. 2006;34:880–886.
Palmer CN, Hsu MH, Griffin KJ, et al. Peroxisome proliferator activated receptor α expression in human liver. *Mol Pharmacol*. 1998;53:14–22.
Palmer RB, Brent J. Derivation of a chemical-specific adjustment factor (CSAF) for use in the assessement of risk from chronic exposure to ethylene glycol: application of international programme for chemical safety guidelines. *Toxicol Appl Pharmacol*. 2005;207:576–584.
Park J, Kamendulis LM, Klaunig JE. Effects of 2-butoxyethanol on hepatic oxidative damage. *Toxicol Lett*. 2002;126:19–29.
Pastino GM, Conolly RB. Application of a physiologically based pharmacokinetic model to estimate the bioavailability of ethanol in male rats: distinction between gastric and hepatic pathways of metabolic clearance. *Toxicol Sci*. 2000;55:256–265.
Pastino GM, Flynn EJ, Sultatos LG. Genetic polymorphisms in ethanol metabolism: issues and goals for physiologically based pharmacokinetic modeling. *Drug Chem Toxicol*. 2000;23:179–201.
Patel AS, Talbott EO, Zborowski JV, et al. Risk of cancer as a result of community exposure to gasoline vapors. *Arch Environ Health*. 2004;59:497–503.
Patel KG, Yadav PC, Pandya CB, Saiyed HN. Male exposure mediated adverse reproductive outcomes in carbon disulphide exposed rayon workers. *J Environ Biol*. 2004;25:413–418.
Paulu C, Aschengrau A, Ozonoff D. Tetrachloroethylene-contaminated drinking water in Massachusetts and the risk of colon-rectum, lung, and other cancers. *Environ Health Perspect*. 1999;107:265–271.
Pereira MA. Route of administration determines whether chloroform enhances or inhibits cell proliferation in the liver of B6C3F1 mice. *Fund Appl Toxicol*. 1994;23:87–92.
Pereira MA, Grothaus M. Chloroform in drinking water prevents hepatic cell proliferation induced by chloroform administered by gavage in corn oil to mice. *Fundam Appl Toxicol*. 1997;37:82–87.
Pereira MA, Kramer PM, Conran PB, Tao L. Effect of chloroform on dichloroacetic acid and trichloroacetic acid-induced hypomethylation and expression of c-myc gene and on their promotion of liver and kidney tumors in mice. *Carcinogenesis*. 2001;22:1511–1519.

Pesch B, Haerting J, Ranft U, et al. Occupational risk factors for renal cell carcinoma: Agent-specific results from a case–control study in Germany. *Int J Epidemiol*. 2000;29: 1014–1024.

Pfaff J, Parton K, Lantz RC, et al. Inhalation exposure to JP-8 jet fuel alters pulmonary function and substance P levels in Fischer 344 rats. *J Appl Toxicol*. 1995;15:249–256.

Pfaff JK, Tollinger BJ, Lantz RC, et al. Neutral endopeptidase (NEP) and its role in pathological pulmonary change with inhalation exposure to JP-8 jet fuel. *Toxicol Ind Health*. 1996;12:93–103.

Pfeifer GD, Roper JM, Dorman D, Lynam DR. Health and environmental testing of manganese exhaust products from use of methylcyclopentadienyl manganese tricarbonyl in gasoline. *Sci Total Environ*. 2004;334-335:397–408.

Philip BK, Anand SS, Palkar PS, et al. Subchronic chloroform priming protects mice from a subsequently administered lethal dose of chloroform. *Toxicol Appl Pharmacol*. 2006;216:108–12106.

Philip BK, Mumtaz MM, Latendresse JR, Mehendale HM. Impact of repeated exposure on toxicity of perchloroethylene in Swiss Webster mice. *Toxicology*. 2007;232:1–14.

Phillips CF, Jones RK. Gasoline vapor exposures during bulk handling operations. *Am Ind Hyg Assoc J*. 1978;39:118–128.

Phillips M. Detection of carbon disulfide in breath and air: a possible new risk factor for coronary artery disease. *Int Arch Occup Environ Health*. 1992;64:119–123.

Pikaar NA, Wedel M, Hermus RJJ. Influence of several factors on blood alcohol concentrations after drinking alcohol. *Alcohol Alcoholism*. 1988;23:289–297.

Pisinger C, Dossing M. A systematic review of health effects of electronic cigarettes. *Prev Med*. 2014;69:248–260.

Plaa GL. Chlorinated methanes and liver injury: highlights of the past 50 years. *Annu Rev Pharmacol Toxicol*. 2000;40:43–65.

Poet TS, Soelberg JJ, Weitz KK, et al. Mode of action and pharmacokinetic studies of 2-butoxyethanol in the mouse with an emphasis on forestomach dosimetry. *Toxicol Sci*. 2003;71:176–189.

Pohl HR, Scinicariello F. The impact of CYP2E1 genetic variability on risk assessment of VOC mixtures. *Regul Toxicol Pharmacol*. 2011;59:364–374.

Pohl HR, Tarkowski S, Buczynska A, et al. Chemical exposures at hazardous waste sites: Experiences from the United States and Poland. *Environ Toxicol Pharmacol*. 2008;25: 283–291.

Poldelski V, Johnson A, Wright S, et al. Ethylene glycol-mediated tubular injury: identification of critical metabolites and injury pathways. *Am J Kidney Dis*. 2001;38:339–348.

Pomierny B, Krzyzanowska W, Neidzielska E, et al. Ethylene glycol ethers induce apoptosis and disturb glucose metabolism in the rat brain. *Pharmacol Rep*. 2016;68:162–171.

Ponomarkov V, Tomatis L. Effects of long-term oral administration of styrene to mice and rats. *Scand J Work Environ Health*. 1978;4(suppl 2):127–135.

Poon R, Chu IH, Bjarnason S, et al. Short-term inhalation toxicity of methanol, gasoline, and methanol/gasoline in the rat. *Toxicol Ind Health*. 1995;11:343–361.

Pottenger LH, Carney EW, Bartels MJ. Dose-dependent nonlinear pharmacokinetics of ethylene glycol metabolites in pregnant (GD 10) and nonpregnant Sprague–Dawley rats following oral administration of ethylene glycol. *Toxicol Sci*. 2001;62:10–19.

Powley MW, Carlson GP. Species comparison of hepatic and pulmonary metabolism of benzene. *Toxicology*. 1999;139:207–217.

Prah J, Ashley D, Blount B, et al. Dermal, oral, and inhalation pharmacokinetics of methyl tertiary butyl ether (MTBE) in human volunteers. *Toxicol Sci*. 2004;77:195–205.

Preedy VR, Ohlendieck K, Adachi J, et al. The importance of alcohol-induced muscle disease. *Clin Sci*. 2003;104:287–294.

Prescott-Matthews JS, Wolf DC, Wong BA, Borghoff SJ. Methyl *tert* -butyl ether causes α_{2u}-globulin nephropathy and enhanced renal cell proliferation in male Fischer-344 rats. *Toxicol Appl Pharmacol*. 1997;143:301–314.

Price B, Bergman TS, Rodriguez M, et al. A review of carbon disulphide exposure data and the association between carbon disulfide exposure and ischemic heart disease mortality. *Regul Toxicol Pharmacol*. 1997;26:119–128.

Price B, Berner T, Henrich RT, et al. A benchmark concentration for carbon disulfide: analysis of the NIOSH carbon disulfide exposure database. *Regul Toxicol Pharmacol*. 1996;24:171–176.

Price PS, Conolly RB, Chaisson CF, et al. Modeling interindividual variation in physiological factors used in PBPK models in humans. *Crit Rev Toxicol*. 2003;33:469–503.

Proctor SP, Heaton KJ, Smith KW, et al. The occupational JP8 exposure neuroepidemiology study (OJENES): repeated workday exposure and central nervous system functioning among US Air Force personnel. *Neurotoxicology*. 2011;32:799–808.

Pryor GT, Rebert CS. Interactive effects of toluene and hexane on behavior and neurophysiologic responses in Fischer-344 rats. *Neurotoxicology*. 1992;13:225–234.

Purohit V, Brenner DA. Mechanisms of alcohol-induced hepatic fibrosis: a summary of the Ron Thurman Symposium. *Hepatology*. 2006;43:872–878.

Purohit V, Khalsa J, Serrano J. Mechanisms of alcohol-associated cancers: introduction and summary of the symposium. *Alcohol*. 2005;35:155–160.

Qui J, Chien Y-C, Bruckner JV, Fisher JW. Bayesian analysis of a physiologically based model for perchloroethylene in humans. *J Toxicol Environ Health A*. 2010;73:74–91.

Qin LQ, Wang Y, Xu J-Y, et al. One-day dietary restriction changes hepatic metabolism and potentiates the hepatotoxicity of carbon tetrachloride and chloroform. *Tohoku J Exp Med*. 2007;212:379–387.

Quast JF, Calhoun LL, Frauson LE. 1,1,1-Trichlooethane formulation: a chronic inhalation toxicity and oncogenicity study in Fischer 344 rats and B6C3F1 mice. *Fund Appl Toxicol*. 1988;11:611–625.

Raabe GK. Review of the carcinogenic potential of gasoline. *Environ Health Perspect*. 1993;101:35–38.

Raaschou-Nielsen O, Hansen J, McLaughlin JK, et al. Cancer risk among workers at Danish companies using trichloroethylene: a cohort study. *Am J Epidemiol*. 2003;158:1182–1192.

Rajendram R, Preedy VR. Effect of alcohol consumption on the gut. *Dig Dis*. 2005;23:214–221.

Ramdhan DH, Kamijima M, Yamada N, et al. Molecular mechanism of trichloroethylene-induced hepatotoxicity mediated by CYP2E1. *Toxicol Appl Pharmacol*. 2008;231: 300–207.

Ramos G, Kazimi N, Nghiem DX, et al. Platelet activating factor receptor binding plays a critical role in jet fuel-induced immune suppression. *Toxicol Appl Pharmacol*. 2004;195:331–338.

Ramos G, Limono-Flores AY, Ullrich SE. Dermal exposure to jet fuel suppresses delayed-type hypersensitivity: A critical role for aromatic hydrocarbons. *Toxicol Sci*. 2007;100:415–422.

Ramos G, Limon-Flores AY, Ullrich SE. JP-8 induces immune suppression via a reactive oxygen species NF-$_{\kappa}$β-dependent mechanism. *Toxicol Sci*. 2009;108:100–109.

Ramos G, Nghiem DX, Walterscheid JP, Ullrich SE. Dermal application of jet fuel suppresses secondary immune reactions. *Toxicol Appl Pharmacol*. 2002;180:136–144.

Ramsay M. Genetic epigenetic insights into fetal alcohol spectrum disorders. *Genome Med*. 2010;2:27–35.

Ramsey JC, Andersen ME. A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol Appl Pharmacol*. 1984;73:159–175.

Rao PS, Mangipudy RS, Mehendale HM. Tissue injury and repair as parallel and opposing responses to CCl4 hepatotoxicity: a novel dose–response. *Toxicology*. 1997;118:181–193.

Rappaport SM, Kim S, Lan Q, et al. Evidence that humans metabolize benzene via two pathways. *Environ Health Perspect*. 2009;117:946–952.

Rappaport SM, Kim S, Lan Q, et al. Human benzene metabolism following occupational and environmental exposures. *Chem-Biol Interact*. 2010;184:189–195.

Ratcliffe JM, Schrader SM, Clapp DE, et al. Semen quality in workers exposed to 2-ethoxyethanol. *Br J Ind Med*. 1989;46:399–406.

Raucy JL, Kraner JC, Lasker JM. Bioactivation of halogenated hydrocarbons by cytochrome P4502E1. *Crit Rev Toxicol*. 1993;23:1–20.

Raucy JL, Lasker JM, Kraner JC, et al. Induction of cytochrome P450IIE1 in the obese overfed rat. *Mol Pharmacol*. 1991;39:275–280.

Raunio H, Husgafvel-Pursianinen K, Anttila S, et al. Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility: a review. *Gene*. 1995;159:113–121.

Rawlings SJ, Shuker DE, Webb M, Brown NA. The teratogenic potential of alkoxy acids in post-implantation rat embryo culture: structure–activity relationships. *Toxicol Lett*. 1985;28:49–58.

Reaney SH, Bench G, Smith DR. Brain accumulation and toxicity of Mn(II) and Mn(III) exposures. *Toxicol Sci*. 2006;93:114–124.

Recknagel RO, Glende EA Jr, Dolak JA, Waller RL. Mechanisms of carbon tetrachloride toxicity. *Pharmacol Therap*. 1989;43:139–154.

Reddy NJ, Sudini M, Lewis LD. Delayed neurological sequelae from ethylene glycol, diethylene glycol and methanol poisoning. *Clin Toxicol*. 2010;48:967–973.

Reese E, Kimbrough RD. Acute toxicity of gasoline and some additives. *Environ Health Perspect*. 1993;101(suppl 6):115–131.

Reeve E, Wiese MD, Mangoni AA. Alterations in drug disposition in older adults. *Expert Opinion Drug Metab Toxicol*. 2015;11:491–508.

Rehm J, Room R, Monteiro M, et al. Alcohol use. In: Ezzati M, Lopez A, Rodgers A, Murray C, eds. *Comparative Quantification of Health Risks. Global and Regional Burden of Disease Attritutable to Selected Major Risk Factors*. Geneva: WHO; 2004:960–1108.

Reigel AC, Ali SF, Torinese S, French ED. Repeated exposure to the abused inhalant toluene alters levels of neurotransmitters and generates peroxynitrite in nigrostriatal and mesolimbic nuclei in rat. *Ann NY Acad Sci*. 2004;1025:543–551.

Reigel AC, French ED. Abused inhalants and central reward pathways: electrophysiological and behavioral studies in the rat. *Ann NY Acad Sci*. 2002;965:281–291.

Reinhardt CT, Mulllin LS, Maxfield ME. Epinephrine-induced cardiac arrhythmia potential of some common industrial solvents. *J Occup Med*. 1973;15:953–955.

Reitz RH, McDougal JN, Himmelstein MW, et al. Physiologically-based pharmacokinetic modeling with methylchloroform: implications for interspecies, high dose/low dose, and dose route extrapolations. *Toxicol Appl Pharmacol*. 1988;95:185–199.

Reitz RH, Mendrala AL, Guengerich FP. In vitro metabolism of methylene chloride in human and animal tissues: use in physiologically based pharmacokinetic models. *Toxicol Appl Pharmacol*. 1989;97:220–246.

Richardson PJ, Patel VB, Preedy VR. Alcohol and the myocardium. *Novartis Found Symp*. 1998;216:35–45.

Richter R. Degeneration of the basal ganglia in monkeys from chronic carbon disulfide poisoning. *J Neuropathol Exp Neurol*. 1945;4:324–353.

Rifas L, Towler DA, Avioli LV. Gestational exposure to ethanol suppresses msx2 expression in developing mouse embryos. *Proc Natl Acad Sci*. 1997;94:7549–7554.

Riihimaki V, Kivisto H, Peltonen K, et al. Assessment of exposure to carbon disulfide in viscose production workers from urinary 2-thiothiazolidine-4-carboxylic acid determinations. *Am J Ind Med*. 1992;22:85–97.

Rikans LE, Hornbrook KR. Age-related susceptibility to hepatotoxicants. *Environ Toxicol Pharmacol*. 1997;4:339–344.

Rinn JL, Rozowsky JS, Laurenzi IJ, et al. Major molecular differences between mammalian sexes are involved in drug metabolism and renal function. *Dev Cell*. 2004;6:791–800.

Riordan SM, Williams R. Alcohol exposure and paracetamol-induced hepatotoxicity. *Addict Biol*. 2002;7:191–206.

Ritchie GD, Rossi J III, Nordholm AF, et al. Effects of repeated exposure to JP-8 jet fuel vapor on learning of simple and difficult operant tasks by rats. *J Toxicol Environ Health*. 2001;64:385–415.

Ritchie GD, Still KR, Rossi J, et al. Biological and health effects of exposure to kerosene-based jet fuels and performance additives. *J Toxicol Environ Health B*. 2003;6:357–451.

Ritschel WA. Physiology of the aged respiratory system. In: Ritschel WA, ed. *Gerontokinetics: The Pharmacokinetics of Drugs in the Elderly*. Caldwell, NJ: The Telford Press; 1988:7.

Robb TM, Rogers MJ, Woodward SS, et al. *In vitro* time- and dose-effect response of JP-8 and S-8 jet fuel on alveolar type II epithelial cells of rats. *Toxicol Ind Health*. 2010;26:367–374.

Roberts LG, Bevans AC, Schreiner CA. Development and reproductive toxicity evaluation of toluene vapor in the rat. I. Reproductivity toxicity. *Repro Toxicol*. 2003;17:649–658.

Roberts LG, Gray TM, Marr MC, et al. Health assessment of gasoline and fuel oxygenate vapors: Developmental toxicity in mice. *Regul Toxicol Pharmacol*. 2014a;70:S58–S68.

Roberts LG, Gray TM, Trimmer GW, et al. Health assessment of gasoline and fuel oxygenate vapors: Developmental toxicity in rats. *Regul Toxicol Pharmacol*. 2014b;70:S69–S79.

Robertson OH, Loosli CG, Puck TT, et al. Tests for the chronic toxicity of propylene glycol and triethylene glycol on monkeys and rats by vapor inhalaton and oral administration. *J Pharmacol Exp Therap*. 1947;91:52–76.

Robinson CN, Latimer B, Abreo F, et al. *In-vivo* evidence of nephrotoxicity and altered hepatic function in rats following administration of diglycolic acid, a metabolite of diethylene glycol. *Clin Toxicol.* 2017;55:196–205.

Robinson V, Bergfeld WF, Belsito DV, et al. Final report on the safety assessment of PPG-2 methyl ether, PPG-3 methyl ether, and PPG-2 methyl ether acetate. *Int J Toxicol*. 2009;28:162S–174S.

Robledo RF, Barber DS, Witten ML. Modulation of bronchial epithelial cell barrier function by in vitro jet propulsion fuel 8 exposure. *Toxicol Sci*. 1999;51:119–125.

Robledo RF, Witten ML. NK1-receptor activation prevents hydrocarbon-induced lung injury in mice. *Am J Physiol*. 1999;276:L229–L238.

Rodrigues EG, Smith K, Maule AL, et al. Urinary polycyclic aromatic hydrocarbon (OH-PAH) metabolite concentrations and the effect of GST polymorphisms among US Air Force personnel exposed to jet fuel. *J Occup Environ Med*. 2014;56:465–471.

Rodriguez-Antona C, Ingelman-Sundberg M. Cytochrome P450 pharmacogentics and cancer. *Oncogene*. 2006;25:1679–1691.

Rodriguez CE, Mahle DA, Gearhart JM, et al. Predicting age-appropriate pharmacokinetics of six volatile organic compounds in the rat utilizing physiologically based pharmacokinetic modeling. *Toxicol Sci*. 2007;98:43–56.

Roe FJ. Styrene: Toxicity studies—what do they show? *Crit Rev Toxicol*. 1994;24(suppl 1):S117–S125.

Roe FJC, Palmer AK, Worden AN, Van Abbe NJ. Safety evaluation of toothpaste containing chloroform. I. Long-term studies in mice. *J Environ Pathol Toxicol*. 1979;2:799–819.

Rogers JV, Gunasekar PG, Garrett CM, et al. Detection of oxidative species and low-molecular weight DNA in skin following dermal exposure with JP-8 jet fuel. *J Appl Toxicol*. 2001;21:521–525.

Rosenberg NL. Neurotoxicity of organic solvents. In: Rosenberg NL, ed. *Occupational and Environmental Neurology*. Newton, MA: Butterworth-Heinemann, 1995:71–113.

Rosenberg NL, Grigsby J, Dreisbach J, et al. Neuropsychologic and MRI abnormalities associated with chronic solvent abuse. *J Toxicol Clin Toxicol*. 2002;40:21–34.

Rosenberg NL, Kleinschmidt-DeMasters BK, Davis KA, et al. Toluene abuse causes central nervous system white matter changes. *Ann Neurol*. 1988a;23:611–614.

Rosenberg NL, Spitz MC, Filley CM, et al. Central nervous system effects of chronic toluene abuse—Clinical, brainstem evoked response and magnetic resonance imaging studies. *Neurotoxicol Teratol*. 1988b;10:489–495.

Rosenthal DS, Simbulan-Rosenthal CMG, Liu WF, et al. Mechanisms of JP-8 jet fuel cell toxicity. II. Induction of necrosis in skin fibroblasts and keratinocytes and modulation of levels of Bcl-2 family members. *Toxicol Appl Pharmacol*. 2001;171:107–116.

Roskos KV, Maibach HI, Guy RH. The effect of aging on percutaneous absorption in man. *J Pharmacokin Biopharmaceut*. 1989;17:617–630.

Ross D, Siegel D, Schattenberg DG, et al. Cell-specific activation and detoxification of benzene metabolites in mouse and human bone marrow: Identification of target cells and potential role for modulation of apoptosis in benzene toxicity. *Environ Health Perspect*. 1996;104(suppl 6):1177–1182.

Rossi J III, Nordholm AF, Carpenter RL, et al. Effects of repeated exposure of rats to JP-5 or JP-8 jet fuel vapor on neurobehavioral capacity and neurotransmitter levels. *J Toxicol Environ Health A*. 2001;63:397–428.

Roth RA, Harkema JR, Pestka JP, Ganey PE. Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? *Toxicol Appl Pharmacol*. 1997;147:300–311.

Rowe BL, Toccalino PL, Moran MJ, et al. Occurrence and potential human-health relevance of volatile organic compounds in drinking water from domestic wells. *Environ Health Perspect*. 2007;115:1539–1546.
Roychowdhury S, McMullen MR, Pisano SG, et al. Absence of receptor interacting protein kinase 3 prevents ethanol-induced liver injury. *Hepatology*. 2013;57:1773–1783.
Ruder AM, Ward EM, Brown DP.: Mortality in dry-cleaning workers: an update. *Am J Ind Med*. 2001;39:121–132.
Rumack BH. Acetaminophen misconceptions. *Hepatology*. 2004;40:10–15.
Russo D, Purohit V, Foudin L, Salin M. Workshop on alcohol use and health disparities 2002: A call to arms. *Alcohol*. 2004;32:37–43.
Rusyn I, Chiu WA, Lash LH, et al. Trichloroethylene: mechanistic, epidemiologic and supporting evidences of carcinogenic hazard. *Pharmacol Therap*. 2014;141:55–68.
Saavedra D, Arteaga M, Tena M. Industrial contamination with glycol ethers resulting in teratogenic damage. *Ann NY Acad Sci*. 1997;837:126–137.
Saito A, Sasaki T, Kasai T, et al. Hepatotumorigenicity of ethyl *tertiary*-butyl ether with 2-year inhalation exposure in F344 rats. *Arch Toxicol.* 2013;87:905–914.
Sakurai K, Mikamoto K, Shirai M, et al. MicroRNA profiling in ethylene glycol monomethyl ether-induced monkey testicular toxicity model. *J Toxicol Sci*. 2015;40:375–382.
Salvini M, Binaschi S, Riva M. Evaluation of the psychological functions in humans exposed to the Threshold Limit Value of 1,1,1-trichloroethane. *Br J Industr Med*. 1971;28: 286–292.
Sanzgiri UY, Kim HJ, Muralidhara S, et al. Effect of route and pattern of exposure on the pharmacokinetics and acute hepatotoxicity of carbon tetrachloride. *Toxicol Appl Pharmacol*. 1995;134:148–154.
Sanzgiri UY, Srivatsan V, Muralidhara S, et al. Uptake, distribution, and elimination of carbon tetrachloride in rat tissues following inhalation and ingestion exposures. *Toxicol Appl Pharmacol*. 1997;143:120–129.
Sarangapani R, Gentry PR, Covington TR, et al. Evaluation of the potential impact of age- and gender-specific lung morphology and ventilation rate on the dosimetry of vapors. *Inhal Toxicol*. 2003;15:987–1016.
Sarangapani R, Teeguarden JG, Cruzan G, et al. Physiologically based pharmacokinetic modeling of styrene and styrene oxide respiratory-tract dosimetry in rodents and humans. *Inhal Toxicol*. 2002;14:789–834.
Sasso AF, Schlosser PM, Kedderis GL, et al. Application of an updated physiologically based pharmacokinetic model for chloroform to evaluate CYP2E1-mediated renal toxicity in rats and mice. *Toxicol Sci*. 2013;131:360–374.
Sato A, Endoh K, Kaneko T, Johanson G. Effects of consumption of ethanol on the biological monitoring of exposure to organic solvent vapors: a simulation study with trichloroethylene. *Br J Ind Med*. 1991;48:548–556.
Sato A, Nakajima T. Enhanced metabolism of volatile hydrocarbons in rat liver following food deprivation, restricted carbohydrate intake, and administration of ethanol, phenobarbital, polychlorinated biphenyl and 3-methylcholanthrene: a comparative study. *Xenobiotica*. 1985;15:67–75.
Sato A, Nakajima T, Fujiwara Y, Murayama N. Kinetic studies on sex difference in susceptibility to chronic benzene intoxication with special reference to body fat content. *Br J Ind Med*. 1975;32:321–328.
Sato N, Lindros KO, Baraona E, et al. Sex difference in alcohol-related organ injury. *Alcohol Clin Exp Res*. 2001;25:40S–45S.
Sato TK, Yamada RG, Ukai H, et al. Feedback repression is required for mammalian clock function. *Nat Genet.* 2006;38:312–319.
Savolainen K, Riihimaki V, Laine R, Kekoni J. Short-term exposure of human subjects to *m*-xylene and 1,1,1-trichloroethane. *Int Arch Occup Environ Health*. 1981;49:89–98.
Sawant SP, Dnyanmote AV, Shankar K, et al. Potentiation of carbon tetrachloride hepatotoxicity and lethality in Type 2 diabetic rats. *J Pharmacol Exp Therap*. 2004;308:694–704.
Scalley RD, Ferguson DR, Piccaro JC, et al. Treatment of ethylene glycol poisoning. *Am Fam Physician*. 2002;66:807–812.
Schaumburg HH, Spencer PS. Organic solvent mixtures. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. 2nd ed. New York: Oxford University Press, 2000, pp. 894–897.
Schenker MB, Gold EB, Beaumont JJ, et al. Association of spontaneous abortion and other reproductive effects with work in the semiconductor industry. *Am J Ind Med*. 1995;28:639–59.
Schep LJ, Slaughter RJ, Temple WA, Beasley DMG. Diethylene glycol poisoning. *Clin Toxicol*. 2009;47:525–536.
Scheuplein R, Charnley G, Dourson M. Differential sensitivity of children and adults to chemical toxicity. I. Biological basis. *Regul Toxicol Pharmacol*. 2002;35:429–447.
Schmucker DL. Age-related changes in liver structure and function: implications for disease? *Exper Gerontol*. 2005;40:650–659.
Schramm A, Uter W, Brandt M, et al. Increased intima-media thickness in rayon workers after long-term exposure to carbon disulfide. *Int Arch Occup Environ Health*. 2016;89:513–519.
Schreiner CA, Hoffman GM, Gudi R, Clark CR. Health assessment of gasoline and fuel oxygenate vapors: micronucleus and sister chromatid exchange evaluations. *Regul Toxicol Pharmacol*. 2014;70:S29–S34.
Schlender JF, Meyer M, Thelen K, et al. Development of a whole-body physiologically based pharmacokinetic approach to assess the pharmacokinetics of drugs in elderly individuals. *Clin Pharmacokinet*. 2016;55:1573–1589.
Schmucker DL. Age-related changes in liver structure and function: implications for disease? *Exper Gerontol*. 2005;40:650–659.
Schumann AM, Fox TR, Watanabe PG. [^{14}C] Methyl chloroform (1,1,1-trichlorothane): pharmacokinetics in rats and mice following inhalation exposure. *Toxicol Appl Pharmacol*. 1982;62: 390–401.
Schwartz JB. The influence of sex on pharmacokinetics. *Clin Pharmacokinet*. 2003;42:107–121.
Schwetz BA, Leong BK, Gehring PJ. Embryo- and fetotoxicity of inhaled chloroform in rats. *Toxicol Appl Pharmacol*. 1974;28:442–451.
Scofield EH, Henderson WM, Funk AB, et al. Diethylene glycol monomethyl ether, ethylene glycol monomethyl ether and the metabolite, 2-methoxyacetic acid affect *in vitro* chondrogenesis. *Reprod Toxicol.* 2006;22:718–724.
Scott WJ, Fradkin R, Wittfoht W, Nau H. Teratologic potential of 2-methoxyethanol and transplacental distribution of its metabolite, 2-methoxyacetic acid, in non-human primates. *Teratology*. 1989;39:363–373.
Seaton MJ, Schlosser PM, Bond JA, Medinsky MA. Benzene metabolism by human liver microsomes in relation to cytochrome P450 2E1 activity. *Carcinogenesis*. 1994;15:1799–1806.
Seidler A, Mohner M, Berger J, et al. Solvent exposure and malignant lymphoma: a population-based case–control study in Germany. *J Occup Med Toxicol*. 2007;2:2–12.
Seiji K, Inoue O, Jin C, et al. Dose excretion relationship in tetrachloroethylene-exposed workers and the effect of tetrachloroethylene co-exposure on trichloroethylene exposure. *Am J Ind Med*. 1989;16:675–684.
Seitz HK, Egerer G, Simanowski UA, et al. Human gastric alcohol dehydrogenase activity: effect of age, sex, and alcoholism. *Gut*. 1993;34:1433–1437.
Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Natl Rev Cancer*. 2007;7:599–612.
Seme MT, Summerfelt P, Henry MM, et al. Formate-induced inhibition of photoreceptor function in methanol intoxication. *J Pharmacol Exp Therap*. 1999;289:361–370.
Semple S. Dermal exposure to chemicals in the workplace: just how important is skin absorption? *Occup Environ Med*. 2004;61:376–382.
Serota DG, Thakur AK, Ulland BM, et al. A two-year drinking-water study of dichloromethane in rodents. I. Rats. *Food Chem Toxicol*. 1986a;24: 951–958.
Serota DG, Thakur AK, Ulland BM, et al. A two-year drinking-water study of dichloromethane in rodents. II. Mice. *Food Chem Toxicol*. 1986b;24: 959–963.
Sexton K, Adgate JL, Church TR, et al. Children's exposure to volatile organic compounds as determined by longitudinal measurements in blood. *Environ Health Perspect*. 2005;113: 342–349.
Shamsky S, Samimi B. Organic vapors at underground gasoline tank removal sites. *Appl Ind Hyg*. 1987;2:242–245.
Sharma R, Locke BR. Jet fuel toxicity: skin damage measured by 900MHz MRI skin microscopy and visualization by 3D MR image processing. *Magn Reson Imaging*. 2010;28:1030–1048.
Shehab N, Lewis CL, Streetman DD, Donn SM. Exposure to the pharmaceutical excipients benzyl alcohol and propylene glycol among critically ill neonates. *Pediatr Crit Care Med*. 2009;10:256–259.

Sherratt PJ, Williams S, Foster J, et al. Direct comparison of the nature of mouse and human GST T1-1 and the implications on dichloromethane carcinogenicity. *Toxicol Appl Pharmacol*. 2002;179:89–97.

Shi J, Aisaki K, Ikawa Y, Wake K. Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride. *Am J Pathol*. 1998;153:515–525.

Shibley IA, Pennington SN. Metabolic and mitotic changes associated with the fetal alcohol syndrome. *Alcohol Alcoholism*. 1997;32:423–434.

Shimada T, Yamazaki H, Mimura M, et al. Interindividual variations in human liver P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Therap*. 1994;270:414–423.

Short BG, Steinhagen WH, Swenberg JA. Promoting effects of unleaded gasoline and 2,2,4-trimethylpentane on the development of atypical cell foci and renal tubular cell tumors in rats exposed to *N*-ethyl-*N* hydroxyethylnitrosamine. *Cancer Res*. 1989;49:369–378.

Shroads AL, Langaee T, Coats BS, et al. Human polymorphisms in the glutathione transferase zeta 1/maleylacetoacetate isomerase gene influence the toxicokinetics of dichloroacetate. *J Clin Pharmacol*. 2012;52(6): 837–49.

Siesky AM, Kamendulis LM, Klaunig JE. Hepatic effects of 2-butoxyethanol in rodents. *Toxicol Sci*. 2002;70:252–260.

Sills RC, Harry GJ, Valentine WM, Morgan DL. Interdisciplinary neurotoxicity inhalation studies: carbon disulfide and carbonyl sulfide research in F344 rats. *Toxicol Appl Pharmacol*. 2005;207:S245–S250.

Silvaggio T, Mattison D. Setting occupational health standards: toxicokinetic differences among and between men and women. *J Occup Med*. 1994;36: 849–854.

Singh S, Singh J. Dermal toxicity and microscopic alteration by JP-8 jet fuel components in vivo in rabbit. *Environ Toxicol Phar*. 2004;16:153–161.

Singh S, Zhao K, Singh J. In vivo percutaneous absorption, skin barrier perturbation, and irritation from JP-8 jet fuel components. *Drug Chem Toxicol*. 2003;26:135–146.

Singh SP, Ehmann S, Snyder AK. Ethanol-induced changes in insulin-like growth factors and IGF gene expression in the fetal brain. *Proc Soc Exp Biol Med*. 1996;212:349–354.

Sitarek K, Gromadzinska J, Lutz P, et al. Fertility and developmental toxicity studies of diethylene glycol monobutyl ether (DGBE) in rats. *Int J Occup Med Environ Health*. 2012;25:404–417.

Sivilotti ML, Burns MJ, McMartin KE, Brent J. Toxicokinetics of ethylene glycol during fomepizole therapy: implications for management. For the Methylpyrazole for Toxic Alcohols Study Group. *Ann Emerg Med*. 2000;36:114–125.

Skrzypinska-Gawrysiak M, Piotrowski JK, Sporny S. Circadian variations in hepatotoxicity of carbon tetrachloride in mice. *Internat J Occup Med Environ Health*. 2000;13:165–173.

Smialowicz RJ, Riddle MM, Luebke RW, et al. Immunotoxicity of 2-methoxyethanol following oral administration in Fischer 344 rats. *Toxicol Appl Pharmacol*. 1991a;109:494–506.

Smialowicz RJ, Riddle MM, Rogers RR, et al. Evaluation of the immunotoxicity of orally administered 2-methoxyacetic acid in Fischer 344 rats. *Fundam Appl Toxicol*. 1991b;17:771–781.

Smialowicz RJ, Riddle MM, Williams WC, et al. Species and strain comparisons of immunosuppression by 2-methoxyethanol and 2-methoxyacetic acid. *Int J Immunopharmacol*. 1994;16:695–702.

Smialowicz RJ, Williams WC, Riddle MM, et al. Comparative immunosuppression of various glycol ethers orally administered to Fischer 344 rats. *Fundam Appl Toxicol*. 1992;18:621–627.

Smith EN, Taylor RT. Acute toxicity of methanol in the folate-deficient acatalasemic mouse. *Toxicology*. 1982;25:271–287.

Smith JH, Maita K, Sleight SD, Hook JB. Effect of sex hormone status on chloroform nephrotoxicity and renal mixed function oxidases in mice. *Toxicology*. 1984;30:305–316.

Smith KW, Proctor SP, Ozonoff A, McClean MD. Urinary biomarkers of occupational jet fuel exposure among Air Force personnel. *J Exp Sci Environ Epidemiol*. 2012;22:35–45.

Smith MT. The mechanism of benzene-induced leukemia: A hypothesis and speculations on the causes of leukemia. *Environ Health Perspect*. 1996;104(suppl 6):1219–1225.

Snawder JE, Lipscomb JC. Individual variance of cytochrome P450 forms in human hepatic microsomes: correlation of individual forms with xenobiotic metabolism and implications in risk assessment. *Regul Toxicol Pharmacol*. 2000;32:200–209.

Snellings WM, Corley RA, McMartin KE, et al. Oral reference dose for ethylene glycol based on oxalate crystal-induced renal tubule degeneration as the critical effect. *Regul Toxicol Pharmacol*. 2013;65:229–241.

Snyder R. Benzene and leukemia. *Crit Rev Toxicol*. 2002;32:155–210.

Snyder R. Xenobiotic metabolism and the mechanism(s) of benzene toxicity. *Drug Metab Rev*. 2004;36:531–547.

Snyder R, Hedli CC. An overview of benzene metabolism. *Environ Health Perspect*. 1996;104:S1165–S1171.

Soffritti M, Belpoggi F, Cevolani D, et al. Results of long-term experimental studies on the carcinogenicity of methyl alcohol and ethyl alcohol in rats. *Ann NY Acad Sci*. 2002;982:46–69.

Sohn MD, McKone TE, Blancato JN. Reconstructing population exposure from dose biomarkers: Inhalation of trichloroethylene (TCE) as a case study. *J Expos Sci Environ Epidemiol*. 2004;14:204–213.

Song F, Zhang C, Wang Q, et al. Alterations in neurofilaments content and calpains activity of sciatic nerve of carbon disulfide-treated rats. *Arch Toxicol*. 2009;83:587–594,

Spencer PJ. New toxicity data for the propylene glycol ethers—a commitment to public health and safety. *Toxicol Lett*. 2005;156:181–188,

Spencer PJ, Crissman JW, Stott WT, et al. Propylene glycol monomethyl ether (PGME): Inhalation toxicity and carcinogenicity in Fischer 344 rats and B6C3F1 mice. *Toxicol Pathol*. 2002;30:570–579.

Spiller HA. Epidemiology of volatile substance abuse (VSA) cases reported to US poison control centers. *Am J Drug Alcohol Abuse*. 2004;30:155–165.

Spitzer HL. An analysis of the health benefits associated with the use of MTBE reformulated gasoline oxygenated fuels in reducing atmospheric concentrations of selected volatile organic compounds. *Risk Anal*. 1997;17:683–691.

Sprankle CS, Larson JL, Goldsworthy SM, Butterworth BE. Levels of *myc*, *fos*, Ha-*ras*, *met* and hepatocyte growth factor mRNA during regenerative cell proliferation in female mouse liver and male rat kidney after a cytotoxic dose of chloroform. *Cancer Lett*. 1996;101:97–106.

Spurgeon A, Glass DC, Calvert IA, et al. Investigation of dose related neurobehavioral effects in paintmakers exposed to low levels of solvents. *Occup Environ Med*. 1994;51:626–630.

Stacey NH. Toxicity of mixtures of trichloroethylene, tetrachloroethylene and 1,1,1-trichloroethane: Similarity of in vitro to in vivo responses. *Toxicol Ind Health*. 1989;5:441–450.

Standeven AM, Blazer DG, Goldsworthy TL. Investigation of antiestrogenic properties of unleaded gasoline in female mice. *Toxicol Appl Pharmacol*. 1994a;127:233–240.

Standeven AM, Goldsworthy TL. Promotion of preneoplastic lesions and induction of CYP2B by unleaded gasoline vapor in female B6C3F1 mouse liver. *Carcinogenesis*. 1993;14:2137–2141.

Standeven AM, Goldsworthy TL. Identification of hepatic mitogenic and cytochrome P-450-inducing fractions of unleaded gasoline in B6C3F1 mice. *J Toxicol Environ Health*. 1994;43:213–224.

Standeven AM, Wolf DC, Goldsworthy TL. Interactive effects of unleaded gasoline and estrogen on liver tumor promotion in female B6C3F1 mice. *Cancer Res*. 1994b;54:1198–1204.

Standeven AM, Wolf DC, Goldsworthy TL. Promotion of hepatic preneoplastic lesions in male B6C3F1 mice by unleaded gasoline. *Environ Health Perspect*. 1995;103:696–700.

Staples CA, Williams JB, Craig GR, Roberts KM.Fate, effects and potential environmental risks of ethylene glycol: a review. *Chemosphere*. 2001;43:377–383.

Starr TB, Matanoski G, Anders MW, Andersen ME. Workshop overview: reassessment of the cancer risk of dichloromethane in humans. *Toxicol Sci*. 2006;91:20–28.

Stein Y, Finkelstein Y, Levy-Nativ O, et al. Exposure and susceptibility: schizophrenia in a young man following prolonged high exposures to organic solvents. *Neurotoxicology*. 2010;31:603–607.

Steinmaus C, Smith MT. Steinmaus and Smith respond to "proximity to gasoline stations and childhood leukemia. *Am J Epidemiol*. 2017;185:5–7.

Stenback F, Shubik P. Lack of toxicity and carcinogenicity of some commonly used cutaneous agents. *Toxicol Appl Pharmacol*. 1974;30:7–13.

Stewart RD, Gay HH, Schaffer AW, et al. Experimental human exposure to methyl chloroform vapor. *Arch Environ Health*. 1969;19:467–472.

Stewart RD, Hake CL, Forster HV, et al. Tetrachloroethylene: Development of a biologic standard for the industry worker by breath analysis. Contract No. HSM 99-72-83. NIOSH-MCOW-ENVM-PCE-74-6. NTIS No. PB82-152166. National Institute of Occupational Safety and Health, Cincinnati, OH, 1981.

Stewart RD, Hake CL, Wu A, et al. Effects of perchloroethylene/drug interaction on behavior and neurological function. Final Report PB-83-17460. National Institute of Occupational Safety and Health, Washington, DC, 1977.

Stenner RD, Merdink JL, Stevens DK, et al. Enterohepatic recirculation of trichloroethanol glucuronide as a significant source of trichloroacetic acid. *Drug Metab Dispos*. 1997;25:529–535.

Stoica BA, Boulares AH, Rosenthal DS, et al. Mechanisms of JP-8 jet fuel toxicity. I. Induction of apoptosis in rat lung epithelial cells. *Toxicol Appl Pharmacol*. 2001;171:94–106.

Stott WT, McKenna MJ. The comparative absorption and excretion of chemical vapors by the upper, lower and intact respiratory tract of rats. *Fundam Appl Toxicol*. 1984;4:594–602.

Stoyanovsky DA, Cederbaum AI. Thiol oxidation and cytochrome P450-dependent metabolism of CCl_4 triggers Ca^{+2} release from liver microsomes. *Biochemistry*. 1996;35:15839–15845.

Sturtevant RP, Sturtevant FM, Pauly JE, Scheving LE: Chronopharmacokinetics of ethanol. *Int J Clin Pharmacol*. 1978;16:594–599.

Su C, Chen K, Zou Y, et al. Chronic exposure to manganese sulfate leads to adverse dose-dependent effects on the neurobehavioral ability of rats. *Environ Toxicol*. 2016;31:1571–1579.

Sulsky SI, Hooven FH, Burch MT, Mundt KA: Critical review of the epidemiological literature on the potential cardiovascular effects of occupational carbon disulfide exposure. *Int Arch Occup Environ Health*. 2002;75:365–380.

Sun D-Q, Li A-W, Li J, et al. Changes in lipid peroxidation in carbon disulfide-treated rat nerve tissues and serum. *Chem-Biol Interact*. 2009;179:110–117.

Sun Y, Dai B, Wu Y, et al. Carbon disulfide exposure at peri-implantation disrupts embryo implantation by decreasing integrin β3 expression in the uterine tissue of pregnant mice. *Chem-Biol Interact*. 2013;206:126–133.

Suzuki M, Yamazaki K, Kano H, et al. No carcinogenicity of ethyl *tertiary*-butyl ether by 2-year oral administration in rats. *J Toxicol Sci*. 2012;37:1239–1246.

Swaen GM, van Amelsvoort L, Twisk JJ, et al. Low level occupational benzene exposure and hematological parameters. *Chem-Biol Interact*. 2010;184:94–100.

Swann HE Jr, Kwon BK, Hogan GK, Snellings WM. Acute inhalation toxicology of volatile hydrocarbons. *Am Ind Hyg Assoc J*. 1974;35:511–518.

Sweeney LM, Tyler TR, Kirman CR, et al. Proposed occupational exposure limits for select ethylene glycol ethers using PBPK models and Monte Carlo simulations. *Toxicol Sci*. 2001;62:124–139.

Sweeting JN, Siu M, McCallum GP, et al. Species differences in methanol and formic acid pharmacokinetics in mice, rabbits and primates. *Toxicol Appl Pharmacol*. 2010;247:28–35.

Sweeting JN, Siu M, Wiley MJ, Wells PG. Species- and strain-dependent teratogenicity of methanol in rabbits and mice. *Reprod Toxicol*. 2011;31:50–58.

Swenberg JA. α2μ-Globulin nephropathy: Review of the cellular and molecular mechanisms involved and their implications for human risk assessment. *Environ Health Perspect*. 1993;101:39–44.

Taieb D, Malicet C, Garcia S, et al. Inactivation of stress protein 8 increases murine carbon tetrachloride hepatotoxicity via preserved CYP2E1 activity. *Hepatology*. 2005;42:176–182.

Takebayashi T, Nishiwaki Y, Uemura T, et al. A six-year follow-up study of the subclinical effects of carbon disulphide exposure on the cardiovascular system. *Occup Environ Med*. 2004;61:127–134.

Takei M, Ando Y, Saitoh W, et al. Ethylene glycol monomethyl ether-induced toxicity is mediated through the inhibition of flavoprotein dehydrogenase enzyme family. *Toxicol Sci*. 2010;118:643–652.

Taketa Y, Inomata A, Hosokawa S, et al. Histopathological characteristics of luteal hypertrophy induced by ethylene glycol monomethyl ether with a comparison to normal luteal morphology in rats. *Toxicol Pathol*. 2011;39:372–380.

Talbott EO, Xu X, Youk AO, et al. Risk of leukemia as a result of community exposure to gasoline vapors: A follow-up study. *Environ Res*. 2011;111:597–602.

Tan H-H, Schiano TD. Effects of liver disease on drug metabolism in humans. *Encyclopeda Drug Metab Interact*. 2012;X:1–46.

Tan X, Peng X, Wang F, et al. Cardiovascular effects of carbon disulfide: meta-analysis of cohort studies. *Int J Hyg Environ Health*. 2002;205:473–477.

Tan Y-M, Butterworth BE, Gargas ML, Conolly RB. Biologically motivated modeling of chloroform cytolethality and regenerative cellular proliferation. *Toxicol Sci*. 2003;75:192–200.

Tan Y-M, Clewell H, Campbell J, Andersen M. Evaluating pharmacokinetic and pharmacodynamic interactions with computational models in supporting cumulative risk assessment. *Int J Environ Res Public Health*. 2011;8: 1613–1630.

Tan Y-M, Liao KH, Clewell HJ. Reverse dosimetry: Interpreting trihalomethanes biomonitoring data using physiologically based pharmacokinetic data. *J Expos Sci Environ Epidemiol*. 2007;17:591–603.

Tang C, Lin JH, Lu AYH. Metabolism-based drug-drug interactions: what determines individual variability in cytochrome P450 induction? *Drug Metab Dispos*. 2005;33:603–613.

Tao L, Kramer PM, Ge R, Pereira MA. Effect of dichloroacetic acid and trichloroacetic acid on DNA methylation in liver and tumors of female B6C3F1 mice. *Toxicol Sci*. 1998;43:139–144.

Tao L, Li Y, Kramer PM, et al. Hypomethylation of DNA and the insulin-like growth factor-II gene in dichloroacetic and trichloroacetic acid-promoted mouse liver tumors. *Toxicology*. 2004;196:127–136.

Tao L, Wang W, Li L, et al. DNA hypomethylation induced by drinking water disinfection by-products in mouse and rat kidney. *Toxicol Sci*. 2005;87:344–352.

Tao L, Yang S, Xie M, et al. Effect of trichloroethylene and its metabolites, dichloroacetic acid and trichloroacetic acid, on the methylation and expression of c-Jun and c-Myc protooncogenes in mouse liver: Prevention by methionine. *Toxicol Sci*. 2000;54:399–407.

Tapin D, Kennedy G, Lambert J, Zayed J. Bioaccumulation and locomotor effects of manganese sulfate in Sprague–Dawley rats following subchronic (90 days) inhalation exposure. *Toxicol Appl Pharmacol*. 2006;211:166–174.

Tardif R, Lapare S, Plaa GL, Brodeur J. Effect of simultaneous exposure to toluene and xylene on their respective biological exposure indices in humans. *Int Arch Occup Environ Health*. 1991;63:279–284.

Taylor MD, Clewell HJ, Andersen ME, et al. Update on a pharmacokinetic-centric alternative tier II program for MMT – Part II: physiologically based pharmacokinetic modeling and manganese risk assessment. *J Toxicol*. 2012;2012:1–17.

Teboul M, Grechez-Cassiau A, Guillaumond F, Delaunnay F. How nuclear receptors tell time. *J Appl Physiol*. 2009;107:1965–1971.

Tephly TR. The toxicity of methanol. *Life Sci*. 1991;48:1031–1041.

TERA (Toxicology Excellence for Risk Assessment). Workplace Environmental Exposure Level, Deithylene Glycol 2016. Available at: http://www.tera.org/OARS/. Accessed June 13, 2017.

Terry KK, Elswick BA, Welsch F, Conolly RB. Development of a physiologically based pharmacokinetic model describing 2-methoxyacetic acid disposition in the pregnant mouse. *Toxicol Appl Pharmacol*. 1995;132:103–114.

Thamilselvan S, Khan SR. Oxalate and calcium oxalate crystals are injurious to renal epithelial cells: results of in vivo and in vitro studies. *J Nephrol*. 1998;11:66–69.

Thier R, Wiebel FA, Hinkel A, et al. Species differences in the glutathione transferase GSTT1-1 activity towards the model substrates methyl chloride and dichloromethane in liver and kidney. *Arch Toxicol.* 1998;72:622–629.

Thompson CM, Johns DO, Sonawane B, et al. Database for physiologically based pharmacokinetic (PBPK) modeling: physiological data for healthy and healthy-impaired elderly. *J Toxicol Environ Health Part B*: 2009;12(1):1–24.

Thompson CM, Sonawane B, Barton HA, et al. Approaches for applications of physiological models in risk assessment. *J Toxicol Environ Health Part B*. 2008;11:519–547.

Thompson DJ, Warner SD, Robinson VB. Teratology studies on orally administered chloroform in the rat and rabbit. *Toxicol Appl Pharmacol*. 1974;29:348–357.

Thrall KD, Weitz KK, Woodstock AD. Use of real-time breath analysis and physiologically based pharmacokinetic modeling to evaluate dermal absorption of aqueous toluene in human volunteers. *Toxicol Sci*. 2002;68:280–287.

Thurman RG. Mechanisms of hepatic toxicity II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. *Am J Physiol*. 1998;275:G605–G611.

Thurman RG. Sex-related liver injury due to alcohol involves activation of Kupffer cells by endotoxin. *Can J Gastroenterol*. 2000;14:129D–135D.

Timchalk C, Kousba AA, Poet TS. An age-dependent physiologically based pharmacokinetic/pharmacodynamic model for the organophosphorus insecticide in the preweanling rat. *Toxicol Sci*. 2007;98:348–365.

Tinston DJ. Perchloroethylene: multigeneration inhalation study in the rat. Report No. CTL/P/4907. Zenca Central Laboratory, Alderly Park, Cheshire, UK, 1995.

Tonkin EG, Erve JC, Valentine WM. Disulfiram produces a non-carbon disulfide-dependent schwannopathy in the rat. *J Neuropathol Exp Neurol*. 2000;59:786–797.

Toraason M, Breitenstein M. Prenatal ethylene glycol ether (EGME) exposure produces electrocardiographic changes in the rat. *Toxicol Appl Pharmacol*. 1988;95:321–327.

Tornambe A, Manfra L, Mariani L, et al. Toxicity evaluation fo diethylene glycol and its combined effects with produced waters of off-shore gas platforms in the Adriatic Sea (Italy): Bioassays with marine/estuarine species. *Mar Environ Res*. 2012;77:141–149.

Tornero-Velez R, Mirfazaelian A, Kim K-B, et al. Evaluation of deltamethrin kinetics and dosimetry in the maturing rat using a PBPK model. *Toxicol Appl Pharmacol*. 2010;244:208–217.

Tornero-Velez R, Rappaport SM. Physiological modeling of the relative contributions of styrene-7,8-oxide derived from direct inhalation and from styrene metabolism to the systemic dose in humans. *Toxicol Sci*. 2001;64:151–161.

Toso L, Roberson R, Woodard J, et al. Prenatal alcohol exposure alters GABA(A)alpha(5) expression: a mechanism of alcohol-induced learning dysfunction. *Am J Obstet Gynecol*. 2006;195:1038–1044.

Toth B, Hornung D, Scholz S, et al. Peroxisome proliferator-activated receptors: new players in the field of reproduction. *Am J Reprod Immunol*. 2007;58:289–310.

Tracey R, Manikkam M, Guerrero-Bosagna C, Skinner MK. Hydrocarbons (Jet Fuel JP-8) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *Reprod Toxicol*. 2013;36:104–116.

Traiger GJ, Bruckner JV. The participation of 2-butanone in 2-butanol-induced potentiation of carbon tetrachloride hepatotoxicity. *J Pharmacol Exp Therap*. 1976;196:493–500.

Traphagen N, Tian Z, Allen-Gipson D. Chronic ethanol exposure: Pathogenesis of pulmonary disease and dysfunction. *Biomolecules*. 2015;5:2840–2853.

Travis CC, Fox MT, Simmons WM, Lyon BF. Co-exposure to gasoline vapor decreases benzene metabolism in Fischer-344 rats. *Toxicol Lett*. 1992;62:231–240.

Treichel JL, Henry MM, Skumatz CM, Eells JT, Burke JM. Formate, the toxic metabolite of methanol, in cultured ocular cells. *Neurotoxicology*. 2003;24:825–834.

Tremblay RT, Martin SA, Fisher JW. Novel characterization of the aerosol and gas-phase composition of aerosolized jet fuel. *Inhal Toxicol*. 2010a;22:394–401.

Tremblay RT, Martin SA, Fisher JW. Chapter 12: Evaluation of methods used to generate and characterize jet fuel vapor and aerosol for inhalation toxicology studies. In: Witten ML, Zeigler E, Ritchie GD, eds. *Jet Fuel Toxicology*. Boca Raton, FL: CRC Press, Taylor and Francis Group; 2011:219–238.

Triebig G, Claus D, Csuzda I, et al. Cross-sectional epidemiological study on neurotoxicity of solvents in paints and lacquers. *Int Arch Occup Environ Health*. 1988;60:233–241.

Triebig G, Hallermann J. Survey of solvent related chronic encephalopathy as an occupational disease in European countries. *Occup Environ Med*. 2001;58:575–581.

Troutman JA, Rick DL, Stuard SB, et al. Development of a physiologically-based pharmacokinetic model of 2-phenoxyethanol and its metabolite phenoxyacetic acid in rats and humans to address toxicokinetic uncertainty in risk assessment. *Regul Toxicol Pharmacol*. 2015;73:530–543.

Trush MA, Twerdok LE, Rembish SJ, et al. Analysis of target cell susceptibility as a basis for the development of a chemoprotective strategy against benzene-induced hematotoxicities. *Environ Health Perspect*. 1996;104(suppl 6):1227–1234.

Tsai SP, Fox EE, Ransdell JD, et al. A hematology surveillance study of petrochemical workers exposure to benzene. *Regul Toxicol Pharmacol*. 2004;40:67–73.

Turini A, Amato G, Longo V, Gervasi PG. Oxidation of methyl- and ethyl-tertiary-butyl ethers in rat liver microsomes: role of the cytochrome P450 isoforms. *Arch Toxicol*. 1998;72:207–214.

Turtletaub KW, Mani C. Benzene metabolism in rodents at doses relevant to human exposure from urban air. *Res Report Health Effects Inst*. 2003;113:1–26.

Tyl RW, Ballantyne B, France KA, et al. Evaluation of the developmental toxicity of ethylene glycol monohexyl ether vapor in Fischer 344 rats and New Zealand white rabbits. *Fundam Appl Toxicol*. 1989;12:269–280.

Tyson CA, Hawk-Prather K, Story DL, et al. Correlations of *in vitro* and *in vivo* hepatotoxicity for five haloalkanes. *Toxicol Appl Pharmacol*. 1983;70:289–302.

Uboh FE, Ebong PE, Umoh IB. Comparative hepatoprotective effect of vitamins A and E against gasoline vapor toxicity in male and female rats. *Gastroenterol Res*. 2009;2:295–302.

Uchida Y, Nakatsuka H, Ukai H, et al. Symptoms and signs in workers exposed predominantly to xylenes. *Int Arch Occup Environ Health*. 1993;64:597–605.

Udden MM. Hemolysis and deformability of erythrocytes exposed to butoxyacetic acid, a metabolite of 2-butoxyethanol: II. Resistance in red blood cells from humans with potential susceptibility. *J Appl Toxicol*. 1994;14:97–102.

Udden MM. Effects of diethylene glycol butyl ether and butoxyethoxyacetic acid on rat and human erythrocytes. *Toxicol Lett*. 2005;156:95–101.

Udden MM, Patton CS. Hemolysis and deformability of erythrocytes exposed to butoxyacetic acid, a metabolite of 2-butoxyethanol: I. Sensitivity in rats and resistance in normal humans. *J Appl Toxicol*. 1994;14:91–96.

Udden MM, Patton CS. Butoxyacetic acid-induced hemolysis of rat red blood cells: effect of external osmolarity and cations. *Toxicol Lett*. 2005;156:81–93.

Ullrich SE. Dermal application of JP-8 jet fuel induces immune suppression. *Toxicol Sci*. 1999;52:61–67.

Ullrich SE, Lyons HJ. Mechanisms involved in the immunotoxicity induced by dermal application of JP-8 jet fuel. *Toxicol Sci*. 2000;58:290–298.

Umulis DM, Gurmen NM, Singh P, Fogler HS. A physiologically based model for ethanol and acetaldehyde metabolism in human beings. *Alcohol*. 2005;35:3–12.

UNEP (United Nations Environment Programme). *SIDS Initial Assessment Report for SIAM 2 - Tripropylene Glycol*, July, 1994. Available at: http://www.inchem.org/documents/sids/sids/24800-44-0.pdf.

UNEP (United Nations Environment Programme). *SIDS Initial Assessment Report for 11th SIAM—Dipropylene Glycol (Mixed Isomers and Dominant Isomer)*, January, 2001. Available at: http://www.inchem.org/documents/sids/sids/25265-71-8.pdf.

Uzma N, Kumar BS, Hazari MAH. Exposure to benzene induces oxidative stress, alters the immune response and expression of p53 in gasoline filling workers. *Am J Ind Med*. 2010;53:1264–1270.

Vahter M, Gochfeld M, Casati B, et al. Implications of gender differences for human health risk assessment and toxicology. *Environ Res*. 2007;104:70–84.

Valentine JL, Lee SS-T, Seaton MJ, et al. Reduction of benzene metabolism and toxicity in mice that lack CYP2E1 expression. *Toxicol Appl Pharmacol*. 1996;141:205–213.

Valentine WM, Amarnath V, Amarnath K, et al. Covalent modification of hemoglobin by carbon disulfide: III. A potential biomarker of effect. *Neurotoxicology*. 1998;19:99–108.

Valentine WM, Amarnath V, Graham DG, et al. CS2-mediated cross-linking of erythrocyte spectrin and neurofilament protein: dose response and temporal relationship to the formation of axonal swellings. *Toxicol Appl Pharmacol*. 1997;142:95–105.

Valentine WM, Graham DG, Anthony DC. Covalent cross-linking of erythrocyte spectrin by carbon disulfide in vivo. *Toxicol Appl Pharmacol*. 1993;121:71–77.

Valpey R, Sumi SM, Copass MK, Goble GJ. Acute and chronic progressive encephalopathy due to gasoline sniffing. *Neurology*. 1978;28:507–510.

Vamvakas S, Bruning T, Thomasson B, et al. Renal cell cancer correlated with occupational exposure to trichloroethene. *J Cancer Res Clin Oncol*. 1998;124:374–382.

van der Hoek JAF, Verberk MM, Hageman G. Criteria for solvent-induced chronic toxic encephalopathy: a systematic review. *Int Arch Occup Environ Health*. 2000;73:362–368.

van der Hoek JAF, Verberk MM, van der Laan G, Hageman G. Routine diagnostic procedures for chronic encephalopathy induced by solvents: survey of experts. *Occup Environ Med*. 2001;58:382–385.

van Valen E, van Thriel C, Akila R, et al. Chronic solvent-induced encephalopathy: European consensus of neuropsychological characteristics, assessment, and guidelines for diagnostics. *Neurotoxicology*. 2012;33:710–726.

van Valen E, Wekking E, van der Laan G, et al. The course of chronic solvent induced encephalopathy: a systematic review. *Neurotoxicology*. 2009;30:1172–1186.

Vanhoorne MH, De Bacquer D, De Backer G. Epidemiological study of the cardiovascular effects of carbon disulphide. *Int J Epidemiol*. 1992;21:745–752.

Vasiliou V, Pappa A, Estey T. Role of human aldehyde dehydrogenases in endobiotic and xenobiotic metabolism. *Drug Metab Rev*. 2004;36:279–299.

Verberk MM, van der Hoek J, van Valen E, et al. Decision rules for assessment of chronic solvent-induced encephalopathy: Results in 2370 patients. *Neurotoxicology*. 2012;33:742–752.

Varela-Rey M, Woodhoo A, Lu SC. Alcohol, DNA methylation, and cancer. *Alcohol Res*. 2013;35:25–35.

Verhulst A, Asselman M, Persy VP, et al. Crystal retention capacity of cells in the human nephron: Involvement of CD44 and its ligands hyaluronic acid and osteopontin in the transition of a crystal binding—into a non-adherent epithelium. *J Am Soc Nephrol*. 2003;14:107–115.

Verplanke AJ, Leummens MH, Herber RF. Occupational exposure to tetrachloroethylene and its effects on the kidneys. *J Occup Environ Med*. 1999;41:11–16.

Veulemans H, Steeno O, Maschelein R, Groeseneken D. Exposure to ethylene glycol ethers and spermatogenic disorders in man: a case–control study. *Br J Ind Med*. 1993;50:71–78.

Vidali M, Stewart SF, Albano E. Interplay between oxidative stress and immunity in the progression of alcohol-mediated liver injury. *Trends Mol Med*. 2008;14: 63–71.

Visser I, Lavini C, Booij J, et al. Cerebral impairment in chronic solvent-induced encephalopathy. *Ann Neurol*. 2008;63:572–580.

Visser I, Wekking EM, de Boer A, et al. Prevalence of psychiatric disorders in patients with chronic solvent induced encephalopathy (CSE). *Neurotoxicology*. 2011;32:916–922.

Visser I, de Boer A, Wekking EM, et al. The coping with attention and memory complaints questionnaire (CAMQ): psychometric evaluation of an instrument in suspected chronic solvent-induced encephalopathy (CSE). *Neuropsychol Rehab*. 2012;22:794–808.

Vlaanderen J, Straif K, Ruder A, et al. Tetrachloroethylene exposure and cancer risk: a meta-analysis of dry-cleaning-worker studies. *Environ Health Perspect*. 2014;122:661–666.

Volkel W, Friedewald M, Lederer E, et al. Biotransformation of perchloroethene: dose-dependent excretion of trichloroacetic acid, dichloroacetic acid, and *N*-acetyl-*S*-(trichlorovinyl)-l-cysteine in rats and humans after inhalation. *Toxicol Appl Pharmacol*. 1998;153:20–27.

von Euler G. Toluene and dopaminergic transmission. In: Isaacon RL, Jensen KF, eds. *The Vulnerable Brain and Environmental Risk. Toxins in Air and Water*. Vol 3. New York: Plenum Press; 1994:301–321.

Vrca A, Bozicevic D, Karacic V, et al. Visual evoked potentials in individuals exposed to long-term low concentrations of toluene. *Arch Toxicol*. 1995;69:337–340.

Vyskocil A, Emminger S, Tejral J, et al. Study on kidney function in female workers exposed to perchloroethylene. *Human Exp Toxicol*. 1990;9: 377–380.

Wade MG, Kawata A, Williams A, Yauk C. Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. *Biol Reprod*. 2008;78:822–831.

Walgren JL, Kurtz DT, McMillan JM. Lack of direct mitogenic activity of dichloroacetate and trichloroacetate in cultured rat hepatocytes. *Toxicology*. 2005;211: 220–230.

Wallace L. Environmental exposure to benzene: an update. *Environ Health Perspect*. 1996;104(suppl 6):1129–1136.

Wallace LA, Pellizzari ED, Hartwell TD, et al. A TEAM study: personal exposures to toxic substances in air, drinking water, and breath of 400 residents of New Jersey, North Carolina, and North Dakota. *Environ Res*. 1987;43:290–307.

Wang FI, Kuo ML, Shun CT, et al. Chronic toxicity of a mixture of chlorinated alkanes and alkenes in 1CR mice. *J Toxicol Environ Health*. 2002a;65:279–291.

Wang PY, Kaneko T, Tsukada H, et al. Dose- and route-dependent alterations in metabolism and toxicity of chemical compounds in ethanol-treated rats: Difference between highly (chloroform) and poorly (carbon tetrachloride) metabolized hepatotoxic compounds. *Toxicol Appl Pharmacol*. 1997;142:13–21.

Wang S, Sun Y, Wu Y, et al. Down-regulation of uterine LIF expression induced by the hormonal level disorder causes embryo implanation loss after mice exposed to carbon disulfide at peri-implantation. *Biochem Biophys Res Commun*. 2015;467:7–13.

Wang S, Young RS, Sun NN, Witten ML. In vitro cytokine release from rat type II pneumocytes and alveolar macrophages following exposure to JP-8 jet fuel in co-culture. *Toxicology*. 2002b;173:211–219.

Wang W, Chapin RE. Differential gene expression detected by suppression subtractive hybridization in the ethylene glycol monomethyl ether-induced testicular lesion. *Toxicol Sci*. 2000;56:165–174.

Wang R-S, Ohtani K, Suda M, Nakajima T. Inhibitory effect of ethylene glycol monoethyl ether on rat sperm motion. *Ind Health*. 2006;44:665–668.

Wang W, Wine RN, Chapin RE. Rat testicular Src: Normal distribution and involvement in ethylene glycol monomethyl ether-induced apoptosis. *Toxicol Appl Pharmacol*. 2000;163:125–134.

Ward E, Hornung R, Morris J, et al. Risk of low red or white blood cell count related to estimated benzene exposure in a rubberworker cohort. *Am J Ind Med*. 1996;29:247–257.

Warren DA, Bowen SE, Jennings WB, et al. Biphasic effects of 1,1,1-trichloroethane on the locomotor activity of mice: relationship to blood and brain solvent concentrations. *Toxicol Sci*. 2000;56:365–373.

Warren DA, Graeter LJ, Channel SR, et al. Trichloroethylene, trichloroacetic acid, and dichloroacetic acid: do they affect eye development in the Sprague–Dawley rat? *Int J Toxicol*. 2006;25:279–284.

Warren DA, Reigle TG, Muralidhara S, Dallas CE. Schedule-controlled operant behavior of rats during 1,1,1-trichloroethane inhalation: relationship to blood and brain solvent concentrations. *Neurotoxicol Teratol*. 1998;20:143–153.

Warren KR, Hewitt BG, Thomas JD. Fetal alcohol spectrum disorders: research challenges and opportunities. *Alcohol Res Health*. 2011;34: 4–14.

Wartenberg D, Reyner D, Scott CS. Trichloroethylene and cancer: epidemiologic evidence. *Environ Health Perspect*. 2000;108(suppl 2):161–176.

Watanabe A, Nakano Y, Endo T, et al. Collaborative work to evaluate toxicity on male reproductive organs by repeated dose studies in rats 27. Repeated toxicity study on ethylene glycol monomethyl ether for 2 and 4 weeks to detect effects on male reproductive organs in rats. *J Toxicol Sci*. 2000;25:259–266.

Watanabe K, Guengerich FP. Limited reactivity of formyl chloride with glutathione and relevance to metabolism and toxicity of dichloromethane. *Chem Res Toxicol*. 2006;19:1091–1096.

Watanabe M, Tanaka M, Tateishi T, et al. Effects of the estrous cycle and the gender differences on hepatic drug metabolizing enzyme activities. *Pharmacol Res*. 1997;35:477–480.

Watson RE, Jacobson CF, Williams AL, et al. Trichloroethylene-contaminated drinking water and congenital heart defects: a critical analysis of the literature. *Reprod Toxicol*. 2006;21:117–147.

Watson WA, Litovitz TL, Rodgers GC Jr, et al. 2004 Annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. *Am J Emerg Med*. 2005;23:589–666.

Watson WA. Ethylene glycol toxicity: closing in on rational, evidence-based treatment. *Ann Emerg Med*. 2000;36:139–141.

Wauthier V, Verbeeck RK, Calderan PB. Age-related changes in the protein and mRNA levels of CYP2E1 and CYP3A isoforms as well as in their hepatic activities in Wistar rats. What role for oxidative stress? *Arch Toxicol*. 2004;78:131–138.

Wax PM. Elixirs, diluents, and the passage of the 1938 Federal Food, Drug and Cosmetic Act. *Ann Intern Med*. 1995;122:456–461.

Weber LWD, Boll M, Stampfl A. Hepatotoxicity and mechanisms of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol*. 2003;33:105–136.

Weisel CP, Jo WK. Ingestion, inhalation, and dermal exposures to chloroform and trichloroethene from tap water. *Environ Health Perspect*. 1996;104:48–51.

Weiss NS. Cancer in relation to occupational exposure to perchloroethylene. *Cancer Causes Control*. 1995;6:257–266.

Welch LS, Schrader SM, Turner TW, Cullen MR. Effects of exposure to ethylene glycol ethers on shipyard painters: II. Male reproduction. *Am J Ind Med*. 1988;14:509–526.

Wenker MAM, Kerzic S, Monster AC, de Wolff FA. Metabolic capacity and interindividual variation in toxicokinetics of styrene in volunteers. *Human Exp Toxicol*. 2001;20:221–228.

West R, Banton M, Hu J, Klapacz J. The distribution, fate, and effects of propylene glycol substances in the environment. *Rev Environ Contam Toxicol*. 2014;232:107–138.

White INH, Razvi N, Gibbs AH, et al. Neoantigen formation and clastogenic action of HCFC-123 and perchloroethylene in human MCL-5 cells. *Toxicol Lett*. 2001;124:129–138.

White KL, Peachee VL, Armstrong SR, et al. Health assessment of gasoline and fuel oxygenate vapors. Immunotoxicity evaluation. *Regul Toxicol Pharmacol*. 2014;70:S43–S47.

Wilson ME, Karaoui M, Al Djasim L, et al. The safety and efficacy of chloral hydrate sedation for pediatric ophthalmic procedures: a retrospective review. *J Pediatr Ophthalmol Strabismus*. 2014;51:154–159.

WHO (World Health Organization)/Nordic Council of Ministers. *Chronic Effects of Organic Solvents on the Central Nervous System and Diagnostic Criteria*. Copenhagen, 1985.

WHO (World Health Organization of the United Nations). *Environmenta Health Criteria 206: Methyl Tertiary-Butyl Ether*. Geneva, 1998.

WHO (World Health Organization). *Concise International Chemical Assessment Document 46—Carbon Disulfide*. Geneva, 2002:1–64. Available at: http://www.inchem.org/documents/cicads/cicads/cicad46.htm.

WHO (World Health Organization). *Guidelines for Drinking Water Quality*, 3rd ed., Geneva, 2004. Available at: http://www.who.int/water_sanitation_health/drug/gdwq3/en/.

Wiener HL, Richardson KE. The metabolism and toxicity of ethylene glycol. *Res Commun Subst Abuse*. 1988;9:77–87.

Wiener HL, Richardson KE. Metabolism of diethylene glycol in male rats. *Biochem Pharmacol*. 1989;38:539–541.

Wiesenhutter B, Selinski S, Golka K, et al. Re-assessment of the influence of polymorphisms of phase-II metabolic enzymes on renal cell cancer risk of trichloroethylene-exposed workers. *Int Arch Occup Environ Health*. 2007;81: 247–251.

Wilkins-Haug L. Teratogen update: Toluene. *Teratology*. 1997;55: 145–151.

Williams J, Reel JR, George JD, Lamb JC IV. Reproductive effects of diethylene glycol and diethylene glycol monoethyl ether in Swiss CD-1 mice assessed by a continuous breeding protocol. *Fundam Appl Toxicol*. 1990;14:622–635.

Williams JF, Storck M. Inhalant abuse. *Pediatrics*. 2007;119:1009–1017.

Williams PR, Cushing CA, Sheehan PJ. Data available for evaluating the risks and benefits of MTBE and ethanol as alternative fuel oxygenates. *Risk Anal*. 2003;23:1085–1115.

Williams PRD, Scott PK, Sheehan PJ, Paustenbach DJ. A probabilistic assessment of household exposures to MTBE in California drinking water. *Human Ecol Risk Assess*. 2000a;6:827–849.

Williams TM, Cattley RC, Borghoff SJ. Alterations in endocrine responses in male Sprague–Dawley rats following oral administration of methyl *tert*-butyl ether. *Toxicol Sci*. 2000b;54:168–176.

Williams TM, Borghoff SJ. Induction of testosterone biotransformation enzymes following oral administration of methyl *tert* -butyl ether to male Sprague–Dawley rats. *Toxicol Sci*. 2000;57:147–155.

Williams TM, Borghoff SJ. Characterization of *tert*-butyl alcohol binding to alpha2u-globulin in F-344 rats. *Toxicol Sci*. 2001;62:228–235.

Williams WC, Riddle MM, Copeland CB, et al. Immunological effects of 2-methoxyethanol administered dermally or orally to Fischer 344 rats. *Toxicology*. 1995;98:215–223.

Wilmarth KR, Viana ME, Abou-Donia MB. Carbon disulfide inhalation increases Ca^{2+}/calmodulin-dependent kinase phosphorylation of cytoskeletal proteins in the rat central nervous system. *Brain Res*. 1993;628:293–300.

Wilson KC, Reardon C, Farber HW. Propylene glycol toxicity in a patient receiving intravenous diazepam. *N Engl J Med*. 2000;343:815.

Wilson KC, Reardon C, Theodore AC, Farber HW. Propylene glycol toxicity—a severe iatrogenic illness in ICU patients receiving IV benzodiazepines: A case series and prospective, observational pilot study. *Chest*. 2005;128:1674–1681.

Wine RN, Ku WW, Li LH, Chapin RE. Cyclophilin A is present in rat germ cells and is associated with spermatocyte apoptosis. Reproductive Toxicology Group. *Biol Reprod*. 1997;56:439–446.

Winek CL, Shingleton DP, Shanor SP. Ethylene and diethylene glycol toxicity. *Clin Toxicol*. 1978;13:297–324.

Win-Shwe TT, Fujimaki H: Neurotoxicity of toluene. *Toxicol Lett*. 2010;198:93–99.

Win-Shwe TT, Hojo R, Mitsushima D, et al. Establishment of mouse model to assess brain neurotransmitter level and learning performance simultaneously following toxic chemical exposure: Using in vivo microdialysis and schedule-controlled operant behavior. *J UOEH*. 2009;31:1–11.

Witten ML, Zeigler E, Ritchie GD, eds. *Jet Fuel Toxicology*. Boca Raton, FL: CRC Press, Taylor and Francis Group; 2011.

Witz G, Zhang Z, Goldstein BD. Reactive ring-opened aldehyde metabolites in benzene hematotoxicity. *Environ Health Perspect*. 1996;104(suppl 6):1195–1199.

Witzmann FA, Bauer MD, Fieno AM, et al. Proteomic analysis of simulated jet fuel exposure in the lung. *Electrophoresis*. 1999;20:3659–3669.

Witzmann FA, Bauer MD, Fieno AM, et al. Proteomic analysis of the renal effects of simulated occupational jet fuel exposure. *Electrophoresis*. 2000a;21:976–984.

Witzmann FA, Carpenter RL, Ritchie GD, et al. Toxicity of chemical mixtures: proteomic analysis of persisting liver and kidney protein alterations induced by repeated exposure of rats to JP-8 jet fuel vapor. *Electrophoresis*. 2000b;21:2138–2147.

Witzmann FA, Bobb A, Briggs GB, et al. Analysis of rat testicular protein expression following 91-day exposure to JP-8 jet fuel vapor. *Proteomics*. 2003;3:1016–1027.

Witzmann FA, Monteiro-Riviere NA, Inman AO, et al. Effect of JP-8 jet fuel exposure on protein expression in human keratinocyte cells in culture. *Toxicol Lett*. 2005;160:8–21.

Wong FW-Y, Chan W-Y, Lee SS-T. Resistance to carbon tetrachloride-induced hepatotoxocity in mice which lack CYP2E1 expression. *Toxicol Appl Pharmacol*. 1998;153:109–118.

Wong O. Carcinogenicity of trichloroethylene: an epidemiologic assessment. *Clin Occup Environ Med*. 2004;4:557–589.

Wong SS, Thomas A, Barbaris B, et al. Pulmonary evaluation of permissible exposure limit of syntroleum S-8 synthetic jet fuel in mice. *Toxicol Sci*. 2009;109:312–320.

Wormhoudt LW, Commandeur JNM, Vermeulen NPE. Genetic polymorphisms of human N-acetyltransferase, cytochrome P450, glutathione-S-transferase, and epoxide hydrolase enzymes: Relevance to xenobiotic metabolism and toxicity. *Crit Rev Toxicol*. 1999;29: 59–124.

Wronska-Nofer T. Various disorders of cholesterol metabolism and their effect on the development of experimental arteriosclerosis in rats exposed to carbon disulfide. *Med Proc*. 1979;30:121–134.

Wronska-Nofer T, Chojnowska-Jezierska J, Nofer JR, et al. Increased oxidative stress in subjects exposed to carbon disulfide (CS_2)—an occupational coronary risk factor. *Arch Toxicol*. 2002;76:152–157.

Wu L-T, Pilowsky DJ, Schlenger WE. Inhalant abuse and dependence among adolescents in the United States. *J Am Acad Child Adolesc Psych*. 2004;43:1206–1214.

Xu H, Tanphaichitr N, Forkert PG, et al. Exposure to trichloroethylene and its metabolites causes impairment of sperm fertilizing ability in mice. *Toxicol Sci*. 2004;82:590–597.

Yamada T, Tanaka Y, Hasegawa R, et al. Development of a category approach to predict the testicular toxicity of chemical substances structurally related to ethylene glycol methyl ether. *Reg Toxicol Pharmacol*. 2014;70:711–719.

Yamada T, Tanaka Y, Zhang H, et al. A category approach to predicting the hemolytic effects of ethylene glycol alkyl ethers in repeated-dose toxicity. *J Toxicol Sci*. 2012;37:503–515.

Yamamoto T, Horii I, Yoshida T. Ingegrated NMR-based metabonomic investigation of early metabolic effects of ethylene glycol monomethyl ether (EGME) on male reproductive organs in rats. *J Toxicol Sci*. 2007;32:515–528.

Yang AM, Inamine T, Hochrath K, et al. Intestinal fungi contribute to development of alcoholic liver disease. *J Clin Invest*. 2017;127:2829–2841.

Yang F, Luo J. Endoplasmic reticulum stress and ethanol neurotoxicity. *Biomolecules*. 2015;5:2538–2553.

Yang JH, Lee CH, Monteiro-Riviere NA, et al. Toxicity of jet fuel aliphatic and aromatic hydrocarbon mixtures on human epidermal keratinocytes: evaluation based on in vitro cytotoxicity and interleukin-8 release. *Arch Toxicol*. 2006;80:508–523.

Yang J, Bai W, Niu P, et al. Aberrant hypomethylated STAT3 was identified as a biomarker of chronic benzene poisoning through integrating DNA methylation and mRNA expression data. *Exp Mol Pathol*. 2014;96:346–353.

Yang Q, Nagano T, Shah R, et al. The PPARα-humanized mouse: a model to investigate species differences in liver toxicity mediated by PPARα. *Toxicol Sci*. 2008;101: 132–139.

Yang Y, Xu X, Georgopoulos PG. A Bayesian population PBPK model for multiroute chloroform exposure. *J Expos Sci Environ Epidemiol*. 2010;20:326–341.

Ye LL, Zhang GH, Huang JW, et al. Are polymorphisms in metabolism protective or a risk for reduced white blood cell counts in a Chinese population with low occupational benzene exposures? *Int J Occup Environ Health*. 2015;21:232–240.

Yin M, Ikejima K, Wheeler MD, et al. Estrogen is involved in early alcohol-induced liver injury in a rat enteral feeding model. *Hepatology*. 2000;31:117–123.

Yin SJ, Han CL, Lee AI, Wu CW. Human alcohol dehydrogenase family. Functional classification, ethanol/retinol metabolism, and medical implications. *Adv Exp Med Biol*. 1999;463:265–274.

Yin S-N, Hayes RB, Linet MS, et al. A cohort study of cancer among benzene-exposed workers in China: overall results. *Am J Ind Med*. 1996;29:227–235.

Yokley K, Tran HT, Pekari K, et al. Physiologically based pharmacokinetic (PBPK) modeling of benzene in humans: a Bayesian approach. *Risk Anal*. 2006;26:925–942.

Yoon BI, Hirabayashi Y, Kawasaki Y, et al. Aryl hydrocarbon receptor mediates benzene-induced hematotoxicity. *Toxicol Sci*. 2002;70:150–156.

Yuan R, Venitz J. Effect of chronic renal failure on the disposition of highly hepatically metabolized drugs. *Internat J Clin Pharmacol Therap*. 2000;38:245–253.

Yue J, Khokhar J, Miksys S, Tyndale RF. Differential induction of ethanol-metabolizing CYP2E1 and nicotine-metabolizing CYP2B1/2 in rat liver by chronic nicotine treatment and voluntary ethanol intake. *Europ J Pharmacol*. 2009;609:88–95.

Zangar RC, Benson JM, Burnett VL, Springer DL. Cytochrome P450 2E1 is the primary enzyme responsible for low-dose carbon tetrachloride metabolism in human liver microsomes. *Chem-Biol Interact*. 2000;125:233–243.

Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Therap*. 2013;138:103–141.

Zar T, Graeber C, Perazella MA. Recognition, treatment, and prevention of propylene glycol toxicity. *Semin Dial*. 2007;20:217–219.

Zhang B, Shen C, Yang L, et al. DNA damage and apoptosis of endometrial cells cause loss of the early embryo in mice exposed to carbon disulfide. *Toxicol Appl Pharmacol*. 2013;273:381–389.

Zhang Y-KJ, Yeager RL, Klaassen CD. Circadian expression of drug-processing genes and transcription factors in mouse liver. *Drug Metab Dispos*. 2009;37:106–115.

Zhao Y, Krishnadasan A, Kennedy N, et al. Estimated effects of solvents and mineral oils on cancer incidence and mortality in a cohort of aerospace workers. *Am J Ind Med*. 2005;48:249–258.

Zimniak P. Detoxification reactions: relevance to aging. *Ageing Res Rev*. 2008;7:281–300.

Zintzaras E, Stefanidis I, Santos M, Vidal F. Do alcohol-metabolizing enzyme gene polymorphisms increase the risk of alcoholism and alcoholic liver disease? *Hepatology*. 2006;43:352–361.

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chapter 25

Toxic Effects of Radiation and Radioactive Materials

David G. Hoel

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INTRODUCTION

Ionizing radiations such as gamma-rays and x-rays are radiations that have sufficient energy to displace electrons from molecules. These freed electrons then have the capability of damaging other molecules and, in particular, DNA. Thus, the potential health effects of low levels of radiation are important to understand in order to be able to quantify their effects. For example, it has been estimated that 10% of lung cancers are attributable to radon exposures. In recent years the amount of radiation that the public receives has greatly increased due to medical applications, especially the higher doses associated with computed tomography (CT) scans. Currently 50% of radiation exposures are from medical, 48% from environmental (primarily radon), and 2% from consumer products. The average yearly total effective exposure to individuals is 6.2 mSv. For an extensive analysis, the reader is referred to National Council on Radiation Protection (NCRP) Report 160 (NCRP, 2009). Fig. 25-1 taken from the report gives a summary breakdown of exposure sources.

Biological effects of radiation are primarily damage to the DNA. Atoms of the DNA target may be directly ionized or indirectly affected by the creation of a free radical that can interact with the DNA molecule. In particular, the hydroxyl radical is predominant in DNA damage. For radiation particles such as neutrons and α particles the damage is primarily direct, whereas for photons such as x-rays, about two-thirds of the DNA damage in mammalian cells is due to hydroxy radicals. The study of health effects of ionizing radiation is complicated by the fact that there are various types of radiation, from x-ray photons to heavy charged particles encountered in space. Within any type of radiation the potential damage also depends on the energy level of the photons or particles.

Cancer has been the major adverse health effect of ionizing radiation. It has been well studied epidemiologically, as well as in the laboratory and in animal toxicological studies. More recently, there has been a concern with possible cardiovascular effects, cataractogenesis, and possibly immunosenescence. At one time there was considerable concern about the possible heritable effects, but the risks now appear to be small. The issues with chemicals are the risks at low doses, and with radiation the effects of acute versus chronic exposures. Most radiation risk analyses depend on epidemiological studies. There have been many that have large populations and varied exposures. There is, however, a need for better radiobiological understanding of potential health effects in order to estimate low-dose effects, sensitive subgroups, and radiation types and energies that have not been adequately studied.

RADIATION BACKGROUND

Types of Ionizing Radiation

When ionizing radiation passes through matter, it has the energy to ionize atoms so that one or more of its electrons can be dislodged and chemical bonds broken. Ionizing radiation is of two types: particulate and electromagnetic waves. Particulate radiation may either be electrically charged (α, β, proton) or have no charge (neutron). Ionizing electromagnetic radiation (photons) in the form of x-rays or gamma-rays has considerably more energy than nonionizing radiation, such as ultraviolet and visible light.

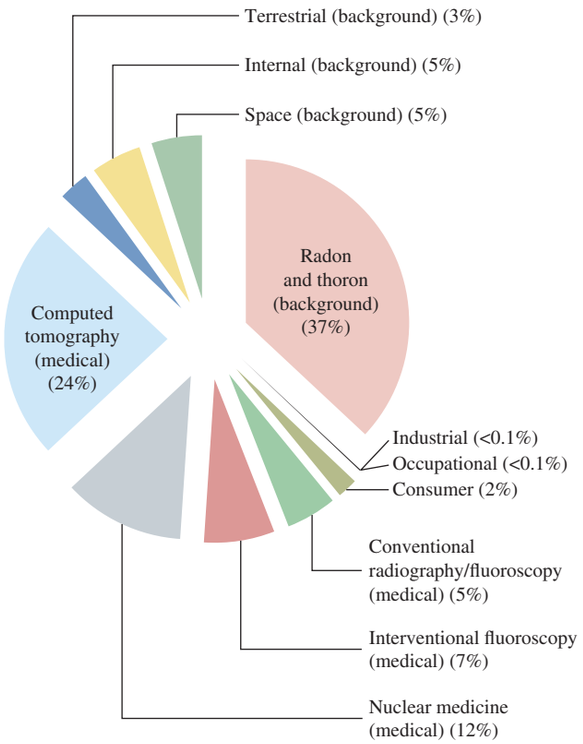

Figure 25-1. *Percent contribution of total effective dose to individuals (NCRP Report 160).*

Radionuclides (i.e., radioactive atoms), being unstable, release both electromagnetic and particulate radiation during their radioactive decay. The radionuclides decay into either stable elements or through a decay chain of successive radionuclides called decay daughters. The types of radiation emitted, its rate of decay, and the energies of the released radiation are unique to each type of radionuclide. The charged particles are directly ionizing because they ionize molecules by direct collisions with the molecules' electrons. Photons can travel relatively long distances, and after interacting with an electron in a material, the photon transfers some or all of its energy to the electron. The energetic electrons (i.e., photoelectrons or Compton electrons) are released, producing an indirect ionization. There are three basic radionuclide decay series: thorium, neptunium, and uranium series. The uranium decay series is illustrated in Fig. 25-2, and Table 25-1 gives the details.

The rate of energy dissipation by a single event is referred to as linear energy transfer (LET). The LET of a charged particle is the average energy lost due to interactions per unit length of its trajectory given as kiloelectron volts per micrometer (keV/μm). X-rays, gamma-rays, and β particles of similar energies produce sparse ionization tracks and are classified as low-LET radiation. Particulate radiation (e.g., neutrons and α particles) causes interactions with large amounts of energy being dissipated within short distances. Alpha particles (helium nucleus), which are released from the nucleus of some radionuclides, are slow-moving with a positive charge. Although they cannot penetrate a piece of paper or skin, they are of concern if ingested or inhaled. The most recognized example is the lung cancer risk from the inhalation of radon (Rn 222) and its daughter products. LET depends on the energy of the particle; for example, the LET of a 5-MeV α particle would be 100 keV/μm. Neutrons released during nuclear chain reactions have a greater range and lower LET (20 for a 2.5-MeV neutron). A 250-keV x-ray has an LET of 2 to 3 and a Cobalt 60 gamma-ray 0.2 to 0.3. A β particle is a simple electron that is released from the nucleus when a neutron converts into a proton, and it has similar low-LET values depending again on its energy.

The rate of decay of a radionuclide is exponential with a decay rate constant λ that is then simply $Ae^{-\lambda t}$ for time t and initial quantity of the radionuclide A. The radiological half-life is the time required for the radionuclide to lose 50% of its activity by decay. Each radionuclide has its own unique half-life as illustrated in Table 25-1. For example, the half-life of iodine 131 is 8 days while for cesium 137 it is 20 years. If a radionuclide is internally deposited in an individual, the actual half-life is obtained by taking the reciprocal of the sum of the reciprocals of the radiological half-life and the biological half-life; that is, $1/T = 1/T_r + 1/T_b$ where T is the half-life, T_r is the radiological half-life, and T_b is the biological half-life. For more extensive and detailed information the reader is referred to Biological Effects of Ionizing Radiation (BEIR) VII and the text by Turner (2007).

Relative Biological Effectiveness and Quality Factors

The various types of ionizing radiation have similar biological effects that occur because of the ionization of molecules. However, without knowing the type of radiation, one cannot specify how much radiation is needed to produce a specific biological effect. This is because a given absorbed dose (energy per unit mass) of x-rays does not have the same biological effect as an identical dose of neutrons. The relative effectiveness of different types of radiation in producing biological changes depends on deposition of energy, that is, LET. In order to deal with this problem the International Council of Radiation Protection (ICRP) developed the concept of relative biological effectiveness (RBE). It is defined in BEIR V as follows:

Biological potency of one radiation as compared with another to produce the same biological endpoint. It is numerically equal to the inverse of the ratio of absorbed doses of the two radiations required to produce equal biological effects. The reference radiation is often 200-kV x-rays.

The difficulty is that the RBE may differ depending on the biological endpoint and it may also be dose-dependent. When dose is not specified, it is assumed that the RBE is at the low-dose limit (see BEIR VII). The RBE increases as a function of LET, reaching a maximum at about 100 keV/μm. Interestingly, it was pointed out by Hall and Giaccia (2005) that at the density of ionization of 100 keV/μm, the average separation between ionization events just coincides with the diameter of the DNA double helix. Thus, radiation with this density has the highest probability of causing a double-stranded break by the passage of a single charged particle.

For regulatory purposes, the ICRP introduced the related concept of "quality factor" (Q) or weighting factor w_R. It is defined as an LET-dependent factor by which doses are multiplied to obtain a quantity that corresponds to the degree of biological effect produced by x-rays and low-energy gamma-rays. Table 25-2 gives the current weighting factors, whereas Fig. 25-3 plots the values for various neutron energy levels (taken from ICRP 103; ICRP, 2007).

The "equivalent dose" to an organ or tissue is then defined as $H_T = \Sigma_R w_R D_{TR}$ where D_{TR} is the dose to the organ from radiation type R. For public health purposes, ICRP further defines "effective dose" as $E = \Sigma_T w_T H_T$ where w_T are tissue-weighting factors (Table 25-3). It should be noted that these are the currently recommended

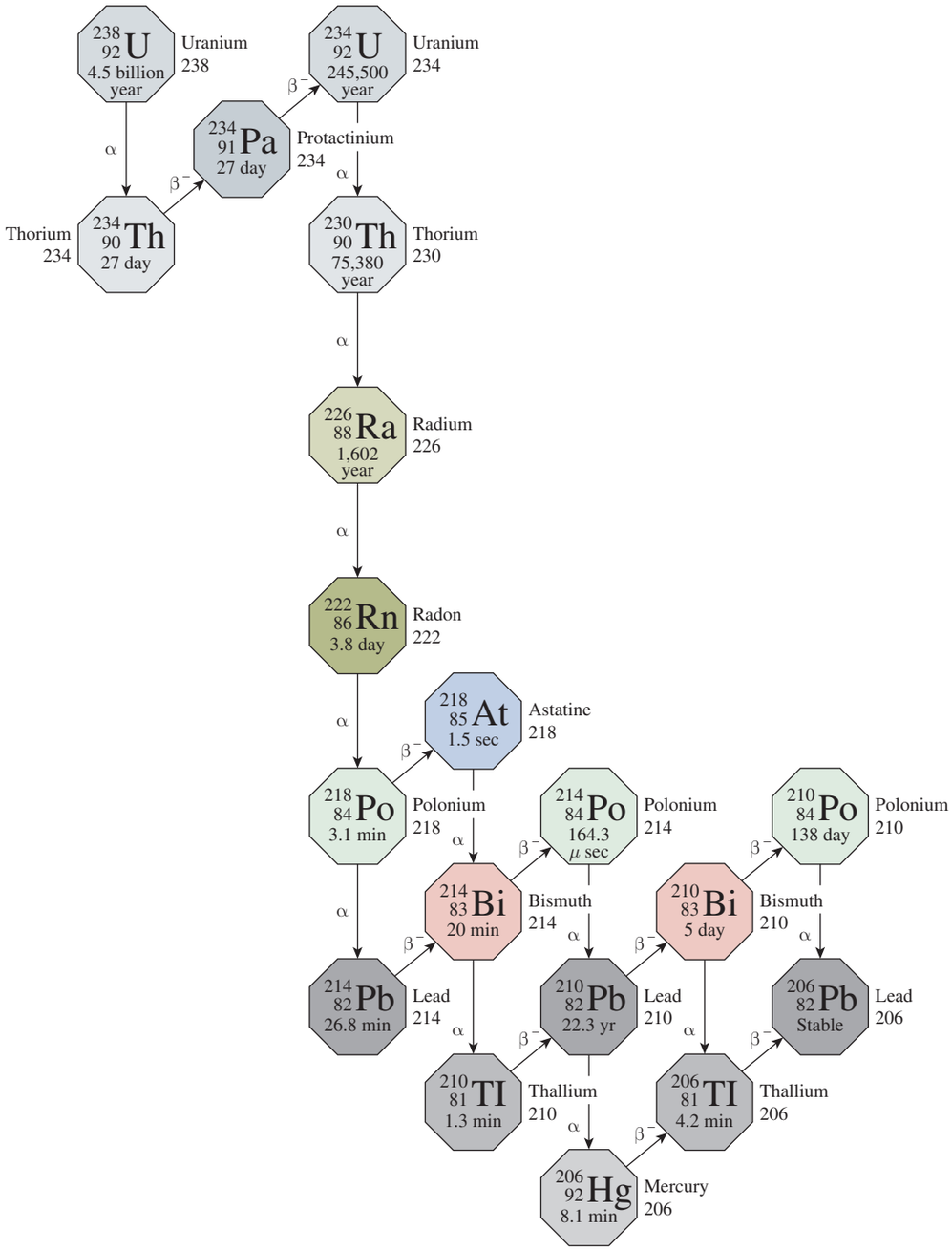

Figure 25-2. *Uranium decay chain.*

ICRP values although the Nuclear Regulatory Agency is using previous values (e.g., 0.25 instead of 0.08 for gonads possibly because of previous concerns about genetic effects). For the radionuclides there are also the terms "committed equivalent dose" and "committed effective dose," which simply means the total accumulative dose for 50 years after the exposure. Finally "total effective dose equivalent" (TEDE) means the sum of the effective dose equivalent (for external exposures) and the committed effective dose equivalent (for internal exposures).

Units of Radiation Activity and Dose

The basic unit of radiation activity is the Becquerel (Bq), which is nuclear disintegrations per second. The older unit of activity is the Curie (Ci), which corresponds to the number of disintegrations in 1 second from 1 g of radium 226 or 1 Ci = 3.7×10^{10} decays per second; thus, 1 Bq = 2.7×10^{-11} Ci. The EPA continues to use the old unit of activity with regard to radon. For example, the EPA's level of concern for radon in the home currently is 4 pCi/L, which is equivalent to 148 Bq/m^3.

Table 25-1

Radioisotopes in the Uranium Decay Series

NUCLIDE	DECAY MODE	HALF-LIFE (a = YEAR)	ENERGY RELEASED (MeV)	PRODUCT OF DECAY
^{238}U	α	4.468 × 10^{9}a	4.270	^{234}Th
^{234}Th	β$^-$	24.10 days	0.273	^{234m}Pa
^{234m}Pa	β− 99.84%	1.16 min	2.271	^{234}U
	IT 0.16%		0.074	^{234}Pa
^{234}Pa	β$^-$	6.70 h	2.197	^{234}U
^{234}U	α	245,500 a	4.859	^{230}Th
^{230}Th	α	75,380 a	4.770	^{226}Ra
^{226}Ra	α	1602 a	4.871	^{222}Rn
^{222}Rn	α	3.8235 days	5.590	^{218}Po
^{218}Po	α 99.98%	3.10 min	6.115	^{214}Pb
	β$^-$ 0.02%		0.265	^{218}At
^{218}At	α 99.90%	1.5 s	6.874	^{214}Bi
	β$^-$ 0.10%		2.883	^{218}Rn
^{218}Rn	α	35 ms	7.263	^{214}Po
^{214}Pb	β$^-$	26.8 min	1.024	^{214}Bi
^{214}Bi	β$^-$ 99.98%	19.9 min	3.272	^{214}Po
	α 0.02%		5.617	^{210}Tl
^{214}Po	α	0.1643 ms	7.883	^{210}Pb
^{210}Tl	β$^-$	1.30 min	5.484	^{210}Pb
^{210}Pb	β$^-$	22.3 a	0.064	^{210}Bi
^{210}Bi	β$^-$	5.013 days	1.426	^{210}Po
	99.99987%		5.982	^{206}Tl
	α 0.00013%			
^{210}Po	α	138.376 days	5.407	^{206}Pb
^{206}Tl	β$^-$	4.199 min	1.533	^{206}Pb
^{206}Pb	—	Stable	—	—

Table 25-2

Recommended Radiation Weighting Factors

RADIATION TYPE	RADIATION WEIGHTING FACTOR (W_R)
Photons	1
Electrons and muons	1
Protons and pions	2
Alpha particles, fission fragments, heavy ions	20
Neutrons	See Fig. 25-3

The "equivalent dose" to an organ or tissue is then defined as $H_T = \Sigma_R w_R D_{TR}$, where D_{TR} is the dose to the organ from radiation type R. For public health purposes.

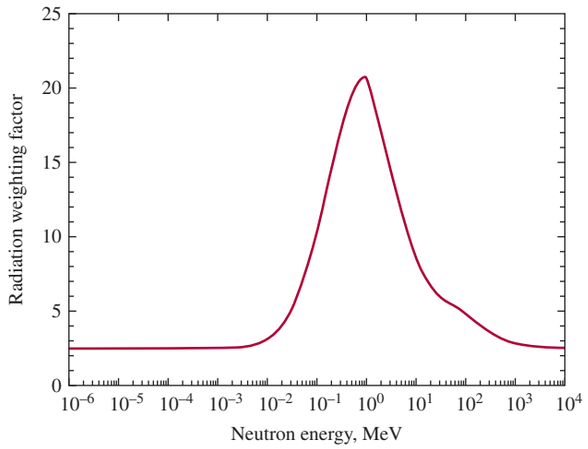

Figure 25-3. *Neutron weighting factor by energy level.*

The basic unit of dose is the Gray (Gy), which is the amount of energy released in a given mass of tissue. One Gray is defined as 1 joule of energy released in 1 kg of tissue. The older dose unit of rad (100 erg/g) is 1/100 of a Gray. The other common measure is the Sievert (Sv), which is a dose equivalent; that is, the dose in Gray multiplied by the appropriate quality factor. The dose equivalent rem is rad multiplied by the quality factor. Because ^{60}Co gamma-rays and 240 keV x-rays have a quality factor of one, we see Sv and Gy are used interchangeably in studies. Less common is the roentgen (R) that quantifies exposure, and is the number of ionizations in a cubic meter of air produced by x-rays or gamma radiation. Because 1 roentgen of gamma- and x-ray gives an absorbed dose to tissue of about 1 rad (10 mGy), they are nearly equivalent. For radon and its daughters, a different exposure system is used. Considering the uranium miner studies, exposure is expressed as a working level (WL), which is defined by BEIR VI as follows:

Any combination of the short-lived progeny of radon in 1 L of air, under ambient temperature and pressure, that results in the ultimate emission of 1.3 × 10^5 MeV of α particle energy. This is approximately the total amount of energy released over a long period of time by the short-lived progeny in equilibrium with 100 pCi of radon. 1 WL = 2.08 × 10^{-5} Jm^{-3}.

For cumulative exposure, a working level month (WLM) is used, which is the exposure to 1 WL for 1 working month of 170 hours.

Table 25-3

Recommended Tissue Weighting Factors

TISSUE	W_T	$\Sigma_T W_T$
Bone-marrow, colon, lung, stomach, breast, remainder tissues*	0.12	0.72
Gonads	0.08	0.08
Bladder, esophagus, liver, thyroid	0.04	0.16
Bone surface, brain, salivary glands, skin	0.01	0.04
	Total	1.00

**Remainder tissues: adrenals, extrathoracic (ET) region, gall bladder, heart, kidneys, lymphatic nodes, muscle, oral mucosa, pancreas, prostate, small intestine, spleen, thymus, and uterus/cervix.*

RADIOBIOLOGY

Radiation biology has made significant progress in our understanding of radiation effects at low doses. BEIR VII and, more recently, Dauer et al. (2010) go into great detail describing our current knowledge as it pertains to radiation-induced cancer. Currently radiation cancer risk extrapolations make two assumptions: namely that the basic mode of action is linearly related to dose and that the individual cell is the unit of risk. However, effects occurring in nontargeted cells, such as with induced genomic instability and bystander effects, suggest that responses can occur nonuniformly over time at the tissue level. How cells in tissues react to radiation effects is not well understood. There are many complexities in the cancer process that are not explained by the "single-cell biophysical hit" model of radiation-induced cancer, which is the basis for the linear no-threshold (LNT) model used for low-dose cancer risk assessments. Damage from ionizing radiation appears to increase linearly with dose, but recent data suggest that the processing and repair of induced damage could be nonlinear. The understanding of these processes is necessary for low-dose risk estimation. Following irradiation, various protective cellular processes occur that depend on the degree of damage and the tissue type. These mechanisms include DNA repair, intracellular metabolic oxidation/reduction reactions, cell cycle checkpoint controls, cellular signaling, senescence, and apoptosis (see Dauer et al., 2010).

The BEIR VII report reviewed in detail the effects of DNA damage after exposure to low doses of ionizing radiation. After an exposure to 5 mGy of low-LET radiation (average background per year), each cell nucleus is on average hit by one electron, resulting in 5 to 10 damaged bases, 2.5 to 5 single-strand breaks and 0.25 double-strand breaks.

Nontargeted Radiation Effects

Exposure to ionizing radiation can result in direct damage to the irradiated cells as well as producing effects in cells that were not irradiated (bystander effects). These nontargeted effects can occur in the nonirradiated neighbors of irradiated cells and at sites distant from the irradiated cells. Effects can also be observed in the progeny of an irradiated cell (genomic instability). Both targeted and nontargeted effects can result in DNA mutations, gene amplifications, chromosomal rearrangements, carcinogenesis, and cell death.

Bystander Effects Radiation-induced bystander effects are those in which cells that have not been directly exposed to ionizing radiation react as though they have been exposed by receiving a biochemical signal from a radiation-exposed cell. That is, they show chromosomal instability and other abnormalities, or die. BEIR VII concluded that for high-LET radiation a bystander effect has been shown for inducing cell lethality, chromosome aberrations, sister-chromatid exchanges, mutations, genomic instability, signal transduction pathways, and in vitro transformation. For low-LET radiation, the bystander effect has been limited to cell lethality and lethal mutations.

These bystander cells can be either adjacent or at some distance from the radiation-exposed cell. Not all cells can produce bystander signals or react to them. The important issue from a risk assessment view is whether bystander effects are beneficial (e.g., adaptive response and apoptosis [removal of damaged cells]) or detrimental to the nonexposed cells, and what impact they may have on dose response at low doses. It should be noted that most observed effects are detrimental, but beneficial effects are more difficult to measure. However, bystander effects demonstrate that the organism and tissues communicate and are responding as an organized structure to radiation insult. Large DNA deletions are the major type of radiation-induced mutations. In bystander cells, however, the types of mutations are similar to those that occur spontaneously, with the majority being point mutations. These differences are important in understanding risk at low doses (Dauer et al., 2010).

BEIR VII quotes Sawant et al. (2001) on high-LET bystander in vitro studies that "These results, if applicable in vivo, would have significant consequences in terms of radiation risk extrapolation to low doses, implying that the relevant target for radiation oncogenesis is larger than an individual cell, and that the risk of carcinogenesis would increase more slowly, if at all, at higher doses—an effect seen in vivo, as well as epidemiologically. Thus, a simple linear extrapolation of radiation risk from high doses (where they can be measured) to lower doses (where they must be inferred) would be of questionable validity." In other words, it is speculated that there could be a convex, downward-curving dose–response relationship at low doses, and that extrapolation of data from high doses could lead to an underestimate of the effect at low doses of high-LET radiation. Finally, BEIR VII concludes that "until molecular mechanisms of the bystander effect are elucidated, especially as related to an intact organism, and until reproducible bystander effects are observed for low-LET radiation in the dose range of 1 to 5 mGy, where an average of about one electron track traverses the nucleus, a bystander effect of low dose, low-LET radiation that might result in a dose–response curving either upward or downward should not be assumed." For an in-depth review of bystander effects both in vitro and in vivo see the papers by Morgan (2003a, 2003b) as well as Mothersill and Seymour (2004, 2005) and Liu et al. (2006).

Genomic Instability Genomic instability has been defined as the increase in rate of acquiring genetic change, and induced genomic instability can be observed in the progeny of irradiated cells and can persist for many generations (Morgan, 2003a, 2003b). When a cell is saturated in repairing radiation damage it may change its gene-product profile without any specific genetic damage. This has been suggested as a cause of genomic instability, which is an anti-inflammatory response, and is a risk for malignancy (Barcellos-Hoff, 2005). There is a need for the understanding of the molecular targets and processes responsible for genomic instability in order to understand the dose–response and why the frequency saturates at 10% to 30% of the surviving cells. This may be due to the fact that only a fraction of cells in a particular part of the cell cycle is susceptible. It is believed that genomic instability could be linked to the loss of telomere maintenance and too short telomeres as a mechanism in cancer development. Chromosome instability can also be initiated by double-strand breaks that result in the loss of a telomere that protects the chromosome end and prevents chromosome fusion. The induction of chromosomal aberrations plays an important role in genomic instability. There is also some evidence that reactive oxygen species (ROS) may play a role. BEIR VII concludes, "However, until the molecular mechanisms responsible for genomic instability and its relationship to carcinogenesis are understood, extrapolation of the limited dose–response data for genomic instability to radiation-induced cancers in the low-dose range <100 mGy is not warranted."

Adaptive Response Cells that are exposed to a low priming dose of radiation (e.g., 10 to 20 mGy) followed in a short time interval with a larger challenge dose (e.g., 1 Gy), the frequency of chromosomal aberrations induced by the challenge dose was found to be less than that from the challenge dose given alone. This effect is referred to as "adaptive response" (Tapio and Jacob, 2007). Studies have also shown that low doses of radiation may reduce the biological background effect. This has been shown for cell transformation

and chromosomal damage. It has also been observed that the normal rate of cell transformation (Redpath, 2006) and chromosome damage can be decreased to below the normal background level after an initial low-dose radiation exposure. Adaptive responses have been observed both in vitro and in vivo for both cancer and genetic effects, which suggests that low doses may decrease radiation risk. These adaptive responses suggest that enhancing normal repair or protective processes make it possible to decrease the risk for low-dose radiation-induced cancer (Dauer et al., 2010).

In BEIR VII's assessment of adaptive response, they state that data are needed when both the priming and challenging doses are low (less than 100 mGy) and when doses are delivered over several weeks or months at low dose rates or with fractionated exposures. They also suggest that the adaptive response studies using malignant transformation assays using immortalized cell lines may not be relevant to normal nonimmortalized cells. Finally BEIR VII states, "Thus, it is concluded that any useful extrapolations for dose–response relationships in humans cannot be made from the adaptive responses observed in human lymphocytes or other mammalian cellular systems. Therefore, at present, the assumption that any stimulatory effects of low doses of ionizing radiation substantially reduce long-term deleterious radiation effects in humans is unwarranted."

Hormesis

The BEIR VII committee defined hormesis as "the stimulating effect of small doses of substances which in larger doses are inhibitory" (see Calabrese et al. [2007] for a review). The committee concluded that the preponderance of available experimental data does not support the idea that low levels of ionizing radiation have a beneficial effect, and the possible mechanisms remain obscure. Further, they state, "At this time, the assumption that any stimulatory hormetic effects from low doses of ionizing radiation will have a significant health benefit to humans that exceeds potential detrimental effects from radiation exposure at the same dose is unwarranted." Research actively continues in this area and the BEIR committee recommends the need for research on the possible mechanisms for hormesis at low radiation doses.

Gene Expression

Gene expression profiling for monitoring ionizing radiation exposure has become an active area of research. It has been shown that dose, dose rate, radiation quality, and time since exposure result in variations in the response of genes, so that gene expression signatures may be markers of radiation exposure. Changes in gene expression in human cell lines occur after as little as 0.02 Gy gamma-rays (Amundson and Fornace, 2001; Amundson et al., 2003). Using gene expression methods, Ory et al. (2011) and Ugolin et al. (2011) were able to distinguish a number of post-Chernobyl thyroid tumors and postradiotherapy thyroid tumors from their sporadic counterparts. These techniques, if applicable to low-dose radiation exposures, could be very useful in radiation epidemiological studies (see Maenhaut et al., 2011).

Summary

Cancer being the primary health concern from exposure to ionizing radiation, there is a focus on mechanisms and dose–response as they relate to the induction of chromosomal aberrations and gene mutations because cancer is believed to be associated with these cellular responses. Experimental data indicate that the dose–response relationship over a range of 20 to 100 mGy is most likely to be linear, and not significantly affected by either an adaptive or a bystander effect. Data in this dose range for genomic instability are generally not available. The question of the shape of the dose–response relationship up to 20 mGy remains uncertain. The future of understanding low-dose radiation cancer risks will depend on the continued advancement of molecular biology, gene expression analysis, and computational biology.

CANCER EPIDEMIOLOGY

Epidemiological studies have been extensive and provide the basis for our understanding of radiation-induced cancer effects. These studies provide a wide range of exposures including populations at high natural background exposures, occupational studies, populations exposed to nuclear testing and reactor accidents, A-bomb survivor studies, and the follow-up of patients having had high-dose medical radiation therapies. Radiation cancer studies are no different from other types of occupational and environmental cancer studies in that radiation-induced cancers are not distinguishable pathologically, and there are usual issues of exposure confounding, long latencies (e.g., 10 to 20 years for solid tumors), levels of exposure, and study size. Generally for acute exposures only epidemiological studies with exposures to relatively high doses of radiation (greater than 0.15 Sv) have shown such an excess of cancer. Because of these difficulties, the most informative studies are those that involve a large number of individuals with large radiation doses and follow-up of several decades.

Epidemiological assessment of cancer in populations exposed to radiation has been the principal source of information used by regulatory groups. The National Academies series of BEIR reports provides this information through expert committee analyses. The most recent report BEIR VII is restricted to the health effects of low-LET radiation (e.g., gamma-ray and x-ray). The BEIR VI report is solely devoted to radon, whereas BEIR IV is focused on radionuclides. BEIR V is a previous version of BEIR VII. The BEIR reports, besides reviewing the scientific literature on radiation health effects, develop quantitative cancer risk models for low-LET exposures as well as radon exposures. The United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) reports also review the literature on cancer and noncancer effects of radiation exposures. The committee developed quantitative cancer risk models and applied them to various populations. More recently, the International Agency for Research on Cancer (IARC), in its monograph series, evaluated cancer effects for various types of radiation exposures (IARC report 100D; IARC, 2009). This qualitative analysis determined that for each radiation type which cancer sites are considered to be causal. A brief summary was published after the meeting and the findings are reproduced here in Table 25-4 (El Ghissassi et al., 2009). We see from Table 25-4 that there are numerous Class 1 cancer sites for gamma-ray and x-ray, while only lung cancer is associated with radon exposures.

A-bomb Survivor Studies

The Radiation Effects Research Foundation (RERF) and its predecessor the Atomic Bomb Casualty Commission (ABCC) have reported a series of mortality studies on a fixed population (Life Span Study [LSS] cohort) of 120,321 individuals. These included A-bomb survivors and residents who were not present at the time of the bombing (NIC [not in city] 26,529). The most recent report is the 14th in the series and covers the years 1950 to 2003, and includes both cancer and noncancer mortality (Ozasa et al., 2012). There are also studies on cancer incidence, with the most recent for solid cancers during the period 1958 to 1998 and for hematopoietic

Table 25-4

IARC: Tumor Sites with Sufficient Evidence of Human Carcinogenicity

RADIATION TYPE	MAJOR STUDY POPULATIONS	TUMOR SITES (AND TYPES) ON WHICH SUFFICIENT EVIDENCE IS BASED
α-Particle and β-particle emitters		
Radon-222 and decay products	General population (residential exposure), underground miners	Lung
Radium-224 and decay products	Medical patients	Bone
Radium-226, radium-228, and decay products	Radium-dial painters	Bone, paranasal sinus, and mastoid process (radium-226 only)
Thorium-232 and decay products	Medical patients	Liver, extrahepatic bile ducts, gall bladder, and leukemia (excluding CLL)
Plutonium	Plutonium-production workers	Lung, liver, and bone
Phosphorus-32	Medical patients	Acute leukemia
Fission products, including strontium-90	General population, following nuclear reactor accident	Solid cancers and leukemia
Radioiodines, including iodine-131	Children and adolescents, following nuclear reactor accident	Thyroid
X radiation or gamma radiation	Atomic-bomb survivors, medical patients; in utero exposure (offspring of pregnant medical patients and of atomic-bomb survivors)	Salivary gland, esophagus, stomach, colon, lung, bone, skin (BCC), female breast, urinary bladder, brain and CNS, leukemia (excluding CLL), thyroid, kidney (atomic-bomb survivors, medical patients); multiple sites (in utero exposure)
Solar radiation	General population	Skin (BCC, SCC, and melanoma)
UV-emitting tanning devices	General population	Skin (melanoma) and eye (melanoma, particularly choroid and ciliary body)

Abbreviations: BCC, basal cell carcinoma; CLL, chronic lymphocytic leukemia; CNS, central nervous system; SCC, squamous cell carcinoma.

tumors during 1950 to 2001 (Preston et al., 2007; Hsu et al., 2013). RERF also publishes analyses of individuals in the Adult Health Study (AHS), which is a subcohort of LSS with 22,400 individuals and an additional 1000 in utero exposed. This subcohort is oversampled with more individuals who were exposed to the higher doses. Members of the AHS are given physical examinations every 2 years, and this provides much clinical data as well as biological samples. Details of the RERF study cohorts, publications, and databases can be accessed at the RERF Web site.

The LSS cohort's exposures are a whole-body exposure, which then allows for comparisons between specific cancer sites and their relative risks. The cohort is not likely to have many confounding issues because the basis of the dose estimation is the distance from the hypocenter. Finally the AHS subcohort allows for clinical, lifestyle, and biological measurements, which help to assure the lack of confounding and effect modifiers in the dose calculations. The problem with the cohort is that the results are extrapolated from a group of Japanese who survived the bombing and have different background cancer rates than other populations (e.g., Pierce et al., 2007).

The number of individuals in the LSS cohort is shown by city and colon dose (Gy) in Table 25-5 (adapted from Ozasa et al. [2012]). Although most individuals have low estimated doses, there is a good range of doses, with sufficient numbers at the higher doses

Table 25-5

Number of LSS Cohort Members by Colon Dose (Gy)

	TOTAL*	<0.005	0.005–	0.1–	0.2–	0.5–	1.0–	2.0–	UNKNOWN†	NIC‡	TOTAL§
Hiroshima	58,494	21,697	22,733	5037	5067	2373	1152	435	3442	20,179	82,214
Nagasaki	28,117	16,812	7228	937	1289	1051	611	189	3616	6350	38,107
Total	86,611	38,509	29,961	5974	6356	3424	1763	624	7058	26,529	120,321

**Total number of subjects with a reconstructed dose.*
†Those subjects with an unknown dose.
‡Not in the cities of Hiroshima or Nagasaki at the time of the bombing.
§Total of those with either a reconstructed dose, an unknown dose, or were NIC.

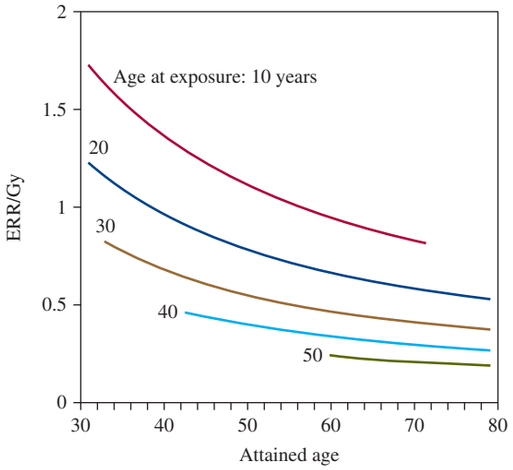

Figure 25-4. *LSS solid tumor mortality excess relative risk.*

for analysis and risk modeling. The individual dose reconstructions have evolved over the years with the current system completed in 2002 and referred to as DS02 (dosimetry system 2002). The uncertainty in the dosimetry is estimated to be about 30% and has been modeled for risk assessment purposes (see Pierce et al. [1990] and Little et al. [2008]).

The results of the mortality data show that for total solid cancers, the excess relative risk decreases with attained age and age at exposure. The modeled data are illustrated in Fig. 25-4, which is extracted from Ozasa et al. (2012). The excess relative risks for individual cancer sites and other causes of mortality are shown in Fig. 25-5, again from the Ozasa et al. (2012) analysis.

There have been sufficient incidence data so that both BEIR VII and UNSCEAR (2006) can model cancer incidence risk by cancer site. BEIR V previously used mortality data for their risk analyses. The most recent cancer incidence data for solid tumors, used by BEIR VII in its risk modeling, included the years 1958 to 1998, given in the publication by Preston et al. (2007). Each individual cancer site is analyzed and discussed in detail in the article by Preston. For total solid tumor incidence the estimated excess relative risk per Gy is 0.47 (0.40 to 0.54). Coincidently it is also 0.47 for solid tumor mortality study as shown in Fig. 25-5. Recently, the dosimetry at RERF has been revised (Cullings et al., 2017). One result of this change has been to add more curvature to the estimated dose–response function for solid cancer mortality.

Occupational Studies

Nuclear Worker Studies There have been numerous worker studies over the years among nuclear workers, primarily at governmental facilities. In most of these studies, mortality rates were compared with those in the general population. In most cases, the cancer mortality rates were less than those for the general public, which may be due to the healthy worker effect and differences between nuclear workers and the public. For those studies with external radiation dose estimates solid cancer risk estimates either increased or decreased with dose and had large confidence intervals (CIs; see BEIR VII for details). As an example in a nested case–control study of workers at the Portsmouth Naval Shipyard (PNS), 1097 lung cancer deaths were age-matched with 3291 controls among the cohort of 37,853 civilian workers (Yiin et al., 2007). After adjusting for confounders such as asbestos and welding fumes, lung cancer risk was associated with radiation exposure. However when work-related medical x-rays, which occur more frequently among radiation monitored workers, were included, lung cancer was no longer associated with radiation dose. The medical x-rays were of the same magnitude as the work exposures of the monitored workers. Because the individual covariates were associated with lung cancer risk, this study shows the importance of incorporating potential confounders in occupational radiation studies.

To address the problem of varied results, IARC carried out a joint analysis of seven nuclear facilities in Canada, the United Kingdom, and the United States, and is referred to as the three-country study (Cardis et al., 1995). This analysis involved a total of 95,673 workers and 3830 cancer deaths. The study estimated a slightly negative ERR per Sv of −0.9 (90% CI –0.4, 0.3) for total solid cancers, but observed a significant increase in mortality of both acute and chronic myelogenous leukemia. Also, multiple myeloma was significantly increased with increasing dose in the study. Although negative for total solid cancers, this result was not inconsistent with the risk estimates derived from the A-bomb studies.

The three-country study was followed by the 15-country study that involved a joint analysis of over 400,000 nuclear workers from 154 facilities (Cardis et al., 2007). As seen in Table 25-6 the number of workers and person-years at risk (pyr) are much greater in the 15-country study. However, the number of workers at the higher cumulative exposures is much lower. This is due to the elimination of workers in the 15-country cohorts who were monitored for internal exposures and also those who may have received an acute exposure of some significance. The study reported significant increases in smoking-related solid cancer mortality but not for leukemias, which was essentially the opposite of the three-country findings. There are a number of issues with the 15-country study as discussed in Dauer et al. (2010), namely, questions about the exclusion of workers included in the previous (three-country) study, such as the Idaho National Engineering Laboratory cohort, and exclusion of workers who were considered to have had potential for high internal dose. Also of concern is the inability to deal with smoking and the undue influence given to the results for one country, namely Canada, where risk estimates are very high and error-prone (Ashmore et al., 2010). The increase in overall solid cancer incidence would no longer be significant if the Canadian cohort were excluded; however, it would be consistent with risk estimates projected from the A-bomb studies.

Recently, a newer study of 308,297 nuclear workers in France, the United Kingdom, and the United States has been reported. This program is referred to as INWORKS (International Nuclear Workers Study) and has 8.2 million person-years of follow-up with 66,632 worker deaths. The excess relative risk (ERR) for solid cancer deaths was estimated to be 0.47 /Gy, which is the same as was observed in the LSS study. For this cohort it was reported that the estimated ERR for leukemia mortality was 2.96/Gy, which was primarily due to chronic myeloid leukemia (CML) (see Leuraud et al. [2015] and Richardson et al. [2015]). Risk estimates for the individual cancer sites are expected to be published in the near future. In an accompanying editorial, Little (2015) discussed the problems with using epidemiological data to estimating low-dose radiation risks.

A series of analyses of workers at the Russian nuclear facility at Mayak have been published. The Mayak workers generally experienced very high doses from both internal (plutonium, α particles) and external radiation exposures. Plutonium body burdens were based on urine analyses. Worker exposures of Pu239 and Pu240 and external exposures have been reassessed by Vasilenko et al. (2007).

Cause of death	ERR/Gy [*] (95%CI [†])	Cases
All causes	0.22 (0.18, 0.26)	50,620
All solid cancer	0.47 (0.38, 0.56)	10,929
Cancers of specific sites [‡]		
Esophagus	051 (0.11, 1.06)	339
Stomach	0.28 (0.14, 0.42)	3125
Colon	0.54 (0.23, 0.93)	621
Rectum	0.17 (–0.17, 0.64)	427
Liver	0.36 (0.18, 0.58)	1519
Gallbladder	0.45 (0.10, 0.90)	419
Pancreas	0.08 (–0.18, 0.44)	513
Other digestive system	1.29 (0.14, 3.25)	84
Lung	0.63 (0.42, 0.88)	1558
Breast	1.60 (0.99, 2.37)	324
Uterus	0.22 (–0.09, 0.64)	547
Ovary	0.79 (0.07, 1.86)	157
Prostate	0.33 (NA [‖], 1.25)	130
Bladder	1.12 (0.33, 2.26)	183
Kidney parenchyma	0.52 (–0.15, 1.75)	80
Renal pelvis and ureter	2.62 (0.47, 7.25)	33
Other solid cancer	0.47 (0.24, 0.76)	864
Lymphoid and hematopoietic malignancies [‡, §]		
Malignant lymphoma	0.16 (–0.13, 0.59)	284
Multiple myeloma	0.54 (–0.04, 1.58)	93
Other neoplasms [‡]	0.65 (0.26, 1.14)	518
Non-neoplastic diseases and other causes		
Blood diseases	1.70 (0.96, 2.70)	238
Circulatory diseases	0.11 (0.05, 0.17)	19,054
Respiratory diseases	0.21 (0.10, 0.33)	5119
Digestive diseases	0.11 (–0.01, 0.24)	3394
Genitourinary diseases	0.14 (–0.06, 0.38)	1309
Infectious diseases	–0.02 (–0.15, 0.13)	1962
Other diseases	0.01 (–0.1, 0.12)	4847
External causes	–0.11 (–0.21, 0.02)	2432

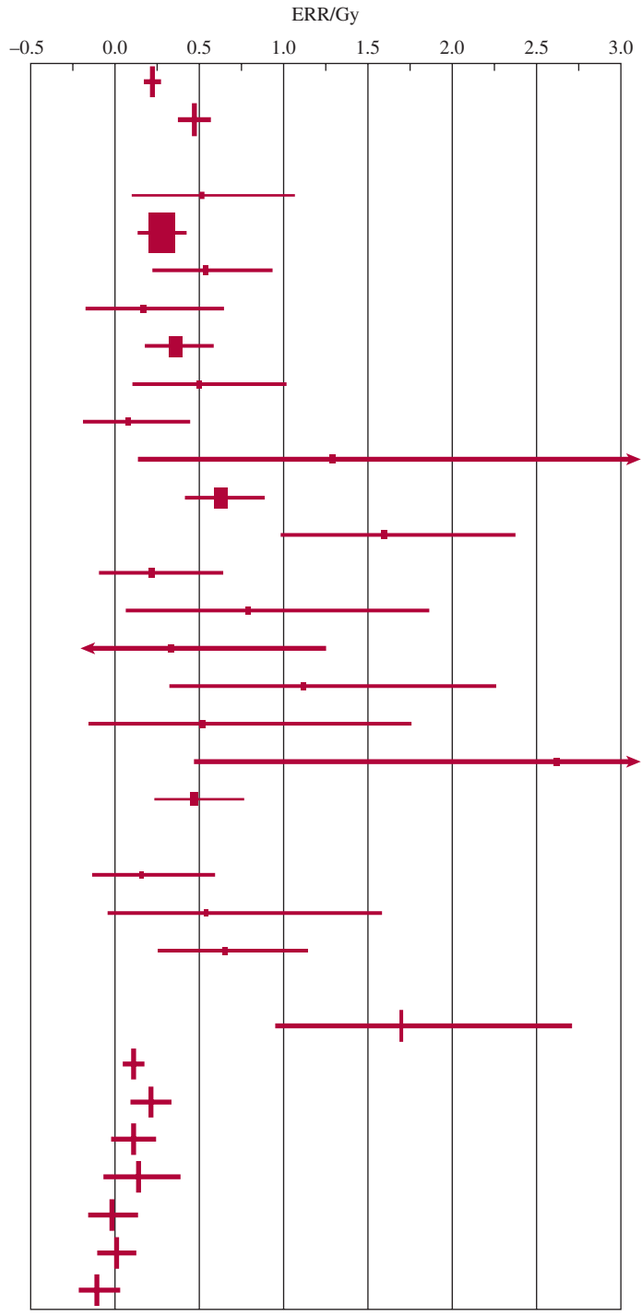

Figure 25-5. *LSS mortality 1950 to 2003: excess relative risk (ERR).* *ERR was estimated using the linear dose model, in which city, sex, age at exposure, and attained age were included in the background rates, but not allowing radiation effect modification by those factors. [†]Confidence interval. Horizontal bars show 95% confidence intervals. [‡]The size of plots for site-specific cancers was proportional to the number of cases. [§]ERR (95% CI) of leukemia was 3.1 (1.8, 4.3) at 1 Gy and 0.15 (–0.01, 0.31) at 0.1 Gy based on a linear quadratic model with 318 cases (not displayed in the figure). [‖]The lower limit of 95% CI was lower than zero, but not specified by calculation.

High levels of body burdens of plutonium greater than 7.4 kBq (mean 76 kBq) were found to have a relative risk of liver cancer of 17 (Gilbert et al., 2000) and 7.9 for bone cancer (Koshurnikova et al., 2000). Small nonsignificant increases were seen at low doses. These studies were based on the mortality experience of 11,000 workers. In a larger group of 21,000 workers 655 lung cancer deaths were evaluated (Gilbert et al., 2004). In contrast to the high-dose effects on the liver and bone, the effects of plutonium on lung cancer were found to be consistent with a linear dose–response. Recently, Sokolnikov et al. (2015) reported an ERR/Gy = 0.16 (0.07, 0.26) for solid cancer deaths other than those associated with plutonium exposures, namely liver, bone, and lung. Plutonium exposures to

Table 25-6

IARC Nuclear Worker Studies

	STUDY	SUBJECT	pyr	DEATHS		
	3-country	95,673	2.1 million	18,993		
	15-country	407,291	5.2 million	15,825		
			DEATHS PER mSv INTERVAL			
	0–10	10–50	50–100	100–200	200–400	400+
3-Country	9582	3728	989	702	586	238
15-Country	13,608	3728	855	492	268	42

Abbreviation: pyr, person-years at risk.

workers in the United States and the United Kingdom were much lower, and increase in cancer was not observed except possibly for a nonsignificant increase in lung cancer (Wilkinson et al., 1987; Wiggs et al., 1994; Omar et al., 1998). Also, experimental studies of plutonium administered to beagle dogs have predicted these high-dose effects in workers (e.g., Wilson et al., 2010). The dogs overpredicted the bone and liver cancer risks, but the lung cancers were similar.

Medical Radiation Workers It is estimated that there are 2.3 million medical radiation workers worldwide. Radiologists and radiological technologists have been studied epidemiologically for many years. These workers were some of the earliest exposed to radiation with the first finding in 1902 that radiation can cause skin cancer. It was recognized in the 1940s that radiologists had increased rates of leukemia (e.g., Ulrich, 1946). More recently, Yoshinaga et al. (2004) at the NCI reviewed cancer mortality from eight international cohorts involving 270,000 subjects. By far the greatest number of workers were from the study of 146,000 U.S. technologists (Mohan et al., 2003). In this study there were increases in breast cancer and non-chronic lymphocytic leukemias (non-CLLs), based on years worked for those employed before 1950 when exposures were much higher than today. The cohorts consistently show increases in leukemias for the early years with less consistency for skin cancer and breast cancer (Yoshinaga et al., 2004).

The U.S. Radiologic Technologists (USRT) health study is a large study (90,000 subjects with about 75% females), first contacted in 1983 to 1998, and is representative of the population of technologists with good employment history information and data on other risk factors such as smoking. Radiation monitoring (badges) was introduced in the 1950s and is beginning to be used in the analyses (Simon et al., 2006). Recently, Rajaraman et al. (2006) analyzed lung cancer in the cohort and did not find increases after adjustments for smoking. The USRT health study will continue with improved dosimetry and longer follow-up times. These studies will continue to be important because they involve low doses of continuous exposure in contrast to the acute exposure of the A-bomb survivors.

Chernobyl Cleanup Workers The Chernobyl cleanup workers are of interest because of their higher exposures compared with other nuclear workers. It has been estimated that there were 600,000 workers, with 240,000 of the workers exposed during 1986 to 1987 and having an average exposure of 100 mSv (Cardis et al., 2006). Ivanov et al. (2006) reported an analysis of 55,718 workers who had yearly physical examinations. The analysis covered two 5-year periods 1991 to 1995 and 1996 to 2001, with a total of 1370 cancer cases. The average dose for these workers during 1985 to 1986 was 130 mSv. Although there was an increase in the incidence of solid tumors, it was not significant. Cancer mortality was evaluated in a smaller cohort of workers (29,003 with 651 cancer deaths). The excess relative risk for solid tumors was significant (ERR = 1.52 per Gy) and the increase was observed in the highest dose interval (mean 200 mSv) with no increase in the lower-dose interval (mean 100 mSv). Besides solid tumors, a significant increase in leukemia incidence was observed for those with exposures greater than 0.15 Gy, compared with workers with lower exposures (Ivanov, 2007). Also, increases in the incidence of cardiovascular disease were observed among those at higher exposures.

A smaller cohort (10,332) of Chernobyl cleanup workers from the Baltic States was reported by Rahu et al. (2006). The mean exposure was 109 mGy, and 155 incidental cancer cases were observed. Although there were increases in thyroid (7 cases) and brain cancer (11 cases), they were not dose-related and were likely the result of close surveillance and multiple comparisons. More recently, Kashcheev et al. (2015) reported on 67,568 emergency workers' cancer incidence and mortality. The follow-up period was longer, from 1992 to 2009, resulting in 4002 solid cancer cases and 2442 cancer deaths. The estimated ERR/Gy for solid cancer incidence was 0.47, while for cancer mortality the ERR/Gy was 0.58 with both estimates being statistically significant.

Other than for leukemias, the follow-up period has been relatively short and it is thus too soon to conclude much about solid cancer risks. The relatively high reconstructed doses make it important to continue the follow-up of the cohorts and to evaluate the exposures and their uncertainties.

Nuclear Worker Registries The National Dose Registry of Canada includes 206,620 workers for the period 1951 to 1987. Ashmore et al. (1998) analyzed 5426 deaths and reported an increase in total cancer mortality among males (ERR = 3% per 10 mSv), which was marginally significant but not for any particular cancer, including leukemia. There was not a significant increase among females who comprised about half of the workers, but generally had much lower recorded doses. Sont et al. (2001) analyzed the Registry for cancer incidence of 191,333 workers for the period 1951 to 1988. Using national rates, the standardized incidence ratio (SIR) for all cancer was significantly less than 1 (SIR = 0.88 [90% CI 0.85, 0.92]). Leukemia was also significantly less than 1. When doses were used, the ERR for all cancer was 2.5 (90% CI 1.2, 4.0) and 5.4 (90% CI 0.2, 20.0) for leukemia. One difficulty with these analyses is that the cohort includes dental (0.3 mSv mean accumulative dose), medical (3.95 mSv), industrial (4.78 mSv), and nuclear workers (31.98 mSv), whose lifestyles may well differ as do their radiation exposures.

Muirhead et al. (2009) analyzed both mortality and incidence of the U.K. National Registry of Radiation Workers (124,723 workers). Total solid tumors were increased, ERR = 0.28 per Sv among 23,326 deaths and ERR = 0.27 among 11,165 malignant incident cases. Leukemias were also increased with ERR = 1.7 for mortality and 1.8 for incidence. Other than leukemia, individual cancer sites were not significantly increased. The increases for solid tumors and leukemias were significant using a 90% CI but not for a 95% CI.

Nonoccupationally Exposed Groups

Studies of the Techa River Exposures A joint U.S. and Russian program has been evaluating the health effects of those living near the Techa River. During the early 1950s radioactive waste from the Mayak nuclear production facility was released into the river. A dose reconstruction system has been developed (Degteva et al., 2000, 2007) and applied to a cohort of 30,000 people. Exposures were 55% internal from food and water, primarily of Cs 137 and Sr 89, 90. A mortality analysis based on 1842 solid cancer deaths resulted in an estimated ERR = 0.92 (0.2, 1.7) per Gy, and 6.5 (1.8, 24) based on 61 leukemia deaths (Krestinina et al., 2007). Cancer incidence for a subcohort of 17,000 individuals with 1836 cases reported an ERR = 1.0 per Gy with a mean cumulative exposure of 40 mGy stomach dose and mean bone marrow dose of 30 mGy. Recently, Davis et al. (2015) estimated the solid cancer incidence rate as ERR/Gy = 0.77 (0.13, 1.5). There are some uncertainties about the exposures in the early years, which may have been underestimated and, if increased, would reduce the risk estimates (Cardis, 2007).

High Natural Background Radiation Areas In 2000, a series of articles were published in a supplement issue of the *Journal of Radiation Research* that described the results of a joint China–Japan collaborative research program, which studied the population living in the high background radiation areas in Yangjiang, China. Cancer mortality was analyzed for the period 1979 to 1995 for 125,000 individuals, which accumulated 1.7 million person-years with 10,415 deaths including 1003 cancer deaths and a population average annual effective exposure of 6.4 mSv (Tao et al., 2000). Comparing individuals in the high background area to those in a control area, the relative risk was 0.99 (0.87, 1.14) for total cancer mortality. Dividing the high background area into low, medium, and high radiation exposures, the relative risks for nonleukemia cancer mortality actually decreased with dose (1.07, 0.98, and 0.91, respectively).

In an additional analysis, Sun et al. (2000) modeled the cancer risk using Poisson regression. The cumulative dose groups were in five 100-mSv intervals with the largest being 400+ mSv. The modeled solid tumor mortality risk did not increase with dose and was estimated to be negative but not significantly different from zero.

Recently Tao et al. (2012) analyzed cancer mortality (956 cases) among 31,604 people in the Yangjiang area between 1979 and 1998. The mean cumulative radiation dose was 84.8 mGy, with 21.6 mGy for those in the control area. The excess relative risk for solid cancers was ERR = –1.01 per Gy (95% CI –2.93, 0.95). Liver cancer is a major cancer that is negatively correlated with dose and is difficult to correctly diagnose. On removing the liver cancer from the analysis the ERR for solid cancers becomes 0.19 (–1.87, 3.04).

To evaluate the dose reconstruction, Jiang et al. (2000) calculated the correlation between estimated radiation exposure and frequency of dicentric and ring chromosomes, which are recognized as a good biomarker of radiation exposure. They observed that for those in the high radiation background area, the incidence of these markers agrees with what has been observed in other studies of radiation exposures and chromosome aberrations. This result provides some evidence in support of the program's exposure estimates. In conclusion, the high background Chinese studies have not shown an increase in cancer incidence at low dose and dose rate exposures.

The coastal area of Kerala, India, is a region of high natural radiation background that has been well studied. Nair et al. (2009) published an analysis of cancer incidence and individually determined gamma exposures in the region. A cohort of 70,000 individuals (age 30 to 84 years) were followed for 10.5 years, which resulted in 1379 cancer cases including 30 leukemias. The researchers also obtained individual data on smoking, education, occupation, and other possible cancer risk factors for their analysis. Cumulative dose intervals ranged from 0 to 49 mGy to 500+ mGy. For total cancers and for leukemia there were no increases observed among any of the dose intervals compared to the lowest dose interval. Excluding leukemia, the overall excess relative risk for cancer was estimated to be –0.13/Gy (95% CI –0.58, 0.46). Leukemia was not increased. The analysis included tobacco use and other risk factors that usually are not available in most other radiation epidemiological studies.

Although not a high background study, Kendall et al. (2012) studied childhood cancers in relation to natural background radiation in Great Britain. Using the National Registry of Childhood Tumours, the study involved over 27,000 cancer cases and matched controls with radiation exposures estimated on the basis of the mother's residence at time of birth. The mean cumulative dose from birth until diagnosis was 4 mSv. The study found a significant increase in leukemias with an estimated ERR of 12% (95% CI 3, 22) per mSv of gamma exposure to the red marrow. Other cancers were not significantly increased and radon was not a significant risk factor.

Semipalatinsk Fallout-Related Exposures The Semipalatinsk area of Kazakhstan has been studied for possible cancer effects resulting from radiation exposures from local fallout of the Soviet atomic nuclear weapons testing program. Bauer et al. (2005) studied six rural districts that had measurable levels of exposure and compared the individuals in the districts with six villages that were several hundred kilometers east of the nuclear test site. Follow-up of the populations began 5 to 10 years after the nuclear testing. Using soil samples taken in 1963 of strontium and cesium as well as information on the types of weapons, a dose reconstruction was developed for external and internal exposures. This dose reconstruction was based on the eight most dose-contributing nuclear tests.

For the purpose of analysis the approximate 10,000 individuals in the exposed area were matched with a group of 10,000 in the distant comparison area.

Cancer rates were considerably higher in the exposed groups of individuals, which resulted in significant dose–response relationships for most solid tumors. There appeared, however, to be an undefined bias in the distant control group since the rate ratios increased relatively little among the exposure categories. The authors' estimate for total solid tumors was ERR/Sv = 0.81 (0.46, 1.33), which is similar to the A-bomb cancer risk estimates. The CIs are fairly narrow but the uncertainty in the dose reconstruction was not included in the analysis. The lack of a dose–response is possibly explained by the fact that the study was ecological in nature.

Other Nonoccupational Studies

Buildings with Cobalt 60 A group of 7271 individuals who resided in buildings with high levels of Cobalt 60–contaminated steel in Taiwan have been studied by Hwang et al. (2006). The average cumulative exposure was about 50 mSv, and after a follow-up

period of 16 years, a total of 141 cancer cases were identified. For total solid cancers (82 cases) compared to the national rates, the total cancers were less than expected, but thyroid cancer (7 cases) and non-Hodgkin lymphoma (5 cases) were significantly increased. This is a small study that shows little effect of continuous exposure. It is also difficult to interpret because the exposed population was likely of a higher social economic status (SES) than the general comparison population.

More recently, Hwang et al. (2008) reported significant increases in leukemia excluding CLL, and marginally significant increases in breast cancer. This was based on a dose–response analysis using an exposure assessment system developed to reconstruct the doses to the individuals. The average cumulative dose was 48 mGy.

Populations Residing Near Nuclear Facilities Ecological cancer studies have been carried out in the vicinity of nuclear power plants in many countries. There has often been reported an unexplained increase in childhood leukemia (Laurier et al., 2002; Baker and Hoel, 2007). Spix et al. (2008) and Kaatsch et al. (2008) conducted a childhood cancer case–control study in the areas in the vicinity of 16 German nuclear power plants. They observed 1592 cancer cases with 593 cases of leukemia among children under the age of five during the period 1983 to 2003. Children within 5 km of a nuclear facility had a significant increase in leukemias, but not for other cancers (37 leukemia cases and 54 controls). It was not possible to measure for possible confounders. The authors stated that radiation exposures near these German nuclear power plants are less than the natural background radiation by a factor of 1000 to 100,000.

In France a study of childhood leukemia among children living in the vicinity of the 29 French nuclear sites reported 670 cases, with 729 cases expected from national rates (White-Koning et al., 2004). They observed no trend in leukemia rates with respect to distance. For those within 5 km of a facility, there were 65 leukemia cases, with 75 expected, and for children less than 5 years of age, there were 39 leukemia cases with 40 cases expected.

Evrard et al. (2006) analyzed childhood leukemia, which included three additional years of follow-up from the White-Koning study. Instead of the usual distance surrogate for dose, the researchers estimated doses to the bone marrow based upon the nuclear plants' radioactive discharges. The Institute for Radiation Protection and Nuclear Safety combined the radionuclide discharge data and the local climate data to model the environmental exposure levels. Estimated doses to the red marrow for those in the vicinity of the nuclear sites ranged from 0.06 to 1.33 μSv per year. There were a total of 750 leukemia cases (age less than 15 years) with 795 expected and 394 among those less than 5 years of age (415 expected). There was no trend of leukemia incidence based on yearly exposure rates. The authors concurred with the authors of the German study by estimating that the doses due to releases in the vicinity of the nuclear facilities are approximately 1000 to 100,000 times lower than the average dose due to natural radiation sources.

The recent COMARE (2011) report, focused on leukemias in children around nuclear power plants. The report discusses in detail the German studies as well as studies in other countries. Importantly Kinlen (1988, 2011) describes the concept of population mixing, which basically is the idea that workers arriving in a typically rural area bring foreign infectious agents that in turn will affect local childhood leukemias. In his article Kinlen (2011) focuses on the nuclear power plant studies and the German results in particular. The Kinlen hypothesis of population mixing has been discussed by some authors to explain increased leukemia effects in children when there is no evidence of radiation exposures from the nuclear plants.

The National Research Council (2012) released a review of studies around nuclear facilities with recommendations on how a study could be carried out in the United States using cancer registry data. Previously the NCI (Jablon et al., 1991) analyzed cancer mortality rates in those counties with nuclear power reactors compared with control counties. Basically no differences were found; however, the use of counties as the analysis unit is likely to be too great a geographical area to detect any possible small effects.

Radionuclides

Radon There have been some human studies involving exposures to radionuclides but the data are limited other than for radon, which has been extensively studied. Radon is a natural radioactive gas produced by the decay of uranium and thorium. Originally, exposures to radon and its daughter radionuclides among uranium miners and some other groups of miners established that high exposures were a clear risk for lung cancer. Cancer causation has been restricted to lung cancer but there are also some suggestions that leukemia risk may also be an issue (see the BEIR VI [1999] and IARC [2009] reports). More recently there have been many case–control studies in the United States and Europe of lung cancer and radon exposure in the home. These studies have been brought together for joint analyses of those studies carried out in Europe (13 studies; Darby et al., 2006) and for those studies from North America (7 studies; Krewski et al., 2006). The analysis by Darby, as well as Krewski, observed the data to be best fit by a linear function in dose, with Darby estimating the excess relative risk of lung cancer to be 0.08 per 100 Bq/m^3 and 0.10 in the Krewski analysis. The lung cancer risk was also significantly increased when the cases were restricted to exposures less than 200 Bq/m^3. The lung cancer effects were also consistent, with the risks projected downward from the higher exposed uranium miners, that is, 0.12 per 100 Bq/m^3. When Darby adjusted the data for uncertainties in the radon exposure estimates, the excess relative risk increased to 0.16. BEIR IV, which also includes chapters on radionuclides other than radon, provides tables giving the excess lifetime relative risk for various ages at first exposure, exposure level, and period of exposure. This is done for both males and females and for smokers and never smokers. These tables were not included in the newer BEIR VI report on radon; however, Chen (2005) published similar tables using the newer BEIR VI risk models. Currently (e.g., Darby et al., 2006), the risk analyses find that the relative risk for lung cancer and radon exposure is independent of smoking status. Average radon levels in the home for various countries are given in the *WHO Handbook on Indoor Radon 2009* and it is estimated that 3% to 16% of lung cancers are attributable to radon in the home depending on the risk model used. From Table 25-7 we see that for the United States, with an average level of 46 Bq/m^3, and using BEIR VI risk models, the attributable lung cancer risk is 10% to 14%.

Radium There are 25 isotopes of radium of which 4 occur naturally (radium 223, 224, 226, and 228); the others are man-made or decay products of man-made radionuclides. Radium 226 with a half-life of 1601 years is by far the common natural form, followed by 228 with a half-life of 5.75 years. Radium 223 and 224 have half-lives of only a few days. Except for radium 228, which is a β emitter, the other three are all α emitters. The different isotopes have been used both occupationally as luminescent paint on watches and instruments (radium 226 and 228) and in medical applications (radium 223 and 224). These uses, as well as radium found environmentally in drinking water, have provided material for many

Table 25-7

WHO's Estimated Proportion of Lung Cancer Attributable to Radon

COUNTRY	MEAN INDOOR RADON (Bq/m^3)	RISK ESTIMATE USED IN CALCULATION	PERCENTAGE OF LUNG CANCER ATTRIBUTED TO RADON (%)	ESTIMATED NO. OF DEATHS DUE TO RADON-INDUCED LUNG CANCER EACH YEAR
Canada (Brand et al., 2005)	28	BEIR VI	7.8	1400
Germany (Menzler et al., 2008)	49	European pooling study*	5	1896
Switzerland (Menzler et al., 2008)	78	European pooling study*	8.3	231
United Kingdom (AGIR, 2009)	21	European pooling study* BEIR IV	3.3 6	1089 2005
France (Catelinois et al., 2006)	89	European pooling study BEIR VI	5 12	1234 2913
United States (BEIR VI, 1999)	46	BEIR VI	10–14	15,400–21,800

**With adjustment for year-to-year variation in indoor radon concentrations.*

epidemiological studies. Beginning in the 1920s, young women worked painting the dials of watches with paint containing radium 226 and 228. Many of them "pointed" the tips of their paintbrushes by mouth resulting in ingestion of relatively large amounts of radium for some of the women. A cohort of 1476 of these dial painters has been established with careful exposure reconstruction (Rowland et al., 1978). Radium as a bone seeker resulted in increases in bone cancer as well as paranasal sinus cancers. Although about one-half of the cohort had cumulative exposures less than 1 Gy, there were no bone cancers for those who were exposed to less than 10 Gy, which using a quality factor of 20 for α irradiation gives 200 Sv. In a statistical modeling of the bone cancers, it was estimated that 5 Gy was a threshold level (Hoel and Carnes, 2004). During the 1940s instrument dials were painted in Britain without the tipping of brushes by mouth (Baverstock and Papworth, 1989). In a follow-up of this cohort, cancers and bone cancers in particular were not increased.

Bone sarcomas were also the major cancer effect among patients with tuberculosis and ankylosing spondylitis who were treated with high doses of radium 224 (mean bone surface dose of 30 Gy) in two cohort studies in Germany (Wick et al., 1999; Nekolla et al., 2010). There were increases in bone cancer in both studies, but there were also some increases in other cancer sites (Nekolla et al., 2010).

Plutonium Plutonium is used for nuclear weapons production, and in the production of mixed oxide fuels. Most of the exposure to plutonium is to workers involved in the processing of plutonium in nuclear weapons (Pu 239) and in nuclear power generation (Pu 238). Several groups of workers exposed to plutonium have been studied in the United States, the United Kingdom, and Russia. The major exposure to plutonium is by inhalation and is retained primarily in the lung, liver, and bone, and has been studied in the United States, the United Kingdom, and Russia. The most informative studies are those of workers employed at the Russian Mayak plant where exposures to plutonium were substantial. Dose–response relationships have been shown for cancers of the lung, liver, and bone over a wide range of doses (see Gilbert et al., 2000, 2004; Koshurnikova et al., 2000; Sokolnikov et al., 2008). Internal estimated plutonium doses were very high: up to 5+ Gy for the lung, 23 Gy for the liver, and 144 Gy for the bone surface.

Radioiodine Releases from nuclear facilities of fission-product radionuclides deposited in the environment as well as internal doses from the ingestion of foods containing fission products have been the result of the Chernobyl and Fukushima accidents. Cesium 134 and 137 as well as Iodine 131 were the most important radionuclide exposures that potentially are health risks. Besides nuclear accidents, there are studies of the health effects from exposures to nuclear testing fallout from the Nevada test site and the testing in the South Pacific. The major observable health effect has been childhood thyroid cancer resulting from the β emitter Iodine 131, which has a half-life of only 8 days. From studies of external radiation exposures in the A-bomb survivor studies as well as the children who were treated by radiation for tinea capitis (ring worm present on the scalp), it is clear that radiation is a risk for thyroid cancer for exposures to adolescents. Ron et al. (1995), in a pooled analysis of the studies of external exposures, observed for childhood exposures that the excess relative risk per Gy was 7.7 (95% CI 2.1, 28.7). Iodine 131 is of particular concern because it is taken up by the thyroid gland. Cardis et al. (2005) studied 276 case patients with thyroid cancer and 1300 controls, ages less than 15 years at the time of the Chernobyl accident. Individual doses were estimated for each subject based on their location and diets at the time of the accident. A dose–response relationship was observed between Iodine 131 dose to the thyroid received in childhood and thyroid cancer risk. For a dose of 1 Gy, the estimated odds ratio (OR) of thyroid cancer ranged from 5.5 to 8.4 depending on the risk model. The risk of radiation-related thyroid cancer was three times higher in iodine-deficient areas and the use of potassium iodide as a supplement reduced this risk of radiation-related thyroid cancer by a factor of 3. Cardis et al. (2006) further predict that within Europe by 2065 about 16,000 cases of thyroid cancer and 25,000 cases of other cancers may be expected due to radiation from the accident, whereas several hundred million cancer cases are expected from other causes. Ron (2007) estimated that 90% of the thyroid cancer was due to the ingestion of iodine 131.

NONCANCER EPIDEMIOLOGY

Cardiovascular Disease

Fig. 25-5 shows that there are mortality effects of radiation exposure in the A-bomb studies besides cancer. What is of particular interest is cardiovascular disease (CVD) mortality, because although it may have a lower relative risk from radiation exposure than solid tumors, it accounts for more total background deaths. Atherosclerosis is an inflammatory disease of the arteries, which can lead to ischemia of the heart. In a study of inflammatory biomarkers (TNF-α, IL-10, IgM, IgA, and IFN-γ) as well as erythrocyte sedimentations rates among A-bomb survivors, it was shown that these markers were associated with radiation exposure. It has been estimated that an exposure of 1 Sv was equivalent to an increase in immunological age of 9 to 10 years (see Hoel [2006] for a discussion). The LSS data show a linear dose–response for heart disease mortality but curvilinear for stroke mortality (Shimizu et al., 2010; Takahashi et al., 2017). It is not clear whether there are effects at acute exposures below 0.5 Gy. A current review of what we scientifically understand about CVD and radiation can be found in Darby et al. (2010), and a meta-analysis of the epidemiological studies in Little et al. (2012).

Cataracts

Cataracts were one of the earliest radiation-associated effects found after the discovery of x-rays. It has long been believed that it results from only high doses of radiation to the lens of the eye. For example, the ICRP estimates that acute exposures of 0.5 to 2 Sv are required to cause detectable lens opacities and 5 Sv for vision-impairing cataracts. For protracted exposures, they estimate the dose thresholds as 5 and 8 Sv. There are basically three types of cataracts that are described by Asbell et al. (2005). These types are nuclear or nuclear sclerosis, cortical, and posterior-subcapsular (PS) cataracts. Each of these clinical types has its known risk factors such as cigarette smoking for nuclear and possibly PS cataracts, while UV-B is a risk factor for cortical cataracts. Ionizing radiation is a risk factor for both cortical and PS cataracts but not nuclear sclerotic cataracts (Rafnsson et al., 2005; Worgul et al., 2007). Recent studies have, however, suggested that radiation exposures to the lens confer the risk of opacities at doses well under 1 Sv. The most carefully studied population for relating ionizing radiation and cataracts is the A-bomb survivors. In an ophthalmological screen of members of the adult heath study, the researchers found a statistically significant dose response for PS opacities OR = 1.41 (threshold 0.7 Gy) and for cortical opacities OR = 1.29 (threshold 0.6 Gy). A greater risk for PSC opacities was observed for those exposed at a younger age. In a subsequent study of survivors who had lens removal surgery the radiation risk was estimated to be OR = 1.39 with a low estimated threshold of 0.1 Gy (10 rem). There was no evidence of an association of nuclear cataracts with radiation exposure.

Shore et al. (2010) have shown that among Japanese A-bomb survivors, the risks for cataracts requiring lens surgery are at doses under 1 Gy. The CI on the dose threshold for cataract surgery indicated that the data are consistent with a dose threshold ranging from 0 to 0.8 Gy. What is not known is what are the risks at continuous or protracted exposures. Also there is limited evidence that those exposed at a younger age are at greater risk. The question of thresholds of effect continues to be undecided, with no consensus at this point in time. Classically, PSC opacities were considered to be the risk of ionizing radiation exposure to the lens. Several studies have also found that radiation affects cortical opacities. As far as the degree of risk is concerned, the typical estimate from the studies suggests that 1 Gy of exposure to the lens would correspond to a relative risk of about 1.4 for PSC and somewhat less for cortical opacities. For a general discussion of the scientific issues see Blakely et al. (2010). Also, the NCRP (2017) has completed a committee review of the risks as well as the possible mechanisms. The report is summarized in the published article by Dauer et al. (2017).

Mental Effects

In the A-bomb survivor analyses, significant effects on the developing brain were observed among those exposed during the period of the 8th week through 25th week of gestation. During the most sensitive period of 8 to 15 weeks, there was an increased frequency of severe mental retardation, a diminution in IQ scores and school performances, as well as an increase in the occurrence of seizures. During this sensitive period there is a rapid increase in the number of neurons; they migrate to the cerebral cortex where they lose their capacity to divide, becoming perennial cells. Of the 30 cases of severe retardation, 60% had small head sizes and of those with small head sizes about 10% were retarded. Also the highest IQ score among the 30 cases was 64. For retardation, the dose–response appears linear with an estimated threshold at about 0.2 Gy. At about 1 Gy the statistical regression estimates about 50% retardation. The data also suggest that at 1 Gy there is a loss of 25 to 31 IQ points with no observed effect below 0.1 Gy (Otake et al., 1990, 1991; also see COMARE, 2004).

DISCUSSION

The major issue in radiation health effects is the causation of cancer. We now see noncancer effects such as CVD at low-dose and also low dose-rate exposures that occur at environmental levels and in diagnostic medical screening. Currently, the LNT model is used to estimate these effects well below what can be observed in epidemiological studies. The simple defense of the LNT model is that the physical energy deposition of ionizing radiation increases cancer risk linearly with increasing dose, and the carcinogenic effectiveness is constant independent of dose. The French National Academy of Sciences and the French National Academy of Medicine (Tubiana et al., 2005) have taken an opposing view on the appropriateness of the use of the LNT model. It is generally agreed that epidemiological studies have been unable to detect increases in cancer below exposures of 100 mSv. The French Academy's reason for believing that thresholds may be present and that the LNT overestimates risk is based on radiobiology. It is recognized that a cell is not passively affected by the accumulation of lesions induced by ionizing radiation. The cell reacts through at least three main mechanisms: first by reacting against radiation-induced ROS; second by eliminating damaged cells by either apoptosis or through cell death during mitosis of unrepaired cells; and third by immunosurveillance systems that eliminate clones of transformed cells. The Academy states that experimental data strongly suggest that a practical threshold exists, but the current data do not indicate at what dose or dose rate of radiation it becomes trivial, probably somewhere between 10 and 50 mGy (Tubiana, 2005). For a recent debate see Brenner (2009) and Averbeck (2009). The BEIR VII committee concluded:

The committee judged that the linear no-threshold model (LNT) provided the most reasonable description of the relation between low-dose exposure to ionizing radiation and the incidence of solid cancers that are induced by ionizing radiation.

The answer awaits further progress in radiobiology.

A second question is whether there is a dose and dose-rate effectiveness factor (DDREF) greater than 1. In other words, are chronic exposures less of a cancer risk than an acute exposure such as from the A-bomb studies that provide the quantitative risk estimates? Previously a value of 2 had been used until BEIR VII concluded that it should be 1.5. Recently it has been argued that it should probably be equal to 1 (Jacob et al., 2009). Animal studies have generally suggested that a DDREF is appropriately between 2 and 10 (BEIR V); however, these studies were generally conducted at relatively high doses.

For a much more in-depth reading of health effects of ionizing radiation the reader is referred to two outstanding text books by Hall and Giaccia (2005) and Mettler and Upton (2008). For an understanding of radiation exposures there are several available health physics texts (e.g., Cember and Johnson, 2009). Finally, BEIR VII is a very good source of the current scientific understanding of biology, toxicology, and epidemiology of radiation exposures. Further committee conclusions about health effects can be found in IARC (2009) and UNSCEAR (2006).

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REFERENCES

Advisory Group on Ionising Radiation, AGIR. Radon and public health radiation, chemicals and environmental hazards. Health Protection Authority. 2009. Available at: http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1243838496865.

Amundson SA, Fornace AJ Jr. Gene expression profiles for monitoring radiation exposure. *Radiat Prot Dosim*. 2001;97(1):11–16.

Amundson SA, Lee RA, Koch-Paiz CA, et al. Differential responses of stress genes to low dose-rate gamma irradiation. *Mol Cancer Res*. 2003;1(6):445–452.

Asbell PA, Dualan I, Mindel J, Brocks D, Ahmad M, Epstein S. Age-related cataracts. *Lancet*. 2005;365:599–609.

Ashmore JP, Gentner NE, Osborne RV. Incomplete data on the Canadian cohort may have affected the results of the study by the International Agency for Research on Cancer on the radiogenic cancer risk among nuclear industry workers in 15 countries. *J Radiol Prot*. 2010;30(2):121–129.

Ashmore JP, Krewski D, Zielinski JM, Jiang H, Semenciw R, Band PR. First analysis of mortality and occupational radiation exposure based on the National Dose Registry of Canada. *Am J Epidemiol*. 1998;148:564–574.

Averbeck D. Does scientific evidence support a change from the LNT model for low-dose radiation risk extrapolation? *Health Phys*. 2009;97(5):493–504.

Baker PJ, Hoel DG. Meta-analysis of standardized incidence and mortality rates of childhood leukaemia in proximity to nuclear facilities. *Eur J Cancer Care (Engl)*. 2007;16(4):355–363.

Barcellos-Hoff MH. Integrative radiation carcinogenesis: interactions between cell and tissue responses to DNA damage. *Semin Cancer Biol*. 2005;15(2):138–148.

Bauer S, Gusev BI, Pivina LM, Apsalikov KN, Grosche B. Radiation exposure due to local fallout from Soviet atmospheric nuclear weapons testing in Kazakhstan: solid cancer mortality in the Semipalatinsk historical cohort, 1960–1999. *Radiat Res*. 2005;164(4 pt 1):409–419.

Baverstock KF, Papworth DG. The UK radium luminizer survey. *BIR report*. 1989;21:72–79.

BEIR IV. *Health Effects of Radon and Other Internally Deposited Alpha-Emitters*. Washington, DC: Advisory Committee on the Biological Effects of Ionizing Radiation, National Academy of Sciences; 1988.

BEIR V. *Health Effects of Exposure to Low Levels of Ionizing Radiation*. Washington, DC: National Research Council Advisory Committee on the Biological Effects of Ionizing Radiation, National Academy of Sciences; 1990.

BEIR VI. *Health Effects of Exposure to Radon*. Washington, DC: National Research Council Advisory Committee on the Biological Effects of Ionizing Radiation, National Academy of Sciences; 1999.

BEIR VII, Phase 2. *Health Risks from Exposure to Low Levels of Ionizing Radiation*. Washington, DC: National Research Council Advisory Committee on the Biological Effects of Ionizing Radiation, National Academy of Sciences; 2006.

Blakely EA, Kleiman NJ, Neriishi K, et al. Radiation cataractogenesis: epidemiology and biology. *Radiat Res*. 2010;173(5):709–717.

Brand K, Zielinski J, Krewski D. Residential radon in Canada: an uncertainty analysis of population and individual lung cancer risk. *Risk Anal*. 2005;25:253–269.

Brenner DJ. Extrapolating radiation-induced cancer risks from low doses to very low doses. *Health Phys*. 2009;97(5):505–509.

Calabrese EJ, Bachmann KA, Bailer AJ, et al. Biological stress response terminology: integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. *Toxicol Appl Pharmacol*. 2007;222(1):122–128.

Cardis E. Commentary: low dose-rate exposures to ionizing radiation. *Int J Epidemiol*. 2007;36(5):1046–1047.

Cardis E, Gilbert ES, Carpenter L, et al. Effects of low doses and low dose rates of external ionizing radiation: cancer mortality among nuclear industry workers in three countries. *Radiat Res*. 1995;142(2):117–132.

Cardis E, Howe G, Ron E, et al. Cancer consequences of the Chernobyl accident: 20 years on. *J Radio Prot*. 2006;26:127–140.

Cardis E, Kesminiene A, Ivanov V, et al. Risk of thyroid cancer after exposure to ^{131}I in childhood. *J Natl Cancer Inst*. 2005;97:724–732.

Cardis E, Vrijheid M, Blettner M, et al. The 15-Country Collaborative Study of Cancer Risk among Radiation Workers in the Nuclear Industry: estimates of radiation-related cancer risks. *Radiat Res*. 2007;167(4):396–416.

Catelinois O, Rogel A, Laurier D, et al. Lung cancer attributable to indoor radon exposure in France: impact of the risk models and uncertainty analysis. *Environ Health Perspect*. 2006;114(9):1361–1366.

Cember H, Johnson TE. *Introduction to Health Physics*. 4th ed. New York: McGraw-Hill Medical; 2009.

Chen J. Estimated risks of radon-induced lung cancer for different exposure profiles based on the new EPA model. *Health Phys*. 2005;88: 323–333.

COMARE, Committee on Medical Aspects of Radiation in Environment (COMARE). *Review of Pregnancy Outcomes Following Preconceptional Exposure to Radiation*. Eighth report. Chilton, WI: National Radiological Protection Board; 2004.

COMARE, Committee on Medical Aspects of Radiation in the Environment (COMARE). *Further Consideration of the Incidence of Childhood Leukemia Around Nuclear Power Plants in Great Britain*. Fourteenth report. London: Health Protection Agency for the Committee on Medical Aspects of Radiation in the Environment; 2011.

Cullings HM, Grant EJ, Egbert SD, et al. DS02R1: improvements to atomic bomb survivors' input data and implementation of Dosimetry System 2002 (DS02) and resulting changes in estimated doses. *Health Phys*. 2017;112(1):56–97.

Darby S, Hill D, Deo H, et al. Residential radon and lung cancer: detailed results of a collaborative analysis of individual data on 7148 subjects with lung cancer and 14208 subjects without lung cancer from 13 epidemiologic studies in Europe. *Scand J Work Environ Health*. 2006;32(suppl 1):1–83.

Darby SC, Cutter DJ, Boerma M, et al. Radiation-related heart disease: current knowledge and future prospects. *Int J Radiat Oncol Biol Phys*. 2010;76(3):656–665.

Dauer LT, Ainsbury E, Dynlacht JR, et al. Guidance on radiation dose limits for the lens of the eye: overview of the recommendations in NCRP Commentary No. 26. *Int J Radiat Bio*. 2017;93(10):1015–1023.

Dauer LT, Brooks AL, Hoel DG, et al. Review and evaluation of updated research on the health effects associated with low-dose ionising radiation. *Radiat Prot Dosim*. 2010:140:103–136.

Davis FG, Krestinina LY, Preston D, et al. Solid cancer incidence in the Techa River Incidence Cohort: 1956-2007. *Radiat Res*. 2015;184:56–65.

Degteva MO, Kozheurov VP, Tolstykh EI, et al. Dose reconstruction system for the exposed population living along the Techa River. *Health Phys*. 2000;78(5):542–554.

Degteva MO, Shagina NB, Tolstykh EI, et al. An approach to reduction of uncertainties in internal doses reconstructed for the Techa River population. *Radiat Prot Dosim*. 2007;127(1–4):480–485.

El Ghissassi F, Baan R, Straif K, et al. Special report: policy a review of human carcinogens—Part D: radiation. *Lancet Oncol*. 2009;10:751–752.

Evrard AS, Hemon D, Morin A, et al. Childhood leukaemia incidence around French nuclear installations using geographic zoning based on gaseous discharge dose estimates. *Br J Cancer*. 2006;94(9):1342–1347.

Gilbert ES, Koshurnikova NA, Sokolnikov M, et al. Liver cancers in Mayak workers. *Radiat Res*. 2000;154, 246–252.

Gilbert ES, Koshurnikova NA, Sokolnikov ME, et al. Lung cancer in Mayak workers. *Radiat Res*. 2004;162(5):505–516.

Hall E, Giaccia A. *Radiobiology for the Radiologist*. 6th ed. Lippincott Williams & Wilkins; 2005.

Hoel DG. Ionizing radiation and cardiovascular disease. *Ann N Y Acad Sci*. 2006;1076:309–317.

Hoel DG, Carnes B. Cancer dose–response analysis of the radium dial workers. *Proceedings from HEIR 2004 9th International Conference on Health Effects Incorporated Radionuclides*. 2004:169–173.

Hsu W, Preston D, Soda M, et al. The incidence of leukemia, lymphoma, and multiple myeloma among atomic bomb survivors: 1950–2001. *Radiat Res*. 2013;179:361–382.

Hwang SL, Guo HR, Hsieh WA, et al. Cancer risks in a population with prolonged low dose-rate gamma-radiation exposure in radiocontaminated buildings, 1983–2002. *Int J Radiat Biol*. 2006;82(12):849–858.

Hwang SL, Hwang JS, Yang YT, et al. Estimates of relative risks for cancers in a population after prolonged low-dose-rate radiation exposure: a follow-up assessment from 1983 to 2005. *Radiat Res*. 2008;170:143–148.

International Commission on Radiological Protection (ICRP). The 2007 recommendations of the International Commission on Radiological Protection. ICRP publication 103. *Ann ICRP*. 2007;37(2–4):1–332.

IARC. A review of human carcinogens: Part D radiation. *IARC Monogr Eval Carcinog Risks Hum*. 2009;100D:1–362.

Ivanov VK. Late cancer and noncancer risks among Chernobyl emergency workers of Russia. *Health Phys*. 2007;93(5):470–479.

Ivanov VK, Tsyb AF, Vasilenko EK, et al. Cancer morbidity and mortality among the liquidators of the Chernobyl accident: estimation of radiation risks. *Radiats Biol Radioecol*. 2006;46(2):159–166.

Jablon S, Hrubec Z, Boice JD Jr. Cancer in populations living near nuclear facilities. A survey of mortality nationwide and incidence in two states. *JAMA*. 1991;265(11):1403–1408.

Jacob P, Ruhm W, Walsh L, Blettner M, Hammer G, Zeeb H. Is cancer risk of radiation workers larger than expected? *Occup Environ Med*. 2009;66:789–796.

Jiang T, Hayata I, Wang C, et al. Dose–effect relationship of dicentric and ring chromosomes in lymphocytes of individuals living in the high background radiation areas in China. *J Radiat Res (Tokyo)*. 2000;41(suppl):63–68.

Kaatsch P, Spix C, Schulze-Rath R, Schmiedel S, Blettner M. Leukaemia in young children living in the vicinity of German nuclear power plants. *Int J Cancer*. 2008;122(4):721–726.

Kashcheev VV, Chekin SY, Maksioutov MA, et al. Incidence and mortality of solid cancer among emergency workers of the Chernobyl accident: assessment of radiation risks for the follow-up period of 1992–2009. *Radiat Environ Biophys*. 2015;54:13–23.

Kendall GM, Little MP, Wakeford R, et al. A record-based case–control study of natural background radiation and the incidence of childhood leukaemia and other cancers in Great Britain during 1980–2006. *Leukemia*. 2012; 1–7.

Kinlen L. Evidence for an infective cause of childhood leukaemia: comparison of a Scottish new town with nuclear reprocessing sites in Britain. *Lancet*. 1988;2(8624):1323–1327.

Kinlen L. Childhood leukemia, nuclear sites, and population mixing. *Br J Cancer*. 2011;104:12–18.

Koshurnikova NA, Gilbert ES, Sokolnikov M, et al. Bone cancers in Mayak workers. *Radiat Res*. 2000;154:237–245.

Krestinina LY, Davis F, Ostroumova EV, et al. Solid cancer incidence and low-dose-rate radiation exposures in the Techa River cohort: 1956-2002. *Int J Epidemiol*. 2007;36(5):1038–1046.

Krewski D, Lubin JH, Zielinski JM, et al. A combined analysis of North American case-control studies of residential radon and lung cancer. *J Toxicol Environ Health A*. 2006;69:533–597.

Laurier D, Grosche B, Hall P. Risk of childhood leukaemia in the vicinity of nuclear installations—findings and recent controversies. *Acta Oncol*. 2002;41(1):14–24.

Leuraud K, Richardson DB, Cardis E, et al. Ionizing radiation and risk of death from leukaemia and lymphoma in radiation-monitored workers (INWORKS): an international cohort study. *Lancet Haematol*. 2015;2:e276–e281.

Little MP. Ionizing radiation in the workplace. *BMJ*. 2015;351:h5405.

Little MP, Azizova TV, Bazyka D, et al. Systematic review and meta-analysis of circulatory disease from exposure to low-level ionizing radiation and estimates of potential population mortality risks. *Environ Health Perspect*. 2012;120:1503–1511.

Little MP, Hoel DG, Molitor J, Boice JD, Wakeford R, Muirhead CR. New models for evaluation of radiation-induced lifetime cancer risk and its uncertainty employed in the UNSCEAR 2006 report. *Radiat Res*. 2008;169(6):660–676.

Liu Z, Mothersill CE, McNeill FE, et al. A dose threshold for a medium transfer bystander effect for a human skin cell line. *Radiat Res*. 2006;166:19–23.

Maenhaut C, Detours V, Dom G, Handkiewicz-Junaky D, Oczko-Wojciechowska M, Jarzab B. Gene expression profiles for radiation-induced thyroid cancer. *Clin Oncol*. 2011;23:282–288.

Menzler S, Piller G, Gruson M, Rosario AS, Wichmann H, Kreienbrock L. Population attributable fraction for lung cancer due to residential radon in Switzerland and Germany. *Health Phys*. 2008;95:179–189.

Mettler F, Upton AC. *Medical Effects of Ionizing Radiation*. 3rd ed. Philadelphia, PA: WB Saunders; 2008.

Mohan AK, Hauptmann M, Freedman DM, et al. Cancer and other causes of mortality among radiologic technologists in the United States. *Int J Cancer*. 2003;103:259–267.

Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. *Radiat Res*. 2003a;159(5):567–580.

Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, clastogenic factors and transgenerational effects. *Radiat Res*. 2003b;159(5):581–596.

Mothersill C, Seymour C. Radiation-induced bystander effects: are they good, bad or both? *Med Confl Surviv*. 2005;21(2):101–110.

Mothersill C, Seymour CB. Radiation-induced bystander effects—implications for cancer. *Nat Rev Cancer*. 2004;4(2):158–164.

Muirhead CR, O'Hagan JA, Haylock RGE, et al. Mortality and cancer incidence following occupational radiation exposure: third analysis of the National Registry for Radiation Workers. *Br J Cancer*. 2009;100:206–212.

Nair RRK, Rajan B, Akiba S, et al. Background radiation and cancer incidence in Kerala, India—Karanagappally cohort study. *Health Phys*. 2009;96(1):55–66.

NCRP. *Commentary No. 26: Guidance on Radiation Dose Limits for the Lens of the Eye*. Bethesda, MD: National Council on Radiation Protection and Measurements; 2017.

NCRP. *Report No. 160: Ionizing Radiation Exposure of the Population of the United States*. Bethesda, MD: National Council on Radiation Protection and Measurements; 2009.

National Research Council. *Analysis of Cancer Risks in Populations Near Nuclear Facilities: Phase 1*. Washington, DC: National Academy Press; 2012.

Nekolla EA, Walsh L, Spiess H. Incidence of malignant diseases in humans injected with radium-224. *Radiat Res*. 2010;174:377–386.

Omar RZ, Barber JA, Smith PJ. Cancer mortality and morbidity among plutonium workers at the Sellafield plant of British Nuclear Fuels. *Br J Cancer*. 1998;79:1288–1301.

Ory C, Ugolin N, Levalois C, et al. Gene expression signature discriminates sporadic from post-radiotherapy-induced thyroid tumors. *Endocr Relat Cancer*. 2011;18:193–206.

Otake M, Schull WJ, Neel JV. Congenital malformations, stillbirths, and early mortality among the children of atomic bomb survivors: a reanalysis. *Radiat Res*. 1990;122:1–11.

Otake M, Schull WJ, Yoshimaru T. Brain damage among the prenatally exposed. *J Radiat Res*. 1991;32(suppl):249–264.

Ozasa K, Shimizu Y, Suyama A, et al. Studies of the mortality of atomic bomb survivors, report 14, 1950–2003: an overview of cancer and noncancer diseases. *Radiat Res*. 2012;177(3):229–243.

Pierce DA, Stram DO, Vaeth M. Allowing for random errors in radiation dose estimates for the atomic bomb survivor data. *Radiat Res*. 1990;123(3):275–284.

Pierce DA, Vaeth M, Shimizu Y. Selection bias in cancer risk estimation from A-bomb survivors. *Radiat Res*. 2007;167(6):735–741.

Preston DL, Ron E, Tokuoka S, et al. Solid cancer incidence in atomic bomb survivors: 1958–1998. *Radiat Res*. 2007;168:1–64.

Rafnsson V, Olafsdottir E, Hrafnkelsson J, et al. Cosmic radiation increases the risk of nuclear cataract in airline pilots: a population based case-control study. *Arch Ophthalmol*. 2005;123:1102–1105.

Rahu M, Rahu K, Auvinen A, et al. Cancer risk among Chernobyl cleanup workers in Estonia and Latvia, 1986–1998. *Int J Cancer*. 2006;119(1):162–168.

Rajaraman P, Sigurdson AJ, Doody MM, et al. Lung cancer risk among US radiologic technologists, 1983–1998. *Int J Cancer*. 2006;119(10):2481–2486.

Redpath JL. Suppression of neoplastic transformation in vitro by low doses of low let radiation. *Dose Response*. 2006;4(4):302–308.

Richardson DB, Cardis E, Daniels RD, et al. Risk of cancer from occupational exposure to ionising radiation: retrospective cohort study of workers in France, the United Kingdom, and the United States (INWORKS). *BMJ*. 2015;351:h5359.

Ron E. Thyroid cancer incidence among people living in areas contaminated by radiation from the Chernobyl accident. *Health Phys*. 2007;93(5):502–511.

Ron E, Lubin JH, Shore RE, et al. Thyroid cancer after exposure to external radiation: a pooled analysis of seven studies. *Radiat Res*. 1995;141:259–277.

Rowland RE, Sterhney AF, Lucas HF. Dose–response relationships for female radium dial workers. *Radiat Res*. 1978;76:368–383.

Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ, Hall EJ. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. *Radiat Res*. 2001;155:397–401.

Shimizu Y, Kodama K, Nishi N, et al. Radiation exposure and circulatory disease risk: Hiroshima and Nagasaki atomic bomb survivor data, 1950–2003. *BMJ*. 2010;340:193.

Shore RE, Neriishi J, Nakashima E. Epidemiological studies of cataract risk at low to moderate radiation doses: (not) seeing is believing. *Radiat Res*. 2010;174:889–894.

Simon SL, Weinstock RM, Doody MM, et al. Estimating historical radiation doses to a cohort of U.S. radiologic technologists. *Radiat Res*. 2006;166(1 pt 2):174–192.

Sokolnikov ME, Gilbert ES, Preston DL, et al. Lung, liver and bone cancer mortality in Mayak workers. *Int J Cancer*. 2008;123:905–911.

Sokolnikov M, Preston D, Gilbert E, et al. Radiation effects on mortality from solid cancers other than lung, liver, and bone cancer in the Mayak worker cohort: 1948–2008. *PLoS One*. 2015;10(2):e0117784.

Sont WN, Zielinski JM, Ashmore JP, et al. First analysis of cancer incidence and occupational radiation exposure based on the National Dose Registry of Canada. *Am J Epidemiol*. 2001;153(4):309–318.

Spix C, Schmiedel S, Kaatsch P, Schulze-Rath R, Blettner M. Case–control study on childhood cancer in the vicinity of nuclear power plants in Germany 1980–2003. *Eur J Cancer*. 2008;44(2):275–284.

Sun Q, Akiba S, Tao Z, et al. Excess relative risk of solid cancer mortality after prolonged exposure to naturally occurring high background radiation in Yangjiang, China. *J Radiat Res (Tokyo)*. 2000;41(suppl):43–52.

Takahashi I, Shimizu Y, Grant EJ, et al. Heart disease mortality in the life span study, 1950–2008. *Radiat Res*. 2017;187(3):319–332.

Tao Z, Akiba S, Zha Y, et al. Cancer and non-cancer mortality among inhabitants in the high background radiation area of Yangjiang, China (1979–1998). *Health Phys*. 2012;102(2):173–181.

Tao ZF, Zha YR, Akiba S, et al. Cancer mortality in the high background radiation areas of Yangjiang, China during the period between 1979 and 1995. *J Radiat Res (Tokyo)*. 2000;41(suppl):31–41.

Tapio S, Jacob V. Radioadaptive response revisited. *Radiat Environ Biophys*. 2007;46(1):1–12.

Tubiana M. Dose–effect relationship and estimation of the carcinogenic effects of low doses of ionizing radiation: the Joint Report of the Académie des Sciences (Paris) and of the Académie Nationale de Medicine. *Int J Radiat Oncol Biol Phys*. 2005;63:317–319.

Tubiana M, Aurengo A, Averbeck D, et al. *Dose–effect Relationships and Estimation of the Carcinogenic Effects of Low Doses of Ionizing Radiation*. Académie des Sciences Report March 30, 2005, Académie Nationale de Medicine (France). Académie Nationale de Médicine Report; 2005.

Turner JE. *Atoms, Radiation, and Radiation Protection*. 3rd ed. Weinheim, Germany: Wiley; 2007.

Ugolin N, Ory C, Lefevre E, et al. Strategy to find molecular signatures in a small series of rare cancers: validation for radiation-induced breast and thyroid tumors. *PLoS ONE*. 2011;6(8):e23581.

Ulrich H. The incidence of leukemia in radiologists. *N Engl J Med*. 1946;234:45–46.

United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR). *Volume 1: Report to the General Assembly, Scientific Annexes A and B; Volume II UNSCEAR*. New York: United Nations; 2006.

Vasilenko EK, Khokhryakov VF, Miller SC, et al. Mayak workers dosimetry study: an overview. *Health Phys*. 2007;93(3):190–206.

White-Koning ML, Hemon D, Laurier D, et al. Incidence of childhood leukaemia in the vicinity of nuclear sites in France, 1990–1998. *Br J Cancer*. 2004;91(5):916–922.

Wick RR, Nekolla EA, Gossner W, Kellerer AM. Late effects in ankylosing spondylitis patients treated with 224Ra. *Radiat Res*. 1999;152(suppl):S8–S11.

Wiggs LD, Johnson ER, Cox-DeVore CA, Voelz GL. Mortality through 1990 among white male workers at the Los Alamos National Laboratory: considering exposures to plutonium and external ionizing radiation. *Health Phys*. 1994;67:577–588.

Wilkinson GS, Tietjen GL, Wiggs LD, et al. Mortality among plutonium and other radiation workers at a plutonium weapons facility. *Am J Epidemiol*. 1987;125:231–250.

Wilson DA, Mohr L, Frey GD, Lackland D, Hoel DG. Lung, liver and bone cancer mortality after plutonium exposure in beagle dogs and nuclear workers. *Health Phys*. 2010;98:42–52.

Worgul BV, Kundiyev YI, Sergiyenko NM, et al. Cataracts among Chernobyl clean-up workers: implications regarding permissible eye exposures. *Radiat Res*. 2007;167:233–243.

Yiin JH, Silver SR, Daniels RD, et al. A nested case-control study of lung cancer risk and ionizing radiation exposure at the Portsmouth Naval Shipyard. *Radiat Res*. 2007;168(3):341–348.

Yoshinaga S, Mabuchi K, Sigurdson AJ, Doody MM, Ron E. Cancer risks among radiologists and radiology technologists: review of epidemiology studies. *Radiology.* 2004;233(2):313–321.

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chapter 26

Toxic Effects of Plants and Animals

John B. Watkins, III

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Introduction to Plant Toxicities

Toxic Effects by Organ

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Clinical Study of Plant Poisons

Summary of Plant Toxicities

Introduction to Animal Venoms

Properties of Animal Toxins

Arthropods

Arachnida

- Scorpions
- Spiders
  - Agelenopsis *Species (American Funnel Web Spiders)*
  - Latrodectus *Species (Widow Spiders)*
  - Loxosceles *Species (Brown or Violin Spiders)*
  - Steatoda *Species*
  - Cheiracanthium *Species (Running Spiders)*
  - Theraphosidae *Species (Tarantulas)*
- Ticks

Chilopoda (Centipedes)

Diplopoda (Millipedes)

Insecta

- Heteroptera (True Bugs)
- Hymenoptera (Ants, Bees, Wasps, and Hornets)
  - *Formicidae (Ants)*
  - *Apidae (Bees)*
  - *Vespidae (Wasps)*
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Mollusca (Cone Snails)

Reptiles

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Conclusion

Acknowledgments

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INTRODUCTION

The earliest humans have used plant extracts and animal venoms for hunting, war, assassination, and political intrigue for millennia. The Ebers Papyrus, which dates to around 1550 BC, describes concoctions using plant substances as primary ingredients. The toxic properties of plants and animals often enhance their ability to survive in their surroundings and against predators. Some toxic compounds are used primarily to aid an animal in obtaining food, whereas plants have developed toxic properties to specifically ward off being used as food. These toxic compounds are invaluable as tools and in the insight that they provide into the systems that they disrupt and poison. One major complication to the study of plant and animal poisons arises from their complexity as mixtures. Studies readily separate and evaluate individual components, but it is very difficult to use purified components to make the original toxin or venom. Nevertheless, extensive study of many toxins has contributed to a greater understanding of their biology and chemistry. Toxins have been utilized as tools to study human biochemistry and physiology in order to pave the way for new pharmaceuticals. Clinical evaluation of human poisoning is complicated by questionable identification of plant or animal species and the inability to quantify the level of exposure. In this chapter, an overview of specific plant and animal toxins and their effects will precede a

short discussion of the considerable effort to harness the natural pharmacopeia for clinical use.

INTRODUCTION TO PLANT TOXICITIES

The plant kingdom contains potentially 300,000 species, and the toxic effects of plants serve primarily as defense mechanisms against natural predators. Toxicity in humans can result from simply touching as well as ingesting plants to cause a truly wide array of deleterious effects. Toxic effects on humans can range from simple hay fever caused by exposure to plant pollen all the way to serious systemic reactions caused by ingestion of specific plants. Table 26-1 lists some of the poisoning syndromes that plants can produce (Nelson et al., 2007).

Many variables can affect the concentration of a plant's toxin and can be a major factor in the severity of reaction one will experience on exposure. These factors include what part of the plant exposure is from, the age of the plant, amount of sunlight and soil quality that the plant has grown in, and genetic differences within a species. Also, plant toxins fall under a number of different chemical structures, which is useful in understanding related toxins. Table 26-2 lists some of the common classifications.

The number and variety of plants that have some level of use or toxicity are far too many to be discussed here (see Table 1 in Polito et al. [2016]). Criteria for inclusion for discussion are based on how frequently human exposure occurs, the seriousness of these exposures, and also how well the toxic agent is scientifically understood. Excellent source material can be found in the comprehensive texts by Barceloux (2008), Dart (2004), Cameron et al. (2015), Leikin and Paloucek (2008), and Nelson et al. (2007).

TOXIC EFFECTS BY ORGAN

Skin

Irritant Contact Dermatitis Plants causing irritation of the skin on contact are rather common (Nelson et al., 2007). A list of such plants containing their family, genus, and common names can be found in Table 26-3. The trichomes, or barb-like hairs (Fig. 26-1), found on stinging nettles (*Urtica* species, Urticaceae) puncture skin on contact and release an irritating sap containing a mixture of formic acid, histamine, acetylcholine, and serotonin (Kavalali, 2003). Various species of *Urtica* can be found all over the world; however, the most dangerous is *U. ferox* (poisonous tree nettle), which is most common in New Zealand. Exposure has been reported to cause death in humans and animals. *Mucuna pruriens* (cowhage), which also deploys its toxin via barbed trichomes on contact, may cause pain, itching, erythema, and vesication. Mucinain, contained

Table 26-1

Poisoning Syndromes Caused by Plants

SYNDROME	GENERA	MECHANISM(S)
Antimuscarinic	*Atropa, Datura, Hyoscyanmus, Solanum*	Blockade of muscarinic cholinoceptors
Cardiotoxic	*Adenium, Digitalis, Convallaria, Nerium*	Inhibition of cellular Na^+, K^+-ATPase increases contractility, enhanced vagal effect
Convulsants	*Anemone, Conium, Labrunum, Nicotinia, Ranunculus*	Blockade of gamma-aminobutyric acid (GABA) receptor on the neuronal chloride channel, alteration of acetylcholine homeostasis, mimic excitatory amino acids, sodium channel alteration, hypoglycemia
Cyanogenic	*Eriobotrya, Hydrangea, Prunus*	Gastric acid hydrolysis of cyanogenic glycosides releases cyanide
Dysrhythmia	*Acotinum, Rhododendron, Veratrum*	Sodium channel activation
Nicotinic	*Conium, Laburnum, Lobelia, Nicotinia*	Stimulation of nicotinic cholinoceptors
Pyrrolizidine	*Crotalaria, Heliotropium, Senecia*	Pyrroles injure endothelium of hepatic or pulmonary vasculature leading to veno-occlusive disease and hepatic necrosis
Toxalbumin	*Abrus, Ricinus*	Protein synthesis inhibitors leading to multiple organ system failure

Table 26-2

Chemical Classification of Plant Toxins

CHEMICAL CATEGORY	GENERA	EXAMPLES
Alkaloids	*Atropa, Senecio, Nicotinia, Coffea, Papaver, Solanum, Acotinum*	Tropines, pyrrolizidines, pyridines, purines, isoquinolines, steroids, diterpines
Glycosides	*Digitalis, Aesculus*	Steroids, coumarins
Proteinaceous compounds	*Abrus, Amanitin, Lathyrus*	Toxalbumins (abrin, ricin), polypeptides (amatoxins, phallotoxins, phalloidin), amines (aminopropionitrile)
Organic acids	*Caladium, Dieffenbachia, Rheum*	Oxalates
Alcohols	*Cicuta, Eupatorium*	Cicutoxin, tremetol
Resins and resinoids	*Cannabis, Rhus*	Tetrahydrocannabinol, urushiol

Table 26-3

Selective Plants Producing Contact Dermatitis

BOTANICAL FAMILY	GENUS SPECIES	COMMON NAME
Amaryllidaceae	*Narcissus*	Narcissus
Apocynaceae	*Nerium oleander*	Oleander
Bromeliaceae	*Ananas comosus*	Pineapple
Asteraceae	*Ambrosia, Aster, Chrysanthemum, Rudbeckia hirta, Tagetes minuta*	Ragweed, aster, chrysanthemum, black-eyed Susan, Mexican marigold
Euphorbiaceae	*Ricinus communis*	Castor bean
Fumariaceae	*Dicentra spectabilis*	Bleeding heart
Ginkgoaceae	*Ginkgo biloba*	Ginkgo
Liliaceae	*Allium cepa*	Onion
Myrtaceae	*Eucalyptus globulus*	Eucalyptus
Pinaceae	*Abies balsamea*	Balsam fir
Saxifragaceae	*Hydrangea*	Hydrangea
Solanaceae	*Lycopersicon esculentum, Solanum carolinense, S. turerosum*	Tomato, horse nettle, potato
Umbelliferae	*Daucus carota, Heracleum lanatum*	Carrot, cow parsnip
Urticaceae	*Urtica dioica, U. urens*	Stinging nettle

Figure 26-1. *Stinging hairs of Urtica ferox (nettles).*

in the toxin, is the proteinase responsible for causing the pruritus (Southcott and Haegi, 1992).

Certain species of *Ranunculus* (buttercup) contain a compound known as ranunculin, which is enzymatically broken down into the toxin protoanemonin. On contact with skin, protoanemonin found in *Anemone* (buttercup) is converted to anemonin, which is the

Figure 26-2. *Euphorbia pulcherrima (poinsettia).*

irritant directly responsible for the resulting dermatitis. If ingested, protoanemonin may cause severe irritation of the gastrointestinal tract (Kelch et al., 1992).

Damage to the stems or leaves of the genus *Euphorbia* (Euphorbiaceae, spurge family) causes exudation of a milky latex that contains diterpene esters that are irritating to the skin. *Euphorbia marginata* (snow-on-the-mountain) is a common plant in the United States that is used in flower arrangements by florists. Dermal contact with its latex can cause skin irritation (Urushibata and Kase, 1991). In addition, serious eye irritation has been reported (Frohn et al., 1993). The poinsettia (*Euphorbia pulcherrima*; Fig. 26-2), which is ubiquitous at holiday times, may cause contact dermatitis (Massermanian, 1998).

Allergic Contact Dermatitis Many people have experienced allergic dermatitis (see Chap. 19, "Toxic Responses of the Skin"), most frequently from contact with poison ivy. Allergic dermatitis is an actual allergic reaction occurring within the skin as opposed to just a response to the presence of an irritant. Due to this immunological component, the severity of the reaction can range widely.

Philodendron scandens (Araceae, arum family) and the toxicodendron group of plants, which contains *Rhus radicans* (poison ivy; Fig. 26-3), *Rhus diversiloba* (poison oak), and *Rhus vernix* (poison sumac), are all known to cause allergic dermatitis. *P. scandens* is a common houseplant that produces allergenic resorcinols, especially 5-*n*-heptadecatrienyl resorcinol (Knight, 1991). In the *Rhus* species, the allergen is a fat-soluble substance called urushiol that can penetrate the stratum corneum and then bind Langerhans cells in the epidermis. These haptenated cells then migrate to lymph nodes, where T cells are activated resulting in the allergic response (Kalish and Johnson, 1990). Ingestion of *Rhus* species has been reported to cause generalized dermatitis (Oh et al., 2003). Sap of the mango fruit (*Magnifera indica*, Anacardiaceae) can also cause allergic dermatitis due to the presence of oleoresins that, with repeated exposure, will cross-react with allergens of poison ivy (Tucker and Swan, 1998).

Figure 26-3. *Toxicodendron radicans (poison ivy).*

Alkaloids present in the sap of daffodils, hyacinths, and tulips can sometimes cause irritation. Irritation can also be caused by contact with needle-like crystals of calcium oxalate, also known as raphides, which are present on these plants' bulbs (Gude et al., 1988). The major culprit is the compound tulipalin-A, which causes "tulip fingers" from handling tulip bulbs (Christensen and Kristiansen, 1999). Tulipalin-A can be found in concentrations up to 2%. A safe threshold for this allergen is considered to be 0.01% (Hausen et al., 1983). Table 26-4 contains more plants that cause irritation due to oxalates.

"Latex-fruit syndrome" is the result of cross-sensitivity to latex in rubber gloves and some fruits. *Hevea brasiliensis* (the latex tree) produces prohevein, a chitin-binding polypeptide that is also found in several plants. Hevein, a 43 amino acid N-terminal fragment of prohevein, is the major binding component (Kolarich et al., 2005). Individuals who are allergic to rubber latex may become sensitized to fruits containing a chitinase with a hevein-like domain, such as banana, kiwi, tomato, and avocado (Blanco et al., 1999).

Lichens, such as species of *Usnea* and *Cladonia*, are known to cause dermatitis due to the production of usnic acid (a benzofuran) and related acids (Aalto-Korte et al., 2005). Usnic acid has also been implicated in hepatotoxicity following use of certain nonprescription weight loss supplements (Han et al., 2004).

Photosensitivity Dermatitis does not necessarily have to be caused by skin contact. Consumption of *Hypericum perforatum* (St. John's wort) by animals can lead to serious dermatitis and even may be life threatening. The toxic agent is hypericin (a bianthraquinone) that once ingested and dispersed systemically causes photosensitization of the animal's skin. On exposure to sunlight, edematous lesions form on areas of skin that are not protected by hair such as the nose and ears (Sako et al., 1993). Photosensitization caused by St. John's wort in humans is a rare occurrence; however, an increased response to therapeutic exposure to ultraviolet therapy has been reported (Beattie et al., 2005). However, compounds in *Hypericum perforatum* cause induction of cytochrome P450s, leading to potential interactions with many drugs (Russo et al., 2014).

Respiratory Tract

Allergic Rhinitis Rhinitis from inhalation of plant pollens is a seasonal problem for many individuals. A chromosomal association in these individuals is under investigation (Blumenthal et al., 2006). Trees, grasses, and weeds are all responsible to contributing to airborne pollen. Grass species *Poa* and *Festuca* are major contributors along with pollen from several weed genera in the Asteraceae (e.g., mugwort, *Artemisia vulgaris*, in Europe, and ragweed, *Ambrosia* sp., in North America; Fig. 26-4). The common denominator in the various pollen allergens is the conserved binding domain known as profiling, which is also found in birch pollen (Hirschwehr et al., 1998). Asthma and rhinitis have been linked to individuals who are exposed to cascara sagrada (*Rhamnus purshiana*) (Giavina-Bianchi et al., 1997), or workers in greenhouses in which bell peppers are growing (Groenewoud et al., 2006).

Table 26-4

Selective Plants Causing Gastrointestinal Irritation Due to Release of Raphides of Oxalates

BOTANICAL FAMILY	SCIENTIFIC NAME	COMMON NAME
Amaranthaceae	*Halogeton glomeratus*	Saltlover, halogeton
Araceae	*Alocasia macrorrhiza*	Giant taro
	Anthurium andreanum	Flamingo Lily
	Caladium bicolor	Caladium
	Dieffenbachia sp.	Dumbcane
	Epipremnum sp.	Pothos, Devil's Ivy
	Monstera sp.	Shingle plant, Swiss Cheese plant
	Philodendron scandens	Philodendron
	Scindapsus aureus, Pothosaureus sp.	Marble queen
	Spathiphyllum sp.	Peace Lily
	Syngonium podophyllum	Arrowhead plant
Chenopodiaceae	*Spinacia oleracea*	Spinach
Commelinaceae	*Tradescantieae* sp.	Spiderworts
Onagraceae	*Fushsia* sp.	Fuchsia
Oxalidaceae	*Oxalis* sp.	Wood sorrel
Palmae	*Caryotamitis* sp.	Fishtail palm
Polygonaceae	*Rheum rhaponticum*	Rhubarb
Portulacaceae	*Portulaca* sp.	Purslane
Vitaceae	*Parthenocissus quinquefolia*	Virginia creeper
	Parthenocissus triscupidata	Boston ivy, Japanese creeper

Cough Reflex Workers who process peppers have a significantly increased incidence of coughing when specifically handling *Capsicum annuum* (sweet pepper) and *Capsicum frutescens* (red pepper). These two types of peppers produce the major irritants capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (Surh et al., 1998). Specific nerves in the airway have been found to be capsaicin-sensitive, which leads to the irritation and cough (Blanc et al., 1991).

Toxin-Associated Pneumonia The pneumotoxin, 4-ipomeanol, is produced in sweet potato roots (*Ipomea batatas*, Convolvulaceae) by the mold *Fusarium solani*. 4-Ipomeanol is activated by human

Figure 26-4. *Ambrosia psilostachya (western ragweed).*

cytochrome P450s to an intermediate that binds to DNA (Alvarez-Diez and Zheng, 2004). In cattle and rabbits, the major P450 activator is CYP4B1 found in the lung that results in pneumonia. In the mouse, CYP4B1 is most abundant in the kidneys that results in renal toxicity, and in humans multiple subsets of liver P450 enzymes are responsible for activating 4-ipomeanol (Baer et al., 2005).

Gastrointestinal System

Direct Irritant Effects Ingestion of a toxic plant can cause irritation of the gastrointestinal tract, often resulting in nausea, vomiting, and diarrhea. Many different compounds produced by plants can cause mild to severe versions of these effects. A list of plants known to cause these effects is provided in Table 26-5. Ingestion of ripe tung nuts (*Aleurites fordii*) causes abdominal pain, vomiting, and diarrhea. Outbreaks of poisoning are most likely to occur in children (Lin et al., 1996).

Toxic quinolizidine alkaloids are found in buffalo beans, also known as buffalo peas (*Thermopsis rhombifolia*), which grow naturally in the western United States. Ingestion by children causes nausea, vomiting, dizziness, and abdominal discomfort (McGrath-Hill and Vicas, 1997). In addition, consumption by livestock of the mature plant with seeds has been reported to be fatal.

Nuts from *Aesculus hippocastanum* (horse chestnut; Fig. 26-5) and *Aesculus glabra* (Ohio buckeye) contain a white crystalline coumarin glucoside called esculin. Ingestion by humans causes gastroenteritis, which increases in severity with the number of nuts consumed. Fortunately, esculin is poorly absorbed by the gastrointestinal tract of humans and its systemic effects are usually limited. However, in cattle, esculin may be hydrolyzed in the rumen, resulting in the release of aglycone to cause systemic effects. This can lead to nervous system stimulation—marked by a stiff-legged gait and, in severe poisoning, tonic seizures with opisthotonus (Casteel et al., 1992). The triterpene saponin β-aescin in horse chestnut seed extract may have value in the treatment of venous insufficiency in humans (Siebert et al., 2002).

Antimitotic Effects Podophyllotoxin is found in *Podophyllum peltatum* (May apple, Berberidaceae) especially in its foliage and roots. In low doses, mild purgation occurs; however, overdose results in nausea and severe paroxysmal vomiting (Frasca et al., 1997). By binding microtubules, podophyllotoxin blocks mitosis from proceeding. This has made podophyllotoxin of interest for the treatment of cancer (Schacter, 1996).

Found in the bulbs of *Colchicum autumnale* (autumn crocus, Liliaceae), colchicine blocks the formation of microtubules, ultimately preventing successful mitosis. Ingestion of these bulbs causes severe gastroenteritis (nausea, vomiting, diarrhea, and dehydration). Severe poisoning can result in confusion, hematuria, neuropathy, renal failure, and cardiotoxicity (Mendis, 1989).

Table 26-5

Selective Plants Causing Gastrointestinal Irritation

COMMON NAME	SCIENTIFIC NAME	TOXIC PART	TOXIN
Amaryllis	*Hippeastrum equestre*	Bulb	Lycorine
Barberry	*Berberis vulgaris*	Root	Protoberberine and other isoquinoline alkaloids
Boxwood	*Buxus* sp.	Leaves, stems	Steroidal alkaloids
Buttercup	*Ranunculus* sp.	All parts	Ranunculin, protoanemonin
Crown of thorns	*Euphorbia milii*	All parts	Resiniferatoxin
Daffodil	*Narcissus* sp.	All, especially bulb	Lycorine, narcissin, phenanthridine alkaloids
English Ivy	*Hedera helix*	All parts	Hederin from hederagenin
Euonymus	*Euonymus* sp.	All parts	Alkaloids
Hyacinth	*Hyacinthus orientalis*	Bulb	Calcium oxalate, lycorine
Iris	*Iris*	Bulb	Irritant resin
Mayapple	*Podophyllum peltatum*	Green fruit, roots	Podophyllotoxin
Mistletoe	*Phoradendron flavescens*	Berries, other parts	Phoratoxin
Pokeweed	*Phytolacca americana*	All parts	Phytolaccatoxin, related triterpines
Purging nut	*Jatropha curcas*	Seeds	Jatrophin (curcin) (toxalbumin)
Tung nut	*Aleurites fordii*	Nut	Derivative of phorbol, saponins, toxalbumins
Wiseria	*Wisteria sinensis*	Pods	Wistarine (glycoside)

Figure 26-5. *Aesculus hippocastanum.*

Figure 26-6. *Ricinus communis (castor bean plant).*

Protein Synthesis Inhibition There are many plants that produce ribosomal inhibiting proteins (Bolognesi et al., 2016; Polito et al., 2016). These ribosome-inhibiting proteins are broadly distributed among different plant genera; various tissues express different isoforms and most are single-chain proteins (Schrot et al., 2016). The family Euphorbiaceae contains several genera that are known to be very toxic. The castor bean (*Ricinus communis*; Fig. 26-6) is an ornamental plant that produces seeds that, if eaten by children or adults, causes no symptoms of poisoning for several days after ingestion. Gradually, gastroenteritis develops, resulting in some loss of appetite, with nausea, vomiting, and diarrhea. If a fatal dose is ingested, the gastroenteritis becomes extremely severe and is marked by persistent vomiting, bloody diarrhea, and icterus followed by death within 6 to 8 days. A fatal dose for a child can be as few as 5 seeds and may be as low as 20 seeds for an adult. However, fatality occurs in less than 10% of ingestions when a "fatal" dose is consumed, owing to the fact that the toxic protein is largely destroyed during digestion. There are two lectins found in the beans: ricin I and ricin II of which ricin II is more toxic. Ricin II is made up of an A-chain and a B-chain. The B-chain, composed of

Figure 26-7. *Phytolacca americana (pokeweed).*

262 amino acids, binds terminal galactose residues on cell surfaces allowing the A-chain to be endocytosed (Wu et al., 2006). Once inside, the 267 amino acid A-chain inactivates the 60S ribosomal subunit of cells by catalytic depurination of an adenosine residue within the 28S rRNA, thereby blocking protein synthesis (Bantel et al., 1999; Spooner and Lord, 2015).

The seeds of *Abrus precatorius* (jequirity bean, Leguminosae) contain lectins known as abrins. Abrin-a, one of four isoabrins produced by the plant, has the highest inhibitory effect on protein synthesis and consists of an A-chain and a B-chain (Tahirov et al., 1994). Similar to ricin, the A-chain directly inhibits protein synthesis while the B-chain is responsible for getting the A-chain inside the cell (Ohba et al., 2004). The LD_{50} of abrin when injected in mice is less than 0.1 μg/kg, making abrin one of the most toxic substances known.

Plants that produce only A-chains are not nearly as toxic as those that pair them with a B-chain. Young shoots of pokeweed (*Phytolacca americana*, Phytolaccaceae; Fig. 26-7) can be ingested without toxicity; however, consumption of mature leaves and berries may cause nausea and diarrhea. The plant produces three isozymes of single-chain lectins that are capable of inhibiting protein synthesis in cells and have antiviral properties (Di and Tumer, 2015; Polito et al., 2016). However, these do not readily pass through a cell's membrane but are capable of doing so with the help of a virus (Monzingo et al., 1993).

Wisteria floribunda (Leguminosae) is a common ornamental climbing vine that develops seeds in the fall. Ingestion of these seeds can cause severe gastroenteritis. Just a few seeds can produce

headache, nausea, and diarrhea within hours, followed by dizziness, confusion, and hematemesis (Rondeau, 1993). The toxic lectin binds *N*-acetylglucosamine on mammalian neurons.

Sambucus ebulus (dwarf elder) has medicinal and food properties that are limited by inherent toxicity (Jimenez et al., 2015). Medicinal applications include antioxidant action owing to polyphenols, and inhibition of tumor cell growth by anthocyanidins including cyanidin-3-O-glucoside. Lectins with and without antiribosomal activity have been isolated. Single-chain ribosome-inhibiting proteins have *N*-glycosidase activity on the 28S rRNA subunit, thereby inhibiting polypeptide chain elongation. Ebulin and nigrin have been tested in targeted therapy (Ferreras et al., 2011).

Cardiovascular System

Cardioactive Glycosides The best known cardioactive glycoside is *Digitalis purpurea* (foxglove, Scrophulariaceae; Fig. 26-8). However, squill (*Scilla maritima*), which contains scillaren, and lily of the valley (*Convallaria majalis*), which contains convallatoxin in the bulbs, have actions similar to digitalis. Also, milkweeds (*Asclepias* spp., Asclepiadaceae) contain glycosides (Roy et al., 2005). Other cardiotoxic plants can be found in Table 26-6. The cardiac glycoside desglucouzarin in *Asclepias asperula*, like digitalis, inhibits Na^+,K^+-ATPase (Abbott et al., 1998). Two plants in the Apocynaceae (oleander family) also contain cardioactive glycosides. *Thevetia peruviana* (yellow oleander) is a common ornamental plant in the United States whose seeds contain the highest concentration of cardiac glycosides. The fatal dose to an adult is 8 to 10 seeds (Prabhasankar et al., 1993). Clinical aspects of oleander toxicosis have been described in sheep (Aslani et al., 2004) and humans (Pietsch et al., 2005).

Table 26-6

Selective Plants Causing Cardiotoxicity

COMMON NAME	SCIENTIFIC NAME	TOXIC PART	TOXIN
Azalea	*Rhodendron* sp.	All	Grayanotoxins
Death camus	*Zigadenus* sp.	All	Zygadenine, veratrine
Foxglove	*Digitalis* sp.	Leaves, seeds	Digitalis glycosides
Larkspur	*Delphenium ambiguum*	All	Delphinine
Lily of the valley	*Convallaria majalis*	All	Convallarin, convallamarin
Milkweed	*Asclepias* sp.		(Hydroxycinnamoyl) desglucouzarin
Monkshood	*Aconitum* sp.	Leaves, roots, seeds	Aconitine, aconine
Oleander	*Nerium oleander*	All	Oleandrin, oleandrosine

Figure 26-8. *Digitalis purpurea (common foxglove).*

Figure 26-9. *Veratrum viride (American hellebore).*

Actions on Cardiac Nerves Toxic alkaloids found in *Veratrum viride* (American hellebore, Liliaceae; Fig. 26-9), *Veratrum album* (European hellebore), and *Veratrum californicum* cause nausea, emesis, hypotension, and bradycardia on ingestion. *Veratrum album* has been used for centuries to "slow and soften the pulse." The mixture of alkaloids includes protoveratrine, veratramine, and jervine that affects the heart by causing a repetitive response to a single stimulus resulting from prolongation of the sodium current (Jaffe et al., 1990). The bulbs of the wild camas (*Zigadenus paniculatus*

Figure 26-10. *Rhododendron ponticum (rhododendron).*

and other species of *Zigadenus*, Liliaceae) contain veratrum-like alkaloids (Peterson and Rasmussen, 2003).

Aconitum species, which have been used in western and eastern medicine for centuries, produce the toxic alkaloids aconitine, mesaconitine, and hypoaconitine. Poisoning may occur on ingestion but severity varies with the concentration of the alkaloids, which depends on species, place of origin, time of harvest, and processing procedures (Chan et al., 1994). Along with cardiac arrhythmias and hypotension, ingestion causes gastrointestinal upset and neurological symptoms. The alkaloids work by causing a prolonged sodium current with slowed repolarization in cardiac muscle (Peper and Trautwein, 1967) and in nerve fibers (Murai et al., 1990).

Grayanotoxins are produced exclusively by several genera of Ericaceae (heath family) and in particular they are found in *Rhododendron ponticum* (Onat et al., 1991; Fig. 26-10), *R. macrophyllum* (Casteel and Wagstaff, 1989), and *Kalmia angustifolia* (Burke and Doskotch, 1990). Ingestion of honey contaminated with grayanotoxins, brought by bees, can produce a severe reaction called "mad honey poisoning." The poisoning resembles aconitine poisoning, in that there is bradycardia, hypotension, oral parasthesia, and gastrointestinal upset. Severe poisoning can result in respiratory depression and eventually loss of consciousness. Grayanotoxins bind to sodium channels in cardiac and muscle cells resulting in increased sodium conductance (Maejima et al., 2002).

Vasoactive Chemicals American mistletoe (*Phoradendron flavescens*) and European mistletoe (*Viscum album*, Loranthaceae) are members of the same family and both produce a toxin that is marked for its effect on the cardiovascular system. Both phoratoxin (produced by American mistletoe) and viscotoxin (produced by European mistletoe) cause hypotension, vasoconstriction of the vessels in skin and skeletal muscle, and bradycardia resulting from negative inotropic actions on heart muscle. Phoratoxin is only one-fifth as active as the viscotoxins (Rosell and Samuelsson, 1988). Although serious poisoning from the plants is rare, it is possible to induce anaphylaxis with repeated injections of a mistletoe extract (Bauer et al., 2005).

Ingestion of the fungus *Claviceps purpurea* (ergot), which grows on grains that are used for food, causes vasoconstriction. The "ergot gene cluster" is required for the production of ergot alkaloids (Coyle and Panaccione, 2005; Haarmann et al., 2005). Ergot poisoning was called "St. Anthony's fire" due to the blackened appearance of the limbs of some victims. In extreme cases, the vasoconstriction was severe enough that gangrene would develop in the extremities. Abortion in pregnant women is also common after ingestion of ergot-contaminated grains. *Acremonium coenophialum*, a fungus which grows on the grass tall fescue (*Festuca arundinacea*), produces some ergot alkaloids. Grazing cattle that ingest the contaminated grass develop "fescue toxicosis" (Hill et al., 1994; Blodgett, 2001). This condition results in decreased weight gain, decreased reproductive performance, and increased peripheral vasoconstriction. Pulmonary infection from *Acremonium strictum* has been noted in a horse (Pusterla et al., 2005).

Liver

Hepatocyte Damage Pyrrolizidine alkaloids can be found in *Senecio* (groundsel, Asteraceae) and within four genera of Boraginaceae: *Echium* (bugloss), *Cynoglossum* (hound's tongue), *Heliotropium* (heliotrope), and *Symphytum* (comfrey) (Altamirano et al., 2005; Mei et al., 2005). Ingestion of significant concentrations of these alkaloids causes liver damage in the form of hepatic veno-occlusive disease associated with lipid peroxidation (Bondan et al., 2005). Cattle that graze on grass contaminated with *Senecio* have been found to develop hepatitis that can progress to death if allowed to continue grazing. Human deaths have also been reported in several countries, and in Afghanistan, an epidemic of hepatic veno-occlusive disease arose from consumption of a wheat crop contaminated by *Heliotropium* (Tandon et al., 1978). The liver damage caused by ingestion clinically appears to be similar to cirrhosis and some hepatic tumors that can easily be mistaken to be the source of the disease (McDermott and Ridker, 1990). Consumption of these plants leads to a form of the Budd–Chiari syndrome, which is hallmarked by portal hypertension and obliteration of the small hepatic veins. Human consumption can occur from drinking "comfrey tea" that contains *Symphytum* (Rode, 2002). There are species differences in metabolism of the pyrrolizidine alkaloids (Huan et al., 1998).

Lantana camara (Verbenaceae), a shrub native to Jamaica, has been shown to poison livestock, particularly in India. Cattle grazing on the plant develop bile-related disorders including cholestasis and hyperbilirubinemia. Several triterpenoids have been isolated from the plant and, in particular, lantadene A (22-β-angeloyloxy-3-oxo-olean-12-en-28-oic acid) has been shown to be hepatotoxic (Sharma et al., 1991).

Mushroom Toxins Most nonedible mushrooms may cause mild discomfort and are not life threatening; however, repeated ingestion of the false morel, *Gyromitra esculenta*, has been found to cause hepatitis (Michelot and Toth, 1991). The toxin gyromitrin is inactivated generally by boiling. Most fatal poisonings related to wild mushrooms are from ingestion of different species within *Amanita*, *Galerina*, and *Lepiota* (Karlson-Stiber and Persson, 2003). The dangerousness of *Amanita phalloides* (Fig. 26-11) and *Amanita ocreata* is why they are named "death cap" and "death angel," respectively. Two types of toxins, phalloidin and amatoxins, can be found within *A. phalloides*. Phalloidin is capable of binding actin in muscle cells; however, it is not readily absorbed during digestion, which limits its harmful effects (Cappell and Hassan, 1992). Parenterally administered phalloidin produces toxicity in wild-type mice but not in Oatp1b2-null mice, which is consistent with phalloidin being a substrate for uptake into hepatocytes by the Oatp1b2 carrier (Lu et al., 2008). Unfortunately, α-, β-, and γ-amanitins are readily absorbed due to being molecularly much

Figure 26-11. *Amanita phalloides (death cap).*

smaller than phalloidin. Of the amatoxins, α-amanitin is the most toxic as it inhibits protein synthesis in hepatocytes by binding to RNA polymerase II (Jaeger et al., 1993). In addition to the liver, the intestinal mucosa and kidneys are also affected and serious clinical signs develop about 3 days after ingestion. In cases of severe poisoning, a liver transplant may be required. Amatoxin-α irreversibly inhibits acetylcholinesterase (Hyde and Carmichael, 1991).

Mycotoxins Fumonisin toxins are produced by the fungus *Fusarium* that is known to grow on corn. Consumption of contaminated corn by horses leads to equine leukoencephalomalacia that is marked by lethargy, ataxia, convulsions, and ultimately death (Norred, 1993). The liver is the most affected organ in many species including horses, pigs, chickens, and rats (Riley et al., 1994). Ingestion in humans has been suggested to be associated with esophageal cancer (Yoshizawa et al., 1994). Fumonisins are diesters of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane containing a primary amino acid (Gurung et al., 1999) that is similar to sphingosine. This similarity is responsible for their toxicity as they block the enzymes involved in sphingolipid biosynthesis (Norred, 1993). In contrast, mycoestrogenic zearalenone induces CYP3A enzymes (Ding et al., 2006). Aflatoxin B1 has been shown to form guanine adducts and induce apoptosis in human hepatocytes (Reddy et al., 2006).

Kidney and Bladder

Carcinogens The bracken fern (*Pteridium aquilinum*), which is extremely common worldwide, is the only higher plant known to be carcinogenic in animals under natural feeding conditions. The commonest bladder tumors in cattle are epithelial and mesenchymal neoplasms (Kim and Lee, 1998). Ptaquiloside, a norsesquiterpene glucoside, is the known carcinogen present in the fern and it has been found to alkylate adenines and guanines of DNA (Shahin et al., 1999; Rasmussen et al., 2003). Bovine consumption of bracken fern has been shown to significantly increase chromosomal aberrations (Lioi et al., 2004). In addition, evidence has been found that consumption of young bracken fern shoots by humans is associated with cancers of the mouth and throat (Alonso-Amelot and Avendano, 2002).

Kidney Tubular Degeneration Species of *Xanthium* (cocklebur, Asteraceae) have been found to contain the toxin carboxyatractyloside, which causes microvascular hemorrhages in multiple organs (Turgut et al., 2005). Livestock poisoning has been noted to cause the outward signs of depression and dyspnea; however, internally the toxin causes tubular degeneration and necrosis in the kidney and centrilobular necrosis in the liver (del Carmen Mendez et al., 1998).

Consumption of the mushroom species *Cortinarius* has been found to cause acute renal failure but different species vary widely in toxicity and, therefore, edibility. In an investigation of a series of 135 poisonings related to *Cortinarius* ingestion, where death occurred in almost 15% of the cases, renal biopsy showed acute degenerative tubular lesions with inflammatory interstitial fibrosis (Bouget et al., 1990). *Cortinarius orellanus* and *C. rubellus* (Fig. 26-12) contain the deadly toxin orellanin, which triggers renal failure. Table 26-7 lists the common and scientific names and toxins for selective plants that are capable of inducing nephrotoxicity.

Figure 26-12. *Cortinarius rubellus (deadly webcap).*

Table 26-7
Selective Plants Producing Nephrotoxicity

COMMON NAME	SCIENTIFIC NAME	PART	TOXIN
Autumn crocus	*Colchicum autumnale*	Bulb	Colchicine
Castor bean	*Ricinus communis*	All	Ricin, recinine
Daphne	*Daphne mezereum*	Leaves, fruits	Daphnin, mezerein (diterpenoid)
Impila	*Callilepis laureola*	Tubers	Atractyloside
Mushrooms	*Amanita phalloides*	All	Amatoxin
	Cortinarius sp.	All	Orellanine
Water hemlock	*Cicuta maculata*	Roots	Cicutoxin

Blood and Bone Marrow

Anticoagulants Fungal infections in sweet clover (*Melilotus alba*) have been found to produce dicumarol, a coumarin derivative that is a potent anticoagulant. Deaths in cattle have been reported and are caused by hemorrhages (Puschner et al., 1998). The dicumarol derivative warfarin is a widely used rodenticide and as an anticoagulant drug.

Bone Marrow Genotoxicity *Argemone* (Papaveraceae), a species of poppy, produces sanguinarine, a benzophenanthridine alkaloid that is known to intercalate DNA and have carcinogenic potential (Das et al., 2005). Studies in mice have shown that a single low dose of argemone oil increases chromosomal aberrations in bone marrow cells (Ansari et al., 2004).

Cyanogens Cyanogens are found in a wide variety of plants including the kernels of apples, cherries, and peaches. The highest concentrations are found in the seeds of the bitter almond, *Prunus amygdalus* var *amara*. However, small children are susceptible to amygdalin poisoning if they consume sufficient peach (*Prunus persica*) kernels. Fortunately, the concentration present in the seeds of apples is low enough that they are unlikely to cause a problem. Metabolism of amygdalin releases hydrocyanic acid that binds to the ferric ion in methemoglobin and the cytochrome oxidases, which, if severe enough, results in cyanide poisoning with death from asphyxiation (Bromley et al., 2005; Jorgensen et al., 2005). Severe cyanide poisoning can occur with cyanogen-containing vitamin supplements (O'Brien et al., 2005).

Cassava produced from *Manihot esculenta* (Euphorbiaceae) is a major food source for some regions of Africa. The raw root contains a cyanogenic glucoside linamarin that degrades during processing of the root for human consumption. Unfortunately, local processing may be inadequate and that can lead to ingestion of linamarin. Chronic ingestion has been suggested to be the cause of epidemics of konzo, a form of tropical myelopathy with sudden onset of spastic paralysis (Tylleskar et al., 1992).

Nervous System

Epileptiform Seizures The common and scientific names for selective plants that produce neurotoxins can be found in Table 26-8. Within the family Apiaceae, which contains carrots, the fleshy tubers of *Cicuta maculata* (water hemlock; Fig. 26-13) produce neurotoxic cicutoxin (a C17-polyacetylene). Consumption of a single tuber can result in fatal poisoning, characterized by tonic–clonic convulsions, owing to the cicutoxin binding to GABA-gated chloride channels (Uwai et al., 2000).

Members of the mint family (Labiatae) such as pennyroyal (*Hedeoma*), sage (*Salvia*), and hyssop (*Hyssopus*) are well known for their essential oils containing monoterpenes. Ingestion of these monoterpenes in concentrations much higher than those used for flavoring can cause tonic–clonic convulsions. In particular, menthol is a selective modulator of inhibitory ligand-gated channels (Hall et al., 2004).

Certain species within *Strychnos* (Loganiaceae) produce strychnine and brucine, which are known to cause increased central nervous system (CNS) stimulation by blocking glycine-gated chloride channels. *Strychnos nux vomica*, a small tree native to India, produces seeds that have been implicated in cases of unintentional poisoning (Wang et al., 2004).

Excitatory Amino Acids Red algae (*Digenia simplex*) under certain conditions can proliferate rapidly leading to the notorious beach vacating "red tide" and producing kainic acid. Kainic acid

Table 26-8

Selective Plants Producing Neurotoxicity

COMMON NAME	SCIENTIFIC NAME	PART	TOXIN	MECHANISM
Acacia tree	*Acacia willardiana*	Seeds	Willardiine	Glutamate receptor agonist
Alga	*Digenea simplex* *Chondria armata*	All	Kainic acid Domoic acid	Depolarization of glutamate-gated channels
Betel nut	*Areca catechu*	Nut	Guvacine Arecoline	GABA uptake inhibitor, anticonvulsant Stimulates muscarinic cholinoceptors; CNS stimulation
Buckthorn; Coyotillo	*Karwinskia humboldtiana*	Seeds, leaves	Tullidinol	Demyelination of motor neurons leading to paralysis
Chrysanthemum	*Chrysanthemum cinerarifolium*	Seeds	Pyrethrins	Stimulate sodium efflux from neurons in insects
Deadly nightshade	*Atropa belladonna*	Berries	Tropine alkaloid	Blockade of muscarinic cholinoceptors
Fly agaric mushroom	*Amanita muscaria*	All	Muscarine Muscimol	Stimulates muscarinic cholinoceptors; CNS stimulation GABA receptor agonist
Rhododendron	*Rhododendron* sp.	Leaves	Grayanotoxins	Stimulate sodium channel and membrane depolarization
Ryania	*Ryania speciosa*	Stems	Ryanodine	Stimulates calcium channels and muscle contraction
Poison nut tree	*Strychnos nux vomica*	All, especially seeds	Strychnine	Glycine receptor antagonist that produces convulsions
Tobacco	*Nicotiana tabacum*	Leaves	Nicotine	Nicotinic cholinoceptor agonist (low doses) or antagonist (high doses); CNS stimulation

Figure 26-13. *Cicuta maculata (water hemlock).*

Figure 26-14. *Amanita muscaria (fly agaric).*

may be ingested by humans who eat filter-feeding mussels that have eaten red algae. Acute symptoms are most notably gastrointestinal distress, headache, hemiparesis, confusion, and seizures. Severe exposure can result in severe memory deficits and sensorimotor neuropathy (Teitelbaum et al., 1990). Isodomoic acid from *Nitzschia* sp. has seizure-inducing properties (Kotaki et al., 2005).

The fungi *Amanita muscaria* (fly agaric; Fig. 26-14) and *Amanita pantherian* (panther agaric) produce the excitatory amino acid ibotenic acid (isoxazole amino acid) and its derivative muscimol that is neurotoxic (Li and Oberlies, 2005). Poisoning produces CNS depression, ataxia, hysteria, and hallucinations. Myoclonic twitching and seizures sometimes develop (Benjamin, 1992). Other genera of fungi have been marked for their hallucinogenic actions, notably *Psilocybe*, which contains psilocin and psilocybin (Tsujikawa et al., 2003).

Willardiine, an agonist on glutamate receptors, has been isolated from *Acacia willardiana*, *Acacia lemmoni*, *Acacia millefolia*, and *Mimosa asperata* (Gmelin, 1961) and causes excitation of the nervous system. Seeds from the legume *Lathyrus sativus* (grass pea) also contain an excitatory amino acid known as β-*N*-oxalyl-L-α,β-diaminopropionic acid (Warren et al., 2004). Consumption of seeds over long periods can cause lathyrism with severe spastic muscle weakness and atrophy but little sensory involvement. Thiocyanate from linamarin can stimulate neuronal glutamate receptors, leading to degeneration of corticospinal motor pathways (Spencer, 1999).

Motor Neuron Demyelination *Karwinskia humboldtiana* is a shrub found in the southwestern United States, Mexico, and Central America that produces anthracenones in its seeds. Both human and livestock poisonings have been known to occur. Several days following ingestion, ascending flaccid paralysis develops with demyelination of large motor neurons in the legs and eventually leads to bulbar paralysis in fatal cases (Martinez et al., 1998). In addition to neurotoxicity, the anthracenones in *Karwinskia*, especially peroxisomicine A_2, cause lung atelectasis, emphysema, and massive liver necrosis. Inhibition of catalase in peroxisomes has been proposed as the mechanism of cell toxicity (Martinez et al., 1997).

Cerebellar Neurons The legumes *Swainsonia cansescens*, *Astragalus lentiginosus* (spotted locoweed), and *Oxytropis sericea* (locoweed) produce a toxic indolizidine alkaloid called swainsonine. These weeds can be accidentally consumed by grazing cattle, causing aberrant behavior with hyperexcitability and locomotor difficulty (James et al., 1991). In fatal cases, there is cytoplasmic foamy vacuolation of cerebellar neurons. Swainsonine causes marked inhibition of liver lysosomal and cytosomal α-mannosidase and Golgi mannosidase II resulting in abnormal accumulation of brain glycoproteins and mannose-rich oligosaccharides that ultimately causes cell death (Tulsiani et al., 1988). *Embellisia* fungi from locoweed produce a locoweed-like toxicosis (McLain-Romero et al., 2004).

Parasympathetic Stimulation Certain mushrooms of the genera *Inocybe*, *Clitocybe*, and *Omphalatus* contain significant amounts of muscarine, which mimics the action of acetylcholine, the principal neurotransmitter in the parasympathetic nervous system. Consumption of any of these species results in extreme parasympathetic activation resulting in miosis, diarrhea, sweating, salivation, lacrimation, urination, bradycardia, and bronchoconstriction (de Haro et al., 1999).

Parasympathetic Block Atropine, L-hyoscyamine, and scopolamine are belladonna alkaloids that can be found in varying concentrations in several genera of Solanaceae such as *Datura stramonium* (jimson weed; Fig. 26-15), *Hyoscyamus niger* (henbane), *Atropa belladonna* (deadly nightshade; Fig. 26-16), and *Duboisia myoporoides* (pituri). These alkaloids all effectively block the muscarinic receptor, essentially turning off the parasympathetic drive at

Figure 26-15. *Datura stramonium (jimson weed).*

Figure 26-16. *Atropa belladonna (deadly nightshade).*

the target organ. This explains why tachycardia, dry mouth, dilated pupils, and decreased gastrointestinal motility all occur on ingestion of these toxins. Of the three alkaloids, scopolamine is the most potent; however, sizable doses of L-hyoscyamine or atropine can produce similar effects. Large doses can cause confusion, bizarre behavior, hallucinations, and subsequent amnesia, and in severe intoxication, tachycardia may be completely absent (Caksen et al., 2003).

The seeds of *Solanum dulcamara* (bittersweet, which is often used in flower arrangements) contain the glycoalkaloid solanine that on ingestion causes tachycardia, dilated pupils, and hot and dry skin—symptoms similar to atropine poisoning (Ceha et al., 1997).

Sensory Neuron Block Capsaicin found in *C. annuum* (sweet pepper) and *C. frutescens* (red pepper) causes a burning sensation on vanilloid-type (VR1) sensory receptors. It also desensitizes the transient receptor potential vanilloid 1 (TRPV1) receptor of sensory endings of C-fiber nociceptors to stimuli, a property that has therapeutic use in treating chronic pain (Szalcsany, 2002). Capsaicin also can relax ileal smooth muscle (Fujimoto et al., 2006). Fortunately, desensitization produced by capsaicin is not due to cell death (Dedov et al., 2001). Polygodial, a sesquiterpene found in *Polygonum hydropiper*, also desensitizes the TRPV1 (Andre et al., 2006), whereas resiniferatoxin activates TRVP1 (Raisinghani et al., 2005). Pyrethum from chrysanthemum flowers blocks sodium channels in the insect nervous system (McGovern and Barkley, 1999).

Skeletal Muscle and Neuromuscular Junction

Neuromuscular Junction Anabasine, an isomer of nicotine, is present in *Nicotiana glauca* (tree tobacco, Solanaceae) and produces prolonged depolarization of the junction after a period of excessive stimulation. Consumption of *N. glauca* leaves can result in flexor muscle spasm and gastrointestinal irritation, followed by severe, generalized weakness, and respiratory compromise (Mellick et al., 1999). Curare, which is used as a poison placed on the tips of arrows, is also a potent neuromuscular blocking agent and is obtained from tropical species of *Strychnos toxifera* and *Chondrodendron tomentosum*. *Anabaena flosaquae*, a species of alga, can produce under the right conditions a neurotoxin anatoxin A that is known to be fatal. Anatoxin A, ingested by animals that drink pond water with the alga present, depolarizes and blocks the animal's nicotinic and muscarinic acetylcholine receptors, which can cause death from respiratory arrest within minutes to hours (Short and Edwards, 1990).

Delphinium barbeyi (tall larkspur, Ranunculaceae), which grows naturally in the western United States, contains methyllycaconitine. Ingestion of the toxin by cattle results in muscle tremors and ataxia followed by prostration and can ultimately lead to respiratory arrest in fatal cases. Methyllycaconitine is similar to curare, in that it has a high affinity for the acetylcholine receptor at the neuromuscular junction. Physostigmine, an inhibitor of acetylcholinesterase that increases acetylcholine at the neuromuscular nicotinic receptor, has been used successfully as an antagonist to treat some cases of methyllycaconitine poisoning (Pfister et al., 1994).

Skeletal Muscle Damage Certain species of *Thermopsis* produce seeds that contain quinolizidine alkaloids. Human poisoning from eating the seeds is rare, but cases have been reported in young children who experienced abdominal cramps, nausea, vomiting, and headache lasting up to 24 hours (Spoerke et al., 1988). Livestock grazing on *Thermopsis montana* (false lupine, mountain goldenbanner; Fig. 26-17) develop locomotor depression and recumbency due to areas of necrosis in skeletal muscle that have been found on autopsy (Keeler and Baker, 1990).

Consumption of *Cassia obtusifolia* (sicklepod, Leguminosae) seeds by livestock causes a degenerative myopathy in cardiac and skeletal muscle. Extracts of *C. obtusifolia* have been found to inhibit NADH-oxidoreductase in bovine and swine mitochondria in vitro (Lewis and Shibamoto, 1989).

White snakeroot (*Ageratina altissima*, Asteraceae; Fig. 26-18), a common plant in the central and western United States, can be accidentally eaten by grazing cattle. On ingestion, the cattle exhibits tremors and humans who drink the milk of an affected cow can get "milk sickness" (Beier et al., 1993). The toxic effects are attributed to tremetone, a benzofuran, which blocks gluconeogenesis from lactate, resulting in acidosis, tremor, and ultimately death (Polya, 2003).

Bone and Tissue Calcification

Bone and Soft Tissue Consumption of *Solanum malacoxylon* (Solanaceae) by sheep and cows can cause a marked decrease in bone calcium and calcification of the entire vascular system due to the presence of a water-soluble vitamin D–like substance. In severe

Figure 26-17. *Thermopsis montana (mountain goldenbanner).*

Figure 26-18. *Ageratina altissima (white snakeroot).*

cases other organs can also be affected such as the lungs, joint cartilage, and kidney.

Consumption of *Cestrum diurnum* (day-blooming jasmine, Solanaceae) and *Cestrum laevigatum* has been found to cause hypercalcemia and soft tissue calcification in grazing cattle (Durand et al., 1999) and chickens (Mello and Habermehl, 1992), which is due to the presence of a dihydroxyvitamin D_3 glycoside in the leaves (Durand et al., 1999). Hay that had been contaminated with *C. laevigatum* caused a marked centrilobular and midzonal hepatic necrosis in goats (Peixato et al., 2000).

Reproduction and Teratogenesis

Abortifacients Besides its actions on the nervous system, swainsonine, the active alkaloid in the legumes *Astragalus* and *Oxytropus*, also causes abortions in pregnant livestock that accidentally ingest locoweeds (Bunch et al., 1992).

Foliage and seeds of *Leucaena leucocephala*, *Leucaena glauca*, and *Mimosa pudica* contain a toxic amino acid, mimosine, which on ingestion by cattle leads to uncoordinated gait, goiter, and reproductive disturbances including infertility and fetal death (Kulp et al., 1996). Mimosine has been found to arrest the cell cycle in late G1 phase that helps explain its toxic effects (Perry et al., 2005).

Lectins present in bitter melon seeds (*Momordica charantia*, Cucurbitaceae) have antifertility, abortifacient, and embryotoxic actions on ingestion. Components of the lectins known as momorcharins are known to induce midterm abortion in humans (Wang and Ng, 1998).

Caulophyllum thalictroides (blue cohosh, Berberidaceae) contains a toxin known as caulophylline that is teratogenic in rats (Kennelly et al., 1999) and herbal preparations of blue cohosh have been used to terminate pregnancy (Jones and Lawson, 1998). In fact, nicotine toxicity may result from blue cohosh use as an abortifacient (Rao and Hoffman, 2002).

Teratogens Ingestion of *V. californicum* (California false hellebore, Liliaceae; Fig. 26-19) by pregnant sheep is known to cause malformations in its offspring that can include cyclopia, exencephaly, and microphthalmia. As with most teratogens, the severity of malformations depends on what developmental stage the fetus is in at the time of exposure. Limb defects are common with exposure during the fourth to fifth weeks of gestation; fetal stenosis of the trachea will occur with ingestion on days 31 to 33 (Omnell et al., 1990). Many other species of animals are susceptible to *V. californicum* poisoning, including cows, goats, chickens, rabbits, rats, and mice (Omnell et al., 1990), hamsters (Gaffield and Keeler, 1993), lambs (Keeler, 1990), and rainbow trout embryos (Crawford and Kocan, 1993). The toxic alkaloid called jervine causes teratogenesis by blocking cholesterol synthesis that, among other things, prevents a proper response of fetal target tissue to the sonic hedgehog gene (Shh). Shh has an important role in proper developmental

Figure 26-19. *Veratrum californicum (California false hellebore).*

patterning of head and brain, and blocking cholesterol synthesis has been shown experimentally to cause a loss of midline facial structures (Cooper et al., 1998).

Pregnant cattle grazing on *Lupinus caudatus* and *Lupinus formosus* (lupines, Leguminosae), *N. glauca* (tree tobacco, Solanaceae), and *Conium maculatum* (poison hemlock, Solanaceae) have been found to experience a myriad of fetal deformations if ingestion occurs during a sensitive gestational period. The toxic alkaloids in these plants are anagyrine (*L. caudatus*), ammodendrine (*L. formosus*), anabasine (*N. glauca*), and coniine (*C. maculatum*; Forsyth et al., 1996). It is thought that these alkaloids depress fetal movements that can lead to malformations (Lopez et al., 1999).

CLINICAL STUDY OF PLANT POISONS

Recent research concerning the use of plant toxins in a clinical setting has blossomed (Ball and Kowdley, 2005). A number of factors make plant toxins of particular interest, including their diverse effects, availability, and cost. With a growing group of people who wish to return to a more "natural" way of life, a medicine that is derived straight from a plant is much more marketable than one that is completely chemically synthesized. For example, root extract of the African Uzara plant has been used as an antidiarrheal remedy for centuries; however, its mechanism of action was only speculative. Recent research has shown that Uzara root extract reduced chloride secretion by the gut specifically by inhibiting Na^+,K^+-ATPase. This effect was seen even in the presence of cholera toxin that causes potent diarrhea by increasing chloride secretion in the gut (Schulzke et al., 2011). Interestingly, anemonin, which is the active skin irritant produced by species of *Ranunculus* (buttercup), has been found to show potent anti-inflammation effects under certain conditions. The compound was found to reduce nitric oxide production that resulted in a lessened inflammatory response to inflammatory stimuli (Lee et al., 2008). This mechanism could provide a new anti-inflammatory treatment modality and is being tested for possible medical use. Silymarin and usnic acid may have promise as anticancer drugs (Mayer et al., 2005a, 2005b). Finally, native Nigerians use an extract from *M. pruriens* seeds as a pretreatment for snakebites from *Naja sputatrix* (Javan spitting cobra). When tested in rats, the seed extract helped protect against the cardiorespiratory and neuromuscular depressant effects of the snake venom (Fung et al., 2011). A recent review on the ethnopharmacology of aconitine, strychnine, scopolamine, and anisodamine contrasted the medicinal value of these chemicals with their high toxicity (Ma et al., 2015). In addition, tenuifolide B, which is extracted from the stem of the plant Cinnamomum tenuifolium, may selectively reduce cell viability of oral cancer cells (Chen et al., 2016). These toxic plants have great potential for pain reduction and other purposes. Thus, old herbal remedies are a ripe field of study, for many of their effects can be beneficial yet toxic at high enough concentrations. A goal for new research is to elucidate the mechanism of action so that treatments can be tailored to the individual needs and toxic effects can be avoided or interactions with conventional drugs can be minimized (Izzo and Ernst, 2001). As our knowledge of plant toxins and their mechanisms of action expands, the number of new and clinically useful plant remedies and medical products is bound to increase (Polito et al., 2016). Technological advances in chemical purification and identification will permit better toxic characterization as well (Arilla et al., 2006).

Plant engineering with ribosome-inhibiting proteins can increase resistance to pests and microbial pathogens. Moreover, conjugation with carriers such as antibodies and nanoparticles can help direct the active protein to target cells for destruction, such as cancer cells (Gilabert-Oriol et al., 2014; Pizzo and Di Maro, 2016). A challenge to the use of these ribosomal inhibitor-conjugates, or immunotoxins, is that endosomal destruction can occur upon entry into target cells. Considerable effort to produce endosomal escape enhancers has been reviewed (Fuchs et al., 2016). These enhancers may work by increasing cell penetrance, protection of the catalytic domain, conversion to secondary metabolites, and complexing with light-sensitive substances for activation. Of more than 500-targeted toxins from disparate species, very few have been approved for therapeutic application (Fuchs et al., 2016).

SUMMARY OF PLANT TOXICITIES

Toxic chemicals produced by plants can cause something as innocuous as a mild rash on the skin all the way to death by respiratory arrest. It is important to remember that these toxins are primarily defense mechanisms against natural predators. However, we benefit greatly from plants as many have produced potent antibiotics and pharmacologic therapies for myriad diseases that afflict humans.

INTRODUCTION TO ANIMAL VENOMS

The animal kingdom consists of more than 100,000 species spread through major phyla including arthropods, mollusks, and chordates (Mebs, 2002). Venomous animals are capable of producing a poison in a highly developed exocrine gland or group of cells and can deliver their toxin during a biting or stinging act. The venom is the sum of all natural venomous substances produced in the animal (Ménez et al., 2006). Conversely, poisonous animals have no specific mechanism or structure for the delivery of their poisons, and poisoning usually takes place through ingestion. Venomous or poisonous animals are widely distributed throughout the animal kingdom, from the unicellular protistan *Alexandrium* (*Gonyaulax*) to certain mammals, including the platypus and the short-tailed shrew. At least 400 species of snakes are considered dangerous to humans. Myriad venomous and poisonous arthropods exist, and toxic marine animals are found in almost every sea and ocean (Russell and Nagabhushanam, 1996; Mebs, 2002; Mackessy, 2010).

Animal venom may play a role in offense (as in the capture and digestion of food), in the animal's defense (as in protection against predators or aggressors), or in both functions. Venoms used in an offensive posture are generally associated with the oral pole, as in the snakes and spiders, while those used in a defensive function are usually associated with the aboral pole or with spines, as in the stingrays and scorpion fish. In the snake, the venom provides a food-getting mechanism. Its secondary function is its defensive status. In contrast, in venomous spiders, toxin is used to paralyze the prey before the extraction of hemolymph and body fluids. The venom is not primarily designed to kill the prey, but it is only to immobilize the organism for feeding. The same can be said for scorpions, although they do use their venom in defense. In fish, such as the scorpion fish and stone fish, and in elasmobranches, such as the stingray, the venom apparatus is generally used in the animal's defense. In contrast, poisonous animals usually derive their toxins through the food chain. As such, poison is often a metabolite produced by microorganisms, plants, or animals. Poisons are sometimes concentrated as they pass through the food chain from one animal to another. It is also worth noting that animal and plant toxins tend to differ significantly in their chemical complexity, yet both are capable of causing massive harm. Plant toxins tend to be smaller compounds or proteins, and often times a single offending substance can be pinpointed. Conversely, animal toxins must be studied in the context of the entire venom or poison that typically is very complex and contains many individual toxic compounds and very large proteins that essentially work together to cause their effects. Just the same as the preceding section on plant toxins, certain criteria were employed to effectively narrow down the animal toxins discussed. Specifically considered were how often does human exposure occur, how significant is an exposure, and in general how much is known about the animal's toxic properties.

PROPERTIES OF ANIMAL TOXINS

Venoms are very complex, containing polypeptides, high- and low-molecular-weight proteins, amines, lipids, steroids, aminopolysaccharides, quinones, glucosides, and free amino acids, as well as serotonin, histamine, and other substances. Some venoms are known to consist of more than a hundred proteins. The venom is a source of peptides and proteins that act on myriad exogenous targets such as ion channels, receptors, and enzymes within cells and on cell membranes (Ménez et al., 2006). Studying venom is important for several reasons. First, venoms offer valuable insight into the systems they act on such as the cardiovascular system, nervous system, coagulation, and homeostasis. Second, venoms are useful as a source of potential new drugs, with at least five chemicals already on the market and dozens undergoing preclinical or clinical trials. Third, a greater understanding of venoms favors development of improved protection against envenomation (Ménez et al., 2006).

Novel instrument developments have permitted the greater application of mass spectrometry, coupled with various separation technologies, to tease out the complexity of natural venoms, thereby identifying the peptide and protein components of venoms (Escoubas, 2006). The technology allows considerable resolution of extremely small amounts of venom. Fig. 26-20 demonstrates the application of gel filtration and high-pressure liquid chromatography (HPLC), as cone snail venom was fractionated into numerous peptides with varying activities (Olivera et al., 1990). Similar fractionations have been performed on many other types of venom to identify the individual components. Unfortunately, studying the chemistry, pharmacology, and toxicology of venoms requires isolating and dismantling the venoms and losing the synergy among multiple components. Nevertheless, advanced technology will permit peptide sequencing, and the characterization of posttranslational modifications, such as glycosylation, and the discovery of new pharmacophores. Most venoms probably exert their effects on almost every cell and tissue, and their principal pharmacologic properties are usually determined by the amount of a fraction that accumulates at an activity site. Table 26-9 reveals the extremely large range in the LD_{50} of different toxic compounds and venoms injected intravenously into mice.

The bioavailability of a venom is determined by its composition, molecular size, amount or concentration gradient, solubility, degree of ionization, and the rate of blood flow into that tissue, as well as the properties of the engulfing surface itself. The venom can be absorbed by active or passive transport, facilitated diffusion, or pinocytosis, among other physiologic mechanisms. Besides the bloodstream, the lymph circulation not only carries surplus interstitial fluid produced by the venom but also transports larger molecular components and other particulates back to the bloodstream. Thus, the larger toxins of snake venoms, particularly those of Viperidae, probably enter the lymphatic network preferentially and then are transported to the central venous system in the neck (Russell, 2001). Because lymphatic capillaries, unlike blood capillaries, lack a basement membrane and have fibroelastic "anchoring filaments," they can readily adjust their shape and size, facilitating absorption of excess interstitial fluid along with macromolecules contained in a venom.

The site of action and metabolism of venom is dependent on its diffusion and partitioning along the gradient between the plasma and the tissues where the components are deposited. Once the toxin reaches a particular site, its entry to that site is dependent on the rate of blood flow into that tissue, the mass of the structure, and the partition characteristics of the toxin between the blood and the particular tissue. Receptor sites appear to have highly variable degrees of sensitivity. In the case of complex venoms, there may be several, if not many, receptor sites. There is also considerable variability in the sensitivity of those sites for the different components of a venom.

A venom may also be metabolized in several or many different tissues before undergoing excretion. Some venom components are metabolized distant to the receptor site(s) and may never reach the primary receptor in a quantity sufficient to affect that site. The amount of a toxin that tissues can metabolize without endangering the organisms may also vary. Organs or tissues may contain enzymes that catalyze a host of reactions, including deleterious ones. Once a venom component is metabolically altered, the end substance is excreted primarily through the kidneys. The intestines

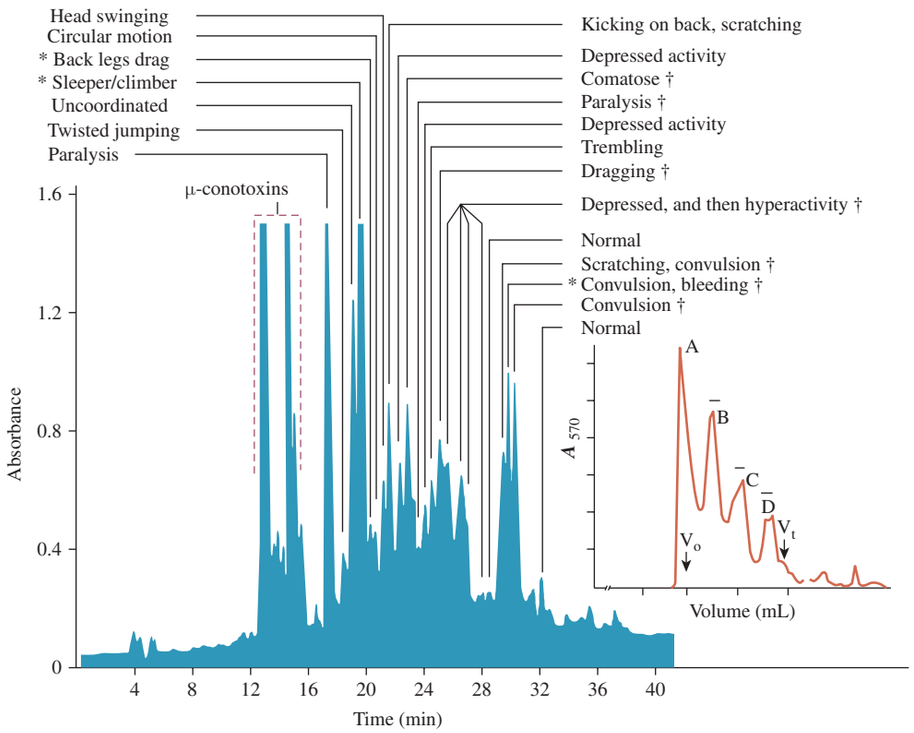

Figure 26-20. *Multiple biologically active components were obtained from Conus geographus venom by first subjecting the venom to gel filtration on Sephadex G-25 into four fractions and then separation of fraction B (which contains the α-conotoxins) by high-pressure liquid chromatography on a VYDAC C18 column using a trifluoroacetic acid–acetonitrile gradient.* Various peak fractions were then injected intracerebrally into mice and different responses were noted. (†) The fraction was lethal in at least one injected animal. (Reprinted with permission from Olivera et al. [1990].)

Table 26-9

Intravenous LD_{50} Values of Selected Toxins Determined in Mice

TOXIN SOURCE	COMMON NAME	LD_{50} (μg/kg)
Clostridium botulinum	Botulinum toxin	0.0003
Crotalus viridis helleri	Southern pacific rattlesnake	1.3
Crotalus adamanteus	Eastern diamondback	1.5
Oxyuranus scutellatus	Australian taipan	2
Crotalus atrox	Western diamondback	2.2
Agkistrodon piscivorus	Eastern cottonmouth	4
Agkistrodon contortrix	Copperhead	11
Androctonus australis	North African scorpion	17
Notechis scutatus	Australian tiger snake	25
Naja siamensis	Indochinese spitting cobra	75

SOURCES: Data from Mebs (2002) and Russell (2001).

play a minor role, and the contributions by the lungs and biliary system have not been determined. Excretion may be complicated by the direct action of the venom on the kidneys themselves.

ARTHROPODS

There are more than 1 million species of arthropods, generally divided into 25 orders, of which only about 10 orders are of significant venomous or poisonous importance. These include the arachnids (scorpions, spiders, whip scorpions, solpugids, mites, and ticks), the myriapods (centipedes and millipedes), the insects (water bugs, assassin bugs, and wheel bugs), beetles (blister beetles), Lepidoptera (butterflies, moths, and caterpillars), and Hymenoptera (ants, bees, and wasps). Several texts and papers that deal with venomous and poisonous arthropods are available (Piek, 1986; Cohen and Quistad, 1998; Russell, 2001; Kuhn-Nentwig, 2003; Isbister et al., 2004; Heinen and Gorini da Veiga, 2011).

The number of deaths from arthropod stings and bites is unknown. In Mexico, parts of Central and South America, North Africa, and India, deaths from scorpion stings, for instance, exceed several thousand a year. Spider bites probably do not account for more than 200 deaths a year worldwide. A common problem faced by physicians in suspected spider bites relates to the differential diagnosis. The arthropods most frequently involved in the misdiagnoses were ticks (including their embedded mouthparts), mites, bedbugs, fleas (infected flea bites), Lepidoptera insects, flies, vesicating beetles, water bugs, and various stinging Hymenoptera. Among the disease states that were confused with spider or arthropod bites or stings were erythema chronicum migrans, erythema

nodosum, periarteritis nodosum, pyroderma gangrenosum, kerion cell-mediated response to a fungus, Stevens–Johnson syndrome, toxic epidermal necrolysis, herpes simplex, and purpura fulminans. Any arthropod may bite or sting and not eject venom. Finally, some arthropod venom poisonings accentuate the symptoms and signs of an existing undiagnosed subclinical disease that further complicates diagnosis.

ARACHNIDA

Scorpions

Of the more than 1000 species of scorpions, the stings of more than 75 can be considered of sufficient importance to warrant medical attention (Keegan, 1980; Polis, 1990; Russell, 2001). Some of the more important scorpions are noted along with their location in Table 26-10. In addition, members of the genera *Pandinus*, *Hadrurus*, *Vejovis*, *Nebo*, and some of the others are capable of inflicting painful and often erythematous lesions.

Many scorpion venoms contain low-molecular-weight proteins, peptides, amino acids, nucleotides, and salts, among other components (Possani et al., 2000; Goldin, 2001; Rodriguez de la Vega and Possani, 2004, 2005). The neurotoxic fractions are generally classified on the basis of their molecular size. The short-chain toxins with 20 to 40 amino acid residues have three or four disulfide bonds and appear to affect potassium or chloride channels. The long-chain toxins have 58 to 76 amino acid residues (6500 to 8500 Da) with four disulfide bonds and affect mainly the sodium channels (Mouhat et al., 2004). These particular toxins may have an effect on other voltage-dependent channels. The amino acid content is known for more than 90 species, and there appears to be a high number of cysteine residues in most of these venoms. The toxins can selectively bind to a specific channel of excitable cells, thus impairing the initial depolarization of the action potential in the nerve and muscle that results in their neurotoxicity. It appears that the way some scorpion venoms differently affect mammalian, as opposed to insect tissues, is related to the structural basis of the gates in the two organisms. Not all scorpions, however, have fractions that affect neuromuscular transmission. In fact, Rodriguez de la Vega and Possani (2005) constructed a phylogenetic tree using 191 different amino acid sequences from long-chain peptides and discussed their functional divergence and extant biodiversity. The effects of scorpion venom on various potassium channels have been reviewed (Rodriguez de la Vega and Possani, 2004).

The symptoms and signs of scorpion envenomation differ considerably depending on the species (Russell, 2001). Common offenders are members of the family Vejovidae, generally found in the southwestern and western United States, Central America, and South America. Their sting gives rise to localized pain, swelling, tenderness, and mild parasthesia. Systemic reactions are rare, although weakness, fever, and muscle fasciculations have been reported.

Envenomations by some members of the genus *Centruroides* are clinically the most important, particularly in the western United States. The bark scorpion, *C. exilicauda*, is often found hiding under the loose bark of trees or in dead trees or logs, and may frequent human dwellings. Straw to yellowish-brown or reddish-brown in color, it is often easily distinguishable from other scorpions in the same habitat by its long, thin telson, or tail, and its thin pedipalps, or pincer-like claws. In children, their sting may produce initial pain, although some children do not complain of pain and are unaware of the injury. The area becomes sensitive to touch, and merely pressing lightly over the injury will elicit an immediate retraction. Usually there is little or no local swelling and only mild erythema. The child becomes tense and restless and shows abnormal and random head and neck movements. Often the child will display roving eye movements. *Centruroides vittatus*, the striped bark scorpion (Fig. 26-21), is commonly involved in envenomenation but fatalities are rare. In their review of *Centruroides sculpturatus* stings, Rimsza et al. (1980) noted visual signs, including nystagmus roving eye and oculogyric movements, in 12 of 24 patients stung by this scorpion. Tachycardia is usually evident within 45 minutes as well as some hypertension. Although this is not seen in children as early or as severely as in adults, it is often present within an hour following the sting. Respiratory and heart rates are increased, and by 90 minutes the child may appear quite ill. Fasciculations may be seen over the face or large muscle masses, and the child may complain of generalized weakness and display some ataxia or motor weakness. Opisthotonos is not uncommon. The respiratory distress may proceed to respiratory paralysis. Excessive salivation is often present and may further impair respiratory function. Slurring of speech may

Table 26-10

Location of Some Medically Important Scorpions

GENUS	DISTRIBUTION
Androctonus species	North Africa, Middle East, Turkey
Buthus species	France and Spain to Middle East and north Africa, Mongolia, China
Buthotus species	Africa, Middle East, central Asia
Centruroides species	North, Central, South America
Heterometrus species	Central and Southeast Asia
Leiurus species	North Africa, Middle East, Turkey
Mesobuthus species	Turkey, India
Parabuthus species	Southern Africa
Tityus species	Central and South America

Figure 26-21. *Centruroides vittatus (striped bark scorpion).*

be present, and convulsions may occur. If death does not occur, the child usually becomes asymptomatic within 36 to 48 hours.

In adults, the clinical picture is somewhat similar, but there are some differences (Russell, 2001). Almost all adults complain of immediate pain after the sting, regardless of the *Centruroides* species involved. Adults do not show the restlessness that is seen in children. Instead, they are tense and anxious. They develop tachycardia and hypertension, and respirations are increased. They may complain of difficulties in focusing and swallowing. In some cases, there is some general weakness and pain on moving the injured extremity. Convulsions are very rare, but ataxia and muscle incoordination may occur. Most adults are asymptomatic within 12 hours, but may complain of generalized weakness for 24 hours or more.

Spiders

Of the 30,000 or so species, at least 200 have been implicated in significant bites on humans. Spiders are predaceous, polyphagous arachnids that generally feed on insects or other arthropods. Additional information on spider bites can be found elsewhere (Gertsch, 1979; Maretiĉ and Lebez, 1979; Russell, 2001). Table 26-11 provides a short list of spiders with their associated toxins and the targets of their toxins.

All spiders except the Uloboridae family possess a venom apparatus that produces neurotoxins designed to paralyze or kill prey. Spider venoms are complex mixtures of low-molecular-weight components, including inorganic ions and salts, free acids, amino acids, biogenic amines and neurotransmitters, and polypeptide toxins. The acylpolyamines, composed of a hydrophobic aromatic carboxylic acid linked to a lateral chain of one to nine aminopropyl, aminobutyl, or aminopentyl units, are voltage-dependent open-channel blockers (sodium, calcium, and potassium channels) and/or blockers of the ion channel associated with glutamate receptors. They also act on nicotinic acetylcholine receptors. The acylpolyamines possess insecticidal activity and induce fast insect paralysis via a reversible block of the insect neuromuscular junction. Polypeptide toxins include the ion channel blockers, pore-forming peptides, and enzymes. In particular, hanatoxins 1 and 2 have allowed characterization of voltage-dependent potassium channels (Swartz and MacKinnon, 1995; Yellen, 2002). The ω-agatoxins block voltage-sensitive vertebrate calcium channels (Corzo and Escoubas, 2003). The ω-atracotoxins have greater selectivity on insect voltage-sensitive calcium channels (Wang et al., 2001). Many more spiders and the spider peptide toxins have been studied to date (Fatehi et al., 1997; Liu et al., 1997; Corzo and Escoubas, 2003; Adams, 2004; Kuhn-Nentwig et al., 2004; Rodriguez de la Vega and Possani, 2004; Ushkaryov et al., 2004; Wilson and Alewood, 2006). The small spider peptide toxins are relatively easy to produce by chemical synthesis or by recombinant means. Peptide toxins from spiders have proved useful in discriminating between different cellular components of native ion channel currents and for the molecular isolation and designation of cellular receptors (Escoubas et al., 2000; Corzo and Escoubas, 2003; Adams, 2004).

Poor case definition and spider identification contribute to the controversy regarding the clinical consequences and inappropriate diagnosis of spider bites worldwide. Many patients never see the spider, yet many skin lesions or areas of necrosis are attributed to spider bites. The medically important spiders include the *Agelenopsis* sp., *Latrodectus* sp., *Loxosceles* sp., *Atrax* sp., *Hadronyche* sp., and *Phoneutria* sp. Table 26-12, summarized from Isbister and White (2004), lists local and systemic effects for known cases of major spider groups in Australia.

Table 26-11

Some Significant Spiders, Their Toxins, and the Targets of the Toxins

SPIDER	PEPTIDE	TARGET
Acanthoscurria gomesiana	Gomesin	PLM
Agelenopsis aperta	ω-AfaI-IVA μ-Afatoxin 1-6	Ca^{2+} Na^{+}
Grammostola spatula	HaTx1,2 GsMTx2,4 GSTxSIA	K^{+} MS Ca^{2+}
Hadronyche versuta	ω-ACTX-Hv1a ω-ACTX-Hv2a δ-ACTX-Hv1a	Ca^{2+} Ca^{2+} Na^{+}
Heteroscodra maculate	HmTx1,2	K^{+}
Ornithoctonus huwena	Huwentoxin I Huwentoxin IV	Ca^{2+} Na^{+}
Psalmopoeus cambridgei	PcTx1	ASIC
Phrixotrichus auratus	PaTx1,2	K^{+}
Thrixopelma pruriens	ProTxI,II	Na^{+}

Abbreviations: ASIC, acid-sensing ion channels; $Ca2^{+}$, K^{+}, and Na^{+}, calcium, potassium, and sodium ion channels; MS, mechanosensitive ion channels; PLM, phospholipid membranes.

Additional species, their toxins, and their targets may be obtained from the article by Corzo and Escoubas (2003).

Agelenopsis Species (American Funnel Web Spiders)

The American funnel web spider (*Agelenopsis aperta*) contains three classes of agatoxins that target ion channels (Adams, 2004). The α-agatoxins appear to be use-dependent, noncompetitive antagonists of the glutamate receptor channels. These are low-molecular-weight acylpolyamines that are devoid of amino acids. Mass spectrometry analysis has identified more than 33 α-agatoxins in *A. aperta* venom (Chesnov et al., 2001). The μ-agatoxins are 36 to 37 amino acid, C-terminal amidated, peptides with four internal disulfide bridges (Skinner et al., 1989). These μ-agatoxins cause increased spontaneous release of neurotransmitter from presynaptic terminals and repetitive action potentials in motor neurons. In addition, the μ-agatoxins are specific for insect sodium channels. The ω-amatoxins are a structurally diverse group of peptides that are selective for voltage-activated calcium channels. There are four types of ω-amatoxins that can be distinguished by sequence similarity and their spectrum of action against insect and vertebrate calcium channels. The μ-agatoxins causing channels to open at the normal resting potentials synergize the action of the α-agatoxins. It is interesting to note that the agatoxins are used as selective pharmacologic probes to characterize ion channels in organs such as brain and heart, and have been evaluated as candidate biopesticides.

Latrodectus Species (Widow Spiders)

Found throughout the world in all continents with temperate or tropical climates, these spiders are commonly known as the black widow, brown widow, or red-legged spider. They, however, have many other common names in English: hourglass, poison lady, deadly spider, red-bottom spider, T-spider, gray lady spider, and shoe-button spider. Although both male and female widow spiders are venomous, only the female has fangs that are large and strong enough to penetrate the human skin. Mature *Latrodectus mactans* females range in body length from

Table 26-12

Comparison of Clinical Effects of Bites by Some Spiders of Australia

CLINICAL EFFECTS	*LACTRODECTUS* REDBACK SPIDERS	*STEATODA* CUPBOARD SPIDERS	LAMPONIDAE WHITE-TAIL SPIDERS	MYGALOMORPHAE FUNNEL WEB SPIDERS, MOUSE SPIDERS, TRAPDOOR SPIDERS
Severe pain (%)	62	26	27	49
Duration of pain	36 hours	6 hours	0.8 hours	1 hour
Fang marks (%)	6	17	17	58
Initial erythema	74	96	83	36
Swelling (%)	7	9	8	13
Itchiness (%)	38	48	44	0
Nausea, vomiting, headache, malaise (%)	35	30	9	36
Distal limb bite (%)	46	52	82	91

SOURCE: Data from Isbister and White (2004).

Figure 26-22. *Latrodectus mactans (female black widow spider).*

10 to 18 mm, whereas males range from 3 to 5 mm (Fig. 26-22). These spiders have a globose abdomen varying in color from gray to brown to black, depending on the species. In the black widow, the abdomen is shiny black with a red hourglass or red spots and sometimes with white spots on the venter (Russell, 2001).

The latrotoxins, a family of high-molecular-weight proteins that are found in *Latrodectus* venoms, target different classes of animals including vertebrates, insects, and crustaceans (Grishin, 1998, 1999; Ushkaryov et al., 2004). The toxins are synthesized as large precursors containing around 1000 amino acid residues (around 132 to 156 kDa) that undergo proteolytic processing to 110 to 130 kDa and activation in the lumen of the venom gland. Mature latrotoxins are structurally conserved and contain multiple ankyrin repeats. At least seven different latrotoxins have been isolated and all are large acidic proteins (pI approximately 5.0 to 6.0). Some are called latroinsectotoxins because they affect insects but not vertebrates, and one protein called α-latrocrustatoxin is active only in crayfish. All latrotoxins stimulate massive release of neurotransmitters after binding to specific neuronal receptors.

α-Latrotoxin is the most studied protein that is toxic only to vertebrates and not to insects or crustaceans (Ushkaryov et al., 2004). A presynaptic toxin is said to exert its toxic effects on the vertebrate central nervous system depolarizing neurons by increasing intracellular [Ca^{2+}] and by stimulating exocytosis of neurotransmitters from nerve terminals (Holz and Habener, 1998). The purported three-dimensional structure of α-latrotoxin consists of tetrameric complexes with a central channel that inserts into the lipid bilayer. A G protein–coupled receptor latrophilin and a single-transmembrane receptor neurenin are high-affinity binding sites for α-latrotoxin (Ushkaryov et al., 2004). The latrotoxins act by both calcium-dependent and calcium-independent mechanisms. In fact, a mutant recombinant α-latrotoxin that does not form pores has been invaluable in furthering our understanding of the multiple actions of the toxin (Volynski et al., 2003). α-Latrotoxin and its mutants are versatile tools for the study of exocytosis. In particular, studies with this toxin have helped confirm the vesicular hypothesis of transmitter release (Hurlbut et al., 1990), establish the requirement of calcium ion for endocytosis (Ceccarelli and Hurlbut, 1980), characterize individual neurotransmitter sites in the central nervous system (Auger and Marty, 1997), and identify two families of important neuronal cell surface receptors (Krasnoperov et al., 1997).

Bites by the black widow are described as sharp and pinprick-like, followed by a dull, occasionally numbing pain in the affected extremity and by pain and cramps in one or several of the large muscle masses (Russell, 2001). Rarely is there any local skin reaction except during the first 60 minutes following the bite. Muscle fasciculations frequently can be seen within 30 minutes of the bite. Sweating is common, and the patient may complain of weakness and pain in the regional lymph nodes, which are often tender on palpation and occasionally are enlarged; lymphadenitis is frequently observed. Pain in the low back, thighs, or abdomen is a common complaint, and rigidity of the abdominal muscles is seen in most cases in which envenomation has been severe. Severe paroxysmal muscle cramps may occur, and arthralgia has been reported. Hypertension is a common finding, particularly in the elderly after moderate to severe envenomations. Blood studies are usually normal.

***Loxosceles* Species (Brown or Violin Spiders)** These primitive spiders are variously known in North America as the fiddleback spider or the brown recluse (Fig. 26-23). There are over 100 species of *Loxosceles*. The abdomen of these spiders varies in

Figure 26-23. *Loxosceles reclusa (male brown recluse spider) with the violin pattern on the dorsal cephalothorax.*

color from grayish to orange and reddish-brown to blackish and is distinct from the pale yellow to reddish-brown background of the cephalothorax. This spider has six eyes grouped in three dyads. Females average 8 to 12 mm in body length, whereas males average 6 to 10 mm. Both males and females are venomous (Russell, 2001).

The venom of *Loxosceles* spiders appears to contain phospholipase, protease, esterase, collagenase, hyaluronidase, deoxyribonuclease, ribonuclease, dipeptides, dermonecrosis factors, and sphingomyelinase D. The venom has coagulation and vasoconstriction properties and it causes selective vascular endothelial damage. There are adhesions of neutrophils to the capillary wall with sequestration and activation of passing neutrophils by the perturbed endothelial cells (Patel et al., 1994). In *Loxosceles intermedia*, the toxic effects appear to be associated with a 35-kDa protein that demonstrates a complement-dependent hemolytic activity and a dermonecrotic-inducing factor. ^{31}P-nuclear magnetic resonance assay of the four bands representing proteins, measuring 34 kDa in the venom, produced three proteins with sphingomyelinase D activity (Merchant et al., 1998). An endotoxemic-like shock, showing eosinophilic material in the proximal and distal tubules and tubular necrosis, was the most common histopathological finding, preceded in mice by prostration, acute cachexia, hypothermia, neurological changes, and hemoglobinuria (Tambourgi et al., 1998).

The bite of this spider produces about the same degree of pain as does the sting of an ant, but sometimes the patient may be unaware of the bite. In most cases, a local burning sensation, which may last for 30 to 60 minutes, develops around the injury. Pruritus over the area often occurs, and the area becomes red, with a small blanched area surrounding the reddened bite site. Skin temperature usually is elevated over the lesion area. With significant envenomations, the reddened area enlarges and becomes purplish during the subsequent 1 to 8 hours. It often becomes irregular in shape, and as time passes, hemorrhages may develop throughout the area. A small bleb or vesicle may form at the bite site, increase in size, and rupture with subsequent pustule formation. The red hemorrhagic area continues to enlarge, as does the pustule. The whole area may become swollen and painful, and lymphadenopathy is common. During the early stages, the lesion often takes on a bull's-eye appearance, with a central white vesicle surrounded by the reddened area and ringed by a whitish or bluish border. The central pustule ruptures, and necrosis to various depths can be visualized (Russell, 2001).

In serious bites, the lesion can measure 8 × 10 cm^2 with severe necrosis invading muscle tissue. On the face, large lesions resulting in extensive tissue destruction and requiring subsequent plastic surgery sometimes are seen after bites by *Loxosceles laeta* in South America. Systemic symptoms and signs include fever, malaise, stomach cramps, nausea and vomiting, jaundice, spleen enlargement, hemolysis, hematuria, and thrombocytopenia. Fatal cases, while rare, usually are preceded by intravascular hemolysis, hemolytic anemia, thrombocytopenia, hemoglobinuria, and renal failure (Russell, 2001).

***Steatoda* Species** These spiders are variously known as the false black widow, combfooted, cobweb, or cupboard spiders. The female of *Steatoda grossa* differs from *L. mactans* and *Latrodectus hesperus* in having a purplish-brown abdomen rather than a black one. It is less shiny, and its abdomen is more oval than round. It may have pale yellow or whitish markings on the dorsum of the abdomen, and no markings on the venter. The abdomen of some species is orange, brown, or chestnut in color, and often bears a light band across the anterior dorsum (Russell, 2001).

The venom of *Steatoda paykulliana* stimulates the release of transmitter substances similar to *Latrodectus* venom (Cavalieri et al., 1987). The venom is said to form ionic channels permeable for bivalent and monovalent cations, and the duration of time in the open state depends on the membrane potential (Sokolov et al., 1984). *S. paykulliana* venom induces strong motor unrest, clonic cramps, exhaustion, ataxia, and then paralysis in guinea pigs. Bites by *S. grossa* or *Steatoda fulva* in the United States have been followed by local pain, often severe; induration; pruritus; and the occasional breakdown of tissue at the bite site (Russell, 2001).

***Cheiracanthium* Species (Running Spiders)** The 160 species of this genus have an almost circumglobal distribution, although only four or five species have been implicated in bites on humans (Russell, 2001). *Cheiracanthium punctorium*, *Cheiracanthium inclusum*, *Cheiracanthium mildei*, *Cheiracanthium diversum*, and *Cheiracanthium japonicum* are often implicated in envenomations. The abdomen is convex and egg shaped and varies in color from yellow, green, or greenish-white to reddish-brown; the cephalothorax is usually slightly darker than the abdomen. The chelicerae are strong, and the legs are long, hairy, and delicate. The spider ranges in length from 7 to 16 mm. Like *Phidippus* jumping spiders but even more so, *Cheiracanthium* tends to be tenacious and sometimes must be removed from the bite area. For that reason, there is a high degree of identification following the bite of these spiders. The most toxic venom fraction is said to be a protein of 60 kDa, and the venom is high in norepinephrine and serotonin.

The patient usually describes the bite by *C. inclusum* as sharp and painful, with the pain increasing during the first 30 to 45 hours. The patient complains of dull pain over the injured part. A reddened wheal with a hyperemic border develops. Small petechiae may appear near the center of the wheal. Skin temperature over the lesion is often elevated, but body temperature is usually normal. Lymphadenitis and lymphadenopathy may develop. *C. japonicum* produces more severe manifestations, including severe local pain, nausea and vomiting, headache, chest discomfort, severe pruritus, and shock (Russell, 2001).

***Theraphosidae* Species (Tarantulas)** True tarantulas are members of the family Theraphosidae and there are around 800 species that are distributed worldwide, but especially in tropical or semitropical regions. Tarantulas are predators and they feed on various vertebrate and invertebrate preys that are captured after envenomation with venoms that act rapidly and irreversibly on the central and peripheral nervous systems. In humans, reported bites elicit mild to severe local pain, strong itching, and tenderness that may last for several hours. Edema, erythema, joint stiffness, swollen limbs, burning feelings, and cramps are common. In more

severe cases, strong cramps and muscular spasms lasting up to several hours may be observed. *Poecilotheria* and *Stromatopelma* spp. appear to be most toxic to humans, although no fatalities have been reported (Escoubas and Rash, 2004).

Tarantula venoms have been extensively studied, and modern purification often uses orthogonal HPLC separations that combine reversed-phase and ion-exchange chromatography. Most spider peptide toxins appear to have basic pI in the 9 to 11 range (Escoubas et al., 2000). At least 33 peptide toxins have been described from various tarantula venoms. These have a molecular weight of 3000 to 5700 Da, and targets include voltage-gated potassium, sodium, and calcium channels, tetrodotoxin-sensitive channels, and acid-sensing ion channels, which are sensitive to extracellular pH (reviewed by Escoubas and Rash, 2004). At least two distinct structural motifs have been characterized: the inhibitory cystine knot (ICK) with a consensus sequence of C_IX_{3-7}–$C_{II}X_{3-8}$–$C_{III}X_{0-7}$–$C_{IV}X_{1-4}$–C_VX_{4-13}–C_{VI} and disulfide bond pairing of C_I–C_{IV}, C_{II}–C_V, and C_{III}–C_{VI} (Craik et al., 2001), and the disulfide-directed β-hairpin (DDH) motif that comprises an antiparallel β-hairpin stabilized by two disulfide bridges (Wang et al., 2000). Tarantula toxins have been classified as long-loop ICK, short-loop ICK, or DDH toxins (Escoubas and Rash, 2004).

Theraphosid spiders contain several toxins that are being evaluated for development as antiarrhythmic or as antinociceptive drugs. In particular, *Grammostola* mechanotoxin 4 from *Grammostola spatulata* has considerable promise as an antiarrhythmic. Protoxin I and II from *Thrixopelma pruriens* have promise as analgesics because they inhibit the tetrodotoxin-resistant sodium channels (Middleton et al., 2002).

Future work on the toxins of tarantulas will encompass genomic and proteomic approaches. Expanding knowledge suggests trends in the association of primary and three-dimensional structures with pharmacologic activity. Continued improvement in isolation and purification technologies plus the combination of cDNA library screening with mass spectrometry will prove invaluable in characterization of toxin function and definition of toxin targets.

Ticks

Many of the approximately 900 species of ticks are associated with disease in humans and wild and domesticated animals (Rash and Hodgson, 2002; Barker and Murrell, 2004; Steen et al., 2006). The saliva of certain ticks of the families Ixodidae, Argasidae, and Nuttalliellidae causes tick paralysis. The tick bite involves insertion of cutting, tube-like mouthparts through the host's skin with anchoring so that the tick can feed for hours, days, or weeks. Saliva from the salivarium flows outward initially and the blood meal flows inward afterward. Ticks are known to transmit the organisms causing Lyme disease, Rocky Mountain spotted fever, babesiosis, leptospirosis, Q fever, ehrlichiosis, typhus, tick-borne encephalitis, and others. In fact, the import of immunosuppression by tick saliva in the transmission of flaviviruses has been discussed (Nuttall and Labuda, 2003).

Tick saliva contains a number of active constituents (Steen et al., 2006). For example, saliva from *Ixodes scapularis* contains apyrase (ATP diphosphohydrolase), which hydrolyzes ADP that is released at the bite site thereby inhibiting ADP-induced platelet aggregation (Mans et al., 1998); kininase (ACE-like protein or angiotensin-converting enzyme-like protein), which hydrolyzes circulating kinins and reduces the host inflammatory response (Francischetti et al., 2003); glutathione peroxidase (Das et al., 2001); serine protease inhibitors, which inhibit coagulation enzymes (Valenzuela, 2004); an anticomplement protein that inhibits an enzyme in the alternative pathway for complement (Valenzuela et al., 2000); an amine-binding protein that binds serotonin, histamine, and other biogenic amines (Sangamnetdej et al., 2002); and prostanoids (PGE_2 and $PGF_{2\alpha}$) (Inokuma et al., 1994). A discussion of toxins from other species may be found elsewhere (Cavassani et al., 2005; Steen et al., 2006).

Potentially 50 species of ticks are associated with clinical paralysis (Russell, 2001). As tick bites are often not felt, the first evidence of envenomation may not appear until several days later, when small macules 3 to 4 mm in diameter develop that are surrounded by erythema and swelling, often displaying a hyperemic halo. The patient often complains of difficulty with gait, followed by paresis and eventually locomotor paresis and paralysis. Problems in speech and respiration may ensue and lead to respiratory paralysis if the tick is not removed. The saliva of *Ixodes holocyclus* has yielded a peptide holocyclotoxin-1 that may cause paralysis (Masina and Broady, 1999). Peak paralytic activity was found between 60 and 100 kDa, and was a trimer of a neurotoxic protein subunit of 23 kDa (Crause et al., 1993). Symptoms resolve rapidly on removal of the tick (Russell, 2001).

CHILOPODA (CENTIPEDES)

These elongated, many-segmented brownish-yellow arthropods have a pair of walking legs on most segments, and they are fast moving, secretive, and nocturnal and are found worldwide. They feed on other arthropods and even small vertebrates and birds. The first pair of legs behind the head is modified into poison jaws. Centipedes range in length from 3 to almost 300 mm. In the United States, the prevalent biting genus is a *Scolopendra* species. The venom is concentrated within the intracellular granules, discharged into vacuoles of the cytoplasm of the secretory cells, and moved by exocytosis into the lumen of the gland; from thence ducts carry the venom to the jaws (Ménez et al., 1990).

Centipede venoms contain high-molecular-weight proteins, proteinases, esterases, 5-hydroxytryptamine, histamine, lipids, and polysaccharides (Mebs, 2002). Such venom contains a heat-labile cardiotoxic protein of 60 kDa that produces, in humans, changes associated with acetylcholine release (Gomes et al., 1983). The bite produces two tiny punctures, sharp pain, immediate bleeding, redness, and swelling often lasting for 24 hours. Localized tissue changes and necrosis have been reported, and severe envenomations may cause nausea and vomiting, changes in heart rate, vertigo, and headache. In the most severe cases, there can be mental disturbances (Bush et al., 2001; Russell, 2001; Mebs, 2002).

DIPLOPODA (MILLIPEDES)

Ranging in length from 20 to 300 mm, these arthropods are cylindrical, worm-like creatures, mahogany to dark brown or black in color, and bearing two pairs of jointed legs per segment. In Australia and New Guinea particularly, the repellent secretions expelled from the sides of their bodies contain a toxin of benzoquinone derivatives plus a variety of complex substances such as iodine and hydrocyanic acid, which the animal makes use of to produce hydrogen cyanide (Kuwahara et al., 2002). Some species can spray these defensive secretions, and eye injuries are not uncommon. The lesions produced by millipedes consist of a burning or prickling sensation and development of a yellowish or brown-purple lesion; subsequently, a blister containing serosanguinous fluid forms, which may rupture. Eye contact can cause acute conjunctivitis, periorbital edema, keratosis, and much pain; such an injury must be treated immediately (Russell, 2001).

INSECTA

Heteroptera (True Bugs)

The clinically most important of the true bugs are the Reduviidae (the reduviids): the kissing bug, assassin bug, wheel bug, or cone-nose bug of the genus *Triatoma* (Russell, 2001). Generally, they are parasites of rodents and common in the nests of wood rats or in wood piles. These are elongated bugs with freely movable, cone-shaped heads, and straight beaks. The most commonly involved species appear to be *Triatoma protracta*, *Triatoma rubida*, *Triatoma magista*, *Reduvius personatus*, and *Arilus cristatus*. The average length of these bugs is 19 mm. The venom of these bugs appears to have apyrase activity and to lack 5-nucleotidase, inorganic pyrophosphatase, phosphatase, and adenylate kinase activities, but it is fairly rich in protease properties. It inhibits collagen-induced platelet aggregation. Three peptides isolated from the saliva of predatory reduviids are 34 to 36 amino acid residues in size, are calcium channel blockers similar to ω-conotoxins, and belong to the four-loop disulfide bridge scaffold structural class (Corzo et al., 2001). The bites of *Triatoma* species are painful and give rise to erythema, pruritus, increased temperature in the bitten part, localized swelling, and—in those allergic to the saliva—systemic reactions such as nausea and vomiting and angioedema. With some bites the wound area will slough, leaving a depression.

The water-dwelling true bugs are of at least three families, Naucoridae, Belostomatidae, and Notonectidae, which are capable of biting and envenomating humans (Russell, 2001). They are found in lakes, ponds, marshes, quiet fresh water, and swimming pools. *Lethocerus americanus*, a Belostomatidae, ranges in length from 12 to 70 mm, but some water bugs may reach 150 mm. The dorsal side is usually tan or brown, but it may be brightly colored, while the ventral side is brown. They are very strong insects and can immobilize snails, tadpoles, salamanders, and even small fish and water snakes. Water bug saliva is said to contain digestive enzymes, neurotoxic components, and hemolytic fractions. ApoLp-III isolated from the hemolymph of *Lathocerus medius* is about 19 kDa and has an amino acid composition high in methionine. If molested, water bugs will bite, and their bites give rise to immediate pain, some localized swelling, and possibly induration and formation of a small papule.

Hymenoptera (Ants, Bees, Wasps, and Hornets)

Formicidae (Ants) Most ant species sink their powerful mandibles into the flesh, providing leverage, and then drive their stings into the victim. Most ants have stings, but those that lack them can spray a defensive secretion from the tip of the gaster, which is often placed in the wound of the bite. Ants of the different species vary considerably in length, ranging from less than 1.5 to over 35 mm. Clinically important stinging ants are the harvesting ants (*Pagonomyrmex*), fire ants (*Solenopsis*), and little fire ants (*Ochetomyrmex*). The harvester ants are large red, dark brown, or black ranging in size from 6 to 10 mm and having fringes of long hairs on the posterior of their heads (Russell, 2001).

The venoms of the ants vary considerably. The venoms of the Ponerinae, Ecitoninae, and *Pseudomyrmex* are proteinaceous in character. The Myrmicinae venoms are a mixture of amines, enzymes, and proteinaceous materials, histamine, hyaluronidase, phospholipase A, and hemolysins, which hemolyze erythrocytes and mast cells. Formicinae ant venom contains about 60% formic acid. Fire ant venoms are poor in polypeptides and proteins, but are rich in alkaloids such as solenopsin (Russell, 2001; Mebs, 2002). The sting of the fire ant gives rise to a painful burning sensation, after which a wheal and localized erythema develop, leading in a few hours to a clear vesicle. Within 12 to 24 hours, the fluid becomes purulent and the lesion turns into a pustule. It may break down or become a crust or fibrotic nodule. In multiple stings, there may be nausea, vomiting, vertigo, increased perspiration, respiratory difficulties, cyanosis, coma, and even death. Cross-exposure to the venom of other species of ants is possible. Allergic reactions and fatal anaphylactic shock are seen in sensitized victims (Hoffman, 2006). Treatment of ant stings is dependent on the number of bites, on whether an allergic reaction is involved, and on whether there are possible complications.

Apidae (Bees) This family includes the bumble bees, honeybees, carpenter bees, and yellow jackets. The commonest stinging bees are *Apis mellifera* and the Africanized bee, *Apis mellifera adansonii*, and the incidence of Hymenoptera poisonings is increasing. The venom of the Africanized bee is not remarkably different from that of the European bee, *A. m. mellifer* (Fig. 26-24). The former bee is smaller and gives less venom, but its aggressiveness is such that attacks of 50 to hundreds of bees are not unusual (Russell, 2001).

The venom contains biologically active peptides, such as melittin, apamine, mast cell–degranulating peptide, and others, as well as phospholipases A_2 and B, hyaluronidase, histamine, dopamine, monosaccharides, and lipids (Mebs, 2002). Melittin, which is secreted as the 70 amino acid prepromelittin, consists of 26 amino acids with no cysteines that have natural detergent-like properties and causes erythrocyte lysis. Melittin also forms tetramers with pores, thereby facilitating ion transport through membranes. In particular, melittin tetramers cause a breakdown of the resting potential and rapid depolarization of nociceptors, which induces pain (Demsey, 1990; Bechinger, 1997). The compound apamine contains 18 amino acids cross-linked by two disulfide bridges. Apamine is a blocker of calcium-dependent potassium channels and is thought to be the "lethal factor" (Habermann, 1984). In addition to apamine, mast cell–degranulating peptide is also a basic peptide containing 22 amino acids with two disulfide bonds. Besides stimulating release of histamine, this peptide specifically inhibits voltage-dependent potassium channels (Dreyer, 1990; Baku, 1999).

Bee stings typically produce immediate, sharp or burning pain, slight local erythema, and edema followed by itching. The edema may vary depending on location of the sting. It is said that 50 stings can be serious and lead to respiratory dysfunction, intravascular hemolysis, hypertension, myocardial damage, hepatic changes, shock, and renal failure. With 100 or more stings, death can occur. In patients who are allergic to bee stings, immediate allergic reaction with the risk of anaphylactic shock requires urgent medical treatment (Mebs, 2002; Hoffman, 2006).

Figure 26-24. *Apis mellifera mellifera (honey bee).*

Vespidae (Wasps) This family includes wasps and hornets. These venoms contain a high content of peptides, which include mastoparan in wasps and hornets and crabolin from hornet venom. These peptides release histamine from mast cells and consist of 13 to 17 amino acids with no disulfide bridges. Other peptides named wasp kinins cause immediate pain, vasodilation, and increased vascular permeability leading to edema. These venoms also contain phospholipases and hyaluronidases, which contribute to the breakdown of membranes and connective tissue to facilitate diffusion of the venom. These proteins also contribute to the allergenicity of the venoms (Mebs, 2002; Hoffman, 2006).

Lepidoptera (Caterpillars, Moths, and Butterflies)

The urticating hairs, or setae, of caterpillars are effective defensive weapons that protect some species from predators. The setae are attached to unicellular poison glands at the base of each hair. Both the larvae and the adults are capable of stinging, either by direct contact with the setae or indirectly when the creature becomes irritated. It appears that contraction of the caterpillar's abdominal muscles is sufficient to release the barbs from their sockets, allowing them to become airborne. The toxic material found in the venom glands contains aristolochic acids, cardenolides, kallikrein, and histamine among other substances. Fibrinolytic activity has been found in 16 and 18 kDa components (isoelectric point of 8.5); coagulation defects such as prolonged prothrombin and partial thromboplastin times have been detected, and decreases in fibrinogen and plasminogen have been noted. It is thought that the hemorrhagic syndrome cannot be classified as being either totally fibrinolytic or a syndrome such as disseminated intravascular coagulopathy. The spicules of *Thaumetopoea pityocampa* contain a 28-kDa toxin called thaumetopoein, which is a strong dermal irritant and highly allergenic peptide (Kawamoto and Kumada, 1984; Russell, 2001; Mebs, 2002).

In some parts of the world, the stings of several species of Lepidoptera give rise to a bleeding diathesis, often severe and sometimes fatal. Envenomation by members of the family Saturniidae, the buck moths, the grapeleaf skeletonizer (family Zygaenidae), the puss moth (family Megalopygidae), and the brown-tailed moth (*Euproctis* species) generally gives rise to little more than immediate localized itching and pain, usually described as burning, followed in some cases by urticaria, edema, and occasionally fever. The hemolymph and spicules contain highly active clotting enzymes, a protease with fibrinolytic activity and an enzyme that activates prothrombin. In the more severe cases—often due to *Megalopyge*, Dioptidae, *Automeris*, and *Hemileuca* spp.—there is localized pain as well as papules (sometimes hemorrhagic) and hematomas; on occasions there may also be headache, nausea, vomiting, hematuria, lymphadenitis, and lymphadenopathy. Contacts with larvae of the saturnid moths in South America (*Lonomia acheolus* and *Lonomia oblique*, Fig. 26-25) can cause severe coagulopathy, due to inhibition of clotting factor XIII by a venom component called lonomine V. Severe envenomation can cause cerebral hemorrhage and death (Guerrero et al., 1999).

Figure 26-25. *Lonomia oblique (giant silkworm moth).*

Figure 26-26. *Conus geographus (geography cone).*

MOLLUSCA (CONE SNAILS)

Human interest in this group of mollusks has been due to the beautiful patterns on their shells. Cone snails were known to Roman scholars and natural history collectors, as the shells were often made into jewelry. The first record of fatality from cone snail sting may be found in the book of Rumphius from 1705. The genus *Conus* is a group of approximately 500 species of carnivorous predators found in marine habitats that use venom as a weapon for prey capture (Fig. 26-26). Cone snails have a venom duct for synthesis and storage of venom and hollow harpoon-like teeth for injection of the venom (Rockel et al., 1995).

Cone snails may be divided into three groups depending on preferred prey. The largest group contains worm-hunting species that feed on polychaetes (segmented marine worms in the phylum Annelida). The second group is molluscivorous and hunts other gastropods. The final group is piscivorous and has venoms that rapidly immobilize fish (Olivera, 1997, 2002; Mebs and Kauferstein, 2005).

There are probably over 100 different venom components per species (Terlau and Olivera, 2004). Components have become known as conotoxins, which may be rich in disulfide bonds, and conopeptides. Molecular targets include G protein–coupled receptors, neurotransmitter transporters, and ligand- or voltage-gated ion channels. Some components have enzymatic activity.

Fig. 26-27 provides an overview of peptidic *Conus* venom components, indicating gene superfamilies, disulfide bond characteristics, and general targets (McIntosh et al., 1999; Olivera, 2002). The two major divisions of *Conus* toxins are the disulfide-rich conotoxins and the peptides that lack multiple disulfide cross-links. The arrangement of cysteines in the primary sequence is restricted to only a few patterns, which may be diagnostic of the gene superfamily. Most disulfide-rich conotoxins are small and consist of 12 to 30 amino acids. These toxins contain an unusually diverse complement of posttranslationally modified amino acids, including hydroxyproline, *O*-glycosylated serine or threonine, γ-carboxyl-glutamate, sulfated tyrosine, and D-amino acids (Craig et al., 1999). Apparently, the posttranslational modification enzyme γ-carboxylase is present in their venom ducts (Bandyopadhyay et al., 2002). The enzyme has a recognition signal in the pro region of the precursor that instructs the enzyme to modify specific amino acid residues in the mature toxin region. Thus, the pro region of conopeptide precursors provides potential anchor-binding sites for posttranslational modification enzymes (Hooper et al., 2000).

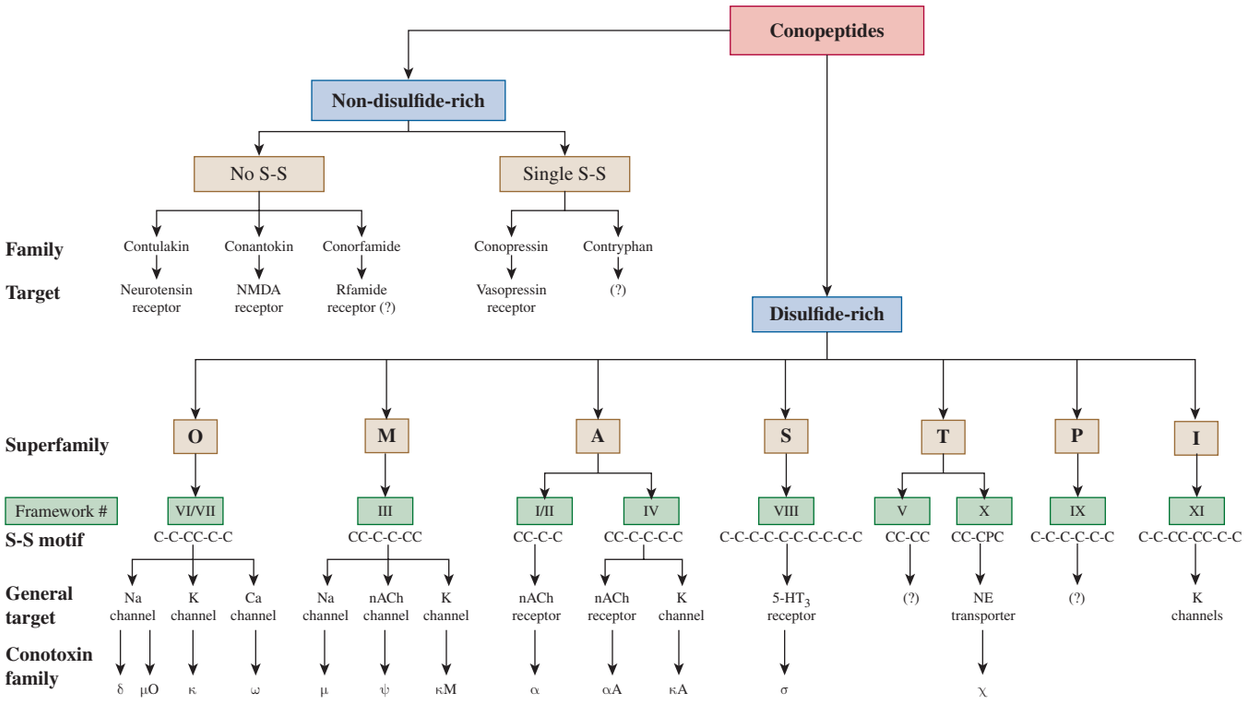

Figure 26-27. *Organizational diagram for Conus peptides, indicating gene superfamilies, disulfide patterns, and known pharmacologic targets.* Only the superfamilies of the disulfide-rich peptides are shown. (Used with permission from Terlau and Olivera [2004].)

Cone snails could be called sophisticated practitioners of combination drug therapy. After injection, multiple conopeptides act synergistically to affect the targeted prey. The term "toxin cabal" has been applied to this coordinated action of the conopeptide mixture. The fish-hunting species *Conus purpurascens* apparently has two distinct cabals whose effects differ in time and space. The "lightning-strike cabal" causes immediate immobilization of the injected prey because various venom components inhibit voltage-gated sodium channel inactivation and block potassium channels, resulting in massive depolarization of axons near the injection site and a tetanic state. The second physiologic cabal, the "motor cabal," acts more slowly as conotoxins must distribute throughout the body of the prey. The overall result is total inhibition of neuromuscular transmission. Various conopeptides inhibit presynaptic calcium channels that control neurotransmitter release, the postsynaptic neuromuscular nicotinic receptors, and the sodium channels involved in the muscle action potential (Terlau et al., 1996).

Conopeptides can affect many ion channels (Olivera et al., 1994; Terlau and Olivera, 2004). For example, μ-conotoxins are peptides with 22 to 25 amino acids and six cysteine residues that block sodium currents by acting at a site that overlaps the tetrodotoxin-sensitive site of sodium channels. The main effect of the unusually hydrophobic δ-conotoxins, which are peptides with an inhibitory cysteine knot motif pattern of disulfide bridges, is the inhibition of the fast inactivation of sodium currents, which affects the shape and duration of the action potential. This can lead to a massive electrical hyperexcitation of the complete organism. The κ-, κA-, and κM-conotoxins target potassium channels. The ω-conotoxins are peptides that target calcium channels, and these toxins have been widely used in neuroscience research. A characteristic feature of the ω-conotoxins is their high content of basic amino acids. One peptide, ω-conotoxin MVIIA from *Conus magus*, has been developed as ziconotide (Prialt®) for the treatment of intractable pain (Olivera, 2000). Other conotoxins are being studied as potential antinociceptive agents.

Conopeptides also target ligand-gated ion channels that mediate fast synaptic transmission (Kandel et al., 2013). One major group of ligand-gated ion channels is activated by acetylcholine, serotonin, γ-aminobutyric acid, or glycine. These proteins differ in ligand specificity and selectivity for the permeant ion. The second family activates the glutamate receptors, *N*-methyl-D-aspartate and kainate/AMPA receptors. A third group activates synaptic transmission at certain synapses via the ATP receptors. It is evident from Fig. 26-27 that there are conopeptides that target these three different families of ligand-gated ion channels. It is clear that our understanding of the structure and function of conopeptides is rapidly expanding and additional information regarding these toxins is available in some excellent reviews (Terlau and Olivera, 2004; Lewis et al., 2012; Kumar et al., 2015; Lebbe and Tytgat, 2016; Mir et al., 2016).

REPTILES

Lizards

The Gila monster (*Heloderma suspectum*; Fig. 26-28) and the beaded lizards (*Heloderma horridum*) are divided into five subspecies. These large, corpulent, relatively slow moving, and largely nocturnal reptiles have few enemies other than humans. They are far less dangerous than is generally believed. Their venom is transferred from venom glands in the lower jaw through ducts that discharge their contents near the base of the larger teeth of the lower jaw. The venom is then drawn up along grooves in the teeth by capillary action (Brown and Carmony, 1991). The venom of this

Figure 26-28. *Heloderma suspectum (Gila monster).*

lizard has serotonin, amine oxidase, phospholipase A, a bradykinin-releasing substance, helodermin, gilatoxin, and low-proteolytic as well as high-hyaluronidase activities, but lacks phosphomonoesterase and phosphodiesterase, acetylcholinesterase, nucleotidase, ATPase, deoxyribonuclease, ribonuclease, amino acid oxidase, and fibrinogenocoagulase activities. The clinical presentation of a helodermatid bite can include algogenia, edema, hypotension, nausea, vomiting, weakness, and diaphoresis. No antivenin is commercially available. Treatment is supportive (Strimple et al., 1997).

The venom has been shown to contain a 25-kDa protein, helothermine, containing 223 amino acids and four pairs of disulfide bonds, which appears to inhibit Ca^{2+} flux from the sarcoplasmic reticulum (Morrissette et al., 1995; Nobile et al., 1996). A fraction causing hemorrhage in internal organs and the eye, a glycoprotein of 210 amino acid residues with plasma kallikrein-like properties, has also been described (Datta and Tu, 1997). A 35 amino acid residue, helodermin produces hypotension that is partially attributed to activation of glibenclamide-sensitive K^+ channels (Horikawa et al., 1998). In fact, helospectin I and II and helodermin are nonamidated peptides similar to vasoactive intestinal peptide that are isolated from the salivary gland venom of the lizards *H. suspectum* and *H. horridum*, which have strong and potent vasodilator effects (Uddman et al., 1999).

Snakes

General Information and Classification Snakes have a three-chambered heart and rely almost exclusively on an enlarged right lung (which spans approximately half of the body length) for respiration. Of the approximately 2700 known species of snakes, about 20% are considered to be venomous (Mebs, 2002; Mackessy, 2010). Venomous snakes primarily belong to the following families: Viperidae (vipers), Elapidae, Atractaspididae, and Colubridae. The vipers are further divided into subfamilies, an example of which is the Crotalinae, or pit vipers, which possess a pit between the eyes and nostrils that serves as a heat sensor to detect warm-blooded animals. Some of the subfamilies are regarded as separate families altogether depending on the classification scheme. Overall, the Colubridae are considered the largest venomous family, and are composed of nearly 60% of all snakes. The Atractaspididae family is known for burrowing into the ground and possessing the ability to expose their fangs without opening their mouth.

Another characteristic that is often used for self-defense or hunting prey is mimicry (O'Shea, 2011). Scale pattern and coloration provide distinct boundaries in the wild and often carry unspoken warnings based on a reputation for snake venom toxicity for a given species. If a relatively harmless species is able to mimic the physical characteristics, especially color patterns, of a well-known highly toxic counterpart, then other potential predators may recognize both snakes as toxic and not pursue either.

Fang anatomy and dentition are good indicators of such factors as snake habitat and feeding habits (O'Shea, 2011). In general, the anatomical structure of fangs makes it nearly impossible for snakes to chew their prey. The distinct curvature of the fangs is engineered not only for puncturing skin and delivering venom, but also for swallowing whole prey as well. The teeth and jaw structure are relatively mobile and effectively facilitate the positioning of whole prey for swallowing. The skull and jaw possess an extremely high degree of responsiveness to stimuli. The jaw does not actually dislocate; however, it is able to rapidly reposition itself to capture, contain, and swallow prey. In addition to the capabilities of the teeth, fangs, and jaws, the classic fork-shaped tongue is another tool for identifying prey (O'Shea, 2011).

The Viperidae fang structure is regarded as the most developed and efficient means of venom, or toxin, delivery to prey. The venom gland is positioned at the base of a long (about 30 mm) hollow retractable fang (Mebs, 2002; Weinstein et al., 2010). Muscle pressure on the gland determines the amount of venom released. Another highly developed venom delivery apparatus is characteristic of the spitting cobras, aptly named for their ability to project venom via glands that protrude from the base of the fang opening (Mebs, 2002). Venom is carried toward the prey, or target, via forceful exhalation that is accompanied by a hissing sound. Toxin delivery via venom exposure is the primary mechanism by which snakes immobilize and kill their prey. Toxin composition and toxicity differ depending on the species as most venoms consist of complex mixtures of components that affect variable organ systems and interact with one another increasing the overall potency.

It is estimated that there are over 2.5 million snakebites annually, and that over 100,000 victims will die (White, 2005; Mackessy, 2010). Information resources available to physicians on management of snakebite victims may be found at the Clinical Toxinology Resources Web site—www.toxinology.com—or other appropriate references (Dart, 2004; Tintinalli et al., 2016; Corbett and Clark, 2017).

Snake Venoms These venoms are complex mixtures: proteins and peptides, consisting of both enzymatic and nonenzymatic compounds, make up over 90% of the dry weight of the venom (Phui Yee et al., 2004). Snake venoms also contain inorganic cations such as sodium, calcium, potassium, magnesium, and small amounts of zinc, iron, cobalt, manganese, and nickel. The metals in snake venoms are likely catalysts for metal-based enzymatic reactions. For example, in the case of some elapid venoms, zinc ions appear to be necessary for anticholinesterase activity, and calcium may play a role in the activation of phospholipase A and the direct lytic factor. Some proteases appear to be metalloproteins. Some snake venoms also contain carbohydrates (glycoproteins), lipids, and biogenic amines, such as histamine, serotonin, and neurotransmitters (catecholamines and acetylcholine) in addition to positively charged metal ions (Russell, 2001; Mebs, 2002; Ménez, 2003; Ramos and Selistre-de-Araujo, 2006; McCleary and Kini, 2013; Nery et al., 2016; Boldrini-França et al., 2017; Silva et al., 2017). The complexity of snake venom components is illustrated nicely in Fig. 26-29 (Ramos and Selistre-de-Araujo, 2006).

Actions of snake venoms can be said to be broad ranging in several areas (O'Shea, 2011). A simplistic approach would group toxin components as neurotoxins, coagulants, hemorrhagins,

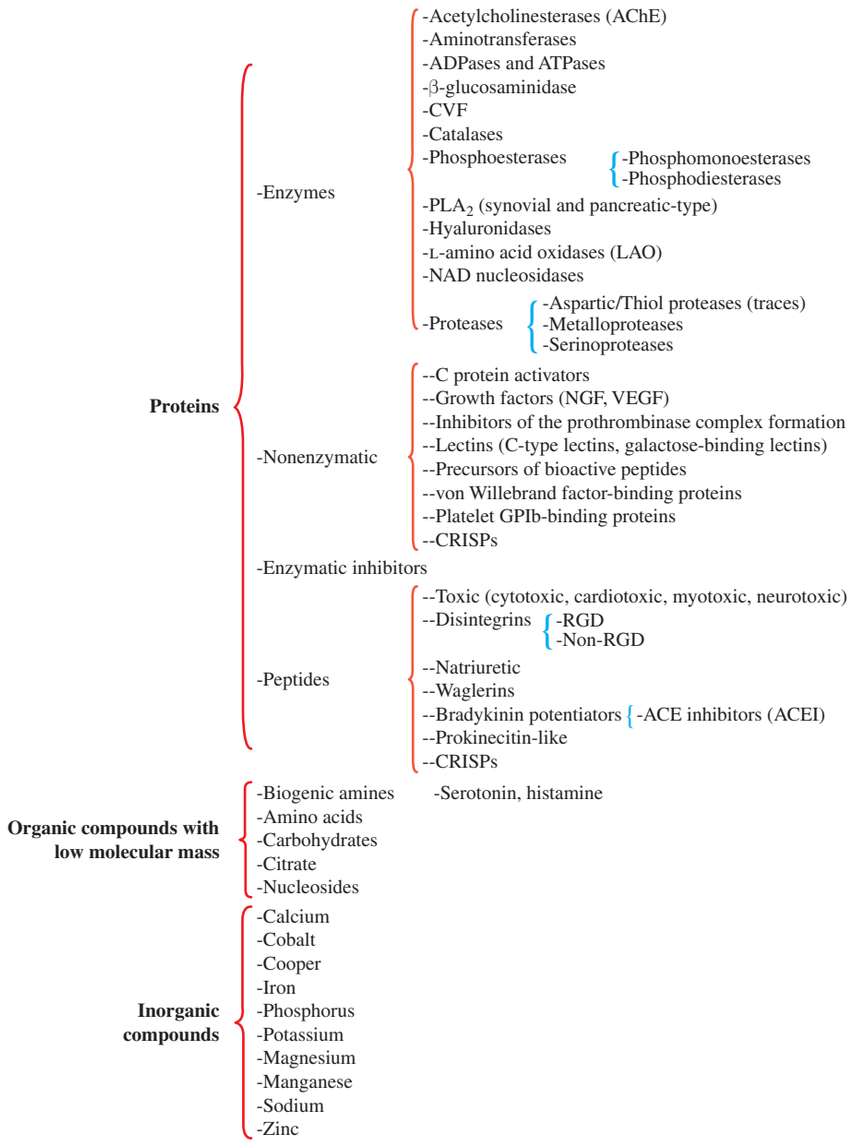

Figure 26-29. *Components of snake venoms.* ACE, angiotensin-converting enzyme; CRISP, cysteine-rich secretory protein; CVF, cobra venom factor–like proteins; LAO, L-amino acid oxidase; PLA_2, phospholipase A2; RGD, arginine–glycine–aspartate. (Used with permission from Ramos and Selistre-de-Araujo [2006].)

hemolytics, myotoxins, cytotoxins, and nephrotoxins. Neurotoxins produce neuromuscular paralysis ranging from dizziness to ptosis; to ophthalmoplegia, flaccid facial muscle paralysis, and inability to swallow; to paralysis of larger muscle groups; and finally to paralysis of respiratory muscles and death by asphyxiation. Coagulants may have initial procoagulant action that uses up clotting factors leading to bleeding. Coagulants may directly inhibit normal clotting at several places in the clotting cascade or via inhibition of platelet aggregation. In addition, some venom components may damage the endothelial lining of blood vessels leading to hemorrhage. Bite victims may show bleeding from nose or gums, from the bite site, and in saliva, urine, and stools. Myotoxins can directly affect muscle contraction leading to paralysis or cause rhabdomyolysis or the breakdown of skeletal muscle. Myoglobinuria, or a dark brown urine, and hyperkalemia may be noted. Cytotoxic agents have proteolytic or necrotic properties leading to the breakdown of tissue. Typical signs include massive swelling, pain, discoloration, blistering, bruising, and wound weeping. Sarafotoxins, which are found only in burrowing asps of Afro-Arabia, cause coronary artery constriction that can lead to reduced coronary blood flow, angina, and myocardial infarction. Finally, nephrotoxins can cause direct damage to kidney structures leading to bleeding, damage to several parts of the nephron, tissue oxygen deprivation, and renal failure.

Enzymes At least 26 different enzymes have been isolated from snake venoms, which can be a sequence of 150 to 1500 amino acids (Ménez, 2003). No single snake venom contains all 26 enzymes and some important snake venom enzymes are shown in Fig. 26-29.

Proteolytic enzymes that catalyze the breakdown of tissue proteins and peptides include peptide hydrolases, proteases, endopeptidases, peptidases, and proteinases. Several proteolytic enzymes may be in a single venom. The proteolytic enzymes have molecular

Figure 26-30. *Crotalus atrox (western diamondback rattlesnake).*

Figure 26-31. *Oxyuranus scutellatus (Australian costal taipan).*

weights between 20,000 and 95,000. Some are inactivated by ethylenediaminetetraacetic acid (EDTA) and certain reducing agents. Metals appear to be intrinsically involved in the activity of certain venom proteases and phospholipases. The crotalid venoms examined so far appear to be rich in proteolytic enzyme activity. Viperid venoms have lesser amounts, whereas elapid and sea snake venoms have minimal, if any, proteolytic activity. Venoms that are rich in proteinase activity are associated with marked tissue destruction (Russell, 2001; Serrano, 2013).

Collagenase is a specific kind of proteinase that digests collagen. This activity has been demonstrated in the venoms of a number of species of crotalids and viperids. The venom of *Crotalus atrox* (Fig. 26-30) digests mesenteric collagen fibers but no other proteins. EDTA inhibits the collagenolytic effect, but not the arginine esterase effect.

Hyaluronidase cleaves internal glycoside bonds in certain acid mucopolysaccharides resulting in a decrease in the viscosity of connective tissues. The breakdown in the hyaluronic barrier allows other fractions of venom to penetrate the tissues, causing hyaluronidase to be called "spreading factor." The degree to which hyaluronidase contributes to the extent of edema produced by the whole venom is not known (Kemparaju et al., 2010).

Phospholipase A_2 (PLA_2) enzymes are widely distributed throughout the tissues of animals, plants, and bacteria, and have been well studied in snake venoms (Ohno et al., 2003; Soares and Giglio, 2003; Doley et al., 2010). Mammalian PLA_2 enzymes are involved in fertilization and cell proliferation, and have been implicated in respiratory ailments such as asthma, as well as skin conditions such as psoriasis (Kini, 2003; Soares and Giglio, 2003). PLA_2 catalyzes the Ca^{2+}-dependent hydrolysis of the 2-acyl ester bond, producing free fatty acids and lysophospholipids. However, liberation of pharmacologically active products and effects independent of enzymatic action may contribute to their overall action. In general, mammalian PLA_2s are considered to be nontoxic and do not elicit the same pharmacologic effects as similar enzymes in snake venoms.

As of 2003, over 280 PLA_2 enzymes had been classified, sharing at least 40% identity in their amino acid sequences (Kini, 2003; Soares and Giglio, 2003). Many PLA_2s have been sequenced. They have approximately 120 amino acids and 14 cysteine residues forming seven disulfide bonds. Surface residue recognition and covalent/noncovalent bonds stabilize complexes for receptor binding. Specific amino acid residues play an important role in the diversification and overall function of the PLA_2s. Of note, histidine, lysine, cysteine, and methionine have been well studied for their contribution to enzyme structure and function (Soares and Giglio, 2003). For example, alkylation of His48 diminishes the hydrolytic capabilities of certain toxins. Alkylation of amino acids has proven to effectively mitigate PLA_2 toxicity and has been proposed as an antivenom treatment (Soares and Giglio, 2003). PLA_2s interact with other toxins in the venom as well, often resulting in synergistic reactions. Similarly, snake venom PLA_2 enzymes can be separated into three major groupings depending on their pharmacologic activities: low-toxicity enzymes ($LD_{50} > 1$ mg/kg), high-toxicity enzymes ($1 \text{ mg/kg} > LD_{50} > 0.1$ mg/kg), and presynaptically acting toxins ($LD_{50} < 0.1$ mg/kg) (Rosenberg, 1990). Although the sequences of these enzymes are homologous and their enzymatically active sites are identical, they differ widely in their pharmacologic properties. For example, taipoxin, a PLA_2 enzyme from the venom of the Australian elapid *Oxyuranus scutellatus* (Fig. 26-31), has an intravenous LD_{50} in mice of 2 μg/kg, whereas the neutral PLA_2 from *Naja nigricollis* (Fig. 26-32) has an LD_{50} of 10,200 μg/kg, even though *N. nigricollis* PLA_2 is enzymatically more active (Russell, 2001).

Arginine ester hydrolase is one of a number of noncholinesterases found in snake venoms. The substrate specificities are directed to the hydrolysis of the ester or peptide linkage, to which an arginine residue contributes the carboxyl group. This activity

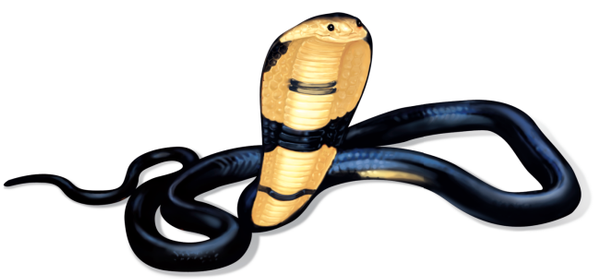

Figure 26-32. *Naja nigricollis (black necked spitting cobra).*

Figure 26-33. *Agkistrodon contortrix contortrix (copperhead).*

Table 26-13

Representative Properties of Some α-Chain and β-Chain Fibrin(ogen)ases

PROPERTIES	α-CHAIN FIBRINOGENASE	β-CHAIN FIBRINOGENASE
Common name	Fibrolase	β-Fibrinogenase
Class of enzyme	Metalloproteinase	Serine protease
Chain length	203 amino acids	232 amino acids
Molecular weight	~22.7 kDa	~26 kDa
pI	6.8	~3
Carbohydrate content	None	>30%
pH optimum	7.1–7.4	8.5–9.5

SOURCE: Data from Swenson and Markland (2005).

is found in many crotalid and viperid venoms and some sea snake venoms, but is lacking in elapid venoms with the possible exception of *Ophiophagus hannah*. Some crotalid venoms contain at least three chromatographically separable arginine ester hydrolases. The bradykinin-releasing and perhaps bradykinin-clotting activities of some crotalid venoms may be related to esterase activity.

Two distinct classes of fibrin(ogen)olytic enzymes, the metalloproteinases and the serine proteinases, have been isolated from venom of Viperidae, Elapidae, and Crotalidae snake families (Swenson and Markland, 2005). These two classes of proteinases differ in mechanism of action and their target in fibrin(ogen), but ultimately they break down fibrin-rich clots and help to prevent further clot formation. The properties of fibrolase, an α-chain fibrinolytic metalloproteinase from *Agkistrodon contortrix contortrix* (Fig. 26-33) venom, and β-fibrinogenase, a β-chain fibrinogenase from *Vipera lebetina*, are provided in Table 26-13. Properties of some additionally characterized fibrin(ogen)ases are listed in Table 26-14. It is apparent that there are major differences in the properties of these enzymes from different snakes even though they have similar catalytic properties. An exciting development from the research on these enzymes is that one specific recombinant fibrinolytic enzyme derived from fibrolase called alfimeprase is progressing through clinical trials for the treatment of peripheral arterial occlusions.

The snake venom metalloproteinases (SVMPs) are enzymes that disrupt the hemostatic system and they are characterized by their domain structure into four primary classes, PI to PIV (Fox and Serrano, 2010; Markland and Swenson, 2013). SVMPs are synthesized in vivo as multimodular proteins that comprise a signal peptide, a prodomain, and a metalloprotease domain (SVMP PI). SVMP potency tends to increase by class and those within the PIV class are larger and comprise additional disulfide bonds (Calvette et al., 2003, 2005). Class PII proteins exhibit a C-terminal disintegrin domain. The PII metalloproteases block the function of integrin receptors, a function that could alleviate a variety of pathological conditions such as inflammation, tumor angiogenesis and metastasis, and thrombosis. The integrin-blocking specificity of this class of metalloproteins is highly dependent on the conformation of the inhibitory loop, and thus the placement and bonding of cysteine residues. More specifically, within the inhibitory loops, RGD-containing disintegrins are specific to the class PII metalloproteases

Table 26-14

Biological and Physiological Properties of Specific Fibrin(ogen)ases

PROPERTIES	α-CHAIN FIBRINOGENASE	α-CHAIN FIBRINOGENASE	α-CHAIN FIBRINOGENASE	β-CHAIN FIBRINOGENASE
Genus species	*Bothops neuwiedi*	*Agkistrodon halys*	*Lachesis stenophrys*	*Agkistrodon blomhoffi brevicadus*
Common name	Neuwiedase	Brevilysin L6	LSF	Brevinase
Chain length	198 amino acids	203 amino acids	>200 amino acids	233 amino acids
Molecular weight	22.5 kDa	22.7 kDa	24 kDa	25.7 kDa
pI	5.9	4.8	ND	5.5
Carbohydrate content	<1%	None	Glycosylated	ND
pH optimum	7.4–8.0	8.5–9.5	ND	5.5–8.5

Abbreviation: ND, not determined.
SOURCE: Data from Swenson and Markland (2005).

(Calvette et al., 2003, 2005). Class PIII SVMPs exhibit the disintegrin-like domain and the C-type lectin-like domain is present in PIV SVMPs. The metalloproteinase domain or catalytic domain is composed of about 215 amino acids and has metal-dependent endopeptidase activity (Calvette et al., 2005). SVMPs degrade proteins such as laminin, fibronectin, type IV collagen, and proteoglycans from the endothelial basal membrane; degrade fibrinogen and von Willebrand factor enhancing the hemorrhagic action; and inhibit platelet aggregation and stimulate release of cytokines (Ramos and Selistre-de-Araujo, 2006).

The proteolytic action of thrombin and thrombin-like snake venom enzymes is shown in Table 26-15. This table compares ancrod (from *Calloselasma rhodostoma*), batroxobin (from *Bothrops moojeni*), crotalase (from *Crotalus adamanteus*), gabonase (from *Bitis gabonica*), and the enzyme from *A. contortrix*. Table 26-16 shows the molecular size of some thrombin-like enzymes. A recent contribution on snake toxins, using mass spectrometric immunoassay and bioactive probe techniques, has been published by Ramirez et al. (1999a, 1999b). Considerable study has been given to the hemostatic properties of venoms (Markland, 1998; Phillips et al., 2010). The hemostatically active components are summarized in Table 26-17.

Phosphomonoesterase (phosphatase) is widely distributed in the venoms of all families of snakes except the colubrids (Dhananjaya et al., 2010). It has the properties of an orthophosphoric monoester phosphohydrolase. There are two nonspecific phosphomonoesterases, and they have optimal pH at 5.0 and 8.5. Many types of venom contain both acid and alkaline phosphatases, whereas others contain one or the other.

Phosphodiesterase has been found in the venoms of all families of poisonous snakes. The enzyme is an orthophosphoric diester phosphohydrolase that releases 5-mononucleotide from the polynucleotide chain, and thus acts as an exonucleotidase, attacking DNA and RNA. More recently, it has been found that it also attacks derivatives of arabinose.

Table 26-16

Comparison of Snake Venom Thrombin-Like Enzymes

VENOM ENZYME	MOLECULAR WEIGHT	CARBOHYDRATE CONTENT (%)	ACTIVE SITE SERINE
Agkistrodon contortrix contortrix	100,000	ND	+
Bitis gabonica	32,500	ND	ND
Bothrops marajoensis	31,400	High	+
Bothrops moojeni	36,000	5.8	+
Calloselasma rhodostoma	59,000	36.0	+
Crotalus adamantus	32,700	8.3	+
Crotalus horridus horridus	19,400	Very low	ND
Deinagkistrodon acutus	33,500	13.0	+
Trimeresurus gramineus	27,000	25.0	+
Trimeresurus okinavensis	34,000	6.0	+

Abbreviation: ND, not determined.
SOURCE: Data from Russell (2001).

Table 26-15

Comparison of Thrombin and Thrombin-Like Snake Venom Enzyme Actions

	ACTION ON HUMAN FIBRINOGEN				
ENZYMES	FIBRINOPEPTIDES RELEASED	CHAIN DEGRADATION	ACTIVATION OF FACTOR XIII	PROTHROMBIN FRAGMENT CLEAVAGE	PLATELET AGGREGATION AND RELEASE
Thrombin	A − B	α(A)	Yes	Yes	Yes
Thrombin-like enzymes	A*	α(A)† or β(B)‡	No	Yes or no§	No
Agkistrodon c. contortrix venom	B	ND	Incomplete	ND	No
Bitis gabonica venom	A + B	ND	Yes	ND	ND

Abbreviation: ND, not determined.
**Includes ancrod, batroxobin, crotalase, and the enzyme from T. okinavensis.*
†Ancrod (batroxobin degrades α(A) chain of bovine but not human fibrinogen).
‡Crotalase.
§Fragment I released by crotalase and Agkistrodon contortrix venom, but not by ancrod or batroxobin.
SOURCE: Data from Russell (2001).

Table 26-17

Snake Venom Proteins Active on the Hemostatic System

GENERAL FUNCTIONAL ACTIVITY	SPECIFIC BIOLOGICAL ACTIVITY
Procoagulant	Activates factors II, V, IX, X, and protein C Fibrinogen clotting
Anticoagulant	Factor IX/factor X–binding protein Thrombin inhibitor Phospholipase A
Fibrinolytic	Fibrin(ogen) degradation Plasminogen activation
Vessel wall interactive	Hemorrhagic

SOURCES: Data from Markland (1998) and Russell (2001).

Acetylcholinesterase was first demonstrated in cobra venom and is widely distributed throughout the elapid venoms (Ahmed et al., 2010). It is also found in sea snake venoms but is totally lacking in viperid and crotalid venoms. It catalyzes the hydrolysis of acetylcholine to choline and acetic acid. The role of the enzyme in snake venoms is not clear, but may function to catalyze the hydrolysis of acetylcholine, thereby facilitating tetanic paralysis and capture of prey.

RNase is present in some snake venoms in small amounts as the endopolynucleotidase RNase. It appears to have specificity toward pyrimidine-containing pyrimidyladenyl bonds in DNA. The optimum pH is 7 to 9 when ribosomal RNA is used as the substrate. This enzyme in *Naja oxiana* venom has a molecular weight of 15,900.

DNase acts on DNA to produce predominantly trinucleotides or higher oligonucleotides that terminate in 3′-monoesterified phosphate. *C. adamanteus* venom contains two DNases, with optimum pH at 5 and 9.

5′-Nucleotidase is a common constituent of all snake venoms; in most instances, it is the most active phosphatase in snake venoms. It specifically hydrolyzes phosphate monoesters, which link with a 5′ position of DNA and RNA. It is found in greater amounts in crotalid and viperid venoms than in elapid venoms. The molecular weight as determined from amino acid composition and gel filtration with *Naja naja atra* venom has been estimated at 10,000. The enzyme from *N. naja* venom is enhanced by Mg^{2+}, is inhibited by Zn^{2+}, is inactivated at 75°C at pH 7.0 or 8.4, and has an isoelectric point of about 8.6. The enzyme from *Agkistrodon halys blomhoffi* shows a pH optimum of 6.8 to 6.9, with activity being enhanced by Mg^{2+} and Mn^{2+} and inhibited by Zn^{2+}. The enzyme has a low order of lethality (Russell, 2001).

Nicotinamide adenine dinucleotide (NAD) nucleotidase has been found in a number of snake venoms. This enzyme catalyzes the hydrolysis of the nicotinamide *N*-ribosidic linkage of NAD, yielding nicotinamide and adenosine diphosphate riboside. Its optimum pH is 6.5 to 8.5; it is heat labile, losing activity at 60°C. Nucleotidases function as ADP scavengers, thereby acting as potent inhibitors of platelet aggregation.

L-Amino acid oxidase has been found in all snake venoms examined so far and it is what gives the characteristic yellow color to the venom. This enzyme catalyzes the oxidation of L-α-amino and α-hydroxy acids. This activity results from a group of homologous enzymes with molecular weights ranging from 85,000 to 150,000. The mouse intravenous LD_{50} of the enzyme from *C. adamanteus* venom was 9.13 mg/kg body weight, approximately four times less than the lethal value of the crude venom, and this enzyme had no effect on nerve, muscle, or neuromuscular transmission (Russell, 2001; Tan and Fung, 2010).

Polypeptides Snake venom polypeptides are low-molecular-weight proteins that do not have enzymatic activity. More than 80 polypeptides with pharmacologic activity have been isolated from snake venoms. Interested readers will find definitive reviews on these peptides in the works of Lee (1979), Eaker and Wadström (1980), and Gopalakrishnakone and Tan (1992). Most of the lethal activity of the poison of the sea snake *Laticauda semifasciata* was recovered as two toxins, erabutoxin-a and erabutoxin-b, using carboxymethylcellulose chromatography; 30% of the proteins were erabutoxins. More recently, erabutoxin-a, a short-chain curamimetic, has been crystallized in monomeric and dimeric forms (Nastopoulos et al., 1998). Erabutoxin-b is said to be relatively ineffective at the mammalian neuromuscular junction (Vincent et al., 1998). Another curamimetic, a long-chain polypeptide, is α-cobratoxin, while a novel "neurotoxin" from *N. naja atra*, having 61 amino acid residues and eight cystine residues, has been isolated by Chang et al. (1997).

Disintegrins are a family of short cysteine-rich polypeptides and are divided into five subgroups based on the combination of length and number of disulfide bonds of polypeptides. In general, the disintegrins comprise 40 to 100 amino acids. Their small size coupled with a relatively dense network of disulfide bonds contributes to the tertiary structure of these compounds and high potency of such small compounds. Disintegrins are released in venoms via proteolytic processes of PII metalloproteinases, whereas structures similar in form and function to disintegrins, or disintegrin-like, are subject to PIII processes (Calvette et al., 2005). Monomeric disintegrins can vary from about 50 residues and four disulfide bonds as in echistatin and obtustatin to around 70 amino acid residues and 6 disulfide bridges as in albolabrin, barbourin, and halysin, to over 84 amino acids and 7 disulfide bonds for bitistatin and salmosin-3. Dimeric disintegrins are about 67 amino acids long and contain 4 intrachain disulfide linkages and 2 between-chain bonds. Examples include contortrostatin and acostatin. The monomeric disintegrin-like chemicals contain around 100 amino acids and 8 disulfide bonds, and include trimelysin-I, bothropasin, and jararhagin (Calvette et al., 2003; Ramos and Selistre-de-Araujo, 2006).

RGD (argine–glycine–asparagine) and non-RGD-containing disintegrins coexist in certain venoms and exhibit affinities for variable ligand receptors. In such cases, one copy of the gene encodes for the more conserved RGD function of platelet aggregation, whereas the duplicated genes have drifted toward facilitating other biological functions. Modeling and structure analysis of cyclic RGD peptides has implicated the importance of amino acid sequences on the C-terminal of the RGD sequence; furthermore, the physical features such as size of the integrin-binding loop contribute to the receptor-binding capabilities (Calvette et al., 2005). In general, the amino acid residues of this region of the RGD sequence are not well conserved and are thought to play a key role in determining integrin receptor-binding specificity. There are additional mechanisms within the C-terminal region, which include conformational epitopes that are utilized to alter receptor-binding capabilities.

The small basic polypeptide myotoxins are widely distributed in *Crotalus* snake venoms. The specific agent crotamine from *Crotalus durissus terrificus* venom induces skeletal muscle spasms

and paralysis by changing the inactivation process of sodium channels, which are inhibited by tetrodotoxin and potentiated by veratridine and grayanotoxin, leading to depolarization of the neuromuscular junction. Crotamine is composed of 42 amino acid residues and 3 disulfide bonds. The crotamine gene contains 1.8 kbp and has three exons that are separated by a long phase-1 and a short phase-2 intron and mapped to chromosome 2. In addition, the three-dimensional structure has been published, and the structural topology is similar to that of other three disulfide bridge containing peptides such as human β-defensins and scorpion sodium channel toxin. These structural properties enable crotamine to have a unique cell-penetrating ability allowing the toxin to concentrate in the nucleus by means of a probable receptor-independent mechanism. It is interesting to note that topology and diversification of functional folds are common themes in animal venom peptides acting on ion channels and other targets (Ménez, 1998; Mouhat et al., 2004; Oguiura et al., 2006).

Toxicology In general, the venoms of rattlesnakes and other New World crotalids produce alterations in the resistances and often in the integrity of blood vessels, changes in blood cells and blood coagulation mechanisms, direct or indirect changes in cardiac and pulmonary dynamics, and—with crotalids such as *C. durrissus terrificus* and *C. scutulatus*—serious alterations in the nervous system and changes in respiration. In humans, the course of the poisoning is determined by the kind and amount of venom injected; the site where it is deposited; the general health, size, and age of the patient; the kind of treatment; and those pharmacodynamic principles noted earlier in this chapter. Death in humans may occur within less than 1 hour or after several days, with most deaths occurring between 18 and 32 hours. Hypotension or shock is the major therapeutic problem in North American crotalid bites (Russell, 2001).

Snakebite Treatment The treatment of bites by venomous snakes is now so highly specialized that almost every envenomation requires specific recommendations. However, three general principles for every bite should be kept in mind: (1) snake venom poisoning is a medical emergency requiring immediate attention and the exercise of considerable judgment; (2) the venom is a complex mixture of substances of which the proteins contribute the major deleterious properties, and the only adequate antidote is the use of specific or polyspecific antivenom; and (3) not every bite by a venomous snake ends in an envenomation. Venom may not be injected. In almost 1000 cases of crotalid bites, 24% did not end in a poisoning. The incidence with the bites of cobras and perhaps other elapids is probably higher. The reader is referred to other appropriate texts for appropriate treatment of snakebites (Dart, 2004; Singletary and Holstege, 2006; Smith and Bush, 2010; Tintinalli et al., 2016; Auerbach et al., 2017; see also Clinical Toxinology Resources, www.toxinology.com.).

Snake Venom Evolution Considerable efforts are being expended to examine the complex process by which snake venom components are thought to have changed over the years. This evaluation involves tracing the ancestral roots of toxins, which is made even more cumbersome due to the distinct differences in the speed at which individual components of a venom evolve. The current assemblage of snake venoms with regard to functionality and ancestral protein activity is outlined in Table 26-18 (Fry, 2005). In general, the toxins from ancestral proteins that were constructed of dense networks of cysteine cross-linkages are considered among the most diverse today in terms of toxicological insult.

The evolution of the PLA_2s appears to be directed toward modifying the molecular surface in order to maximize the diversity of the pharmacologic properties of each isozyme component (Kini and Chan, 1999; Li et al., 2005). As PLA_2s are not crucial for snake survival, mutations in some PLA_2 isozymes may be ignored and allowed to progress, thereby expediting the evolution in comparison to other more critical enzymes in which mutations are monitored more closely and not allowed to progress. The functionality of PLA_2 isozymes can be significantly hindered by increased rates of mutations within highly conserved regions of the amino acid sequence, which could in turn displace cysteine residues and alter the tertiary protein structure. However, increased mutation of a particular subset of enzymes, such as the PLA_2s, may be a result of a shift in ecological niche. A recent study (Li et al., 2005) reports a shift in the diet composition of the marbled sea snake as the root cause of decelerated evolution of the PLA_2 enzymes. The diet change from live prey to fish eggs essentially negated the need for PLA_2 enzymes to immobilize live prey for swallowing and digestion. Future research could focus on the declining need for PLA_2s, and on the altered digestive enzyme composition that has likely occurred as a result of diet shift.

Table 26-18

Basal Bioactivities of Some Toxin Types

TOXIN	ACTIVITY
3FTx	α-Neurotoxicity, blocks nicotinic acetylcholine receptor
ADAM	In Viperidae venoms, proteolytic cleavage of C-terminal domains results in direct fibrinolytic activity, liberation of disintegrins, which inhibit platelet aggregation
Cobra venom factor	Causes unregulated activation of complement cascade, hemolysis, and cytolysis
Crotamine	Myonecrosis, modifies voltage-gated sodium channels
Factor V	In taipan and brown snake venom, combines with toxic form of factor X to convert prothrombin to thrombin
Kallikrein	Increases vascular permeability, stimulates inflammation, and reduces blood pressure
Kunitz	Inhibits plasmin and thrombin and other serine proteases, blocks L-type calcium channels
L-Amino oxidase	Induces apoptosis, decreases platelet aggregation, inhibits blood factor IX
PLA_2	Releases arachidonic acid from phospholipids, resulting in inflammation and tissue destruction
VEGF	Increases permeability of vascular bed causing hypotension and shock
Whey acidic proteins	Inhibit leukoproteinases

SOURCE: Data from Fry (2005).

ANTIVENOMS

Antivenoms have been produced against most medically important snake, spider, scorpion, and marine toxins. Animals immunized with venom develop a variety of antibodies to the many antigens

in the venom. Antivenom consists of venom-specific antisera or antibodies concentrated from immune serum to the venom. Antisera contain neutralizing antibodies: one antigen (monospecific) or several antigens (polyspecific). Monovalent antivenoms have a high neutralization capacity, which is desirable against the venom of a specific animal. Polyvalent antisera are typically used to cover several venoms, such as snakes from a geographic region. Polyvalent preparations usually require higher doses or volumes than monovalent antivenoms. Neutralization capacity of antivenom is highly variable as there are no enforced international standards. Antivenom may cross-react with venoms from distantly related species and may not react with venom from the intended species. Nevertheless, in general, the antibodies bind to the venom molecules, rendering them ineffective.

Antivenoms are available in several forms: intact IgG antibodies or fragments of IgG such as $F(ab)_2$ and Fab. The molecular weight of the intact IgG is about 150,000, whereas that of Fab is approximately 50,000. The molecular size of IgG prevents its renal excretion and produces a volume of distribution much smaller than that of Fab. The elimination half-life of IgG in the blood is approximately 50 hours. Its ultimate fate is not known, but most IgG is probably taken up by the reticuloendothelial system and degraded with the antigen attached. Fab fragments have an elimination half-life of about 17 hours, and are small enough to permit renal excretion.

All antivenom products may produce hypersensitivity reactions. Type I (immediate) hypersensitivity reactions are caused by antigen cross-linking of endogenous IgE bound to mast cells and basophils. Binding of antigen by a mast cell may cause the release of histamine and other mediators, producing an anaphylactic reaction. Once initiated, anaphylaxis may continue despite discontinuation of antivenom administration. Type III hypersensitivity (serum sickness) may develop several days after antivenom administration. In these cases, antigen–antibody complexes are deposited in different areas of the body, often producing inflammatory responses in the skin, joints, kidneys, and other tissues. Fortunately, these reactions are rarely serious. The risks of anaphylaxis should always be considered when one is deciding whether to administer antivenom, and thus antivenom should be given only by intravenous infusion under medical supervision (Heard et al., 1999; Russell, 2001; Mebs, 2002; Dart, 2004; Tintinalli et al., 2016; Clinical Toxinology Resources, n.d.).

POTENTIAL CLINICAL APPLICATION OF VENOMS

Animal venoms are being used as research and clinical tools based on their high affinity for specific targets and well-studied pharmacologic properties (Ménez, 1998, 2003; Dimarcq and Hunneyball, 2003; Escoubas and Rash, 2004; Cherniack, 2010, 2011; Mayer et al., 2010; McCleary and Kini, 2013). Toxin specificities for receptors and channels that facilitate the interface and coordination of neuromuscular activity are utilized and manipulated to study, model, diagnose, and sometimes treat acute and degenerative conditions. On closer examination of α-bungarotoxin and candoxin nicotinic acetylcholine receptor specificity, plans are under way to utilize the reversible and irreversible receptor binding in muscular and neuronal tissues, respectively, in Alzheimer patients (Phui Yee et al., 2004). In addition to treating neurological diseases, specific α-toxins (longer chained) are also studied for their antiangiogenic capabilities in treating malignant tumor growth in patients suffering from small cell lung carcinoma (Tsetlin and Hucho, 2004). In cases such as this, there is an inherent trade-off between promoting some degree of neurological deficit in light of combating tumor growth. Toxins such as the snake venom thrombin–like enzymes are valuable tools in both research and therapeutic applications. Similarly, fibrin(ogen)olytic enzymes that break down fibrin-rich clots preventing further clot formation may be useful as controls in blood clotting research or to treat heart attacks and strokes (Castro et al., 2004; Marsh and Williams, 2005; Swenson and Markland, 2005). To facilitate research, the complement-activating cobra venom factor has been produced by recombinant techniques (Vogel et al., 2004). These pharmazooticals include reptilase-R, bothrocethin, stypven, ecarin, and protac.

Active research efforts have noted that animal venoms contain components that can reduce pain, can selectively kill specific cancers, may reduce the incidence of stroke via effects on blood coagulability, and function as antibiotics. Numerous compounds have been isolated from marine animals (Newman et al., 2009; Bingham et al., 2010), including the anticancer agents plinabulin, tasidotin, bryostatin 1, hemiasterlin, and salinosporamide A (also known as marizomib). The compound epibatidin comes from the skin of the South American frog, *Epipedobates tricolor*, and a synthetic derivative ABT-594 appears to be more effective than morphine without being addictive. TM 601 is derived from the Israeli yellow scorpion and attacks malignant brain tumors called glioma tumors responsible for two-thirds of the cases of brain cancer, without harming healthy cells. ET 743, which comes from sea squirts, is being tested for treatment of ovarian cancer and soft tissue sarcoma. Ancrod is an anticoagulant with potential to prevent cell damage and death when someone suffers a stroke. The active ingredient comes from the venom of the Malaysian pit viper. In Germany, where Ancrod has been marketed for a number of years, a specially built facility houses about 3000 snakes. Several other sources of anticoagulants are being examined. A substance called magainin 2, which comes from the skin of frogs, is an effective antibiotic to which bacteria do not appear to develop resistance. The clotting enzyme batroxobin is an ingredient in reptilase and has been used in the development of fibrin glue, which is used in surgery to stop diffuse bleeding from liver or lung by covering the surface with a thin layer of fibrin.

Another major area of investigation and success involves the venom components that act as enzyme inhibitors. In particular, venom peptides from *Bothrops jararaca* were initially called bradykinin-potentiating peptides and lowered blood pressure. After further research, it became clear that these peptides were inhibitors of angiotensin I–converting enzyme, and chemical modification leads to orally active agents such as captopril.

Venom toxins can also be used as a component of the toxin–receptor–antibody complex for diagnosis of autoimmune disorders (Ménez, 2003). In addition to providing a promising means for researching and treating muscular and neurological diseases and cancer, work is being conducted on designing methods by which to consistently reconstruct and conform the overall toxin structure to bind to a specific protein, such as HIV (Ménez, 2003).

The venoms of the spider *Phoneutria nigriventer* and the yellow South American scorpion *Tityus serrulatus* are known to cause priapism. While erectile dysfunction is a growing health problem, the use of animal venoms and toxins as pharmacological tools could not only illuminate the mechanisms involved in erectile function, but also potentially create a possible model for new drugs to treat erectile dysfunction (Nunes et al., 2013).

It has been shown that leeches, earthworms, helminths, snails, centipedes, spiders, and ticks all produce substances with potential clinical applications, such as osteoarthritis, deep vein thrombosis, antimicrobial action, inflammatory bowel disease, analgesia, and hyperlipidemia (Cherniack, 2011). In particular, approximately 14

anticoagulant chemicals have been isolated from the leech, including hirudin and lepirudin, apyrase, collagenase, hyaluronidase, and eglin (Lubenow and Greinacher, 2002; Massart et al., 2009). Tick saliva contains proteins that inhibit human kallikrein and factors XIa and XIIa (Mazzuca et al., 2007). Earthworms contain proteins with anticoagulant, antimicrobial, and anti-inflammatory bowel disease properties (Joo et al., 2009; Reddy and Fried, 2009; Ruyssers et al., 2010). Bees can provide honey, royal jelly, and propolis, which all have antimicrobial properties, and the venom contains apamin and mellitin, which have anti-inflammatory properties. Additional details of the clinical applications of animal saliva and venom may be found in Cherniack (2010, 2011).

There have been numerous success stories: captopril, an angiotensin converting enzyme inhibitor for hypertension was patterned after a Brazilian viper venom peptide (Koh and Kini, 2012); ziconotide, a conotoxin from Conus magus, is used to treat pain (Webster and Fakata, 2005); and exenatide from Gila monster venom is used in type 2 diabetes treatment (Furman, 2012). Much exciting work will no doubt lead to other new therapeutic compounds as the exploration and harvest of natural products continues (Harvey, 2014; Verdes et al., 2016)

Additional work is being conducted on animals such as the mongoose, hedgehog, and opossum, which all embody a high level of resistance to snakebites. Blood from these animals contains proteins between 400 and 700 amino acids long that inhibit hemorrhagins. The exact mechanism of the many components in animal venoms that produce toxicity or resistance to certain toxins has yet to be determined. Further research will require a multidisciplinary approach involving techniques from parasitology, chemistry, molecular biology, genomics, proteomics, physiology, pharmacology, and toxicology.

CONCLUSION

The myriad toxins produced by plants and animals range from the relative simplicity of small chemicals to the exceedingly complex proteinaceous toxins. The effects of these compounds are amazingly diverse and can range from local irritation to systemic destruction and death. The interplay between toxin and organism is often difficult to study due to difficulty involved in recreating the interaction in the laboratory. One must also not forget the interactions that arise between toxins and substances already present in the organism. These interactions can act to worsen or reduce the poisonous outcome. As laboratory techniques become more sophisticated and new methods are developed, research concerning toxins and their effects will continue to grow.

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REFERENCES

Aalto-Korte K, Lauerma A, Alanko K. Occupational allergic contact dermatitis from lichens in present-day Finland. *Contact Dermatitis*. 2005;52:36–38.

Abbott AJ, Holoubek CG, Martin RA. Inhibition of Na^+,K^+-ATPase by the cardenolide 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin. *Biochem Biophys Res Commun*. 1998;251:256–259.

Adams ME. Agatoxins: ion channel specific toxins from the American funnel web spider, *Agelenopsis aperta*. *Toxicon*. 2004;43:509–525.

Ahmed M, Rocha JBT, Morsch VM, Schetinger MRC. Snake venom acetylcholinesterase. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:207.

Alonso-Amelot ME, Avendano M. Human carcinogenesis and bracken fern: a review of the evidence. *Curr Med Chem*. 2002;9:675–686.

Altamirano JC, Gratz SR, Wolnik KA. Investigation of pyrrolizidine alkaloids and their *N*-oxides in commercial comfrey-containing products and botanical materials by liquid chromatography electrospray ionization mass spectrometry. *JAOAC Int*. 2005;88:406–412.

Alvarez-Diez TM, Zheng J. Mechanism-based inactivation of cytochrome P450 3A4 by 4-ipomeanol. *Chem Res Toxicol*. 2004;17:150–157.

Andre E, Campi B, Trevisani M, et al. Pharmacological characterisation of the plant sesquiterpenes polygodial and drimanial as vanilloid receptor agonists. *Biochem Pharmacol*. 2006;71:1248–1254.

Ansari KM, Chauhan LK, Dhawan A, et al. Unequivocal evidence of genotoxic potential of argemone oil in mice. *Int J Cancer*. 2004;112:890–895.

Arilla MC, Gonzalez-Rioja R, Ibarrola I, et al. A sensitive monoclonal antibody-based enzyme-linked immunosorbent assay to quantify *Parietaria judaica* major allergens, Par j1 and Par j2. *Clin Exp Allergy*. 2006;36:87–93.

Aslani MR, Movassaghi AR, Mhori M, et al. Clinical and pathological aspects of experimental oleander (*Nerium oleander*) toxicosis in sheep. *Vet Res Commun*. 2004;28:609–616.

Auerbach PS, Cushing TA, Harris NS. *Auerbach's Wilderness Medicine*. 7th ed. Philadelphia, PA: Elsevier, 2017.

Auger C, Marty A. Heterogeneity of functional synaptic parameters among single release sites. *Neuron*. 1997;19:139–150.

Baer BR, Rettie AE, Henne KR. Bioactivation of 4-ipomeanol by CYP4B1: adduct characterization and evidence for an enedial intermediate. *Chem Res Toxicol*. 2005;18:855–864.

Baku A. Mast cell degranulating (MCD) peptide: a prototypic peptide in allergy and inflammation. *Peptides*. 1999;20:415–420.

Ball KR, Kowdley KV. A review of *Silybum marianum* (milk thistle) as a treatment for alcoholic liver disease. *J Clin Gastroenterol*. 2005;39:520–528.

Bandyopadhyay PK, Garrett JE, Shetty RP, et al. γ-Carboxylation: an extra-cellular post-translational modification that antedates the divergence of molluscs, arthropods and chordates. *Proc Natl Acad Sci U S A*. 2002;99:1264–1269.

Bantel H, Engels IH, Voelter W, et al. Mistletoe lectin activates caspase-8/FLICE independently of death receptor signaling and enhances anticancer drug-induced apoptosis. *Cancer Res*. 1999;59:2038–2090.

Barceloux DG. *Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants and Venomous Animals*. New York: Wiley; 2008.

Barker SC, Murrell A. Systematics and evolution of ticks with a list of valid genus and species names. *Parasitology*. 2004;129:S15–S36.

Bauer C, Oppel T, Rueff F, et al. Anaphylaxis to viscotoxins of mistletoe (*Viscum album*) extracts. *Ann Allergy Asthma Immunol*. 2005;94:86–89.

Beattie PE, Dawe RS, Traynor NJ, et al. Can St. John's wort (*Hypericin*) ingestion enhance the erythremal response during high-dose ultraviolet A1 therapy? *Br J Dermatol*. 2005;153:1187–1191.

Bechinger B. Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. *J Membr Biol*. 1997;156:197–211.

Beier RC, Norman JO, Reagor JC, et al. Isolation of the major component in white snakeroot that is toxic after microsomal activation: possible explanation of sporadic toxicity of white snakeroot plants and extracts. *Nat Toxins*. 1993;1:286–293.

Benjamin DR. Mushroom poisoning in infants and children. *J Toxicol Clin Toxicol*. 1992;30:13–22.

Bingham J-P, Mitsunaga E, Bergeron ZL. Drugs from slugs—past, present and future perspectives of conotoxin research. *Chem Biol Interact*. 2010;183:1–18.

Blanc P, Liu D, Juarez C, et al. Cough in hot pepper workers. *Chest*. 1991;99:27–32.

Blanco C, Diaz-Perales A, Collada C, et al. Class I chitinases as potential panallergens involved in the latex-fruit syndrome. *J Allergy Clin Immunol*. 1999;103(pt 1):507–513.

Blodgett DJ. Fescue toxicosis. *Vet Clin North Am Equine Pract*. 2001;17:567–577.

Blumenthal MN, Langefeld CD, Barnes KC, et al. A genome-wide search for quantitative trait loci contributing to variations in seasonal pollen reactivity. *J Allergy Clin Immunol*. 2006;117:79–85.

Boldrini-França J, Cologna CT, Pucca MB, et al. Minor snake venom proteins: structure, function and potential applications. *Biochim Biophys Acta*. 2017;1861:824–838.

Bolognesi A, Bortolotti M, Maiello S, et al. Ribosome-inactivating proteins from plants: a historical overview. *Molecules*. 2016. 21:1627–1646.

Bondan C, Soares JC, Cecim M, et al. Oxidative stress in the erythrocytes of cattle intoxicated with *Senecio* sp. *Vet Clin Pathol*. 2005;34:353–357.

Bouget J, Bousser J, Pats B, et al. Acute renal failure following collective intoxication by *Cortinarius orellanus*. *Intensive Care Med*. 1990;16:506–510.

Bromley J, Hughes BG, Leong DC, et al. Life-threatening interaction between complementary medicines: cyanide toxicity following ingestion of amygdalin and vitamin C. *Ann Pharmacother*. 2005;39:1566–1569.

Brown DE, Carmony NB. *Gila Monster*. Silver City, NM: High Lonesome Books; 1991.

Bunch TD, Panter KD, James LK. Ultrasound studies of the effects of certain poisonous plants on uterine function and fetal development in livestock. *J Anim Sci*. 1992;70:1639–1643.

Burke JW, Doskotch RW. High field I H- and 13 C-NMR assignments of grayanotoxins I, IV and XIV isolated from *Kalmia angustifolia*. *J Nat Prod*. 1990;53:131–137.

Bush SP, King BO, Norris RL, et al. Centipede envenomation. *Wilderness Environ Med*. 2001;12:93–99.

Caksen H, Odabas D, Akabayram S, et al. Deadly nightshade (*Atropa belladonna*) intoxication: an analysis of 49 children. *Hum Exp Toxicol*. 2003;22:665–668.

Calvette JJ, Marcinkiewicz C, Monleon D, et al. Snake venom disintegrins: evolution of structure and function. *Toxicon*. 2005;45:1063–1074.

Calvette JJ, Moreno-Murciano MP, Theakston RDG, et al. Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulfide bond engineering. *Biochem J*. 2003;372:725–734.

Cameron P, Jelinek G, Kelly A-M, Murray L, Brown AFT, Little M. *Textbook of Adult Emergency Medicine*. 4th ed. New York: Churchill Livingstone; 2015.

Cappell MS, Hassan T. Gastrointestinal and hepatic effects of *Amanita phalloides* ingestion. *J Clin Gastroenterol*. 1992;15:225–228.

Casteel SW, Johnson GC, Wagstaff DJ. *Aesculus glabra* intoxication in cattle. *Vet Hum Toxicol*. 1992;34:55.

Casteel SW, Wagstaff DJ. *Rhododendron macrophyllum* poisoning in a group of sheep and goats. *Vet Hum Toxicol*. 1989;31:176–177.

Castro HC, Zingali RB, Albuquerque MG, et al. Snake venom thrombin-like enzymes: from retilase to now. *Cell Mol Life Sci*. 2004;61:843–856.

Cavalieri M, D'Urso D, Lassa A, et al. Characterization and some properties of the venom gland extract of a theridiid spider (*Steatoda paykulliana*) frequently mistaken for black widow spider (*Latrodectus tredecimguttatus*). *Toxicon*. 1987;25:965–974.

Cavassani KA, Aliberti JC, Dias ARV, et al. Tick saliva inhibits differentiation, maturation and function of murine bone-marrow derived dendritic cells. *Immunology*. 2005;114:235–245.

Ceccarelli B, Hurlbut WP. Ca^{2+}-dependent recycling of synaptic vesicles at the frog neuromuscular junction. *J Cell Biol*. 1980;87:297–303.

Ceha LJ, Presperin C, Young E, et al. Anticholinergic toxicity from night-shade berry poisoning responsive to physostigmine. *J Emerg Med*. 1997;15:65–69.

Chan TYF, Tomlinson B, Crichley JAJH, et al. Herb-induced aconitine poisoning presenting as tetraplegia. *Vet Hum Toxicol*. 1994;36:133–134.

Chang LS, Chou YC, Lin SR, et al. A novel neurotoxin, cobrotoxin b, from *Naja naja atra* venom: purification, characterization and gene organization. *J Biochem*. 1997;122:1252–1259.

Chen CY, Yen CY, Wang HR, et al. Tenuifolide B from *Cinnamomum tenuifolium* stem selectively inhibits proliferation of oral cancer cells via apoptosis, ROS generation, mitochondrial depolarization, and DNA damage. *Toxins*. 2016;8:319.

Cherniack EP. Bugs as drugs, part 1: insects. The "new" alternative medicine for the 21st century? *Altern Med Rev*. 2010;15:124–135.

Cherniack EP. Bugs as drugs, part two: worms, leeches, scorpions, snails, ticks, centipedes and spiders. *Altern Med Rev*. 2011;16:50–58.

Chesnov S, Bigler L, Hesse M. The acylpolyamines from the venom of the spider *Agelenopsis aperta*. *Helv Chim Acta*. 2001;84:2178–2197.

Christensen LP, Kristiansen K. Isolation and quantification of tuliposides and tulipalins in tulips (*Tulipa*) by high-performance liquid chromatography. *Contact Dermatitis*. 1999;40:300–309.

Clinical Toxinology Resources. Department of Toxinology, Women's Children's Hospital, Adelaide, Australia. n.d. Available at: www.toxinology.com.

Cohen E, Quistad GB. Cytotoxic effects of arthropod venoms on various cultured cells. *Toxicon*. 1998;36:353–358.

Cooper MK, Porter JA, Young RE, et al. Teratogen-mediated inhibition of target tissue response to Shh signalling. *Science*. 1998;280:603–1610.

Corbett B, Clark RF. North American snake envenomation. *Emerg Med Clin North Am*. 2017;35:339–354.

Corzo G, Adachi-Akahane S, Nagao T, et al. Novel peptides from assassin bugs (Hemiptera: Reduviidae): isolation, chemical and biological characterization. *FEBS Lett*. 2001;499:256–261.

Corzo G, Escoubas P. Pharmacologically active spider peptide toxins. *Cell Mol Life Sci*. 2003;60:2409–2426.

Coyle CM, Panaccione DG. An ergot alkaloid biosynthesis gene and clustered hypothetical genes from *Aspergillus fumigatus*. *Appl Environ Microbiol*. 2005;71:3112–3118.

Craig AG, Bandyopadhyay P, Olivera BM. Post-translationally modified peptides from *Conus* venoms. *Eur J Biochem*. 1999;264:271–275.

Craik DJ, Daly NL, Waine C. The cysteine knot motif in toxins and implications for drug design. *Toxicon*. 2001;39:43–60.

Crause JC, Vershoor JA, Coetzee J, et al. The localization of paralysis toxin in granules and nuclei of prefed female *Rhipicephalus evertsi evertsi* tick salivary gland cells. *Exp Appl Acarol*. 1993;17:357–363.

Crawford L, Kocan RM. Steroidal alkaloid toxicity to fish embryos. *Toxicol Lett*. 1993;66:175–181.

Dart RC, ed. *Medical Toxicology*. 3rd ed. Philadelphia, PA: Lippincott; 2004.

Das M, Ansari KM, Dhawan A, et al. Correlation of DNA damage in epidemic dropsy patients to carcinogenic potential of argemone oil and isolated sanguinarine alkaloid in mice. *Int J Cancer*. 2005;117:709–717.

Das S, Bannerjee G, DePonte K, et al. Salp25D, an *Ixodes scapularis* antioxidant, is 1 of 14 immunodominant antigens in engorged tick salivary glands. *J Infect Dis*. 2001;184:1056–1064.

Datta G, Tu AT. Structure and other chemical characterizations of gila toxin, a lethal toxin from lizard venom. *J Pept Res*. 1997;50:443–450.

de Haro L, Prost N, David JM, et al. Syndrome sudorien ou muscarinien. *Presse Med*. 1999;28:1069–1070.

Dedov VN, Mondadi S, Armati PJ, et al. Capsaicin-induced depolarisation of mitochondria in dorsal root ganglion neurons is enhanced by vanilloid receptors. *Neuroscience*. 2001;103:219–226.

del Carmen Mendez M, dos Santos RS, Riet-Correa F. Intoxication by *Xanthium cavanillesii* in cattle and sheep in southern Brazil. *Vet Hum Toxicol*. 1998;40:144–147.

Demsey CE. The actions of melittin on membranes. *Biochim Biophys Acta*. 1990;1031:143–161.

Dhananjaya BL, Vishwanath BS, D'Souza CJM. Snake venom nucleases, nucleotidases and phosphomonoesterases. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:155.

Di R, Tumer NE. Pokeweed anitviral protein: its cytotoxicity mechanism and applications in plant disease resistance. *Toxins*. 2015;7:755–772.

Dimarcq J-L, Hunneyball I. Pharma-entomology: when bugs become drugs. *Drug Discov Today*. 2003;8:107–110.

Ding X, Lichti K, Staudinger JL. The mycoestrogen zearalenone induces CYP3A through activation of the pregnane X receptor. *Toxicol Sci*. 2006;91:448–455.

Doley R, Zhou X, Kini RM. Snake venom phospholipase A_2 enzymes. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:173.

Dreyer F. Peptide toxins and potassium channels. *Rev Physiol Biochem Pharmacol*. 1990;115:93–116.

Durand R, Figueredo JM, Mendoza E. Intoxication in cattle from *Cestrum diurnum*. *Vet Hum Toxicol*. 1999;41:26–27.
Eaker D, Wadström T, eds. *Natural Toxins*. Elmsford, NY: Pergamon Press; 1980.
Escoubas P. Mass spectrometry in toxinology: a 21st-century technology for the study of biopolymers from venoms. *Toxicon*. 2006;47:609–613.
Escoubas P, Diochot S, Corzo G. Structure and pharmacology of spider venom neurotoxins. *Biochimie*. 2000;82:893–907.
Escoubas P, Rash L. Tarantulas: eight-legged pharmacists and combinatorial chemists. *Toxicon*. 2004;43:555–574.
Fatehi M, Rowan EG, Harvey AL, et al. Polyamine FTX-3.3 and polyamine amide sFTX-3.3 inhibit presynaptic calcium currents and acetylcholine release at mouse motor nerve terminals. *Neuropharmacology*. 1997;36:185–194.
Ferreras JM, Citores L, Iglesias R, et al. Use of ribosome-inactivating proteins from *Sambusus* for the construction of immunotoxins and conjugates for cancer therapy. *Toxins*. 2011;3:420–441.
Forsyth CS, Speth RC, Wecker L, et al. Comparison of nicotinic receptor binding and biotransformation of coniine in the rat and chick. *Toxicol Lett*. 1996;89:175–183.
Fox JW, Serrano SMT. Snake venom metalloproteinases. In: Mackessy SP. ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:95.
Francischetti IBM, Mather TN, Ribeiro JMC. Cloning of a salivary gland metalloprotease and characterization of gelatinase and fibrino(gen)lytic activities in the saliva of the lyme disease tick vector *Ixodes scapularis*. *Biochem Biophys Res Commun*. 2003;305:869–875.
Frasca T, Brett AS, Yoo SD. Mandrake toxicity. *Arch Intern Med*. 1997;157:2007–2009.
Frohn A, Frohn C, Steuhl KP, et al. Wolfsmilchveratzung. *Ophthalmology*. 1993;90:58–61.
Fry BG. From genome to "venome": molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. *Genome Res*. 2005;15:403–420.
Fuchs H, Weng A, Gilabert-Oriol R. Augmenting the efficacy of immunotoxins and other targeted protein toxins by endosomal escape enhancers. *Toxins*. 2016;8:200–228.
Fujimoto S, Mori M, Tsushima H, et al. Capsaicin-induced, capsazepine-insensitive relaxation of the guinea-pig ileum. *Eur J Pharmacol*. 2006;530:144–151.
Fung SY, Tan NH, Sim SM, et al. *Mucuna pruriens* seed extract pretreatment protects against cardiorespiratory and neuromuscular depressant effects of *Naja sputatrix* (Javan spitting cobra) venom in rats. *Indian J Exp Biol*. 2011;49:254–259.
Furman BL. The development of Byetta (exenatide) from the venom of the Gila monster as an anti-diabetic agent. *Toxicon*. 2012;59:464–471.
Gaffield W, Keeler RF. Implications of C-5, C-6 unsaturation as a key structural factor in steroidal alkaloid-induced mammalian teratogenesis. *Experientia*. 1993;49:922–924.
Gertsch WJ. *American Spiders*. 2nd ed. New York: Van Nostrand Reinhold; 1979.
Giavina-Bianchi PF Jr, Castro FF, Machado ML, et al. Occupational respiratory allergic disease induced by *Passiflora alata* and *Rhamnus purshiana*. *Ann Allergy Asthma Immunol*. 1997;79:449–454.
Gilabert-Oriol R, Weng A, von Mallinckrodt B, et al. Immunotoxins constructed with ribosome-inactivating proteins and their enhancers. *Curr Pharm Des*. 2014;20:6584–6643.
Gmelin R. Isolierung von Willardiin [3-(1-uracyl)-L-alanin] aus den samen von *Acacia millefolia*, *Acacia lemmoni* und *Mimosa asperata*. *Acta Chem Scand*. 1961;15:1188–1189.
Goldin AL. Resurgence of sodium channel research. *Annu Rev Physiol*. 2001;63:871–894.
Gomes A, Datta A, Sarangi B, et al. Isolation, purification and pharmacodynamics of a toxin from the venom of centipede, *Scolopendra subspinipes dehaani* Brandt. *Ind J Exp Biol*. 1983;21:203–207.
Gopalakrishnakone P, Tan CK, eds. *Recent Advances in Toxinology Research*. Singapore: National University of Singapore; 1992.
Grishin E. Polypeptide neurotoxins from spider venoms. *Eur J Biochem*. 1999;264:276–280.
Grishin EV. Black widow spider toxins: the present and the future. *Toxicon*. 1998;36:1693–1701.
Groenewoud GC, de Groot H, van Wijk RG. Impact of occupational and inhalant allergy on rhinitis-specific quality of life in employees of bell pepper greenhouses in the Netherlands. *Ann Allergy Asthma Immunol*. 2006;96:92–97.
Gude M, Hausen MD, Heitsch H, et al. An investigation of the irritant and allergenic properties of daffodils (*Narcissus pseudonarcissus*, Amaryllidaceae): a review of daffodil dermatitis. *Contact Dermatitis*. 1988;19:1–10.
Guerrero B, Perales J, Gil San Juan A, Arocha-Pinango CL. Effect on platelet FXIII and partial characterization of Lonomin V, a proteolytic enzyme from *Lonomia achelous* caterpillars. *Thromb Res*. 1999;93:243–252.
Gurung NK, Rankens DL, Shelby RA. In vitro ruminal disappearance of fumonisin B1 and its effects on in vitro dry matter disappearance. *Vet Hum Toxicol*. 1999;41:196–199.
Haarmann T, Machado C, Lubbe Y, et al. The ergot alkaloid gene cluster in *Claviceps purpurea*: extension of the cluster sequence and intra species evolution. *Phytochemistry*. 2005;66:1312–1320.
Habermann E. Apamin. *Pharmacol Ther*. 1984;25:255–270.
Hall AC, Turcotte CM, Betts BA, et al. Modulation of human GABAA and glycine receptor currents by menthol and related monoterpenoids. *Eur J Pharmacol*. 2004;506:9–16.
Han D, Matsumaru K, Rettori D, et al. Usnic acid-induced necrosis of cultured mouse hepatocytes: inhibition of mitochondrial function and oxidative stress. *Biochem Pharmacol*. 2004;67:439–451.
Harvey AL. Toxins and drug discovery. *Toxicon*. 2014;92:193–200.
Hausen BM, Prater E, Shubert H. The sensitizing capacity of *Alstroemeria* cultivars in man and guinea pig. *Contact Dermatitis*. 1983;9:46–54.
Heard K, O'Malley GF, Dart RC. Antivenom therapy in the Americas [review]. *Drugs*. 1999;58:5–15.
Heinen TE, Gorini da Veiga AB. Arthropod venoms and cancer. *Toxicon*. 2011;57:495–511.
Hill NS, Thompson FN, Dawe DL, et al. Antibody binding by circulating ergot alkaloids in cattle grazing tall fescue. *Am J Vet Res*. 1994;55:419–424.
Hirschwehr R, Heppner C, Spitzauer S, et al. Allergens, IgE mediators, inflammatory mechanisms. *J Allergy Clin Immunol*. 1998;101:196–206.
Hoffman DR. Hymenoptera venom allergens. *Clin Rev Allergy Immunol*. 2006;30:109–128.
Holz GG, Habener JF. Black widow spider alpha-latrotoxin: a presynaptic neurotoxin that shares structural homology with the glycogen-like peptide-1 family of insulin secretagogic hormones. *Comp Biochem Physiol B Biochem Mol Biol*. 1998;121:177–184.
Hooper D, Lirazon MB, Schoenfeld R, et al. Post-translational modification: a two dimensional strategy for molecular diversity of *Conus* peptides. In: Fields GB, Tam JP, Barany G, eds. *Peptides for the New Millennium: Proceedings of the Sixteenth American Peptide Symposium*. Dordrecht, The Netherlands: Kluwer; 2000.
Horikawa N, Kataha K, Watanabe N, et al. Glibenclamide-sensitive hypotension produced by helodermin assessed in the rat. *Biol Pharm Bull*. 1998;21:1290–1293.
Huan J-Y, Mironda CL, Buhler DR, et al. Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids. *Toxicol Lett*. 1998;99:127–137.
Hurlbut WP, Iezzi N, Fesce R, et al. Correlation between quantal secretion and vesicle loss at the frog neuromuscular junction. *J Physiol*. 1990;425:501–526.
Hyde EG, Carmichael WW. Amatoxin-a(s), a naturally occurring organophosphate, is an irreversible active site-directed inhibitor of acetylcholinesterase (E.C.3.1.1.7). *J Biochem Toxicol*. 1991;6:195–201.
Inokuma H, Kemp DH, Willadsen P. Comparison prostaglandin E2 in salivary gland of *Boophilus microplus*, *Haemaphysalis longicornis* and *Ixodes holocyclus* and quantification of PGE2 in saliva, hemolymph, ovary and gut of B. microplus. *J Vet Med Sci*. 1994;56:1217–1218.
Isbister GK, Volschenk ES, Seymour JE. Scorpion stings in Australia: five definite stings and a review. *Int Med J*. 2004;34:427–430.
Isbister GK, White J. Clinical consequences of spider bites: recent advances in our understanding. *Toxicon*. 2004;43:477–492.

Izzo AA, Ernst E. Interactions between herbal medicines and prescribed drugs: a systematic review. *Drugs*. 2001;61:2163–2175.
Jaeger A, Fehl F, Flesch F, et al. Kinetics of amatoxins in human poisoning: therapeutic implications. *Clin Toxicol*. 1993;31:63–80.
Jaffe AM, Gephardt D, Courtemanche L. Poisoning due to ingestion of *Veratrum viride* (false hellebore). *J Emerg Med*. 1990;8:161–167.
James LF, Panter KE, Broquist HP, et al. Swainsonine-induced high mountain disease in calves. *Vet Hum Toxicol*. 1991;33:217–219.
Jimenez P, Tejero J, Cordoba-Diaz D, et al. Ebulin from dwart elder (*Sambucus ebulus* L.): A mini-review. *Toxins*. 2015;7:648–658.
Jones TK, Lawson BM. Profound neonatal congestive heart failure caused by maternal consumption of blue cohosh herbal medication. *J Pediatr*. 1998;132:550–552.
Joo SS, Won TJ, Kim JS, et al. Inhibition of coagulation activation and inflammation by a novel factor Xa inhibitor synthesized from the earthworm *Eisenia andrei*. *Biol Pharm Bull*. 2009;32:253–258.
Jorgensen K, Bak S, Busk PK, et al. Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. *Plant Physiol*. 2005;139:363–374.
Kalish RS, Johnson KL. Enrichment and function of urushiol (poison ivy)-specific T lymphocytes in lesions of allergic contact dermatitis to urushiol. *J Immunol*. 1990;145:3706–3713.
Kandel ER, Schwartz JH, Jessel TM, Siegelbaum SA, Hudspeth AJ. *Principles of Neural Science*. 5th ed. New York: McGraw-Hill; 2013.
Karlson-Stiber C, Persson H. Cytotoxic fungi—an overview. *Toxicon*. 2003;42:339–349.
Kavalali G. The chemical and pharmacological aspects of *Urtica*. In: Kavalali GM, ed. *Urtica*. New York: Taylor & Francis; 2003:25–39.
Kawamoto F, Kumada N. Biology and venoms of Lepidoptera. In: Tu A, ed. *Insect Poisons, Allergens, and Other Invertebrate Venoms*. Vol 2. New York: Marcel Dekker; 1984:292.
Keegan HL. *Scorpions of Medical Importance*. Jackson, MI: University Press of Mississippi; 1980.
Keeler RF. Early embryonic death in lambs induced by *Veratrum californicum*. *Cornell Vet*. 1990;80:203–207.
Keeler RF, Baker DC. Myopathy in cattle induced by alkaloid extracts from *Thermopsis montana*, *Laburnum anagyroides* and a *Lupinus* sp. *J Comp Pathol*. 1990;103:169–182.
Kelch WJ, Kerr LA, Adair HS, et al. Suspected buttercup (*Ranunculus bulbosus*) toxicosis with secondary photosensitization in a Charolais heifer. *Vet Hum Toxicol*. 1992;34:238–239.
Kemparaju K, Girish KS, Nagaraju S. Hyaluronidases, a neglected class of glycosidases from snake venom: beyond a spreading factor. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:237.
Kennelly EJ, Flynn TJ, Mazzola EP, et al. Detecting potential teratogenic alkaloids from blue cohosh rhizomes using an in vitro rat embryo culture. *J Nat Prod*. 1999;62:1385–1389.
Kim D-Y, Lee Y-S. Ovine copper poisoning and *Pteridium aquilinum*-associated bovine urinary bladder tumor in Korea. *J Toxicol Soc*. 1998;23(suppl 4):645–646.
Kini RM. Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. *Toxicon*. 2003;42:827–840.
Kini RM, Chan YM. Accelerated evolution and molecular surface of venom phospholipases A2 enzymes. *J Mol Evol*. 1999;48:125–132.
Knight TE. Philodendron-induced dermatitis: report of cases and review of the literature. *Cutis*. 1991;48:375–378.
Koh CY, Kini RM. From snake venom toxins to therapeutics—cardiovascular examples. *Toxicon*. 2012;59:497–506.
Kolarich D, Altmann F, Sunderasan E. Structural analysis of the glycoprotein allergen Hev b 4 from natural rubber latex by mass spectrometry. *Biochim Biophys Acta*. 2005;46:946–953.
Kotaki Y, Furio EF, Satake M, et al. Production of isodomoic acids A and B as major toxin components of a pennate diatom *Nitzschia navis-varingica*. *Toxicon*. 2005;46:946–953.
Krasnoperov VG, Bittner MA, Beavis R, et al. α-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. *Neuron*. 1997;18:925–937.
Kuhn-Nentwig L. Antimicrobial and cytolytic peptides of venomous arthropods. *Cell Mol Life Sci*. 2003;60:2651–2668.
Kuhn-Nentwig L, Schaller J, Nentwig W. Biochemistry, toxicology and ecology of the venom of the spider *Cupiennius salei* (Ctenidae). *Toxicon*. 2004;43:534–553.
Kulp KS, Valliet PR, Richard P. Mimosine blocks cell cycle progression by chelating iron in asynchronous human breast cancer cells. *Toxicol Appl Pharmacol*. 1996;139:356–364.
Kumar PS, Kumar DS, Umamaheswari S. A perspective on toxicology of *Conus* venom peptides. *Asian Pac J Trop Med*. 2015;8:337–351.
Kuwahara Y, Omura H, Tanabe T. 2-Nitroethenylbenzenes as natural products in millipede defense secretions. *Naturwissenschaften*. 2002;89:308–310.
Lebbe EK, Tytgat J. In the picture: disulfide-poor conopeptides, a class of pharmacologically interesting compounds. *J Venom Anim Toxins Incl Trop Dis*. 2016;22:30.
Lee C-Y, ed. *Snake Venoms*. New York: Springer-Verlag; 1979.
Lee TH, Huang NK, Lai TC, et al. Anemonin, from *Clematis crassifolia*, potent and selective inducible nitric oxide synthase inhibitor. *J Ethnopharmacol*. 2008;116:518–527.
Leikin JB, Paloucek FP. *Poisoning and Toxicology Handbook*. New York: Informa Healthcare; 2008.
Lewis DC, Shibamoto T. Effects of *Cassia obtusifolia* (sicklepod) extracts and anthraquinones on muscle mitochondrial function. *Toxicon*. 1989;27:519–529.
Lewis RJ, Dutertre S, Vetter I, Christie MJ. Conus venom peptide pharmacology. *Pharmacol Rev*. 2012;64:259–298.
Li C, Oberlies NH. The most widely recognized mushroom: chemistry of the genus *Amanita*. *Life Sci*. 2005;78:532–538.
Li M, Fry BG, Kini RM. Putting the brakes on snake venom evolution: the unique molecular evolutionary patterns of *Aipysurus eydouxii* (marbled sea snake) phospholipase A2 toxins. *Mol Biol Evol*. 2005;22:934–941.
Lin TJ, Hsu CI, Lee KH, et al. Two outbreaks of acute tung nut (*Aleurites fordii*) poisoning. *J Toxicol Clin Toxicol*. 1996;34:87–92.
Lioi MB, Barbieri R, Borzacchiello G, et al. Chromosome aberrations in cattle with chronic enzootic haematuria. *J Comp Pathol*. 2004;131:233–236.
Liu M, Nakazawa K, Inoue K, et al. Potent and voltage-dependent block by philanthotoxin-343 of neuronal nicotinic receptor/channels in PC12 cells. *Br J Pharmacol*. 1997;122:379–385.
Lopez TA, Cid MS, Bianchini ML. Biochemistry of hemlock (*Conium maculatum*) alkaloids and their acute and chronic toxicity in livestock: a review. *Toxicon*. 1999;37:841–865.
Lu H, Choudhuri S, Ogura K, et al. Characterization of organic anion transporting polypeptide 1b2-null mice: essential role in hepatic uptake/toxicity of phalloidin and microcystin-LR. *Toxicol Sci*. 2008;35–45.
Lubenow N, Greinacher A. Hirudin in heparin-induced thrombocytopenia. *Semin Thromb Hemost*. 2002;28:431–438.
Ma L, Gu R, Tang L, et al. Important poisonous plants in Tibetian ethnomedicine. *Toxins*. 2015;7:138–155.
Mackessy SP. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010.
Maejima H, Kinoshita E, Yuki T, et al. Structural determinants for the action of grayanotoxin in D1 S4-S5 and D4 S4-S5 intracellular linkers of sodium channel alpha-subunits. *Biochem Biophys Res Commun*. 2002;295:452–457.
Mans BJ, Gasper AR, Louw AI, Neitz AW. Purification and characterization of apyrase from the tick, *Ornithodoros savignyi*. *Comp Biochem Physiol B Biochem Mol Biol*. 1998;120:617–624.
Mareti Z, Lebez D. *Araneism*. Belgrade, Yugoslavia: Nolit Belgrade; 1979.
Markland FS. Snake venoms and their hemostatic system. *Toxicon*. 1998;36:1749–1800.
Markland FS, Swenson S. Snake venom metalloproteinases. *Toxicon*. 2013;62:3–18.
Marsh N, Williams V. Practical applications of snake venom toxins in haemostasis. *Toxicon*. 2005;45:1171–1181.
Martinez FJ, Duron RR, de Torres NW, et al. Experimental evidence for toxic damage induced by a dimeric anthracenon: diast T-514 (peroxisome A2). *Toxicol Lett*. 1997;90:155–162.
Martinez HR, Bermudez MV, Rangel-Guerra RA, et al. Clinical diagnosis in *Karwinskia humboldtiana* polyneuropathy. *J Neurol Sci*. 1998;154:49–54.

Masina S, Broady KW. Tick paralysis: development of a vaccine. *Int J Parasitol*. 1999;29:535–541.

Massart D, Sohawon S, Noordally O. Medicinal leeches. *Rev Med Brux*. 2009;30:533–536.

Massermanian A. Contact dermatitis due to *Euphorbia pulcherrima* Willd, simulating a phototoxic reaction. *Contact Dermatitis*. 1998;38:113–114.

Mayer AM, Glaser KB, Cuevas C, et al. The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends Pharmacol Sci*. 2010;31:255–265.

Mayer KE, Myers RP, Lee SS. Silymarin treatment of viral hepatitis. *J Viral Hepat*. 2005a;12:559–567.

Mayer M, O'Neill MA, Murray KE, et al. Usnic acid: a non-genotoxic compound with anti-cancer properties. *Anticancer Drugs*. 2005b;16:805–809.

Mazzuca M, Heurteaux C, Alloui A, et al. A tarantula peptide against pain via ASIC1a channels and opioid mechanisms. *Nat Neurosci*. 2007;10:943–945.

McCleary RJ, Kini RM. Non-enzymatic proteins from snake venoms: a gold mine of pharmacological tools and drug leads. *Toxicon*. 2013;62:56–74.

McDermott WV, Ridker PM. The Budd–Chiari syndrome and hepatic venoocclusive disease. *Arch Surg*. 1990;125:525–527.

McGovern TW, Barkley TM. Botanical briefs: chrysanthemum—*Dendranthema* spp. *Cutis*. 1999;63:319–320.

McGrath-Hill CA, Vicas IM. Case series of *Thermopsis* exposures. *J Toxicol Clin Toxicol*. 1997;35:659–665.

McIntosh JM, Santos AD, Olivera BM. *Conus* peptides targeted to specific nicotinic acetylcholine receptor subtypes. *Annu Rev Biochem*. 1999;68:59–88.

McLain-Romero J, Creamer R, Zepeda H, et al. The toxicosis of *Embellisia* fungi from locoweed (*Oxytropis lambertii*) is similar to locoweed toxicosis in rats. *J Anim Sci*. 2004;82:2169–2174.

Mebs D. *Venomous and Poisonous Animals*. Stuttgart: Medpharm; 2002.

Mebs D, Kauferstein S. Ichthyotoxity caused by marine cone snail venoms? *Toxicon*. 2005;46:355–356.

Mei N, Guo L, Fu PP, et al. Mutagenicity of comfrey (*Symphytum officinale*) in rat liver. *Br J Cancer*. 2005;92:873–875.

Mellick LB, Makowski T, Mellick GA, et al. Neuromuscular blockade after ingestion of tree tobacco (*Nicotiana glauca*). *Ann Emerg Med*. 1999;34:101–104.

Mello JR, Habermehl GG. Calcinogenic plants and the incubation effect of rumen fluid. *Dtsch Tierarztl Wochenschr*. 1992;99:371–376.

Mendis S. Colchicine cardiotoxicity following ingestion of *Gloriosa superba* tubers. *Postgrad Med J*. 1989;65:752–755.

Ménez A. Functional architectures of animal toxins: a clue to drug design? *Toxicon*. 1998;36:1557–1572.

Ménez A. *The Subtle Beast: Snakes, from Myth to Medicine*. New York: Taylor & Francis; 2003.

Ménez A, Stocklin R, Mebs D. "Venomics" or: the venomous systems genome project. *Toxicon*. 2006;47:255–259.

Ménez A, Zimmerman K, Zimmerman S, et al. Venom apparatus and toxicity of the centipede *Ethmostigmus rubripes* (Chilopoda, Scolopendridae). *J Morphol*. 1990;206:303–312.

Merchant ML, Hinton JF, Geren CR. Sphingomyelinase D activity of brown recluse spider (*Loxosceles reclusa*) venom as studied by 31 P-NMR: effects on the time-course of sphingomyelin hydrolysis. *Toxicon*. 1998;36:537–545.

Michelot D, Toth B. Poisoning by *Gyromitra esculenta*—a review. *J Appl Toxicol*. 1991;11:235–243.

Middleton RE, Warren VA, Kraus RL, et al. Two tarantula peptides inhibit activation of multiple sodium channels. *Biochemistry*. 2002;41:14734–14747.

Mir R, Karim S, Kamal MA, Wilson CM, Mirza Z. Conotoxins: structure, therapeutic potential and pharmacological applications. *Curr Pharm Des*. 2016;22(5):582–589.

Monzingo AF, Collins EJ, Ernst SR, et al. The 2.5 A structure of pokeweed antiviral protein. *J Mol Biol*. 1993;233:705–715.

Morrissette J, Kratezschmar J, Haendler B, et al. Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors. *Biophys J*. 1995;68:2280–2288.

Mouhat S, Jouirou B, Mosbah A, et al. Diversity of folds in animal toxins acting on ion channels. *Biochem J*. 2004;378:717–726.

Murai M, Kimura I, Kimura M. Blocking effects of hypoaconitine and aconitine on nerve action potentials in phrenic nerve–diaphragm muscles of mice. *Neuropharmacology*. 1990;29:567–572.

Nastopoulos D, Kanellopoulos PN, Tsernoglov D. Structure of dimeric and monomeric erabutoxin a refined at 1.5 A resolution. *Acta Crystallogr D Biol Crystallogr*. 1998;54:964–974.

Nelson LS, Shih RD, Balik MJ. *Handbook of Poisonous and Injurious Plants*. New York: Springer; 2007.

Nery NM, Luna KP, Fernandes CF, Zuliani JP. An overview of *Bothrops erythromelas* venom. *Rev Soc Bras Med Trop*. 2016;49:680–686.

Newman DJ, Cragg GM, Battershill CN. Therapeutic agents from the sea: biodiversity, chemo-evolutionary insight and advances to the end of Darwin's 200th year. *Diving Hyperbaric Med*. 2009;39:216–225.

Nobile M, Noceti F, Prestipino G, et al. Helothermine, a lizard venom toxin, inhibits calcium current in cerebellar granules. *Exp Brain Res*. 1996;110:15–20.

Norred WP. Fumonisins—mycotoxins produced by *Fusarium moniliforme*. *J Toxicol Environ Health*. 1993;38:309–328.

Nunes KP, Torres FS, Borges MH, et al. New insights on arthropod toxins that potentiate erectile function. *Toxicon*. 2013;69:152–159.

Nuttall PA, Labuda M. Dynamics of infection in tick vectors and at the tick–host interface. *Adv Virus Res*. 2003;60:233–272.

O'Brien B, Quigg C, Leong T. Severe cyanide toxicity from "vitamin supplements." *Eur J Emerg Med*. 2005;12:257–258.

Oguiura N, Boni-Mitake M, Radis-Baptista G. New view on crotamine, a small basic polypeptide myotoxin from South American rattlesnake venom. *Toxicon*. 2006;46:363–370.

Oh SH, Haw CR, Lee MH. Clinical and immunologic features of systemic contact dermatitis from ingestion of *Rhus* (Toxicodendron). *Contact Dermatitis*. 2003;48:251–254.

Ohba H, Moriwaki S, Bakalova R, et al. Plant-derived abrin-a induces apoptosis in cultured leukemic cell lines by different mechanisms. *Toxicol Appl Pharmacol*. 2004;195:182–193.

Ohno M, Chijiwa T, Oda-Ueda N, et al. Molecular evolution of myotoxic phospholipases A2 from snake venom. *Toxicon*. 2003;42:841–854.

Olivera BM. *Conus* venom peptides, receptor and ion channel targets and drug design: 50 million years of neuropharmacology. *Mol Biol Cell*. 1997;8:2101–2109.

Olivera BM. ω-Conotoxin MVIIA: from marine snail venom to analgesic drug. In: Fusetani N, ed. *Drugs from the Sea*. Basel, Switzerland: Karger; 2000.

Olivera BM. *Conus* venom peptides: reflections from the biology of clades and species. *Annu Rev Ecol Syst*. 2002;33:25–42.

Olivera BM, Miljanich G, Ramachandran J, et al. Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. *Annu Rev Biochem*. 1994;63:823–867.

Olivera BM, Rivier J, Clark C, et al. Diversity of *Conus* neuropeptides. *Science*. 1990;249:257–263.

Omnell ML, Sun FRP, Keeler RF, et al. Expression of *Veratrum* alkaloid teratogenicity in the mouse. *Teratology*. 1990;42:105–119.

Onat FY, Yegen BC, Lawrence R, et al. Mad honey poisoning in man and rat. *Rev Environ Health*. 1991;9:3–9.

O'Shea M. *Venomous Snakes of the World*. Princeton, NJ: Princeton University Press; 2011.

Patel KA, Modur V, Zimmerman GA. The necrotic venom of the brown recluse spider induces dysregulated endothelial cell-dependent neutrophil activation. Differential induction of GM-CSF, IL-8, and E-selectin expression. *J Clin Invest*. 1994;94:631–642.

Peixato PV, Brust LC, Duarte MD, et al. *Cestrum laevigatum* poisoning in goats in southeastern Brazil. *Vet Hum Toxicol*. 2000;42:13–14.

Peper K, Trautwein W. The effect of aconitine on the membrane current in cardiac muscle. *Pflugers Arch*. 1967;296:328–336.

Perry C, Sastry R, Nasrallah IM, et al. Mimosine attenuates serine hydroxymethyltransferase transcription by chelating zinc. Implications for inhibition of DNA replication. *J Biol Chem*. 2005;280:396–400.

Peterson MC, Rasmussen GJ. Intoxication with foothill camas (*Zigadenus paniculatus*). *J Toxicol Clin Toxicol*. 2003;41:63–65.

Pfister JA, Panter KE, Manners GD. Effective dose in cattle of toxic alkaloids from tall larkspur (*Delphinium barberi*). *Vet Hum Toxicol*. 1994;36:10–11.

Phillips DJ, Swernson SD, Markland FS. Thrombin-like snake venom serine proteinases. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:139.

Phui Yee JS, Nanling G, Afifiyan F, et al. Snake postsynaptic neurotoxins: gene structure, phylogeny and applications in research and therapy. *Biochimie*. 2004;86:137–149.

Piek T, ed. *Venoms of the Hymenoptera*. London: Academic Press; 1986.

Pietsch J, Oertel R, Trautmann S, et al. A non-fatal oleander poisoning. *Int J Legal Med*. 2005;119:236–240.

Pizzo E, Di Maro A. A new age for biomedical applications of ribosome inactivating proteins: from bioconjugate to nanoconstructs. *J Biomed Sci*. 2016;23:54–62.

Polis GA, ed. *The Biology of Scorpions*. Stanford, CA: Stanford University Press; 1990:482.

Polito L, Bortolotti M, Maiello S, et al. Plants producing ribosome-inactivating proteins in traditional medicine. *Molecules*. 2016;21:1560–1587.

Polya G. *Biochemical Targets of Plant Bioactive Compounds. A Pharmacological Reference Guide to Sites of Actions and Biological Effects*. London: Taylor & Francis; 2003:583.

Possani LD, Merino E, Corona M, et al. Peptides and genes encoding for scorpion toxins that affect ion-channels. *Biochimie*. 2000;82:861–868.

Prabhasankar P, Raguputhi G, Sundaravadivel B, et al. Enzyme-linked immunosorbent assay for the phytotoxin thevetin. *J Immunoassay*. 1993;14:279–296.

Puschner B, Galey FD, Holstege DM, et al. Sweet clover poisoning in California. *J Am Vet Med Assoc*. 1998;212:857–859.

Pusterla N, Holmberg TA, Lorenzo-Figueras M, et al. *Acremonium strictum* pulmonary infection in a horse. *Vet Clin Pathol*. 2005;34:413–416.

Raisinghani M, Pabbidi RM, Premkumar LS. Activation of transient receptor potential vanilloid 1 (TRPV1) by resiniferatoxin. *J Physiol*. 2005;567(pt 3):771–786.

Ramirez M, Rivera E, Ereu C. Fifteen cases of atropine poisoning after honey ingestion. *Vet Hum Toxicol*. 1999a;41:19–20.

Ramirez MS, Sanchez EE, Garcia-Prieto C, et al. Screening for fibrinolytic activity in 8 viperid venoms. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol*. 1999b;124:91–98.

Ramos OHP, Selistre-de-Araujo HS. Snake venom metalloproteases—structure and function of catalytic and disintegrin domains. *Comp Biochem Physiol C Toxicol Pharmacol*. 2006;142:328–346.

Rao RB, Hoffman RS. Nicotinic toxicity from tincture of blue cohosh (*Caulophyllum thalictroides*) used as an abortifacient. *Vet Hum Toxicol*. 2002;44:221–222.

Rash LD, Hodgson WC. Pharmacology and biochemistry of spider venoms. *Toxicon*. 2002;40:225–254.

Rasmussen LH, Jensen LS, Hansen HC. Distribution of the carcinogenic terpene ptaquiloside in bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. *J Chem Ecol*. 2003;29:771–778.

Reddy A, Fried B. An update on the use of helminths to treat Crohn's and other autoimmunune diseases. *Parasitol Res*. 2009;104:217–221.

Reddy L, Odhav B, Bhoola K. Aflatoxin B1-induced toxicity in HepG2 cells inhibited by carotenoids: morphology, apoptosis and DNA damage. *Biol Chem*. 2006;387:87–93.

Riley RT, Hinton DM, Chamberlain WJ, et al. Dietary fumonisin B1 induces disruption of sphingolipid metabolism in Sprague–Dawley rat: a new mechanism of nephrotoxicity. *J Nutr*. 1994;124:594–603.

Rimsza ME, Zimmerman DR, Bergeson PS. Scorpion envenomation. *Pediatrics*. 1980;66:298–302.

Rockel D, Korn W, Kohn AJ. *Manual of the Living Conidae*. Weisbaden, Germany: Verlag Christa Hemmen; 1995.

Rode D. Comfrey toxicity revisited. *Trends Pharmacol Sci*. 2002;23:497–499.

Rodriguez de la Vega RC, Possani LD. Current views on scorpion toxins specific for K^+-channels. *Toxicon*. 2004;43:865–875.

Rodriguez de la Vega RC, Possani LD. Overview of scorpion toxins specific for Na^+ channels and related peptides: biodiversity, structure–function relationships and evolution. *Toxicon*. 2005;46:831–844.

Rondeau ES. Wisteria toxicity. *J Toxicol*. 1993;31:107–112.

Rosell S, Samuelsson G. Effect of mistletoe viscotoxin and phoratoxin on blood circulation. *Toxicon*. 1988;26:975–987.

Rosenberg P. Phospholipases. In: Shier WT, Mebs D, eds. *Handbook of Toxinology*. New York: Marcel Dekker; 1990:67.

Roy MC, Chang FR, Huang HC, et al. Cytotoxic principles from the formosan milkweed, *Asclepias curassavica*. *J Nat Prod*. 2005;68: 1494–1499.

Russell FE. Toxic effects of animal venoms. In: Klaassen CD, ed. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. 6th ed. New York: McGraw-Hill; 2001:945.

Russell FE, Nagabhushanam R. *The Venomous and Poisonous Marine Invertebrates of the Indian Ocean*. Enfield, NH: Science Publications; 1996:271.

Russo E, Scicchitano F, Whalley BJ, et al. *Hypericum perforatum*: pharmacokinetic, mechanism of action tolerability and clinical drug–drug interactions. *Phytotherap Res*. 2014;28:643–655.

Ruyssers NE, De Winter BY, De Man JG, et al. *Schistosoma mansoni* proteins attenuate gastrointestinal motility disturbances during experimental colitis in mice. *World J Gastroenterol*. 2010;16:703–712.

Sako MDN, Al-Sultan II, Saleem AN. Studies on sheep experimentally poisoned with *Hypericum perforatum*. *Vet Hum Toxicol*. 1993;35: 298–300.

Sangamnetdej S, Paesen GC, Slovak M, et al. A high affinity serotonin- and histamine-binding lipocalin from tick saliva. *Insect Mol Biol*. 2002;11:79–86.

Schacter L. Etopside phosphate: what, why, where and how? *Semin Oncol*. 1996;23(suppl 13):1–7.

Schrot J, Weng A, Melzig MF. Ribosome-inactivating and related proteins. *Toxins*. 2016:1556–1615.

Schulzke JD, Andres S, Amasheh M, Fromm A, Günzel D. Anti-diarrheal mechanism of the traditional remedy Uzara via reduction of active chloride secretion. *PLoS One*. 2011;6(3):e18107.

Serrano SM. The long road of research on snake venom serine proteinases. *Toxicon*. 2013;62:19–26.

Shahin M, Smith BL, Prakash AS. Bracken carcinogens in the human diet. *Mutat Res*. 1999;443:69–79.

Sharma OP, Dawra RK, Pattabhi V. Molecular structure, polymorphism, and toxicity of lantadene A, the pentacyclic triterpenoid from the hepatotoxic plant *Latana camara*. *J Biochem Toxicol*. 1991;6:57–63.

Short SO, Edwards WC. Blue green algae toxicosis in Oklahoma. *Vet Hum Toxicol*. 1990;32:558–560.

Siebert U, Brach M, Sroczynski G, et al. Efficacy, routine effectiveness, and safety of horse chestnut seed extract in the treatment of chronic venous insufficiency. A meta-analysis of randomized controlled trials and large observational studies. *Int Angiol*. 2002;21:305–315.

Silva A, Hodgson WC, Isbister GK. Antivenom for neuromuscular paralysis resulting from snake envenoming. *Toxins*. 2017;9:143–160.

Singletary EM, Holstege CP. Bites and stings: insects, marine, mammals and reptiles. In: Aghababian RV, ed. *Essentials of Emergency Medicine*. Sudbury, MA: Jones and Bartlett; 2006.

Skinner WS, Adams ME, Quistad GB, et al. Purification and characterization of two classes of neurotoxins from the funnel web spider *Agelenopsis aperta*. *J Biol Chem*. 1989;264:2150–2155.

Smith J, Bush S. Envenomations by reptiles in the United States. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:475.

Soares AM, Giglio JR. Chemical modifications of phospholipases A2 from snake venoms: effects on catalytic and pharmacological properties. *Toxicon*. 2003;42:855–868.

Sokolov IuV, Chanturiia AN, Lishko VK. Channel-forming properties of *Steatoda paykulliana* spider venom [Russian]. *Biofizika*. 1984;29:620–623.

Southcott RV, Haegi LAR. Plant hair dermatitis. *Med J Aust*. 1992;156: 623–628.

Spencer PS. Food toxins, AMPA receptors and motor neuron diseases. *Drug Metab Rev*. 1999;31:561–587.

Spoerke DG, Murphy MM, Wruk KM, et al. Five cases of *Thermopsis* poisoning. *J Toxicol Clin Toxicol*. 1988;26:397–406.

Spooner RA, Lord JM. Ricin trafficking in cells. *Toxins*. 2015. 7:49–65.

Steen NA, Barker SC, Alewood PF. Proteins in the saliva of the *Ixodida* (ticks): pharmacological features and biological significance. *Toxicon*. 2006;47:1–20.

Strimple PD, Tomassoni AJ, Otten EJ, et al. Report on envenomation by a Gila monster (*Heloderma suspectum*) with a discussion of venom apparatus, clinical findings, and treatment. *Wilderness Environ Med*. 1997;8:111–116.

Surh Y-J, Lee E, Lee JM. Chemoprotective properties of some pungent ingredients present in red pepper and ginger. *Mutat Res*. 1998;404:259–267.

Swartz KJ, MacKinnon R. An inhibitor of the Kv2.1 potassium channel isolated from the venom of a Chilean tarantula. *Neuron*. 1995;15:35–42.

Swenson S, Markland FS Jr. Snake venom fibrin(ogen)olytic enzymes. *Toxicon*. 2005;45:1021–1039.

Szalcsany J. Capsaiscin receptors as target molecules on nociceptors for development of novel analgesic agents. In: Keri G, Toth I, eds. *Molecular Pathomechanisms and New Trends in Drug Research*. London: Taylor & Francis; 2002:319–333.

Tahirov THO, Lu T-H, Liaw Y-C, et al. A new crystal form of abrina from the seeds of *Abrus precatorius*. *J Mol Biol*. 1994;235:1152–1153.

Tambourgi DV, Petricevich VL, Magnoli FC, et al. Endotoxemic-like shock induced by *Loxosceles* spider venoms: pathological changes and putative cytokine mediators. *Toxicon*. 1998;36:391–403.

Tan N-H, Fung S-Y. Snake venom l-amino acid oxidases. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:221.

Tandon HD, Tandon BN, Mattocks AR. An epidemic of veno-occlusive disease of the liver in Afghanistan. *Am J Gastroenterol*. 1978;70:607–613.

Teitelbaum JS, Zatorre RJ, Carpenter S, et al. Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. *N Engl J Med*. 1990;322:1781–1787.

Terlau H, Olivera BM. *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiol Rev*. 2004;84:41–68.

Terlau H, Shon K, Grilley M, et al. Strategy for rapid immobilization of prey by a fish-hunting cone snail. *Nature*. 1996;381:148–151.

Tintinalli JE, Stapczynski JS, Ma OJ, et al., eds. *Emergency Medicine: A Comprehensive Study Guide*. 8th ed. New York: McGraw-Hill; 2016.

Tsetlin VI, Hucho F. Snake and snail toxins acting on nicotinic acetylcholine receptors: fundamental aspects and medical applications. *FEBS Lett*. 2004;557:9–13.

Tsujikawa K, Kanamori T, Iwata Y, et al. Morphological and chemical analysis of magic mushrooms in Japan. *Forensic Sci Int*. 2003;138:85–90.

Tucker MO, Swan CR. The mango–poison ivy connection. *N Engl J Med*. 1998;339:235.

Tulsiani DR, Broquest HP, James LF, et al. Production of hybrid glycoprotein and accumulation of oligosaccharides in the brain of sheep and pigs administered swainsonine or locoweed. *Arch Biochem Biophys*. 1988;264:607–617.

Turgut M, Alhan CC, Gurgoze M, et al. Carboxyatractyloside poisoning in humans. *Ann Trop Paediatr*. 2005;25:125–134.

Tylleskar T, Banca M, Bikongi N, et al. Cassava cyanogens and konzo, an upper motoneuron disease found in Africa. *Lancet*. 1992;339:208–211.

Uddman R, Goadsby PJ, Jansen-Olesen I, et al. Helospectin-like peptides: immunochemical localization and effects on isolated cerebral arteries and on local cerebral blood flow in the cat. *J Cerebral Blood Flow Metab*. 1999;19:61–67.

Urushibata O, Kase K. Irritant contact dermatitis from *Euphorbia marginata*. *Contact Dermatitis*. 1991;24:155–157.

Ushkaryov YA, Volnski KE, Ashton AC. The multiple actions of black widow spider toxins and their selective use in neurosecretion studies. *Toxicon*. 2004;43:527–542.

Uwai K, Ohashi K, Takaya Y, et al. Exploring the structural basis of neurotoxicity in C(17)-polyacetylenes isolated from water hemlock. *J Med Chem*. 2000;43:4508–4515.

Valenzuela JG. Blood-feeding arthropod salivary glands and saliva. In: Marquardt WC, ed. *Biology of Disease Vectors*. Amsterdam: Elsevier; 2004:chap 28.

Valenzuela JG, Charlab R, Mather TN, et al. Purification, cloning and expression of a novel salivary anticomplement protein from the tick *Ixodes scapularis*. *J Biol Chem*. 2000;275:18717–18723.

Verdes A, Anand P, Gorson J, et al. From mollusks to medicine: a venomics approach for the discovery and characterization of therapeutics from Terebridae peptide toxins. *Toxins*. 2016;8:117–147.

Vincent A, Jacobson L, Curran L. Alpha-bungarotoxin binding to human muscle acetylcholine receptor. *Neurochem Int*. 1998;332:427–433.

Vogel C-W, Fritsinger DC, Hew BE. Recombinant cobra venom factor. *Mol Immunol*. 2004;41:191–199.

Volynski KE, Capogna M, Ashton AC, et al. Mutant α-latrotoxin (LTXN4C) does not form pores and causes secretion by receptor stimulation. This action does not require neurexins. *J Biol Chem*. 2003;278:31058–31066.

Wang H, Ng TB. Ribosome inactivating protein and lectin from bitter melon (*Momordica charantia*) seeds: sequence comparison with related proteins. *Biochem Biophys Res Commun*. 1998;253:143–146.

Wang X, Connor M, Smith R, et al. Discovery and characterization of a family of insecticidal neurotoxins with a rare vicinal disulfide bridge. *Nat Struct Biol*. 2000;7:505–513.

Wang X, Connor M, Wilson D, et al. Discovery and structure of a potent and highly specific blocker of insect calcium channels. *J Biol Chem*. 2001;276:40306–40312.

Wang Z, Zhao J, Xing J, et al. Analysis of strychnine and brucine in postmortem specimens by RP-HPLC: a case report of fatal intoxication. *J Anal Toxicol*. 2004;28:141–144.

Warren BA, Patel SA, Nunn PB, et al. The *Lathyrus* excitotoxin beta-*N*-oxalyl-L-alpha,beta-diaminopropionic acid is a substrate of the L-cystine/L-glutamate exchanger system xc-. *Toxicol Appl Pharmacol*. 2004;200:83–92.

Webster LR, Fakata KL. Ziconotide for chronic severe pain. *Pr Pain Mgmt*. 2005;5:47–51, 55–56.

Weinstein SA, Smith TL, Kardong KV. Reptile venom glands: form, function and future. In: Mackessy SP, ed. *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press; 2010:65.

White J. Snake venoms and coagulopathy. *Toxicon*. 2005;45:951–967.

Wilson D, Alewood PF. Taxonomy of Australian funnel-web spiders using rp-HPLC/ESI-MS profiling techniques. *Toxicon*. 2006;47:614–627.

Wu JH, Singh T, Herp A, et al. Carbohydrate recognition factors of the lectin domains present in the *Ricinus communis* toxic protein (ricin). *Biochemie*. 2006;88:201–217.

Yellen G. The voltage-gated potassium channels and their relatives. *Nature*. 2002;419:35–42.

Yoshizawa T, Yamashita A, Luo Y. Fumonisin occurrence in corn from high- and low-risk areas for human esophageal cancer in China. *Appl Environ Microbiol*. 1994;60:1626–1629.

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chapter 27

Food Toxicology: Fundamental and Regulatory Aspects*

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Introduction to Food Toxicology

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*The opinions expressed in this chapter are the authors' personal opinions and do not necessarily reflect those of FDA, DHHS, or the federal government.

Some Heavy Metal Contaminants in Food
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Food Allergy
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INTRODUCTION TO FOOD TOXICOLOGY

Food is one of the most essential materials for the survival of living organisms, in addition to oxygen and water. Food toxicology deals with the substances found in food that, when consumed, may cause harm to the consumers. Therefore, the practice of food toxicology involves detecting toxic substances in food, characterizing their properties, studying their fate in the body (absorption, distribution, metabolism, and excretion), and investigating their adverse health effects. Toxic substances can be naturally present in food, formed when the food is cooked, added directly to food, or they can find their way into food from the immediate environment, such as packaging. Among various subdisciplines of toxicology, food toxicology has received wider public attention in recent years. This has been driven by an increased awareness of the health effects of foods, foodborne illness, as well as the rapid availability of information to consumers, thanks to the world-wide-web.

Typically, a food contains hundreds of substances. Apart from the most obvious constituents, a majority of the substances in various foods have not been fully characterized. The assumption that food is safe to consume is based on the history of its consumption. In recent years, there has been increasing interest on the part of consumers regarding the "health-promoting" properties of various naturally occurring constituents of food, such as phytosterols from vegetable oils and isoflavones from soy. The addition of substances to foods that do not contain them naturally has raised questions about how they should be regulated. There is a debate whether the concept of nutrients should be expanded to include a growing number of food constituents that seemingly produce health benefits (Sansalone, 1999). Because federal dietary guidance applies to the total diet and there are no widely accepted standards to judge the "healthfulness" of individual foods, fortification of foods with new substances should require a thorough evaluation of safety at the intended level of intake for the general population (Mackey and Kotsonis, 2002).

NATURE AND COMPLEXITIES OF FOOD

Food is a very complex mixture of nutrient and non-nutrient substances, whether it is consumed uncooked (e.g., raw agricultural product) or in a cooked and highly processed ready-to-eat form (Tables 27-1 and 27-2). Many of the non-nutrient substances are necessary for the growth and survival of the plant (from which they are derived), and for the plant's adaptation to the environment. Some of these substances may act as antinutrients rather than frank toxins, such as trypsin and chymotrypsin inhibitors, phytates that bind minerals, anti-thiamines in fish and plants, etc. Traditionally, the nutrients in food are classified as calorigenic *macronutrients*, such as carbohydrates, proteins, and fats, and the non-calorigenic *micronutrients*, such as vitamins and minerals.

The Food and Nutrition Board of the Institute of Medicine (IOM), which is a division of the U.S. National Academies of Sciences, Engineering and Medicine (NAS), has developed Dietary Reference Intakes (DRIs) that serve as a guide for good nutrition. Since 1994 the IOM's Food and Nutrition Board has been involved in developing an expanded approach to establish dietary reference standards. This approach, the DRIs, provides a set of four nutrient-based reference values designed to replace the

Table 27-1
Food as a Complex Mixture

NUTRIENTS		NON-NUTRIENTS
Macronutrients	Micronutrients	Naturally occurring substances
Carbohydrates	Vitamins	Food additives (direct, indirect)
Total fiber	Minerals	Contaminants
Proteins		Products of food processing
Fats		

Table 27-2
Non-nutrient Substances in Some Common Food

FOOD	NUMBER OF IDENTIFIED NON-NUTRIENT CHEMICALS
Cheddar cheese	160
Orange juice	250
Banana	325
Tomato	350
Wine	475
Coffee	625
Beef (cooked)	625

SOURCE: From Smith (1991), with permission from Medical Society of London.

Recommended Dietary Allowances (RDAs) in the United States and the Recommended Nutrient Intakes (RNIs) in Canada. These reference values include Estimated Average Requirement (EAR), RDA, Adequate Intake (AI), and Tolerable Upper Intake Level (UL). To date, several volumes in this series have been published (http://nationalacademies.org/hmd/reports/2000/dietary-reference-intakes-applications-in-dietary-assessment.aspx).

The EAR is the average daily nutrient intake level estimated to meet the requirements of 50% of the healthy individuals in a group, and the RDA is the daily dietary intake level sufficient to meet the nutrient requirements of nearly all (97% to 98%) healthy individuals in a group. RDA is calculated from the EAR; the RDA is the EAR plus two standard deviations (RDA = EAR + 2SD). If sufficient information is not available to establish an EAR and calculate the RDA, an AI level is developed; AI represents the approximate estimates of nutrient intake by healthy people. The UL is the highest level of daily nutrient intake that is not likely to pose any risk of adverse health effects (IOM, 2003). Therefore, nutrient intake levels beyond the UL represent a transition from nutrition to potential toxicity in an exposure-dependent manner.

For adults, the average DRIs (average of values for males and females) for carbohydrate and protein are 130 and 51 g/day, respectively. The acceptable ranges for the source of energy in adults are 45% to 65% from carbohydrate, 10% to 35% from protein, and 20% to 35% from fat (IOM, 2003). The IOM recommendation includes total dietary fiber as a macronutrient and recommends its AI for different age groups in males and females, such as 38 g/day for males and 25 g/day for females, between the age of 19 and 50 years. Dietary fiber is a complex carbohydrate that is primarily a non-calorigenic macronutrient, but it is claimed to have beneficial effects on health and well-being. It is resistant to digestion by the gut enzymes in humans, but can be broken down by gut bacteria.

IMPORTANCE OF THE GASTROINTESTINAL TRACT

Humans consume approximately 1500 g of food and an equal amount of liquid per day; therefore, within a year, humans ingest approximately 550 kg of food (or about 1200 lbs). All these foods are digested, the products of digestion are absorbed from the intestine, and the undigested remains are excreted on a regular basis. The digestive system of all animals has evolved to handle this job efficiently. The gastrointestinal (GI) tract is a large, complex, and dynamic organ with a vast absorptive surface that has been estimated in humans to be 250 square meters or more (Hall, 2011). The digestive system consists of the GI tract (digestive tract), and the liver, pancreas, and gallbladder. The salivary glands, liver, pancreas, and gall bladder all play a direct role in the digestive process. Current understanding of the digestive process demonstrates an important role of the gut microbiome as well. The recently revised estimate of gut microbiome in adult humans is roughly 40 trillion organisms (Sender et al., 2016), which changes the old 10:1 microbe:body cell ratio to roughly 1:1.

Because chemicals and microorganisms from the environment can get direct entry into the GI tract, it has developed the capacity to perform various important functions in addition to digestion. The enterocytes of the intestinal epithelium are capable of extensive metabolism of xenobiotics that gain entry through the GI tract. The ability of the GI tract to metabolize xenobiotics may be second only to the liver, as it possesses a full complement of phase I and phase II xenobiotic metabolizing enzymes and transporters. The GI tract is also an immunologic organ and is constantly exposed to a large number of antigens in food (approximately 88 kg of protein annually), commensal and ingested bacteria. Therefore, the immune system maintains a strong presence at the intestinal mucosal barrier. The immune system of the GI tract is referred to as the gut-associated lymphoid tissue (GALT). It is heavily populated by lymphocytes, macrophages, and other cells that participate in immune responses (Doe, 1989). One cell layer away from these antigens is the lamina propria of the GI tract, which contains the mucosal-associated lymphoid tissue (MALT), comprising lymphocytes and antigen-presenting cells, as well as unique dendritic cells, which interact with dietary antigens and ultimately determine whether an antigen is tolerated or an immune response is initiated (Brandtzaeg, 2010; Vickery et al., 2011).

The combination of a large absorptive surface area and the rich vascularization of the intestine further aids in efficient absorption. The normal rate of blood flow in the portal vein is approximately 1.2 L/h/kg; however, after a meal, there is a 30% increase in blood flow through the splanchnic area (Concon, 1988). Consequently, the substances that affect blood flow also tend to affect the rate of absorption of other materials. An example is alcohol, which tends to increase blood flow to the stomach and thus enhances its own absorption as well as other substances. Lymph circulation is important in the transfer of fats, large molecules, such as botulinum toxin, benzo[*a*]pyrene, 3-methylcholanthrene, and *cis*-dimethylaminostilbene (Chhabra and Eastin, 1984). Lymph has a flow rate of about 1 to 2 mL/h/kg in humans. Tripalmitin has been shown to double the lymph flow rate and therefore double the absorption of materials, such as *p*-aminosalicylic acid and tetracycline (Chhabra and Eastin, 1984). Thus, understanding the changes substances undergo in the GI tract, their possible effect on the GI tract, and whether they are absorbed or affect the absorption of other substances is critical to an understanding of food toxicology and safety assessment (Kotsonis and Burdoch, 2013).

Transporters and Absorption

The constituents of food and other ingesta (e.g., drugs, contaminants, inhaled pollutants dissolved in saliva and swallowed) are physicochemically heterogeneous and, as a result, the intestine has evolved into a relatively impermeable membrane with specialized mechanisms of absorption. Recent progress in transporter research has changed our understanding of the role of various membrane transport proteins in regulating the absorption, distribution, and excretion of food, drugs, and other substances. The role of some transporters in the absorption of various macro- and microingredients in food is shown in Table 27-3. The strategic localization of transporters on the apical versus the basolateral side of the cells is crucial for proper directional transport. It has been known for a long time that hydrophilic molecules need specialized mechanisms for transport through the cell membrane. Even hydrophobic molecules need to be transported by specific transporters in order to maintain a tightly regulated cellular homeostasis. This is contrary to the traditional thinking that assumed that hydrophobic molecules could simply diffuse through the plasma membrane. The transporters may be active transporters or facilitative transporters. In *primary active transport* (1° active), ATP directly provides the energy needed to move a molecule against its concentration gradient. In *secondary active transport* (2° active), the electrochemical gradient of an ion (e.g., Na^+ or H^+), already established through primary active transport, is utilized to move a molecule across the membrane. In *facilitative (facilitated) or passive transport*, transporters passively transport substrates down the concentration gradient from high to low concentration (Choudhuri and Chanderbhan, 2016).

Table 27-3

An Overview of Some Nutritionally Relevant Transporters

TRANSPORTER	*SLC* (GENE) FAMILY DESIGNATION	KNOWN SUBSTRATES
	Sugar Transporters	
SGLT (2° Active) SGLT1—high-affinity but low-capacity glucose transporter that also transports galactose SGLT2—low-affinity but high-capacity glucose transporter that does not transport galactose	*SLC5A*	Glucose, galactose
GLUT (Facilitative) The major glucose transporter across the basolateral membrane	*SLC2A*	Glucose, fructose, myo-inositol, glucosamine, dehydroascorbate
	Amino Acid Transporters	
AA TRANSPORTERS (2° Active or Facilitative depending on the transporter)	*SLC 1A, 6A, 7A, 16A, 36A, 38A, 43A*	Amino acids, monocarboxylates (e.g., lactate, pyruvate)
	Peptide Transporters	
PEPT (2° Active)	*SLC15A*	Dipeptides, tripeptides
	Fatty Acid Transporters	
MCT (2° Active)	*SLC16A*	Short-chain fatty acids (monocarboxylates, e.g., acetate, lactate, pyruvate, butyrate, propionate, succinate)
FATP	*SLC27A*	Long-chain fatty acids
	Nucleoside Transporters	
CNT (2° Active)	*SLC28A*	Nucleosides
ENT (Facilitative)	*SLC29A*	Nucleosides
	Water-Soluble Vitamin Transporters	
SVCT (2° Active)	*SLC23A*	Vitamin C (L-ascorbate)
SMVT (2° Active)	*SLC5A6*	Biotin, pantothenate, α-lipoate
PCFT (2° Active)	*SLC46A1*	Folate, antifolate (e.g., methotrexate)
RFC1 (2° Active)	*SLC19A1*	Folate, antifolate (e.g., methotrexate)
THTR (Facilitative)	*SLC19A2 & SLC19A3*	Thiamine
	Fat-Soluble Vitamin Transporters	
SR-B1		Vitamin D, vitamin E, lutein, lycopene, cholesterol, cholesteryl esters
NPC1L1		Vitamin D, vitamin E, cholesterol
	Other Transporters	
OCTN, CT2 (2° Active or Facilitative depending on the transporter)	*SLC22A*	L-Carnitine
TAUT	*SLC6A6*	Taurine
ZIP	*SLC39*	Metals (e.g., Zn)
ZnT	*SLC30*	Metals (e.g., Zn)
Nramp (Nramp1; Nramp2 also known as DCT1/DMT1)	*SLC11A*	Divalent metals and iron

SOURCE: Modified from Choudhuri and Chanderbhan (2016, chap. 7). © Elsevier. With permission from Elsevier.

Once inside the cell, the nutrients exert a multitude of effects from providing energy to regulating specific gene expression and cellular homeostasis, and the survival/death of the cell. Readers are referred to http://slc.bioparadigms.org/ for the detailed classification of the solute carrier (SLC) family of transporters.

Like food, water is absorbed by special means in the GI tract. Fluid transport is a major function of the GI tract, with more than 9 L of fluid being absorbed or secreted across epithelia in human salivary gland, stomach, the hepatobiliary tract, pancreas, small intestine, and colon (Ma and Verkman, 1999). The movement of water across the cell membrane can occur by several mechanisms. The membrane phospholipid bilayer is basically permeable to small polar molecules, and water can slowly permeate the bilayer by simple diffusion. But simple (passive) diffusion is a

slow process that does not account for the transcellular transport of large volumes of water on a daily basis. The general paradigm of water absorption in the GI tract is that water is cotransported along with ions and solutes. For example, Loo et al. (1996) expressed Na^+/glucose cotransporter SGLT1 in *Xenopus* oocyte and demonstrated that the transport of 260 water molecules was directly coupled to each glucose molecule transported. Water is also transported by water channels (aquaporins). Aquaporins are a family of small, integral membrane proteins. The primary function of most aquaporins is to serve as channels in the transfer of water across cell membranes, and in some cases, small solutes. Water transport through aquaporins occurs in response to osmotic gradients created by active solute transport. In addition to water, aquaporins serve in the permeation of small molecules such as glycerol and urea. The mammalian aquaporins are expressed in many cell types involved in fluid transport, including epithelia and endothelia in the kidney, lung, exocrine glands, eye, and GI organs (Takata et al., 2004; Verkman, 2013).

Sometimes a transporter may transport multiple food ingredients as substrates but with vastly different affinities. For example, biotin (vitamin B7) transport is mediated by the Na^+-dependent multivitamin transporter (SMVT; gene *SLC5A6*), which also transports pantothenic acid (vitamin B5) and α-lipoic acid (a metabolic antioxidant) (Prasad and Ganapathy, 2000). The SMVT is a very high affinity transporter of α-lipoic acid, which can inhibit the transport of both biotin and pantothenic acid with an inhibition constant (K_i) of about 5 μM (Choudhuri and Chanderbhan, 2016). Lipoic acid is present in many dietary supplements. It seems logical that separating the intakes of such nutrients/ingredients by several hours might ensure proper absorption from the small intestine. An example of how a drug/xenobiotic transporter may play a protective role from the toxicity of normal food constituents comes from the study of *Abcg2*$^{-/-}$ mice. ABCG2 in humans (Abcg2 in mice), also referred to as BCRP (Breast Cancer Resistance Protein), is an ATP-binding cassette (ABC) transporter that plays an important role in the efflux of many drugs and xenobiotics. Jonker et al. (2002) showed that *Abcg2*$^{-/-}$ mice were extremely sensitive to pheophorbide-a, which is a breakdown product of dietary chlorophyll. These mice had severe phototoxic lesions on light-exposed skin. Because Abcg2 extrudes pheophorbide-a from cells, the lack of Abcg2 activity results in the accumulation of pheophorbide-a in cells. Therefore, low or no Abcg2 activity (due to inherited genetic mutations) may make an organism susceptible to developing diet-dependent photosensitization and even phototoxicity.

FOOD CONSTITUENTS AND GENOTYPIC VARIATIONS: NUTRITIONAL IMPLICATIONS OF INTER-INDIVIDUAL GENETIC DIFFERENCES

There are inter-individual genetic differences in taste preference, food tolerance, metabolism, absorption of food constituents, and other nutrition-related characteristics in human population (Lampe, 2009). The evolution of *Homo sapiens* was intimately associated with adaptive radiation to different environments. Many genotypic variations that conferred selective advantage in an environment were fixed in a population. In terms of food, the impact of selection pressures was reciprocal; that is, just as a genotypic variation could determine the ability to utilize food constituents, food habits could also drive the evolution of traits necessary for nutritional adaptation. However, not all genotypic variants are adaptive in nature; some could even be maladaptive. Certain null alleleic variants can make an individual unable to utilize a food constituent. A classic example discussed in all genetics textbooks is phenylketonuria (PKU), which is an inherited disorder caused by mutations in both alleles of the gene for phenylalanine hydroxylase. Individuals afflicted with PKU have increased levels of the amino acid phenylalanine in the blood because they are not able to metabolize dietary phenylalanine. If PKU is not treated, phenylalanine can build up to harmful levels in the body, causing intellectual disability and other serious health problems.

Many of these genotypic variations are allelic variants created through single nucleotide polymorphisms (SNPs; pronounced *snips*). SNPs and copy number variations (CNVs) are the two common types of heritable variations that are responsible for many apparent inter-individual genetic differences in a population. In the human genome, about 65% of all SNPs involve C→T (transition) mutations. It is thought that many of the SNPs and CNVs can predispose people to disease or influence their response to drugs and xenobiotics. The frequency of occurrence of SNPs in the human genome is about one per 1000 bases, but SNPs that make up about 90% of all human genetic variations occur every 100 to 300 bases in the genome. The inter-individual differences in the ability to produce functional enzymes, transporters, and other proteins that aid in the digestion, transport, and assimilation of nutrients are important determinants of personalized nutrition (Choudhuri, 2009).

Genotypic Variations and the Ability to Utilize Food Constituents

There are a number genotypic variants that determine an individual's ability to utilize nutrients in food—both macro- and micronutrients. The lack of ability to utilize the nutrients often results in mild-to-severe pathological conditions. For example, two polymorphisms in the methylenetetrahydrofolate reductase (*MTHFR*) gene [C677T→Ala222Val (A222V) and A1298C→Glu429Ala (E429A)[1]] are associated with higher homocysteine levels in the blood; the C677T polymorphism being the most common. The C677T variant encodes the thermolabile form of the MTHFR enzyme and it is recognized as the most common genetic cause of hyperhomocysteinemia. The TT homozygous individuals (C677T mutation in both alleles) have mildly elevated homocysteine levels and low-serum folate levels. The frequency of this polymorphism is the highest in Mexico (32%) and the least in Africa (6%). The A1298C does not cause increased homocysteine levels in AC heterozygous or CC homozygous individuals, but combined heterozygosity of A1298C and C677T results in an outcome similar to TT homozygous individuals (van der Put et al., 1998; Wilcken et al., 2003). Carriers of these mutations could be at higher risk of cardiovascular disease (Choudhuri and Chanderbhan, 2016).

Another example of a genotypic variation that impacts the ability to use food ingredients is glutathione *S*-transferase M1 (GSTM1)

[1]A1298C→Glu429Ala means that base #1298 in the open reading frame (ORF) of the cDNA has mutated from Adenine (A) to Cytosine (C). This SNP can also be written as 1298A>C. This A1298C mutation in the ORF has changed the amino acid #429 in the protein from Glutamic Acid to Alanine; hence, Glu429Ala (E429A). The recommendations for designating changes in DNA and protein have been developed by the Human Genome Variation Society, which is an affiliate of the International Federation of Human Genetics Society, and the Human Genome Organization (commonly referred to as HUGO). In the current text, the use of SNP designations is based on that of the original article.

deficiency. Mutations downregulating GSTM1 expression is associated with more rapid excretion of sulforaphane, an isothiocyanate with anti-tumor effects. Because sulforaphane is obtained from cruciferous vegetables, such as broccoli, brussel sprout, and cabbage, individuals with this mutation may not be able to derive the full benefit of consuming cruciferous vegetables. Likewise, polymorphisms in the genes encoding UDP-glucuronosyltransferase (*UGT*) and sulfotransferase (*SULT*) may contribute to variability in phytochemical (e.g., flavonoid) clearance and efficacy (Lampe, 2009; Lampe et al., 2013).

Human milk contains about 200 oligosaccharides that contain 3 to 22 sugar residues (German et al., 2008). These oligosaccharides modulate the microbiota of breast-fed infants (Wang et al., 2015). However, some mothers are deficient in glycosyl transferase enzymes involved in the synthesis of specific oligosaccharides. For example, 2′-fucosyllactose (2′-FL) is a highly abundant oligosaccharide in the milk of most mothers. However, mothers who are deficient in the enzyme α-(1→2)-fucosyltransferase (FUT2) do not produce the 2′-FL in their milk, leading to the nonsecretor phenotype. The human milk oligosaccharides apparently provide protection and enhance the development of the GI and immune systems of breast-fed infants. Thus, genetic differences among mothers can determine a baby's exposure/lack of exposure to specific oligosaccharides from mother's milk (Muñoz et al., 2013).

Lactose malabsorption, commonly also referred to as lactose intolerance,[2] is due to the cessation of expression of the enzyme lactase with age in certain populations. In certain populations the lactase expression is maintained throughout life. Such difference is determined by genotypic difference involving specific SNPs. It has been proposed that the evolution of this trait was the result of nutritional adaptation driven by the food habit (discussed below in detail).

Dietary iron is found in heme and ionic (non-heme) forms and their absorption occurs at the apical surface of the enterocytes of the small intestine by different mechanisms. Heme iron is absorbed by an as yet unidentified, heme receptor (Siah et al., 2006). The metabolism of non-heme iron involves a complex interplay of several proteins, starting from the intestinal divalent metal transporter 1 (DMT1) to the intracellular iron storage protein ferritin. Interestingly, DMT1 is upregulated in iron-deficient diet and it also transports cadmium and lead. Therefore, long-term consumption of iron-deficient diet contaminated with cadmium (such as, the rice in the diet) or lead may result in a significant increase in the body burden of these heavy metal contaminants through food (Park et al., 2002; Bressler et al., 2004). The dysregulation of many of these proteins can cause iron overload in the body and precipitate hemochromatosis. There are several forms of hemochromatosis of which Type 1 hemochromatosis is known as the primary hereditary hemochromatosis. It is an autosomal recessive disorder involving the *HFE* gene on chromosome 6. Two mutations in the *HFE* gene most relevant to the onset of this disease are G845A→Cys282Tyr (C282Y) and C187G→His63Asp (H63D). About 85% to 90% of the affected individuals are homozygous for C282Y mutation (genotype AA) whereas about 5% of the affected individuals are compound heterozygous for C282Y and H63D mutations (genotype: GA at position 845 and CG at position 187) (Siah et al., 2006). Another mutation A193T→Ser65Cys (S65C) localized in the vicinity of H63D also contributes to the etiology of primary hereditary hemochromatosis (Mura et al., 1999). Primary hemochromatosis mainly affects Caucasians of Northern European descent, and is extremely rare in other ethnic groups.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a genetic disorder that occurs mostly in males because the gene is X-linked. Normally RBCs have a life span of 120 days in the circulation. In individuals affected by G6PD deficiency the life span of RBCs is shortened because of premature hemolysis, resulting in hemolytic anemia. In people with G6PD deficiency, hemolytic anemia can be triggered by certain foods, drugs, and infection. For example, eating fava beans or inhaling pollen from fava plants (*Vicia faba*) can trigger hemolytic anemia. This adverse reaction to fava beans is called favism. Males of Asian, Middle Eastern, or African descent are more prone to this hereditary disorder. Many missense mutations causing single amino acid substitutions of G6PD protein have been described (Vulliamy et al., 1993). Oxidative stress plays an important role in the development of favism. Because G6PD helps in the formation of NADPH, and NADPH helps in the regeneration of GSH from GSSG, G6PD-deficient erythrocytes are NADPH-deficient, high in GSSG, and are consequently prone to oxidative stress-induced damage. Oxidative stress mediates the opening of cation channels that triggers a series of events, such as the entry of Ca^{2+} into the erythrocytes, activation of scramblases, loss of membrane asymmetry, and ultimately hemolysis (Ganesan et al., 2012).

Another example of genotypic variations limiting the uptake of the micronutrient zinc (Zn) comes from the mutations in Zn transporters. At least two zinc transporters, ZIP4 and ZnT2, are now known to be involved in the genetics of zinc deficiency. ZIP4 is an uptake transporter that is involved in intestinal zinc uptake. Mutations in ZIP4, inherited as an autosomal recessive trait, can cause acrodermatitis enteropathica (AE). In contrast, mutations in ZnT2 cause transient neonatal zinc deficiency (TNZD) with symptoms similar to AE but with autosomal dominant inheritance. This is because ZnT2 is responsible for supplying human milk with zinc. The two diseases can be distinguished in affected infants. AE is fatal if zinc is not supplied to the infant after weaning, whereas TNZD is a genetic defect of the mother limiting the supply of zinc in the milk, and therefore the infant usually will obtain enough zinc once weaned (Kasana et al., 2015). Some mutations in ZIP4 and ZnT2 are discussed as follows.

A total of 34 mutations (missense, nonsense, splice-site, and frameshift insertion–deletion mutations) have been reported in the *SLC39A4* gene that codes for the ZIP4 protein. A vast majority of these mutations exist in a homozygous or compound heterozygous state (Küry et al., 2016). Several mutations in mother's *SLC30A2* gene-encoding mutant ZnT2 have also been reported. Heterozygous mutations resulting in His54Arg (H54R) and Gly87Arg (G87R) mutations in ZnT2 lead to a 75% reduction in the secretion of zinc into the human milk. Infants fed on such Zn-deficient milk develop TNZD (Chowanadisai et al., 2006; Lasry et al., 2012). Itsumura et al. (2013) identified two additional missense mutations in the *SLC30A2* gene in a Japanese mother, resulting in a 90% reduction in Zn concentrations in her milk. These were T454C transition in exon 4 causing a Trp152Arg (W152R) mutation, and C887T transition in exon 7 causing a Ser296Leu (S296L) mutation in the ZnT2 protein.

Food Habits Driving Nutritional Adaptation During Evolution

Just as the genotype could determine the ability to utilize food constituents/nutrients, food habits could also drive the evolution of traits necessary for nutritional adaptation. The focus of research

[2]Lactose malabsorption is a common condition caused by reduced expression or activity of lactase in the small intestine. The genetic basis of lactose malabsorption has been established. In contrast, lactose intolerance is less well understood, and could be driven by a number of factors, such as lactase expression, the dose of lactose, intestinal flora, and the sensitivity of the gastrointestinal tract.

in this area has been to identify genetic adaptations to dietary specializations. The obvious candidate genes are those that encode enzymes, which play important roles in nutrient metabolism. To date, the genes coding for amylase (*AMY1*), lactase (*LCT*), and alcohol dehydrogenase (*ADH*) enzymes provide three of the most interpretable examples of genetic adaptations to dietary specializations in human metabolism (Luca et al., 2010).

Starch Digestion An example of how gene CNV may play a role in nutritional adaptation comes from the study by Perry et al. (2007). The alpha-amylase (*AMY1*) gene encodes salivary alpha-amylase enzyme that helps digest starch. Salivary amylase can persist in the stomach and intestine after swallowing, thereby augmenting the enzymatic activity of pancreatic amylase (Luca et al., 2010). There is a direct correlation between the *AMY1* gene copy number and salivary amylase content. When seven different populations were examined for their salivary amylase content and the diploid *AMY1* gene copy number, it was found that the high-starch consuming populations (e.g., agricultural populations of European Americans, Japanese, and Hazda hunter-gatherers) had a higher diploid *AMY1* gene copy number (median 7) compared to the low-starch consuming populations (e.g., Biaka and Mbuti rainforest hunter-gatherers, Datog pastoralists, and Yakut pastoralist and fishing society) (median 5). Further comparison of chimpanzee, Bonobo (an ape), and human *AMY1* genes suggest that (1) the expansion of *AMY1* gene copy number took place in human lineage, suggesting that a shift toward high-starch diet resulted in positive selection of the high CNVs of the *AMY1* gene in the population, and (2) the selection appears to be ongoing even after the evolution of *Homo sapiens* (Choudhuri and Chanderbhan, 2016).

Lactose Digestion An independent evolution of convergent adaptive traits occurred in response to selection pressure related to milk consumption. The lactase gene (*LCT*) is expressed in the intestine, and it produces the enzyme lactase that digests the lactose in milk. Most mammals, including humans, lose the ability to digest lactose with age due to reduced lactase expression. The persistence of lactase expression phenotype, known as lactase persistence (LP), and the continuing ability to digest milk lactose is inherited as a dominant genetic trait. The LP is a common phenotype only in populations with a long history of pastoralism and milk production (Luca et al., 2010). The LP phenotype is high among northern Europeans (more than 90% in Swedes and Danes), decreases across southern Europe and the Middle East (about 50% in Spanish, French, and pastoralist Arab populations), and is low in the African and Asian agriculturalists (about 5% to 20% in West African and about 1% in Chinese). Notably, LP is also prevalent in African pastoralist groups, like the Tutsi (about 90%) and Fulani (about 50%) (Ye and Gu, 2011). Two SNPs, C/T(−13910)[3] and G/A(−22018) identified in the enhancer region of the *LCT* gene are associated with LP phenotype in European populations. The C/T(−13910) is proposed to be the causal regulatory site because of its strong correlation with the LP phenotype (Ye and Gu, 2011). Surprisingly, C/T(−13910) is present in low frequency in a few West African pastoralist populations and absent in others, suggesting the existence of different genetic variants for the LP phenotype in Africans. The genetic variants determining the LP phenotype in Africans were identified through a genotype–phenotype association study by Tishkoff et al. (2007). The authors identified three SNPs [G/C(−14010), T/G(−13915), and C/G(−13907)] that are associated with the LP phenotype, as confirmed by enhanced in vitro transcription of the alleles containing these SNPs. These SNPs originated on different haplotype backgrounds compared to that of the European C/T(−13910) SNP. Subsequently, Enattah et al. (2008) identified two new population-specific SNPs determining the LP phenotype among Middle Easterners: T/G(−13915) within the −13910 enhancer region and a synonymous T/C(−3712) SNP in exon 17 of the *MCM6* gene. It is interesting to note that the *MCM6* gene, which is a different gene, contains some of the regulatory regions controlling *LCT* gene expression. The absence of the European C/T(−13910) SNP in these populations was also confirmed by the authors. Because the SNPs responsible for the LP phenotypes are different in geographically isolated populations, it suggests the independent evolution of convergent adaptive traits in response to similar selection pressure (milk consumption).

Alcohol Metabolism Natural selection has led to metabolic adaptation that is protective of alcohol overconsumption related to fermentation of food. Ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH), and acetaldehyde is subsequently oxidized to acetic acid by aldehyde dehydrogenase (ALDH). The metabolism of ethanol varies widely among individuals due to genetic variations in these two genes. One of the best-studied polymorphisms influencing ethanol metabolism is *ADH1B* Arg47His (Peng et al., 2010). The *ADH1B*47His* allele (previously called *ADH2*2*) causes enhanced catalytic activity, resulting in an increased metabolism of ethanol, and increased blood levels of acetaldehyde. If acetaldehyde is not metabolized rapidly by ALDH, chronic tissue exposure to acetaldehyde could result in significant adverse consequences including cancer. Therefore, ALDH deficiency, which is seen among East Asians, together with an increased catalytic activity of ADH results in an increased blood level of acetaldehyde. This causes flushing, intolerance, and discomfort associated with alcohol drinking. Such individuals are most likely to avoid alcohol. Therefore, the *ADH1B*47His* allele indirectly serves to protect from alcoholism (Li et al., 2007). A global investigation of the *ADH1B*47His* allele frequency shows a strong geographic distribution; it is dominant in East Asian populations, but rare in European and African populations. The regionally restricted enrichment of this allele in southern China and the adjacent areas suggests positive selection. Molecular dating suggests that the emergence of this allele coincides with the origin of rice domestication in East Asia, and that there is a strong correlation between *ADH1B*47His* frequency and the age of rice domestication (Peng et al., 2010). It has been proposed that the rise of *ADH1B*47His* allele frequency was an adaptation to rice domestication and the subsequent production and consumption of fermented food and/or beverages. Because *ADH1B*47His* is involved in protection from alcohol overconsumption-related adverse effects, the fixation of this allele was favored by natural selection (Peng et al., 2010).

FOOD CONSTITUENTS AND REGULATION OF GENE EXPRESSION

One of the earliest examples of nutrient-mediated modulation of gene expression came from the discovery of the lactose operon in *Escherichia coli*. Growing *E. coli* in lactose instead of glucose led to the de novo transcription and expression of three enzymes:

[3]C/T(−13910) means that base #13910 upstream of the transcription start site in the gene (i.e., to the left of the transcription start site in the linear sequence) has mutated from C to T. This can also be represented as −13910C>T or C−13910T or C/T_{-13910}. Therefore, SNP notation with a "−" sign represents a mutation in the regulatory region of the gene. Depending on the distance from the transcription start site, the mutation may fall in the proximal or distal promoter, or further upstream in the enhancer. Obviously −13910 position falls in the enhancer region of the gene.

β-galactosidase, *trans*-acetylase, and permease that are all involved in the uptake and utilization of lactose as the carbon source. Likewise, in higher eukaryotes nutrients such as glucose, amino acids, fatty acids, as well as vitamins and minerals are known to regulate gene expression (Choudhuri and Chanderbhan, 2016).

Carbohydrates and Gene Expression

Glucose homeostasis is tightly regulated in the body to meet the energy demands during the starvation/feeding cycle in animals. Red blood cells and the brain primarily rely on a direct supply of glucose as their fuel source. Therefore, for most genes regulated by a rich carbohydrate diet, the induction or inhibition of their mRNAs is substantial and relatively rapid, and is reversible once the carbohydrate availability decreases. Both insulin and glucose contribute to the coordinated regulation of carbohydrate and lipid metabolism in the liver (Vaulont et al., 2000; Dentin et al., 2005).

Fasting causes low-insulin levels, and activates glucose production and lipolysis. In contrast, feeding a carbohydrate-rich diet after fasting activates fat synthesis by enhancing the rate of fatty acid synthase transcription (Fukuda et al., 1999). Many enzymes involved in carbohydrate metabolism are induced in the liver and other tissues by a diet high in carbohydrates. For example, Iynedjian et al. (1987) demonstrated that in adult rats, *glucokinase* mRNA was low during fasting but it increased more than 50-fold above the fasting level within 6 hours of an oral glucose load, and the mRNA level returned toward basal values by 18 hours after refeeding. A high carbohydrate (65% sucrose) diet also induces *L-type pyruvate kinase* (*L-PK*) and *aldolase B* mRNAs in rats (Weber et al., 1984). In livers from starved rats, *L-PK* mRNA was practically undetectable. A carbohydrate-rich diet increased both mRNA concentrations, with a maximum being between 12 and 18 hours, and the increase being about 4 to 8 times for *aldolase B* mRNA and 40 to 100 times for *L-PK* mRNA. Transcription was maximal at 12 hours after refeeding, decreasing to 10% of maximum at 72 hours. The level of transcribed *L-PK* mRNA remained constant at 50% of maximum for at least 120 hours (Vaulont et al., 1986). Glucose also induces the expression of glucose transporters and lipogenic enzymes, such as acetyl CoA carboxylase (ACC) (Pape et al., 1988) and fatty acid synthase (FAS) (Paulauskis and Sul, 1989), and represses the expression of genes of the gluconeogenetic pathway, such as phosphoenolpyruvate carboxykinase (PEPCK) (Kahn et al., 1989). Both insulin and glucose can independently repress *PEPCK* gene expression. Normally, PEPCK expression is induced by glucagon but is dominantly inhibited by glucose-induced increases in insulin secretion upon feeding (Kahn et al., 1989).

Based on a number of observations, Mourrieras et al. (1997) proposed that *glucose-6-phosphate (G6P) is the most likely signaling molecule regulating the glucose-induced transcription of the target genes*. The conversion of glucose to G6P is carried out by glucokinase, whose expression is strongly activated by insulin. Glucose-6-Phosphatase (G6Pase), the enzyme that breaks down G6P to release glucose, is active in the liver, kidneys, and intestines, and is the main regulator of glucose production in the liver. Insulin reduces G6Pase expression (mRNA and protein levels) in the fed and refed state where insulin levels are elevated (Argaud et al., 1996). In contrast, a low-glucose level dramatically increases G6Pase expression (mRNA and protein levels) so that more glucose can be obtained from the glycogen stores to meet the cellular energy need. Therefore, in a fed and refed state, when both insulin and glucose levels are high, insulin exerts a dominant negative effect. The dominant negative effect of a high-insulin level on glucagon-mediated *PEPCK* gene expression and *G6Pase* gene expression is yet another testimony of insulin's central role in regulating carbohydrate metabolism.

Two transcription factors—the sterol regulatory element (SRE)-binding protein 1c (SREBP-1c) and the carbohydrate response element (ChRE)-binding protein (ChREBP)—play crucial roles in insulin- and glucose-mediated transcriptional regulation of genes involved in carbohydrate metabolism (Foufelle and Ferré, 2002; Dentin et al., 2005; Shankar et al., 2010).

SREBP-1c is a major mediator of insulin action on hepatic glucokinase and lipogenic gene expression. It is rapidly and robustly induced by a carbohydrate-rich diet (resulting in a high-insulin level) and downregulated by fasting (resulting in a low-insulin level). The expression of *SREBP-1c* gene itself is under the control of the nuclear receptor transcription factor liver-X-receptor (LXR) (Espenshade, 2006). For nuclear receptors (NRs) that form heterodimers with the retinoid-X-receptor (RXR), the NR–RXR (in this case LXR-RXR) heterodimer binds to the corresponding response element (NR-RE; in this case LXRE) in the promoter of the target genes. The induced SREBP-1c binds to the SREs in the promoter of the target genes, such as *glucokinase*, *ACC*, *FAS* (Dentin et al., 2005). Fig. 27-1 shows the relationship between the glycolytic and lipogenic pathways.

The ChREBP is another important regulator that mediates the effects of glucose; it is predominantly expressed in the liver, kidney, and white and brown adipose tissue. ChREBP forms a heterodimer with Max-like protein X (Mlx) that binds to the carbohydrate response element (ChRE) located in the promoters of glucose target genes, such as the *L-PK*, *FAS*, and *ACC*. The ChREBP–Mlx complex is able to discriminate between the E-box sites that are glucose-responsive and those that are not (Stoeckman et al., 2004). Fig. 27-2 shows possible signaling pathways for transcriptional regulation of target genes in the liver mediated by glucose and insulin.

Studies in humans with high- and low-carbohydrate diets showed differential gene expression in subcutaneous fat as well. In individuals fed a rye-pasta diet (low postprandial insulin response), 71 genes showed decreased expression and none showed increased expression. In contrast, in individuals fed an oat–wheat–potato diet (high postprandial insulin response), 62 genes showed increased expression and none showed decreased expression (Kallio et al., 2007). Two of the noteworthy downregulated genes were the hormone-sensitive lipase (*HSL*) gene and the transcription factor *TCF7L2* gene. Downregulation of HSL, which is a key enzyme in the release of fatty acids from adipose tissue, is associated with resistance to genetic or diet-induced obesity. The transcription factor TCF7L2 is the strongest known genetic predictor of type 2 diabetes. In humans, the stable but differential expression pattern of many genes involved in carbohydrate metabolism may be rooted in geographic ancestral lineage (Schisler et al., 2009).

Amino Acids and Gene Expression

On an average, the daily protein turnover (synthesis and breakdown of proteins) in humans is more than 300 g/day; about half of the daily protein turnover comes from protein synthesis (Waterlow, 1984; Poortmans et al., 2012). About 75% of the amino acids are utilized for the resynthesis of tissue proteins. Unlike carbohydrates and fats, excess amino acids cannot be stored, but are degraded primarily in the liver. Most of the nitrogen in amino acids is converted to urea and excreted, while the carbon skeletons are either oxidized to form carbon dioxide and water or utilized for gluconeogenesis and ketogenesis.

Amino acid availability regulates the expression of many genes both in vivo and in vitro. Some examples of genes upregulated by

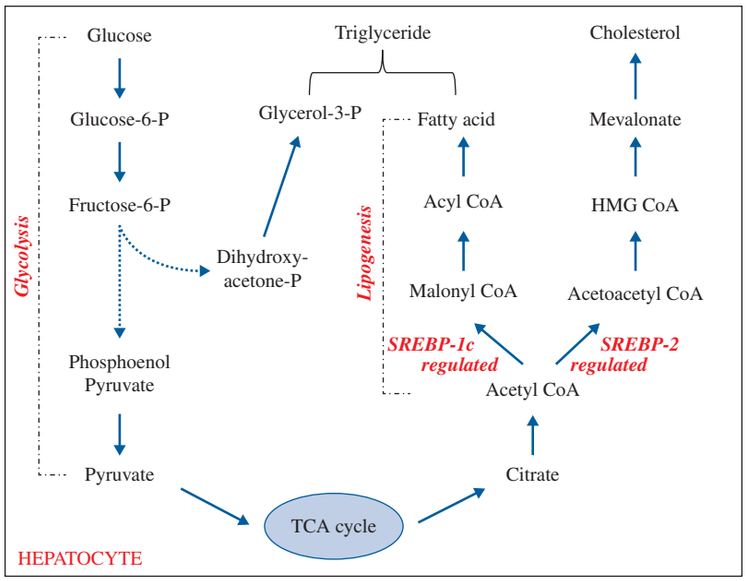

Figure 27-1. *The liver is the major site of carbohydrate metabolism (glycolysis and glycogen synthesis) and fatty acid synthesis (lipogenesis).* The precursor of fatty acid synthesis is acetyl CoA. The SREBP-1c preferentially regulates genes involved in de novo lipogenesis, but SREBP-2 preferentially regulates genes involved in cholesterol synthesis and uptake, at least in the liver.

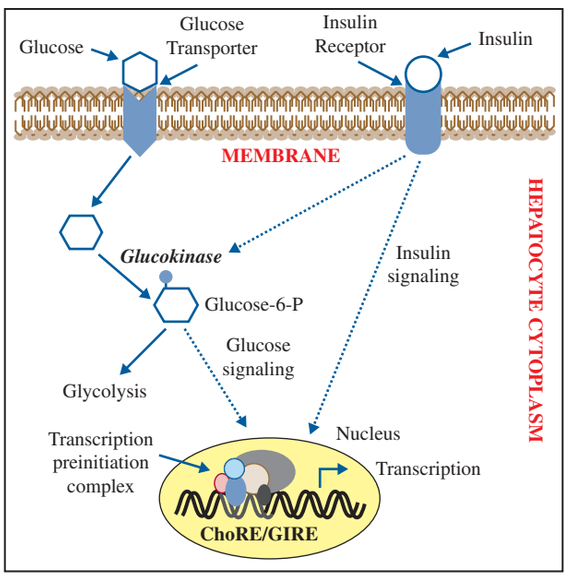

Figure 27-2. *Transcriptional regulation by glucose and insulin signaling in the liver.* Glucose-6-phophate is the most likely signaling molecule regulating glucose-induced transcription, and its formation is an important step in glucose-signaling pathway. Insulin-signaling pathway may involve insulin-responsive transcription factor SREBP-1c.

amino acid starvation include the enzymes of amino acid synthesis pathway (e.g., asparagine synthetase, glutamine synthetase), enzymes of the urea cycle (e.g., argininosuccinate synthetase), the rate-controlling enzyme in polyamine biosynthesis pathway (e.g., ornithine decarboxylase), transporters (e.g., sodium-coupled neutral amino acid transporter SNAT2, cationic amino acid transporter CAT1, amino acid transporter ATA2), ribosomal proteins (e.g., L17, L35, S13, and S25), transcriptional activator proteins, such as CCAAT/enhancer-binding protein (C/EBP) and C/EBP homologous protein (CHOP), insulin-like growth factor-binding protein-1 (IGFBP-1), and proto-oncogenes (jun and myc) (Kilberg et al., 1994, 2012; Fafournoux et al., 2000; Jousse et al., 2004; Brasse-Lagnel et al., 2009).

Overexpression of IGFBP-1 (e.g., in transgenic mice) causes growth defect. Growth defect is also seen in amino acid deficiency. Consistent with these facts was the finding that strong induction of *IGFBP-1* mRNA in the liver was seen following feeding rats with a low-protein diet for 10 days. Amino acid starvation has also been reported to induce *IGFBP-1* mRNA in cultured cells (Jousse et al., 2004). Amino acid supplementation results in the upregulation of a number of genes. For example, a high concentration of tryptophan enhances the expression of collagenase and tissue inhibitors of metalloproteinases. The expression of argininosuccinate synthetase and ornithine decarboxylase, which are induced by amino acid starvation, is also upregulated in response to high amino acid supplementation. The majority of the mRNA increase by amino acid starvation or supplementation is through an increase in de novo transcription (mRNA synthesis), but mRNA stabilization also plays important roles. For example, the increase in *CHOP* mRNA, at least in part, involves mRNA stabilization. High glutamine concentration induces the accumulation of *argininosuccinate synthetase* mRNA in isolated hepatocytes. Similarly, high asparagine concentration induces *ornithine decarboxylase* mRNA expression primarily by post-transcriptional stabilization of the mRNA, but also by stimulating the synthesis and suppressing the degradation of ornithine decarboxylase protein (Fafournoux et al., 2000). The translation of mRNAs of some of these amino acid–regulated genes is initiated through internal ribosomal entry sites (IRES).[4]

[4]IRES facilitate cap-independent translational initiation, and are located at the 5′-untranslated region of certain "survival" mRNAs whose expression is upregulated during severe cellular stress. Many viral mRNAs employ the same mechanism to hijack the host's translation machinery.

The current knowledge on the amino acid regulation of gene expression has developed mostly from detailed studies on the upregulation of two genes, *CHOP* and asparagine synthetase (*AS*), in response to amino acid starvation. *It may be emphasized here that cellular stressors like nutrient and energy deprivation activate autophagy, which is the primary catabolic program.*

The *CHOP* gene expression is strongly induced by cellular stress, such as oxidative stress, DNA damage, and amino acid starvation. CHOP is a nuclear protein related to the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors. Leucine limitation in human cell lines results in the induction of *CHOP* mRNA and protein in a dose-dependent fashion (Averous et al., 2003). The *CHOP* gene promoter contains the amino acid response element (AARE), which binds in vitro the activating transcription factor 2 (ATF-2) under both starved and unstarved conditions. The expression of *CHOP* mRNA during amino acid starvation is mediated by ATF-2 and ATF-4 (Bruhat et al., 2000; Jousse et al., 2004). The expression of *CHOP* mRNA is also induced by endoplasmic reticulum (ER) stress response (ERSR), also known as unfolded protein response (Patil and Walter, 2001), and is mediated by the ER stress response element (ERSRE)-binding protein ATF-6.

Asparagine synthetase (AS) is responsible for the biosynthesis of asparagine from aspartate and glutamate. It is ubiquitously expressed in most mammalian cells. The transcription of the *AS* gene is also increased in response to both amino acid and glucose starvation, the latter resulting in an ERSR as discussed above. The *AS* gene contains five specific *cis*-elements: three GC boxes (GC-I, GC-II, and GC-III) and two nutrient-sensing response elements (NSRE-1 and NSRE-2) (Leung-Pineda and Kilberg, 2002). All three GC boxes are required for basal transcription and also for the maximal induction of the *AS* gene by amino acid limitation. The genomic unit containing both NSRE-1 and NSRE-2 is called nutrient-sensing response unit (Jousse et al., 2004). Even though amino acid starvation does not cause ER stress (unfolded protein response), the amino acid starvation response pathway and the ER stress pathway appear to closely overlap. This may be due to the involvement of some common transcriptional *trans*-activators that are involved in both pathways, such as ATF-4 (Jousse et al., 2004).

The amino acid deficiency response involves a number of signaling pathways that include many proteins, but the central pathway of general control nonderepressible 2 (GCN2)–eukaryotic initiation factor eIF2–ATF4 appears to be involved in all tissues (Kilberg et al., 2012; Choudhuri and Chanderbhan, 2016).

Fatty Acids and Gene Expression

Fatty acids and their acyl-CoA derivatives serve many biological functions within the cell. They serve as metabolic energy sources, substrates for membranes, signaling molecules for metabolic regulation, and regulators of gene expression. Fatty acid regulation of gene transcription occurs in unicellular as well as complex organisms (Duplus et al., 2000).

While controversy still exists regarding the contribution of passive diffusion versus protein-mediated fatty acid transport, both processes are now widely accepted (Kalant and Cianflone, 2004). A completely passive diffusion of fatty acids across the plasma membrane is not conducive to maintaining cellular homeostasis. The uptake of fatty acid across the plasma membrane is mediated by distinct transport proteins (Table 27-3). Once within the cell, most fatty acids are non-covalently bound to fatty acid-binding proteins (FABPs). FABPs are involved in reversibly binding intracellular hydrophobic ligands, and trafficking them throughout cellular compartments, including the peroxisomes, mitochondria, endoplasmic reticulum, and nucleus (Smathers and Petersen, 2011).

The regulation of gene expression by fatty acids is dictated by the type, quantity, and the duration of exposure to dietary fats. Among short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, the effects of butyrate on gene expression have been studied by many laboratories. Butyrate is produced by anaerobic bacterial fermentation of dietary fibers. It was shown by the mid-1970s that sodium butyrate halts DNA synthesis, arrests cell proliferation, alters cell morphology, and increases or decreases gene expression (Prasad and Sinha, 1976). The observation that butyrate increased the level of acetylated histones in cultured HeLa and Friend erythroleukemic cells was a turning point in understanding the molecular mechanisms of action of butyrates in gene regulation (Davie, 2003). Butyrates were found to increase gene expression by inhibiting histone deacetylase activity, thereby increasing histone acetylation, which promotes transcriptional activation (Davie, 2003).

A large number of studies investigating the role of fatty acids on gene expression have focused on the long-chain fatty acids (LCFAs). Analyses of fatty acid regulation of gene expression have focused on several possible mechanisms, including peroxisome proliferator-activated receptor (PPAR) and prostanoid-regulated processes (Jump and Clarke, 1999). PPAR is a ligand-dependent transcription factor that heterodimerizes with RXR. Three different PPAR subtypes have been cloned—PPARα, PPARβ (or δ), and PPARγ. All three forms of PPARs bind polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA), docosahexanoic acid (DHA), and others. In addition to PUFAs that are bona fide PPAR ligands, numerous fatty acid-derived compounds showing a structural resemblance to fatty acids, such as acyl-CoAs, oxidized fatty acids, eicosanoids, endocannabinoids, and phytanic acid, have also been shown to activate PPARs (Clark, 2002).

Surprisingly, circulating free fatty acids (FFAs) that primarily originate from adipose tissue lipolysis (so-called *old fat*) cannot activate PPARα in the liver. In contrast, dietary and endogenously synthesized fatty acids (so-called *new fat*) can. This difference may be related to the existence of (as yet unknown) differential signaling properties between the two forms of FFAs. However, the plasma FFAs (the *old fat*) can activate hepatic PPARγ (Georgiadi and Kersten, 2012).

Studies in rodents demonstrated that PPARα mediates the effects of dietary fatty acids on hepatic gene expression (Choudhuri and Chanderbhan, 2016). The importance of PPARα in overall glucose and fatty acid homeostasis was demonstrated by the observations that PPARα-knockout mice lack the ability to increase fatty acid oxidation during periods of food deprivation and they develop characteristics of adult-onset diabetes, including fatty livers, elevated blood triglycerides, and hyperglycemia (Clark, 2002). *Because PPARα plays a pivotal role in both fatty acid and glucose metabolism, it was proposed to be a "master switch" transcription factor that is targeted by PUFAs to coordinately suppress genes encoding the enzymes of lipid synthesis and induce genes encoding the enzymes of fatty acid oxidation and thermogenesis* (Clark, 2002). It should be borne in mind, however, that peroxisome proliferation is a rodent-specific response mediated by PPARα. Although humans have a functional PPARα, its expression and ligand-binding affinities are different from that of mouse PPARα.

The PPARs play a critical physiological role as lipid sensors and regulators of lipid metabolism (Rogue et al., 2010). The major groups of target genes for the PPARs are genes involved in lipid and glucose metabolism. PPARα exerts its effects on the liver and muscle, whereas PPARγ exerts its effects on the adipose tissues and tumor cells. PPARα ligands include both ω-3 and ω-6 PUFAs, but ω-3 PUFAs seem to be more potent (Price et al., 2000). PPARγ is expressed at the highest level in adipose tissue whereas

its expression in the liver is low, being only 10% to 30% of the level in adipose tissue in both mice and humans. Prostaglandin PGJ_2 is a PPARγ ligand; it binds and activates PPARγ. The effects of PPARγ on lipid metabolism are mainly mediated by genes expressed in adipocytes.

Fatty acids also regulate the expression of various transcription factors, such as SREBP-1c, LXR, farnesoid-X-receptor (FXR, the bile acid–activated nuclear receptor), hepatic nuclear factor 4α (HNF-4α), and NF-κB (Georgiadi and Kersten, 2012), thus regulating the expression of the target genes of these transcription factors. Mammalian cells produce several isoforms of SREBPs. The importance of SREBP-1a, SREBP-1c, and SREBP-2 in regulating the expression of genes relevant to carbohydrate and lipid metabolism has been studied more extensively. SREBP-1a and -1c are the products of alternative splicing but SREBP-2 is encoded by a different gene (Hua et al., 1995). Unlike typical transcription factors, SREBPs are synthesized as integral membrane proteins localized in the endoplasmic reticulum. The N- and the C-terminal domains are cytosolic (Fig. 27-3). *The N-terminal domain of the SREBP is the actual transcription regulatory part of the protein and it contains a basic helix–loop–helix (bHLH)–Zip domain*. This domain is cleaved from the membrane by a sterol-regulated protease and allows it to enter into the nucleus. In the nucleus, this transcription regulatory bHLH-Zip domain binds to the enhancer sequences known as the sterol response elements (SREs) of the lipogenic genes, and activates transcription (Ye and DeBose-Boyd, 2011). The proteolytic pathway that liberates the N-terminal bHLH-Zip domain from membranes in response to intracellular levels of cholesterol and fatty acids is known as the SREBP pathway (Brown and Goldstein, 1997).

Dietary PUFAs inhibit hepatic lipogenesis through inhibition of both SREBP-1c and SREBP-2, in particular SREBP-1c (Georgiadi and Kersten, 2012). Interestingly, the ω-3 long-chain PUFAs (e.g., EPA and DHA) and the ω-6 PUFA (e.g., ARA) have far more inhibitory capacity on SREBP than do the shorter chain unsaturated fatty acids (e.g., 18:1 oleic acid, 18:2 linoleic acid), and saturated fatty acids have almost no effect on SREBP (Georgiadi and Kersten, 2012; Choudhuri and Chanderbhan, 2016). The SREBP-1c preferentially activates genes involved in de novo lipogenesis, but SREBP-2 preferentially activates genes involved in cholesterol synthesis and uptake, at least in the liver (Fig. 27-1). Because PUFAs antagonize LXR, PUFAs inhibit the transcription of LXR-regulated genes, such as *SREBP-1a* and *SREBP-1c*, in humans (Hannah et al., 2001).

Dietary fatty acids can also regulate the expression of proteins associated with micronutrient metabolism. It is well known that vitamin A absorption in the intestine is stimulated by the presence of fat. The modulation of intestinal vitamin A absorption is the result of a dietary fat-inducible cellular retinol-binding protein II (CRBPII), which plays a pivotal role in the intestinal absorption and metabolism of retinol and β-carotene. In mice, unsaturated fatty acids (e.g., oleic, linoleic, and α-linolenic acids) enhance *CRBPII* mRNA levels, whereas medium-chain fatty acids and saturated fatty acids have little effect. Consistent with this finding, feeding a fat-free diet repressed *CRBPII* mRNA accumulation in the small intestine by 50% within a day, and this low level was sustained over the following 9 days. The molecular mechanism of such dietary fatty acid regulation was shown to involve PPARα (Takase et al., 2000).

Vitamins and Gene Expression

Like the macronutrients (protein, carbohydrate, and fat), micronutrients (vitamins and minerals) can also modulate gene expression. There are several publications discussing the potential role of vitamins in regulating gene expressions. These studies primarily relied on microarray to investigate global gene expression profiling; hence, these findings need to be tested more comprehensively in order to establish the causality. The following discussion focuses on two examples, vitamins A and D.

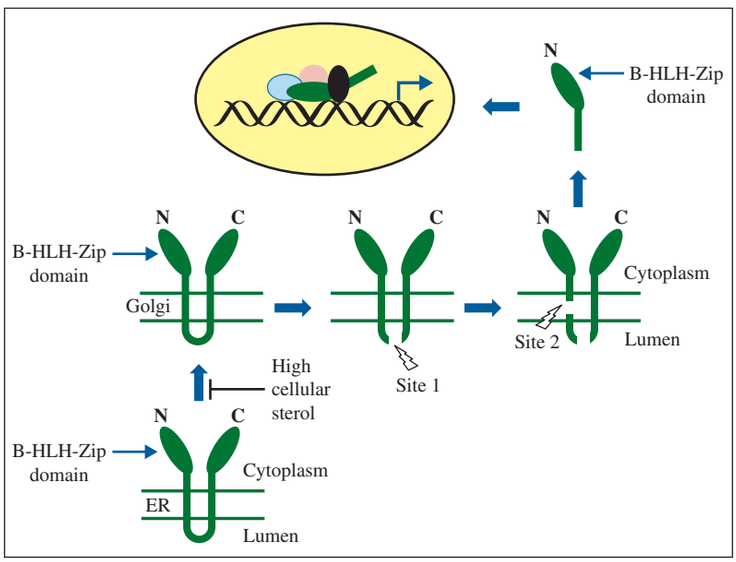

Figure 27-3. *The N-terminal domain of SREBP is the actual transcription regulatory part of the protein and it contains a basic helix–loop–helix (bHLH)–Zip domain.* The precursor of SREBP is synthesized in the endoplasmic reticulum (ER), where it is in complex with another protein, the SREBP cleavage activating protein (SCAP). SCAP contains a sterol sensory domain. In low intracellular sterol concentration, SCAP helps in the translocation of SREBP to the Golgi. This translocation is prevented when the intracellular sterol concentrations are high. Once in Golgi, the SREBP undergoes two proteolytic cleavage steps to release the mature, biologically active transcription factor that translocates to the nucleus. The nuclear SREBP binds to the sterol response element (SRE) in the promoter/enhancer regions of target genes to activate transcription.

Vitamin A-mediated regulation of gene expression is a well-known example of direct gene regulation by micronutrients. Retinoic acid, a bioactive form of vitamin A, is a ligand for the nuclear retinoic acid receptor (RAR). Ligand-activated RAR heterodimerizes with RXR; the RAR–RXR complex binds to the cognate retinoic acid response element (RARE) in the regulatory region of the target genes and regulates gene expression. As mentioned above, PEPCK-catalyzed reaction is the rate-limiting step of gluconeogenesis in animals. The expression of *PEPCK* gene is a target of retinoic acid. Retinoic acid plays an important role in development, but high levels of retinoic acid exposure can be teratogenic to the developing embryo. Thus, it is imperative that retinoic acid targets many genes including the developmentally important homeobox (*Hox*) genes, such as *Hoxa1, Hoxb1, Hoxb4,* and *Hoxd4*. All the target genes harbor RARE (Rhinn and Dollé, 2012). Dietary vitamin A levels can also modulate the expression of genes encoding insulin-like growth factor I (IGF-I), IGF-I receptor (IGF-IR), and insulin receptor (IR) in many different tissues. Serum IGF-I concentrations and tissue *IGF-I* mRNA are downregulated by vitamin A deficiency (Choudhuri and Chanderbhan, 2016).

The model of vitamin D (1,25-dihydroxyvitamin D_3)-mediated gene expression involves similar steps of events as for vitamin A, that is, vitamin D binding-mediated activation of vitamin D receptor (VDR), formation of VDR–RXR complex, its binding to VDRE, and modification of transcriptional output. Genome-wide mapping of vitamin D receptor binding revealed that vitamin D could influence over 200 genes, highlighting links to disease states (Ramagopalan et al., 2010). Vitamin D plays an important role in regulating genes involved in bone remodeling and the maintenance of bone health, such as *VDR*, *TNFSF11*, *LRP5*, *CBS*, and *CYP24a1*.

Vitamin D and the Genetics of Bone Remodeling

During bone remodeling osteoclasts resorb old bone tissue, osteoblasts deposit new bone tissue, and some osteoblasts that become trapped within the bone matrix differentiate into osteocytes. When the two functions are out-of-balance, bone health is affected. For example, when osteoclasts remove more bone than osteoblasts can deposit, the result is osteoporosis. In contrast, if osteoblasts deposit more bone than osteoclasts can remove, the result is osteopetrosis (hyper dense bones). By regulating the *VDR* gene, vitamin D regulates a wide variety of target genes that contain the VDRE. The *VDR* gene is highly expressed in osteoblasts, terminally differentiated osteocytes and probably in osteoclasts as well. The *TNFSF11* gene produces the protein RANKL that regulates osteoclast differentiation, function, and activation. In normal bone remodeling, the RANKL (ligand) is secreted by osteoblasts and binds to the RANK receptor on both osteoclast precursor and mature osteoclast cells. The RANK–RANKL interaction triggers cellular signaling events that trigger osteoclast differentiation, function, and activation. This can be inhibited by osteoprotegerin (OPG), which is produced by osteoblasts and stromal cells and acts as a decoy receptor that sequesters RANKL. Sequestration of RANKL by OPG inhibits the differentiation and activation of osteoclasts. Evidently, the RANKL/OPG ratio acts as an important determinant of bone mass and skeletal integrity by regulating the balance between bone resorption and bone deposition processes. This is also demonstrated in animal models—RANK-null ($RANK^{-/-}$) and OPG-null ($OPG^{-/-}$) mice. While RANK-null mice develop osteopetrosis-like conditions (Dougall et al., 1999), OPG-null mice develop osteoporosis-like conditions (Bucay et al., 1998). The *LRP5* gene encodes LRP5 protein that regulates osteoblast proliferation and differentiation. A loss-of-function mutation in *LRP5* gene causes a decrease in bone mass and osteoporosis, whereas a gain-of-function mutation causes drastic increases in bone mass. The *CBS* gene encodes the enzyme cystathionine β-synthase that converts homocysteine to cystathionine, which is an intermediate in the synthesis of cysteine. High homocysteine levels are a risk factor for osteoporotic fracture. Thus, individuals with cystathionine β-synthase deficiency that results in a reduction in the enzyme activity are prone to osteoporosis (Kruger et al., 2003).

Mencej-Bedrac et al. (2009) reported certain SNPs in the human *VDR*, *OPG*, and *TNFSF11* genes that are associated with bone mineral density. These SNPs predispose the carriers to osteoporosis. Vitamin D is inactivated by the vitamin D 24-hydroxylase, which is encoded by the *CYP24A1* gene. Because vitamin D upregulates the expression of *CYP24A1,* it autoregulates its own inactivation. Vitamin D also regulates a network of genes involved in bile acid metabolism in the colon, the degradation of xenobiotic compounds in several tissues, the differentiation of keratinocytes in skin, the development and cycling of dermal hair follicles, and the functions of key cell types involved in both innate and adaptive immunity (Pike and Meyer, 2010; Pike et al., 2014).

Some vitamins may also regulate gene expression through epigenetic modification of the chromatin. For example, using Jurkat lymphoma cells, Gralla et al. (2008) demonstrated that when biotin concentration in Jurkat cells was high because of high levels of biotin supplementation in the media, the lysine 12 of histone H4 (H4K12) at the SMVT promoter had high levels of biotinylation (H4K12Bio). Biotinylation of histone H4 is a transcription repressing modification. Therefore, high levels of intracellular biotin downregulated the expression of SMVT (by 86%) and resulted in a significant decrease in further biotin uptake. Thus, biotin may autoregulate its own intracellular concentration by regulating its own uptake through epigenetic regulation of SMVT.

Minerals and Gene Expression

In recent years, several nutrigenomics studies investigated global changes in gene expression by a number of minerals. The changes in gene expression profiles by the same mineral reported in different studies are not the same. Therefore, these findings are yet to be interpreted for their overall biological relevance to be able to develop a coherent narrative. The following discussion focuses on iron.

Dietary non-heme ferric iron Fe(III) is first reduced by enterocyte membrane-bound ferrireductases into ferrous Fe(II) form. The apical transporter divalent metal transporter 1 (DMT1) transports Fe(II) from the intestine into the enterocytes, thus helping in non-heme iron absorption. Heme iron is taken up by enterocytes by an as yet undefined mechanism. The Fe(II) is translocated through the enterocyte and is exported into the circulation by the basolateral exporter ferroportin. *Ferroportin is the only iron export protein known*. During export, Fe(II) is oxidized by hephaestin, which is a membrane-bound ferrooxidase. Thus, hephaestin is necessary for effective iron transport from enterocytes into the circulation. In the circulation, Fe(III) binds to plasma transferrin (Tf) to be distributed throughout the body, and taken up primarily by the liver. In the liver, the Fe(III)–Tf complex binds to the membrane transferrin receptor (TfR). The Fe(III)–Tf–TfR complex is endocytosed inside the cell. The acidic pH of the endosome releases the Fe(III), which is again reduced to Fe(II) by the intracellular ferrireductase STEAP3 (Fig. 27-4). The intracellular iron storage is regulated at multiple levels.

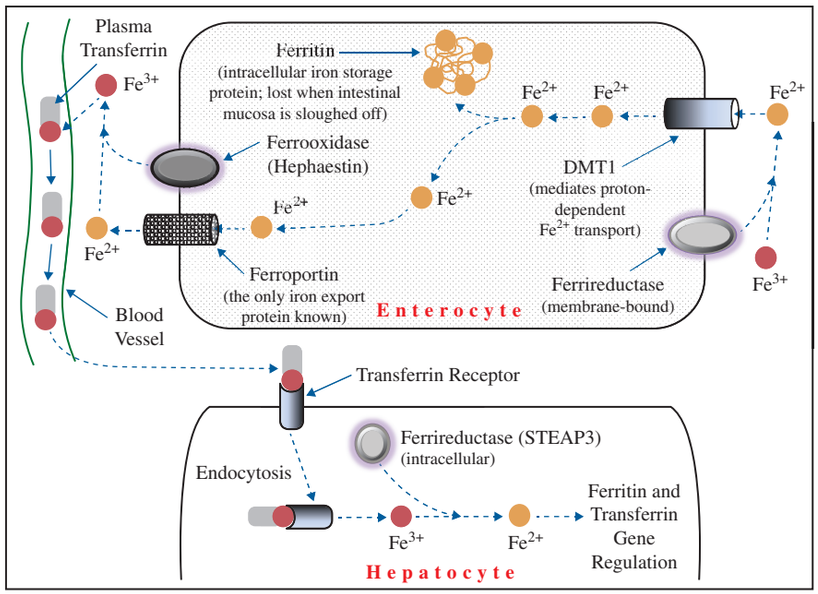

Figure 27-4. *The transport of dietary iron from the intestine to the liver.* Non-heme ferric iron Fe(III) is first reduced by enterocyte membrane–bound ferrireductases into ferrous Fe(II) form. The apical transporter divalent metal transporter 1 (DMT1) transports Fe(II) into the enterocytes. The Fe(II) is translocated through the enterocyte and is exported into the circulation by the basolateral exporter ferroportin. During export, Fe(II) is oxidized by hephaestin, which is a membrane-bound ferrooxidase. In the circulation, Fe(III) binds to plasma transferrin to be distributed throughout the body, and taken up primarily by the liver. In the liver, the Fe(III)–Tf complex binds to the membrane transferrin receptor (TfR). The Fe(III)–Tf–TfR complex is endocytosed inside the cell. The acidic pH of the endosome releases the Fe(III), which is again reduced to Fe(II) by the intracellular ferrireductase STEAP3.

When body iron stores and erythropoiesis are adequate, iron export from enterocytes into the circulation is reduced by hepcidin-mediated internalization and degradation of Fe–ferroportin complex. The iron thus released from ferroportin is stored in ferritin. Under such conditions, the hepatic iron uptake is also reduced by downregulating the expression of the transferrin receptor through degradation of the transferrin receptor mRNA. In the enterocytes, the stored iron in ferritin is lost after 3 days by desquamation of intestinal cells (Anderson et al., 2012). Iron regulation of gene expression requires the presence of a 30-nucleotide long iron response element (IRE) in the 5′-untranslated or 3′-untranslated region of the target mRNAs. The IRE forms a special stem-loop structure. The IRE binds the iron regulatory protein (IRP), also called IRE-binding protein (IRE-BP). The IRPs are cellular iron sensors. The mRNAs associated with iron transport, distribution, and storage all contain IREs, such as DMT1, ferroportin, transferrin, and ferritin mRNAs. Some other mRNAs containing IREs include that of mitochondrial aconitase and succinate dehydrogenase. Fig. 27-5 shows how the binding of the IRP–Fe complex to IRE affects mRNA stability based on the location of the IRE in the 5′-UTR versus the 3′-UTR of the target mRNA. Other dietary minerals, such as zinc (Zn) and copper (Cu), regulate gene expression by activating metal regulatory transcription factor 1 (MTF1).

Dietary Polyphenols, Indoles, Carotenoids, and Gene Expression

Dietary polyphenolic constituents present in fruits, vegetables, and spices can mediate nutrient–gene interactions by activating or inhibiting specific transcription factor pathways (Ashida et al., 2008). These include both flavonoid polyphenols (e.g., quercetin, kaempferol, apigenin, tangeretin), and non-flavonoid polyphenols (e.g., resveratrol, curcumin). Indole-3-carbinol (I3C), present in cruciferous vegetables, and dietary carotenoids (e.g., canthaxanthin, astaxanthin) also mediate nutrient–gene interactions. I3C is derived from glucobrassicin (glucosinolate) (Fig. 27-11). These dietary constituents regulate gene expression through modulation of the aryl hydrocarbon receptor (AhR) pathway. Although AhR is known for its role in effecting the toxicity of dioxins and other environmental pollutants, it also plays important roles in normal biological processes, such as regulating anti-inflammatory response, and the development of the immune system.

Table 27-4 shows some transcription factor pathways involved in nutrient–gene interactions.

FOOD–DRUG INTERACTIONS

The food and drug interactions are in most part the results of food-induced changes in drug bioavailability or metabolism, although some are the result of altered drug concentrations at the site of action and the resulting effect. In other words, food–drug interactions can alter pharmacokinetic and pharmacodynamic parameters of the drug. For drugs with a narrow therapeutic index and the need for dose titration, even small changes in the dose–response effects can have great consequences (Schmidt and Dalhoff, 2002).

Examples of dietary constituents that may produce an effect on the overall pharmacokinetics of drugs would include food or food components that change the pH of urine or simply the presence of fiber in the intestine. Examples of pharmacodynamic interactions might include the effect of unsaturated fatty acids in the diet on anticoagulants or membrane potentials of the membranes in which they become incorporated, or high potassium intake from potassium-rich foods and the risk of hyperkalemia during therapy with angiotensin-converting enzyme inhibitors or spironolactone (Schmidt and Dalhoff, 2002). Some food–drug interactions, such as the effects of dietary constituents on the induction or inhibition of CYP3A4, may be potentially important because CYP3A4 metabolizes more than

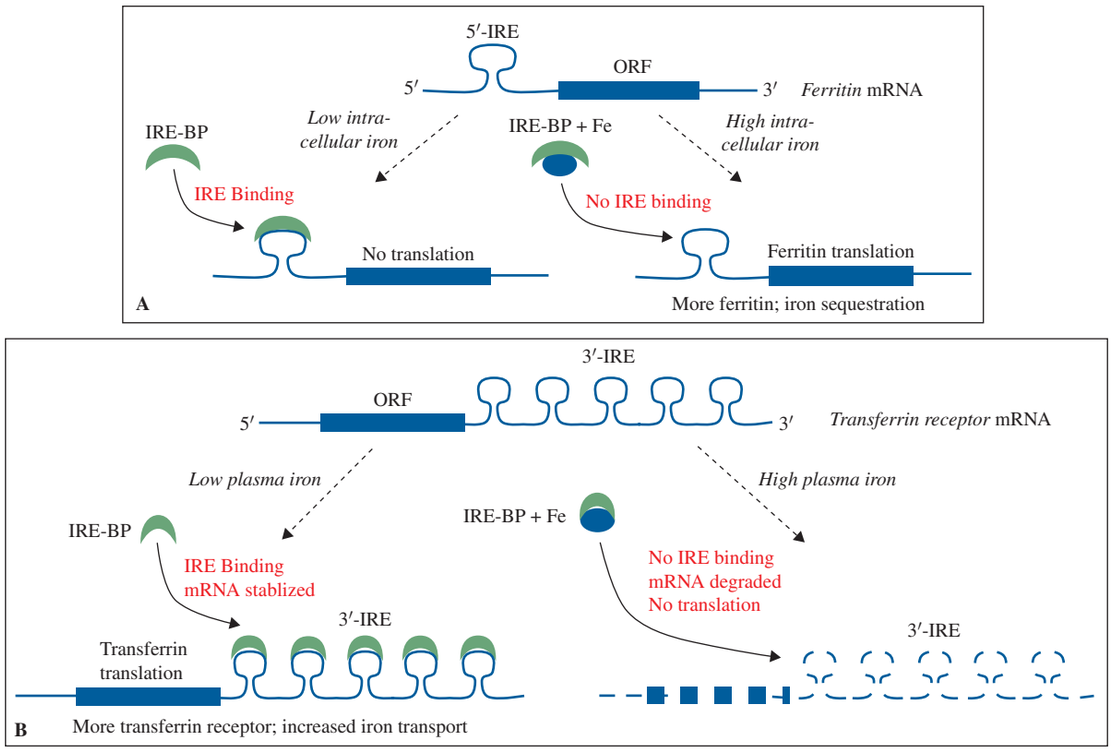

Figure 27-5. *When intracellular iron levels are high, IRP binds iron (Fe) and the IRP–Fe complex cannot bind to IRE in the 5′-UTR of ferritin mRNA.* **(A)** This allows ferritin mRNA to be translated to ferritin, which then sequesters excess intracellular iron. When intracellular iron levels are low, the IRP does not bind iron; hence it can bind to the IRE. As a result, the translation of the ferritin mRNA is prevented. When iron levels are low, transferrin receptor mRNAs are stabilized by the binding of IRP with the IREs in its 3′-UTR so that more transferrin receptors can be translated and cells can take up more iron from transferring. **(B)** Unlike ferritin mRNA which has a single IRE in 5′-UTR, transferrin receptor mRNA has multiple IREs in 3′-UTR. When iron levels are high, IRP binds iron and cannot interact with IREs; as a result, transferrin receptor mRNA is degraded at a faster rate to prevent over-uptake (thus, over-accumulation) of iron in the cell.

Table 27-4

Some Dietary Constituents That Mediate Nutrient–Gene Interactions by Modulating Specific Transcription Factor Pathways (Partial List)

FOOD/FOOD INGREDIENTS (SOURCE)	ACTIVATING CONSTITUENTS	TARGET TRANSCRIPTION FACTORS
Proteins	Amino acids	C/EBP, CHOP, IGFBP-1
Carbohydrates	Glucose	SREBPs, ChREBP, LXR
Fats	Fatty acids	SREBPs, ChREBP, PPARs, HNF-4, NF-κB, LXR, RXR
	Cholesterol	SREBPs, LXR, FXR, RXR
Vitamins	A	RAR, RXR
	D	VDR, RXR
Minerals	Fe	IRPs (IREBPs)
	Zn, Cu	MTF1
Vegetables and fruits	Flavonoid polyphenols (e.g., quercetin, kaempferol, apigenin, tangeretin)	AhR, Nrf2
Spices and wine	Non-flavonoid polyphenols (e.g., curcumin in turmeric, resveratrol in grapes and wine)	
Cruciferous vegetables	Indole-3-carbinol	
Salmon, shrimp	Carotenoids (e.g., canthaxanthin, astaxanthin)	

Table 27-5

Food–Drug Interactions (Activity May be Enhanced or Inhibited)

ENZYME OR TRANSPORTER	FOOD	DRUG
CYP1A2	Caffeine, theophylline, grapefruit juice (naringen and furanocoumarins bergamottin and dihydroxybergamotin), grape juice, cruciferous vegetables, apiaceous vegetables, cooked meat	Clozapine, fluvoxamine, imipramine
CYP2E1	Watercress and possibly other isothiocyanate-containing cruciferous vegetables; polyunsaturated fatty acids (corn oil, menhaden oil)	Ethanol, halothane, enflurane
CYP3A4	Grapefruit juice, orange juice, red wine, possibly other polyphenol-containing substances, St Johns wort, garlic	Ketoconazole, cyclosporin, erythromycin, protease inhibitors, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors
UGT and GST	Brussel sprouts, cabbage, watercress, broccoli	Acetaminophen, oxazepam, morphine, ibuprofen
P-glycoprotein and OATP	Vegetables, fruit juice, St Johns wort	Digoxin, cyclosporine, pravastatin

Abbreviations: GST, glutathione-S-transferases; OATP, organic anion–transporting polypeptides; UGT, uridine diphosphate glucuronosyltransferases.

50% of all drugs consumed by humans. Thus, induction of CYP3A4 might enhance the clearance and reduce the efficacy of a drug. In contrast, inhibition of CYP3A4 might prevent the drug's metabolism, resulting in a gradual build-up of its serum concentration and causing toxicity (Choudhuri and Valerio, 2005; Kirby and Unadkat, 2007). Examples of foods affecting a number of phase I and phase II enzymes, and transporters are provided in Table 27-5.

FOOD SAFETY REGULATIONS IN THE UNITED STATES

The U.S. Food and Drug Administration

The United States (U.S.) Food and Drug Administration (FDA) has a wide responsibility for protecting public health that includes ensuring the safety of the nation's food supply. FDA began as the Division of Chemistry under the U.S. Department of Agriculture (USDA). The name changed to the Bureau of Chemistry in 1901. In 1927, the Bureau of Chemistry was reorganized into two separate entities; the regulatory functions became the responsibility of Food, Drug, and Insecticide Administration, and nonregulatory research became the responsibility of the Bureau of Chemistry and Soils. In 1930 the name was changed again to the Food and Drug Administration (Swan, 1998; http://www.fda.gov/AboutFDA/WhatWeDo/History/Milestones/ucm128305.htm). Within FDA, the Center for Food Safety and Applied Nutrition (CFSAN) is the center that is responsible for protecting the food supply. CFSAN's food safety mission involves all of the areas noted below:

- The safety of substances added to food, e.g., food additives (including sources of radiation) and color additives
- The safety of foods and ingredients developed through biotechnology
- Seafood and juice Hazard Analysis and Critical Control Point (HACCP) regulations
- Regulatory and research programs to address health risks associated with foodborne, chemical, and biological contaminants
- Regulations and activities dealing with the proper labeling of foods (e.g., ingredients, nutrition health claims)
- Regulations and policy governing the safety of dietary supplements, infant formulas, and medical foods
- Food industry postmarket surveillance and compliance
- Industry outreach and consumer education
- Cooperative programs with state, local, and tribal governments
- International food standard and safety harmonization efforts

Chemical risk assessment is a critical aspect of food toxicology; the chemicals of interest are natural toxicants found in food, substances intentionally added to food, substances that unintentionally become part of food, and contaminants. Microbial risk assessment, also critically important to food safety, will not be discussed here.

Historical Perspective on the Science of Food Toxicology

Food toxicology involves the study of the toxic substances in food and any adverse impact in humans that results from their ingestion. Early on in history, humans recognized that many plants including plants used for food contained poisonous substances. Such plants were either avoided or prepared in ways that mitigated concerns for toxicity. However, the history of food toxicology as it relates to substances intentionally added to food or becoming part of food via food processing or contamination began in the last decades of the 19th century.

In 1883, Dr Harvey Washington Wiley of Purdue University was named the Chief Chemist of the USDA. That era saw the beginning of numerous debates about the use of substances added to food, including chemical preservatives, such as benzoates and salicylates; flour-bleaching agents, such as nitrites and nitrogen peroxide; artificial sweeteners, such as saccharin; and food colors, such as synthetic coal-tar dyes. The issues raised in those debates formed the basis for the development of food toxicology during the early part of the 20th century. Dr Wiley recruited healthy male volunteers, the so-called "poison squad," whose job was to ingest various foods containing chemical additives under controlled conditions to test their purported health effects. The volunteers were fed many chemicals including borax; salicylic, sulfurous, and benzoic acids; and formaldehyde. The experiments went on for 5 years. Wiley and the public became convinced that chemical preservatives

should be used in food only when necessary. Wiley intended for his experiments to lead to the regulation of food additives but he was opposed by the food lobby. Nevertheless, the Pure Food and Drug Act was enacted by Congress in 1906 as the first federal law to regulate food; the poison squad was disbanded one year later (Janssen, 1981; Swan, 1998; Watson, 2013; http://www.fda.gov/AboutFDA/WhatWeDo/History/Milestones/ucm128305.htm). Relatively speaking, food toxicology as a scientific discipline did not significantly advance for another 30 years.

The 50 years between 1930 and 1980 witnessed considerable development of toxicology (Guengerich, 2004). After World War II, there was a significant growth in both the food and the chemical industry, which led to the development of chemicals such as pesticides. The beginning of toxicology as "the science of poisons" implied that death was an important endpoint measured in toxicology studies. In order to find out the adverse effects of a chemical, toxicologists of the time relied mainly on acute toxicity studies with a LD_{50} value as the endpoint. The concepts of longer-term studies with repeat dosing, the importance of various biochemical endpoints, species differences, and other factors determining toxicity were not appreciated until later.

During the post–World War II period, the concept of longer term, repeat dose, toxicity studies, and their utility took root in experimental toxicology. Using various short- and long-term toxicity studies with apical endpoints, food toxicology at FDA advanced within the Division of Pharmacology under the leadership of Dr O. Garth Fitzhugh. During the 1950s further advances were made under the new director of the Division of Pharmacology, Dr Arnold J. Lehman. FDA established an organized approach to evaluating the safety of chemical constituents of foods, drugs, and cosmetics in making safety decisions. In 1959, FDA published the document *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics* (Hattan and Rulis, 1999). In 1982, the FDA Center for Food Safety and Applied Nutrition (CFSAN), then known as the Bureau of Foods, published a document titled *Toxicological Principles for the Safety Assessment of Food Additives and Color Additives Used in Food,* often referred to as the "FDA Redbook." The safety standard applicable to food ingredients is *reasonable certainty of no harm under the conditions of intended use*. The Redbook has been updated several times since then, and FDA has published numerous additional guidance related to diverse aspects of food safety assessment. A web search using the search term "FDA Redbook" returns many relevant FDA links including a link to download the Redbook.

History of Food Law

The current federal law governing food safety is the result of incremental legislative actions over a period spanning more than 100 years. It began with the passage of the Pure Food and Drug Act of 1906 by the U.S. Congress, which prohibited interstate commerce in misbranded and adulterated foods, drinks, and drugs. The law declared a food to be adulterated if it contained "any added poisonous or other added deleterious ingredient which may render such article injurious to health" (Hattan and Rulis, 1999). This spurred efforts to investigate the potential adverse effects of inappropriate food chemicals. Although several publications reported various toxicological investigations during the first decade of the 20th century (when the law was passed), the science of toxicology was in its infancy. Therefore, the evaluation of food chemical safety using the toxicology testing paradigms currently practiced did not evolve until much later as described above. *It is interesting to note that the two concepts, "adulterated" and "misbranded," that were part of the 1906 Act, still provide the legal basis for food safety regulations*.

In 1933, FDA recommended a complete revision of the obsolete 1906 Pure Food and Drug Act. The first bill was introduced in the Senate, launching a five-year legislative battle. In 1937, the Elixir of Sulfanilamide containing the poisonous solvent diethylene glycol killed 107 individuals. This unfortunate event accelerated the passage of the 1938 Food, Drug, and Cosmetic Act (Janssen, 1981; Swan, 1998; http://www.fda.gov/AboutFDA/WhatWeDo/History/Milestones/ucm128305.htm). The Food, Drug, and Cosmetic Act (FD&C Act) of 1938 clarified the regulation of poisonous and deleterious substances in foods. Foods under this statute are considered adulterated if they contain added poisonous or deleterious substances that "may render the food injurious to health." In case a substance is not added but is naturally present, the food would be adulterated only if the substance present "ordinarily renders" the food injurious. The FD&C Act of 1938 introduced the concept of premarket approval. However, premarket approval by FDA at the time was required only for drugs, not food ingredients.

The 1938 FD&C Act provided FDA with the regulatory authority to stop the use of known poisons. However, the vast effort needed to assure that all food chemicals were safe was clearly beyond the available resources and regulatory authority. In 1949, FDA Commissioner Paul B. Dunbar presented the problem to Rep. Frank B. Keefe of Wisconsin. Mr Keefe introduced a resolution to investigate chemicals in food, and later, cosmetics. The hearings, chaired by Rep. James T. Delaney of New York, went on for 2 years. From the committee's work came three amendments that fundamentally changed the character of the U.S. food and drug law: the Pesticide Amendment (1954), the Food Additives Amendment (1958), and the Color Additive Amendments (1960) (Janssen, 1981).

The FDA Modernization Act (FDAMA) of 1997 further amended the 1958 FD&C Act to enhance FDA's mission. The FDAMA focused largely on reforming certain aspects of the regulation of medical products and cosmetics, and to a lesser extent it addressed the regulation of food additives by streamlining the review process for the food contact substances (FCSs). The FCSs are defined in Section 409 of the Act as "any substance intended for use as a component of materials used in manufacturing, packing, packaging, transporting, or to hold food if such use is not intended to have any technical effect in such food" [sec. 409(h)(6) of the Act] (FDA, 1997).

Current U.S. Food Law to Ensure Safety of Foods, Food Ingredients, and Food Contaminants

The FD&C Act along with its amendments provides the necessary regulatory tools to ensure the safety of food and food ingredients. The recent amendments include The Food Additives Amendment of 1958, the Color Additive Amendments of 1960, and FDAMA of 1997. The following discussion briefly focuses on the regulatory definitions and safety evaluation processes of food additives, generally recognized as safe (GRAS) ingredients, and color additives.

FD&C Act, FDA Regulations, and FDA Guidance

The United States Code (U.S.C.) is a compilation of all codified federal laws enacted by Congress. Each "Title" in the U.S.C. represents the subject matter for which federal laws have been enacted. Title 21 of the U.S.C. pertains to Food and Drug laws. Therefore, the FD&C Act (referred to as "the Act" below) is a federal law enacted by Congress and contained in Title 21 of the United States Code (21 U.S.C.). The Act provides the legal framework within which FDA

operates. The Act along with all its provisions can be accessed at http://uscode.house.gov/browse/&edition=prelim.

Once the Congress enacts a federal law, executive departments and agencies can publish further regulations based on the legal framework set forth in the federal law. FDA develops regulations based on the laws set forth in the Act and other laws under which FDA operates. An example of "other laws" is the Administrative Procedure Act. FDA follows the procedures required by the Administrative Procedure Act (5 U.S.C.) to issue FDA regulations. This typically involves a process known as "notice and comment rulemaking" that allows for public input on a proposed regulation before FDA issues a final rule. The rulemaking procedures also come from Executive Orders and memoranda issued by the President, and FDA's own regulations. The rules are first published in the *Federal Register*, the federal government's official publication. Rules are then codified into regulations (laws) in the code of federal regulations (CFR). *Therefore, FDA regulations are also federal laws, but they are not part of the FD&C Act*. FDA regulations can be found in Title 21 of the Code of Federal Regulations (21 CFR).

In contrast to FDA regulations that are federal laws, FDA guidance describes the agency's current thinking on a regulatory issue, and is not legally binding on the public or FDA. FDA follows the procedures required by its "Good Guidance Practice" regulation to issue FDA guidance. The Good Guidance Practice regulation can be found in 21 CFR 10.115 (http://www.fda.gov/AboutFDA/Transparency/Basics/ucm194909.htm).

Food Additives

Sections 201(s) of the 1958 Food Additives Amendment defines the term "food additive" very broadly. Food additive is defined as follows:

… any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food (including any substance intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food; and including any source of radiation intended for any such use), if such substance is not generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use …

The 1958 Food Additives Amendment covers both "direct additives" and "indirect additives." Direct additives are described in 21 CFR Part 172 as substances added deliberately to food for a specific technical purpose; such substances usually remain in food as consumed. The technical effects could be intended to maintain or improve safety and freshness; improve or maintain nutritional value; or, improve taste, texture, and appearance. Secondary direct additives are described in 21 CFR Part 173 as substances added to food or food components during the manufacture or processing, but which are usually removed from the food before the product is consumed. Small amounts of these substances may remain in the final food. Examples of secondary direct additives include processing agents such as solvents, sanitizers, and defoaming agents. Indirect additives are described in 21 CFR Part 174 as substances that may become components of food, such as materials that may migrate from food contact surfaces during production, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food. For premarket approval by FDA, a food additive must be demonstrated to be safe at levels of requested use (the Act, Section 409(c)(3)(A)). The actual addition of a substance to food should be at levels no higher than necessary to fulfill the desired technical effect, even if the safety data support a higher level of exposure (21 CFR 171.l(c)(C)).

Both indirect and some secondary direct additives are now referred to as food contact substances (FCSs). Therefore, FCSs are legally food additives. Some FCSs may still be evaluated by FDA under the premarket paradigm depending on the complexity and exposure. In 1958 the FD&C Act was amended by adding Section 409, which requires FDA to approve new food additives before they can be used in foods. Section 409-mandated premarket approval also applies to the FCSs. Section 309 of the FDAMA amended Section 409 of the Act (21 U.S.C. 348) to establish a food contact notification (FCN) process as the primary method by which FDA regulates food additives that are FCSs. In doing so, the FDAMA eliminated the food additive petition process for most packaging and other substances that come in contact with food and migrate into food, and established a notification program whereby a notifier notifies FDA about its intended use of the FCS(s). The notifier may use the FCSs unless FDA objects to the intended use. By law, FDA must respond within 120 days. The amendment did not alter the safety standard applied to all food additives. Therefore, the notification for an FCS must contain sufficient scientific information demonstrating that the substance is safe for the intended use. On January 18, 2000, the Agency began accepting new food contact notifications, and in July 2000 FDA published proposed regulations for the program. The regulations were finalized in May 2002 and codified in 21 CFR Part 170.100-106 (Shanklin and Sanchez, 2005). Table 27-6 shows some direct food additives based on functionality.

Table 27-6

Some Direct Food Additives by Functionality

NUMBER	DESIGNATION	DESCRIPTION	EXAMPLES
170.3(o) (1)	Anticaking agents and free-flow agents	Substances added to finely powdered or crystalline food products to prevent caking, lumping, or agglomeration	Glucitol, sodium ferrocyanide, silicon dioxide
(3)	Antioxidants	Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation	Butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate
(9)	Enzymes	Enzymes used to improve food processing and the quality of the finished food	Papain, rennet, pepsin
(12)	Flavor agents and adjuvants	Substances added to impart or help impart a taste or aroma in food	Cinnamon, citral, *p*-cresol, thymol, Zingerone
(19)	Non-nutritive sweeteners	Substances having less than 2% of the caloric value of sucrose per equivalent unit of sweetening capacity	Aspartame, neotame, sucralose, saccharin

Substances Generally Recognized as Safe (GRAS) While developing the definition of a food additive, it became clear that there are many foods and food ingredients that have a long history of safe use, such as butter, egg, salt, milk, vinegar, sugar, and others. Thus, the concept of generally recognized as safe (GRAS) was born. Under the 1958 Food Additives Amendment to the Act, any substance intentionally added to food is a food additive and is subject to premarket approval by FDA unless the use of the substance is generally recognized as safe (GRAS; the GRAS provision) or otherwise exempted from the definition of food additive (e.g., color additive). By 1961, FDA had amended its regulations to include a list of food substances that are GRAS under certain conditions of use ("the GRAS list") (Gaynor et al., 2006). The GRAS status of an ingredient can be based on either a safe history of "common use in food prior to 1958" (history of use) or "scientific procedures" that demonstrate safety.

The GRAS provision removed the requirement of premarket review by FDA, and provided for independent conclusions of GRAS status. In 1969, FDA removed cyclamate salts from its GRAS list as a result of safety questions, and then-President Nixon directed FDA to reexamine the safety of GRAS substances. In the 1970s, FDA began a "comprehensive review" of presumed GRAS substances and established rulemaking procedures to affirm the GRAS status of substances that were either on the GRAS list or the subject of a GRAS affirmation petition. If FDA concluded that the available data and information supported GRAS status, FDA issued a final rule affirming GRAS status by amending 21 CFR 184 (direct food ingredients) or 186 (indirect food substances). By affirming the GRAS status through rulemaking, FDA owned the GRAS conclusions. However, meeting this self-imposed regulatory burden became resource-intensive and was difficult to meet. To eliminate the resource-intensive rulemaking procedures, FDA proposed to replace the GRAS affirmation petition process with a notification procedure ("GRAS notification") (Gaynor et al., 2006).

On April 17, 1997 FDA proposed to move away from the GRAS affirmation petition process and establish the GRAS notification process, whereby a notifier could voluntarily inform FDA of its determination that the use of a substance is GRAS under the intended conditions of use (62 FR 18938; April 17, 1997). By law, premarket approval by FDA is not required for GRAS. The initial GRAS notification process was established under an interim policy and pilot program. It operated from 1997 until mid-August, 2016. By that time FDA had already reviewed more than 600 GRAS notices. On August 17, 2016 FDA published the final rule to amend and clarify the criteria for when the use of a substance in human food or animal feed is not subject to the premarket approval requirements of the Act because the substance is GRAS under the conditions of its intended use (81 FR 54959; August 17, 2016). The final rule became effective on October 17, 2016.

The GRAS notification process is voluntary like the GRAS affirmation petition process. However, from the regulatory perspective, the main differences are that the affirmation petition process was a premarket approval process and the onus of GRAS affirmation was on FDA, whereas the notification process is not premarket approval and the onus of GRAS determination is on the notifier. Because the onus of GRAS determination was on FDA in the GRAS affirmation petition process, it involved rulemaking (discussed above).

Color Additives

Section 201(t) of the 1960 Color Additives Amendment defines the term "color additive" as follows:

A color additive is any material, not exempted under section 201 (t) of the Act, that is a dye, pigment, or extracted, isolated, or otherwise derived with or without intermediate or final change of identity, from vegetable, animal, mineral, or other source and that, when added or applied to a food, drug, or cosmetic or to the human body or any part thereof, is capable (alone or through reaction with another substance) of imparting a color thereto. Substances capable of imparting a color to a container for foods, drugs, or cosmetics are not "color additives" unless the customary or reasonably foreseeable handling or use of the container may reasonably be expected to result in the transmittal of the color to the contents of the package or any part thereof. Food ingredients such as cherries, green or red peppers, chocolate, and orange juice which contribute their own natural color when mixed with other foods are not regarded as color additives; but where a food substance such as beet juice is deliberately used as a color, as in pink lemonade, it is a color additive.

Color additives are defined in 21 CFR, Section §70.3. Section §70.5 describes general restrictions on the use of color additives, such as use in the area of the eye, injections, and surgical sutures. The use of an unlisted color additive, the improper use of a listed color additive, or the use of a color additive that does not conform to the purity and identity specifications of the listing regulation may cause a product to be adulterated and could be subject to enforcement action by FDA. Under the Color Additive Amendments, approximately 200 color additives that were in commercial use at the time were provisionally listed and could be used on an interim basis until they were either permanently listed or terminated due to safety concerns or lack of commercial interest. After 1960, FDA gradually removed color additives from the provisional list either by permanent listing or by termination of listing. Today about half of the 1960 color additives remain listed (Barrows et al., 2003). Table 27-7 shows per capita intakes of certified color additives for the U.S. population.

Table 27-7

Per Capita Intakes of Certified Color Additives for the U.S. Population

COLOR ADDITIVE	U.S. POPULATION PER CAPITA EDI (mg/PERSON/DAY)	U.S. POPULATION PER CAPITA ADI (mg/person/day) (60-kg PERSON)	CHILDREN PER CAPITA ADI (mg/person/day) (30-kg CHILD)
FD&C Blue No. 1	1.72	720	360
FD&C Blue No. 2	1.95	150	75
FD&C Green No. 3	0.038	150	75
FD&C Red No. 3	0.61	150	75
FD&C Red No. 40	17.91	420	210
FD&C Yellow No. 5	12.06	300	150
FD&C Yellow No. 6	10.74	225	113

SOURCE: Kotsonis and Burdoch (2013).

The Delaney Clause and Its Applications The Delaney clause [Section 409(c)(3)(A) of the Act] is a provision in the Food Additives Amendment of 1958. It states that no cancer-causing substance, as demonstrated in humans or animals, may be added to food. The 1960 Color Additive Amendments also contained the Delaney clause [Section 706(b)(5)(B) of the Act] that prohibited the use of a color additive shown to be a carcinogen in animal feed or human food.

A perusal of the scientific literature reveals that there is disagreement among experts regarding the utility of the blanket application of the Delaney clause with its zero-risk standard. Many scientists are of the opinion that it was needed at a time when the biology of carcinogenesis was poorly understood and the limits of detection of various residues in food were not nearly as sensitive as today's techniques, which have advanced from detection at the level of parts per thousand to parts per trillion or beyond. Advances in science ultimately triggered careful consideration of the zero-risk standard for carcinogens and action from the Congress through the passage of The Food Quality Protection Act (FQPA) of 1996 (21 U.S.C, §346A). The new law amended the zero-risk standard of the Delaney clause as it applies to pesticide residues in food, which are regulated by the U.S. Environmental Protection Agency (EPA). The new standard was based on quantitative risk assessment and determination of the lifetime risk of developing cancer from exposure to the pesticide residues in food. In practice, EPA targets a risk level of less than one in one million people (10^{-6}) over the course of a typical human life-span.

Except for the situation of pesticide residues in food discussed above, the Delaney clause still applies to food safety in general. The most conservative assumptions for the carcinogenic risk assessment result in an overestimation of the risk at each step ultimately leading to a combined overestimate of perhaps a million-fold or more. Therefore, an upper bound lifetime risk of one cancer or less in a million individuals is considered to be the biological equivalent of zero. Historically, FDA has employed a high threshold for establishing that a food or color additive has been found to induce cancer when ingested by humans or animals. Two indirect food additives (Flectol H and mercaptoimidazoline) that migrate from packaging material were banned. Among direct additives, safrole, cinnamyl anthranilate, thiourea, and diethylpyrocarbonate were banned because of the Delaney clause, and diethylpyrocarbonate because it forms urethane (Kotsonis and Burdoch, 2013).

Nevertheless, a number of substances (e.g., butylated hydroxyanisole [BHA], xylitol, methylene chloride, sorbitol, trichloroethylene, nitrilotriacetic acid [NTA], diethylhexyl phthalate, melamine, formaldehyde, bentonite) are listed in the CFR as food additives, but they are also listed as carcinogens by NTP, the International Agency for Research on Cancer (IARC), or the state of California (under the Safe Drinking Water and Toxic Enforcement Act of 1986, also known as Proposition 65). The reasoning applied by FDA in almost every case is based on secondary carcinogenesis, such as irritation and restorative hyperplasia in the forestomach in the case of BHA, mineral imbalance in the case of sugar alcohol, etc. The one exception is formaldehyde, which is carcinogenic only on inhalation, and inhalation is not an appropriate route for food additives (Kotsonis and Burdoch, 2013).

In an effort to restrict the applicability of the Delaney clause, FDA has narrowed the definition of food additive to exempt constituents and contaminants that pose a de minimis risk. According to FDA's constituents' policy, food additive refers to the added substance as a whole and not to each of its individual constituents. For example, the constituents' policy was employed to approve the use of FD&C Green No. 5 and Green No. 6, both of which contain a nonfunctional contaminant *p*-toluidine that by itself causes cancer (Picut and Parker, 1992).

For new animal drugs, safety assessment is concerned primarily with residues that occur in animal food products (milk, cheese, etc.) and edible tissues (muscle, liver, etc.). Toxicity studies require data on metabolism and the nature of metabolites, along with data on the drug's pharmacokinetics. During this phase, the parent drug and its metabolites are evaluated both qualitatively and quantitatively in the animal products of concern (eggs, milk, meat, etc.).

To comply with the no residue provision of the Delaney clause, FDA addressed the issue of residues of metabolites of animal drugs known to induce cancer in humans or animals. Because not every metabolite could be accurately tested, FDA considered threshold assessment. Threshold assessment combines information on the structure and in vitro biological activity of a metabolite for the purpose of determining whether carcinogenicity testing is necessary. If testing is necessary and if the substance is found to induce cancer, the FDA's definition states that a lifetime risk of one in a million is equivalent to the meaning of "no residue" as intended by Congress (Kotsonis and Burdoch, 2013).

The Safety Standard and Toxicological Testing Recommendations for Food Ingredients The following narrative is a general discussion on the toxicological testing recommendations for food ingredients, such as food additives, color additives, GRAS ingredients, and FCSs. Section 409 of the 1958 Food Additives Amendment states that before a food additive can be added to food, it must be demonstrated to be safe under its intended conditions of use. This statute is the foundation of the premarket approval of food additives, including FCSs. Congress, however, realized that it is impossible to assure the absolute safety of food additives under all circumstances. Thus, Section 409 states, "Safety requires proof of *a reasonable certainty* that *no harm* will result from the intended use of an additive." In other words, Congress recognized that absolute certainty of no harm (zero risk) was impossible to achieve. Thus, the safety standard for food and color additives is *reasonable certainty of no harm*. The same safety standard also applies for the other food ingredients.

In 1982 the FDA published a document titled *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*. This is popularly known as "the Redbook." In the Redbook, FDA provided its recommendations for the minimum set of toxicological data normally needed for evaluating the safety of the use of direct food additives. The 1982 Redbook was updated in 1993 and 2000 (Revised October 2001, November 2003, April 2004, February 2006, and July 2007) (FDA Guidance for industry). The original Redbook of 1982 is referred to as Redbook I, the 1993 updated version is referred to as Redbook II or Redbook 1993, and the 2000 version is called Redbook 2000. The Redbook is available to the public on the world-wide-web.

The recommendations in the Redbook utilize the concept of structure–activity relationships of chemicals to assign categories to substances added to food. Additives with functional groups with a high order of toxicity are assigned to category C, those with low potential for toxicity are assigned to category A, while those of unknown or intermediate toxicity are assigned to category B. Further, based on structure assignment and calculated exposure, "concern levels" are assigned. Concern levels are relative measures of the degree to which the use of an additive may present a hazard to human health. Concern level III represents the highest probable risk to human health, Concern level I represents the lowest probable risk, and Concern level II is intermediate between high and low risk (Table 27-8). Each concern level is associated with a different level

Table 27-8

Assignment of Concern Levels Based on Structure Category and Exposure

STRUCTURE CATEGORY A	STRUCTURE CATEGORY B	STRUCTURE CATEGORY C	CONCERN LEVEL
<0.05 ppm (= <50 ppb) in the total diet	<0.025 ppm (= <25 ppb) in the total diet	<0.0125 ppm (= <12.5 ppb) in the total diet	**I**
or	or	or	
≥0.05 ppm (= ≥50 ppb) in the total diet	≥0.025 ppm (= ≥25 ppb) in the total diet	≥0.0125 ppm (= ≥12.5 ppb) in the total diet	**II**
or	or	or	
≥1 ppm in the total diet	≥0.5 ppm in the total diet	≥0.25 ppm in the total diet	**III**

SOURCE: The Redbook. Available at: http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm.

Table 27-9

Recommended Toxicological Testing Based on Concern Level Assignment

TOXICITY STUDIES	CONCERN LEVELS		
	I	II	III
Genetic toxicity tests	X	X	X
Short-term toxicity tests in rodents	X	X	X
Subchronic toxicity studies in rodents		X	X
Subchronic toxicity studies in nonrodents		X	X
One-year toxicity studies in nonrodents			X
Chronic toxicity or combined chronic toxicity/carcinogenicity tests in rodents			X
Carcinogenicity study in rodents			X
Reproduction studies with teratology phase		X	X
Developmental toxicity studies		X	X
Metabolism and pharmacokinetic studies		X	X
Human studies			X

SOURCE: The Redbook. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm054658.htm.

of initial toxicological testing. Listed in Table 27-9 are the recommended initial testing batteries for substances assigned to Concern levels I, II, and III. These represent the minimum testing recommendations; more extensive testing may be needed to resolve scientific questions that may arise during research and development. While testing in human subjects is not required for the safety assessment of many food additives, certain circumstances or scientific issues arise that may profit from this type of testing. For example, comparison of data from metabolism and pharmacokinetic studies in human subjects and test animals may be used to identify the species that provides the most accurate reflection of a toxicological effect in human subjects. The FDA expects that any toxicological testing that is conducted to provide data to support the safety of food additives will comply with its regulations entitled *Good Laboratory Practice for Nonclinical Laboratory Studies* that are found in 21 CFR 21.58 (Hattan and Rulis, 1999).

FDA scientists review the data and information submitted by the sponsor in support of the safety of a new additive or a new use of an additive or GRAS ingredient and they independently determine whether the intended use is consistent with the safety standard of reasonable certainty of no harm. Once the FDA determines that the intended use of the food/color additive is safe, it issues a regulation permitting the addition of the new food additive to food. In the case of GRAS ingredients, it is the notifier that determines whether the ingredient is GRAS for its intended use in food. FDA reviews the data and information provided by the notifier and determines whether it has no questions to the notifier's conclusion that the ingredient is GRAS for its intended use, or whether it finds that there is no basis to conclude that the ingredient is GRAS for its intended use. Therefore, FDA does not have to write a regulation for GRAS ingredients because FDA does not "own" the safety conclusion.

In the revised guidance for industry for the FCSs, FDA provided the minimum safety testing recommendations that depend on the exposure to the FCS in the diet. For example, no safety studies are recommended for an FCS (or a constituent, as appropriate) if exposure for a single use is at or less than 0.5 ppb [see the section "Threshold of Regulation (TOR)"]. If the cumulative exposure is between 0.5 ppb and 50 ppb, the potential carcinogenicity of the FCS (and/or a constituent, if appropriate) should be evaluated using genetic toxicity tests, such as (1) a test for gene mutations in bacteria and (2) an in vitro test with cytogenetic evaluation of chromosomal damage using mammalian cells or an in vitro mouse lymphoma $tk^{\pm}$ (thymidine kinase) assay. If the cumulative exposure is between 50 ppb and 1 ppm the potential carcinogenicity of the FCS (and/or a constituent, if appropriate) should be evaluated using genetic toxicity tests, such as (1) a test for gene mutations in bacteria; (2) an in vitro test with cytogenetic evaluation of chromosomal damage using mammalian cells *or* an in vitro mouse lymphoma $tk^{\pm}$ assay (the mouse lymphoma assay is preferred); and (3) an in vivo test for chromosomal damage using rodent hematopoietic cells. If the estimated exposure to an FCS or a constituent is 1 ppm or greater, FDA recommends that a food additive petition be submitted for the FCS (FDA, 2002).

ADI, EDI, and Reasonable Certainty of No Harm The goal of the toxicological testing of a food additive is to determine the acceptable daily intake (ADI) of the additive. The ADI is compared to the estimated daily intake (EDI) of the additive to determine whether the proposed use of the additive falls within the bounds of "reasonable certainty of no harm." In order to calculate the ADI, FDA uses the longest duration study in the most appropriate animal species to establish the no-observed-adverse-effect-level (NOAEL). The NOAEL is the highest dose that does not cause any observed adverse effects. By applying an appropriate safety factor to this NOAEL, the ADI is obtained. The standard safety factor is usually 100, which is derived by multiplying a factor of 10 to account for interspecies variation with an additional factor of 10 to account for intraspecies variation in the sensitivity of response to a chemical. Hence, ADI = NOAEL /100.

The default safety factor of 100 may be modified (lowered or increased) based on various considerations, including the number of

long-term studies, number of species in which these studies are conducted, the nature of the toxicological effect found, the nature of the food additive, or the extent and the quality of supporting data. For micronutrients, for example, it may not be possible to apply a 100-fold safety factor to a NOAEL and have an adequate dietary intake to allow them to fulfill their physiological roles. In such instances, a 10-fold or lower safety factors are not uncommon. The same is true for *macroingredients*, which are food additives intended to replace macronutrients (e.g., fats) in the diet. Macroingredients are added to food in appreciable quantities in order to impart their technological purpose. An example is olestra, which is a fat substitute. Animals were fed up to about 6 g/kg body weight/day whereas the human 90th percentile intake of olestra for just one food category, snack foods, is 0.33 g/kg body weight/day (Hattan and Rulis, 1999). The traditional safety factors are not applied to substances that have been demonstrated to be carcinogenic in humans or animals. This is because Delaney clause prohibits the addition of carcinogenic food additives to the food supply.

Typically, if the EDI is less than the ADI (in the same units, such as mg/kg body weight/day), the use of the additive or a GRAS ingredient is considered to lie within the bounds of "reasonable certainty of no harm," for its intended use in food. GRAS ingredients are often macroingredients and it is not possible to feed enough to experimental animals in order to calculate an ADI based on 100-fold safety factor.

Dietary Intake Estimate The FDA's pre-market approval processes for food and color additives require an estimate of the *probable* consumer intake of the additive to determine whether its use or presence in a food at a given concentration is safe. The basis for this requirement is stated in the Act as amended in 1958 (Section 409—food additives) and 1960 (Section 721—color additives). In addition, FDA also considers consumer intake of contaminants that may be present in foods. Under Section 402(a)(1)-adulterated food, a food shall be deemed to be adulterated if it bears or contains any poisonous or deleterious substance which may render it injurious to health (FDA, 2006a). In 2006 FDA revised its 1995 guidance on estimating dietary intake of substances in food. The document uses the term "ingredient" to refer generically to substances intentionally added to food, including food additives, GRAS ingredients, color additives, and prior-sanctioned substances.

The dietary intake (dietary exposure) is an important contributor to the development of the concern level. Estimates of intake of an ingredient or chemical constituent of food require two key pieces of information: (1) the concentration of the substance in a food, and (2) consumer intake of foods that will or might contain the substance (FDA, 2006a).

The concentration of an ingredient in food can be obtained from (1) the intended use levels of the substance in target foods; (2) the measured concentration in food as consumed; (3) the limit of detection (LOD) or limit of quantification (LOQ) of the analytical method; (4) established limits for the substance (e.g., specifications in the CFR or the Food Chemical Codex [FCC] for unavoidable impurities and contaminants in food ingredients, or maximum allowable levels of impurities and contaminants in foods adopted by a recognized standards-setting body, such as the Codex Alimentarius Commission) (FDA, 2006a).

The consumer intake can be obtained from a number of sources that provide intake estimates of substances in the diet, such as (1) food consumption surveys; (2) food/ingredient disappearance data; (3) total diet study; (4) body burden/excretion measurements, or "biomarkers." *Food consumption data* may be collected at the national, household, or individual level. Examples of some frequently used food consumption surveys are Continuing Survey of Food Intakes by Individuals (CSFII), National Health and Nutrition Examination Survey (NHANES), and the integrated CSFII/NHANES, and MRCA 14-Day Survey. *Food/ingredient disappearance data* represent the disappearance of food into the marketing system. Annual amounts of major U.S. commodities entering into the U.S. food supply are determined by subtracting data on exports, year-end inventory, and non-food use from data on production, imports, and beginning inventories. In the United States, information on the poundage of many food commodities entering commerce is available annually from the Economic Research Service (ERS) of the USDA. An online database of poundage-based per capita food consumption is also available from the ERS, which permits customized searches of commodities (e.g., meat) and sub-commodities (e.g., red meat, poultry, fishery products) by year. *FDA's Total Diet Study* (TDS; also called the market basket study) is an ongoing program through which the Agency monitors and analyzes a wide range of foods to determine levels of selected substances (e.g., nutrients, pesticide residues, and industrial contaminants). The TDS has been conducted continuously in the United States since the early 1960s. The program currently uses four regional sample collections (or market baskets) each year. FDA updates the list of foods to be surveyed about every 10 years to reflect changes in food consumption patterns. *The use of biomarkers* for estimating intake has been investigated for methyl mercury, fumonisins, phthalate diesters, and several nutrients (e.g., fat, protein, minerals, and vitamins). The use of biomarkers in exposure assessment methods is the subject of ongoing research. For a detailed discussion of these topics as well as additional sources of information, see FDA (2006a).

The relationship of substance concentration data, portion size, and food consumption frequency to the estimated daily intake (EDI) of a substance X for a single individual is captured in the following equation (FDA, 2006a):

$$\mathrm{EDI}_x = \sum_{f=1}^{F} \frac{\mathrm{Freq}_f \times \mathrm{Port}_f \times \mathrm{Conc}_{xf}}{N}$$

where
F = Total number of foods in which substance "x" can be found
Freq_f = Number of eating occasions of food "f" over "N" survey days
Port_f = Average portion size for food "f"
Conc_{xf} = Concentration of the substance "x" in food "f"
N = Number of survey days

Threshold of Regulation (TOR) In 1995, the FDA introduced the threshold of regulation (TOR) policy and established procedures for exempting indirect food additives (now called FCSs) from premarket evaluation and petition review if the likely dietary exposure to humans from the migrating substances is below 0.5 ppb (21 CFR 170.39). The TOR policy is based upon the notion that below certain dietary exposure levels, additives migrating into food will present only negligible risk. The policy is intended only for substances not known or expected to be carcinogenic, but is based upon the presumption that even if a migrating substance were inadvertently a carcinogen, the upper-bound risk of cancer from exposure to the substance at the low levels permitted would be negligible. The policy is based upon a systematic analysis of the potencies of numerous carcinogens and noncarcinogens (Hattan and Rulis, 1999).

Food from New Plant Varieties FDA regulates the safety of food for humans and animals produced from new plant varieties, including genetically engineered (GE) plants. The safety of new

plant varieties is regulated primarily under the FDA's postmarket authority [section 402(a)(1) of the act (21 U.S.C. 342(a)(1)]. This section, normally applied to occurrences of unsafe levels of toxicants in food, is applied to new plant varieties whose composition has been altered by an added substance. Foods from GE plants must meet the same food safety requirements as foods derived from traditionally bred plants.

The FDA published its "Statement of Policy: Foods Derived from New Plant Varieties", the 1992 policy (FDA, 1992) in the Federal Register in 1992 (57 FR 22984–23005). In the 1992 policy, the FDA explained that under the Act developers of new foods have a responsibility to ensure that the foods they offer to consumers are safe and in compliance with all requirements of the Act. The 1992 policy applied to all foods (such as fruits, vegetables, grains, and their byproducts) derived from all new plant varieties, including varieties that are developed using recombinant DNA technology. The plant varieties that are developed using recombinant DNA technology are referred to as "bioengineered foods."

In the 1992 policy, the FDA provided guidance to the industry regarding scientific considerations for evaluating the safety and nutritional aspects of food from new plant varieties derived by traditional methods (such as hybridization or mutagenesis), tissue culture methods (such as somaclonal variation and protoplast fusion), and recombinant DNA methods. FDA recognized in its 1992 policy statement that it is prudent practice for developers of new varieties to consult with the agency on safety and regulatory questions, especially with regard to products developed through new technology. Because of the rapid advances in recombinant DNA technology (bioengineering), FDA believes that it is in the best interests of the regulated industry and the agency for developers to inform FDA prior to commercial distribution, about foods or feed derived from new plant varieties, including those derived using recombinant DNA technology. FDA issued guidance on the consultation process in 1996, which was revised in 1997 (FDA, 1997a).

In the United States, new plant varieties are regulated not only by FDA, but also by the EPA and USDA. FDA is responsible for the safety and labeling of foods and feeds derived from crops, irrespective of the method used to produce the new plant variety. The EPA is responsible for assuring the safety of pesticides; thus, if a pesticidal protein is produced in a new plant variety, this product would also fall under EPA's jurisdiction. The USDA's Animal and Plant Health Inspection Service (APHIS) has responsibility for the environmental safety of field testing and commercial planting of new plant varieties. The developer of a biotechnology-derived crop variety must obtain registration from not only the country of origin but from importing countries as well (Kotsonis and Burdoch, 2013).

The Food Allergen Labeling and Consumer Protection Act (FALCPA) In 2004, an amendment to the FD&C Act (Public Law 108-282) was introduced mandating the labeling of a food that contains an ingredient that is or contains protein from a "major food allergen." This is the Food Allergen Labeling and Consumer Protection Act (FALCPA), which became effective January 1, 2006. For a series of questions and answers on FALCPA, see FDA (2006b). According to FALCPA, manufacturers are required to identify the presence of ingredients that contain proteins derived from milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, or soybeans, and also specify the source when possible. For example, specify the type of tree nut (e.g., almonds, pecans, or walnuts), the species of fish (e.g., bass, flounder, or cod), and the kind of crustacean shellfish (e.g., crab, lobster, or shrimp). Also, because the source of ingredients such as lecithin, flour, or whey may not be obvious to the consumer, the source must follow the name of the ingredient. For example, lecithin, flour, and whey must be given on the labels as follows: lecithin (soy), flour (wheat), and whey (milk); alternatively, immediately after or next to the list of ingredients in a "contains" statement—for example "Contains wheat, milk, and soy."

Food Safety Through the Hazard Analysis and Critical Control Point (HACCP) System The HACCP is a management system in which food safety is addressed through the analysis and control of biological, chemical, and physical hazards from raw material production, procurement, and handling, to manufacturing, distribution, and consumption of the finished product. The current practice of HACCP was developed over time, but the initial trigger came from the need to provide safe food for space flights (Sperber and Stier, 2009–2010). In the United States, the initial participants were the United States Army Laboratories, the National Aeronautics and Space Administration (NASA), and Pillsbury. Pillsbury and NASA required contractors to identify the critical control points (CCPs), that is, the "critical failure areas" and eliminate them from the system. In the 1970s, several cases of *Clostridium botulinum* contamination were attributed to under-processed, low-acid canned foods. FDA asked Pillsbury to organize a training program for its inspectors. This training program, "Food Safety through the Hazard Analysis and Critical Control Point System," was held in 1972, and appears to be the first published use of the term "HACCP" (Sperber and Stier, 2009–2010).

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) was established in 1988. It is an advisory committee chartered under the USDA and comprises participants from the USDA (Food Safety and Inspection Service), Department of Health and Human Services (FDA and CDC), the Department of Commerce (National Marine Fisheries Service), the Department of Defense (Office of the Army Surgeon General), academia, industry, and state employees. When the NACMCF was established, the HACCP system was already in place. However, one of the first NACMCF efforts was the definition of an HACCP system and guidelines for its application. Its efforts were coordinated with those of the Codex Committee for Food Hygiene (permanently chaired by the United States), leading to reports from each group in 1992, which were further harmonized in 1997. Since its birth in 1959 and formalization in 1972, the HACCP system has grown from three to seven principles (Sperber and Stier, 2009–2010). The seven principles of HACCP are: (1) Hazard analysis; (2) Identification of CCPs; (3) Establishing the critical limits; (4) Monitoring control of the CCPs; (5) Establishing corrective actions; (6) Establishing verification procedures; and (7) Establishing documentation and record-keeping systems. The HACCP principles and application guidelines adopted in 1997 can be accessed at the FDA website (FDA, 1997b). The links to dairy HACCP, juice HACCP, retail and food service HACCP, and seafood HACCP can all be accessed from there.

The Food Safety and Modernization Act (FSMA) The FDA Food Safety and Modernization Act (FSMA) was signed into law by the President on January 4, 2011. It aims to ensure the safety of the U.S. food supply by shifting the focus from responding to contamination to preventing it. In other words, FDA can use regulation to prevent food safety problems before they occur. The FSMA provides FDA with new enforcement authorities related to food safety standards, provides FDA tools to hold imported foods to the same standards as domestic foods, and directs FDA to build an integrated national food safety system in partnership with state and local authorities. One of the most important enforcement tools the FDA has under the FSMA is the power to withdraw a food facility's registration.

The major elements of FSMA include (1) Preventive controls; (2) Inspection and compliance; (3) Imported food safety; (4)

Response; and (5) Enhanced partnerships. The new food safety plan requires the identification and prevention of all reasonably foreseeable food safety hazards that could be natural or unintentionally introduced into the food facility. This goes beyond the practice of HACCP. This new process is called Hazard Analysis and Risk-Based Preventive Controls (HARPC), which is the plan and the process that a food company has to use in each of its facilities to implement its food safety plan. Simply stated, it is the system used to control the different hazards found in various facilities that manufacture food. Each food manufacturing facility owned and operated by a food company must have its own HARPC plan if there are any differences in the foods it manufactures between facilities (King and Ades, 2015). The facilities and the products that are already in compliance with HACCP are exempt from HARPC, such as the juice and seafood HACCP-compliant facilities, and HACCP-compliant facilities involved in packaging thermally processed low-acid foods in hermetically sealed containers. FDA provides a detailed FAQ on FSMA that explains various aspects of this law, including the background of its genesis, utility, and applications (FDA FSMA FAQ). While HACCP is a recognized global standard, HARPC is a U.S. standard set forth by FSMA, although there are similarities. In the FAQ (PC7), it is stated that the HACCP systems that many FDA-regulated manufacturers have in place are the foundation of the preventive controls regulations. Although there are similarities between the FSMA preventive controls requirements and the HACCP system, not every provision is identical. For example, in HACCP systems, controls are applied at CCPs, whereas preventive controls include controls at CCPs or controls other than those at CCPs that are appropriate for food safety (https://www.fda.gov/NewsEvents/PublicHealthFocus/ucm239907.htm; https://www.fda.gov/Food/GuidanceRegulation/FSMA/).

Regulation of the Dietary Supplements A dietary supplement is a product intended for ingestion that contains a dietary ingredient intended to add further nutritional value to (supplement) the diet. A dietary ingredient may be one, or any combination, of the following substances: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by people to supplement the diet by increasing the total dietary intake, a concentrate, metabolite, constituent, or extract. Dietary supplements must be labeled as such and must not be represented for use as a conventional food or as the sole item of a meal or the diet. One way to distinguish dietary supplements from conventional foods is by looking at the nutrition information on the label of the product. Conventional foods must have a *Nutrition Facts* panel on their labels, but dietary supplements must have a *Supplement Facts* panel.

FDA regulates dietary supplements under a different set of regulations than those covering conventional foods. This regulation is called the Dietary Supplement Health and Education Act of 1994 (DSHEA). Under DSHEA, dietary supplement manufacturers and distributors are not required to obtain premarket approval from FDA before marketing dietary supplements, but they are responsible for evaluating the safety and labeling of their products before marketing to ensure that they meet all the requirements of DSHEA and FDA regulations. In other words, manufacturers and distributors are prohibited from marketing products that are adulterated or misbranded. FDA can take action against any adulterated or misbranded dietary supplement product after it reaches the market. In August 2016, FDA issued revised draft guidance for the industry regarding the New Dietary Ingredient (NDI) notification process that replaced the earlier guidance of 2011.

The information discussed above and additional information on various aspects of dietary supplements and their regulation can be obtained from the following link (and all the other links provided therein): https://www.fda.gov/Food/DietarySupplements/.

It should be noted that terms such as "functional foods" or "nutraceuticals" are widely used in the marketplace these days. But these terms are not defined by law, that is, these terms do not have any legal definition for regulatory use. Such foods are regulated by FDA under the authority of the FD&C Act.

Global Food Safety Authoritative Bodies

In parallel with U.S. FDA's effort to assure the safety of food additives, similar efforts were started internationally. The evaluation of food additives at the international level was initiated as a result of a Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Conference on Food Additives held in Geneva, Switzerland in 1955. The Conference recommended to the Directors-General of FAO and WHO that expert committees should be convened to address the safety of chemical additives in food. The outcome of the recommendation was the establishment of an international expert scientific committee known as the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which has been meeting since 1956. JECFA developed the concept and coined the term "acceptable daily intake (ADI)" in 1961 (Lu, 1988; http://www.fao.org/3/a-at863e.pdf).

Several other global food safety authoritative bodies were established since the 1990s, such as the Food Standards Australia and New Zealand (FSANZ), European Food Safety Authority (EFSA), and others. FSANZ was formally established in 2002 but its predecessor, called Australia New Zealand Food Authority (ANZFA), was established in 1996. FSANZ is a bi-national government agency that develops and administers the food standard code for Australia and New Zealand. The food standard code, which is publicly available on the web (http://www.foodstandards.gov.au/code/Pages/default.aspx), lists requirements for foods such as additives, food safety, labeling, and genetically engineered foods. The decisions made by FSANZ have to be approved by both governments (http://foodregulation.gov.au/internet/fr/publishing.nsf/Content/history-and-governance).

The European Food Safety Agency (EFSA) is another relatively recent global food safety authoritative body. The EFSA was legally established by the European Union (EU) under the General Food Law and is funded by the EU. It was set up in 2002 following a series of food crises in the late 1990s and it serves as a source of scientific advice and communication on risks associated with the food chain. The General Food Law created a European food safety system in which the responsibility for risk assessment (science) and for risk management (policy) are kept separate. EFSA is responsible for the risk assessment (science), and also has a duty to communicate its scientific findings to the public. As the risk assessor, EFSA produces scientific opinions and advice that form the basis for European policies and legislation, and it covers Food and feed safety; Nutrition; Animal health and welfare; Plant protection; and Plant health. EFSA's scientific opinions are mostly published in the *EFSA Journal* and are publicly available at https://www.efsa.europa.eu/en/aboutefsa.

TOXIC SUBSTANCES IN FOOD

The requirement for food varies with age and activity level. The average daily consumption of food is about 2 kg/day. Obviously, human beings consume a lot of materials as food on a daily basis. Thus, any hazard present in foods, such as toxic substances,[5]

[5]*Toxic substance* is a general term. Typically, toxic substances produced by living organisms are referred to as *toxins*, whereas toxic substances produced by human activities are referred to as *toxicants*.

antinutrients, or contaminants may have adverse health consequences. Discussed below are certain common toxic substances in food that are either naturally occurring, or formed during cooking, or they find their way into the food as contaminants during processing of foods. The list is by no means complete. The human and animal exposure to many of these toxins/toxicants has been significantly reduced because of our understanding of these toxins and their toxicity.

Some Naturally Occurring Toxic Substances in Food

Plants produce a stunning array of chemicals to protect themselves from invading pathogens, insects, and herbivorous animals. Many of these chemicals are secondary metabolites; they are not essential for growth and reproduction. Some of these secondary metabolites may have beneficial health effects on humans, usually at a low or moderate level of consumption. However, many others are toxic to animals and humans, particularly when consumed at high levels. The secondary metabolites usually belong to one of the three major chemical classes: *terpenoids* (contain isoprene units), *phenolics* (contain phenol group), and *alkaloids* (contain nitrogen). There are also nitrogen-containing non-alkaloids. Some of these toxic substances are fatty acids or proteins. Understanding how plant defense mechanisms work is essential to protecting our food supply as well as developing disease-resistant plant varieties. *In the following description, one representative chemical structure from each group of substances is shown.*

Cycasin Cycasin (an alkaloid; Fig. 27-6) is an azoxyglycoside found in cycads. Cycasin and its metabolite methylazoxymethanol (MAM) can be extracted from seeds and roots of cycad plants (Laqueur and Spatz, 1968). The inhabitants of Guam (Chamorros) have long used various parts of *Cycas circinalis*, including seeds, as a source of food before its toxicity was recognized. They also relished the meat of flying fox, a type of fruit bat that lived on cycad seeds. The consumption of cycad seeds was correlated with a complex neurodegenerative disease known as amyotrophic lateral sclerosis-parkinsonism-dementia (ALS-PDC) that occurred at a far higher rate compared to the rest of the world. Studies trying to identify the putative neurotoxin in Cycad seeds, roots, and leaves identified cycasin as a carcinogenic compound in rabbits, monkeys, and multiple species of rodents, although the neurotoxicity was not confirmed. In the animal studies cycasin induced tumors in multiple organs, such as the liver, kidney, lung, and intestine. Oral doses of cycasin induced tumors in conventional rats but not in germ-free rats, suggesting that intestinal metabolism of cycasin produced the carcinogen. Several reports confirmed that the β-D-glucosidase of intestinal bacteria hydrolyzes cycasin into its aglycone metabolite MAM, and that MAM was the toxicant and the ultimate carcinogen in rodents (Sieber et al., 1980). The incidence of ALS-PDC among Chamorros fell sharply after 1960 because of the awareness of cycad seed toxicity.

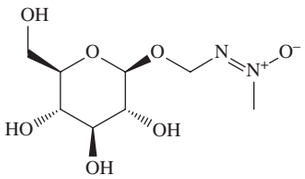

Figure 27-6. *Cycasin.*

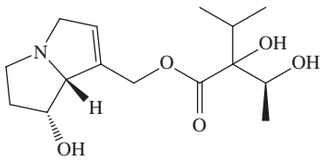

Figure 27-7. *Lycopsamine.*

Pyrrolizidine Alkaloids Pyrrolizidine alkaloids (PAs) are found in many species of flowering plants. Several hundred PAs have been isolated from these plants. PA structure has two parts: the bicyclic necine base (necine) that shares a bridgehead nitrogen atom and the necic acid, a C5-C10 branched chain acid that is esterified to necine. Some examples of PAs are *heliotrine*, *lycopsamine* (Fig. 27-7), *lasciocarpine*, *retrorsine*, and *senecionine*. Animals can be exposed to PA from the consumption of PA containing plant material, whereas human exposure results from the consumption of either the plant material or products of animal origin (Wiedenfeld, 2011). PAs are metabolized in the liver to electrophilic pyrrole derivatives, which cause hepatotoxicity in animals and veno-occlusive disease in humans. Many PAs also have genotoxic and carcinogenic properties (EFSA, 2011). In vitro studies and in vivo studies in big blue transgenic rats demonstrated that upon metabolic activation, PAs form DNA adducts and DNA strand breaks, leading to mutations and chromosomal aberrations. The major type of mutation was GC→TA transversions and tandem base substitutions of G:G to T:T and G:G to A:T, which is consistent with a mechanism involving adduct formation at guanine residues (G). The main carcinogenic target site for PAs in animals is the liver. There are no human epidemiological data on PA carcinogenesis (EFSA, 2011). However, it has been hypothesized that carcinogenesis may be favored by extended exposure to PAs. The U.S. Food and Drug Administration (FDA) issued an advisory in 2001 asking the dietary supplement manufacturers to remove the herb comfrey from their products because comfrey contains PAs.

Ptaquiloside Ptaquiloside (a terpenoid; Fig. 27-8) is a norsesquiterpene glucoside, and is the major carcinogen in bracken fern (*Pteridium aquilinum*). It causes bladder and intestinal cancer in livestock, mammary and intestinal tumors in rats, as well as neurotoxicity in mice and sheep. In addition to the ptaquiloside (PTA), bracken fern contains a number of other toxic substances, such as *prunasin* (a cyanogen glycoside) and *thiaminase*. The thiaminase splits thiamine (vitamin B1) into its two inactive components, pyrimidine and thiazole, causing thiamine deficiency. Bracken-fed cows secrete PTA in the milk and the concentration is dependent on the amount of bracken fern ingested by the cow. After the ingestion of the plant material or contaminated milk, both PTA and its aglycone ptaquilosin are converted by the hepatic CYP enzymes into the unstable dienone, which is a strong electrophile. This dienone

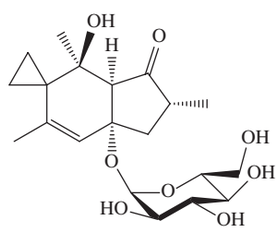

Figure 27-8. *Ptaquiloside.*

is the ultimate carcinogen; it rapidly reacts with DNA, forming covalent adducts and DNA strand breaks. The PTA in milk may be responsible for the incidence of gastric cancer in the populations of farmers that inhabit cattle-range areas in Costa Rica and other countries where bracken growth is dense. Tumors have been demonstrated in rats that were fed the milk of cows that grazed on bracken. However, the dilution resulting from the bulk processing of milk is thought to reduce any risk from milk in most developed countries to negligible levels (Bode and Dong, 2015; Kristanc and Kreft, 2016).

Safrole Safrole (a terpenoid; Fig. 27-9) is typically extracted from the root-bark or the fruit of sassafras plants in the form of sassafras oil. It is also found in a variety of spices, such as cinnamon, nutmeg, black pepper, and herbs such as basil. It was used to flavor root beer and other foods, but was banned by the FDA in 1960 as a food additive. Safrole produces liver tumors in mice and rats by oral administration. The metabolic intermediates of safrole are able to directly react with DNA forming adducts. Safrole is mutagenic and also genotoxic in various in vitro mammalian cell systems. Safrole also induces chromosome aberrations, sister chromatid exchange, and DNA adducts in the liver of rats (EFSA, 2002). In 2011, the National Toxicology Program (NTP) opined that safrole is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals (NTP, 2011). Safrole is a precursor in the synthesis of the illegal recreational drug methylenedioxymethamphetamine (MDMA) or "Ecstasy." Thus, safrole still poses a potential threat to human health.

Cyanogenic Glycosides Cyanogenic glycosides (nitrogen-containing non-alkaloid) account for approximately 90% of the plant toxins known as cyanogens. The toxicity of cyanogenic glycosides is due to their ability to produce hydrogen cyanide (HCN; also known as prussic acid), which inhibits cellular respiration and causes cytotoxic hypoxia. Many common food plants contain cyanogenic glycosides, such as *linamarin* from cassava, flax seed meal; *amygdalin* (Fig. 27-10) from cherry, apple, and peach. The cyanogenic glycosides are present at negligible levels in the fleshy parts of the fruit. Cases of acute poisoning have been associated with misuse, particularly of preparations from apricot pits, bitter almonds, and cyanide rich apple seeds (http://www.foodsafety.govt.nz/elibrary/industry/Cyanogenic_Glycosides-Toxin_Which.pdf). In parts of Africa, cassava is consumed as a dietary staple. Inappropriate handling and processing of cassava prior to consumption can result in a chronic form of cyanide poisoning termed "tropical ataxic neuropathy," the result of demyelinization of the optic, auditory, and peripheral nerve tracts (Dolan et al., 2010).

Glucosinolates Glucosinolates (nitrogen-containing non-alkaloid) are sulfur-containing goitrogenic glycosides found in Brassicaceae and related families (cruciferous vegetables). Enzymatic hydrolysis of glucosinolates produces several different products, such as isothiocyanates, thiocyanates, oxazolidinethiones, and nitriles. These hydrolysis products contribute to the bitter, hot taste of condiments (mustard, horseradish) and are thought to be responsible for the toxicity of glucosinolate including the goitrogenic or antithyroid activity. Thiocyanate and oxazolidinethione anions compete with iodine in two ways, by inhibiting its uptake by the sodium iodide symporter, and also by the binding of iodine to tyrosine residues of thyroglobulin at high concentrations. Some examples of glucosinolates are *sinigrin*, *dehydroerucin*, and *glucobrassicin* (Fig. 27-11). Glucobrassicin is hydrolyzed to form indole-3-carbinol. Glucosinolate toxicity has been reported in ruminants, poultry, and swine. Poultry, swine, and other non-ruminants can tolerate 5% to 10% rapeseed meal in their diets. Symptoms of poisoning include depressed growth, goiters, enlarged livers, perosis (leg deformity in birds), and poor egg production in chicken (EFSA, 2008a). FDA regulates glucosinolate levels in animal feed—glucosinolate calculated as epi-progoitrin, not more than 4%; goitrin, not more than 0.1%; and nitrile calculated as 1-cyano-2-hydroxy-3-butene, not more than 1.4% (21 CFR 573.310).

Glycoalkaloids (α-Solanine and α-Chaconine) Glycoalkaloids (α-solanine, Fig. 27-12; and α-chaconine) in potato are steroidal glycoalkaloids, which together constitute about 95% of the total glycoalkaloids in the edible potato (*Solanum*

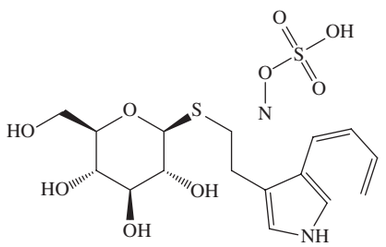

Figure 27-11. *Glucobrassicin.*

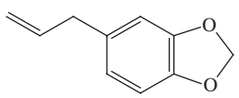

Figure 27-9. *Safrole.*

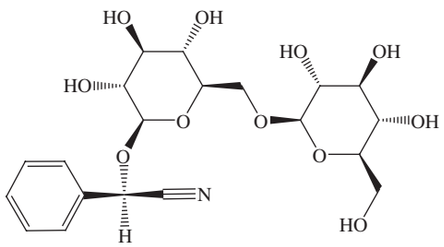

Figure 27-10. *Amygdalin.*

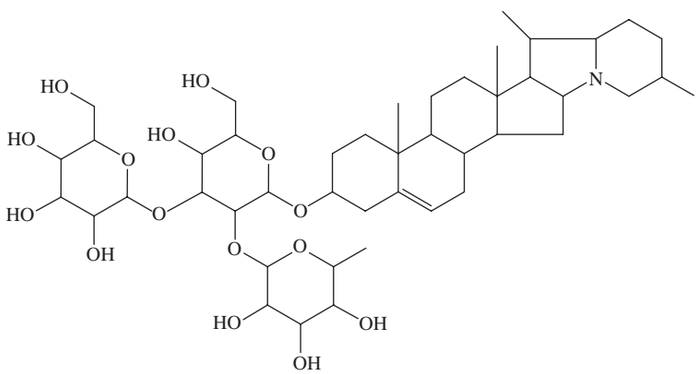

Figure 27-12. *α-Solanine.*

tuberosum). There is usually slightly more α-chaconine than α-solanine. Increases in α-solanine and α-chaconine in the potato peel are closely associated with greening of the peel due to exposure to light, mechanical damage, and improper storage conditions. The α-solanine and α-chaconine each is normally present in amounts less than 5 mg/100 g of tuber fresh weight. Depending on the variety and storage conditions, the concentrations of α-solanine and α-chaconine in potato tubers vary between less than 1 mg and about 25 mg/g potato (Dolan et al., 2010). The content of α-solanine and α-chaconine is not affected much by normal baking, frying, or microwave cooking, but peeling significantly reduces these glycoalkaloids. Acute, short-term, and subchronic animal toxicity studies identified similar adverse effects of α-chaconine, and α-solanine, which is due to their ability to act as inhibitors of acetylcholinesterase and disruptors of cell membranes. Effects on the nervous system included increased heart, pulse, and respiratory rates, sedation, and coma. Effects resulting from cell membrane disruption included internal hemorrhaging, edema, diarrhea, constriction of the abdominal muscles, and lesions of the stomach and duodenum. α-Chaconine is a more potent cholinesterase inhibitor than α-solanine. In some studies, hepatotoxic effects were also induced. It was also determined that the average consumption of glycoalkaloids from potatoes was 12.75 mg glycoalkaloids/person/day (0.18 mg/kg bw based on a bw of 70 kg), which is approximately one-fifth of the lowest dose that has been shown to produce signs of acute toxicity in humans (1 mg/kg bw) (Tice, 1998).

Tannins Tannins are a group of plant phenolics. Tannins are of two types: condensed tannins and hydrolyzable tannins (HT). HTs are polymers of gallic or ellagic acid, and are potentially toxic to ruminants. For example, pyrogallol is a product of HT degradation by ruminal microbes, and has hepatotoxic and nephrotoxic effects (Reed, 1995). Epigallocatechin-3-gallate (EGCG) (Fig. 27-13) and epicatechin gallate (ECG) are the main tannins in green tea. Very high dose of EGCG, such as a single intragastric dose of 1500 mg EGCG/kg, is hepatotoxic in mice (Lambert et al., 2010). Hepatotoxicity in humans has been associated with the consumption of high doses of green tea–containing dietary supplements (10 to 29 mg/kg bw/d) (reviewed by Mazzanti et al., 2009). All cases resolved following cessation of supplement consumption. Based on their analysis, Mazzanti et al. (2009) suggested a causal association between green tea and liver damage, and that the hepatotoxicity was the result of EGCG or its metabolites. Laboratory studies in rodents and dogs using a high bolus dose of green tea–derived preparations rich in EGCG revealed hepatotoxicity and nephrotoxicity (Lambert et al., 2010). However, Isbrucker et al. (2006) demonstrated that dietary administration of EGCG preparation to rats for 13 weeks was not toxic at doses up to 500 mg/kg bw/d. Similarly, no adverse effects were noted when 500 mg EGCG preparation/kg/day was administered to pre-fed dogs in divided doses. The same dose caused morbidity when administered to fasted dogs as a single bolus dose.

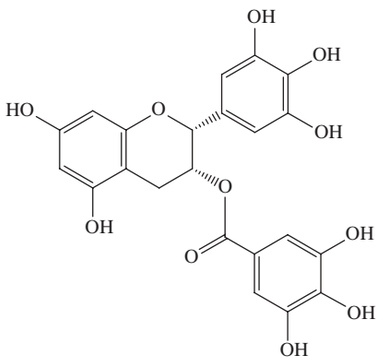

Figure 27-13. *EGCG.*

Furocoumarins Furocoumarins are another group of plant phenolics. Furocoumarins are found mainly in plants belonging to the Rutaceae (e.g., citrus fruits) and Umbelliferae (e.g., parsnip, parsley, celery, carrots) families. Furocoumarins, such as *psoralen* (Fig. 27-14), 5-*methoxypsoralen* (5-MOP, bergapten), and *8-methoxypsoralen* (8-MOP, xanthotoxin or methoxsalen), can form DNA adducts and DNA crosslinks in the presence of UV light (UVA) (320 to 380 nm). The estimated dietary intake of furocoumarins for about 80% of the population is 1.31 mg/day, which is approximately 0.022 mg/kg bw/day for a 60 kg human (Dolan et al., 2010). In humans the phototoxic threshold dose of furocoumarin mixtures is of the order of 10 mg 8-MOP plus 10 mg 5-MOP, which is equivalent to about 15 mg 8-MOP per person (Schlatter et al., 1991). This dose is not reached by the consumption of furocoumarin-containing conventional vegetables under normal dietary habits. Therefore, ordinarily dietary exposure to psoralens is not considered to be a significant risk for the development of photodermatitis, although the margin of safety is low (Schlatter et al, 1991). The 13-week dietary no observable adverse effect level for liver toxicity in the rat (25 mg 8-MOP/kg bw/day) is 1000-fold higher than the estimated dietary intake of furocoumarins in humans. Therefore, the risk of developing liver toxicity in humans due to ingestion of psoralens in the diet is low (Dolan et al., 2010).

Gossypol Gossypol (polyphenolic aldehyde; Fig. 27-15) is a toxic compound found in cotton plants (*Gossypium* sp.). It is concentrated in the cottonseed but is also found in hulls, leaves, and stems. The free form of gossypol is toxic whereas the protein-bound form is relatively nontoxic. The bound form is produced through covalent bonds between gossypol and the free epsilon-amino groups of lysine and arginine. The concentrations of free gossypol contained in feedstuffs such as whole cottonseed and cottonseed meals vary considerably, but gossypol toxicity limits cottonseed use in animal feed. Gossypol poisoning is usually chronic and cumulative. In pigs, poultry, dogs (non-ruminant), pre-ruminants (e.g., young

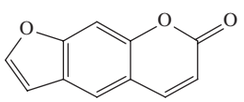

Figure 27-14. *Psoralen.*

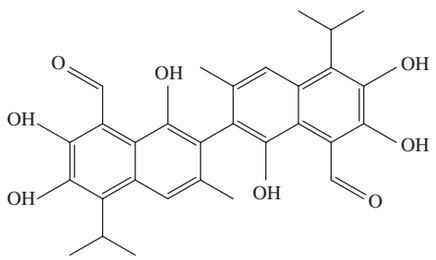

Figure 27-15. *Gossypol.*

calf), and ruminants (e.g., cattle, goat, sheep), the signs of gossypol poisoning are similar and include labored breathing, decreased growth rate, and anorexia. Postmortem findings include generalized edema and congestion of lungs and liver, fluid-filled thoracic and peritoneal cavities, and degeneration of heart fibers. Gossypol also has antifertility effects in many non-ruminant species (Randel et al., 1992). In terms of the severity of toxicity, monogastric animals, such as pigs, birds, and rodents are more susceptible to gossypol toxicity than ruminants. Among ruminants, young ruminants are more sensitive than adult ruminants. However, if the gossypol intake overwhelms the ruminal detoxification capacity, free gossypol may be absorbed at hazardous concentrations even in adult ruminant animals (Gadelha et al., 2014).

Erucic Acid Erucic acid (Fig. 27-16) is a 22-carbon monounsaturated fatty acid with a single double bond at the omega 9 position (C22:1, ω-9). Erucic acid comprises about 30% to 60% of the total fatty acids of rapeseed and mustard seed. The oil extracted from rape (*Brassica napus* or *B. campestris*) and mustard (*Brassica nigra*) seeds have been used in cooking in different parts of the world. Through selective breeding, *B. napus* and *B. campestris* varieties with low erucic acid content were developed in Canada in the 1980s. These improved varieties are popularly known as canola. By definition, canola refers to *B. napus* and *B. campestris* lines containing less than 2% of the total fatty acids as erucic acid. Erucic acid is digested, absorbed, and metabolized like other fatty acids. It undergoes mitochondrial β-oxidation, but is poorly oxidized. Heart muscle seems to be particularly poor at oxidizing erucic acid, which explains the occurrence of myocardial lipidosis in a number of species, such as rat, pigs, and monkeys fed erucic acid in short-term or subchronic studies. Myocardial lipidosis may interfere with the contractile force of heart muscle. Myocardial lipidosis due to erucic acid consumption has not been confirmed in humans, but the results from the animal feeding studies triggered regulatory action in limiting erucic acid exposure in humans (FSANZ, 2003). For example, FDA limits the amount of erucic acid in canola oil to no more than 2% of the component fatty acids (21 CFR 184.1555).

Lectins Lectins are glycoproteins that are known for their ability to agglutinate (clump) cells including erythrocytes in vitro. Hence, lectins are also known as agglutinins (agglutinate cells) and hemagglutinins (agglutinate erythrocytes). Lectins can be found in both plants and animals; those of plant origin are called phytohemagglutinins. Lectins are present in high levels in legumes (e.g., black beans, soybeans, lima beans, kidney beans, and lentils) and grain products. Lectins selectively bind carbohydrates and more importantly, the carbohydrate moieties of the glycoproteins on the surface of most animal cells. High levels of plant lectins in certain improperly cooked food can interfere with nutrient absorption from the intestine and also cause acute GI tract distress. However, not all lectins are toxic, and there are highly toxic lectins without hemagglutinating activity, such as ricin from castor bean. Ricin is one of the most toxic substances known. The seeds of precatory bean *Abrus precatorius* contain the highly toxic lectin *abrin* that has an estimated human fatal dose of 0.1 to 1 μg/kg bw, and has caused death after accidental or intentional poisoning (Wu and Sun, 2012). Less toxic lectins can also be fatal if ingested in high amounts, such as concanavalin A from *Concanavalia ensiformis* (jack bean). Lectins isolated from black beans can produce growth retardation when fed to rats at 0.5% of the diet, and lectin from kidney beans causes death within two weeks when fed to rats at 0.5% of the diet. Soybean lectin produces growth retardation when fed to rats at 1% of the diet (Dolan et al., 2010). Long-term intake of lectins in rodent models shows increased cell turnover, gut hyperplasia, and weight loss. Epithelial cell microvilli are particularly affected by lectin exposure. Areas of epithelial cell necrosis and complete epithelial cell denudation are seen in biopsies of the stomach and intestine. The mechanism of injury of surface-bound lectins may involve inhibition of plasma membrane repair and the exocytosis of mucus that normally accompanies the repair response (Miyake et al., 2007). The combination of soaking and cooking is the best way to reduce the lectin content and its activity in all bean varieties (Nciri et al., 2015).

Phytic Acid (Phytate) Phytic acid (phytate; Fig. 27-17) is inositol hexaphosphate found in edible seeds, nuts, beans/legumes, and grains (mostly in the bran). It serves as the main storage form of phosphorus. When seeds sprout, phytic acid provides the phosphorus needed by the young plant. Ironically, the presence of so many phosphates also enables phytic acid to strongly bind minerals, such as calcium, magnesium, iron, copper, and zinc, making the minerals unavailable for absorption in the intestines. The phytic acid content in plants is highly variable. Phytate has also been shown to inhibit digestive enzymes such as pepsin, trypsin, α-amylase, α-glucosidase. Phytate-rich foods are digested at a slower rate. Several methods reduce the phytic acid content in food, such as fermentation, soaking, germination, and enzymatic treatment of grains with phytase enzyme (Gupta et al., 2015).

Oxalic Acid Oxalic acid (Fig. 27-18) is one of the antinutrients found in the leaves and stems including the corms (underground bulbotubers) of many plants. Some examples include rhubarb, spinach, beet greens, purslane, taro (*Colocasia esculenta*), and many other plants. Ingestion of foods containing high concentrations of oxalic acid results in the formation of insoluble calcium

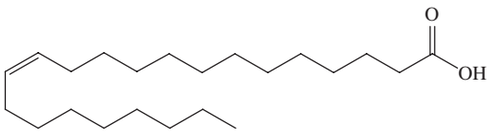

Figure 27-16. *Erucic acid.*

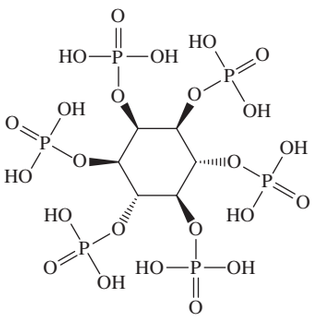

Figure 27-17. *Phytic acid.*

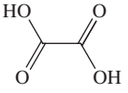

Figure 27-18. *Oxalic acid.*

oxalate and oxalates of other minerals in the systemic circulation. Calcium oxalate precipitates in the kidneys, resulting in renal failure. Sequestration of minerals by oxalate may also decrease their bioavailability, such as reduction in the bioavailability of calcium in the bone. Non-ruminants appear to be more prone to oxalate toxicity than ruminants because rumen bacteria help degrade oxalate. Nevertheless, feeding large amount of oxalate-rich plants at one time may result in oxalate toxicity even in ruminants because the rumen's capacity to degrade oxalate is overwhelmed (Rahman et al., 2013). About 65% of kidney stones consist of calcium oxalate. A randomized controlled trial assigned men with hypercalciuria to follow either a diet low in calcium (400 mg) and oxalate or a diet higher in calcium (1200 mg) with restricted intake of oxalate, protein, and salt. At 5 years, the latter group had a 51% lower rate of stone recurrence than those following a low-calcium diet (Finkelstein and Goldfarb, 2006). Thus, the intake of foods high in oxalate should be limited.

Enzyme Inhibitors Enzyme inhibitors present in many plants (e.g., beans, peas, cereal grains, alfalfa, sunflower, potatoes) and animal foods can inhibit the action of proteases, amylases, and lipases. These inhibitors function as plants' natural defense mechanism against insects and herbivores. For example, kidney beans (*Phaseolus vulgaris*) contain some protease (trypsin and chymotrypsin) inhibitors, and α-amylase inhibitor. The α-amylase inhibitors (α-AI) occur in many plants, and are particularly abundant in cereals. The α-AI inhibits the activity of some mammalian and insect α-amylases, and is a member of a protein family that includes two other defense proteins, phytohemagglutinin and arcelins. These defense proteins are encoded by tightly linked genes. It is likely that this gene family originated from a common ancestral gene through both duplication and divergence (Carlini and Grossi-de-Sá, 2002). Trypsin inhibitor (TI) occurs naturally in many plant-derived foods, notably soybean protein products. Heat treatment inactivates most of the inhibitor activity but 5% to 20% residual inhibitor activity remains. Chronic feeding of food products containing TI can inhibit trypsin and chymotrypsin, stimulate their secretion, cause hypertrophy and hyperplasia of the pancreas, and lead to adenomas and carcinomas of the exocrine pancreas. In the rat, TI promotes pancreatic carcinogenesis initiated by azaserine (Hathcock, 1991). Likewise, most commercially heated soybean meals retain up to 20% of the Bowman–Birk inhibitor (BBI) of chymotrypsin and trypsin, and the Kunitz inhibitor of trypsin (KTI). Toxicology studies in rodents demonstrated that raw soy-based diets had trophic effects on pancreas in rats and hamsters but not in mice. Thus, long-term feeding of raw soy-based diets caused hypertrophy, adenomas, and nodular hyperplasia of the pancreas (Friedman and Brandon, 2001). In contrast, there were no adverse effects on the pancreas of monkeys fed diets containing low levels of trypsin inhibitor for up to 4 years. Specifically, neither pancreatic hypertrophy nor hyperplasia was evident. In addition to monkeys, pancreatic enlargement resulting from the consumption of inhibitors was not observed in dogs, pigs, calves, and baboons (Schneeman and Gallaher, 1986; Friedman and Brandon, 2001). Thus, the consumption of soybeans in amounts normally consumed is unlikely to produce any effect of toxicological significance in humans. Biotin is a coenzyme and a B vitamin (vitamin B7). Biotin acts as a coenzyme of several carboxylase enzymes. Avidin is a tetrameric protein that binds biotin. Avidin is found in raw eggs (egg white) and it binds biotin with a very strong affinity ($K_D \approx 10^{-15}$ M). Thus, consuming raw egg directly or mixing it in other drinks or shakes chronically can cause biotin deficiency. Avidin's ability to bind biotin is largely, but not completely, destroyed by cooking the egg (Durance and Wong, 1992).

Phytoestrogens Phytoestrogens are so named because of their ability to bind to estrogen receptors and stimulate receptor activity. The main groups of phytoestrogen are the lignans, flavonoids (subgroups isoflavones, coumestans, and prenylflavonoids), and stilbenes. Lignans are the main phytoestrogens in a normal diet, and they are found in many cereals, fruits, and vegetables. In vitro assays have shown that most phytoestrogens bind both estrogen receptors (ERs), ERα and ERβ, and activate ER-dependent gene transcription. However, they generally have a higher binding affinity for ERβ. Isoflavones act like selective estrogen receptor modulators (SERMs), such as tamoxifen. Tamoxifen acts as an ER agonist in the uterus and bone but as an antagonist in the breast, where it has proven anticancer activity. In animal models, some phytoestrogens (e.g., isoflavones) produce symptoms of endocrine disruption, which include premature reproductive senescence, compromised fertility, disruption of the lactation and the timing of puberty, the inhibition of sexual behavior, etc. However, the data in humans are not conclusive. The majority of epidemiological studies and meta-analyses concluded that the high intake of soy isoflavones is associated with reduced breast cancer risk in pre- and postmenopausal women. The use of soy isoflavones is seemingly safe in women with a history of breast cancer. Studies even indicate that the isoflavone intake improves the prognosis of breast cancer patients. Thus, the results of the in vitro and animal studies that indicate a potential adverse effect of phytoestrogens are not necessarily corroborated by the findings in humans (Bode and Dong, 2015; Kristanc and Kreft, 2016).

Some Naturally Occurring Toxins in Food of Marine Origin

Toxicity from seafood is due to algal or bacterial toxins that accumulate in shellfish[6] and fish, or due to the production of histamine by spoilage bacteria in fish. These toxin-producing algae are important food source for various shellfish and fish, which accumulate these toxins in specific organs. There are no antidotes for these toxins; they are heat stable and largely unaffected by cooking (Sobel and Painter, 2005). The seafood contaminated with such toxins still look, smell, and taste normal. *Many of these toxins act on sodium channels—some toxins block the channels inducing muscle paralysis; others open the channels, prolong depolarization of the membrane and spontaneous firing causing symptoms like involuntary muscle spasms, tingling paresthesia, etc*. These toxins are usually lethal in small doses.

Saxitoxin Saxitoxin and related toxins are tetrahydropurines mainly produced by the marine dinoflagellate genus *Alexandrium* spp., but also from other dinoflagellate species, such as *Gymnodinium catenatum* and *Pyrodinium bahamense* (Dolan et al., 2010). Saxitoxin causes *paralytic shellfish poisoning* (PSP), which is a severe, rapid onset, life-threatening neurotoxicity caused by the consumption of oysters, mussels, scallops, clams, crustaceans, gastropods, and fish that are contaminated with saxitoxin or closely related marine toxins. *Saxitoxin inhibits the conduction of nerve impulse by blocking the sodium channels* (Daranas et al., 2001). Symptoms of PSP, such as headache, nausea, vomiting, and diarrhea, appear within 30 minutes. In fatal cases respiratory arrest occurs 2 to 12 hours following consumption of the contaminated shellfish. Using the available human data on intoxication in humans

[6]Edible shellfish include two groups: crustaceans and mollusks. Common crustaceans include lobster, crab, crawfish (crayfish), prawn, and shrimp. Common mollusks include clam, mussel, oyster, scallop, squid, and octopus.

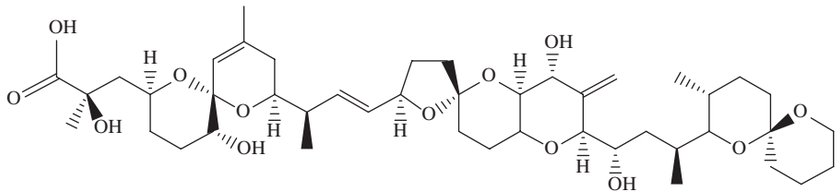

Figure 27-19. *Okadaic acid.*

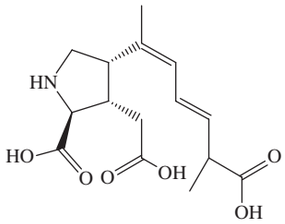

Figure 27-20. *Domoic acid.*

comprising more than 500 individuals, EFSA CONTAM Panel determined a LOAEL of 1.5 μg saxitoxin equivalents/kg bw. Using the uncertainty factor of 3, an ARfD of 0.5 μg saxitoxin equivalents/kg bw was established (EFSA, 2009a). The rationale for using 3 as the safety factor was the fact that documentation of human cases included a large number of affected consumers, including sensitive individuals, and the fact that saxitoxin-induced mild illness is readily reversible. It was determined that in order for a 60 kg adult to avoid exceeding the ARfD of 0.5 μg saxitoxin equivalents/kg bw, there should not be more than 75 μg saxitoxin equivalents/kg of shellfish meat. The EFSA CONTAM Panel recommended significant reduction of the current European Commission (EC) limit of PSP, which is 800 μg/kg (= 80 μg/100 g) of shellfish meat. The FDA consumer guidance recommends that the limit of the saxitoxins in shellfish meat should not exceed 0.8 ppm saxitoxin equivalents, that is, 80 μg saxitoxin equivalents/100 g shellfish meat (FDA, 2011a).

Okadaic Acid (OA), Dinophysistoxin (DTX), Pectenotoxin (PTX), Yessotoxins (YTX) Okadaic acid (OA) (Fig. 27-19), dinophysistoxin (DTX), pectenotoxin (PTX), and yessotoxins (YTX) are lipophilic polyether compounds that all cause *diarrheic shellfish poisoning* (DSP), which is a rapid-onset but self-resolving illness involving the GI system. The toxins are produced by marine dinoflagellates of the genus *Dinophysis* spp. These toxins are known to accumulate in marine shellfish, particularly in the hepatopancreas (Paredes et al, 2011; Reguera et al., 2014). DSP has been reported more often in Japan but also in Europe. OA strongly inhibits the eukaryotic serine/threonine phosphatases 1 and 2A, resulting in hyperphosphorylation of many cellular proteins. *It has been proposed that OA-induced stimulation of phosphorylation of proteins that control sodium secretion by intestinal cells results in diarrhea*. Symptoms of DSP, such as diarrhea and abdominal cramps, usually appear within 30 minutes after exposure but typically resolve within 3 to 4 days. These are not considered to be lethal (Paredes et al, 2011; Valdiglesias et al., 2013). An intake of 40 μg of OA equivalents is the minimum dose required to produce DSP symptoms in human adults. The lowest observed adverse effect level (LOAEL) in mice, by acute oral administration, was calculated to be 75 μg/kg bw. Human data indicate a LOAEL of 1.0 to 1.6 μg/kg bw (Valdiglesias et al., 2013). EFSA concluded that the LOAEL for human illness is approximately 50 μg OA equivalents/person, which is equivalent to 0.83 μg OA equivalent/kg bw for a 60-kg person (EFSA, 2008b). The FDA consumer guidance recommends that the limit of the total OA in shellfish meat should not exceed 0.16 ppm, that is, 16 μg of total OA/100 g shellfish meat (FDA, 2011a).

Domoic Acid (DA) Domoic acid (DA) (Fig. 27-20) is a water-soluble, heat-stable amino acid that causes *amnesic shellfish poisoning* (ASP). DA is produced by marine diatoms (algae) of the genus *Pseudonitzschia* spp., and by marine red algae of the genus *Chondria* spp., particularly when there are severe algal blooms. Because DA toxicity does not always involve amnesia, it is also known as *domoic acid poisoning* (Paredes et al, 2011). Symptoms of ASP included vomiting, nausea, diarrhea, and abdominal cramps within 24 hours of ingestion; neurological symptoms developed within 48 hours. Both shellfish and fish can accumulate DA without apparent ill effects. In mammals including humans, *DA is neurotoxic; high levels of DA cause seizures. DA specifically targets the hippocampus*. DA is structurally similar to but 2 to 3 times more potent than another known neurotoxin kainic acid (KA) found in marine seaweed. Both DA and KA are excitatory amino acid analogues of glutamic acid, and they act through the ionotropic glutamate receptors (GluR), preferentially the KA subtype of GluR (Pulido, 2008). Hyperexcitation of the receptors leads to hyperexcitation of the neurons which ultimately "burn out" and die off. The damage of hippocampal neurons results in permanent loss of the "short-term memory" in susceptible individuals (hence the name amnesic shellfish poisoning). The toxicological database for DA comprises mostly studies on the acute toxicity in rodents and cynomolgus monkeys. Neurotoxicity is the critical toxicological effect identified in experimental animals as well as in humans (EFSA, 2009b). Using the human DA poisoning acute toxicity data, EFSA (2009b) determined the ARfD to be 30 μg DA/kg bw. This ARfD was derived by applying an overall uncertainty factor of 30 to the lowest-observed-adverse-effect-level (LOAEL) for mild signs and symptoms, which was 0.9 mg/kg bw. The basis for the uncertainty factor 30 was a factor of 3 for the extrapolation of LOAEL to NOAEL multiplied by a factor of 10 to account for inter-individual variability in humans. The FDA consumer guidance recommends that the limit of DA in shellfish meat should not exceed 20 ppm except in the viscera of Dungeness crab, where it should not exceed 30 ppm (FDA, 2011a). A 20 and 30 ppm DA are equivalent to 2 mg DA/100 g and 3 mg/100 g of shellfish meat, respectively.

Brevetoxins Brevetoxins are a family of lipophilic cyclic polyether toxins that cause *neurotoxic shellfish poisoning* (NSP), which involves transient GI and neurological symptoms. Brevetoxins are produced by the marine dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve*) (Watkins et al., 2008). *Brevetoxins open voltage-gated sodium channels causing uncontrolled Na^+ influx into the cell, prolonged depolarization of neuronal and muscle cell membranes, and spontaneous firing*. The GI symptoms

of NSP include vomiting, diarrhea, and pain in the abdomen. Neurological symptoms include paresthesia (a pricking, tingling sensation), reversal of hot and cold perception, vertigo (false sense of motion), and ataxia (involuntary muscular movement, or spasms). Symptoms typically occur within 30 minutes to a couple of hours after consuming contaminated shellfish and typically resolve within 2 days. The toxicological database for brevetoxins is limited, and comprises only studies on their acute toxicity following intravenous (i.v.), intraperitoneal (i.p.), and oral administration (EFSA, 2010). Because brevetoxins are lipophilic, they pass through cell membranes including the blood–brain barrier. Brevetoxins are rapidly absorbed and distributed throughout the body, and are metabolized in the liver. They are primarily removed in the bile within the first 48 hours, but urinary excretion plays a role after this time as well (Watkins et al., 2008). The LD50 of different brevetoxins may vary widely and the route of administration also plays a major role. For example, the oral LD50 in mice for brevetoxin-2 is 6600 mg/kg bw and for brevetoxin-3 is 520 mg/kg bw. In contrast, i.v. and i.p. administration causes toxic signs including death almost immediately, whereas toxic signs including death occurred approximately after 5 hours after oral administration (EFSA, 2010). EFSA (2010) concluded that there were insufficient human and animal toxicity data to complete a risk assessment. The FDA consumer guidance recommends that the limit of the brevetoxins in shellfish meat should not exceed 0.8 ppm brevetoxin-2 equivalents, that is, 80 μg brevetoxin-2 equivalents/100 g shellfish meat (FDA, 2011a).

Ciguatoxin Ciguatoxin group of toxins include ciguatera toxin, gambiertoxin, and maitotoxin. They occur in marine fishes. Ciguatoxins are mostly lipophilic, heat-stable polyethers. They are produced by the benthic dinoflagellate *Gambierdiscus toxicus*. Gambiertoxin produced by the dinoflagellate is biotransformed by the fish to produce ciguatera toxin. These dinoflagellates are consumed by small herbivorous fishes that are, in turn, consumed by large carnivorous fishes (e.g., barracuda and grouper fish), resulting in bioconcentration of the toxin along the food chain (Lehane and Lewis, 2000). Ciguatoxins cause *ciguatera fish poisoning* (CFP), which is primarily associated with the consumption of large predatory fishes that have accumulated ciguatoxins by feeding on smaller contaminated fishes. *Ciguatoxins open voltage-gated sodium channels at the neuromuscular junction, resulting in membrane hyperexcitability, spontaneous repetitive neurotransmitter release, blockage of synaptic transmission, and depletion of synaptic vesicles*. Maitotoxin is water-soluble and it opens calcium channels of the cell plasma membrane; it is the most toxic nonproteinaceous molecule known (Lehane and Lewis, 2000; Daranas et al., 2001). CFP is typically a self-limiting illness, although symptoms may last for months and deaths have been reported. Symptoms of CFP include GI (e.g., vomiting, diarrhea, nausea), neurological (e.g., tingling, itching), and cardiovascular (e.g., hypotension, bradycardia) effects. In severe cases the symptoms may begin as soon as 30 minutes after ingestion of contaminated fish, while in milder cases they may be delayed for 24 to 48 hours. Symptoms may last even for months and the administration of mannitol within 24 hours of consumption relieves some symptoms (Dickey and Plakas, 2010). CFP is currently the most common type of marine biotoxin food poisoning with an estimated number of 10,000 to 50,000 people affected annually (Lehane, 2000). The FDA consumer guidance recommends that the limit of ciguatoxins in fish flesh should not exceed 0.01 ppb (0.01 μg/kg fish flesh) for Pacific ciguatoxin, and 0.1 ppb (= 0.1 μg/kg fish flesh) for Caribbean ciguatoxin (FDA, 2011a).

Tetrodotoxin Tetrodotoxin is a heat-stable, water-soluble molecule produced by symbiotic bacteria concentrated in the liver and gonad of puffer fish (Fugu), and in many other marine organisms. *Tetrodotoxin is a potent sodium channel blocker; it reduces the membrane excitability and blocks the conduction of nerve impulse, thereby causing muscle paralysis*. Symptoms of toxicity include perioral paresthesia (burning or prickling sensation) that may spread to the entire body, and could be followed by vomiting, lightheadedness, dizziness. In non-fatal poisoning, symptoms usually begin within 30 minutes after exposure and completely resolve within 24 hours after onset. In fatal cases, progression is precipitous, with death due to respiratory muscle paralysis occurring within 6 hours after onset of symptoms. In Japan, puffer fish is a delicacy but it is cleaned and prepared by specially trained chefs. The intragastric LD50 of tetrodotoxin in mouse was 532 μg/kg (Lago et al., 2015). Even though tetrodotoxin is such a potent sodium channel blocker, it cannot block the sodium ion channel of puffer fish because these sodium channels are insensitive to tetrodotoxin.

Scombroid Toxicity Scombroid toxicity causes *scombroid fish poisoning*. Scombroid poisoning is unique among the seafood toxins because it results from product mishandling rather than contamination from other trophic levels (Hungerford, 2010). *It is mostly caused by the consumption of fish (Scombroidae family) with high histamine levels, such as tuna, mackerel, blue fish, sardines, anchovies,* and *mahi-mahi. Symptoms are essentially the same as those associated with histamine toxicity. Thus, such histamine-induced reactions are often misdiagnosed as IgE-mediated fish allergy.* If the fish with high levels of tissue histidine are not refrigerated properly, the bacteria proliferate and convert the tissue histidine into histamine, which is heat-stable. In most cases, histamine levels in illness-causing fish have been above 200 ppm, often above 500 ppm (FDA, 2011a). Symptoms of toxicity include tingling and burning sensations around the mouth, headache, facial flushing, palpitations, profuse sweating, truncal rash and pruritis, abdominal cramps, nausea, and diarrhea. In most people, these symptoms are self-limiting, but circulatory collapse, shock, and acute pulmonary edema have been described in severe cases (Kerr and Parke, 1998; Becker et al., 2001). Scombroid poisoning is the most common cause of ichythyotoxicosis worldwide. Because more than 80% of fish consumed in the United States is now imported from other countries, the disease is intimately linked with the global fish trade (Feng et al., 2016).

Palytoxin Palytoxin is produced by the zoantharians of the genus *Palythoa* (cnidarians found in coral reefs). Fishes and crabs living in close association with or eating this mass may become contaminated with palytoxin. Palytoxin has been reported in mackerel, parrotfish, and several species of crabs. It is one of the most lethal marine toxins known. *It binds to the Na^+/K^+-ATPase (Na^+/K^+ pump) and turns it into a nonspecific ion channel. This results in K^+ efflux, Na^+ influx, and prolonged membrane depolarization*. Victims exposed to palytoxin report nausea, vomiting, and diarrhea. Within several hours, symptoms include myoglobinuria, a burning sensation around the mouth and extremities, muscle spasms, paresthesia, bradycardia, dyspnea (labored breathing), and dysphonia (difficulty in speaking). Cause of death may be the result of myocardial injury. Palytoxin is known in vitro to be a powerful hemolysin. The Na^+/K^+-ATPase inhibition by palytoxin is similar to that of ouabain, but unlike ouabain, palytoxin has no effect on H^+-, Ca^{2+}-, or H^+/K^+-ATPases (Ramos and Vasconcelos, 2010; Kotsonis and Burdoch, 2013).

Some Microbial Contaminants in Food

Food-related illnesses resulting from microbial contamination can pose a serious threat to public health. Foodborne disease outbreaks are tracked by the Centers for Disease Control and Prevention (CDC). CDC estimates that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3000 die of

foodborne diseases. The 2014 CDC annual report on the surveillance for foodborne disease outbreaks in the United States reports that there were 864 outbreaks involving bacterial, viral, parasitic, chemicals, and toxin etiology. However, the actual frequency may be significantly higher because (1) an outbreak is classified as such only when the source can be identified as affecting two or more people and (2) most home poisonings are mild or have a long incubation time and are therefore not connected to the ingested food, and go unreported.

Food can serve as the vector for the transfer of microbial toxins or live pathogenic microbes into the human GI tract. Bacterial toxins are of two types: exotoxin and endotoxin. *Exotoxins* are not an integral part of the bacterial membrane; they are soluble, usually heat-labile proteins found in the cytoplasm, and are easily secreted into the surrounding medium, usually by gram-positive bacteria. Exotoxins enhance bacterial virulence, and are further subdivided according to their mode of action, such as emetic toxins that trigger severe vomiting; enterotoxins that cause diarrhea; cytotoxins that kill host cells; neurotoxins that interfere with normal transmission of nerve impulse. Table 27-10 lists some important foodborne bacterial exotoxins and their mode of action in causing toxicity. In contrast, *endotoxins* are lipopolysaccharides and membrane constituents. Endotoxins are not secreted but released from dead or dying gram-negative bacteria. Humans have background exposure to endotoxins by the oral and inhalation route, which may vary significantly depending on the living and working conditions (Madsen, 2006; Inagawa et al., 2011). However, higher-than-tolerable exposure to endotoxins can be problematic. Endotoxins are pyrogenic and are not easily destroyed. Endotoxins also stimulate inflammatory responses. Some bacteria, such as *Shigella* spp., *Staphylococcus aureus*, or *Escherichia coli*, can elaborate both endotoxin and exotoxin.

The FDA Bad Bug Book, which is available to the public, provides current information about the major known agents that

Table 27-10

Some Foodborne Bacterial Exotoxins and Their Mode of Action

BACTERIAL EXOTOXIN	PRODUCING ORGANISM	TOXICITY TO THE HOST	MODE OF ACTION
Cereulide (emetic toxin)	*Bacillus cereus*	Vomiting (emesis)	Toxin binds to the 5-HT3 receptors in the stomach/small intestine and stimulates the vagus nerve. Stimulation of the afferent vagal neurocircuit triggers the vomiting center in the hindbrain
Cholera toxin (enterotoxin)	*Vibrio cholerae*	Diarrhea	Stimulates the efflux of ions and water from intestinal epithelial cells. The mechanism involves overstimulation of G proteins which results in persistent activation of adenylate cyclase to produce cAMP. Very high levels of intracellular cAMP prolong the opening of membrane channels that cause the efflux of Cl^- and other ions and water from the infected cells
Staphylococcal enterotoxin (superantigen)	*Staphylococcus aureus*	Vomiting and diarrhea	Stimulates the vomiting center in the brain. Penetrates the gut lining and also activates immune response in the gut. The inflammatory response and the released inflammatory mediators are associated with inflammatory injury to the GI tract, and electrolyte dysregulation
Listeriolysin O (enterotoxin)	*Listeria monocytogenes*	Diarrhea	Pore-forming cytolysin that directly binds to the membranes in a cholesterol-dependent manner, which is followed by oligomerization of monomers, and formation of pores of 20- to 30-nm diameter that facilitate the efflux of ions and water
CPE (enterotoxin)	*Clostridium perfringens*	Diarrhea	Pore-forming cytolysin. Binds to the claudin receptors (tight junction membrane proteins) on enterocytes, forms large complex that forms pores in the membrane, causes a strong influx of calcium, and apoptotic cell death. Enterocyte death disrupts villus integrity, and causes intestinal damage and electrolyte dysregulation
Diphtheria toxin (cytotoxin)	*Corynebacterium diphtheriae*	Cytotoxicity (cell death)	Inhibits protein synthesis by inhibiting the eukaryotic elongation factor 2 (eEF2)
Shiga toxin (cytotoxin)	*Shigella dysenteriae*; shiga toxin–producing *Escherichia coli* (STEC)	Cytotoxicity (cell death)	Inhibits protein synthesis by inhibiting 28S ribosomal RNA (28S rRNA). The same mechanism is also utilized by ricin in castor beans
Botulinum toxin (neurotoxin)	*Clostridium botulinum*	Neurotoxicity	Interferes with normal transmission of nerve impulse by blocking neurotransmitter (e.g., acetylcholine) release at the nerve terminals

SOURCES: Schmitt et al. (1999), Dramsi and Cossart (2002), Pinchuk et al. (2010), and Kotsonis and Burdoch (2013).

cause foodborne illness. The information provided in the book is abbreviated and general in nature, and is intended for practical use. It is not intended to be a comprehensive scientific or clinical reference. In the following narrative, a few of the microbial contaminants in food are briefly discussed. The FDA Bad Bug Book can be accessed at: http://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf.

Clostridium botulinum, C. butyricum, and C. baratti Food botulism is caused by eating foods contaminated with botulinum toxins. Foods associated with botulinum toxin include improperly canned low-acid foods (e.g., green beans, corn, beets, asparagus, chili peppers, mushrooms, spinach, figs, baked potato, cheese sauce, beef stew, olives, and tuna). The toxin also may occur in smoked fish, fermented food (e.g., seal flippers, salmon eggs), and improperly home-cured hams. An increasing source of poisonings is from the use of flavored oils or oil infusion, most typically in garlic-in-oil preparations. All Clostridia are gram-positive, spore-forming anaerobes, and they produce a total of seven serotypically and antigenically distinct botulinum neurotoxins (serotypes A–G) (Rossetto et al., 2014). Human botulism is caused mainly by types A, B, E, and (rarely) F. Types C, D, and also E cause toxicity in other mammals, birds, and fish (WHO, 2013). Type G has not caused any human illness. Botulinum toxin is neurotoxic that interfere with neural transmission by blocking the release of acetylcholine at peripheral nerve endings, causing muscle paralysis. The toxin is elaborated in foods, wounds, and infant gut. Clinical illness is characterized by cranial nerve palsies, followed by descending flaccid muscle paralysis, which can involve the muscles of respiration. Recovery often takes weeks to months (Sobel et al., 2004). The toxins are heat-labile (may be rendered harmless at 80°C to 100°C for 5 to 10 minutes) but the spores are among the most heat-resistant. The case fatality rate in the United States is 4% (Sobel et al., 2004) and the poison is fatal in 3 to 10 days; the lethal dose is approximately 1 ng. Whereas a proteolytic strain of *C. botulinum* (Group I) may cause the food to appear and smell "spoiled" (by-products include isobutyric acid, isovaleric acid, and phenylpropionic acid), this is not the case with nonproteolytic strains, many of which can flourish and elaborate toxin at temperatures as low as 3°C. Current methods for detecting botulinum toxin include a mouse bioassay and an enzyme-linked immunosorbent assay, with the mouse bioassay being the accepted standard. The successful use of nitrates in meat to prevent spoilage by *C. botulinum* resulted in the petitioning of FDA by the USDA to have sodium and potassium nitrate approved for use by "prior sanction" (21 CFR 181.33) (Kotsonis and Burdoch, 2013).

Clostridium perfringens The primary reservoir for *C. perfringens* is the intestinal tract of warm-blooded animals (including humans). Most incidences of *C. perfringens* food poisoning are associated with the consumption of roasted meat that has been contaminated with intestinal contents at slaughter, followed by roasting and inadequate storage. This allows *C. perfringens* growth and the elaboration of *C. perfringens* enterotoxin (CPE). Virtually all food poisoning is produced by type A strain, although a particularly severe form (a necrotic enteritis called "pig-bel" among indigenous peoples of the New Guinea highlands or in Germany known as "Darmbrand") is produced by the type C strain, which has a mortality rate of 15% to 25% even with treatment. The toxin is normally trypsin-sensitive, but people with low intakes of protein or who consume trypsin-inactivating foods (e.g., sweet potatoes) are more at risk than those with normal trypsin levels (Granum and Brynestad, 1999; Granum, 2006). CPE is enterotoxic and follows an ordered series of events, first causing cellular ion permeability, followed by inhibition of macromolecular (DNA, RNA) synthesis, morphological alteration, cell lysis, and villi tip desquamation and severe fluid loss. This is manifested by abdominal cramping and diarrhea; diarrhea occurs within 8 to 16 hours. However, symptoms are of short duration, one day or less. Foods associated with *C. perfringens* poisoning include cooked meat or poultry, gravy, stew, and meat pies. *C. perfringens* is also associated with the production of 11 other toxins, including those associated with gas gangrene (Crane, 1999; Kotsonis and Burdoch, 2013).

Bacillus cereus *B. cereus* produces one emetic toxin and three different enterotoxins. The emetic toxin (a cyclic oligopeptide) is pre-formed in food, and the enterotoxins (proteins) are produced in the small intestine of the host (Granum and Lund, 1997). *B. cereus* is a Gram-positive, spore-forming rod, but is an aerobe or facultative anaerobe. *B. cereus* is a causative agent of emetic or diarrheagenic enterotoxins elaborated in food. The emetic thermostable toxin (surviving 259°F for 90 minutes) is called cerulide (a small cyclic peptide, 1.2 kDa, that acts on 5-HT3 receptors stimulating the vagus afferent nerve) and is produced by serotypes 1, 3, and 8; it is also resistant to pH and proteolysis, but is not antigenic. The diarrheagenic thermolabile toxin (surviving 133°F for 20 minutes) is produced by serotypes 1, 2, 6, 8, 10, and 19 and may also be produced in situ in the lower intestine of the host. The diarrheal form may actually consist of three toxins, one of which is hemolytic (Granum, 2006). Foods associated with this organism and its toxic properties include boiled and fried rice (principally the emetic form), while the diarrheal form has a wider occurrence and may be found in meats, stews, pudding, sauces, dairy products, vegetable dishes, soups, and meat loaf (Granum and Lund, 1997; Crane, 1999). The foods associated with the two types somewhat reflect the geographic distribution of the types. For example, the emetic type predominates in Japan, whereas in North America and Europe, the diarrhea type is most often seen. Evidence is accumulating that other species of *Bacillus* may elaborate food toxins, including *B. thuringiensis*, *B. subtilis*, *B. licheniformis*, and *B. pumilis* (Crane, 1999; Granum, 2006). A notable exception is *B. cereus* var toyoi, a naturally occurring, nontoxigenic, and nonpathogenic strain. This has been approved for addition to swine, bovine, poultry, and rabbit feed in the European communities (Kotsonis and Burdoch, 2013).

Staphylococcus aureus Staphylococcal intoxication includes staphyloenterotoxicosis and staphylococcus food poisoning. *S. aureus* produces a wide variety of exotoxins, including toxic shock syndrome toxin-1 (TSST-1), the exfoliative toxins ETA and ETB, leukocidin, and the staphylococcal enterotoxins A–I (SEA–SEI). SEA is the most common toxin in staphylococcus-related food poisoning. Although enterotoxemia only develops from ingestion of large amounts of SE, emesis is produced as the result of stimulation of the putative SE receptors in the abdominal viscera, followed by a cascade of inflammatory mediator release. All the SE toxins cause emesis and gastroenteritis in primates but the induction of emesis is not directly correlated to superantigenic activity (Granum, 2006). Sources of *Staphylococcus* include nose and throat discharges, hands and skin, infected cuts, wounds, burns, boils, pimples, acne, and feces. The anterior nares of humans are the primary reservoirs. Other reservoirs include mastitis in the udders of cows and ewes (responsible for contamination of unpasteurized milk) and arthritic and bruised tissues of poultry. Foods usually are contaminated after cooking by persons cutting, slicing, chopping, or otherwise handling them, and then keeping them at room temperature for several hours or storing them in large containers. Foods associated with staphylococcal poisoning include cooked ham, meat products including poultry and dressing, sauces and gravy, cream-filled pastry, potatoes, ham, poultry, fish salads, milk, cheese, bread pudding, and generally high-protein leftover foods (Kotsonis and Burdoch, 2013).

Escherichia coli Enterohemorrhagic *E. coli* (EHEC) serotype O157:H7 is a human pathogen responsible for outbreaks of bloody diarrhea and hemolytic uremic syndrome worldwide. There are four main categories of *E. coli* associated with diarrheal disease: enteropathogenic, enterotoxigenic, enteroinvasive, and verocytotoxin-producing *E. coli* (VTEC). VTEC includes "shiga toxin"–producing *E. coli* (STEC) and "shiga-like toxin"–producing *E. coli*. EHEC is a serotype of VTEC. The reference to shiga toxin is the result of the clinical similarity of the bloody diarrhea caused by EHEC to that caused by *Shigella*. The symptoms of *E. coli* STEC infections vary, but commonly include severe stomach cramps, diarrhea (often bloody), and vomiting, and about 5% to 10% of those diagnosed with STEC infections develop hemolytic uremic syndrome, a potentially life-threatening complication because the kidneys may stop working. Cattle are a significant reservoir of *E. coli* O157:H7. Hence, most outbreaks in the United States have been associated with hamburgers and other beef products, although raw vegetables (often fertilized with manure) and unpasteurized apple cider and juice have been reported as sources of outbreaks. Outbreaks in Europe are more often associated with contamination of recreational waters (swimming pools, lakes, etc.). Other sources of contamination include person-to-person contact (especially in families and among institutionalized persons) and contact with farm animals especially following educational farm visits (Karch et al., 1999). Human cases of *E. coli* O157:H7 infections have been reported from the consumption of contaminated lettuce, potatoes, radish sprouts, alfalfa sprouts, cantaloupe, and unpasteurized apple cider and juice (Karch et al., 1999; Kotsonis and Burdoch, 2013).

Like the diarrhea-causing *E. coli*, *Shigella* spp. (*S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*) cause an estimated 165 million cases of *Shigella* diarrhea annually, over 90% of which is in developing countries resulting in over a million deaths annually (Niyogi, 2005). Contamination of food by unhygienic food handlers and consumption of raw vegetables raised in contaminated soils are two main contributors to the incidence of *Shigella* diarrhea.

Listeria monocytogenes *L. monocytogenes* is the causative agent for Listeriosis. *L. monocytogenes* is a gram-positive bacillus found in soil, water, and some animals, including poultry and cattle. It can be present in dairy products, poultry, meat, salads, etc. Food products can be contaminated by contact with soil, feces, discharges, and urine from infected animals and humans. *Listeria* can also live in food processing plants and contaminate a variety of processed foods. *Listeria* can grow even in the refrigerator, but is killed by cooking and pasteurization. The populations that are particularly at risk from *L. monocytogenes* infection include older adults, pregnant women, infants, immunocompromised individuals, organ transplant patients, and people with certain diseases. Listeriosis in pregnant women can result in abortion, fetal death, or premature birth. Listeriosis has a low incidence of infection, although this is undeniably increasing, with a high fatality rate among those infected (Mateus et al., 2013). Since 2008, several Listeriosis outbreaks have been linked to diverse types of fresh produce: sprouts, celery, cantaloupe, stone fruit, and apples. The 2011 cantaloupe-associated outbreak was one of the deadliest foodborne outbreaks in recent U.S. history (Garner and Kathariou, 2016). In 2008, FDA issued a draft guidance for the industry that contains nonbinding recommendations for the control of *L. monocytogenes* in refrigerated or frozen ready-to-eat foods (FDA, 2008). In the recent past there have been multiple reports of food-related Listeria outbreaks in the United States.

Salmonella sp. *Salmonella enterica* serotype Enteritidis is of great public health concern. *Salmonella* sp. in food can cause salmonellosis. Feces of infected humans, animals, and birds containing *Salmonella* can contaminate raw fruits and vegetables as well as meat and milk products. Consuming contaminated food causes nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. The interval between exposure and the onset of the symptoms may be as short as a few hours or it can take a couple of days. The illness is generally self-limiting for people with intact immune system and is resolved within a week. Mortality is rare but occurs in the very young and the very old and in immunocompromised individuals (Coburn et al., 2007). FDA issued a final rule in 2009 that requires shell egg producers to implement measures to prevent *Salmonella* Enteritidis from contaminating eggs on the farm and from further growth during storage and transportation (FDA, 2009). In the recent past there have been several multistate foodborne outbreaks involving *Salmonella*.

Cronobacter sakazakii (Enterobacter sakazakii) *C. sakazakii* was previously known as *E. sakazakii*. *C. sakazakii* is found in milk powder, rice, vegetables, cheese, sausage meat, teas, and various spices. *C. sakazakii* has also been identified in the hospital environment in association with infant bottle brushes and food preparation equipment, such as blenders. Most of the attention to *C. sakazakii*–related contamination of food products has focused on powdered infant formula. In 2002, FDA published a warning regarding the presence of *C. sakazakii* in infant formula (Hunter et al., 2008). This bacterium is an emerging opportunistic pathogen that is associated with rare but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in premature and full-term infants. Infants aged less than 28 days are considered to be most at risk (Drudy et al., 2006).

Some Foodborne Molds and Mycotoxins

Molds have served humans for centuries in the production of foods (e.g., ripening cheese) and have provided various fungal metabolites with important medicinal uses; they also may produce secondary metabolites with the potential to produce severe adverse health effects, including behavioral changes (Cousins et al., 2005). Frank growth of fungi on animal hosts produces *mycoses*, whereas dietary, respiratory, dermal, and other exposures to toxic fungal metabolites produce *mycotoxicoses*. In the context of food toxicology, a discussion of mycotoxins and the mycotoxicosis they cause is more relevant. Mycotoxins are secondary fungal metabolites (i.e., not essential for survival of the mold) secreted into the microenvironment around the mold. Mycotoxins represent a diverse group of chemicals that can occur in a variety of plants used as food, including commodities such as cereal grains (barley, corn, rye, wheat), coffee, dairy products, fruits, nuts, peanuts, and spices. A few mycotoxins also can occur in animal products derived from animals that consume contaminated feed (e.g., milk). However, because commodities are consumed in great amounts, the mycotoxins present in these foods represent the greatest risk (Cousins et al., 2005).

Data from animal studies indicate that the consumption of food contaminated with mycotoxins has the potential to contribute to a variety of human diseases (Miller, 1991). Reports of acute intoxications are few (e.g., consumption of wild *Amanita* mushroom and α-amanitin poisoning); however, prolonged exposure to small quantities of mycotoxin may lead to more deleterious effects including growth retardation, birth defects, impaired immunity, decreased disease resistance, and tumor formation in humans and decreased production in farm animals (CAST, 2003). Importantly, the presence of a toxigenic mold does not guarantee the presence of a mycotoxin, which is elaborated only under certain conditions. Although there are many different mycotoxins and subgroups (Table 27-11), this discussion will focus on aflatoxins, trichothecenes, fumonisins, ochratoxin A, and ergot alkaloids.

Table 27-11

Selected Mycotoxins Produced by Various Molds and Some of Their Effects and Commodities Potentially Contaminated

MUCOTOXIN	SOURCE	EFFECT	COMMODITIES CONTAMINATED
Aflatoxins B1, B2, G1, G2	*Aspergillus flavus*, *A. parasiticus*	Acute aflatoxicosis, carcinogenesis	Corn, peanuts, and others
Aflatoxin M1	Metabolite of AFB1	Hepatotoxicity	Milk
Fumonisins B1, B2, B3, B4, A1, A2	*Fusarium verticillioides*	Renal and liver carcinogenesis	Corn
Trichothecenes (e.g., T-2, deoxynivalenol, diacetoxyscirpenol)	*Fusarium*, myrothecium	Hematopoietic toxicity, meningeal hemorrhage of brain, "nervous" disorder, necrosis of skin, hemorrhage in mucosal epithelia of stomach and intestine, emesis, feed refusal, immune suppression	Cereal grains, corn
Zearalenones	*Fusarium*	Estrogenic effect	Corn, grain
Cyclopiazonic acid	*Aspergillus*, *Penicillium*	Muscle, liver, and splenic toxicity	Cheese, grains, peanuts
Kojic acid	*Aspergillus*	May be hepatotoxic?	Grain, animal feed
3-Nitropropionic acid	*Arthrinium sacchari*, *A. saccharicola, A. phaeospermum*	Central nervous system impairment	Sugarcane
Citreoviridin	*Penicillium citreoviride*, *P. toxicarium*	Cardiac beriberi	Rice
Cytochalasins E, B, F, H	*Aspergillus*, *Penicillium*	Cytotoxicity	Corn, cereal grain
Sterigmatocystin	*Aspergillus versiolar*	Carcinogenesis	Corn
Penicillinic acid	*Penicillium cyclopium*	Nephrotoxicity, abortifacient	Corn, dried beans, grains
Rubratoxins A, B	*Penicillium rubrum*	Hepatotoxicity, teratogenic	Corn
Patulin	*Penicillium patulum*	Carcinogenesis, liver damage	Apple and apple products
Ochratoxin	*Aspergillus ochraceus*, *A. carbonarius*, *Penicillium verrucosum*	Endemic nephropathy, carcinogenesis	Grains, peanuts, grapes, green coffee
Citrinin	*Aspergillus, Penicillium*	Nephrotoxicity	Cereal grains
Penitrem(s)		Tremors, incoordination, bloody diarrhea, death	Moldy cream cheese, English walnuts, hamburger bun, beer
Ergot alkaloids	*Claviceps purpurea*	Ergotism	Grains

Aflatoxins Among the various mycotoxins, the aflatoxins (Fig. 27-21) have been the subject of the most intensive research because of the potent hepatocarcinogenicity and toxicity of aflatoxin B1 in rats. Epidemiological studies conducted in Africa and Asia suggest that it is a human hepatocarcinogen, and various other reports have implicated the aflatoxins in incidences of human toxicity. Aflatoxin B1 is the most potent natural carcinogen known and is the major aflatoxin produced by toxigenic strains (Bennett and Klich, 2003). Generally, aflatoxins occur in susceptible crops as mixtures of aflatoxins B1, B2, G1, and G2, with only aflatoxins B1 and G1 demonstrating carcinogenicity. A carcinogenic hydroxylated metabolite

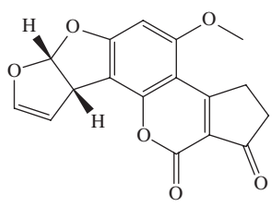

Figure 27-21. *Aflatoxin B1.*

of aflatoxin B1 (termed aflatoxin M1) can occur in the milk from dairy cows that consume contaminated feed. Aflatoxins may occur in a number of susceptible commodities and products derived from them, including edible nuts (peanuts, pistachios, almonds, walnuts, pecans, and Brazil nuts), oil seeds (cottonseed and copra), and grains (corn, grain sorghum, and millet). In tropical regions, aflatoxin can be produced in unrefrigerated prepared foods. The two major sources of aflatoxin contamination of commodities are field contamination, especially during times of drought and other stresses, which allow insect damage that opens the plant to mold attack, and inadequate storage conditions. Aflatoxin B1 is acutely toxic in all species studied, with an LD50 ranging from 0.5 mg/kg for the duckling to 60 mg/kg for the mouse. Death typically results from hepatotoxicity. Aflatoxin B1 is also highly mutagenic, hepatocarcinogenic, and possibly teratogenic. There exists a wide range of species-susceptibility to aflatoxin B1. For instance, aflatoxin B1 appears to be the most hepatocarcinogenic compound known for the rat, but the adult mouse is essentially totally resistant to its hepatocarcinogenicity (Kotsonis and Burdoch, 2013). Such species differences may be due in part to differences in biotransformation (glutathione conjugation) and susceptibility to the initial biochemical lesion (Monroe and Eaton, 1987). Progress has been made in decreasing the level of aflatoxins in specific commodities in developed countries, such as ensuring adequate storage conditions, careful monitoring of susceptible commodities for aflatoxin, and the banning of lots that exceed the action level for aflatoxin B1. The hepatocarcinogenicity of aflatoxin B_1 is associated with the formation of covalent adducts with DNA, RNA, and protein. Damage to DNA is thought to be the initial biochemical lesion.

Trichothecenes The trichothecenes (Fig. 27-22) constitute more than 60 sesquiterpenoid metabolites produced by a number of fungal genera including *Fusarium, Myrothecium*, *Phomopsis, Stachybotrys*, *Trichoderma*, *Trichothecium*, and others. They are commonly found as food and feed contaminants, and consumption of these mycotoxins can result in alimentary hemorrhage and vomiting; direct contact causes dermatitis (Bennett and Klich, 2003). *Fusarium* head blight, caused by trichothecene-producing *Fusarium* species, is a destructive disease of cereal grain crops that has a worldwide economic impact. Consumption of contaminated grain has been associated with intestinal irritation in mammals and can lead to feed refusal in livestock and other toxic responses (Eriksen and Pettersson, 2004). There have been many reported cases of trichothecene toxicity in farm animals and a few in humans. Alimentary toxic aleukia (ATA) is a frequently fatal mycotoxicosis, caused by the ingestion of contaminated overwintered grain or grain by-products. ATA can be caused by trichothecene toxins (e.g., T-2 toxin) in humans and is characterized by leukopenia, agranulocytosis, necrotic angina, a hemorrhagic rash, sepsis, exhaustion of the bone marrow, bleeding from the nose, throat, and gums, and fever. One major outbreak was reported in Russia during 1944 around Orenburg, Siberia. About 10% of the population was affected and mortality rates in the affected population were as high as 60% in some counties. The symptoms of ATA could be induced in cats by administration of the toxins, but not in other experimental models (Bennett and Klich, 2003; Kotsonis and Burdoch, 2013). Trichothecenes bind to ribosomes and inhibit protein synthesis. The acute LD50s of the trichothecenes range from 0.5 to 70 mg/kg. The extent of toxicity associated with the trichothecenes in humans and farm animals is poorly understood, owing in part to the number of entities in this group and the difficulty of assaying for these compounds (JECFA, 2001).

Fumonisins Fumonisins are produced by a number of *Fusarium* species, notably *Fusarium verticillioides* (formerly *F. moniliforme*), *F. proliferatum,* and *F. nygamai.* The major species of economic importance is *F. verticillioides* because it grows in corn, in both vegetative and reproductive tissues. *F. verticillioides* is present in virtually all corn samples (Marasas et al., 2001). Most strains do not produce the toxin, so the presence of the fungus does not necessarily mean that fumonisin is also present (Plumlee and Galey, 1994). The most abundantly produced member of the family is fumonisin B1. Fumonisins affect animals in different ways by interfering with sphingolipid metabolism. They cause leukoencephalomalacia (hole in the head syndrome) in equines and rabbits; pulmonary edema and hydrothorax in swine; and hepatotoxic and hepatocarcinogenic effects, and apoptosis in the liver of rats (Bennett and Klich, 2003). In humans, there is a probable link with esophageal cancer. The occurrence of fumonisin B1 is correlated with the occurrence of a higher incidence of esophageal cancer in regions of Transkei (South Africa), China, and northeast Italy (Peraica et al., 1999). A possible case of acute exposure to fumonisin B1 involved 27 villages in India, where consumption of unleavened bread made from moldy sorghum or corn caused transient abdominal pain, borborygmus, and diarrhea. All those affected recovered fully (Bhat et al., 1997). Fumonisins can cause neural tube defects in experimental animals and thus may also have a role in human cases. The International Agency for Research on Cancer has evaluated the cancer risk of fumonisins to humans and classified them as group 2B (possibly carcinogenic to humans, that is, there is *inadequate evidence* of carcinogenicity in humans, but *sufficient evidence* of carcinogenicity in animals) (IARC, 2002). Fumonisins target different organs in various species, but the underlying mechanism is a disruption of lipid metabolism by inhibition of ceramide synthetase, an enzyme integral to the formation of complex lipids for use in membranes (ICSP, 2000; IARC, 2002).

Ochratoxin A Ochratoxin A (Fig. 27-23) is primarily produced by *Aspergillus ochraceus*, *A. carbonarius*, and *Penicillium verrucosum*. Human exposure occurs through the consumption of contaminated small grains (barley, wheat, and corn), coffee beans,

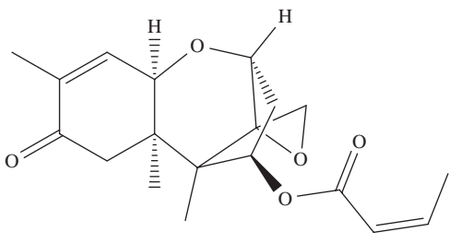

Figure 27-22. *Trichothecene.*

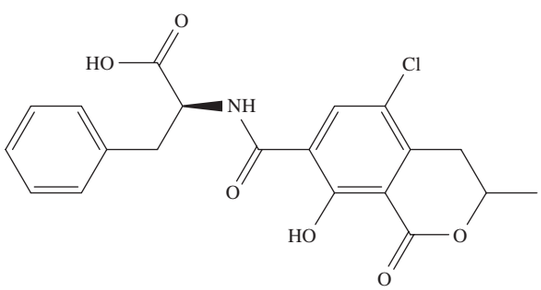

Figure 27-23. *Ochratoxin A.*

and grapes. Ochratoxin A is a nephrotoxin to all animal species studied. It binds tightly to albumin in the blood and can therefore have a very long serum half-life. Of all the ochratoxins studied, ochratoxin A has the longest half-life in humans for elimination (Creppy, 1999). Animal studies indicate that in addition to being a nephrotoxin, ochratoxin A is also a liver toxin, an immune suppressant, a potent teratogen, and a carcinogen (Bennett and Klich, 2003). An important molecular mechanism of action of ochratoxin A is the inhibition of the enzymes involved in phenylalanine metabolism including phenylalanine-tRNA synthetase. In addition, ochratoxin A inhibits mitochondrial ATP production and also stimulates lipid peroxidation (Marquardt and Frohlich, 1992). Initial epidemiological evidence determined that there is an association between exposures to ochratoxin A and the development of Balkan endemic nephropathy (BEN) and renal tumors in parts of eastern Europe. However, recent evidence strongly suggests that the causative agent is aristolochic acid, and not ochratoxin A (Pavlović, 2013).

Ergot Alkaloids Ergot poisoning (ergotism) in humans and domestic animals is one of the oldest known diseases caused by ergot alkaloids. They are produced by the fungus *Claviceps purpurea* and other genera of Claviceps that infect rye and cereals. Ergot alkaloids cause vasoconstriction and reduced blood flow by direct action on the smooth muscles of the arterioles and uterus; the most affected regions being the lower extremities (lower extremity ischemia). The disruption of blood flow causes gangrene, loss of hands and feet, as well as miscarriage of fetuses. Ergot alkaloids also stimulate the central nervous system, resulting in strange hallucinations, itchy and burning skin (Belser-Ehrlich et al., 2013).

Bovine Spongiform Encephalopathy

BSE is a form of transmissible spongiform encephalopathies (TSEs), which are a group of neurodegenerative disorders. In humans, the diseases kuru and Creutzfeldt–Jakob Disease (CJD) are TSEs. The causative agents for TSEs are prions. Normal prion is a cellular protein, designated PrP^{c} and is found on the surface of neurons. The general term for pathogenic (infectious) isoforms of PrP^{c} is PrP^{res} (because they are proteinase K-resistant). The PrP^{res} acts as a template and replicates by converting normal cellular proteins into copies of itself. Other names for PrP^{res} are PrP^{Sc} or PrP^{TSE}. The infective agent can be transferred using preparations of neural tissue from infected animals *across species barriers*. Horizontal transmission from mother to offspring and lateral transmission through biting or injury has been reported. The protein is resistant to heat, antimicrobials, ultraviolet or ionizing radiation, and is not consistently inactivated with alcohol, formaldehyde, glutaraldehyde, or sodium hydroxide. Phenol and sodium hypochlorite disinfection have had variable success.

Clinically, these diseases present neurological deterioration and wasting. The incubation period and interval from clinical onset to inexorable death are determined by the dose of the infective agent, its virulence, and the genetic makeup of the victim. The incubation of BSE in cattle is generally four to five years (range of 20 months to 18 years) and an interval of 1 to 12 months from the presentation of clinical signs to death. Characteristic histological lesions in the brain and spinal cord are vacuolation and "spongiform" changes. BSE fibrils (long strands of the prion protein) in spinal cord preparations may be seen with electron microscopy (Flechsig and Weissmann, 2004). As a response to the concerns about transmission, new enhanced BSE-related feed bans went into effect in Canada in 2007 and in the United States in 2009. The enhanced bans prohibit most proteins, which include potentially BSE infectious tissues from all animal feeds, pet foods, and fertilizers not just from cattle feed as required by bans instituted in 1997 (Kotsonis and Burdoch, 2013).

Some Toxicants Generated in Food During Cooking or Processing

Polycyclic Aromatic Hydrocarbons Polycyclic aromatic hydrocarbons (PAHs) are largely formed when food is cooked using high-temperature methods, such as frying, roasting, barbecuing, grilling, and smoking directly over an open flame. Grilling food, particularly meat, over log fire in which the food is in direct contact with the flame is expected to produce high levels of PAHs. Fat and juices from meat being grilled directly over an open fire drip onto the fire, causing flames. These flames contain PAHs that then adhere to the surface of the meat; hence, charring of meat produces significant amounts of PAHs. PAHs can also be formed during smoking of meats (Cross and Sinha, 2004). Tobacco smoke contains high concentrations of PAHs as well. Therefore, burning of the organic matter in food produces PAHs. One of the widely studied food-associated PAHs is benzo[*a*]pyrene (B[*a*]P) (Fig. 27-24). Studies on PAHs in animals have shown various toxicological effects, such as hematotoxicity, reproductive and developmental toxicity, immunotoxicity, and carcinogenicity. B[*a*]P, for example, is classified as a human carcinogen (Group 1). Estimates of PAH intake from food vary widely, ranging from a few nanograms to a few micrograms per person per day (IARC, 2012).

Heterocyclic Amines Heterocyclic amines (HCAs) are generated during cooking, particularly meat and fish by pan frying or grilling. There are more than 20 HCAs. They are formed when amino acids, sugars, and creatine react at high temperatures. In other words, HCAs are formed as a result of high-temperature cooking of proteins and carbohydrates. Meats cooked at high temperatures (e.g., above 300ºF), as well as well-done meat that are cooked for a long time tend to form more HCAs. Like PAHs, HCAs are also metabolized to bioactivated genotoxic carcinogens that form DNA adducts. HCAs are rapidly absorbed by the GI tract, distributed to all organs, and decline to undetectable levels within 72 hours (Kotsonis and Burdoch, 2013). There are different types of HCAs in cooked foods. The amount of MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) (Fig. 27-25) formed during cooking seems to be

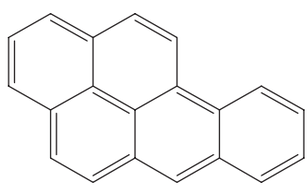

Figure 27-24. *Benzo[a]pyrene.*

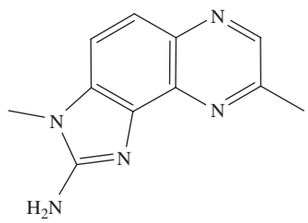

Figure 27-25. *MeIQx.*

higher than any other HCAs formed, being 3.10 ng/g in food-grade beef extract, 2.33 ng/g in broiled chicken, and 2.11 ng/g in broiled beef (Kotsonis and Burdoch, 2013). HCAs are indirect carcinogens because they need to be metabolically activated into electrophilic carcinogen that form DNA adducts. Unreacted HCAs are subject to phase II detoxication reactions and are excreted via the urine and feces.

N-Nitroso Compounds *N*-nitroso compounds (NOCs) are so named because they contain an *N*-nitroso group. Several hundred *N*-nitroso compounds are currently known. There are two major classes of NOCs: nitrosamines and nitrosamides. Nitrosamines are more stable than nitrosamides. Nitrosamines are metabolically activated by hydroxylation, and through a series of steps ultimately form a carbonium ion that alkylates DNA. Nitrosamides spontaneously decompose to a carbonium ion at physiological pH by a similar mechanism. Nitrosamides are potent neurocarcinogens in animal models. Diet is a major source of NOCs, but endogenous NOC formation is estimated to account for 45% to 75% of total exposure to NOCs. Nitrites, amines, and amides found in processed meat are the precursors for the endogenous formation of NOCs in the stomach. In addition, nitrosamines are also found in nitrite-preserved foods, such as meat and fish. NOCs may also be formed endogenously after consumption of nitrate, which is reduced to nitrite by bacteria in the gut. The main sources of dietary nitrate are grain products and many leafy vegetables. The NOCs in food could be volatile or nonvolatile. Volatile NOCs, such as *N*-nitrosodimethylamine (NDMA; Fig. 27-26), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR; Fig. 27-27), and *N*-nitrosopiperidine (NPIP) are known to be carcinogenic, whereas the non-volatile NOCs, such as *N*-nitrosoproline (NPRO) are not known to be mutagenic or carcinogenic. NDMA and NPYR have been commonly reported in food. NOCs originate from two sources: environmental formation and endogenous formation. Environmental sources have declined over the last several years but still include foods (e.g., nitrate-cured meats) and beverages (e.g., malt beverages), occupational exposure, and rubber products. NOCs formed in vivo may actually constitute the greatest exposure and are formed from nitrosation of amines and amides in several areas, including the stomach, where the most favorable conditions exist (pH 2 to 4), although consumption of Histamine H2-receptor blockers or antacids decreases the formation of NOCs (Kotsonis and Burdoch, 2013). In the western countries, the consumption of volatile NOCs is usually 0.3 to 1.0 μg/person/day, whereas that of non-volatile NOCs is usually 10 to 100 μg/person/day. In Asia, the exposure to volatile NOCs is significantly higher because of the consumption of dried and nitrite-salted fish. Cigarette smoke is another significant source of volatile NOCs; an average smoker may be exposed to more than 15 μg volatile NOCs per day (Skog et al., 1998).

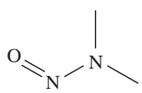

Figure 27-26. *N-nitrosodimethylamine.*

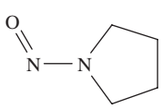

Figure 27-27. *N-nitrosopyrrolidine.*

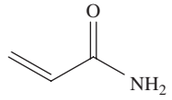

Figure 27-28. *Acrylamide.*

Figure 27-29. *Furan.*

Acrylamide Cooking starchy foods (e.g., potato) at high temperatures (120°C or higher) causes a chemical reaction between certain sugars and the amino acid asparagine present in the food; the reaction forms acrylamide (Fig. 27-28). Longer cooking times and cooking at higher temperatures can increase the amount of acrylamide in foods further. Thus, crunchy, dark brown potato fries are likely to contain higher levels of acrylamide than non-crunchy, non-brown potato fries. Most acrylamide is formed in the final stages of baking, grilling, or frying as the moisture content of the food falls and the surface temperature rises (JECFA, 2005). Acrylamide (the monomer) is known to be a neurotoxin. Repeated dosing of acrylamide in drinking water at 21 mg/kg bw/day for 40 days produces neurotoxicity (JECFA, 2005). Acrylamide was also classified as probably carcinogenic to humans (Group 2A) by IARC (IARC, 1994). Interestingly, the polymer of acrylamide, polyacrylamide, does not have significant toxicity. Acrylamide intake estimates range from 0.3 to 2.0 μg/kg bw/day for the average population. Primary sources include French fries (1% to 30%), potato chips (6% to 46%), coffee (13% to 39%), pastry and cookies (10% to 20%), and bread and rolls/toasts (10% to 30%). The NOEL for morphological change in nerves and for reproductive effects is 200 and 2000 μg/kg bw/day, respectively (JECFA, 2005). Table 27-12 shows the acrylamide content of some common food.

Furan Furan (Fig. 27-29) occurs in a number of foods that undergo heat treatment, such as canned and jarred foods, including baby food. It is considered by IARC (1995) to be possibly carcinogenic to humans. Furan is hepatotoxic and shows clear evidence of carcinogenicity in both sexes in mice and rats (NTP, 1993). Furan is produced in a variety of experimental systems, including heating of sugars (e.g., glucose, lactose, fructose, xylose, rhamnose), heating sugars in the presence of amino acids or protein (e.g., alanine, cysteine, casein), and thermal degradation of vitamins (ascorbic acid, dehydroascorbic acid, thiamine) (FDA, 2004). Furan has been found in a small number of heat-treated foods, including coffee, canned meat, baked bread, cooked chicken, sodium caseinate, hazelnuts, soy protein isolate, hydrolyzed soy protein, rapeseed protein, fish protein concentrate, and caramel (FDA, 2004). Very little information has been developed on furan levels in food.

Some Heavy Metal Contaminants in Food

There are no standard definitions of heavy metals. The term is frequently used to indicate toxic metals with a specific gravity of 5 or above. The heavy metals discussed here are lead, cadmium, mercury, and arsenic. Elsewhere in the book (Chap. 23), these heavy

Table 27-12

Representative Concentrations of Acrylamide in Several Foods

FOOD	MEAN CONC. (μg/kg)	REPORTED MAXIMUM (μg/kg)
Cereal-based products		
Breads and rolls	446	3436
Pastry and cookies	350	7834
Breakfast cereals	96	1346
Roots and tubers		
Baked potato	169	1270
Potato chips	752	4080
French fries	334	5312
Coffee		
Coffee, brewed, ready to drink	13	116
Coffee extracts	1100	4948
Coffee, decaffeinated	668	5399
Coffee substitutes	845	7300
Vegetables		
Raw, boiled, and canned	4.2	25
Processed (toasted, baked, fried, grilled)	59	202
Infant formula	<5	15
Baby food (biscuits)	181	1217

SOURCE: Adapted from JECFA (2005). Available at: http://www.fda.gov/downloads/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Acrylamide/UCM194526.pdf.

metals are discussed along with the history of their human exposure, target organ toxicity, as well as the mechanisms of toxicity. Those are not repeated here.

Lead Lead is a toxic metal present in our environment in small amounts. Everyone is exposed to lead from daily actions such as inhaling dust, eating food, or drinking water. Lead poisoning in children is a potential cause for concern. FDA's provisional total tolerable intake level (PTTIL) for lead by small children is 6 μg/day (FDA, 2006c). FDA also recommends that the lead levels in candy products likely to be consumed frequently by small children do not exceed 0.1 ppm (FDA, 2006c). This recommended maximum level is consistent with the FDA's long-standing goal of reducing lead levels in the food supply.

Cadmium Cadmium is widely dispersed into the environment by industrial and man-made activities. Cadmium released into the environment can be associated with respirable-sized airborne particles and can be carried long distances. It is ultimately deposited onto the Earth by rain or falling out of the air. Once on the ground, cadmium moves easily through soil layers and is taken up into the food chain by uptake into plants such as leafy vegetables, root crops, cereals, and grains (e.g., rice and wheat). Cadmium is primarily toxic to the proximal tubular cells of the kidney and tends to accumulate in the cortex of the kidney, eventually leading to renal failure. Bone demineralization occurs with prolonged exposure. Cadmium absorption in humans is the greatest with pulmonary exposure, but it is relatively low (3% to 5%) after dietary exposure (EFSA, 2009c). Because cadmium has a very long half-life in the body (10 to 30 years), cadmium exposure is still a cause for concern. JECFA (2005) investigated cadmium consumption for several countries including the United States and found that the current total intake of cadmium is 40% to 60% of the Provisional Tolerable Weekly Intake (PTWI) of 7 μg/kg bw/week established several years ago (JECFA, 2005). Many U.S. health agencies have set exposure standards designed to protect the general public from excess cadmium exposure from various sources, such as a maximum limit of 5 μg/L (5 ppb) in drinking water (see ATSDR, 2008).

Mercury Currently, exposure to elemental mercury is relatively rare. Of interest to food toxicology is methylmercury, which is formed in aquatic environments by bacterial action on elemental mercury. Anthropogenic sources that can add elemental mercury to aquatic environments include burning of coal (which contains mercury), chloralkali processes, and other sources of elemental mercury discharge into aquatic environments. Methylmercury is neurotoxic. Developmental delays have been documented in children borne of mothers exposed to methylmercury (Carrington and Bolger, 1992). Methylmercury becomes concentrated in fish including bonito (*Sarda* spp.), halibut (*Hippoglossus* spp.), mackerel (*Scomberomorus* spp.), marlin (*Makaira* spp.), shark (all species), swordfish (*Xiphias* gladius), and bluefin tuna (*Thunnus* spp.). Human exposure to mercury can occur through the consumption of these fish species. The current FDA action level is 1.0 ppm mercury in the edible portion of fish (FDA, 2011b).

Arsenic Arsenic is a naturally occurring element widely distributed in the air, water, and land. In the environment, arsenic may exist as inorganic and organic arsenic compounds. Inorganic arsenic compounds are highly toxic. For most people, food is the largest source of arsenic exposure, although much of this is likely to be in the organic form, which is less toxic. The highest levels of total arsenic (inorganic plus organic) in foods can be found in seafood, rice, rice cereal and other rice products, mushrooms, poultry, and other foods. Rice is of particular concern because it is a major part of the diet in many parts of the world. It is also a major component of many of the cereals consumed by infants and young children. In the United States, an average adult intake of dietary inorganic arsenic was estimated to be 3.2 μg/day, with a range of 1 to 20 μg/day (Hughes et al., 2011). EFSA (2014) reported a mean dietary exposure among European infants, toddlers, and other children ranging from 0.20 to 1.37 μg/kg bw/day, whereas the mean dietary exposure among the adult population ranged from 0.09 to 0.38 μg/kg bw/day. FDA proposed an "action level" of 10 μg/kg (10 ppb) for inorganic arsenic in apple juice. This is the same level set by the United States (EPA) for arsenic in drinking water (FDA, 2013).

FOOD ALLERGY

Definition

In 2003 the World Allergy Organization of the World Health Organization (WHO) proposed that adverse nontoxic reactions to food in general should be termed *food hypersensitivity*. If there is no role of the immune system in the development of food hypersensitivity, it should be called *non-allergic food hypersensitivity* (e.g., lactose intolerance, hypersensitivity toward sulfites). If an immunologic mechanism has been demonstrated, the appropriate term is *food allergy*. Food allergy could further be subdivided into *IgE-mediated food allergy* (e.g., allergy caused by milk, egg, peanut, etc.) and *non-IgE-mediated food allergy* (e.g., celiac disease) (Johansson et al., 2004). The IgE-mediated food allergy involves

the humoral immune response (antibody-mediated), whereas the non-IgE-mediated food allergy involves the cell-mediated immune response.

Occurrence and Symptoms

Food allergies are abnormal responses of the immune system to certain food components (allergens) in some individuals. The vast majority of consumers may not react to the same allergen. *The allergens in foods are typically naturally occurring proteins*. Basically, any food that contains protein has the potential to elicit allergic sensitization in some individuals. There are both genetic and environmental risk factors determining food allergy. Genetic risk factors include a family history of atopic disorders. Environmental factors include the extent and duration of exposure to the allergen from the environment, route of exposure, prior exposure to similar antigens (determining cross-reactivity), and others. The evidence of environmental factors modulating the expression of food allergy is provided by increasing peanut allergy in children (Sicherer et al., 2003; Benedé et al., 2016). Recent findings suggest that in the dual environmental exposure, the scenario of food allergy and the exposure through skin promote sensitization, while early exposure through the GI tract may be tolerogenic. This is supported by clinical findings that early consumption of food, such as peanuts, fish, or wheat is associated with a lower incidence of food allergy (Benedé et al., 2016). The relationship between diet during infancy (other than the allergenic food itself) and the development of allergic diseases is not well understood. Exposure to an increased diversity of foods in early life is inversely associated with allergic diseases including food allergy (Roduit et al., 2014; Benedé et al., 2016). But there are relatively few studies identifying specific dietary components that might be a risk for the development of food allergy (Benedé et al., 2016).

Approximately 6% of young children and 3.7% of adults in the United States have a food allergy. In young children the most common food allergies are those to cow's milk (2.5%), egg (1.3%), peanut (0.8%), wheat (about 0.4%), soy (about 0.4%), tree nuts (0.2%), fish (0.1%), and shellfish (0.1%). Early childhood allergies to milk, egg, soy, and wheat usually resolve by school age (about 80%). Although peanut, tree nut, and seafood allergies are generally considered permanent, about 20% of young children with peanut allergy experience resolution by 5 years of age (although recurrence is also possible). Adults are more likely to have allergies to shellfish (2%), peanut (0.6%), tree nuts (0.5%), and fish (0.4%). Reactions to fruits and vegetables are common (approximately 5%) but usually not severe. Allergy to seeds (e.g., sesame) is being increasingly reported. To summarize, egg and milk allergies are the most common food allergies among infants but are often outgrown. Shellfish allergy is more common among adults than children, while peanut allergy is equally common among children and adults (Sicherer and Sampson, 2006).

The symptoms of food allergies may range from mild discomfort to severe, life-threatening reactions that require immediate medical attention. Symptoms may involve itching, redness, swelling in the skin; itching and swelling of the eyes; swelling of oral cavity; pain in the GI tract; nausea, vomiting, diarrhea; itching and swelling of the nose and throat; asthma; chest pain, abnormal heart rhythm, and very low blood pressure causing fainting. Allergic reactions to foods generally occur within a few minutes to one hour (immediate hypersensitivity) after eating the offending food. Symptoms can last for days or even weeks. The specific symptoms and severity of an allergic reaction are affected by the amount of the allergen consumed and by the sensitivity of the allergic person (WHO-INFOSAN, 2006).

Eosinophilic esophagitis (EoE) is a newly recognized chronic disease that can be associated with food allergies. It affects as many as 56 of every 100,000 people in the United States, and is characterized by inflammation and accumulation of eosinophils in the esophagus. Symptoms include nausea, vomiting, and abdominal pain after eating and can resemble acid reflux. Recently, Davis et al. (2016) identified that calpain 14 is the causative agent of EoE. Calpain 14 is an IL-13-induced protease that mediates esophageal epithelial barrier impairment. EoE involves T-helper type 2 (Th2) response in esophageal mucosal epithelium.

Foods That Elicit Allergic Reactions

Overall, more than 170 foods have been documented as causing food allergies (Hefle et al., 1996). The eight most common foods that elicit allergic reactions are eggs, milk, fish, crustacean shellfish (e.g., crab, lobster, and shrimp), tree nuts (e.g., almonds, walnuts, and pecans), peanuts, wheat, and soybeans, although reactions to many other foods can occur as well. Food-induced anaphylactic reactions are possible, but the more prevalent adverse immunological reactions include eczema, urticaria, angioedema, nausea, vomiting, diarrhea, rhinoconjunctivitis, and asthma (Kotsonis and Burdoch, 2013).

Mechanism of IgE and Non-IgE-Mediated Food Allergic Reactions

IgE-mediated reactions are true allergic responses; these are also known as type I hypersensitivity responses that typically affect the cutaneous, respiratory, GI and/or cardiovascular systems. IgEs are produced by B cells, and the response usually occurs within an hour of exposure and can result in anaphylaxis. Most allergens, including food allergens, are proteins. In IgE-mediated allergic reaction, the allergenic proteins are taken up by the antigen presenting cells (APCs), such as the dendritic cells. The APCs cleave the proteins into pieces in the lysosomes; these pieces are presented at the cell surface in complex with MHC class II molecules. The MHC–antigen (allergen) complex is recognized by the $CD4^+$ T-helper (Th) cells, which then differentiate into T-helper type 2 (Th2) effector cells in individuals who have genetic predisposition to the allergen (atopic individuals). Th2 cells produce a whole array of cytokines of which IL-4 and IL-13 are critical for the synthesis of IgE by B cells. During an allergic response, IgEs bind to the IgE receptors (FcεRI and FcεRII) on mast cells (in tissues) and basophils (in circulation). When two or more IgEs bound to receptors on the mast cells or basophils are cross-linked by the allergen through the allergenic epitope, these cells are activated. Both mast cells and basophils contain special cytoplasmic granules that store many mediators of inflammation (i.e., histamine, tryptase, cysteinyl leukotrienes, prostaglandin D2). The extracellular release of these mediators following activation of these cells is known as degranulation. The released mediators of inflammation trigger allergic response and allergy symptoms. Some examples of IgE-mediated food allergy symptoms include urticaria (hives), eczema, dermatitis, pruritas (severe itching of the skin), rash, nausea, vomiting, diarrhea, abdominal cramps, asthma, wheezing, rhinitis, bronchospasm, anaphylactic shock, hypotension, palatal itching, swelling including tongue and larynx, and methemoglobinemia. The most severe manifestation of food allergy is anaphylaxis, which is an acute reaction that affects two or more organ systems and can be life-threatening (Wang and Sampson, 2009; Benedé et al., 2016).

Non-IgE-mediated food allergy is less frequent and it occurs in the absence of detectable food-specific IgE antibodies in the serum or skin. The mechanisms of non-IgE-mediated food allergy are not well understood, but are thought to be cell-mediated. They occur at least 2 hours after exposure (or later) and mostly involve acute or chronic inflammation in the GI tract. The delayed onset of symptoms makes the clinical association between offending food and the clinical symptoms difficult. The non-IgE-mediated allergies can be classified into different types of hypersensitivity responses, such as Type II (IgG- or IgM-mediated cytotoxic hypersensitivity response), Type III (immune complex–mediated hypersensitivity response), and Type IV (cell-mediated hypersensitivity response). Both Type II and III responses involve activation of the complement proteins. The gut mucosal immune system develops tolerance to macronutrients that could be potentially immunogenic (e.g., proteins). This immune tolerance is maintained by actively suppressing the antigen-specific regulatory T (Treg) cells. In the first few years of life, humans gradually develop an intricate balance between tolerance and immune reactivity in the gut mucosa along with a tremendous expansion of gut-associated lymphoid tissue (GALT). Expectedly, both IgE- and non-IgE-mediated food allergy is frequently seen during this period. Non-IgE-mediated food reactions are classified as negative skin test results or specific IgE to foods, with positive challenge to the offending food. The reactions can vary by system, from GI to cutaneous to respiratory; GI reactions are the most common reactions. Some examples of non-IgE-mediated food allergy symptoms include atopic dermatitis (could be mixed IgE/non-IgE), dermatitis herpetiformis, food protein–induced enterocolitis syndrome, food protein–induced proctitis, eosinophilic esophagitis (could be mixed IgE/non-IgE), celiac disease, and pulmonary hemosiderosis (Heiner syndrome) (Spergel, 2006; Jyonouchi, 2008).

Diagnosis and Treatment of Food Allergy

The first step in diagnosing a food allergy is to obtain/develop a detailed medical history. The next step is to conduct certain tests in order to identify food allergy. The tests that an allergy specialist may conduct are: (1) Skin prick test; (2) Blood test; (3) Oral food challenge; (4) Elimination diet.

The skin prick test (SPT) is conducted by placing a drop of solution (or mashed fresh material, such as fruits or vegetables) containing the food allergen on the forearm or back of the person. Then the area of the skin where the material has been applied is gently pricked to let the applied material enter just below the surface. A positive reaction is indicated by a wheal (a raised bump surrounded by a small circle of itchy red skin) within 30 minutes or so. A positive SPT may prompt further tests by the physician. *Blood test* measures the presence of IgE antibodies to specific food. In the past, the test was called RAST (radioallergosorbent assay). The assay used radiolabeled secondary antibodies that bind and detect human IgE against the food allergen. Modern tests do not use any radioactivity. The RAST has been replaced by more sensitive fluorescence enzyme-labeled immunoassays. As opposed to SPT which is rapid, the blood test takes a few days to perform; however, performing SPT on a person whose skin is full of rashes is impossible; hence, the blood test becomes important for such patients. If the SPT and blood test are not sufficient for the physician to arrive at a conclusion, an *oral food challenge (OFC)* may be required; OFC is a highly accurate diagnostic test for food allergy. Because OFC has the potential to cause a serious reaction, it should be performed under the advice and supervision of a qualified allergy specialist doctor. Briefly, the person is fed the suspected offending food in small, incremental doses until a reaction is observed. The food challenge is then stopped. Depending on the severity of the reactions, the person may be given antihistamine to relieve the symptoms. Opposite to the OFC is the *elimination diet* trial. In this method, the person is advised to avoid the suspected offending food(s) for about a month. The disappearance of symptoms is monitored, and the offending food is identified. Once the offending food is identified by the elimination diet trial, the physician may want to conduct an OFC with the same food to double-confirm the role of the food in precipitating the food allergy (Sicherer and Sampson, 2006).

The best remedy for food allergies is to avoid the foods that cause the allergy. In reality, this may be difficult to practice because the possibility of inadvertent exposure to the offending allergens from the surrounding environment cannot be ruled out. In general, antihistamines should work for mild reactions and epinephrine for serious reactions. However, a person who is suffering from confirmed or suspected food allergy should always consult the doctor about the necessary steps he/she should take, and follow the doctor's advice. The reader may visit the University of Nebraska "Treatment of food allergies" page of the Food Allergy Research and Resource Program for a collection of resources on this topic (http://farrp.unl.edu/resources/gi-fas/treatment-of-food-allergies). In this respect, the FALCPA labeling (discussed above) is an important tool to alert consumers regarding the presence of potential allergic ingredients in the food.

EXAMPLES OF INADVERTENT FOOD CONTAMINATIONS

There have been only a few incidents of mass poisonings *via* food, two of which involved cooking oil contaminated with polychlorinated organics. The first became known as yusho, or rice oil disease, from rice oil contamination originally thought to be confined to polychlorinated biphenyls (PCBs), but has since been revised to include polychlorinated quarterphenyls and polychlorinated dibenzofurans (PCDFs). The poisoning occurred in 1968 in Japan and affected approximately 2000 individuals. The most vulnerable were newborns of poisoned mothers. The liver and skin were the most severely affected. Symptoms included dark brown pigmentation of nails; acne-like eruptions; increased eye discharge; visual disturbances; pigmentation of the skin, lips, and gingiva; swelling of the upper eyelids; hyperemia of the conjunctiva; enlargement and elevation of hair follicles; itching; increased sweating of the palms; hyperkeratotic plaques on the soles and palms; and generalized malaise (Kanagawa et al., 2008; Kotsonis and Burdoch, 2013).

Both polybrominated biphenyls (PBBs) (sold as fire retardant under the trade name FireMaster) and magnesium oxide (sold as a cattle feed supplement under the trade name NutriMaster) were produced at the same site in Michigan. In early 1973, FireMaster was placed in bags labeled NutriMaster because of a mix-up, and several farms received animal feed contaminated by FireMaster. By the time the problem was detected in early 1974, approximately 650 lbs of PBBs had entered the food chain through milk and other dairy products, beef products, and contaminated swine, sheep, chickens, and eggs. Over 500 Michigan farms were quarantined, and approximately 30,000 cattle, 4500 swine, 1500 sheep, and 1.5 million chickens were destroyed, along with over 800 tons of animal feed, 18,000 pounds of cheese, 2500 pounds of butter, 5 million eggs, and 34,000 pounds of dried milk products. PBB-exposed Michigan residents had a variety of complaints including nausea, abdominal pain, loss of appetite, joint pain, fatigue, and weakness, and although a cause–effect relationship could not be established

for these symptoms, there is a fairly strong case for development of chloracne from exposure to PBBs (MDCH, 2011; Kotsonis and Burdoch, 2013).

CONCLUSION

Food toxicology is a unique discipline, in the sense that the words "food" and "toxicology" are apparently not compatible to each other. People take it for granted that food is always nutritious and hedonic or of satiatal value. It should be clear from the preceding discussion that many foods that have been consumed for hundreds of years by humans may naturally contain antinutrients/toxicants that, if consumed in large amounts, could cause health concerns. Microbial food contamination is another problem. There is also the issue of food allergy among a seemingly increasing fraction of the population. The FD&C Act provides some thoughtful and pragmatic solutions through the creative use of specifications, process and manufacturing controls, action levels, tolerances, warning labels, advisories, and outright prohibitions (Kotsonis and Burdoch, 2013).

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REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). Cadmium toxicity: what are the U.S. standards for cadmium exposure? 2008. Available at: https://www.atsdr.cdc.gov/csem/csem.asp?csem=6&po=7.

Anderson CP, Shen M, Eisenstein RS, Leibold EA. Mammalian iron metabolism and its control by iron regulatory proteins. *Biochim Biophys Acta*. 2012;1823:1468–1483.

Argaud D, Zhang Q, Pan W, Maitra S, Pilkis SJ, Lange AJ. Regulation of rat liver glucose-6-phosphatase gene expression in different nutritional and hormonal states: gene structure and 5′-flanking sequence. *Diabetes*. 1996;45:1563–1571.

Ashida H, Nishiumi S, Fukuda I. An update on the dietary ligands of the AhR. *Expert Opin Drug Metab Toxicol*. 2008;4:1429–1447.

Averous J, Bruhat A, Mordier S, Fafournoux P. Recent advances in the understanding of amino acid regulation of gene expression. *J Nutr*. 2003;133:2040S–2045S.

Barrows JN, Lipman AL, Bailey CJ. Color Additives: FDA's regulatory process and historical perspectives. *Food Safety Magazine*. October/November. 2003. Available at: http://www.fda.gov/ForIndustry/ColorAdditives/RegulatoryProcessHistoricalPerspectives/#authors.

Becker K, Southwick K, Reardon J, Berg R, MacCormack JN. Histamine poisoning associated with eating tuna burgers. *JAMA*. 2001;285:1327–1330.

Belser-Ehrlich S, Harper A, Hussey J, Hallock R. Human and cattle ergotism since 1900: symptoms, outbreaks, and regulations. *Toxicol Ind Health*. 2013;29:307–316.

Benedé S, Blázquez AB, Chiang D, Tordesillas L, Berin MC. The rise of food allergy: environmental factors and emerging treatments. *EBioMedicine*. 2016;7: 27–34.

Bennett JW, Klich M. Mycotoxins. *Clin Microbiol Rev*. 2003;16:497–516.

Bhat RV, Shetty PH, Amruth RP, Sudershan RV. A foodborne disease outbreak due to the consumption of moldy sorghum and maize containing fumonisin mycotoxins. *J Clin Toxicol*. 1997;35:249–255.

Bode AM, Dong Z. Toxic phytochemicals and their potential risks for human cancer. *Cancer Prev Res*. 2015;8:1–8.

Brandtzaeg P. Food allergy: separating the science from the mythology. *Nat Rev Gastroenterol Hepatol*. 2010;7:380–400.

Brasse-Lagnel C, Lavoinne A, Husson A. Control of mammalian gene expression by amino acids, especially glutamine. *FEBS J*. 2009;276: 1826–1844.

Bressler JP, Olivi L, Cheong JH, Kim Y, Bannon D. Divalent metal transporter 1 in lead and cadmium transport. *Ann NY Acad Sci*. 2004;1012:142–152.

Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*. 1997;89:331–340.

Bruhat A, Jousse C, Carraro V, Reimold AM, Ferrara M, Fafournoux P. Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. *Mol Cell Biol*. 2000; 20:7192–7204.

Carlini CR, Grossi-de-Sá MF. Plant toxic proteins with insecticidal properties: a review on their potentialities as bioinsecticides. *Toxicon*. 2002;40:1515–1539.

Carrington CD, Bolger PM. An assessment of the hazards of lead in foods. *Regul Toxicol Pharm*. 1992;16:265–272.

Chhabra RS, Eastin WC Jr. Intestinal absorption and metabolism of xenobiotics in laboratory animals. In: Schiller CM, ed. *Intestinal Toxicology*. New York: Raven Press; 1984:145–160.

Choudhuri S. The eukaryotic genome: a brief overview of structure, function and evolution. In: Choudhuri S, Carlson DB, eds. *Genomics: Fundamentals and Applications*. New York: Informa; 2009:49–99.

Choudhuri S, Chanderbhan RF. The biology of nutrients: genetic and molecular principles. In: Gupta RC, ed. *Neutraceuticals: Efficacy, Safety and Toxicity*. London: Elsevier; 2016:209–225.

Choudhuri S, Valerio LG. Usefulness of studies on the molecular mechanism of action of herbals/botanicals: the case of St. John's Wort. *J Biochem Mol Toxicol*. 2005;19:1–11.

Chowanadisai W, Lonnerdal B, Kelleher SL. Identification of a mutation in SLC30A2 (ZnT-2) in women with low milk zinc concentration that results in transient neonatal zinc deficiency. *J Biol Chem*. 2006;281:39699–39707.

Clark RB. The role of PPARs in inflammation and immunity. *J Leukoc Biol*. 2002;71:388–400.

Coburn B, Grassl GA, Finlay BB. *Salmonella*, the host and disease: a brief review. *Immunol Cell Biol*. 2007;85:112–118.

Concon JM. *Food Toxicology Part A: Principles and Concepts*. New York: Marcel Dekker; 1988:1371.

Council for Agricultural Science and Technology (CAST). Mycotoxins: Risks in Plant, Animal, and Human Systems, Task Force Report No. 139. Ames, Iowa; 2003;199 pp.

Cousins MA, Riley RT, Pestka JJ. Foodborne mycotoxins: chemistry, biology, ecology, and toxicology. In: Fratamico PM, Bhunia AK, Smith JL, eds. *Foodborne Pathogens: Microb. Molec. Biol*. Norfolk, UK: Horizon Scientific Press; 2005:164.

Crane JK. Preformed bacterial toxins. *Clin Lab Med*. 1999;19:583–599.

Creppy EE. Human ochratoxicosis. *J Toxicol Toxin Rev*. 1999;18: 277–293.

Cross AJ, Sinha R. Meat-related mutagens/carcinogens in the etiology of colorectal cancer. *Environ Mol Mutagen*. 2004;44:44–55.

Daranas AH, Norte M, Fernandez JJ. Toxic marine microalgae. *Toxicon*. 2001;39:1101–1132.

Davie JR. Inhibition of histone deacetylase activity by butyrate. *J Nutr*. 2003;133:2485S–2493S.

Davis BP, Stucke EM, Khorki ME, et al. Eosinophilic esophagitis-linked calpain 14 is an IL-13-induced protease that mediates esophageal epithelial barrier impairment. *JCI Insight*. 2016;1:e86355.

Dentin R, Girard J, Postic C. Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. *Biochimie*. 2005;87:81–86.

Dickey RW, Plakas SM. Ciguatera: a public health perspective. *Toxicon*. 2010;56:123–136.

Doe W. The intestinal immune system. *Gut*. 1989;30:1679–1685.

Dolan LC, Matulka RA, Burdock GA. Naturally occurring food toxins. *Toxins*. 2010;2:2289–2332.

Dougall WC, Glaccum M, Charrier K, et al. RANK is essential for osteoclast and lymph node development. *Genes Dev*. 1999;13:2412–2424.

Dramsi S, Cossart P. Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. *J Cell Biol*. 2002;156:943–946.

Drudy D, Mullane NR, Quinn T, Wall PG, Fanning S. *Enterobacter sakazakii*: an emerging pathogen in powdered infant formula. *Clin Infect Dis*. 2006;42:996–1002.

Duplus E, Glorian M, Forest C. Fatty acid regulation of gene transcription. *J Biol Chem*. 2000;275:30749–30752.

Durance TD, Wong NS. Kinetics of thermal inactivation of avidin. *Food Res Int*. 1992;25:89–92.

European Food Safety Authority (EFSA). Opinion of the Scientific Committee on Food on the safety of the presence of safrole (1-allyl-3,4-methylene dioxy benzene) in flavorings and other food ingredients with flavoring properties. 2002. Available at: http://ec.europa.eu/food/safety/docs/fs_food-improvement-agents_flavourings-out116.pdf.

EFSA. Glucosinolates as undesirable substances in animal feed: Scientific Panel on Contaminants in the Food Chain. *EFSA J*. 2008a;590:1–76.

EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish—okadaic acid and analogues. *EFSA J*. 2008b;589: 1–62.

EFSA. Marine biotoxins in shellfish—saxitoxin group: scientific opinion of the Panel on Contaminants in the Food Chain. *EFSA J*. 2009a;1019:1–76.

EFSA. Marine biotoxins in shellfish—domoic acid: scientific opinion of the Panel on Contaminants in the Food Chain. *EFSA J*. 2009b;1181:1–61.

EFSA. Cadmium in food: scientific opinion of the Panel on Contaminants in the Food Chain (Question No EFSA-Q-2007-138). *EFSA J*. 2009c;980:1–139.

EFSA. Scientific opinion on marine biotoxins in shellfish—emerging toxins: brevetoxin group. *EFSA J*. 2010;8:1677.

EFSA. Scientific opinion on pyrrolizidine alkaloids in food and feed: EFSA Panel on Contaminants in the Food Chain (CONTAM). *EFSA J*. 2011;9:2406.

EFSA. Dietary exposure to inorganic arsenic in the European population. *EFSA J*. 2014;12:3597.

Enattah NS, Jensen TGK, Nielsen M, et al. Independent introduction of two lactase-persistence alleles into human populations reflects different history of adaptation to milk culture. *Am J Hum Genet*. 2008;82:57–72.

Environmental Protection Agency (EPA). Table of regulated drinking water contaminants. Available at: https://www.epa.gov/ground-water-and-drinking-water/table-regulated-drinking-water-contaminants#Inorganic.

Eriksen GS, Pettersson H. Toxicological evaluation of trichothecenes in animal feed. *Anim Feed Sci Technol*. 2004;114:205–239.

Espenshade PJ. SREBPs: sterolregulated transcription factors. *J Cell Sci*. 2006;119:973–976.

Fafournoux P, Bruhat A, Jousse C. Amino acid regulation of gene expression. *Biochem J*. 2000;351:1–12.

FDA. Guidance to industry for foods derived from new plant varieties. 1992. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096095.htm.

FDA. Consultation Procedures under FDA's 1992 Statement of Policy—foods derived from new plant varieties. 1997a. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm096126.htm.; http://www.fda.gov/Food/IngredientsPackagingLabeling/GEPlants/default.htm.

FDA. HACCP Principles & Application Guidelines. 1997b. Available at: http://www.fda.gov/Food/GuidanceRegulation/HACCP/ucm2006801.htm.

FDA. Guidance for industry: preparation of food contact notifications for food contact substances—toxicology recommendations. 2002. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm081825.htm.

FDA. Furan in food, thermal treatment: request for data and information. *Fed Regist*. 2004;69:25911.

FDA. Guidance for industry: estimating dietary intake of substances in food. 2006a. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm074725.htm#sour.

FDA. Food Allergen Labeling and Consumer Protection Act of 2004: questions and answers. 2006b. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106890.htm#q1.

FDA. Supporting document for recommended maximum level for lead in candy likely to be consumed frequently by small children. 2006c. Available at: http://www.fda.gov/Food/FoodborneIllnessContaminants/Metals/ucm172050.htm.

FDA. Guidance for industry: control of Listeria monocytogenes in refrigerated or frozen ready-to-eat foods; draft guidance. 2008. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/FoodProcessingHACCP/ucm073110.htm.

FDA. Prevention of *Salmonella enteritidis* in shell eggs during production, storage, and transportation; Final Rule 21 CFR Parts 16 and 118. *Federal Register*. 2009;74(130):33030–33101.

FDA. Natural toxins. In: *Fish and Fishery Products: Hazards and Control Guidance*. 2011a:chap 6. Available at: http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM251970.pdf.

FDA. Methyl mercury. In: *Fish and Fisheries Products: Hazards and Controls Guidance*. 4th ed. 2011b:chap 10. Available at: http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM251970.pdf.

FDA. Draft Guidance for Industry: Arsenic in Apple Juice—Action Level. 2013. Available at: http://www.fda.gov/RegulatoryInformation/Guidances/ucm360020.htm.

Feng C, Teuber S, Gershwin ME. Histamine (Scombroid) fish poisoning: a comprehensive review. *Clin Rev Allerg Immunol*. 2016;50: 64–69.

Finkelstein VA, Goldfarb DS. Strategies for preventing calcium oxalate stones. *Can Med Assoc J*. 2006;174:1407–1409.

Flechsig E, Weissmann C. The role of PrP in health and disease. *Curr Mol Med*. 2004;4:337–353.

Food Standards Australia New Zealand (FSANZ). Erucic acid in food: A toxicological review and risk assessment. *Tech. Rep. Ser*. 2003;21:1–25. Available at: https://www.foodstandards.gov.au/publications/documents/Erucic%20acid%20monograph.pdf.

Foufelle F, Ferré P. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem J*. 2002;366:377–391.

Friedman M, Brandon DL. Nutritional and health benefits of soy proteins. *J Agric Food Chem*. 2001;49:1069–1086.

Fukuda H, Iritani N, Sugimoto T, Ikeda H. Transcriptional regulation of fatty acid synthase gene by insulin/glucose, polyunsaturated fatty acids and leptin in hepatocytes and adipocytes in normal and genetically obese rats. *Eur J Biochem*. 1999;260:505–511.

Gadelha IC, Fonseca NB, Oloris SC, Melo MM, Soto-Blanco B. Gossypol toxicity from cottonseed products. *Scientific World J*. 2014;2014:231635.

Ganesan S, Chaurasiya ND, Sahu R, Walker LA, Tekwani BL. Understanding the mechanisms for metabolism-linked hemolytic toxicity of primaquine against glucose 6-phosphate dehydrogenase deficient human erythrocytes: evaluation of eryptotic pathway. *Toxicology*. 2012;294:54–60.

Garner D, Kathariou S. Fresh produce-associated Listeriosis outbreaks: sources of concern, teachable moments, and insights. *J Food Prot*. 2016;79:337–344.

Gaynor PM, Bonnette R, Garcia E Jr, Kahl LS, Valerio LG Jr. FDA's approach to the GRAS provision: a history of processes. 2006. Available at: https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/ucm094040.htm.

Georgiadi A, Kersten S. Mechanisms of gene regulation by fatty acids. *Adv Nutr*. 2012;3:127–134.

German JB, Freeman SL, Lebrilla CB, Mills DA. Human milk oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria. *Nestle Nutr Workshop Ser Pediatr Program*. 2008;62:205–218.

Gralla M, Camporeale G, Zempleni J. Holocarboxylase synthetase regulates expression of biotin transporters by chromatin remodeling events at the *SMVT* locus. *J Nutr Biochem*. 2008;19:400–408.

Granum PE. Bacterial toxins as food poisons. In: Alouf JE, Popoff ME, eds. *The Comprehensive Sourcebook of Bacterial Protein Toxins*. 3rd ed. New York: Academic Press; 2006:949–958.

Granum PE, Brynestad S. Bacterial toxins as food poisons. In: Alouf JE, Freer JH, eds. *The Comprehensive Sourcebook of Bacterial Protein Toxins*. 2nd ed. New York: Academic Press; 1999;669–681.

Granum PE, Lund T. Bacillus cereus and its food poisoning toxins. *FEMS Microbiol Lett*. 1997;157:223–228.

Guengerich FP. Where is toxicology headed in the future? *Drug Metab Rev*. 2004;36:475–486.

Gupta RK, Gangoliya SS, Singh NK. Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *J Food Sci Technol*. 2015;52:676–684.

Hall JE. *Guyton and Hall Textbook of Medical Physiology*. 12th ed. Philadelphia, PA: Saunders; 2011:1091.

Hannah VC, Ou J, Luong A, Goldstein JL, Brown MS. Unsaturated fatty acids down-regulate SREBP isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J Biol Chem*. 2001;276:4365–4372.

Hathcock JN. Residue trypsin inhibitor: data needs for risk assessment. *Adv Exp Med Biol*. 1991;289:273–279.

Hattan DG, Rulis AM. Food toxicology: legal aspects. In: Marquardt H, Schäfer SG, McClellan RO, Welsch F, ed. *Toxicology.* London: Elsevier; 1999;1087–1101.

Hefle SL, Nordlee JA, Taylor SL. Allergenic foods. *Crit Rev Food Sci Nutr*. 1996;36:S69–S89.

Hua X, Wu J, Goldstein JL, Brown MS, Hobbs HH. Structure of the human gene encoding sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1 and SREBF2 to chromosomes 17p11.2 and 22q13. *Genomics*. 1995;25:667–673.

Hughes MF, Beck BD, Chen Y, Lewis AS, Thomas DJ. Arsenic exposure and toxicology: a historical perspective. *Toxicol Sci*. 2011;123:305–332.

Hungerford JM. Scombroid poisoning: a review. *Toxicon*. 2010;56:231–243.

Hunter CJ, Singamsetty VK, Chokshi NK, et al. *Enterobacter sakazakii* enhances epithelial cell injury by inducing apoptosis in a rat model of necrotizing enterocolitis. *J Infect Dis*. 2008;198:586–593.

Inagawa H, Kohchi C, Soma GI. Oral administration of lipopolysaccharides for the prevention of various diseases: benefit and usefulness. *Anticancer Res*. 2011;31:2431–2436.

Institute of Medicine (IOM). *Dietary Reference Intakes: Guiding Principles for Nutrition Labeling and Fortification*. Washington, DC: The National Academies Press; 2003:204.

International Agency for Research on Cancer (IARC). *Acrylamide: Some Industrial Chemicals*. Working Group on the Evaluation of Carcinogenic Risk to Humans; Vol. 60. Lyon, France: IARC Press; 1994:389.

IARC. *Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene*; Vol. 171. IARC Working Group on the Evaluation of Carcinogenic Risk to Humans. Lyon, France: IARC Press; 2002.

IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Dry Cleaning, Some Chlorinated Solvents and Other Industrial Chemicals*; Vol. 63. Lyon, France: IARC Press; 2005:393–407.

IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: A Review of Human Carcinogens; 100F. Lyon, France: IARC Press; 2012:111–144.

International Chemical Safety Programme (ICSP). *Fumonisin B1, Environmental Health Criteria 219*. Geneva: United Nations Environmental Programme, the International Labour Organization and the World Health Organization International Programme on Chemical Safety; 2000:153.

Isbrucker RA, Edwards JA, Wolz E, Davidovich A, Bausch J. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: dermal, acute and short-term toxicity studies. *Food Chem Toxicol*. 2006;44:636–650.

Itsumura N, Inamo Y, Okazaki F, et al. Compound heterozygous mutations in SLC30A2/ZnT2 results in low milk zinc concentrations: a novel mechanism for zinc deficiency in a breast-fed infant. *PLoS ONE*. 2013;8:e64045.

Iynedjian PB, Ucla C, Mach B. Molecular cloning of glucokinase cDNA: developmental and dietary regulation of glucokinase mRNA in rat liver. *J Biol Chem*. 1987;262:6032–6038.

Janssen WF. The story of the laws behind the labels. *FDA Consumer*, June; 1981.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). *Safety Evaluation of Certain Mycotoxins in Food*. WHO Food Additives Series 47. Geneva: WHO; 2001:419.

JECFA. Summary and Conclusions of the Sixty-fourth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA); Vol. 47. 2005. Available at: http://www.fda.gov/downloads/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Acrylamide/UCM194526.pdf.

Johansson SG, Bieber T, Dahl R, et al. Revised nomenclature for allergy for global use: report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol*. 2004;113:832–836.

Jonker JW, Buitelaar M, Wagenaar E, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci U S A*. 2002;99:15649–15654.

Jousse C, Averous J, Bruhat A, Carraro V, Mordier S, Fafournoux P. Amino acids as regulators of gene expression: molecular mechanisms. *Biochem Biophys Res Commun*. 2004;313:447–452.

Jump DB, Clarke SD. Regulation of gene expression by dietary fat. *Annu Rev Nutr*. 1999;19:63–90.

Jyonouchi H. Non-IgE-mediated food allergy. *Inflamm Aller Drug Targ*. 2008;7:173–180.

Kahn CR, Lauris V, Koch S, Crettaz M, Granner DK. Acute and chronic regulation of phosphoenolpyruvate carboxykinase mRNA by insulin and glucose. *Mol Endocrinol*. 1989;3:840–845.

Kalant D, Cianflone K. Regulation of fatty acid transport. *Curr Opin Lipidol*. 2004;15:309–314.

Kallio P, Kolehmainen M, Laaksonen DE, et al. Dietary carbohydrate modification induces alterations in gene expression in abdominal subcutaneous adipose tissue in persons with the metabolic syndrome: the FUNGENUT Study. *Am J Clin Nutr*. 2007;85:1417–1427.

Kanagawa Y, Matsumoto S, Koike S, et al. Association of clinical findings of Yusho patients with serum concentrations of polychlorinated biphenyls, polychlorinated quterphenyls and 2,3,4,7,8-pentachlorodibenzofuran more than 30 years after the poisoning event. *Environ Health*. 2008;7:47–58.

Karch H, Bielaszewska M, Bitzan M, Schmidt H. Epidemiology and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Diagn Microbiol Infect Dis*. 1999;34:229–243.

Kasana S, Din J, Maret W. Genetic causes and gene–nutrient interactions in mammalian zinc deficiencies: acrodermatitis enteropathica and transient neonatal zinc deficiency as examples. *J Trace Elem Med Biol*. 2015;29:47–62.

Kerr GW, Parke TR. Scombroid poisoning—a pseudoallergic syndrome. *J Royal Soc Med*. 1998;91:83–84.

Kilberg MS, Balasubramanian M, Fu L. The transcription factor network associated with the amino acid response in mammalian cells. *Adv Nutr*. 2012;3:295–306.

Kilberg MS, Hutson RG, Laine RO. Amino acid-regulated gene expression in eukaryotic cells. *FASEB J*. 1994;8:13–19.

King H, Ades G. Hazard analysis and risk-based preventive controls (HARPC): the new GMP for food manufacturing. *Food Safety Magazine*. 2015. Available at: http://www.foodsafetymagazine.com/magazine-archive1/octobernovember-2015/hazard-analysis-and-risk-based-preventive-controls-harpc-the-new-gmp-for-food-manufacturing/.

Kirby BJ, Unadkat JD. Grapefruit juice, a glass full of drug interactions? *Clin Pharmacol Ther*. 2007;81:631–633.

Kotsonis FN, Burdoch GA. Food toxicology. In: Klaassen CD, ed. *Casarett and Doull's Toxicology*: *The Basic Science of Poison*. 8th ed. New York: McGraw Hill; 2013;1305–1356.

Kristanc L, Kreft S. European medicinal and edible plants associated with subacute and chronic toxicity part I: Plants with carcinogenic, teratogenic and endocrine-disrupting effects. *Food Chem Toxicol*. 2016;92:150–164.

Kruger WD, Wang L, Jhee KH, Singh RH, Elsas LJ II. Cystathionine beta-synthase deficiency in Georgia (USA): correlation of clinical and biochemical phenotype with genotype. *Hum Mutat*. 2003;22:434–441.

Küry S, Kharfi M, Blouin E, Schmitt S, Bézieau S. Clinical utility gene card for: acrodermatitis enteropathica—update 2015. *Eur J Hum Genet*. 2016;24.

Lago J, Rodríguez LP, Blanco L, Vieites JM, Cabado AG. Tetrodotoxin, an extremely potent marine neurotoxin: distribution, toxicity, origin and therapeutical uses. *Mar Drugs*. 2015;13:6384–6406.

Lambert JD, Kennett MJ, Sang S, Reuhl KR, Ju J, Yang CS. Hepatotoxicity of high oral dose (-)-Epigallocatechin-3-Gallate in mice. *Food Chem Toxicol*. 2010;48:409–416.

Lampe JW. Interindividual differences in response to plant based diets: implications for cancer risk. *Am J Clin Nutr*. 2009;89(suppl):1553S–1557S.

Lampe JW, Navarro SL, Hullar MA, et al. Inter-individual differences in response to dietary intervention: integrating omics platforms towards personalised dietary recommendations. *Proc Nutr Soc*. 2013;72:207–218.

Laqueur GL, Spatz M. Toxicology of cycasin. *Cancer Res*. 1968;28: 2262–2267.

Lasry I, Seo YA, Ityel H, et al. A dominant negative heterozygous G87R mutation in the zinc transporter, ZnT-2 (SLC30A2), results in transient neonatal zinc deficiency. *J Biol Chem*. 2012;287:29348–29361.

Lehane L. Ciguatera update. *Med J Australia*. 2000;172:176–179.

Lehane L, Lewis RJ. Ciguatera: recent advances but the risk remains. *Int J Food Microbiol*. 2000;61:91–125.

Leung-Pineda V, Kilberg MS. Role of Sp1 and Sp3 in the nutrient-regulated expression of the human asparagine synthetase gene. *J Biol Chem*. 2002;277:16585–16591.

Li H, Mukherjee N, Soundararajan U, Tárnok Z, Barta C, et al. Geographically separate increases in the frequency of the derived *ADH1B*47His* allele in eastern and western Asia. *Am J Hum Genet*. 2007;81:842–846.

Loo DDF, Zeuthen T, Chandy G, Wright EM. Cotransport of water by the Na^+/glucose cotransporter. *Proc Natl Acad Sci U S A*. 1996;93:13367–13370.

Lu FC. Acceptable daily intake: inception, evolution, and application. *Regul Toxicol Pharmacol*. 1988;8:45–60.

Luca F, Perry GH, Di Rienzo A. Evolutionary adaptations to dietary changes. *Annu Rev Nutr*. 2010;30:291–314.

Ma T, Verkman AS. Aquaporin water channels in gastrointestinal physiology. *J Physiol*. 1999;517:317–326.

Mackey M, Kotsonis FN. Functional foods: regulatory and scientific considerations. In: Kotsonis F, Mackey M, Hjelle J, eds. *Nutritional Toxicology*. 2nd ed. New York: Taylor & Francis; 2002:243.

Madsen AM. Airborne endotoxin in different background environments and seasons. *Ann Agric Environ Med*. 2006;13:81–86.

Marasas WFO, Miller JD, Riley RT, Visconti A. Fumonisins—occurrence, toxicology, metabolism and risk assessment. In: Summerell BA, Leslie JF, Backhouse D, Bryden WL, Burgess LW, eds. *Fusarium*. St. Paul, MN: Paul E. Nelson Memorial Symposium, APS Press; 2001:332–359.

Marquardt RR, Frohlich AA. A review of recent advances in understanding ochratoxicosis. *J Anim Sci*. 1992;70:3968–3988.

Mateus T, Silva J, Maia RL, Teixeira P. Listeriosis during pregnancy: a public health concern. *ISRN Obstet Gynecol*. 2013;2013:851712.

Mazzanti G, Menniti-Ippolito F, Moro PA, et al. Hepatotoxicity from green tea: a review of the literature and two unpublished cases. *Eur J Clin Pharmacol*. 2009;65:331–41.

MDCH (Michigan Department of Community Health). PBBs (Polybrominated Biphenyls) in Michigan. Frequently asked questions, 2011. Available at: https://www.michigan.gov/documents/mdch_PBB_FAQ_92051_7.pdf. Accessed March 4, 2017.

Mencej-Bedrac S, Prezelj J, Kocjan T, et al. The combinations of polymorphisms in vitamin D receptor, osteoprotegerin and tumour necrosis factor superfamily member 11 genes are associated with bone mineral density. *J Mol Endocrinol*. 2009;42:239–247.

Miller SA. Food additives and contaminants. In: Amdur MO, Doull J, Klaassen CD, eds. *Toxicology: The Basic Science of Poisons*. New York: Taylor & Francis; 1991:819.

Miyake K, Tanaka T, McNeil PL. Lectin-based food poisoning: a new mechanism of protein toxicity. *PLoS One*. 2007;2:e687.

Monroe DH, Eaton DL. Comparative effects of butylated hydroxyanisole on hepatic in vivo DNA binding and in vitro biotransformation of aflatoxin B1 in the rat and mouse. *Toxicol Appl Pharmacol*. 1987;90:401–409.

Mourrieras F, Foufelle F, Foretz M, Morin J, Bouche S, Ferre P. Induction of fatty acid synthase and S14 gene expression by glucose, xylitol and dihydroxyacetone in cultured rat hepatocytes is closely correlated with glucose 6-phosphate concentrations. *Biochem J*. 1997;326:345–349.

Muñoz EC, Martin MJ, Prieto PA. 2′-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. *Nutr Rev*. 2013;71:773–789.

Mura C, Raguenes O, Ferec C. HFE mutations analysis in 711 hemochromatosis probands: evidence for S65C implication in mild form of hemochromatosis. *Blood*. 1999;93:2502–2505.

National Toxicology Program (NTP). *Toxicology and Carcinogenesis Studies of Furan (CAS No. 110-00-9) in F344/N Rats and B6C3F1 Mice (gavage studies)*. NTP Technical Report No. 402. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health; 1993.

National Toxicology Program (NTP). 2016. Safrole. In 14th Report on Carcinogens. https://ntp.niehs.nih.gov/ntp/roc/content/profiles/safrole.pdf.

Nciri N, Cho N, El Mhamdi F, et al. Toxicity assessment of common beans (*Phaseolus vulgaris* L.): widely consumed by Tunisian population. *J Med Food*. 2015;18:1049–1064.

Niyogi SK. Shigellosis. *J Microbiol*. 2005;43:133–143.

Pape ME, Lopez-Casillas F, Kim KH. Physiological regulation ofacetyl-CoA carboxylase gene expression: effects of diet, diabetes, and lactation on acetyl-CoA carboxylase mRNA. *Arch Biochem Biophys*. 1988;267:104–109.

Paredes I, Rietjens IM, Vieites JM, Cabado AG. Update of risk assessments of main marine biotoxins in the European Union. *Toxicon*. 2011;58:336–354.

Park JD, Cherrington NJ, Klaassen CD. Intestinal absorption of cadmium is associated with divalent metal transporter 1 in rats. *Toxicol Sci*. 2002;68:288–294.

Patil C, Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol*. 2001;13:349–355.

Paulauskis JD, Sul HS. Hormonal regulation of mouse fatty acid synthase transcription in liver. *J Biol Chem*. 1989;264:574–577.

Pavlović NM. Balkan endemic nephropathy—current status and future perspectives. *Clin Kidney J*. 2013;6:257-265.

Peng Y, Shi H, Qi X-b, et al. The ADH1B Arg47His polymorphism in East Asian populations and expansion of rice domestication in history. *BMC Evol Biol*. 2010;10:15.

Peraica M, Radic B, Lucic A, Pavlovic M. Toxic effects of mycotoxins in humans. *Bull WHO*. 1999;77:754–766.

Perry GH, Dominy NJ, Claw KG, et al. Diet and the evolution of human amylase gene copy number variation. *Nat Genet*. 2007;39:1256–1260.

Picut CA, Parker GA. Interpreting the Delaney Clause in the 21st century. *Toxicologic Pathol*. 1992;20:617–627.

Pike JW, Lee SM, Meyer MB. Regulation of gene expression by 1,25-dihydroxyvitaminD3 in bone cells: exploiting new approaches and defining new mechanisms. *Bonekey Rep*. 2014;3:482.

Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D3. *Endocrinol Metab Clin North Am*. 2010;39:255–269.

Pinchuk IV, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. *Toxins (Basel)*. 2010;2:2177–2197.

Plumlee KH, Galey FD. Neurotoxic mycotoxins: a review of fungal toxins that cause neurological disease in large animals. *J Vet Intern Med*. 1994;8:49–54.

Poortmans JR, Carpentier A, Pereira-Lancha LO, Lancha A Jr. Protein turnover, amino acid requirements and recommendations for athletes and active populations. *Brazilian J Med Biol Res*. 2012;45:875–890.

Prasad KN, Sinha PK. Effect of sodium butyrate on mammalian cells in culture: a review. *In Vitro*. 1976;12:125–132.

Prasad PD, Ganapathy V. Structure and function of mammalian sodium-dependent multivitamin transporter. *Curr Opin Clin Nutr Metab Care*. 2000;3:263–266.

Price PT, Nelson CM, Clarke SD. Omega-3 polyunsaturated fatty acid regulation of gene expression. *Curr Opin Lipidol*. 2000;11:3–7.

Pulido OM. Domoic acid toxicologic pathology: a review. *Mar Drugs*. 2008;6:180–219.

Rahman MM, Abdullah RB, Wan Khadijah WE. A review of oxalate poisoning in domestic animals: tolerance and performance aspects. *J Animal Physiol Animal Nutr*. 2013;97:605–614.

Ramagopalan SV, Heger A, Berlanga AJ, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res*. 2010;20:1352–1360.

Ramos V, Vasconcelos V. Palytoxin and analogs: biological and ecological effects. *Mar Drugs*. 2010; 8:2021–2037.
Randel RD, Chase CC Jr, Wyse SJ. Effects of gossypol and cottonseed products on reproduction of mammals. *J Anim Sci*. 1992;70:1628–1638.
Reed JD. Nutritional toxicology of tannins and related polyphenols in forage legumes. *J Anim Sci*. 1995;73:1516–1528.
Reguera B, Riobó P, Rodríguez F, et al. *Dinophysis* toxins: causative organisms, distribution and fate in shellfish. *Mar Drugs*. 2014;12:394–461.
Rhinn M, Dollé P. Retinoic acid signalling during development. *Development*. 2012;139:843–858.
Roduit C, Frei R, Depner M, et al. Increased food diversity in the first year of life is inversely associated with allergic diseases. *J Allergy Clin Immunol*. 2014;133:1056–1064.
Rogue A, Spire C, Brun M, Claide N, Guillouzo A. Gene expression changes induced by PPAR gamma agonists in animal and human liver. *PPAR Res*. 2010;2010:325183.
Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: genetic, structural and mechanistic insights. *Nat Rev Microbiol*. 2014;12: 535–549.
Sansalone W, ed. What is a nutrient: defining the Food-Drug Continuum. Washington, DC: Proceedings, Georgetown University, Center for Food and Nutritional Policy; 1999:82.
Schisler JC, Charles PC, Parker JS, et al. Stable patterns of gene expression regulating carbohydrate metabolism determined by geographic ancestry. *PLoS One*. 2009;4:e8183.
Schlatter J, Zimmerli B, Dick R, Panizzon R, Schlatter C. Dietary intake and risk assessment of phototoxic furocoumarins in humans. *Food Chem Toxicol*. 1991;29:523–530.
Schmidt LE, Dalhoff K. Food-drug interactions. *Drugs*. 2002;62: 1481–1502.
Schmitt CK, Meysick KC, O'Brien AD. Bacterial toxins: friends or foes? *Emerg Infect Dis*. 1999;5:224–234.
Schneeman B, Gallaher D. Pancreatic response to dietary trypsin inhibitor: variation among species. *Adv Exp Med Biol*. 1986;199:185–187.
Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol*. 2016;14:e1002533.
Shankar K, Harrell A, Kang P, Singhal R, Ronis MJ, Badger TM. Carbohydrate-responsive gene expression in the adipose tissue of rats. *Endocrinology*. 2010;151:153–164.
Shanklin AP, Sanchez ER. FDA's food contact substance notification program. *Food Safety Magazine*. October/November, 2005.
Siah CW, Ombiga J, Adams LA, Trinder D, Olynyk JK. Normal iron metabolism and the pathophysiology of iron overload disorders. *Clin Biochem Rev*. 2006;27:5–16.
Sicherer SH, Muñoz-Furlong A, Sampson HA. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J Allergy Clin Immunol*. 2003;112:1203–1207.
Sicherer SH, Sampson HA. Food allergy. *Allergy Clin Immunol*. 2006;117:S470–S475.
Sieber SM, Correa P, Dalgard DW, McIntire KR, Adamson RH. Carcinogenicity and hepatotoxicity of cycasin and its aglycone methylazoxymethanol acetate in nonhuman primates. *J Natl Cancer Inst*. 1980;65:177–189.
Skog KI, Johansson MA, Jägerstad MI. Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurrence and intake. *Food Chem Toxicol*. 1998;36:879–896.
Smathers RL, Petersen DR. The human fatty acid-binding protein family: evolutionary divergences and functions. *Hum Genom*. 2011;5:170–191.
Smith RL. Does one man's meat become another man's poison? *Trans Med Soc Lond*. 1991;11:6.
Sobel J, Painter J. Illnesses caused by marine toxins. *Clin Infect Dis*. 2005;41:1290–1296.
Sobel J, Tucker N, Sulka A, et al. Foodborne botulism in the United States, 1990–2000. *Emerg Infect Dis*. 2004;10:1606–1611.
Sperber WH, Stier RF. Happy 50th Birthday to HACCP: retrospective and prospective. *Food Safety Magazine*. 2009–2010. Available at: http://www.foodsafetymagazine.com/magazine-archive1/december-2009january-2010/happy-50th-birthday-to-haccp-retrospective-and-prospective/.
Spergel JM. Nonimmunoglobulin E-mediated immune reactions to foods. *Allergy Asthma Clin Immunol*. 2006;2:78–85.
Stoeckman AK, Ma L, Towle HC. Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. *J Biol Chem*. 2004;279:15662–15669.
Swan JP. FDA's origin. In: George Kurian, ed. *Adapted from a Historical Guide to the U.S. Government*. New York: Oxford University Press; 1998. Available at: http://www.fda.gov/AboutFDA/WhatWeDo/History/Origin/ucm124403.htm.
Takase S, Suruga K, Goda T. Regulation of vitamin A metabolism-related gene expression. *Br J Nutr*. 2000;84(suppl 2):S217–S221.
Takata K, Matsuzaki T, Tajika Y. Aquaporins: water channel proteins of the cell membrane. *Prog Histochem Cytochem*. 2004;39:1–83.
Tice R. α-Chaconine [20562-03-2] and α-Solanine [20562-02-1]. Review of toxicological literature. Prepared for Errol Zeiger, National Institute of Environmental Health Sciences, February, 1998. Available at: http://ntp.niehs.nih.gov/ntp/htdocs/chem_background/exsumpdf/chaconinesolanine_508.pdf.
Tishkoff SA, Reed FA, Ranciaro A, et al. Convergent adaptation of human lactase persistence in Africa and Europe. *Nat Genet*. 2007;39:31–40.
Valdiglesias V, Prego-Faraldo MV, Pásaro E, Méndez J, Laffon B. Okadaic acid: more than a diarrheic toxin. *Mar Drugs*. 2013;11:4328–4349.
van der Put NM, Gabreëls F, Stevens EM, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet*. 1998;62:1044–1051.
Vaulont S, Munnich A, Decaux JF, Kahn A. Transcriptional and post-transcriptional regulation of L-type pyruvate kinase gene expression in rat liver. *J Biol Chem*. 1986;261:7621–7625.
Vaulont S, Vasseur-Cognet M, Kahn A. Glucose regulation of gene transcription. *J Biol Chem*. 2000;275:31555–31558.
Verkman AS. Aquaporins. *Curr Biol*. 2013;23:R52–R55.
Vickery BP, Chin S, Burks AW. Pathophysiology of food allergy. *Pediatr Clin North Am*. 2011;58:363–376.
Vulliamy T, Beutler E, Luzzatto L. Variants of glucose-6-phosphate dehydrogenase are due to missense mutations spread throughout the coding region of the gene. *Hum Mutat*. 1993;2:159–167.
Wang J, Sampson HA. Food allergy: recent advances in pathophysiology and treatment. *Allergy Asthma Immunol Res*. 2009;1:19–29.
Wang M, Li M, Wu S, et al. Fecal microbiota composition of breast-fed infants is correlated with human milk oligosaccharides consumed. *J Pediatr Gastroenterol Nutr*. 2015;60:825–833.
Waterlow JC. Protein turnover with special reference to man. *Quart J Exp Physiol*. 1984;69:409–438.
Watkins SM, Reich A, Fleming LE, Hammond R. Neurotoxic shellfish poisoning. *Mar Drugs*. 2008;6:431–455.
Watson B. The poison squad: an incredible history. 2013. Available at: http://www.esquire.com/food-drink/food/a23169/poison-squad/.
Weber A, Marie J, Cottreau D, et al. Dietary control of aldolase B and L-type pyruvate kinase mRNAs in rat. Study of translational activity and hybridization with cloned cDNA probes. *J Biol Chem*. 1984;259:1798–1802.
WHO−International Food Safety Authorities Network (INFOSAN). Food allergies. INFOSAN Information Note No. 3/2006—food allergies. June 9, 2006.
Wiedenfeld H. Plants containing pyrrolizidine alkaloids: toxicity and problems. *Food Addit Contam*. 2011;28:282–292.
Wilcken B, Bamforth F, Li Z, et al. Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas worldwide. *J Med Genet*. 2003;40:619–625.
World Health Organization. Botulism (updated August 2013). 2013. Available at: http://www.who.int/mediacentre/factsheets/fs270/en/.
Wu W, Sun R. Toxicological studies on plant proteins: a review. *J Appl Toxicol*. 2012;32:377–386.
Ye J, DeBose-Boyd RA. Regulation of cholesterol and fatty acid synthesis. *Cold Spring Harb Perspect Biol*. 2011;3:a004754.
Ye K, Gu Z. Recent advances in understanding the role of nutrition in human genome evolution. *Adv Nutr*. 2011;2:486–496.

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chapter 28

Toxic Effects of Calories

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BIOLOGY OF EATING AND DIGESTION

All biotic organisms derive energy from food to sustain life and this energy "drives" various cellular functions, including digestion, metabolism, pumping blood, and muscle contractions. Nutrients can broadly be defined as chemical substances (typically found in foods or supplements) that are necessary for proper growth and development, reproduction, cellular function and maintenance, and repair following injury. Based on their chemical nature, nutrients can be grouped into organic (carbon-containing) and inorganic classifications. Carbohydrates, proteins, fats, and vitamins comprise the former, while minerals and water are inorganic nutrients essential for life (Stipanuk, 2006). Inorganic nutrients such as minerals can be absorbed into the body through food and are generally incorporated into the food chain through environmental sources (soil, water). Minerals comprise about 4% of the body weight in humans and in combination with water furnish a major part of the obligatory milieu necessary for cellular functioning (pH, osmolarity). Macrominerals are those whose abundance is generally 0.01% of body weight or daily required amounts exceed 100 mg/day. Calcium, phosphorous, sodium, and magnesium fall in this group. Other minerals that are not as abundant can be equally important for an organism. Trace minerals are defined as minerals whose concentration is less than 0.01% of total body weight. Other alternative definitions include nutrients whose requirements are below 1 ppm. Iron, zinc, copper, iodine, selenium, and molybdenum are six essential trace elements with established recommended dietary allowances. Overall, trace nutrients perform a variety of important functions, including transport of oxygen (iron as a part of hemoglobin), catalysis of biological reactions as component of enzymes (iron, zinc), and as part of other organic molecules (selenocysteine). While inorganic compounds serve important roles in physiology, the energy in food is derived from metabolism of organic substances. Organic compounds are generally synthesized by living cells from simpler molecules. For example, green plants and marine phytoplankton utilize photosynthesis to convert the very simple molecule carbon dioxide into more complex, energy-rich compounds such as carbohydrates using the energy from the sunlight. Because most bacteria and higher organisms cannot carry out photosynthesis, they derive their energy by metabolism of preformed organic molecules, such as carbohydrates. In general, bacteria utilize simpler organic molecules and animals and humans require more complex macronutrients (proteins, fats, and carbohydrates) to meet their energy needs.

Digestion of Foods

Common foods are a complex matrix and before the nutrients in food can be utilized they invariably need to undergo digestion. The process of digestion is a remarkable orchestration of many complex biochemical and physiological events, which occurs throughout the gastrointestinal (GI) tract, involving mechanical and chemical breakdown of food into simpler nutrients that are amenable for absorption. The upper GI tract consists of oral cavity, esophagus, and stomach. Breakdown of food begins in the mouth via the actions of enzymes in saliva. Movement of food between sections of the GI tract is restricted via sphincters or valves. In the stomach, food is acted upon by gastric juices, which are secretions from the cardiac, oxyntic, and pyloric glands in the stomach. Gastric juice contains high amounts of hydrochloric acid that is secreted from gastric parietal cells. This along with the enzymes such as pepsin and α-amylase act upon proteins and carbohydrates, respectively, to generate polypeptides and simpler sugars (such as dextrins from starch). Digestion in the small intestine is aided by numerous enzymes supplied by the pancreas, which drains into the proximal portion of the small intestine, the duodenum. Bile is made in the liver and stored in the gall bladder until secreted to aid in the absorption of dietary fats. Pancreatic juice contains digestive enzymes including carboxypeptidases, lipases, amylases, and nucleases. Pancreatic secretions also contain bicarbonate and divalent cations (mostly sodium and potassium) that help neutralize the gastric acid. Almost half of ingested carbohydrates and proteins, and 90% of ingested fat are digested by pancreatic enzymes. In addition to the enzymes, the small intestine itself secretes numerous enzymes including aminopeptidases, lipases, and disaccharidases.

The latter parts of the small intestine, the jejunum and ileum, are primary sites of absorption of nutrients. The surface of the small intestine is uniquely adapted to facilitate the absorption of digested dietary nutrients. The surface area of the intestinal mucosa available for absorption is greatly increased due to a combination of folds called valvulae conniventes (folds of Kerckring) and finger-like projections (villi) that are lined with enterocytes. The luminal surface of the enterocytes is further lined by microvilli that give the mucosal surface brush-border-like appearance. The luminal and basolateral surfaces of the enterocytes are rich in transporters that mediate uptake of nutrients into the enterocytes. Absorption of nutrients into enterocytes may be accomplished by diffusion, facilitated diffusion, or active transport. Luminal digestion of complex carbohydrates involves breakdown via salivary and pancreatic amylases into dextrins that undergo further digestion to simple monosaccharides in the membrane. Furthermore commensal gut microbiota play important roles in breakdown of complex carbohydrates (such as dietary fiber) into substrates that can be utilized by the host. Monosaccharides are transported into the enterocytes by active transport (glucose, galactose) or via facilitated diffusion (fructose). Digestion of proteins begins in the stomach and continues in the lumen of the small intestine. The jejunum is the site of absorption of amino acids and dipeptides and tripeptides. Specific carriers for free amino acid and small peptides in the enterocyte brush border aid absorption. Dietary lipids, such as triacylglycerols, are hydrolyzed by pancreatic and intestinal lipases to free fatty acids or monoacylglycerol. Bile salts along with phospholipids facilitate the absorption of lipids by forming emulsified complexes called micelles, which allows uptake into the enterocyte, where fatty acids are reesterified and packaged into chylomicrons that exit the enterocyte into the lymphatic system. Macronutrient molecules (amino acids, sugars, and fatty acids) that end up in the circulation undergo metabolism in various tissues to be either oxidized to extract energy or stored for future utilization.

Integrated Fuel Metabolism

The requirement for energy to fuel cellular functions in all organisms is continuous. However, food consumption is intermittent. Hence, most organisms have developed processes to store energy from a meal to be utilized until the next meal. Energy in the body is derived from three main nutrient classes: carbohydrates, protein, and fat, which in turn are made up of sugars, amino acids, and free fatty acids, respectively. The principal circulating fuels in the body, glucose and free fatty acids, are stored as glycogen and triglycerides, respectively. Highest concentrations of glycogen are present in the liver and skeletal muscle, it is a macromolecule consisting of branching chains of glucose held together by α-1,4 and 1,6 linkages. Triglycerides are stored in adipose depots in specialized cells (adipocytes) within large lipid droplets. While total body protein constitutes a large percentage of the energy, unlike fats and carbohydrate, protein is not normally utilized as storage form of energy. Proteins are critical in maintaining structure and function and are catabolized for energy only as a last-ditch effort, under extreme conditions. The energetic value of macromolecules per gram of tissue is influenced based on their hydration. While the theoretical energy content of glycogen is approximately 4 kcal/g, due to its highly hydrophilic nature, each gram of glycogen is associated with two to three times its weight of water. Hence, in reality glycogen provides approximately 1 kcal/g of actual energy. This makes storage of energy predominantly in the form of glycogen quite inefficient. In contrast, because triglycerides are stored in a mostly nonaqueous milieu, fat tissue is energy dense, yielding close to the theoretical 9.4 kcal/g of triglyceride. Hence, most animals have developed systems to convert glucose into lipids (lipogenesis) and adipose depots to store de novo synthesized lipids.

The interconversion of fuel sources and release in the time of need is designed to address three priorities of fuel utilization (Shulman et al., 2003). Maintaining a stable supply of substrate for utilization by the brain is the first priority. Because the brain has little to no stored energy in the form of glycogen or triglycerides, it exclusively depends on the liver for maintaining a continuous supply of glucose. Blood glucose concentrations are maintained within a narrow range (55 to 140 mg/dL) via a number of interrelated mechanisms that are highly conserved. Unlike the brain, the heart and to some degree the liver and skeletal muscles derive most of their energy needs through the oxidation of fatty acids. Maintenance of protein reserves and replenishment of proteins (enzymes, cytoskeletal proteins, contractile proteins) following feeding are the next important functions of fuel metabolism. Finally, conversion of existing nutrients into their storage forms (glycogen and triglycerides) is carried via anabolic biological pathways (glycogenesis and lipogenesis).

Hormonal messages generated by the endocrine cells of the pancreas, adipose tissue (adipokines), and GI tract (gut neuropeptides) are critical to orchestrating the multiple processes associated with fuel flux and metabolism. Insulin is the principal hormone required to manage nutrient fuels in both fed and fasted states. The presence and subsequent absorption of food in the GI tract and rise in blood glucose stimulate insulin secretion from the pancreas. Through its actions on various signaling pathways, insulin promotes glucose uptake in peripheral tissues, glycogen synthesis in the liver and muscle, lipid synthesis in adipocytes, and amino acid and protein synthesis in most cells. Insulin also acts to restrain catabolic processes such as lipolysis, gluconeogenesis, and protein degradation, which classically occur under states of low insulin (such as fasting). Following the immediate disposal of absorbed nutrients, the body enters a postabsorptive (early fasting) state, during which the energy derived from glucose supplied directly from the meal is

diminished (Ruderman et al., 2005). In this state, serum levels of insulin start to decrease, and the liver begins metabolic actions to maintain glucose levels by glycogenolysis to release stored glucose and by de novo synthesis of glucose (from gluconeogenic precursors, lactate and fatty acids, and glycerol). Energy for these catabolic and anabolic processes can be derived from metabolism of triglycerides and fatty acids (in adipose tissues). If food remains unavailable for a protracted period, glycogen levels become almost completely depleted in both the muscle and liver, and protein catabolism releases amino acids which become the primary source for glucose production. A rise in glucagon and glucocorticoids (such as cortisol) also characterize this state. These hormones promote lipolysis and breakdown of glycogen.

Set-Point Theory and Neural Control of Energy Balance

A number of redundant feedback mechanisms operate to maintain the homeostasis of energy in living systems. These mechanisms eventually regulate the balance between food intake and energy expenditure to maintain fuel reserves at preset levels. Energy consumed is normally metabolized and utilized to maintain basic metabolic rate and thermogenesis, and carry out cellular processes, organ-specific functions, and movement (muscle contractions). Excess fuels are converted to triglycerides and stored in adipose tissues. Because adipose tissue is the major depot of preserving energy, signals derived from the periphery signal to regions in the brain that coordinate energy balance. When total energy consumed equals the total energy required to meet basal metabolic needs, growth, thermogenesis, and physical activity, the individual is in energy balance, and maintaining this balance will result in relatively stable weight and healthy body composition. When energy intake exceeds the actual energy expenditure, weight gain ensues and body composition can become problematic. The existence of a precise energy balance system is illustrated by considering normal variations in weight among groups of individuals over time. Over the course of a year, the average adult male consumes about 1 million calories, yet body weight of most weight-stable individuals does not fluctuate more than 1 lb. Even a small (about 5%) error in matching intake and expenditure would result in a net change of approximately 6 kg of body weight. Therefore, it is obvious that mechanisms operating within the body can regulate energy balance with exquisite precision.

One theory called the "set-point" theory proposes that food intake and energy expenditure are coordinately regulated by defined regions in the central nervous system that signal to maintain a relatively constant level of energy reserve and body weight (Keesey and Hirvonen, 1997). It is posited that the status of energy stores is sensed by the central nervous system and changes in the afferent signal result in adjustments in food intake and/or energy expenditure. Implicitly, the model requires the existence of four major components of an energy homeostasis system: afferent signals relaying the levels of energy stores, efferent processes regulating energy storage and expenditure, efferent mechanisms controlling ingestive behavior, and integrative centers in the brain to coordinate these processes. Studies have shown that the hypothalamus plays a central role in the control of energy balance, especially food intake. The hormone leptin, secreted in proportion to body fat stores from the adipose tissue, was the first signal to be identified to be a homeostatic regulator of energy balance. Leptin acts on metabolic-sensing neurons in the brain with receptor-mediated actions regulating signaling pathways that in effect decrease food intake (Friedman and Leibel, 1992). Leptin also has potent neurotrophic actions in the brain.

Two populations of neurons are seat of appetite control machinery in the brain, both of which are sensitive to leptin's actions (among other neuropeptides), one expressing orexigenic peptides neuropeptide-Y (NPY) and agouti-related peptide (AGRP) and the other expressing anorexigenic peptides proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). These are located in the arcuate nucleus of the hypothalamus. Downstream projections from these neurons interact with the melanocortin receptor neurons and the neurons in the paraventricular nucleus of the hypothalamus. In addition to the hypothalamic control of appetite per se, reward and hedonic processes of "liking" and "wanting" food occur in the ventral striatum of the midbrain in conjunction with the mesolimbic dopamine system. In addition, the corticolimbic system of reward is controlled by areas in the prefrontal cortex, which integrates sensory, emotional, and cognitive information to coordinate behavioral responses (Lenard and Berthoud, 2008). Hence, the homeostatic control of energy balance fits into the larger decision scheme of choice behavior via a complex neural system.

METHODS TO ASSESS ENERGY BALANCE

Assessing Caloric Intake

In animal studies, caloric intake can be quantitatively monitored by measuring the amount of food consumed by animals in metabolic cages. Caloric intake can be derived by multiplying the quantity (grams per day) of diets consumed with the caloric density of the diet. Knowledge of caloric intake is important in many experimental designs where pair-feeding a parallel cohort of animals is necessary. In clinical studies, assessment of caloric intake can be performed in a number of ways, each with its strengths and limitations. A prospective method to collect information about current intake is maintenance of *food records*. These are usually carried out for a specific duration of time (3 to 7 days, generally including both week and weekend days) during which a written record of all food and beverages consumed is maintained. Quantification is performed by estimation of weights or actual weighing of foodstuff, and hence food records provide both qualitative and quantitative data. Advantages include high reliability, as records do not rely on memory but will be more detailed and accurate records of diet and nutrient intake. However, weighed records are both labor and cost intensive and place higher burden on participants. Habitual eating habits can be influenced by the process of recording, and the reliability of the method decreases over long periods of data collection. Collected data are analyzed using a nutritional database such as the Nutrition Data System for Research (University of Minnesota). Several methods of dietary assessment rely on retrospective collection of food intake. The *diet history* and *food recall* are commonly used methods in clinical research, where a trained individual obtains details of participant's habitual dietary intake in-person or over the phone. Details may include portion sizes, cooking methods, and patterns of eating. The techniques allow a large amount of descriptive data to be collected. Most usually, the interviewer incorporates a 24-hour or 3-day window in which detailed recall is obtained. Burden on the participant is low; however, the technique involves considerable training and time on the part of the researcher. Recall interviews can sometimes be time-consuming and several recalls may be necessary to obtain useful information. Again, analysis of the collected data using a nutritional database is necessary to get quantitative measures of nutrient and caloric intakes. *Food frequency questionnaires* (FFQ) are tools that can be self-administered or provided in an interview setting to participants. FFQ collect information about consumption of each food from a

list of foods over a specified period of time (e.g., last 6 months). They are devoid of specific data regarding the portion sizes and cooking method and are not an appropriate method for quantifying intakes. However, FFQ are useful in gathering information on long-term food patterns, in a low-burden and inexpensive method. Both of these methods are merely estimations and can have significant errors. With the rapid increase in mobile devices and smartphones a number of research and commercial "apps" have been developed to assess and track caloric and nutritional intake by adapting the principles of food diaries and the convenience of digitally capturing images of meals.

Assessing Caloric Content of Foods

Accurate assessment of the caloric value of foods is essential for effective nutritional management in clinical and public policy arenas. Classical investigations by Professor Atwater and his colleagues providing the general calorie factors of 4, 9, and 4 for the major sources of energy—carbohydrate, fat, and protein—have been widely used. Both fats and carbohydrates can be completely oxidized to water and carbon dioxide, while protein is incompletely oxidized and partly excreted from the body as urea. The heat released by combustion of a food in a bomb calorimeter is a measure of its gross energy. However, not all the ingested energy will be available for metabolism and digestible energy refers to the fraction following losses of energy as fecal energy and gases from microbial fermentation. The truly metabolizable energy can be derived by accounting for lost energy in urine (mainly from nitrogen) and on the body surface. Analytical methods to determine the macronutrients in food, protein, fat, and carbohydrates in addition to alcohol and other organic acids have been described widely (Tontisirin et al., 2003). Protein content is mainly determined via estimating nitrogen content by either the Kjeldahl method or hydrolysis of proteins into amino acids and then measuring concentrations using gas or high-pressure liquid chromatography. Fat content can be assessed by measuring the sum of methanol–chloroform extractable total fatty acids that can be expressed as triglyceride equivalents. Total fat can also be assessed by gravimetric methods. Carbohydrate content is generally measured by difference as the remaining energy after accounting for protein, fat, alcohol, and ash. This measure of carbohydrate included energy from fiber as well as some components that may strictly not be carbohydrates (organic acids).

Assessing Energy Expenditure

The process of oxidative phosphorylation couples the oxidation of nutrients with the synthesis of high-energy molecules (viz. adenosine triphosphate [ATP]), the main energy currency of the cell. The energy stored with these molecules is utilized to power all the biochemical processes in cells, which encompass metabolism, growth, and cell division. The total energy expenditure (TEE) or metabolic cost for an average adult ranges from 1500 to 3000 kcal/day. A calorie is defined as a unit of energy that is required to raise the temperature of 1 g of water by 1°C. TEE is primarily composed of three components: (1) basal energy expenditure, (2) thermic effect of food (TEF), and (3) energy expenditure associated with physical activity (EEPA). While most of the variance in daily TEE is accounted for by the three aforementioned components, a small fraction of energy expenditure is accounted for by adaptive thermogenesis (energetic cost of cold adaptation). Basal energy expenditure, also called as resting energy expenditure (REE), is the energy expended when the individual is lying down and at complete rest, generally after sleep in the postabsorptive state. REE accounts for almost 60% of TEE (Gropper et al., 2005). A close surrogate of REE is the resting metabolic rate (RMR). TEF, which accounts for 10% to 15% of TEE, is the energy expenditure associated with the digestion, absorption, and storage of food. EEPA consists of (1) expenditure related to planned exercise and (2) nonexercise activity thermogenesis (NEAT). The former refers to purposeful physical activity, which varies in most individuals and is negligible in individuals who do not participate in exercise. However, even in individuals who do undertake regular exercise, NEAT contributes the greater proportion to EEPA. NEAT includes all energy expenditure from occupational and leisure activities (sitting, climbing stairs, walking, fidgeting, etc.).

Components of energy expenditure can be measured using either (1) direct or (2) indirect calorimetry. The basic principle in direct calorimetry is to measure the actual heat produced by the organism in a highly controlled environment as an estimate of energy expenditure. In large organisms (such as human subjects) direct calorimetry is generally impractical due to the large inherent capacity to store heat. Further, due to limitations in the design, complexity, and cost of carrying out direct calorimetry, this methodology is seldom utilized. The most commonly used methods to estimate energy expenditure, therefore, involve indirect calorimetry. Because most aerobic organisms derive energy from oxidation reactions, consumption of oxygen can serve as reliable surrogate for heat production. Using experimentally derived estimates for energy yields per mole of oxygen for the main fuel substrates, heat production can be calculated based on the quantity of oxygen consumed. Indirect calorimetry can be coupled with specific tracer techniques to examine the utilization and metabolism of specific substrates in both whole-body and tissue-specific compartments. The ratio of carbon dioxide expired and oxygen consumed is called the respiratory quotient (RQ). RQ estimates can be useful in understanding both energy expenditure and overall substrate oxidation. An RQ value of 1.0 suggests that carbohydrate is being oxidized, because under these conditions the amount of oxygen required for oxidation of glucose is equal to the amount of carbon dioxide produced. The representative average RQ values for carbohydrate, fat, and protein are 1.0, 0.7, and 0.8, respectively. Indirect calorimetry can be assessed in animals by placing them in closed chambers in which airflow is controlled at specified flow rates. Oxygen and carbon dioxide concentrations are assessed using gas analyzers to calculate RQ values. Specialized chambers that are commercially available integrate measurement of body weights, food and water intake, activity, and oxygen consumption in a single chamber.

In human subjects, the same principle is utilized to assess oxygen consumption in a variety of designs. A simple metabolic cart can be utilized to assess basal metabolic rate (BMR) in individuals over a short 30-minute period while the individual is in the supine position, but not asleep. Estimates of 24-hour RMR can be calculated by extrapolation. Metabolic carts can be adapted to estimate energy expenditure during physical activity such as treadmill running to estimate maximal oxygen consumption (VO_2 max). However, these measurements only estimate the REE component of TEE. Room calorimeters allow estimating the true TEE by measuring oxygen consumption during all aspects of free living, including postmeal thermic expenditure and during physical activity. These studies require the participant to be isolated in a room calorimeter and are expensive to undertake and maintain. Another method to estimate TEE in free-living subjects utilizes measurement of stable isotopes $^2H_2$ (deuterium) and ^{18}O. In this technique, referred to as "doubly labeled water," a stable isotope of water is given to individuals ($H_2{}^{18}O$ and ^2H_2O) and the disappearance of the labeled tracer in blood and urine is assessed over a 3- to 6-week time period.

The labeled water incorporates into the body water pool and estimates either the water turnover alone (^{2}H label) or the turnover of water and production of CO_2 (^{18}O label). The difference between the two rates of loss of the two labels corresponds to the rate of production of CO_2. The amount of oxygen consumed is indirectly calculated via food records (food quotient) over the assessment period. RQ and energy expenditure can then be calculated using the volumes of oxygen and carbon dioxide. RMR estimates can also be calculated nonexperimentally based on derived equations. Equations such as the Harris–Benedict equation and the Mifflin–St. Jeor equation utilize the person's body weight, body surface area, age, gender, and height (Mifflin et al., 1990).

Assessing Body Composition

Body composition assessments are aimed at describing the overall mass of an individual organism in terms of its molecular or nutrient components: water, fat mass, lean mass, protein, and minerals. In a simple two-compartment model of body composition assessment, total body mass is divided into fat mass (essential and nonessential fat) and fat-free mass (including lean mass and water). Lean mass in this scenario includes protein, carbohydrate, and minerals. A number of techniques have been developed to indirectly assess either specific components or all measures of body composition.

Anthropometric Analysis Body mass is an obvious, but poor surrogate to relative fat mass. While individuals with greater body weight (mass) per height tend to have greater fat mass, total body weight may also be determined by increased muscle mass. Taller people on average have greater weight, and weight and/or body composition also change more dynamically with age. Hence, the simplest indirect measure of body fatness is the relative proportion of weight to height, more commonly referred to as body mass index (BMI). BMI is derived by ratio of mass (in kilograms) and square of height (in meters). The advantage of BMI is that it is easy to compute and large worldwide reference data sets exist. BMI, however, is only an estimate and because BMI does not always reflect fat mass, care must be taken when using BMI as a fat index. Other anthropometric estimates of body composition include measurement of skin folds and circumference at various sites (abdomen, waist, hip). The general assumption is that total body fat is proportional to the fat deposited beneath the skin. Measurements can be analyzed by utilizing several regression models that predict percent body fat. Five commonly employed sites include triceps, subscapula, suprailiac, abdomen, and thigh. While the technique is inexpensive to perform, sites chosen for analysis and skill of the anthropometrist influence the accuracy of the results.

Hydrodensitometry The principle used to calculate body density using hydrostatic weighing has been known since antiquity when the Greek mathematician Archimedes showed that the density of an object is the ratio of its weight in air to its loss of weight in water. Using the density of the whole body and correcting for residual air in the lungs and GI tract, the relative body fat can be estimated using derived equations by Siri or Brozek (Nord and Rayne, 1995). This procedure is also known as underwater weighing.

Air Displacement Plethysmography This procedure employs the same principles as underwater weighing described above, except rather than the body displacing water, it displaces air. This allows the body volume to be calculated and then body fat can be calculated. This is probably the most accurate, precise, and cost-effective measure of total body fat, and is employed widely in clinical research in the United States.

Absorptiometry This is one of the most widely used tools in assessing body composition. In this technique imaging is performed throughout the entire body by a photon beam. Most investigations using this technique use either a dual-photon source (gadolinium) or x-ray at two different energy levels (dual-energy x-ray absorptiometry [DXA]). Hence, this allows imaging of both soft tissues and bone. The attenuation of the x-ray signal received on the detector is proportional to the density of the tissue. Percentage of body fat, lean tissue, and bone mineral density can be computed for the whole body or specific sites based on the analysis of images.

Computerized Tomography While utilizing similar principles of differential x-ray attenuation through a body, computerized tomography is used to produce three-dimensional "slices" or cross-sectional images of the subject. This is made possible by positioning the x-ray source and detectors on opposite poles of a circular tube within which the subject is imaged. The ability to generate 3D images allows regional localization of adipose tissues, muscles, and organs (liver). Using the image data, percent body fat and lean mass can be calculated.

Nuclear Magnetic Resonance Nuclear magnetic resonance (NMR) works by interpreting radio-frequency signals of excited nuclei in an external magnetic field. The physical characteristics of the hydrogen atom differ when the hydrogen is located on protein, fat, or water and this can be detected and quantitated to determine body composition. Magnetic resonance imaging (MRI) is an extremely powerful imaging technique with high-resolution capacity and employed widely as a clinical diagnostic tool. Images acquired are three-dimensional allowing detailed analysis of body composition and regional fat and lean mass distribution with depots and ectopic deposition within tissues. While MRI has several advantages in terms of assessing the distribution of body fat, its expense relative to NMR makes it less used, and other considerations such as the requirement for the participant to lay still during the scanning period and the extreme noise make MRI less applicable than NMR for children.

Electrical Impedance Bioelectrical impedance analysis (BIA) and total body electrical conductivity (TOBEC) measure total body composition based on measuring electrical impedance (the inverse of conductance) of an electric current passed through the body. The conductance of a weak painless current through the body (which serves as an electrolytic medium) is utilized to infer lean body mass and fat mass. Lean mass has more water and greater conductivity than fat mass, and predictive equations are employed to derive fat and lean body mass.

Total Body Water Body fat and lean mass can be calculated by estimating total body water using stable isotopes. Dilution of water containing specific concentrations of labeled isotope (either deuterium or ^{18}O) is usually assessed. Concentrations of isotopes are measured in blood or urine over a timecourse following a 2- to 6-hour equilibration period. Because body water occupies 73.2% of lean mass, fat-free mass can be computed based on total body water. Using a three-compartment model fat mass can be calculated. The method is significantly influenced by hydration status and assumptions are confounded by water present in fat tissue. While the cost of chemicals and collection of samples is not high, analysis of stable isotopes can be expensive.

Assessing Physical Activity

Accurate quantification of physical activity in free-living subjects is challenging. Methods utilizing self-reported diaries and questionnaires tend to overestimate physical activity. Devices such as

accelerometers and pedometers can be utilized to empirically estimate activity. Accelerometers are versatile and cost-effective and can measure activity with little subject burden. Existing accelerometers and wearable fitness trackers come in varying degrees of sophistication from simple pedometers that count steps to models that can assess motion in three dimensions. An important challenge in utilizing accelerometers is to convert the count data into energy expenditure, which is done using different regression models. A widely used and lay-friendly translatable "unit" of ambulatory physical activity is the number of steps in a unit of time (steps/day).

BIOLOGY OF OBESITY

Obesity Risk: Genes, Epigenetics, and Fetal Environment

Access to plentiful food has been unpredictable throughout most of human (and animal) developmental history, marked by periods of feast or famine. In evolutionary terms, fitness and survival of an individual were likely to be closely related to the ability to maximally seek, acquire, consume, and store energy (as fat) when food was available, and to select for mechanisms that reduce energy expenditure during times when food is scarce. Thus, selection favored so-called thrifty genes that orchestrate anabolic processes over energy-consuming ones and provide selective advantage to those who possessed them during periods of food deprivation (Neel, 1962). However, the advent of agrarian lifestyle and recent industrialization has meant that much of the developed and emerging world now has a drastically altered environment. Food is generally available for most people and our lifestyles require less physical activity and exertion. Hence, the genetic legacy of once beneficial thrifty genes placed in an environment of caloric abundance acts as a powerful engine for weight gain, obesity, and its associated metabolic dysfunction.

Recently, questions have been raised whether the selection of thrifty genes occurred because of a true advantage contributing to survival. Speakman has proposed that natural variation and random mutation (genetic drift) in genes controlling hypothalamic energy balance set-points occurred in human evolution as human beings developed fire and social behaviors, and were released from risk of predation. This theory, referred to as the "drifty gene" hypothesis, better explains why even in societies where obesity is high, not everyone becomes obese (Speakman, 2008). Obesity is a highly heritable trait and studies comparing monozygotic with dizygotic twins indicate that 40% to 75% of the interindividual difference in trait is accounted for by genetic variability. Several genes whose disruption causes severe monogenic forms of familial obesity have been described. Remarkably, most of these genes impair central control of food intake. However, the genetic basis of nonsyndromic (common) obesity has remained elusive.

Recent genome-wide association studies (GWAS) have been highly effective in identifying common genetic variants that impact adiposity and other aspects of the metabolic syndrome (MetS). To date 50 such obesity-associated loci have been identified with high confidence (O'Rahilly and Farooqi, 2008). Of these the FTO (fat mass and obesity associated) is unequivocally associated with increased risk of obesity. Individuals who are homozygous for the high-risk (AA) allele weigh on average 3 kg more than individuals who do not carry any risk allele. New meta-analysis of GWAS including about 250,000 subjects has revealed 18 novel loci associated with BMI. Overall, the presently identified loci in combination still explain only a small proportion of obesity. Epigenetic regulation of gene expression is another area that might explain the underlying differences in susceptibility to obesity. Epigenetics is loosely defined as heritable changes in modifications to the DNA that do not alter the DNA sequence. DNA methylation, covalent modification of histone tails that affect chromatin structure and accessibility, and regulation via noncoding RNAs are examples of epigenetic regulation. It is clear that epigenetic mechanisms are involved in basic cell functions including proliferation, differentiation, and growth. Because DNA methylation and histone modifications can also be affected by environmental influences (in utero environment, exposure to xenobiotics, stress), it is plausible that interaction of certain risk alleles and epigenetic mechanisms are responsible for susceptibility to obesity.

The incidence of obesity continues to rise and prevalence even among infants is rapidly rising. As for many chronic diseases, it is now widely accepted that increased susceptibility to obesity can be programmed in utero and early postnatal life. This hypothesis referred to as the "developmental origins of health and disease" is derived from the findings by Professor David Barker and colleagues at the University of Southampton, who discovered an inverse relationship between birth weight and risk of mortality due to coronary heart disease (Barker et al., 1989). Over the last two decades, epidemiological studies have extended the initial associations between birth weight and later cardiovascular disease to include associations between early growth patterns and risk for hypertension, insulin resistance, type 2 diabetes, and obesity in later life. Experimentally manipulating birth weights in a variety of animal models (rats, mice, sheep, pigs) also recapitulates the developmental programming phenotype (McMillen and Robenson, 2005). Another important influence on risk of obesity in later life is maternal body composition (fat mass) at conception and gestational weight gain. Studies in women and experimental models clearly indicate that maternal diet and body composition during pregnancy influence aspects of metabolism and appetite regulation in the offspring (Shankar et al., 2008). The mechanisms of such programming presumably involve epigenetic mechanisms.

TOXICITY RELATED TO EXCESS CALORIC INTAKE/OBESITY

Many of the adaptive, physiological responses to a positive energy balance produced as a result of overeating and inadequate physical activity result in toxicity over the long term. Short-term coordinated changes in metabolic pathways in white adipose tissue in response to overfeeding result in excess energy storage in the form of triglycerides and increased size of preexisting adipocytes (hypertrophy), and this also leads to formation of new adipocytes through hyperplasia (Virtue and Vidal-Puig, 2010). However, management of excess ingested energy over the long term can be complex. Under such conditions, the efficiency of energy storage in adipose tissue is decreased and the body has to resort to several different options to store energy in addition to fat, such as in ectopic sites, which may in fact be detrimental. As plasma concentrations of nonesterified fatty acids (NEFA) increase as a result of ingestion of high-fat diets and as a result of hydrolysis of triglycerides in the fat, triglycerides begin to accumulate in nonadipose tissues such as the liver, skeletal muscle, and the pancreas as lipid droplets. The presence of excess NEFA and lipid metabolites in these tissues results in direct and indirect toxic actions leading to insulin resistance, inflammation, and tissue damage.

In addition, adipose tissue from obese individuals releases chemokines and cytokines, the so-called adipokines, which contribute to a state of "metabolic inflammation" (Dulloo et al., 2010;

Gustafson, 2010). Fat is also an endocrine organ and the pattern of adipokines produced by adipose tissue in obesity differs substantially from that seen in lean individuals (Cornier et al., 2008).

NEFA and the other factors released from adipose tissue contribute to the development of the metabolic syndrome (MetS) in some overweight and obese individuals. This is a cluster of components including insulin resistance, disruptions in lipid homeostasis (dyslipidemia), and elevated blood pressure that substantially increase the risk for development of cardiovascular disease and type 2 diabetes. MetS is also associated with other comorbidities including nonalcoholic fatty liver disease (NAFLD) and reproductive dysfunction and elevated serum insulin in response to insulin resistance contributes to increased cancer risk associated with obesity. As a consequence, overall mortality is increased and life span is shortened with increasing caloric intake, body weight, and adiposity, whereas caloric restriction (CR), at least in animal models, has been shown to substantially increase life span.

Adaptation of Liver and Adipose Tissue to Excess Calories

Triglycerides and glycogen are used by the body to store excess caloric energy. This is a homeostatic mechanism that maintains energy sources such as blood glucose levels between meals. Dietary fats are transported in blood from the gut to the liver and adipose tissue by lipoprotein particles called chylomicrons. In both tissues, local hydrolysis of triglycerides in the capillary bed by the enzyme lipoprotein lipase results in the release of free fatty acids that are subsequently taken up into hepatocytes and adipocytes via fatty acid transport proteins (FATPs) and the scavenger receptor CD36. As they enter the cell, free fatty acids that are toxic are immediately conjugated with acetyl CoA and are bound to intracellular fatty acid–binding proteins before reesterification with glycerol to form triglyceride lipid droplets in the cytosol. Such droplets are highly dynamic and are coated with PAT proteins (named after *p*erilipin, *a*dipophilin, and the *t*ail-interacting protein). Adipophilin and perilipin have important roles in droplet stabilization and regulation of triglyceride turnover. The hepatocyte cytosol contains many small droplets that vary in size depending on the length of time after a meal, dietary fat to carbohydrate ratio, type of dietary fat, and overall caloric intake relative to metabolic requirements. The small hepatic lipid droplets function as a temporary energy storage site, whereas in adipocytes the small lipid droplets fuse to form a single large storage droplet and can serve as a longer-term storage site. In the hours after a meal, triglycerides from the hepatic droplets are incorporated into the lipoprotein VLDL, which is secreted into the plasma and which transports hepatic triglycerides to the adipose tissue for storage. Removal of triglycerides from VLDL by lipoprotein lipase in adipose and other peripheral tissues results in generation of the more dense lipoprotein LDL, which is subsequently recycled by the liver as a result of endocytosis on binding to the LDL receptor (Olson, 1998).

Obesity is often associated with consumption of high-fat diets. In addition to being more energy dense, recent studies have suggested that, at least in part, the obesogenic effects of these diets stem from changes in composition of the gut microbiota (Shen et al., 2014). Transplantation of gut microbiota from donor mice made obese by high fat feeding into germ-free mice has been shown to increase adiposity in these mice relative to mice receiving gut microbes from lean mice. Decreases in populations of Bacteroidetes and increases in populations of Firmicutes in the gut of obese animals change the spectrum of microbial fermentation products, which may in turn change energy harvest and expenditure, alter satiety signals such as the gut peptides YY or ghrelin, and impact insulin action through changes in synthesis of the gut peptide, glucagon-like peptide (GLP-1). In addition, increased bacterial synthesis of short-chain fatty acids (SCFA) may increase obesity as they are substrates for lipid synthesis.

However, obesity can also develop from excessive caloric intake of any food energy source, including carbohydrates and proteins. Although overall consumption of dietary fat has declined in the United States over the past two decades, the proportion of obesity has increased to epidemic levels. In part, this is due to excessive intake of simple carbohydrates that have increased as dietary fat consumption has decreased. Dietary carbohydrates are converted to monosaccharides, mainly glucose and fructose, which are further metabolized in the liver and peripheral tissues. Excess glucose can be stored in the liver in the form of the glucose polymer glycogen that accumulates as cytosolic granules and can make up as much as 7% of liver weight. However, the majority of excess hepatic glucose is metabolized via glycolysis and the citric acid cycle to acetyl CoA and is shunted into de novo fatty acid and triglyceride synthesis.

Recent DNA microarray analysis of gene expression in human adipose tissue biopsies suggests that coordinated upregulation of lipogenesis occurs in fat rapidly and directly as a result of increased caloric intake independent of changes in body weight (Franck et al., 2011). Evidence for increases in adipose tissue glucose transport, and fatty acid and triglyceride biosynthesis has also been obtained from microarray analysis of fat from rats overfed a mixture of simple carbohydrates and fat (Shankar et al., 2010). The increase in triglyceride synthesis in both the liver and fat after consumption of excess calories appears to be driven by activation of two important diet-sensitive transcription factors: sterol regulatory element–binding protein 1c (SREBP-1c) and carbohydrate response element–binding protein (ChREBP) (Postic and Girard, 2008), and this process in fat ultimately drives adipose tissue hypertrophy. In addition to fat cells getting larger, excess calories also trigger proliferation and differentiation of preadipocytes in adipose tissue depots into new adipocytes, a process known as hyperplasia. However, the degree to which hyperplasia contributes to the ability of fat stores to expand in response to the need to store excess energy relative to hypertrophy remains unclear.

What is clear is that in some individuals, there is a limit to which adipocytes can expand safely and that when this limit is reached, toxicity results. This limit appears to depend on which fat depot the adipocytes are in. Subcutaneous adipocytes can attain a greater size than adipocytes in visceral fat and this may explain why metabolic dysfunction and diseases such as type 2 diabetes are linked to visceral adiposity. Hypertrophic adipocytes develop ultrastructural abnormalities including calcium accumulation and cholesterol crystals and show evidence of oxidative and endoplasmic reticulum (ER) stress ultimately leading to cell death and release of intracellular components that can serve as metabolic damage–associated molecular patterns (DAMPs) (Giordano et al., 2013; Khan and Wang, 2014). Recent data from high fat-induced obese mice suggest that DAMP release from dying adipocytes results in activation of the Nod-like receptor NLRP3 inflammasome in remaining hypertrophic adipocytes. This amplifies obese adipocyte cell death by pyroptosis, a form of proinflammatory programmed cell death mediated via NLRP3-dependent caspase 1, and also results in a sterile inflammatory response characterized by activation of interleukin 1β (Giordano et al., 2013). Macrophage infiltration of metabolically stressed adipose tissue to remove cell debris associated with adipocyte death results in further inflammation and the formation of characteristic crown-like structures.

When fat mass increases excessively, adipose tissue also undergoes extensive structural remodeling. An extracellular matrix (ECM) with high concentrations of collagen fibrils and fibronectin appears to be essential for maintenance of the structural integrity of adipocytes and for preadipocyte differentiation (Divoux and Clement, 2011). However, at the point when adipocytes reach a certain size limit within a particular fat pad, hypoxia appears to develop possibly as a result of restricted blood flow. This triggers expression of the transcription factor hypoxia-inducible factor 1α (HIF-1α). Evidence from HIF-1α transgenic and knockout mice suggests that HIF-1α regulates inappropriate ECM remodeling and development of fibrosis in adipose tissue in response to hypoxia and obesity (Halberg et al., 2009). Fibrosis has been reported to be increased in subcutaneous adipose tissue from obese subjects compared with lean subjects both by staining of collagen fibrils and by analysis of col6a3 gene expression. The percentage of fibrosis in white adipose tissue has further been shown to correlate with inflammation in morbidly obese subjects (Divoux and Clement, 2011). Further evidence linking adipose tissue ECM with limitations in the ability of fat pads to expand to store excess caloric energy and for limited adipocyte hypertrophy to precede the development of obesity-linked pathologies such as inflammation and MetS comes from studies with mice in which genes involved in ECM formation have been knocked out. The protein SPARC is required for appropriate collagen synthesis during ECM remodeling. Both SPARC$^{-/-}$ mice and obesity-prone ob/ob mice where the collagen VI gene has been deleted display increased adipocyte and fat pad size, loose ECM structure, and reduced inflammation and metabolic disturbances after high-fat feeding. It is thought that complex interactions between enlarging adipocytes and a fibrotic ECM trigger activation of MAP kinase pathways such as c-Jun N-terminal kinase (JNK) resulting in development of adipocyte insulin resistance (Divoux and Clement, 2011) (see Fig. 28-1).

Ectopic Fat Deposition

In general, there is a positive correlation between increased BMI and inappropriate accumulation of lipids in tissues other than fat. The major sites for this ectopic fat deposition are the liver and skeletal muscle. However, lipid accumulation in other tissues such as small intestine, pancreas, and uterus has also been reported to be associated with chronic consumption of high-fat diets and development of obesity. Correlation between central (visceral) adiposity, waist circumference, and ectopic fat deposition is better than for BMI and is also highly correlated with progressive insulin resistance. However, the relationship between adiposity, ectopic fat accumulation in the liver and muscle, and insulin resistance is highly complex and is strongly affected by diet composition, exercise, and race (Lara-Castro and Garvey, 2008).

In the liver, intrahepatocellular lipid accumulation, also known as fatty liver, or steatosis, is defined as an increase in hepatic lipid content above 5% by weight and is characterized in paraffin-stained sections by the appearance of multiple round empty vacuoles in hepatocytes displacing the nucleus to the periphery of the cell. To confirm that steatosis is actually present, additional staining of frozen sections for triglycerides using stains such as Oil Red O is required. Abnormal

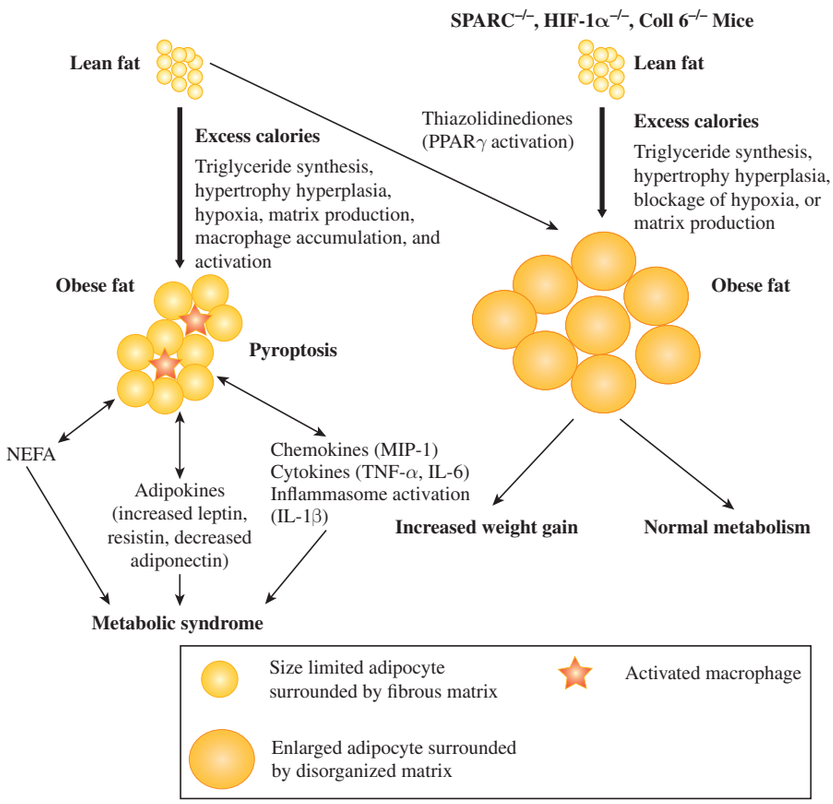

Figure 28-1. *Effects of excess calories (energy) on fat morphology under conditions leading to metabolic syndrome (left) or following stimulation of adipocyte differentiation and hyperplasia by thiazolidinedione treatment/in knockout mice incapable of normal responses to hypoxia (HIF-1α$^{-/-}$)/in knockout mice incapable of normal extracellular matrix production (SPARC$^{-/-}$, Coll 6$^{-/-}$) (right).*

lipid accumulation in the liver in the absence of heavy alcohol usage is referred to as nonalcoholic fatty liver disease (NAFLD) and is associated with a wide spectrum of hepatic dysfunction. The incidence of NAFLD in the general population including children has increased in line with increasing incidence of obesity and is observed in more than 50% of adult obese patients (Browning et al., 2004). Simple steatosis is generally reversible with weight loss and/or lifestyle modification (diet and exercise). However, a small proportion of patients progress to more severe liver pathologies (see the section "Nonalcoholic Steatohepatitis" later in this chapter). Hepatic lipid accumulation can occur as a result of one or more of the following: (1) increased fatty acid supply to the liver and increased fatty acid transporter expression; (2) increased de novo fatty acid and triglyceride synthesis; (3) decreased fatty acid oxidation; and (4) decreased synthesis and/or secretion of VLDL. Which of these processes predominates depends on the degree of obesity, total caloric intake, and diet composition. As the capacity of adipose tissue to expand is compromised with increasing BMI and insulin resistance develops in adipocytes, there is an increase in plasma nonesterified free fatty acids (NEFA) as a result of increased hydrolysis of triglycerides in fat by two enzymes—hormone-sensitive lipase and adipose triglyceride lipase. Increased hepatic exposure to NEFA derived from adipose tissue or from dietary fats has been reported to increase fatty acid transporter expression in hepatocytes (Baumgardner et al., 2008a; Marecki et al., 2011). In contrast, excess calories in the form of simple carbohydrates results in increased de novo hepatic fatty acid synthesis (Shankar et al., 2010). The type of dietary fat can also influence the development of steatosis. For example, it has been shown that saturated and polyunsaturated fatty acids can interfere with VLDL secretion relative to monounsaturated fatty acids via different mechanisms; while polyunsaturated fatty acids provoke oxidative stress and degradation of ApoB100 in hepatocytes, saturated fatty acids appear to block VLDL secretion via stimulation of an unfolded protein response and ER stress (Pan et al., 2004; Caviglia et al., 2011). In addition to increased NEFA, adipose tissue inflammation also appears to significantly contribute to development of NAFLD. Positive correlations have been reported between expression of macrophage markers in adipose tissue and liver fat content independent of total fat mass (Lara-Castro and Garvey, 2008).

Moreover, disrupted adipokine secretion also plays a role. The adipokine adiponectin acts on the liver to inhibit fatty acid synthesis and increase fatty acid degradation through activation of the AMP kinase cascade, downstream inhibition of SREBP-1c, and activation of the transcription factor peroxisome proliferator–activated receptor α (PPARα). Reduced serum concentrations of adipokine adiponectin that accompany development of obesity will result in increased hepatic fatty acid synthesis and reduced fatty acid degradation and thus contribute to development of steatosis (Shankar et al., 2010).

The other major site of ectopic fat deposition in obesity is skeletal muscle in the form of intramyocellular lipid (IMCL). Skeletal muscle contains an intracellular pool of stored triglyceride that exchanges with circulating free fatty acids. Although older methods such as biochemical extraction and computer tomography scans could quantitate muscle tissue triglycerides, more recent approaches such as Oil Red O staining of frozen sections and proton NMR spectroscopy are capable of quantifying IMCL distinct from extramyocellular fat in muscle tissue. Using these methods, IMCL has been shown to positively correlate with visceral adiposity. Various mechanisms have been proposed to underlie accumulation of IMCL in obesity. In adults it has been suggested that changes in intracellular distribution of the fatty acid transporter CD36 from soluble to membrane-associated pools are responsible for increased import of NEFA and triglyceride accumulation (Nickerson et al., 2007). In contrast, data from a pediatric model of obesity demonstrated increased CD36 mRNA and protein expression in skeletal muscle associated with appearance of IMCL (Marecki et al., 2011).

Metabolic Syndrome

The development of central obesity as a result of overnutrition and a sedentary lifestyle leads to a clustering of metabolic and physiological components in some individuals that is associated with a doubling of cardiovascular disease risk and a fivefold increase in incidence of type 2 diabetes originally described as "syndrome X" by Reaven (1988) or "insulin resistance syndrome" and more recently as metabolic syndrome (MetS) (Cornier et al., 2008). Although the definition of MetS differs by health agency, all tend to agree that the core components include central obesity (waist circumference), insulin resistance (increased fasting glucose above 100 mg/dL and increased fasting insulin, as a result of impaired glucose uptake into skeletal muscle/fat and increased glucose output by the liver, resulting from end-organ insensitivity to insulin), dyslipidemia (decreased serum HDL below 40 mg/dL in men and 50 mg/dL in women, and increased serum triglycerides above 150 mg/dL), and hypertension (blood pressure higher than 130/85). The worldwide incidence of MetS is increasing rapidly with the obesity epidemic and is influenced by sex, age, and ethnicity. In the United States, prevalence of MetS is around 30% with higher rates in Mexican Americans than in white non-Hispanics and African Americans and increases with age into the sixth decade. Although there are no established criteria for MetS in children and adolescents and prevalence values depend on age, population studied, and definition, among adolescents, overall incidence has been estimated to be 4.2% rising to 28.7% in those classified as obese (Cook et al., 2008).

Although there does not appear to be a common unifying pathophysiological cause for all components of MetS, central obesity and insulin resistance appear to be the major drivers of this condition. The majority of obese individuals are insulin resistant but the correlation between obesity and insulin resistance is better for abdominal obesity/waist circumference than it is for overall BMI and better for whites than for African Americans. Moreover, weight loss following the feeding of low-calorie diets or following bariatric surgery rapidly leads to marked improvements in insulin sensitivity. However, the relationship between obesity and whole-body insulin resistance is not direct but appears to be mediated through increased circulating fatty acids and ectopic fat deposition particularly in intramyocellular lipid (IMCL) in skeletal muscle (Fig. 28-2). Consumption of a very low calorie diet in obese subjects for as little as 5 days has been shown to produce marked decreases in IMCL and enhanced insulin sensitivity without significant changes in body fat mass (Lara-Castro et al., 2008). It has been suggested that reductions in glucose import into skeletal muscle with IMCL result from inhibition of translocation of the glucose transporter GLUT-4 from cytosolic- to membrane-associated compartments through the action of IMCL metabolites such as diacylglycerol, long-chain fatty acid CoAs, ceramides, and oxidized lipids (Pan et al., 1997). Insulin resistance in muscle is accompanied by evidence of impaired mitochondrial function. However, it is unclear if this is a consequence of or contributor to IMCL (Lara-Castro and Garvey, 2008). Reduced glucose transport also occurs in insulin-resistant adipose tissue itself, and the negative effects of obesity are exacerbated because reduced insulin signaling in adipocytes also enhances expression of hormone-sensitive and adipose triglyceride lipases to further increase the release of NEFA (Cornier et al., 2008).

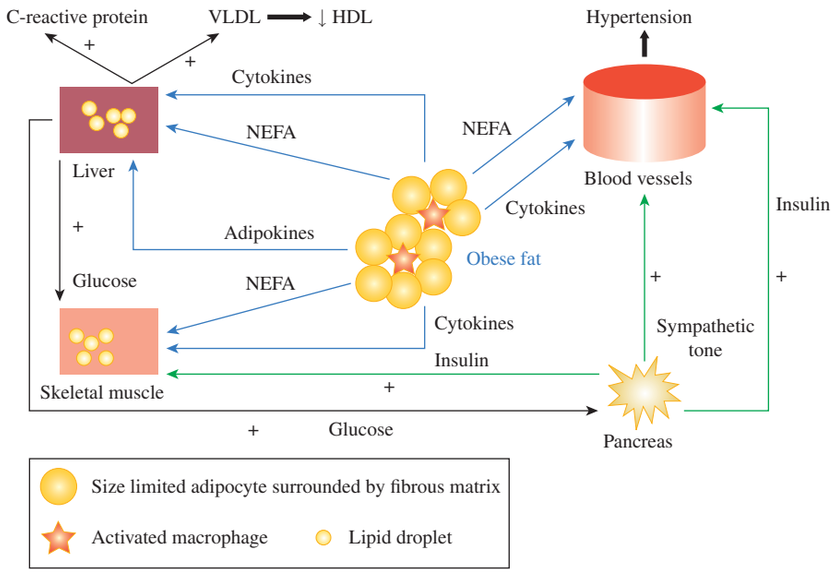

Figure 28-2. *Pathogenesis of metabolic syndrome.*

The relationship between liver steatosis and hepatic insulin resistance is less clear and the question of whether NAFLD is a cause or a consequence of hepatic insulin resistance remains unresolved (Cohen et al., 2011). Insulin resistance in the liver leads to excess glucose production as a result of a reduced ability of insulin to suppress the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK). This contributes to systemic hyperglycemia and increased pancreatic insulin production. Insulin resistance and steatosis are strongly correlated and interventions that lead to lower plasma insulin levels also decrease liver triglyceride content. Moreover, patients producing hepatic insulin as a result of metastatic insulin-secreting tumors develop steatosis in the surrounding hepatocytes. However, the suggestion that steatosis causes hepatic insulin resistance is contradicted by data from genetically manipulated mouse models where reduced fatty acid mobilization, reduced fatty acid oxidation, and defective choline synthesis are all associated with development of steatosis, but where hepatic insulin sensitivity is maintained (Cohen et al., 2011).

The dyslipidemia associated with MetS is thought to be a direct consequence of increased VLDL secretion by the liver. Increased serum triglycerides are generally associated with a change in HDL size and lipid composition, and reduction of serum HDL concentration as a result of increased clearance via the kidney. Several different mechanisms have been proposed to explain the rise in blood pressure associated with MetS. Insulin acts both directly as a vasodilator and secondarily to increase sodium resorption from the kidney. Evidence suggests that under conditions of insulin resistance, the vasodilatory effects of insulin are lost while the renal effect on sodium resorption is maintained. In addition, fatty acids can act directly to mediate vasoconstriction and both fatty acids and insulin can increase the activity of the sympathetic nervous system. Adipokines, such as leptin and resistin, have also been implicated in the pathogenesis of obesity-associated hypertension (Cornier et al., 2008).

Therapeutic Options for Managing Metabolic Syndrome

Because it is unclear that there is a unifying mechanism underlying the constellation of metabolic and physiological abnormalities that make up MetS, management can be problematic. Lifestyle modifications in the obese, including diets producing stable weight loss and long-term increased physical activity or bariatric surgery (discussed in the section "Surgical Interventions" later in this chapter), are of benefit in treating all the components of MetS, but suffer from limited compliance and significant risk of complications in the case of surgery. Therefore, routine clinical management of MetS has focused on pharmaceutical therapies for insulin resistance/hyperglycemia, dyslipidemia, and hypertension to reduce the risks of cardiovascular disease and type 2 diabetes.

Several classes of drugs are used to target insulin resistance. Metformin acts on the liver to reduce hepatic glucose production as a result of activation of the AMP kinase pathway (Boyle et al., 2010). Moreover, metformin is relatively nontoxic and has been shown to reduce progression from hyperglycemia to type 2 diabetes and the incidence of cardiovascular disease (Knowler et al., 2002; Ratner et al., 2005). Thiazolidinediones such as pioglitazone and rosiglitazone also improve insulin sensitivity and slow progression of diabetes in about 50% of patients as a result of activation of the transcription factor PPARγ in extrahepatic tissues and increasing circulating levels of adiponectin (Gerstein et al., 2006; Cho and Momose, 2008). However, increased insulin sensitivity is associated with an expansion of adipose tissue and significantly increased body weight because PPARγ regulates adipocyte differentiation. Moreover, a recently discovered adverse effect of thiazolidinediones is increased bone loss and an increased risk of osteoporosis; PPARγ activation in bone inhibits osteoblastogenesis and stimulates accumulation of fat cells in bone marrow (Lecka-Czernik, 2010). Additional concerns have been raised regarding potential for increased cardiovascular problems in patients taking rosiglitazone (Palee et al., 2011).

Dyslipidemia associated with MetS is a major modifiable risk factor for cardiovascular disease. Increased triglycerides and low HDL levels are often accompanied by increased total and LDL cholesterol and LDL is a primary target for cholesterol-lowering therapy. Standard first-line therapy is statin inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), the rate-limiting step in cholesterol biosynthesis, such as atorvastatin (Lipitor®). These compounds improve overall lipid profiles by decreasing LDL concentrations 20% to 40%, increasing HDL 5% to 10%, and decreasing triglycerides 7% to 30% (McFarlane et al., 2002). Although

generally well tolerated, atorvastatin results in muscle or joint pain in 5% of patients. Muscle pain can be an indication of rhabdomyolysis (muscle breakdown). In addition, liver injury as indicated by elevated serum values of liver enzymes such as alanine aminotransferase (ALT) has been reported in some users (Bakker-Arkema et al., 1996). Statins are often used in combination with ezetimibe or niacin to further reduce LDL and triglycerides and raise HDL levels. In addition to statins, high doses of 2 to 4 g of fish oil omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to reduce serum triglycerides by 20% to 40% (Cornier et al., 2008).

Control of hypertension is also the key to prevention of cardiovascular events. Many antihypertensive drugs are available and are taken chronically. The most commonly prescribed are adrenergic β-blockers, which inhibit sympathetic inputs to reduce cardiovascular output and slow heart rate. Despite substantial decreases in systolic and diastolic blood pressure, long-term prospective studies suggest that treated hypertensive men continue to have a much greater risk of stroke and coronary heart disease than normotensive men of the same age in the second decade of treatment (Deshmukh et al., 2008). This may be due to metabolic side effects, particularly increased serum triglycerides and lowering of HDL. In addition, diuretics are often given in combination with β-blockers. This can result in reductions of serum potassium and increased occurrence of gout in patients with marginal uric acid concentrations. Other frontline antihypertensive drugs are the angiotensin-converting enzyme (ACE) inhibitors. ACE inhibitors work on the renin–angiotensin system by preventing the conversion of angiotensin I to angiotensin II and reducing production of the hormone aldosterone. Aldosterone acts to reduce sodium and water excretion and is a direct vasoconstrictor. ACE inhibitors reduce arteriolar resistance, increase venous capacity, and increase cardiac output while increasing sodium excretion. ACE inhibitors in general have favorable effects on lipid profile.

Data linking obesity development and MetS with dysbiosis of the gut microbiota have resulted in increasing interest in the use of probiotics as a potential therapeutic option. Recent studies of probiotic supplementation with a highly proliferative strain of *Bifidobacterium animalis* spp. *lactis* in mice fed high-fat diets resulted in reduced visceral fat accumulation and improved glucose tolerance associated with increased levels of the most abundant bacterial short-chain fatty acids (SCFA), acetate, and an increase in levels of glucagon-like peptide 1 (GLP-1) in both the gut and the plasma. Acetate has been implicated in the protective effects of probiotics. It has been shown to bind to a G protein–coupled receptor GPR43 to inhibit fat accumulation in adipocytes, to potentiate pancreatic β-cell function, and to reduce appetite via a central hypothalamic mechanism (Aoki et al., 2017).

Nonalcoholic Steatohepatitis

As described above, ectopic fat deposition in the liver is strongly correlated with obesity and insulin resistance. Although NAFLD is not considered a core symptom of MetS, it is often a comorbidity. Fatty liver is very common being found in 30% of autopsies and in more than 80% of the obese. However, for reasons that remain unclear, in the majority of cases, steatosis is reversible and relatively asymptomatic. The disease progression of NAFLD begins with simple steatosis which progresses to nonalcoholic steatohepatitis (NASH), which is characterized by cell death, inflammation, and fibrosis, then to cirrhosis in which liver function is significantly impaired, and ultimately to hepatocellular carcinoma (Cohen et al., 2011) (Fig. 28-3). Only about 30% of individuals with NAFLD show evidence of progression of pathology to NASH, and of these only 20% to 30% progress further to cirrhosis within 10 years. Of those with NASH-induced cirrhosis, 4% to 27% ultimately develop liver cancer. NASH was first described by Ludwig et al. (1980) in a group of obese adults without a history of alcohol abuse and has increased in prevalence with the obesity epidemic to about 2% to 3% of the U.S. population. It is also increasingly observed in the pediatric population including cases in children as young as 2 years of age (Loomba et al., 2009). Although steatosis can be diagnosed noninvasively using ultrasound or imaging, improved methods for early detection of NASH are urgently required. Although elevated serum ALT values increase the likelihood of NASH, up to 59% of

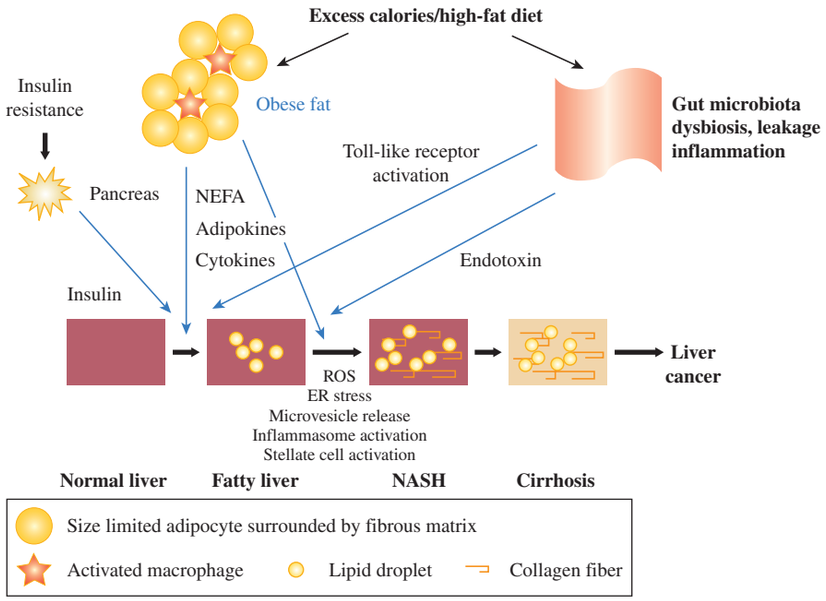

Figure 28-3. *Progression of nonalcoholic fatty liver disease (NAFLD).* ER stress, endoplasmic reticulum stress; ROS, reactive oxygen species.

NASH patients had normal ALT values and at present, only liver biopsy can provide accurate diagnosis.

Our understanding of the molecular mechanisms whereby NAFLD pathology progresses is hampered by lack of an animal model that recapitulates all aspects of the human disease (Hebbard and George, 2011). Until recently, a widely held "two-hit" hypothesis held that progression of NAFLD to NASH requires initial development of steatosis followed by several possible second hits. These include oxidative stress resulting from lipid peroxidation and mitochondrial dysfunction and ER stress triggered by protein misfolding resulting from direct lipotoxicity of saturated fatty acids. In addition, bacterial overgrowth and increased gut permeability resulting from chronic consumption of high-fat diets can increase plasma endotoxin levels resulting in Kupffer cell activation and inflammation. An initiating role for steatosis in NASH progression is supported by human studies of NAFLD heritability. NAFLD, NASH, and cirrhosis cluster in families and one genetic variant consistently associated with appearance of NAFLD is a missense mutation in the *PNPLA3* (adiponutrin) gene. *PNPLA3* has triglyceride hydrolase activity that is lost on mutation, consistent with the accumulation of liver fat. More recent GWA association analysis of SNPs from subjects with biopsy-proven NAFLD has yielded additional genetic variants associated with dysregulated lipid homeostasis. These include lysophospholipase-like 1 (*LYPLAL1*), which like *PNPLA3* is linked to triglyceride breakdown, and glucokinase regulator (*GCKR*), which regulates glucose storage and provides substrates for de novo lipogenesis (Speliotes et al., 2011). Treatment with insulin sensitizers, bariatric surgery, and lifestyle modification (diet and exercise) resulting in weight loss and reduction of hepatic fat content improve NASH liver pathology (Cohen et al., 2011).

More recently the two-hit hypothesis has been modified into a multiple interrelated hit theory as evidence has accumulated that factors derived from obese adipose tissue such as adipokines and the proinflammatory cytokine tumor necrosis factor (TNF-α), activation of the endogenous immune system through Toll-like receptors, ER stress, and signals from the gut microbiota can all increase hepatic fat accumulation (Tilg and Moschen, 2010; Hebbard and George, 2011). The progression of steatosis to necroinflammatory injury and fibrosis in NASH livers appears to depend on oxidative stress mediated by reactive oxygen species generated by cytochrome P450 CYP2E1 and NADPH-oxidase enzymes (Liang et al., 2016; Abdelmegeed et al., 2017). Treatment with dietary antioxidants such as the glutathione precursor *N*-acetylcysteine and vitamin E has recently been shown to prevent increases in serum ALT, suppress hepatic production of TNF, and improve NASH pathology (Baumgardner et al., 2008a, 2008b; Sanyal et al., 2010). Lipid peroxidation resulting from ROS action on accumulated fats has also been linked to NASH progression. Highly reactive short-chain aldehydes such as malondialdehyde and 4-hydroxynonenal (4HNE), which are the end products of lipid peroxidation, accumulate in steatotic livers and can react with cellular proteins to form adducts, especially in the mitochondria. These adducted proteins may have impaired function and may also be recognized as foreign haptens by the immune system triggering an autoimmune response involving the formation of autoantibodies. Consistent with this hypothesis, studies of high fat feeding using genetically manipulated mice made steatotic by loss of the peroxisome proliferator–activated receptor α (PPARα) and unable to degrade 4HNE as a result of deletion of glutathione-*S*-transferase A4-4 have shown greater progression of liver pathology relative to wild-type mice or mice lacking each gene separately (Ronis et al., 2017). In addition, prior immunization with 4HNE-adducted albumin has been shown to exacerbate liver injury in a mouse model of NASH utilizing a methionine- and choline-deficient diet (Sutti et al., 2016). Damage to hepatocytes in steatotic livers has also recently been linked to release of extracellular microvesicles/exosomes containing micro RNAs, and to release of damage-associated molecular patterns (DAMPs), which appear to play a role in development of inflammation including inflammasome activation in other parenchymal cells and resident macrophages (Wan et al., 2016; Cannito et al., 2017).

Development of fibrosis subsequent to NASH appears to involve activation of hepatic stellate cells by the cytokine transforming growth factor β (TGF-β) and factors associated with disruption of lipid homeostasis including the sphingosine metabolite, sphingosine 1-phosphate (Basaranoglu et al., 2013; Mercer et al., 2017). Stellate cell activation is associated with inappropriate secretion of matrix components, particularly collagen, as a result of regenerative wound-healing responses to chronic liver injury. Proliferative regenerative responses also ultimately promote development of hepatocellular carcinomas in NASH patients (Dooley et al., 2009; Matsuzaki, 2009; Nejak-Gowen and Monga, 2011).

Alteration in Drug Pharmacokinetics and Metabolism in Obesity and NAFLD

Obesity and fatty liver disease can have significant effects on drug pharmacokinetics, metabolism, and therapeutic efficacy with the potential to result in adverse drug reactions (Hanley et al., 2010; Merrell and Cherrington, 2011).

The most significant change in drug pharmacokinetics associated with obesity is likely to be in volume of distribution (V_d), and this determines loading dose selection. V_d estimates the extent to which a drug distributes into extravascular tissues and this in turn will depend on the drug's physiochemical properties, binding to plasma proteins, and tissue blood flow (see Chap. 7, "Toxicokinetics"). The V_d of lipid-soluble drugs that partition readily into adipose tissue is likely to be significantly increased in obesity because obese individuals have a larger fat compartment into which the drug can distribute. In contrast, V_d for hydrophilic drugs that do not readily partition into fat may be relatively unaffected (Hanley et al., 2010). For example, while the V_d for the highly lipophilic chemotherapeutic drug docetaxel is increased by more than 400 L in the obese, the V_d for the water-soluble antibiotic daptomycin is increased by only 2 to 4 L. There are little data to suggest that obesity significantly alters drug binding to albumin or other plasma proteins. However, reductions in tissue blood flow and altered cardiovascular function have been observed in obesity and may further alter V_d. Increases in V_d in obesity may significantly alter drug efficacy. For example, the disposition of lipid-soluble oral contraceptives such as ethinyl estradiol has been shown to be altered in obesity and increased weight may be associated with increased risk of failure of contraception. In such circumstances drug loading dose should be adjusted by total body weight or BMI.

The major influence determining the steady-state plasma concentration of a drug in a maintenance dose regimen is clearance (CL). This in turn depends on rate of metabolism and expression of drug transporters. Obesity and NAFLD appear to have variable effects on hepatic phase I and II drug metabolism and on transporters. However, significant species differences in regulation of these enzymes and transporters and lack of appropriate animal models for NAFLD/NASH make extrapolation of much of the experimental data to the clinic problematic (Merrell and Cherrington, 2011). Obesity and NAFLD have been shown to increase expression of the cytochrome P450 enzyme CYP2E1 in both animal models and human clinical studies. CYP2E1 is involved in the metabolism of ethanol, solvents, and volatile anesthetics such as halothane

and enflurane. In contrast, hepatic expression of CYP1A2 that metabolizes approximately 15% of therapeutic drugs including anticoagulants, antidepressants, antihypertensive drugs, and cyclooxygenase-2 inhibitors appears to be consistently suppressed. The effects of NAFLD/NASH on expression of other cytochrome P450 enzymes are inconsistent. However, increases have been observed in expression of other phase I enzymes including NAD(P)H quinone oxidoreductase, the mitochondrial form of epoxide hydrolase, and several aldehyde dehydrogenases in animal models of NAFLD. Less is known about the effects of NAFLD/NASH on drug conjugation and transport. Decreased expression of sulfotransferases has been reported in NASH patients, but effects on drug glucuronidation are inconsistent and glutathione conjugation appears mixed despite observations of GSH depletion in human NAFLD patients. Recent studies in experimental rat models of NAFLD suggest decreased expression of hepatic uptake transporters such as the sodium/taurocholate co-transporting polypeptide (NTCP) and the organic anion transporting polypeptides (OATPs) and increased expression of efflux transporters, such as the multidrug resistance–associated proteins Mrp2, Mrp3, and Mrp4 (Lickteig et al., 2007; Fischer et al., 2009). This was accompanied by a proportional shift in elimination of acetaminophen metabolites from bile to urine (Lickteig et al., 2007).

Drug elimination as measured by half-life is affected by both V_d and CL and thus may also be altered in obesity. Half-life is calculated using the following formula: $t_{1/2} = (\ln 2 \times V_d)/\text{CL}$, and thus changes in both V_d and CL can influence drug half-life. For example, the $t_{1/2}$ of the antianxiety drug diazepam is markedly increased in obese subjects as a result of increased V_d even though CL is unchanged.

Endocrine Dysfunction in Obesity, Metabolic Syndrome, and NAFLD

A wide spectrum of endocrine disruption is associated with obesity and MetS. Obesity in pregnancy can result in many complications including gestational diabetes, pregnancy-associated hypertension, preeclampsia, and fetal abnormalities including neural tube defects, spina bifida, heart defects, and cleft palate (Kulie et al., 2011). In addition, delays in milk production and decreased duration of breast-feeding have been associated with obesity in women as a result of hormonal and metabolic effects on mammary gland development during pregnancy.

As discussed above, hyperglycemia resulting from systemic insulin resistance provokes a compensatory increase in insulin secretion from the pancreas in obese individuals. Hyperinsulinemia then appears to have secondary effects on other endocrine systems. For example, growth hormone (GH) secretion is dramatically suppressed by obesity in both adults and children, but can be reversed by weight loss (Kreitschmann-Andermahr et al., 2010). Mechanisms appear to involve direct feedback effects of insulin on the pituitary and a reduction in secretion of the endogenous GH-releasing peptide ghrelin that is produced by the stomach and hypothalamic centers. In addition, although reductions in GH lead to a decrease in hepatic synthesis and serum concentrations of total insulin-like growth factor 1 (IGF-1) in the obese, paradoxically obesity results in a suppression of IGF-binding proteins and thus an increase in free IGF-1. Free IGF-1 can also exert a negative feedback on GH secretion. In addition to effects on the GH–IGF axis, hyperinsulinemia also appears to increase adrenal androgen production and reduce plasma concentrations of sex hormone–binding globulin (SHBG) in obese prepubertal girls (Burt Solorzano and McCartney, 2010). Increased serum concentrations of free androgens appear to explain, in part, why childhood obesity is associated with earlier pubertal development. Rodent studies of high-fat feeding have demonstrated accelerated vaginal opening that was reversed by both androgen receptor blockade and normalization of glucose homeostasis with metformin. Hyperandrogenization also appears to explain the increased incidence of polycystic ovary syndrome (PCOS) in obese adolescent girls with MetS, anovulatory cycles, and subfertility in obese women of childbearing age. Increased adipose tissue mass also results in increased estrogen production as a result of androgen aromatization in fat tissues. This may also contribute to accelerated puberty in girls. Surprisingly, in obese boys, data suggest that puberty may be delayed. This may also be related to increased aromatization of androgens in adipose tissue because negative feedback of estrogens at the level of the hypothalamic–pituitary axis may result in reduced luteinizing hormone secretion and reduced testosterone production (hypogonadotropic hypogonadism). In addition to suppression in GH and gonadotropin secretion, hypothyroidism is common in individuals with MetS. Reduced thyroid hormone concentrations may exacerbate NASH progression in the liver by increasing triglyceride synthesis, reducing fatty acid oxidation, and increasing hepatic cholesterol concentrations by reducing conversion to bile acids (Loria et al., 2009).

Obesity and Cancer Risk

Increased BMI is well known to be associated with significantly increased risk of a number of cancers. These include sex-steroid-dependent endometrial, breast, and prostate cancer, GI tract cancers such as esophageal adenocarcinoma, and colon cancer and renal cancer (Table 28-1). Several mechanisms have been proposed related to endocrine and metabolic disturbances associated with obesity (Roberts et al., 2010). As described earlier, systemic insulin resistance results in hyperinsulinemia and reduces plasma IGF-1-binding protein levels to increase free IGF-1 concentrations. Insulin and IGF-1 signaling interact to stimulate cell proliferation via activation of MAP-kinase cascades resulting in increased phosphorylation of ERK and via activation of Wnt-β-catenin pathways. In addition, they exert antiapoptotic effects through PI-3 kinase pathways. Increased

Table 28-1

Estimated Risk Ratios* for Cancer in Relation to BMI

CANCER TYPE	MEN	WOMEN
Colon cancer	1.24	1.09
Gallbladder cancer	—	1.59
Leukemia	1.08	1.17
Malignant melanoma	1.17	—
Multiple myeloma	1.11	1.11
Esophageal adenocarcinoma	1.52	1.51
Renal cancer	1.24	1.34
Thyroid cancer	1.33	1.14
Prostate cancer	1.03	—
Postmenopausal breast cancer	—	1.12
Endometrial cancer†	—	1.73

**Per increase in BMI by 5 kg/m² (Roberts et al., 2010).*
†For BMIs above 27 kg/m².

sex steroid concentrations in obesity promote growth of tumors in the mammary gland, endometrium, and prostate. Adipokines may also play a role in promotion of obesity-associated cancers. Leptin that is increased as fat mass increases is proproliferative, antiapoptotic, and proinflammatory and promotes new blood vessel formation (angiogenesis). In contrast, adiponectin that is suppressed in obesity has the opposite properties and is inversely associated with the presence of cancer. Additional potential associations between obesity and cancer may involve depletion of cellular antioxidant systems as a result of the low-grade chronic systemic inflammation that accompanies morbid obesity and the possibility that mesenchymal stromal cells arising from expanding white adipose tissue may be recruited to tumors to promote angiogenesis and drive tumor progression (Roberts et al., 2010).

HEALTH BENEFITS AND LIFE EXTENSION ASSOCIATED WITH CALORIC RESTRICTION

The opposite of overfeeding is caloric restriction (CR) (also known as dietary restriction). Over the past two decades, experimental CR has been repeatedly shown to increase life span and reduce age-related disease in comparison with ad libitum feeding in a wide variety of organisms including yeast, nematodes, fruit flies, fish, many rodent species, and dogs (Smith et al., 2010). Recently, Colman et al. (2009) have reported similar findings in rhesus monkeys and a multisite human randomized clinical research study is currently in progress funded by the National Institute on Aging (NIA): Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy (the CALERIE study, available at: http://calerie.dcri.duke.edu/index.html). Preliminary data suggest replication of many of the results from animal studies, including reduced fat and lean mass; reduced insulin; reduced energy expenditure; lower core body temperature; and improved lipid profiles. It has been suggested that the increased health and longevity associated with CR are related to reduced energy flow, increased insulin sensitivity, and reduced inflammation (Ye and Keller, 2010). Studies in yeast, *Caenorhabditis elegans*, and *Drosophila* have suggested that the life span–enhancing effects of CR are mediated through activation of a family of histone deacetylases known as the sirtuins (Sir), which act as sensors of nutrient flux (Smith et al., 2010). Intensive studies are underway to identify CR mimetics that might have the same benefits as CR on long-term health and longevity without the necessity for imposed food restriction. Most work has focused on the grape polyphenol resveratrol that has been shown to activate Sir2 and to mimic CR effects to increase life span in yeast, *C. elegans*, *Drosophila*, and fish in the absence of nutrient alteration. A second CR mimetic receiving significant attention is rapamycin, an inhibitor of the nutrient signaling mediator target of rapamycin (TOR).

TREATMENT OF OBESITY

Lifestyle Modification: Dieting and Exercise

Attaining a healthy body composition usually requires trimming excess body fat by reductions in total caloric intake, modifications in diet composition, and increases in total energy expenditure (TEE) via increased physical activity. This combined strategy is intended to develop a metabolic state in which energy expenditure exceeds energy intake, and which in turn requires total body energy requirements to come from utilization of body fat stores, thereby burning body fat and improving body composition.

Adhering to almost any commercially available diet plan on the market can achieve body weight loss and reduction of total fat mass. For example, people who use the Atkins diet, which is a diet high in protein and fat and very low in carbohydrates, lose weight. The major problem is sustaining a healthy body composition. Most dieters return to their predieting weight and body composition (or worse) because they never achieve a true lifestyle change that meets their expectations. Comprehensive lifestyle changes that promote sustainability include two major and closely linked components: (1) learning to consume only the number of calories from high-quality foods necessary to support basal body energy requirements, plus energy needs to maintain physical activity; and (2) selecting a reasonable physical activity plan that fits into the dieters' overall lifestyle patterns. Sustaining healthy body composition after significant weight loss seems to occur only in those individuals who can find a way to limit their intake of a healthy diet that they find acceptable in terms of taste, costs, and ease (food availability and ease and speed of preparation), plus an exercise regime that is acceptable in terms of access (e.g., equipment, facilities, and walking trails), appropriateness (e.g., age-related and intensity levels), and time (e.g., duration required and timing within a workday). Thus, losing weight and getting a healthy body composition comes down to energy balance, that is, consuming only enough energy to maintain a given body composition. Maintaining a healthy body composition requires that energy intake equals energy expenditure. Promoting health in the context of a healthy body composition can be best achieved by including a physical activity plan, and this usually leads to healthier muscle, improved insulin sensitivity, normalization of blood lipid profiles, and improved cardiovascular measures.

Determining the energy needs is the easiest part of a plan, whereas limiting caloric intake, increasing physical activity, and sustaining these components of a plan are more difficult. In terms of energy, 3500 cal is stored in 1 lb of fat. So, to lose or take off that pound of fat, the body needs to be forced to "burn" 3500 cal from adipose tissue. That pound of fat was formed over time and under conditions in which calorie intake exceeded caloric output. The energy in food eaten can be "cost accounted" roughly as follows: (1) energy required to digest and absorb food; (2) energy utilized to support basal functions such as pumping blood and breathing; (3) energy for body functions other than basal metabolism, such as walking and playing golf; and (4) nonutilized food calories, such as food components not fully digested or absorbed. Most generally healthy people have similar energy expenditure needs as described in points 1, 2, and 4 above, although there clearly are individual differences. The energy expenditure of a body at rest is termed the basal metabolic rate (BMR) and it can be estimated by the Harris–Benedict equations (Roza and Shizgal, 1984). Energy expenditures also differ by age, gender, and body size (height and weight). For example, per the Harris–Benedict equations, a man and a woman (each healthy and normal body composition) of age 66 years, weighing 160 lb, and 70 in. tall would have a BMR of 1528 and 1372 cal, respectively. However, if they were each age 18 years, their BMR would be 1854 and 1598 cal, respectively. No matter what a person's excess weight might be, there will always be the need for energy to meet the BMR. The energy needs of BMR could be met by diet or, in the case of overweight individuals, body energy stores such as fat. Because there are other nutrient needs besides energy (e.g., nitrogen, amino acids, essential fatty acids, vitamins, minerals, and trace elements), an overweight individual will always have needs for a healthy diet, even for the short term, so fasting is usually not a long-term option to burn all excess stored body fat. Thus, to burn excess body fat, daily energy expenditure needs to exceed the energy expenditure of BMR and this comes mainly from physical activity.

The public tends to overestimate the energy expenditure for various physical activities. It also finds it difficult to practically translate the often-recommended amount of physical activity (30 minutes of activity per day) into a daily regimen. Part of this is due to the difficulty in determining the energy expenditure amount, because the energy expended by an individual on 30 minutes of physical activity differs according to body weight, body composition, health, physical conditioning, the activity itself (e.g., walking, jogging, running, or swimming), and intensity of the activity (e.g., running at 6 mph vs. 4 mph). In addition, many overweight people are usually not accustomed to physical activity at the intensity level and duration needed to induce the body to burn body fat stores that lead to significant body fat reduction. This, when combined with the necessary changes in the diet composition and reductions in caloric intake, makes it difficult to lose body fat and more difficult to maintain a healthy body composition. The most common physical activity used in weight loss programs is walking. Walking 30 minutes at a rate of 4 mi/h for the 160-lb man or woman described above will burn approximately 180 cal. So, if an overweight individual were to maintain the same caloric intake (and same diet) and just increase his or her activity level to 30 minutes of walking at 4 mi/h (a total of 2 mi/day), the calculated fat loss would be 1 lb every 20 days. If he or she also reduced the caloric intake by 180 cal a day and has the same walk schedule, he or she theoretically would reduce the time to lose 1 lb of weight to about 10 days. Thus, the way to reduce body fat (adipose tissue) is to consume less calories than expended (energy deficit) and the rate of fat loss is directly related to the level of the energy deficit (i.e., caloric intake vs. caloric expenditure).

Complicit in any plan to burn body fat stores is that some portion of the total energy expenditure (TEE) must be met by fat stores. This requires a diet intake situation that (1) provides fewer calories than required to meet BMR + any physical activity expenditure; (2) reduces or prevents lipogenesis; and (3) promotes lipolysis. There are several ways and several commercially available diet systems in which this can be accomplished. One of the most widely used diet systems, the Atkins diet, claims to accomplish this by increasing protein (and consequently fat) and limiting carbohydrates.

Following a meal containing an abundance of simple carbohydrates, blood glucose levels rise and glucose gets converted into glycogen by the liver and stored there for future energy use. Maximal glycogen storage is between about 8 and 14 hours, depending on factors such as an individual's metabolism. As glucose enters cells, blood glucose concentrations would fall if not for the liver converting the glycogen back to glucose and releasing it back into the blood. This is a mechanism for maintaining glucose in the normal range between meals. Glucose is the body's energy source of choice. Use of glucose as the primary energy source seems to be prioritized by cell and organ type, with nerve cells having the highest priority. This means that if body glucose stores become scarce, the brain will be the last organ able to use glucose as its primary source. In this case, if another carbohydrate-containing meal is not consumed and blood glucose levels dropped to very low levels, lower priority organs (such as muscle) would need another source of energy and this source is fatty acids released from fat stores (lipolysis). Just like glycogen is the storage form for glucose, fat is the storage source for fatty acids and tissues can easily burn fatty acids when glucose is absent. As liver glycogen stores become minimal, amino acids are converted to glucose (gluconeogenesis). This can support nerve cell metabolism for a while, but eventually the brain will switch from burning pure glucose and start to utilize ketone bodies produced in the liver from fatty acids as an additional energy source. High ketone levels in the blood and tissues are known as ketosis.

Insulin has a major influence on carbohydrate, fat, and protein metabolism. Under conditions of adequate carbohydrate intake, insulin causes excess sugar not utilized as fuel to be stored as fat and prevents utilization of fat as an energy source. Thus, a high-carbohydrate diet. In theory, the low carbohydrate, high protein intake promoted in the Atkins diet forces the body to burn more fat. This would call for the body to switch from using pure carbohydrates for fuel to using more fat for fuel and the source of this fat is the adipose tissue. Thus, when insulin levels are normal, the body will begin to burn its own fat as fuel, thereby resulting in body weight loss. In addition to these potential metabolic effects, the high dietary fat content is thought to reduce appetite and thus total caloric intake. The combination of altered metabolism and reduced caloric intake leads to weight loss and better body composition.

Theoretically, environmental factors (such as diet composition, dietary intake, and physical activity) are the key factors in obesity treatment. However, genetics can also play a major role. For example, Bouchard et al. (1983) have reported that genetic characteristics can be responsible for substantial differences or heterogeneity in the response to training or physical activity and this may be an important obstacle to achieving a healthy body composition in some people. So, although research has shown that obesity can be reversed by the combination of consuming a healthy diet and reducing caloric intake to below calories expended, *maintaining a healthy body composition* with a caloric intake equal to energy expenditure often is as complicated as the factors leading to obesity in the first place.

Clinical and basic research studies have shown that adequate physical activity is essential to maintaining good physical health, and a sedentary lifestyle leads to several common chronic health problems. However, the mechanisms underlying the positive effects of physical activity or the negative effects of insufficient activity are not known. An ongoing NIH study, "Molecular Transducers of Physical Activity in Humans," aims to determine the molecular changes produced by "movement" (i.e., physical activity) and how these changes improve cellular and organ functions (Robinson et al., 2017). Current literature in the field of physical activity as it relates to body composition, health, and disease suggests there is a healthy "fitness range" for individuals based upon genetics and issues such as age, sex, body composition, diet, and total caloric intake. These factors are important in understanding human development, health status, and the effects of medications and toxicants.

Toxic Effects of Dieting

There are potential toxic effects associated with some diet plans. Dieting as prescribed by dieticians, nutritionists, or physicians is based on the use of a healthy diet that meets the daily nutrient needs of the body, but at a reduced caloric intake and with increased moderate physical activity. The overarching premise is to provide adequate nutrients for normal cellular function while reducing caloric intake to a level that forces the use of fat stores as an energy source to meet the energy expenditure above the caloric intake. If the diet does not include all the required nutrients (an imbalanced diet), metabolism will suffer and with time this can result in health problems. This is true in the case of deficiency of specific nutrients (deficiency disorders such as anemia or osteoporosis) or toxicity caused by excesses of a nutrient (such as thyroid impairment, vitamin deficiencies, and mental confusion). Some popular diet plans call for excess intake of a particular food and these can not only alter

metabolism but also interfere with medications. For example, the popular grapefruit diet can lead to inhibition of drug-metabolizing enzymes such as the cytochrome P450 CYP3As that metabolize 60% of all therapeutic medications. This may increase drug concentrations to toxic levels, increase drug–drug interactions, and result in potential health problems (Ameer and Weintraub, 1997; Kiani and Imam, 2007).

Another potential adverse effect of dieting is known as the yo-yo effect or weight cycling. This occurs with repeated dieting interspersed with periods of no dieting. This is caused when a dieter starts one diet and loses a significant amount of body weight and body fat, but cannot maintain the diet and stops to return to the prediet routine. The fat and body weight are regained, often to an even greater body fat content. In fact, the yo-yo experience may result in a condition in which the dieter is more efficient in gaining weight. This causes the dieter to select another diet plan, loss of weight, failure to maintain the diet, and return to overweight or obese conditions. There are often periods of depression and fatigue associated with this cycling behavior and the end results are extreme emotional and physical ramifications.

Drug Therapy for Weight Loss

In addition to the diet plans described earlier, many overweight individuals turn to drug therapy to help lose body weight. Appetite suppressants, for example, sympathomimetics such as diethylpropion, attempt to lessen the psychological motivation for food, usually by acting on central nervous system appetite control centers, such as those in the hypothalamus (Guaraldi et al., 2011). Although sympathomimetics can be used for long periods of time, their appetite-reducing effects tend to decrease after a few weeks in many people. Thus, appetite suppressants are often used in the early stages of a weight loss program. People are likely to lose weight while taking sympathomimetics, but the weight loss is generally temporary without modifications in diet composition, eating behavior, and physical activity. Short-term use is usually accompanied by minor side effects such as thirst, irritability, constipation, stomach pain, dizziness, dryness of mouth, heightened sense of well-being, headache, irritability, nausea, nervousness or restlessness, trembling or shaking, and trouble sleeping. However, long-term use of appetite suppressants often lead to more serious side effects: intracerebral hemorrhage, acute dystonia, myocardial injury, psychosis, cerebral arteritis, cardiac arrhythmias, heart valve damage, and even fatal pulmonary hypertension. These side effects have led to the withdrawal of several such products from the market. Drug interactions are also known to occur, especially with high blood pressure medicine, stimulants, MAO inhibitors (e.g., furazolidone, linezolid, phenelzine, selegiline, and tranylcypromine), any other weight loss medicine, and decongestants such as are commonly found in over-the-counter cough and cold medicines.

Surgical Interventions

Surgery is another avenue to improve body composition in obesity. One of the most radical surgical treatments is the class operations known as bariatric surgeries. The end goal is to limit food intake by reducing the capacity of the stomach, and also having patients feel satiated. Bariatric surgery is a term that includes several surgical procedures (such as sleeve gastrectomy, gastric plication, gastric banding, and gastric bypass surgery); all are aimed at helping obese patients lose weight. Weight loss is achieved by resecting and linking the small intestines to a small stomach pouch (gastric bypass surgery), removal of a portion of the stomach (sleeve gastrectomy), or reducing the size of the stomach with an implanted medical device (gastric banding) or sutures (gastric plication). These procedures are generally credited with significant long-term loss of body fat and body weight, which leads to improvement of secondary conditions caused by obesity, including type 2 diabetes, risk of cardiovascular disease, and the rate of mortality (Robinson, 2009). Very promising preliminary results concerning bariatric surgery bear watching in the future, suggesting the procedure may have effects in a wide range of areas, including changes in the gut microbiome and type 2 diabetes mellitus in nonobese patients (Nguyen and Varela, 2017).

These operations are most commonly used to treat morbid obesity and the comorbidities associated with it. Thus, bariatric surgery may be recommended for people with a BMI greater than 40. These procedures have also been used successfully with obese individuals with BMI 30 to 40, especially when there are serious coexisting medical conditions such as hypertension, impaired glucose tolerance, diabetes mellitus, hyperlipidemia, and obstructive sleep apnea. Most bariatric procedures result in relatively rapid weight loss, which is also associated with gallstones, and many surgeons prefer to remove the gallbladder at the time of bariatric surgery. Malabsorption and nutritional deficiency can occur because of reduced absorptive area. This can reduce calcium and vitamin absorption. Poor calcium absorption can occur because calcium transporters located in areas of the intestine (duodenum) may be removed with bariatric surgery. This can cause metabolic bone disease (osteopenia) and secondary hyperparathyroidism that increase in bone turnover. Combined, these conditions decrease bone mass and increase risk of fracture. Other deficiencies including iron and micronutrients such as vitamin B_{12}, fat-soluble vitamins, thiamine, and folate are common. Many patients will need to take a daily multivitamin pill for life to compensate for reduced absorption of essential nutrients. Because patients cannot eat a large quantity of food, physicians typically recommend a diet that is relatively high in protein and low in fats and alcohol.

Liposuction is another surgical procedure widely used to improve body composition by physically and surgically removing body fat. It is the most common "plastic surgical procedure" in the United States. However, there are significant risks with this procedure and these are mainly associated with (1) the aggressiveness with which the procedure is performed, especially the amount of tissue sucked from the body; (2) the venues in which the procedures are performed; and (3) the amount of anesthesia used to sedate patients during increasingly lengthy procedures. The mortality rate of the late 1990s was approximately 20 per 100,000 or 1 per 5000 cases (de Jong and Grazer, 2001).

ECONOMIC, SOCIOLOGICAL, AND LEGAL ASPECTS OF THE OBESITY EPIDEMIC

Health Insurance and Obesity

Obesity is not considered an illness for most insurance purposes. However, obesity can affect the cost of health insurance because as a group, obese people have a significantly greater risk of cardiovascular disease, hypertension, type 2 diabetes, and other health issues than lean people. Thus, insurance companies factor obesity into the cost of first-time insurance purchasers. Furthermore, people who are already insured may unknowingly not have sufficient insurance to cover health problems associated with obesity. Several health insurance companies use BMI as a measure of obesity and use BMI to compute disease risk and health insurance premiums. Obesity can result in high premiums and in the case of morbidly obese individuals, insurers may decline their application. Obesity is also

regarded by insurance companies as a substantial risk for both life and disability policies.

Mortality statistics for life insurance were the earliest indicator that the cost of obesity to the individual was decreased life span and increased illness, particularly diseases affecting the cardiovascular and musculoskeletal systems. The prevalence of coronary heart disease rises with increases in the BMI in both men and women. Hypertension and diabetes in the obese add further to the risks of vascular disease. Cigarette smoking also greatly augments the health risks of obesity in both sexes.

The risk of death and medical problems increase with the degree or severity of obesity (as indicated by fat mass or BMI). The type of obesity (as indicated by body shape or fat distribution) is another aspect of obesity considered by insurance companies. Abdominal obesity has been positively correlated with the risk of heart disease and stroke. Thus, some insurance companies set rates based in part on fat distribution. Disability insurance rates are also affected by body composition, as cardiovascular disease or musculoskeletal illness associated with obesity greatly increases the chances of disabilities. These factors are used in decision making as to whether to insure an individual and the cost of premiums. Clearly, costs increase proportionally with the degree of obesity.

Changing the Environment: Family and Community Approaches to Healthy Eating and Physical Activity

The development of obesity within an individual, a community, or a country is complex and the central cause(s) or reason(s) underlying the rapid rise in obesity in the United States and the world is not well understood. The end results in terms of body composition and secondary medical problems associated with obesity are better understood than the root causes of the obesity epidemic. One factor, however, that has received much attention relates to basic practices that were common in past decades when the general population was leaner but are lacking now when obesity is so prevalent.

Prior to 1970, the average BMI was 25.1 for men and 24.9 for women in the United States. Physical education (PE) classes were a regular feature of school curriculums; most meals were prepared at home using fresh produce, meats, and dairy products. School lunches were prepared at schools. It was common for children to walk or ride bicycles to school and to participate in games requiring physical activity during school recess and after school and on weekends. There were no computer games and fast food restaurants did not supply such a large proportion of the daily total calories. In the 1990s and 2000s, there are few schools with PE classes and fewer meals cooked from fresh components consumed at home and the lifestyle of today's children and adults is far more sedentary than that prior to 1970. Therefore, by 2002, average BMI for U.S. men had risen to 27.8 and average BMI for U.S. women to 28.1.

One approach to fighting the obesity issue is to bring back many of those practices used in the past. There are initiatives to establish community gardens, build community walking and riding trails, and teach people cooking and shopping skills that lead to healthier meal preparation. School systems are starting to return to PE classes on a regular basis and remove high-density foods and drinks from vending machines. Although these efforts have been increasing in effort throughout the United States, it is still too early to confirm their effectiveness in reversing the obesity trend.

Food security and access to quality foods are an important issue throughout the United States, regardless of whether in rural America or in the inner city. In many areas, there is ready access only to convenience food stores where there is a lack of fresh vegetable and meats and the prices are higher than in a conventional supermarket. The type of food available is poor quality and energy dense, which leads to obesity. These areas have been termed "food deserts." Providing access to higher-quality fresh food to people living in these areas will improve nutrition, and when combined with education on how to select and prepare the food and linked to some form of physical activity it is hoped that body composition and health will improve in these areas.

Food Labels

The Food and Drug Administration (FDA) is responsible for assuring that foods sold in the United States are properly labeled (see Chap. 27, "Food Toxicology: Fundamental and Regulatory Aspects"). This applies to foods produced domestically and foods from foreign countries. The Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Fair Packaging and Labeling Act are the federal laws governing food products under the FDA's jurisdiction. The Nutrition Labeling and Education Act (NLEA), which amended the FD&C Act, requires most foods to bear nutrition labeling. The FDA requires food labels that bear nutrient content claims and certain health messages to comply with specific requirements.

Food labeling is required for most prepared foods, such as breads, cereals, canned and frozen foods, snacks, desserts, and drinks. Nutrition labeling for raw produce (fruits and vegetables) and fish is voluntary. Dietary supplements are a special category of products under the general umbrella of foods, but which has separate labeling requirements. "Functional foods" or "nutraceuticals" are regulated by the FDA under the authority of the FD&C Act, but they are not specifically defined by law. Evidence-based updates of labeling requirements occur when "new data on the associations between nutrients and chronic diseases, health-related conditions, physiological endpoints, and/or maintaining a healthy dietary pattern reflects current public health conditions in the United States." Revisions in the format and appearance of the Nutrition Facts label was last performed in 2016 and full industry-wide compliance with the new regulations must occur by July 2019.

Food labels can be an important factor to help consumers in their food choices that can help prevent obesity and other diseases. Federal law requires that a minimal amount of information be listed on food packaging, including ingredients and nutrition data. The FDA monitors labeling language for such issues as fat, protein, carbohydrate, and other nutrient contents and the purported ability of a particular food to prevent medical problems. Suspicious or doubtful claims or misleading statements about nutrition and health benefits on food packages can be challenged by the FDA and companies that receive warning letters have 15 days to inform the agency of corrective action. Although there has been a formal process by which a health claim can be made, foods generally are not permitted to make disease-fighting claim. The types of claims often challenged by the FDA include warding off maladies such as arthritis, cancer, and heart disease.

The Patient Protection and Affordable Care Act of 2010 establishes requirements for nutrition labeling of standard menu items for chain restaurants, similar retail food establishments, and chain vending machine operators. The most important information that must be provided for standard menu items is the number of calories in each standard menu item and a statement on the menu that puts the calorie information in the context of a recommended total daily caloric intake. In addition to restaurant menus, food sold from vending machines operated by persons who own or operate 20 or more vending machines must disclose the number of calories in a clear and conspicuous manner.

Governmental and Corporate Issues

There has been increasing pressures from local, state, and federal governments to regulate various aspects of food as a means of promoting health and reducing obesity and the secondary consequences of obesity. Food labeling is just one example of government intervention, whereby food processors and restaurants must provide a measure of nutrient and/or caloric content. New York City was the first to pass a law requiring calorie counts to be posted next to prices on menus in some restaurants so consumers would also know the "caloric cost" of each food selection, with the hope that they will make healthy food choices. California became the first state to prohibit restaurants from using "artery-clogging trans fats" in preparing their food. Airlines and the FAA are dealing with complaints from passengers being "infringed on" by overlapping obese passengers and obese passengers complaining about abuse taken from leaner passengers. In an effort to reduce insurance costs and improve work performance, there is also a trend for the business community to set standards associated for overweight and obese workers in a manner similar to that employed for smokers. Civil rights advocates and others are concerned that there is a growing impetus for government intervention and one wonders if the time will come when "big brother" mandates what and how much one can eat.

REFERENCES

Abdelmegeed MA, Choi Y, Godlewski G, et al. Cytochrome P450-2E1 promotes fast food-mediated hepatic fibrosis. *Sci Rep*. 2017;7:39764.

Ameer B, Weintraub RA. Drug interactions with grapefruit juice. *Clin Pharmacokinet*. 1997;33:103–121.

Aoki R, Kamikado K, Suda W, et al. A proliferative probiotic *Bifidobacterium* strain in the gut ameliorates progression of metabolic disorders via microbiota modulation and acetate elevation. *Sci Rep*. 2017;7:43522.

Bakker-Arkema RG, Davidson MH, Goldstein RJ, et al. Efficacy and safety of a new HMG-CoA reductase inhibitor atorvastatin, in patients with hypertriglyeridemia. *JAMA*. 1996;275:128–133.

Barker DJ, Winter PD, Osmond C, et al. Weight in infancy and death from ischemic heart disease. *Lancet*. 1989;2:577–580.

Basaranoglu M, Basaranoglu G, Senturk H. From fatty liver to fibrosis: a tale of "second hit." *World J Gastroenterol*. 2013;19:1158–1165.

Baumgardner JN, Shankar K, Hennings L, et al. A new rat model for nonalcoholic steatohepatitis utilizing overfeeding of diets high in polyunsaturated fat by total enteral nutrition. *Am J Physiol*. 2008a;294:G27–G38.

Baumgardner JN, Shankar K, Hennings L, et al. *N*-Acetylcysteine attenuates progression of liver pathology in a rat model of non-alcoholic steatohepatitis. *J Nutr*. 2008b;138:1872–1879.

Bouchard C. Human adaptability may have a genetic basis. In: Landry F, ed. *Health Risk Estimation, Risk Reduction and Health Promotion. Proceedings of the 18th Annual Meeting of the Society of Prospective Medicine*. Ottawa: Canadian Public Health Association; 1983:463–476.

Boyle JG, Salt IP, McKay GA. Metformin action on AMP-activated protein kinase: a translational research approach to understanding a potential new therapeutic target. *Diabet Med*. 2010;27:1097–1106.

Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology*. 2004;40:1387–1395.

Burt Solorzano CM, McCartney CR. Obesity and the pubertal transition in girls and boys. *Reproduction*. 2010;140:399–410.

Cannito S, Morello E, Bocca C, et al. Microvesicles released from fat-laden cells promote activation of hepatocellular NLRP3 inflammasome: a pro-inflammatory link between lipotoxicity and non-alcoholic steatohepatitis. *PLoS One*. 2017;10:1371.

Caviglia JM, Gayet C, Ota T, et al. Different fatty acids inhibit apolipoprotein B100 secretion by different pathways: unique roles for endoplasmic reticulum stress, ceramide and autophagy. *J Lipid Res*. 2011;52:1636–1651.

Cho N, Momose Y. Peroxisome proliferator-activated receptor gamma agonists as insulin sensitizers: from the discovery to recent progress. *Curr Top Med Chem*. 2008;8:1483–1507.

Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science*. 2011;332:1519–1523.

Colman RJ, Anderson RM, Johnson SC, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science*. 2009;325(5937):201–204.

Cook S, Weitzman M, Auinger P, et al. Prevalence of a metabolic syndrome phenotype in adolescents: findings from the third National Health and Nutrition Examination Survey 1999–2002. *J Pediatr*. 2008;152:160–164.

Cornier MA, Dabelea D, Hernandez TL, et al. The metabolic syndrome. *Endocr Rev*. 2008;29:777–822.

de Jong RH, Grazer FM. Perioperative management of cosmetic liposuction. *Plast Reconstructr Surg*. 2001;107:1039–1044.

Deshmukh M, Lee HW, McFarlane SI, et al. Antihypertensive medications and their effects on lipid metabolism. *Curr Diab Rep*. 2008;8:214–220.

Divoux A, Clement K. Architecture and the extracellular matrix: the still unappreciated components of adipose tissue. *Obes Rev*. 2011;12:e494–e503.

Dooley S, Weng H, Mertens PR. Hypotheses on the role of TGF beta in the onset and progression of hepatocellular carcinoma. *Dig Dis*. 2009;27:93–101.

Dulloo AG, Jacquet J, Solinas G, et al. Body composition phenotypes in pathways to obesity and the metabolic syndrome. *Int J Obes*. 2010;34:S4–S17.

Fischer CD, Lickteig AJ, Augustine LM, et al. Experimental non-alcoholic fatty liver disease results in decreased hepatic uptake transporter expression and function in rats. *Eur J Pharmacol*. 2009;613:119–127.

Franck N, Gummesson A, Jernas M, et al. Identification of adipocyte genes regulated by caloric intake. *J Clin Endocrinol Metab*. 2011;96:E413–E418.

Friedman JM, Leibel RL. Tackling a weighty problem. *Cell*. 1992;69:217–220.

Gerstein HC, Yusuf S, Bosch J, et al. Effect of rosiglitazone on the frequency of diabetes in patients with impaired glucose tolerance or impaired fasting glucose: a randomized controlled trial. *Lancet*. 2006;368:1096–1105.

Giordano A, Murano I, Mondini E, et al. Obese adipocytes show ultrastructural features of stressed cells and die of pyroptosis. *J Lipid Res*. 2013;54:2423–2436.

Gropper SS, Smith JL, Groff JL. Body composition and energy expenditure. In: Gropper SS, Smith JL, Groff JL, eds. *Advanced Nutrition and Human Metabolism*. Belmont, CA: Thomson Wadsworth; 2005:519–550.

Guaraldi F, Pagotto U, Pasquali R. Predictors of weight loss and maintenance in patients treated with antiobesity drugs. *Diabetes Metab Syndr Obes*. 2011;4:229–243.

Gustafson B. Adipose tissue, inflammation and atherosclerosis. *J Atheroscler Thromb*. 2010;17:332–341.

Halberg N, Khan T, Trujillo ME, et al. Hypoxia-inducible factor 1 alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol*. 2009;29:4467–4483.

Hanley MJ, Abernethy DR, Greenblatt DJ. Effect of obesity on the pharmacokinetics of drugs in humans. *Clin Pharmacokinet*. 2010;49:71–87.

Hebbard L, George J. Animal models of nonalcoholic fatty liver disease. *Nat Rev Gastroenterol Hepatol*. 2011;8:35–44.

Khan S, Wang CH. ER stress in adipocytes and insulin resistance: mechanisms and significance. *Mol Med Rep*. 2014;10:2234–2240.

Keesey RE, Hirvonen MD. Body weight set-points: determination and adjustment. *J Nutr*. 1997;127:1875S–1883S.

Kiani J, Imam SZ. Medicinal importance of grapefruit juice and its interaction with various drugs. *Nutr J*. 2007;6:33.

Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med*. 2002;346:393–403.

Kreitschmann-Andermahr I, Suarez P, Jennings R, et al. GH/IGF-1 regulation in obesity—mechanisms and practical consequences in children and adults. *Horm Res Pediatr*. 2010;73:153–160.

Kulie T, Slattengren A, Redmer J, et al. Obesity and women's health. An evidence-based review. *J Am Board Fam Med*. 2011;24:75–85.

Lara-Castro C, Garvey T. Intracellular lipid accumulation in liver and muscle and the insulin resistance syndrome. *Endocrinol Metab Clin North Am*. 2008;37:841–856.

Lara-Castro C, Newcomer BR, Rowell J, et al. Effects of short term very low-calorie diet on intramyocellular lipid and insulin sensitivity in nondiabetic and diabetic subjects. *Metabolism*. 2008;57:1–8.

Lecka-Czernik B. Bone loss in diabetes: use of antidiabetic thiazolidinediones and secondary osteoporosis. *Curr Osteoporosis Res*. 2010;8:178–184.

Lenard NR, Berthoud HR. Central and peripheral regulation of food intake and physical activity: pathways and genes. *Obesity*. 2008;16:S11–S22.

Liang S, Kisseleva T, Brenner DA. The role of NADPH oxidases in liver fibrosis and the activation of myofibroblasts. *Front. Physiol*. 2016;7:17.

Lickteig AJ, Fischer CD, Augustine LM, et al. Efflux transporter expression and acetaminophen metabolite excretion are altered in rodent models of non-alcoholic fatty liver disease. *Drug Metab Dispos*. 2007;25:1970–1978.

Loomba R, Sirlin CB, Schwimmer JB, et al. Advances in pediatric nonalcoholic fatty liver disease. *Hepatology*. 2009;50:1282–1293.

Loria P, Carulli L, Bertolotti M, Lonardo A. Endocrine and liver interaction: the role of endocrine pathways in NASH. *Nat Rev Gastroenterol Hepatol*. 2009;6:236–247.

Ludwig J, Viggiano TR, McGill DB, et al. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc*. 1980;55:434–438.

Marecki JC, Ronis MJJ, Badger TM. Ectopic fat disposition and insulin resistance develop after overfeeding of a high fat diet to prepubertal rats despite increased adiponectin signaling, *J Nutr Biochem*. 2011;22:142–152.

Matsuzaki K. Modulation of TGF beta signaling during progression of chronic liver diseases. *Front Biosci*. 2009;14:2923–2934.

McFarlane SI, Muniyappa R, Francisco R, et al. Clinical review 145: pleiotropic effects of statins: lipid reduction and beyond. *J Clin Endocrinol Metab*. 2002;87:1451–1458.

McMillen IC, Robenson JS. Developmental origins of the metabolic syndrome: prediction, plasticity and programming. *Physiol Rev*. 2005;85:571–633.

Mercer KE, Pulliam CF, Pedersen KB, et al. Soy protein isolate inhibits high fat-induced hepatic tumor promotion in DEN-treated mice. *Exp Biol Med*. 2017; 242:635-644.

Merrell M, Cherrington NJ. Drug metabolism alterations in nonalcoholic steatohepatitis. *Drug Metab Rev*. 2011;43:317–334.

Mifflin MD, St Jeor ST, Hill LA, et al. A new predictive equation for resting energy expenditure in healthy individuals. *Am J Clin Nutr*. 1990;51:241-247.

Neel JV. Diabetes mellitus a "thirfty" genotype rendered detrimental by "progress"? *Am J Hum Genet*. 1962;14:352–362.

Nejak-Gowen KN, Monga SP. Beta catenin signaling, liver regeneration and hepatocellular cancer: sorting the good from the bad. *Semin Cancer Biol*. 2011;21:44–58.

Nguyen NT, Varela JE. Bariatric surgery for obesity and metabolic disorders: state of the art. *Nat Rev Gastroenterol Hepatol*. 2017;14:160–169.

Nickerson JG, Momken I, Benton CR, et al. Protein-mediated fatty acid uptake: regulation by contraction, AMP-activated protein kinase and endocrine signals. *Appl Physiol Nutr Metab*. 2007;32:865–873.

Nord RH, Rayne RK. Dual-energy X-ray absorptiometry vs underwater weighing comparison of strengths and weaknesses. *Asia Pac J Clin Nutr*. 1995;4:173–175.

Olson RE. Discovery of the lipoproteins, their role in fat transport and their significance as risk factors. *J Nutr*. 1998;128:439S–443S.

O'Rahilly S, Farooqi IS. Human obesity: a heritable neurobehavioral disorder that is highly sensitive to environmental conditions. *Diabetes*. 2008;57:2905–2910.

Palee S, Chattipakorn S, Phromminitikul A, et al. PPAR gamma activator, rosiglitazone: is it beneficial or harmful to the cardiovascular system? *World J Cardiol*. 2011;26:144–152.

Pan DA, Lilloja S, Kriketos AD, et al. Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes*. 1997;46:983–988.

Pan M, Cederbaum AI, Zhang YL, et al. Lipid peroxidation and oxidant stress regulate hepatic apolipoprotein B degradation and VLDL production. *J Clin Invest*. 2004;113:1277–1287.

Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest*. 2008;118:829–838.

Ratner R, Goldberg R, Haffner S, et al. Impact of intensive lifestyle and metformin therapy on cardiovascular disease risk factors in the diabetes prevention program. *Diabetes Care*. 2005;26:977–980.

Reaven GM. Banting lecture 1988, role of insulin resistance in human disease. *Diabetes*. 1988;37:1595–1607.

Roberts DL, Dive C, Renehan AG. Biological mechanisms linking obesity and cancer risk: new perspectives. *Annu Rev Med*. 2010;61:301–316.

Robinson MK. Editorial, surgical treatment of obesity—weighing the facts. *N Engl J Med*. 2009;361:520.

Robinson MM, Dasari S, Konopka AR, et al. Enhanced protein translation underlies improved metabolic and physical adaptations to different exercise training modes in young and old humans. *Cell Metab*. 2017: 25:581–592.

Ronis MJJ, Mercer KE, Engi B, et al. Global deletion of glutathione-*S*-transferase A4 exacerbates developmental nonalcoholic steatohepatitis. *Am J Pathol*. 2017;187:418–430.

Roza AM, Shizgal HM. The Harris–Benedict equation reevaluated: resting energy requirements and body cell mass. *Am J Clin Nutr*. 1984;40: 168–182.

Ruderman NB, Myers MG, Chipkin SR, Tornheim K. Hormone–fuel interrelationships: fed state, starvation and diabetes mellitus. In: Kahn CR, Weir GC, King GL, Jacobson AM, Moses AC, Smith RJ, eds. *Joslin's Diabetes Mellitus*. Boston, MA: Lippincott Williams & Wilkins; 2005: 127–144.

Sanyal AJ, Chalasani N, Kowdley KV, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med*. 2010;362:1675–1685.

Shankar K, Harrell A, Kang P, et al. Carbohydrate-responsive gene expression in the adipose tissue of rats. *Endocrinology*. 2010;151:153–164.

Shankar K, Harrell A, Liu X, et al. Maternal obesity at conception programs obesity in the offspring. *Am J Physiol Regul Integr Comp Physiol*. 2008;294:R528–R538.

Shen W, Gaskins HR, McKintosh MK. Influence of dietary fat on intestinal microbes, inflammation, barrier function and metabolic outcomes. *J Nutr Biochem*. 2014;25:270–280.

Shulman GI, Barrett EJ, Sherwin RS. Integrated fuel metabolism. In: Porte D, Sherwin RS, Baron A, Ellenberg M, Rifkin H, eds. *Ellenberg and Rifkin's Diabetes Mellitus*. New York: McGraw-Hill; 2003:1–14.

Smith DL, Nagy T, Allison DB. Calorie restriction: what recent results suggest for the future of aging research. *Eur J Clin Invest*. 2010;40:440–450.

Speakman JR. Thrifty genes for obesity, an attractive, but flawed idea and an alternative perspective: the "drifty gene" hypothesis. *Int J Obes*. 2008; 32:1611–1617.

Speliotes E, Yerges-Armstrong LM, Wu J, et al. Genome-wide association analysis identified variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. *PLoS Genetics*. 2011;7:e1001324.

Stipanuk M. Nutrients: history and definitions. In: Stipanuk M, ed. *Biochemical, Physiological, Molecular Aspects of Human Nutrition*. St. Louis, MO: Saunders Elsevier; 2006:3–12.

Sutti S, Bruzzi S, Albano E. The role of immune mechanisms in alcoholic and nonalcoholic steatohepatitis: a 2015 update. *Expert Rev Gastroenterol Hepatol*. 2016;10:243–253.

Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology*. 2010;52:1836–1846.

Tontisirin K, Maclean WC, Warwick P. *Food Energy: Methods of Analysis and Conversion Factors.* Report of a technical workshop. Vol 77 of FAO Food and Nutrition Paper, Issue 77 of Food Energy, Rome, December 3–6, 2002; 2003. Rome: FAO.

Virtue S, Vidal-Puig A. Adipose tissue expandability, lipotoxicity and the metabolic syndrome—an allostatic perspective. *Biochem Biophys Acta*. 2010;1801:338–349.

Wan X, Xu C, Yu C, Li Y. Role of NLRP3 inflammasome in the progression of NAFLD to NASH. *Can J Gastroenterrol Hepatol*. 2016;2016:6489012.

Ye J, Keller JN. Regulation of energy metabolism by inflammation: a feedback response in obesity and calorie restriction. *Aging*. 2010;2:361–368.

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chapter 29

Nanoparticle Toxicology

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Introduction

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  - *Surface Charge, Steric Interactions, and Agglomeration*
  - *Surface Area and Reactivity*
  - *Surface Chemistry, Surface Morphology, and Surface Impurities*
  - *Unique Properties*
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- Mechanisms of Toxicity

References

INTRODUCTION

Since the classic talk by Richard Feynman (1960) entitled "There Is Plenty of Room at the Bottom," nanotechnology has grown to a multibillion dollar industry worldwide, with 1300 nanotechnology-enabled products in commercial use by 2010 (Woodrow Wilson Center, 2012). The potential of adverse effects from exposure to "nanophase materials" was already pointed out earlier (Oberdörster and Ferin, 1992; Oberdörster et al., 1992), and concerns about human and environmental health and safety of engineered nanomaterials (ENMs) were initially raised in 2003 (Colvin, 2003). Since then, toxicity of high volume, commercial nanomaterials including nanosilver, fullerenes, quantum dots, carbon nanotubes (CNTs), graphene-based materials, and metal oxide nanoparticles (NPs) has been summarized in several reviews (Donaldson et al., 2004; Borm et al., 2006; Nel et al., 2006; Boczkowski and Hoet, 2009; Krug and Wick, 2011; Kunzmann et al., 2011; Sanchez et al., 2012). New ENMs and composites are continually emerging with potential for significant commercial applications in energy generation, environmental sensing and remediation, aerospace and defense, and medical diagnosis and therapy. Examples of nanoscale materials of different shapes and sizes are depicted in Fig. 29-1. Investigations of the magnitude of release of manufactured nanomaterials and their subsequent fate, transport, transformation, and potential for human and environmental exposure and toxicity (Fig. 29-2) have been areas of active research (Mueller and Nowack, 2008; Krug, 2014).

The United States National Nanotechnology Initiative (NNI, http://www.nano.gov/) defines nanotechnology as the understanding and control of matter at the nanoscale at dimensions between approximately 1 and 100 nm, where unique phenomena enable novel applications. Roco (2005) defined the sizes as ranging from the intermediate length scale between a single atom or molecule and

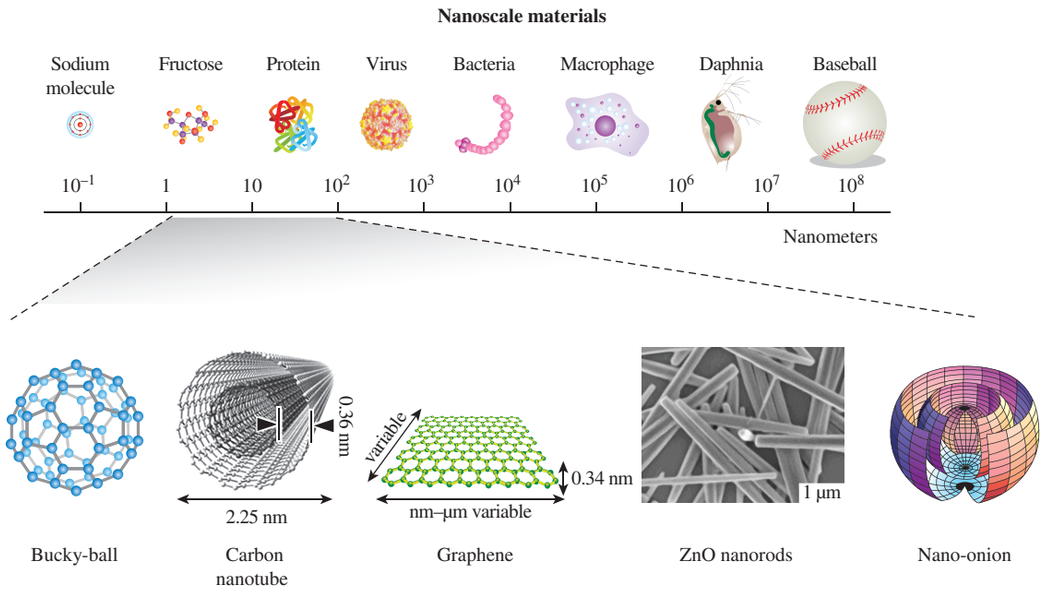


Figure 29-1. *Length scales for natural and synthetic structures (above) and some examples of engineered nanomaterials of varying size and shape (below).*

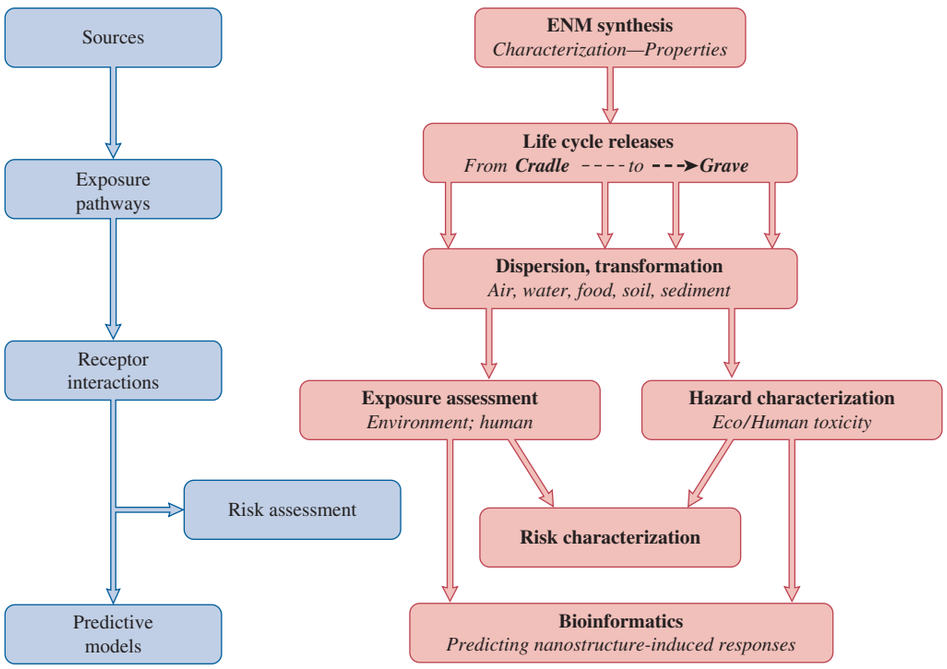


Figure 29-2. *Research phases for assessing human and environmental safety of engineered nanomaterials.*

about 100 molecular diameters or about 100 nm, and emphasizes the ability to measure and transform at this nanoscale and to exploit the properties and functions specific for the nanoscale.

Although the NNI defines nanotechnology as a facilitator of unique properties for novel applications, it is noted that nanomaterials are frequently defined by dimensions alone. For instance, the International Organization for Standardization (ISO) Technical Committee on Nanomaterials (TC 229) defines a nanomaterial as a material with any external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale (ISO/TC, 2015). ISO further describes subsets of nanomaterials and properties resulting from nanomaterials that are nanoenabled and nanoenhanced. These terms emphasize that materials at the nanoscale do not always have dramatically different properties than larger sized materials and that distinctions need to be made between augmented and unique properties. Many material properties may simply be enhanced due to nanoscale size or surface area, whereas a more limited set of properties are uniquely enabled at the nanoscale, for instance, because of quantum confinement effects or the onset of atypical catalytic activity.

Table 29-1

Ultrafine/Nanoparticles (<100 nm): Natural and Anthropogenic Sources

	ANTHROPOGENIC	
NATURAL	*UNINTENTIONAL*	*INTENTIONAL*
Gas-to-particle conversions Forest fires Volcanoes (*hot lava)* Viruses Biogenic magnetite: *magnetotactic bacteria; protoctists, mollusks, arthropods, fish, birds, human brain, meteorite* Ferritin (12.5 nm) Microparticles *(<100 nm) (activated cells)*	Internal combustion engines Power plants Incinerators Airplane jets Metal fumes *(smelting, welding, etc.)* Polymer fumes Other fumes Heated surfaces Frying, broiling, grilling Electric motors	Engineered nanoparticles: *(controlled size and shape, designed for functionality) metals, semiconductors, metal oxides carbon, polymers needles, -tubes, -shells, -rings, -platelets; untreated, coated* *(Nanotechnology applied to many products: cosmetics, medical, fabrics, electronics, optics, displays, etc.)*

SOURCE: From Oberdörster et al. (2007a, 2007b).

Nevertheless, the continuing introduction of nanoscale materials into consumer products, such as TiO_2 in sunscreen creams, antibacterial Ag in textiles, quantum dots in televisions, multiwalled carbon nanotubes (MWCNTs) in sports equipment together with increasing numbers of publications reporting toxic responses mostly observed in in vitro studies, has led to increasing concerns and more public awareness about potential adverse health effects, fostered particularly also by alarmist headlines in the popular press and online media. Unrealistic high doses as well as questionable study designs are most often to blame. Thus, questions should be raised regarding actual versus perceived risk of nanotechnology applications, what is hype, what is reality? To provide answers to these questions is one of the goals of *the subdiscipline of nanotoxicology*.

Nanotoxicology can be defined as the science of studying adverse effects of nanomaterials on living organisms and the environment. As illustrated in Fig. 29-2, there are multiple occasions for exposures to nanomaterials of humans and the environment and there is a serious need for investigation of the potential to cause adverse effects. The need of more toxicological research with the goal of characterizing risks associated with nanotechnology becomes even more urgent when considering the many beneficial and promising applications of nanotechnology for the betterment of human life, also for prophylactic, therapeutic, and diagnostic medical applications. The applications of nanotechnology for human health defines the new field of *nanomedicine*, and the development of new nanoenabled medical technologies must be accompanied by nanotoxicity studies to characterize undesirable effects and ensure the safety of new therapies and medical devices.

The field of nanotoxicology evolved from research on ambient airborne ultrafine particles (UFPs), particles up to about 100 nm in any of three dimensions from diverse anthropogenic and natural emission sources, including tailpipe emissions, power plants, volcanic eruptions, forest fires, and others, as summarized in Table 29-1, which gives examples of three classes: natural NPs, unintentional anthropogenic NPs (or "incidental" NPs), and intentional or engineered anthropogenic NPs. The same toxicological concerns and problems need to be addressed for UFPs and ENMs; they require similar research strategies with respect to study design and characterizing effects and underlying mechanisms at the primary portal of entry and in remote secondary organs. However, not only well-established respiratory tract and air pollution toxicology (see Chaps. 15 and 31) form the basis for nanotoxicology, but findings and knowledge accumulated in other traditional fields of research provide useful complementary information and foundations for nanotoxicology: for example, virology (nanosized structures–cell interactions), metal and polymer-fume toxicology (small particle size and systemic effects), silica toxicology (particle surface reactivity), fiber toxicology (particle shape and biopersistence), radionuclide toxicology (chemistry and biokinetics), and lung deposition studies/modeling (respiratory tract dosimetry) (Oberdörster et al., 2007a, 2007b). Input from these research areas can help in the design of studies and interpretation of results of nanotoxicological research. For example, a major challenge is to correlate the distinct physicochemical characteristics of engineered nanostructures with their toxicological behavior, as will be discussed later.

Of utmost importance for nanotoxicology, as for toxicology in general, are dose-related issues. Administered doses are mostly extraordinarily high when compared to doses achieved in vivo under realistic exposure scenarios. Slikker et al. (2004) stressed that the dose-dependent mechanisms are due to saturation processes underlying toxic responses between typical high acute experimental doses and lower human doses from realistic exposures (Fig. 29-3). Although high doses are justifiable for hazard characterization, ignoring real-world exposure situations may lead to erroneous conclusions about associated risks and causative mechanisms. Thus, consideration of the impact of dose, dose rate (influenced by dosing method in vitro and in vivo), and dosimetry should always be given highest priority when assessing nanomaterial toxicity. Dosimetry involves quantifying or calculating the amount of nanomaterials per body, per organ, per cell (microdosimetry), tissue or airway generation (inhalation route of exposure). Dosimetry should not be confused with "dosemetric," which is defining a dose in terms of an inherent property (physical [number, mass, surface area of ENMs], chemical, reactivity) of nanostructures. Neither should dosimetry or dosemetric be mixed up with "exposure." These issues will be discussed in more detail later.

The goals of nanotoxicology are to identify and characterize a hazard of ENMs for purposes of risk assessment for humans and the environment. This requires a highly multidisciplinary team approach, covering expertise in toxicology, biology, chemistry, physics, material science, geology, exposure assessment, physiological-based pharmacokinetic (PBPK), and fate and transport modeling. Clinical

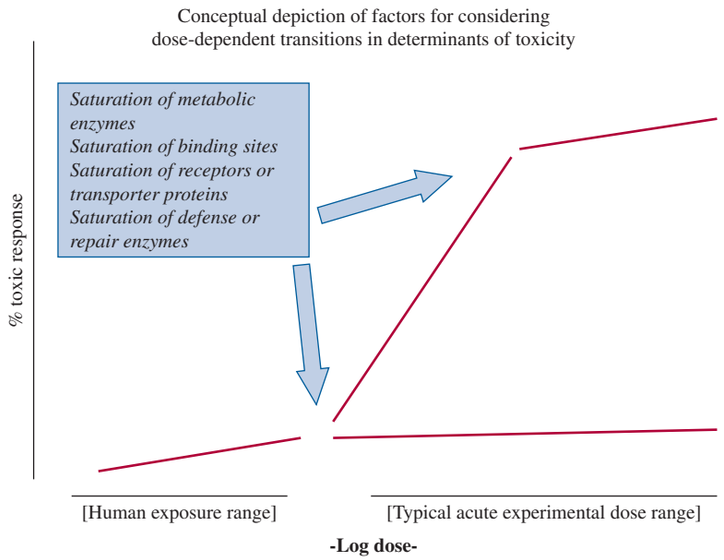


Figure 29-3. *Conceptual depiction of factors for considering dose-dependent transitions in determinants of toxicity.* (From Slikker et al. [2004].)

expertise is also necessary to develop testing strategies, establish toxicity ranking, determine "safe" exposure levels, and derive preventive exposure guidelines. As depicted in Fig. 29-2, exposures throughout the life cycle of ENMs, from their source to their disposal, must be considered. Dispersion state is an important modifier of ENM–receptor interactions, as are physical and chemical transformation of the materials that occur in air, water, food, and soil prior to exposure. Identifying underlying mechanisms of such interactions will aid in assessing risk and establishing preventive measures. Developing predictive models is a distant goal for both human and eco-nanotoxicology.

REGULATORY OVERSIGHT

The uncertainty associated with the potential risks of nanotechnology led the Organization for Economic Co-Operation and Development (OECD) Chemical Committee to establish the Working Party of Manufactured Nanomaterials (WPMN) in 2006. The WPMN remains the leading international forum on regulatory policy and oversight for nanomaterials and addresses issues related to the risk assessment and risk management of nanomaterials regarding human and environmental health and safety. The WPMN comprises international governmental policymakers and subject matter experts including observers from environmental NGOs, unions, UN groups, the International Organization Standardization (ISO), and industry.

In September of 2013, the OECD Council on the Safety Testing and Assessment of Manufactured Nanomaterials recommended that members manage the risks of manufactured nanomaterials, applying the existing international and national chemical regulatory frameworks or other management systems adapted to consider the specific properties of manufactured nanomaterials. It also recommended that in the testing of manufactured nanomaterials, apply the OECD Test Guidelines, adapted as appropriate and in regards to the Mutual Acceptance of Data (MAD) principle in the assessment of chemicals (OECD, 2013).

Member countries have moved forward with the Council Recommendation by integrating nanomaterials into their chemicals regulatory programs. Several jurisdictions have also implemented information collections rules or registries for nanomaterials to acquire information on their prevalence and uses in commerce. However, the application and definition varies largely.

The European Union was the first region to provide a regulatory definition of a nanomaterial in legal text. The European Commission's (EC) Recommended Definition of a Nanomaterial defined nanomaterial as "a natural incidental or manufactured material containing particles, in an unbound state or as an aggregate or an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm" (European Commission, 2011a, 2011b). Because some traditional nanomaterials are less than 1 nm, it was clarified that fullerenes (a form of carbon containing a large spheroidal molecular consisting of a hollow cage of atoms); graphene flakes (free-standing flake consisting of small number of well-defined, stacked graphene layers of extended lateral dimension); and single-walled carbon nanotubes (SWCNTs—having a diameter close to 1 nm with a tube length many times longer) with minimum dimensions below 1 nm are considered by derogation. This definition faced technical hurdles from the start as robust methods for determining number percent distributions of nanomaterials derived from bulk powders and suspensions were not in place (Linsinger et al., 2012; Brown et al., 2013; Amenta et al., 2014; Rauscher et al., 2014, 2015). The number percent metric was also criticized since at 50% by number, the larger sized populations of particles would contribute the most surface area, and potentially the dominant contribution to effects. Fig. 29-4 provides an illustration of the difference between a number percent and a mass or volume percent metric and demonstrates the surface area discrepancy. A revision to this definition is anticipated in late 2018 or in 2019 with accompanying guidance documentation.

Despite uncertain metrology, soon after the release of the EC recommended definition, several countries within Europe began national inventories and reporting rules borrowing aspects of the EC recommended definition of a nanomaterial. This was implemented to avoid capturing specific classes of materials based on applications, potential for release, or incidental production. France,

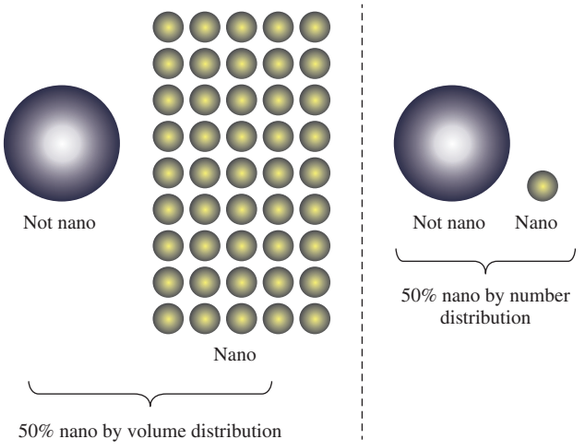


Figure 29-4. *Schematic illustration of the differences between volume and number distribution.* (Reproduced from Brown et al. [2013].)

Belgium, Denmark, and Norway require the reporting of nanomaterials or articles containing nanomaterials. The EC developed a nano-observatory in 2017, for public outreach and information sharing on nanomaterials in commerce. Outside of Europe, both Canada and the United States have also moved forward with reporting rules; however, unlike Europe they did not choose to move forward with a regulatory definition. Other regions of the world are following suit including Taiwan and South Korea.

The regulatory actions that have taken place to date are largely monitoring activities, in part due to marked uncertainty in the sciences as well as the uses, quantities, and diversity of nanomaterials in commerce. After over a decade of research, the vast body of the scientific literature remains questionable. Publishing bias and poor quality research are significant concerns further complicated by increasing information accessibility to the public. Claims of potential adverse effects continue to influence public perception despite related concerns of research quality and human relevance. A large fraction of nanotoxicology research fails to meet basic levels of scientific rigor and reporting (Krug, 2014). The preferential use of non-guideline methods and the reluctance of scientific journals to accept "no effects data" remain concerns hindering a balanced outlook (Warheit and Donner, 2015).

Early outputs from national commercial nanomaterial monitoring efforts indicate that the largest application of nanosized materials are for coatings and inks that have safely been used in commerce for decades. The identification of these applications largely results from the scope of the definitions applied in those reporting efforts being simply focused on the size of the relevant constituent particles. The US EPA's Section 8A rule notably sets a precedent and narrows the reporting requirement to nanoscale materials possessing unique and novel properties while applying a mass percent cutoff including agglomerates and aggregates. These criteria significantly narrow the scope of materials and possess far fewer metrology challenges.

NANOMATERIAL BASICS

Perspectives: Engineered Nanoparticles versus Ambient Particulate Matter

A large body of publications on the toxicology of airborne ambient particulate matter (PM) has shown that they can elicit adverse effects not only in the respiratory tract but also in secondary organs and systemically (see Chap. 31 on Air Pollution). An extensive critical review of the health effects of fine particulate air pollution (Pope and Dockery, 2006) pointed out that exposure to the smallest fraction of PM, referred to as UFPs or $PM_{0.1}$, particles smaller than 0.1 μm or 100 nm, has been associated with effects in the cardiovascular and central nervous system as a consequence of their translocation to and distribution via blood circulation and neurons. It is noteworthy that the definition of UFPs is based on the same maximum dimension (100 nm) as the US NNI and ISO/TC 229 (2015) definition of nanomaterials. As will be discussed later, translocation of UFP and engineered nanoparticles (ENPs) has been clearly demonstrated, but only small amounts are translocating, and thus direct interaction of the translocated particles with organs beyond the lung may not be the sole explanation for their effects. Epidemiological studies have demonstrated that a major factor for inducing adverse effects from ambient particulate air pollution—including UFP—is susceptibility such as pre-existing disease (asthma, cardiovascular disease, diabetes), age (very young, elderly), or genetic background (polymorphism).

A recent comprehensive review assesses and compares epidemiological and toxicological research of ambient UFP versus ENM (Stone et al., 2017). Effects and underlying mechanisms of cardiovascular, CNS, and other organs targeted by inhalation exposures to both types of particles are described, summarized by a workshop of experts who identified 19 lessons which are meant to facilitate knowledge exchange between different disciplines. Gaps in a number of key areas are identified where additional research will facilitate simultaneously both the ambient and engineered nanoparticle research. The authors point out the opportunity to apply knowledge from ENM toxicology to inform ambient PM health risk research and vice versa.

Properties and Behaviors of ENPs versus Larger Particles

Table 29-2 contrasts differences between NPs (<100 nm) and larger particles (>500 nm) in terms of some general characteristics, translocation propensity, interactions with cells, and effects, assuming inhalation exposure and the respiratory tract as the portal of entry. Biological systems do not perceive a precise boundary at the 100 nm size threshold, but rather a gradual transition between nano- and larger-sized particles, and therefore, the 100 to 500 nm size is shown but not covered in the table. Particle scientists tend to refer to this transition zone as the intermediate zone between particle size scale dominated by surface interactions (smaller particles) and size scales predominantly dominated by gravity or internal interactions (larger particles). The general phenomena explain why granular sugar readily flows like a liquid when poured but fine powdered sugar and flour do not. This is also the same consideration that explains why nanoscale particles can form dispersions that do not settle unlike coarser materials. For a variety of reasons, the range of 0 to 100 was used; however, the applicable transition size range depends on the particle shape, density, and other system factors that are beyond the scope of this chapter and not covered in the table.

Marked differences—which will be discussed in the following sections—between the smaller and larger size classes are as follows: The high number and surface area per unit volume of NPs, which enhance the chemical reactivity (e.g., as catalysts) of small particles and potentially increase their biological/toxicological activity toward cells and tissues; the underlying mechanisms for deposition of inhaled particles in the respiratory tract, which is governed by diffusion or Brownian motion for NP versus sedimentation, impaction, and interception for larger particles; and differences

Table 29-2

What Is Different: Nanoparticles versus Larger Particles (*respiratory tract as portal-of-entry*)

	NANOPARTICLES (<100 nm)	LARGER PARTICLES (>500 nm)
General characteristics		
Ratio: number or surface area/volume or mass	High	Low
Agglomeration in air, liquids	Likely (dependent on medium and surface)	Less likely
Deposition mechanism in respiratory tract	Diffusion; throughout resp. tract	Sedimentation, impaction, interception; throughout resp. tract
Protein/lipid adsorption in vitro	Very effective and important	Less effective
Protein/lipid adsorption in vivo	Yes	Some
Translocation to secondary target organs	Yes	Generally not (to liver under "overload")
Clearance		
• Mucociliary	Probably yes	Efficient
• By alveolar macrophages	Poor	Efficient
• Into or across lung epithelium	Yes	Mainly under overload
• Lymphatic	Yes	Under overload
• Blood circulation	Yes	Under overload
• Sensory neurons (uptake + transport)	Yes	Not likely
Cell entry/uptake	Yes (caveolae; clathrin; lipid rafts; diffusion)	Yes (primarily phagocytic cells)
• Mitochondria	Yes	No
• Nucleus	Yes (<40 nm)	No
Effects (*caveat: dose!*)		
At secondary target organs	Yes	(No)
At portal of entry (resp. tract)	Yes	Yes
• Inflammation	Yes	Yes
• Oxidative stress	Yes	Yes
• Activation of signaling pathways	Yes	Yes
• Genotoxicity, carcinogenicity	Probably yes	Some

SOURCE: Data from Oberdörster et al. (2009).

with regard to the extent and impact of adsorptions and adsorbed conformation/activity of proteins and lipids (corona formation) for larger versus smaller particles at the primary portal of entry and secondary target sites.

Mucociliary and alveolar macrophage-mediated elimination following deposition in the respiratory tract is efficient for both nano- and larger particles once they are internalized by macrophages. NPs inhaled and deposited as singlets, however, are too small to be efficiently recognized and phagocytized by alveolar macrophages (Oberdörster et al., 2005; Semmler-Behnke et al., 2007; Geiser et al., 2008)—unless they are aggregated or agglomerated to form larger particles—and thus overall alveolar macrophage-mediated clearance in the lung is poor. In contrast, uptake by epithelial cells and translocation into blood and lymphatic circulation occurs regularly for NPs, and only under heavy overload conditions for larger particles. Uptake of nanosized particles into sensory nerve endings of the tracheobronchial tree has also been described (Hunter and Undem, 1999), which is unlikely to occur for large particles.

Differences in cell entry mechanisms between NPs and larger particles and intracellular distribution to mitochondria and the nucleus are discussed in subsequent sections. With regard to the type of effects induced at the primary site of entry, there does not seem to be a fundamental difference. However, effects in secondary organs are more likely to develop, possibly aided by a direct effect of translocated NP.

Manufactured NPs show a range of other size-dependent properties and behaviors. Among these are nanoenabled properties such as depressed melting points, size-dependent electronic band gaps, quantum mechanical effects giving rise to size-tunable fluorescence in quantum dots (semiconductor NPs), and confined plasmon resonance, which shifts the light absorption and scattering in a way that alters the colors of nanoscale materials from their larger-sized counterparts, giving rise—for example—to nanosilver particles that appear yellow and to gold NPs that appear red. Unique size-dependent properties of NPs form the basis for many of their technological uses, but the significance of many of these properties for biological effects still needs to be evaluated.

Classes of ENMs

Manufactured nanomaterials cover an enormous range of chemical compositions, and geometries, ranging from simple isometric forms (particles), one-dimensional (1D) forms (nanofibers, tubes, rods, wires), and two-dimensional (2D) forms (plate-like or disk-like materials typically referred to as "nanosheets," for very thin and flexible forms, or "nanoplatelets" for thicker stiff materials). A large fraction of the stable elements in the periodic table have now been cast into NP forms, or incorporated as part of a nanocomposite structure. Nanomaterials can be manufactured from metals, metal oxides, sulfides or selenides, carbon, polymers, or from biological molecules including lipids, carbohydrates, peptides, proteins, and nucleic acid oligomers. Fig. 29-5 presents a nanomaterial classification system based on a matrix involving geometry (particles, 1D forms, 2D forms) and chemistry (metals, semiconductors, ceramics, carbons, polymers; Duncan, 2011; Cassee et al., 2011; Feng and Liu, 2011; Rosenthal et al., 2011; Tran and Nguyen, 2011). The combination of geometry and chemistry gives rise to a vast

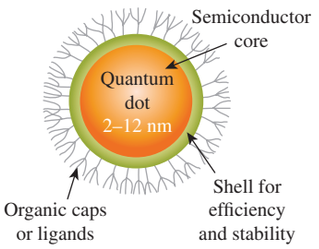
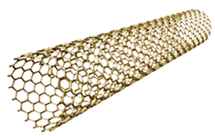
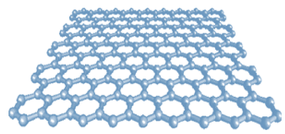
Chemistry \ Geometry	Isometric particles	1D; fibers/tubes	2D; plates, disks
Metals	Silver, gold nanoparticles Iron, cobalt, nickel magnetic NPs Copper NP conducting inks	Gold or platinum nanowires	Silver nanoplates
Semiconductors	CdSe/ZnS quantum dots (see example)	Si, ZnO semiconducting nanowires, nanorods	Plate-like semiconductor nanocrystals
Ceramics	Zinc oxide, titanium dioxide pigments and sun screens, cerium oxide catalysts	Electrospun ceramic nanofibers for composite fillers	Nanoclays MoS_2 nanosheets h-BN nanosheets
Carbons	Fullerenes, carbon black, carbon nanohorns	Carbon nanotubes Carbon nanofibers	Graphene, graphene oxide few-layer graphene (example)
Polymers	Biodegradable polymer nanobeads for medical applications, branched dendrimers	Electrospun polymer nanofibers	
Examples:		 Single-walled carbon nanotube	 Graphene

Figure 29-5. *Classification of nanomaterials by geometry and chemistry.* The examples in this matrix illustrate the diversity in engineered nanomaterials, a diversity that continues to increase as new nanomaterials are synthesized.

number of potential nanomaterials. Not all are, or will be commercially significant and find widespread use, but a great many have been synthesized and characterized in R&D laboratories around the world. Nanomaterials may be adhered to surfaces such as biomedical implants to enhance their function and biocompatibility, or incorporated into nanostructured solids or composites to improve strength, conductivity, and durability. Nanomaterials are often integrated into complex, active structures for chemical or biological sensors or other devices.

There are many approaches for synthesizing and manufacturing nanomaterials. Some methods use high-temperature processing in the vapor phase, such as inert gas condensation, flame synthesis, or "floating catalyst" chemical vapor deposition (Zhang et al., 2011). These processes produce dry powders that can lead to occupational exposures when reactors are opened to extract products or the powdered materials are handled or transferred. Other methods such as physical or chemical vapor deposition grow nanostructures on substrates. Substrate-bound nanostructures are less likely to cause exposures of concern, but can become detached during processing or use. A large class of methods uses solution processing, also known as "colloidal synthesis" (Tran and Nguyen, 2011). These methods involve reagent addition to liquid solvents to nucleate NPs that remain suspended in the liquid. This class of methods is less likely to lead to inhalation exposures, but they can occur if the liquid is sprayed or nebulized to produce mists, or if the NPs are dried into powders or transferred onto substrates and dried for further processing (Hallock et al., 2009).

Finally, most nanotoxicology research has focused on a limited set of simple nanomaterials with high manufacturing volume or the potential for high manufacturing volume in the near term

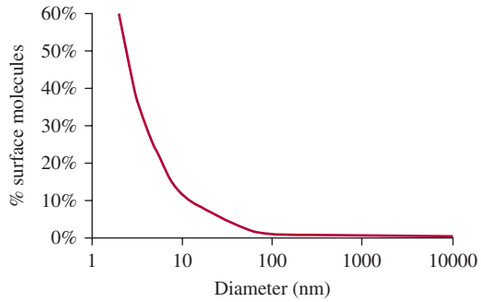


Figure 29-6. *Fraction of molecules in a particle that lie on its surface as a function of particle size.* (From Fissan [2008].)

(including ZnO, TiO_2, fullerenes, CNTs, nanosilver, quantum dots). Increasingly, the nanotoxicology field will have to focus on more complex nanomaterials under development, especially as technological applications for those materials approach commercialization (Maynard et al., 2011). It will be a major challenge for the nanotoxicology field to acquire the data necessary for risk assessment on the many new nanoproducts that are anticipated over the coming decades. Finally, as nanoscience progresses, there will be less emphasis on simple geometries and chemistry (see Fig. 29-6) and more emphasis on complex material structures that combine nanoelements into active or smart structures (Dvir et al., 2011). Early examples of this are the synthesis of two-component or "dumbbell-shaped" NPs composed of gold and iron oxide (Xu et al., 2008) or iron–platinum nanowires (Wang et al., 2007).

Understanding the toxicological profile of complex nanoassemblies will also present a major challenge to the field in the future.

The classification and categorization of nanomaterials for regulatory purposes has proven to be a challenge. Several expert workshops on grouping and categorization of nanomaterials have been conducted. In general, it is concluded that at present nanomaterials need to be considered on a case by case basis, applying information from bulk chemistry, surface chemistry, and shape. The increasing complexity of ENM design involving components from various material classes (e.g., metals, polymers, and metal oxides) integrated in increasingly intricate structural assemblies further challenges universal read-across and categorization schemes.

Physicochemical Properties of Nanomaterials Relevant for Toxicity

For chemical substances existing as liquids or gases, physicochemical frameworks exist for predicting exposure, biopersistence, and for screening potential toxicological consequences primarily through phase partitioning behavior (e.g., water solubility, octanol-water partitioning, vapor pressure, boiling and melting point [USEPA, 1997]). For finely divided solid particulate materials the evaluation becomes more complex. While multi-micrometer-sized particles may have little potential for migration or exposure, nanosized materials share attributes from both larger particles and molecules that draw concerns. Some ENMs can exhibit prolonged dispersion and Brownian motion like molecular solutes; however, they also have solid surfaces that are susceptible to hydration, ionization, adsorption, and transformation process. The later results in the stability and compartmental behavior of ENMs being largely dependent on environmental factors and history. The transport behavior of nanomaterials tends to be extrinsic, stochastic, and relatively irreversible in nature. Moreover, for extremely small nanomaterials (e.g., on the order of 10 nm) traditional, intrinsic, physical–chemical properties such as conductivity and light adsorption can become extrinsic in nature and dependent on interactions with molecules in the environment among other systemic factors. These unique attributes give rise to the use of ENMs for sensing applications, but further complicate their characterization and categorization as chemical substances.

Fig. 29-7 summarizes the nanomaterial properties known or thought to be relevant to biological responses. These properties are discussed in more detail in the section below, or in some cases in later sections on mammalian toxicity and ecotoxicity.

Particle Size, Distributions, Geometry, and Dimensions

Nanomaterials exist as populations of particles with variations in dimensions and morphology. The distribution of sizes and shapes impacts transport and exposure phenomena and can influence cellular uptake. Particle size, although used to define nanomaterials, is not an intrinsic property and can change substantially in the environment through dissolution, precipitation, agglomeration, and heteroagglomeration processes. Agglomeration is the process wherein two or more similar particles weakly stick to one another through physical interactions. Heteroagglomeration is the process where particles of different compositions similarly form weak associations. Monitoring and predicting particle size distributions under real exposure scenarios can be challenging, and typically only size distributions thought to be characteristics of the nanomaterial at key times are reported (e.g., as prepared, as delivered). For biomedical applications, nanoparticles of narrow size distributions are typically desired for consistent delivery or excretion. Dispersed particles in the circulation less than 5 nm are cleared via renal excretion, particles less than 50 nm are excreted via the hepatobiliary route.

The geometry and dimensions of nanomaterials are also important in cellular uptake, systemic translocation, potential

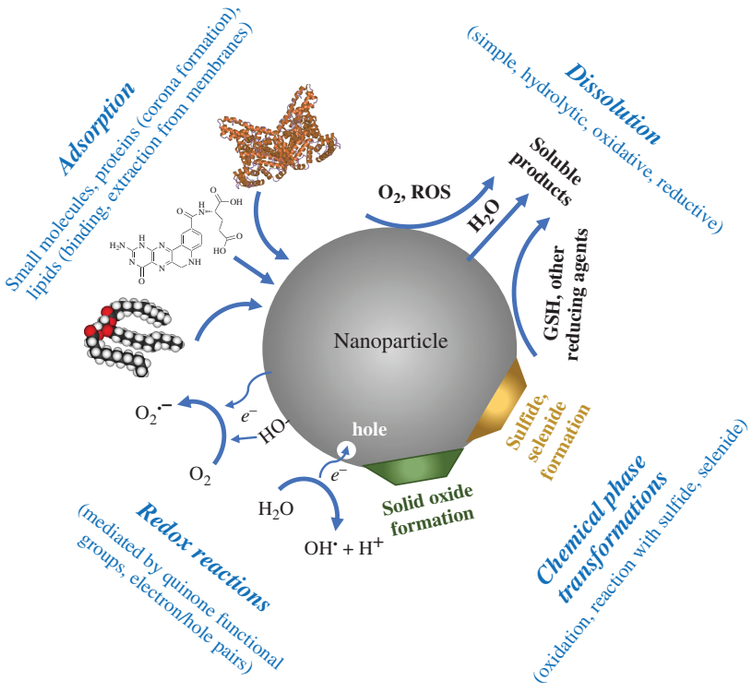


Figure 29-7. *Physicochemical and functional NP properties of relevance for toxicology.* (From Robert Hurt [2018].)

toxicity, and excretion. Nanomaterials can enter target cells by passive diffusion, direct physical penetration, or active, receptor-mediated uptake by endocytosis or phagocytosis depending on their size and extent of agglomeration. For example, small NPs up to 50 nm in diameter appear to enter cells and subcellular organelles, including mitochondria and the nucleus, by passive diffusion (Geiser et al., 2005; Chithrani, 2010). SWCNTs have been shown to directly puncture bacterial cells leading to osmotic lysis and death (Kang et al., 2007; Akhavan and Ghaderi, 2010). SWCNT have been reported to translocate from the alveoli into the interstitium of the lung where they promote collagen deposition and interstitial fibrosis (Wang et al., 2011a). Rigid, long, high-aspect ratio (needle-like) nanomaterials, similar to amphibole asbestos fibers (Palomaki et al., 2011), are recognized by macrophages; however, they undergo incomplete uptake or frustrated phagocytosis if they are longer than the macrophage diameter (Shi et al., 2011), resulting in impaired clearance from the lung, lysosomal disruption, and activation of the inflammasome resulting in the release of pro-inflammatory cytokines (Hamilton et al., 2009; Donaldson et al., 2010). High-aspect ratio materials (i.e., particulates with significantly greater lengths relative to diameters—e.g., fibers) with 2D structure (nanosheets) also show geometry-dependent cellular interactions. These include spontaneous cell membrane penetration initiated at near-atomically thin edges and corners of the 2D sheets (Li et al., 2013), or for nanosheets with large lateral dimension, impaired macrophage uptake (Sanchez et al. 2012), and lung injury at high doses (Roberts et al., 2016).

Surface Charge, Steric Interactions, and Agglomeration

NPs have a strong tendency to form agglomerates in the dry state, and in many cases also in liquid suspension (Fig. 29-8). This tendency is a natural consequence of small size, which leads to intermolecular attractive forces that are strong relative to gravity or to other forces that disrupt aggregates. Attractive forces between particles are determined by size, density, chemistry, electronic structure, morphology, and surface charge. In water, van der Waals forces and hydrophobic forces drive the agglomeration of many NP types. To obtain stable dispersions of non-agglomerated nanomaterials, it is typically necessary to add macromolecular coatings (*caveat*: by doing so it will change particle property potentially affecting toxicity) that prevent particle–particle attachment through steric hindrance, or to impart an electrical charge on the NP surfaces that lead to particle–particle repulsion. A common indicator of surface charge is the "zeta potential" which is the electrical potential at the outer surface of the liquid shell (including, adsorbates, ions, solutes, and solvent molecules) that travels with the particle during its motion. A common rule of thumb is that the zeta potential must be >30 mV or <-30 mV for repulsion to be sufficiently strong to avoid agglomeration and maintain a uniform dispersion. In physiological fluids or seawater, sodium chloride and other ions "screen" the particle surface charge and lower the zeta potential, often causing aggregation. This property is a complicating factor in nanotoxicology assays (discussed below) and is a challenge for some biomedical applications. NPs may also adsorb molecules from their environment that *increase* stability (Nel et al., 2009). Following injection or inhalation, NPs become coated with biomolecules such as serum albumin, an amphiphilic protein that carries poorly soluble molecules in the blood, or phospholipids in lung surfactant lining the alveoli as discussed in detail subsequently. For drug delivery applications (De Jong and Borm, 2008), NPs may be coated with biocompatible surfactants such as sodium cholate, or by biocompatible polymers such as phospholipid-polyethylene glycol (PEG) that stabilize colloidal suspensions of NPs through steric hindrance—a mechanism separate from electrostatic repulsion as indicated by zeta potential.

Surface Area and Reactivity At the nanoscale, chemical and physical forces govern the assembly of atoms or molecules resulting

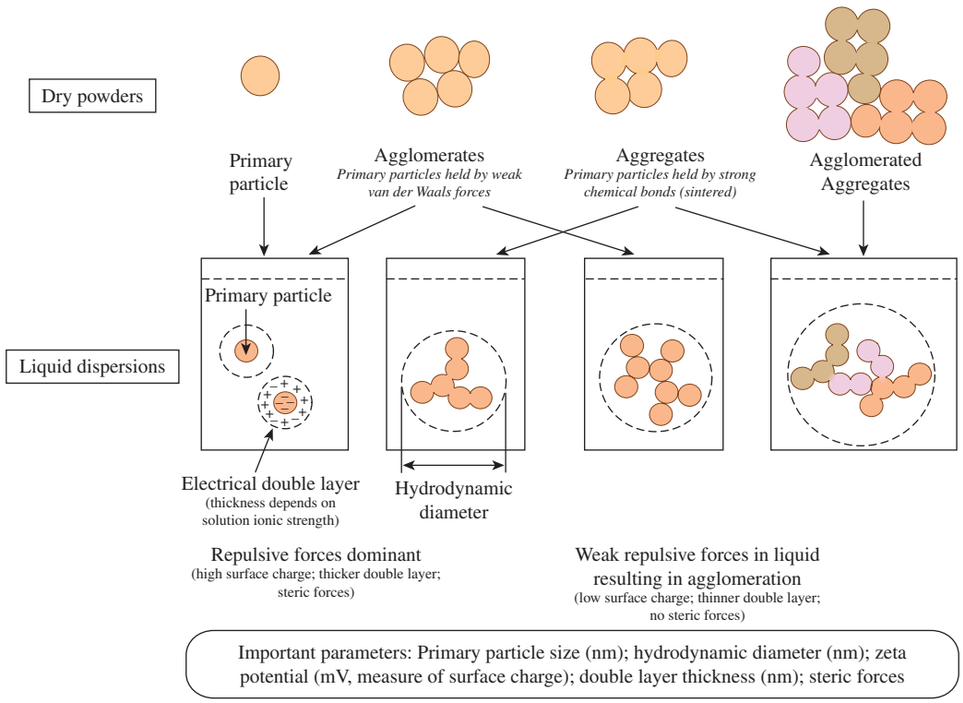


Figure 29-8. *Agglomeration and aggregation of nanoparticles in liquids and as dry powders.* (Modified from Jiang et al. [2009].)

in nanostructures with a high surface-to-volume or surface-to-mass ratio. For example, 5 nm spherical NPs have 1000 times the surface area of 5 μm particles of the same chemical composition and at the same airborne mass concentration. This high surface area of suspended NPs at equivalent mass concentrations is responsible for increased surface reactivity, increased adsorption of chemicals, strong catalytic activity (on an equivalent mass basis), and enhanced dissolution rates. As particle diameter decreases below ~100 nm, the number of exposed surface molecules increases sharply (Fig. 29-9; Oberdörster et al., 2005). The large number of exposed surface molecules or atoms exposes surface defects, vacant sites, and dangling chemical bonds that enhance chemical and redox reactivity. Thus, the NP surface can have higher surface area, higher surface energy (e.g., through surface curvature), and higher surface reactivity relative to the same mass of micron-sized particles of the same chemical composition (Fenoglio et al., 2011). Determining specific surface reactivity—e.g., ROS generating capacity per unit NP surface area—has been suggested as a measure to group ENMs (Oberdörster and Kuhlbusch, 2018).

Surface Chemistry, Surface Morphology, and Surface Impurities The surface chemistry of NPs strongly influences the mode and type of interactions with biotic systems. High surface area, high surface curvature, and exposed surface atoms or molecules promote increased dissolution and release of ions from metallic or metal oxide NPs relative to bulk particles of the same chemical composition in fluids. As an example, Ag^+ ions can be slowly released from nanosilver particles in aqueous environments through oxidation by dissolved O_2 (Liu and Hurt, 2010). This ion release accounts for much of the bactericidal properties of clothes, toys, and medical devices coated or impregnated with nanosilver particles. Metal ions are toxic to bacteria and aquatic organisms by inhibition of enzymes and transport proteins. Silver ions have been shown to induce swelling of the lamellae of trout gills due to inhibition of carbonic anhydrase and Na^+/K^+ ATPase activity (Scown et al., 2010). ZnO NPs are incorporated into sunscreens where they absorb ultraviolet (UV) light; however, in water, Zn^{2+} ions are rapidly released and cause acute toxicity (Xia et al., 2008a). Surface hydrophilicity of charged NPs increases their ability to be suspended in water, whereas surface hydrophobicity of fullerenes or graphene repels water and enables these hydrophobic nanomaterials to partition into lipid membranes and gain entry into target cell (Sayes et al., 2005; Titov et al., 2010). TiO_2 NPs are used in sunscreens because they absorb UV radiation, but their small size reduces the scattering of visible light, and the sunscreens appear transparent to the human eye. UV absorption can also lead to formation of electron/hole pairs that react on the particle surface to generate reactive oxygen species (ROS) such as superoxide anion (O_2^-) from molecular oxygen (O_2) (Schilling et al., 2010). Surface defects, especially on crystalline NPs, expose electron donor/acceptor active groups that donate an electron to molecular oxygen, generating superoxide anions, or accept electrons from water producing the highly reactive hydroxyl radical. Spontaneous or photoactivated generation of electrons can occur based on the semiconducting properties of fullerenes and some metal oxide NPs (Xia et al., 2006).

Industrial nanomaterials are synthesized through a wide range of chemical processes and are purified to differing degrees. It is not uncommon to receive nanomaterials that contain residual surfactants, ions, acids, bases, and other potential reaction contaminants. Due diligence is required to determine whether these residual materials are key contributors to observed outcomes.

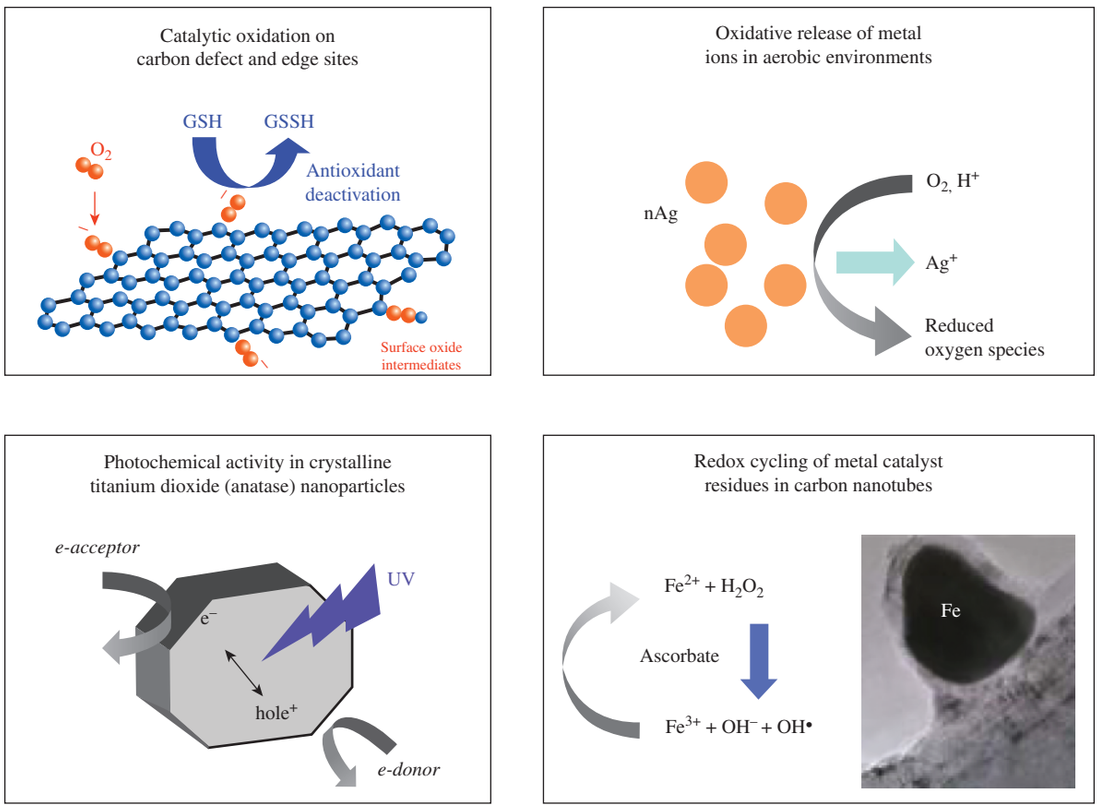


Figure 29-9. *Examples of biological reactivity of nanoparticles.*

For instance, CNTs are synthesized in the presence of metal catalysts, usually iron, nickel, or cobalt, and may contain redox-active metal residues that can undergo redox cycling and iron-catalyzed generation of highly reactive hydroxyl radical ($OH^{\bullet}$) via Fenton chemistry (Kagan et al., 2006; Guo et al., 2007). The high surface area of ENMs can adsorb organic molecules such as polycyclic aromatic hydrocarbons that are potentially carcinogenic (Yang et al., 2006) and quinones that also participate in redox cycling and generation of free radicals (Nel et al., 2006). Cationic NPs that have surface amide groups and cationic dendrimers are especially cytotoxic because they induce membrane damage, especially in lysosomes where the "proton sponge effect" leads to accumulation of water and chloride ions and osmotic rupture (Xia et al., 2008b).

In summary, surface properties are major determinants of biological reactivity due to (1) high surface area, (2) surface charge, (3) hydrophobicity and partitioning into lipid membranes, (4) dissolution and release of metal ions, and (5) redox activity leading to generation of ROS (Nel et al., 2006, 2009; Xia et al., 2008b; Stone et al., 2009).

Unique Properties NPs also have the potential to display unique electronic and magnetic properties relative to micron-sized particles. NPs may interact with energy differently from larger sized materials due to changes in electronic structure, and increasing dominance of surface versus bulk contributions to properties making parameters, commonly thought of as being intrinsically a function of environment. As previously mentioned, it is this surface sensitivity that gives rise to the use of nanomaterials as sensors (e.g., metal-oxide semiconductor field-effect transistors) in a wide range of potential applications. The small size and shape of nanomaterials can also lead to changes in electronic structure that in turn impacts how the nanomaterial interacts with electromagnetic energy. For example, noble metal NPs show preferred wavelengths for absorption and scattering of light due to resonant electron oscillations. This "confined plasmon resonance" phenomenon is shown by 40 to 100 nm diameter gold or silver NPs and leads to color shifts that impart a yellow color to nanosilver and a red color to some types of nanogold (Tran and Nguyen, 2011). Ferromagnetic NPs (e.g., iron, nickel, cobalt, and some oxides) less than about 10 nm in diameter exhibit the property of superparamagnetism. These particles respond strongly to an external magnetic field but have no permanent magnetic moment in the absence of the field because thermal energy is sufficient to cause fluctuations in the small magnetic dipole associated with each particle (Cole et al., 2011). This property is exploited for contrast enhancement in diagnostic MRI and for hyperthermia induced by an external magnetic field to kill tumors targeted by magnetic NPs (Fortin et al., 2007). Although these properties are evident for some nanomaterials, unique properties—distinct from larger sized forms of the material—do not automatically appear with the scaling of size.

Biopersistence Biopersistence of ENMs is an important factor in their environmental and biological toxicity. Biopersistence is determined by mechanical clearance and dissolution processes; the latter not only produces biologically active soluble species (as discussed above), but can also degrade the particle and eventually clear it from biological tissues. ZnO NPs are an excellent example of an ENM that can exhibit acute cellular toxicity due to rapid release of Zn^{2+} ions. The rate of dissolution of metal oxides is increased by natural organic matter in the aqueous environment (Xia et al., 2008b); therefore, these NPs have low biodurability and it is predicted that they would not bioaccumulate in the environment. Following uptake by macrophages at the site of entry, metallic and metal oxide NPs reach the acidic phagolysosomal compartment where release of metal ions is accelerated. Intracellular release of Zn^{2+} or Ni^{2+} ions, for example, is linked with significant acute toxicity to target cells in the lungs (Liu et al., 2007; Xia et al., 2008b; Pietruska et al., 2011). Even metallic nickel NPs which are insoluble can undergo oxidation by O_2 following exposure to produce soluble surface oxides. This slow oxidation dissolution process produces sustained intracellular release of Ni^{2+} ions that cause persistent inflammation (Lu et al., 2009) and activation of the HIF1-α signaling pathway in human lung epithelial cells (Pietruska et al., 2011), which has been linked to nickel carcinogenicity (Costa et al., 2005). Jakubek et al. (2009) reported that release of yttrium ions in trace levels from CNTs displaces Ca^{2+} from voltage-gated calcium channels with implications for neuronal activity. So far, no chronic rodent carcinogenicity assays have been conducted to assess whether inhalation of metal or metal oxide NPs can induce lung cancer or neurological disease.

In contrast, carbon nanomaterials are not easily oxidized under most environmental conditions. They are potentially biodurable, raising concern that they could bioaccumulate in the environment or be biopersistent in the lungs and other organs following inhalation during occupational exposure or direct injection or implantation in biomedical applications (Bianco et al., 2011). Some commercial types of long, rigid, high aspect ratio CNTs have been shown to induce toxicological endpoints similar to carcinogenic asbestos fibers (Sanchez et al., 2009) as discussed in detail below. Biopersistence in the lungs and pleural or peritoneal spaces is an important physicochemical characteristic of asbestos and man-made mineral fibers associated with carcinogenicity (Bernstein et al., 2005). Several recent studies have shown that carboxylated SWCNTs undergo oxidative degradation in the presence of the plant enzyme, horseradish peroxidase, and H_2O_2 (Allen et al., 2008, 2009), or following exposure to phagolysosomal simulant fluid containing H_2O_2 to mimic the lysosomal compartment of macrophages (Liu et al., 2010c). Oxidized or nitrogen-doped MWCNTs also showed both exfoliation of the concentric tubes and shortening in the presence of horseradish peroxidase and H_2O_2 (Zhao et al., 2011). Other studies (Dresselhaus and Terrones, 2013) showed that N-doped MWCNTs did not show an inflammatory response. Oxidatively degraded SWCNTs did not induce lung inflammation or toxicity following pharyngeal aspiration in mice (Kagan et al., 2010), providing proof-of-principle for deliberate design of engineered CNTs that are biodegradable and less likely to induce acute or chronic disease following inhalation or injection for tumor imaging or drug delivery (Bianco et al., 2011). Graphene oxide nanosheets have also been shown to be degraded in the presence of horseradish peroxidase and H_2O_2 (Kotchey et al., 2011) or by *Shewanella* bacteria that chemically reduce graphene oxide to graphene (Salas et al., 2010). These studies provide novel approaches for biodegradation of ENMs and prevention of long-term adverse environmental and health impacts.

Chemical Transformation The dissolution and degradation processes discussed above are a subset of the larger class of "chemical transformation" that nanomaterials can undergo in the natural environment or in biological tissue. Most nanomaterials are synthesized with the input of chemical or thermal energy and when transferred into environmental or biological fluid phases they seek to release this free energy by chemical transformation into more stable phases. Chemical transformations include dissolution to soluble molecular species, degradation to complex solid debris, or chemical reaction to form new solid phases that include sulfides, selenides, or oxides (Liu et al., 2012; Lowry et al., 2012). Such transformations can dramatically alter nanomaterial properties, and require

that studies of hazard and risk be extended beyond the pristine particles to include the transformation products. Transformations can occur between the points of synthesis and exposure during nanomaterial processing, handling, storage, use, or upon release into the natural environment. These transformations complicate nanotoxicology studies, because nanoparticle properties become a complex function of sample history, which in many cases is unknown and uncontrolled. Transformation can also occur after exposure through contact with biological fluids or tissues causing the potential toxic properties of particles to evolve over time.

One of the most studied nanomaterials in the transformation literature is metallic nanosilver. Nanosilver has been shown to undergo oxidative dissolution in the presence of atmospheric O_2 accompanied by Ag^+ ion release (Liu and Hurt, 2010), and because the silver ion is more toxic than the particles in many biological systems, the location and extent of this ion release is a key factor determining nanosilver toxicity. Further transformations include chemical or photochemical reduction of the silver ions back to nanosilver particles, referred to as "secondary particles" (Glover et al., 2011); reaction with reduced sulfur species to form the stable and highly insoluble silver sulfide (Lowry et al., 2012); and reaction to form solid selenides (Liu et al., 2012). These transformations are thought to play a role in the formation of silver-containing solid deposits in tissue that give rise to argyria, which is a skin discoloration phenomenon that has been observed in humans exposed orally to high doses of nanosilver (Liu et al., 2012).

Insights into the novel findings of bioprocessing and biotransformation of ENMs at subcellular, almost atomic levels are addressed by Graham and colleagues (2014, 2017).

THE NANOMATERIAL BIOLOGICAL INTERFACE

The high surface area of NPs provides a platform for adsorption of a variety of biological molecules including proteins, lipids, and nucleic acids. Dawson and his coworkers described in detail rapid binding of proteins in biological fluids to the surface of NPs that they defined as the "protein corona" (Lynch et al., 2007). The protein corona is a dynamic interface between the NP and its local environment that governs its initial interaction with target cells (Dutta et al., 2007; Walczyk et al., 2010). The interaction of nanomaterials with blood plasma proteins has been investigated in most detail due to its importance in drug delivery, circulation time, organ distribution, and clearance (Karmali and Simberg, 2011). Protein adsorption is related to surface charge, hydrophobicity, and radius of curvature; however, NPs of diverse chemical composition and structure (e.g., iron oxide NPs, polymeric NPs, and CNTs) avidly adsorb albumin, complement, fibrinogen, immunoglobulins, and apolipoproteins (Nel et al., 2009). Protein adsorption and dissociation is a complex, dynamic phenomenon (Cedervall et al., 2007a, 2007b). Quantitative techniques to predict and characterize protein adsorption have been developed (Xia et al., 2010). The consequences of protein adsorption to NPs are less clear; although, depending on the NP surface, proteins may denature, resulting in the loss of normal structure and function, or altered enzyme activity, or unfolding that exposes new antigenic determinants. NPs of diverse chemical composition also induce protein fibrillation or aggregation to form amyloid fibrils in an acellular system (Linse et al., 2007). An important potential pathological consequence of serum protein adsorption to NPs is binding of fibrinogen leading to formation of blood clots (Khandoga et al., 2010).

NPs that are inhaled into the respiratory tract or ingested into the gastrointestinal tract encounter a mucus layer that provides a natural barrier to penetration of particulates and microorganisms. Mucus is a complex glycoprotein composed of hydrophilic, dispersed regions and hydrophobic, globular domains. NPs may adhere to mucins causing enlargement of the pore size with increased susceptibility to penetration of microorganisms (Shvedova et al., 2007; Wang et al., 2011b). Smaller, charged NPs may be repelled by the hydrophilic domains and will not be able to penetrate the mucus layer (Cone, 2009; Lai et al., 2009; Wang et al., 2011b). Aquatic organisms (Klaine et al., 2008) and bacterial biofilms (Rodrigues and Elimelech, 2010) are surrounded by similar extracellular barriers that may prevent NP penetration. If inhaled NPs deposit in the alveoli of the lungs, they encounter the lung lining fluid covering a thin film of surfactant lipids. Natural or synthetic lung lining fluid, which is a mixture of serum albumin and dipalmitoylphosphatidylcholine, binds readily to a range of NPs and enhances their dispersion for use in toxicological assays (Sager et al., 2007; Porter et al., 2008).

Some ENP-types also bind nucleic acids and have been proposed as gene delivery devices (Chou et al., 2011). Depending on their chirality, SWCNTs may show sequence-specific DNA binding (Tu et al., 2009). Graphene oxide nanosheets preferentially bind single-stranded RNA or DNA and protect it from enzymatic degradation (Lu et al., 2010). Small graphene oxide nanosheets can also intercalate into double-stranded DNA and induce DNA breaks in the presence of Cu^{2+} ions (Ren et al., 2010). Size-dependent binding of gold NPs into the major groove of DNA has also been reported (Schmid, 2008).

TOXICITY MECHANISMS

The emerging nanotechnology industry has generated fear and distrust among the public about unpredictable toxicity and unintended consequences of human exposure to ENMs (Bainbridge, 2002; Cobb and Macoubrie, 2004). Despite these concerns, there has not yet been an outbreak of human disease associated with exposure to ENMs, although potentially long latency periods between exposure and manifestation of effects require continued vigilance to prevent future problems. The mechanistic pathways associated with toxicity that have been discovered using various in vitro and in vivo assays may be predictable based on the physicochemical properties of ENMs (Meng et al., 2009; Nel et al., 2009). Some of these toxicity pathways were identified by studying UFPs associated with ambient air pollution (Xia et al., 2006), whereas others are the consequence of intracellular release of toxic metal ions. These are in vitro results, but not validated with relevant exposures (Xia et al., 2008b). Oxidative stress due to direct generation of ROS at the surface of NPs or indirectly by target cells following internalization of NPs (Unfried et al., 2007) is a common mechanism responsible for toxicity of ENMs. The most vulnerable subcellular organelles and physiological functions that can be perturbed by exposure to ENPs are summarized in Table 29-3. Depending on the dose, duration of exposure, type of NP, and the target cell, the cellular responses may be minimal and reversible, involve activation of adaptive responses, or severe, leading to significant changes in cell structure and function that may culminate in cell death (Meng et al., 2009). Specific examples of toxicity endpoints induced by exposure to ENMs will be used to summarize our current understanding of mechanisms relevant for nanotoxicology. Most of the mechanistic studies use a variety of in vitro cellular toxicity assays and relatively high concentrations for 24 to 48 hours of exposure. As discussed in detail below, extrapolation from these short-term, high-concentration exposures in vitro to chronic, low-dose exposures in vivo is problematic. Given the wide range of ENMs currently in commercial

Table 29-3

In Vitro Mechanisms of Nanoparticle Toxicity

1. Damage to cell wall and plasma membrane
2. Interference with electron transport and aerobic respiration
3. Induction of oxidant stress
4. Activation of cell signaling pathways
5. Perturbed ion homeostasis
6. Release of toxic metal ions from internalized nanoparticles
7. Disruption of lysosomal membrane integrity
8. Incomplete uptake or frustrated phagocytosis
9. Interference with cytoskeletal function
10. DNA and chromosomal damage

production or in the early development stages, understanding toxicity pathways is essential for developing a rationale strategy for in vitro screening and prioritization for chronic animal testing based on new principles for toxicity testing in the 21st century (Simmons et al., 2009; Walker and Bucher, 2009; Silbergeld et al., 2011).

The cell wall of bacteria and the plasma membrane of eukaryotic cells are the initial barriers to penetration of NPs into target cells. Both gram-negative and gram-positive bacteria have thick cell walls composed of complex lipids and peptidoglycans, respectively. Bacteria do not actively take up particulates; however, several investigators have shown direct physical penetration of bacterial cell walls especially by SWCNTs (Kang et al., 2007, 2008; Liu et al., 2009), graphene and graphene oxide nanowalls (Akhavan and Ghaderi, 2010), and zinc oxide NPs (Liu et al., 2009). Carbon nanomaterials are proposed to act as "nanodarts" creating holes in the plasma membrane, resulting in extracellular release of cytoplasmic contents as assessed by efflux of ribosomal RNA and decreased survival (Liu et al., 2009).

A wide range of NPs have been designed to facilitate delivery of imaging agents, genes, proteins, and drugs into mammalian cells: metallic and iron oxide NPs, silica NPs, quantum dots, biocompatible polymers, liposomes, micelles, and dendrimers (Chou et al., 2011). Target cells may recognize untargeted NPs that bind to surface scavenger receptors (Kanno et al., 2007; Hirano et al., 2008), lectin receptors (Zhang and Monteiro-Riviere, 2010), and Toll-like receptors that recognize hydrophobic domains (Seong and Matzinger, 2004). NPs can also be designed to target specific cell surface receptors triggering internalization by phagocytosis, macropinocytosis, or endocytosis mediated by clathrin, caveolae, or lipid rafts (Nel et al., 2009; Lajoie and Nabi, 2010). In order to facilitate gene, protein, or drug delivery, NPs can be engineered to escape from endosomes or lysosomes by coating with pH-sensitive polymers, viral capsids, cations, or biodegradable carriers (Chou et al., 2011).

Eukaryotic cells can actively internalize a variety of ENPs using energy-dependent, receptor-mediated endocytosis or phagocytosis (Zhao et al., 2011). Following endocytosis or phagocytosis, NPs follow a well-defined intracellular pathway usually leading to fusion with lysosomes (Oh and Swanson, 1996). The kinetics and mechanisms of cell uptake vary, depending on the target cell, size, shape, and surface modifications (Chithrani, 2010). Most NPs and microparticles are stored in membrane-bound vesicles, endosomes, or phagosomes; however, a subset of NPs including mesoporous silica NPs and superparamagnetic iron oxide NPs coated with chitosan undergo exocytosis or release from endothelial cells (Slowing et al., 2011) and macrophages (Serda et al., 2010). Exosomes are derived from sorting endosomes and multivesicular bodies; smaller NPs show greater exocytosis than particles in the range of 30 to 50 nm (Chithrani, 2010; Serda et al., 2010). Selective coating of NPs to favor delivery to exosomes can be exploited for drug delivery to vascularized tumors or to promote cell–cell transfer of genes and proteins (Slowing et al., 2011).

NPs that are recognized by surface receptors may activate cell-type-specific signaling pathways leading to cell proliferation or death by apoptosis, stress-related signaling, or calcium-mediated signal transduction events (Unfried et al., 2007). Dysregulated intracellular calcium ion homeostasis may be the consequence of influx across a damaged plasma membrane permeability barrier or release of calcium ions from the major intracellular storage sites, mitochondria, and endoplasmic reticulum. Sustained elevation in intracellular calcium can cause cell death by necrosis (Xia et al., 2006).

In addition, small polystyrene NPs and metallic NPs and dendrimers have been shown to enter a variety of target cells by passive diffusion in an energy-independent process (Lesniak et al., 2005; Geiser et al., 2005). Following uptake by diffusion, these small NPs are not compartmentalized into membrane-bound vesicles or lysosomes but are found free in the cytoplasm where they may secondarily enter the mitochondria or even the nucleus.

Mitochondria are the site of aerobic respiration and generation of adenosine triphosphate (ATP). Small NPs have been observed inside mitochondria (Li et al., 2003; Xia et al., 2006) where they may disrupt electron transport in the inner mitochondrial membrane and generate excess endogenous ROS. Direct or indirect generation of ROS in target cells exposed to a variety of engineered or ambient NPs is a major mechanism leading to cell toxicity and cell death (Nel et al., 2006; Unfried et al., 2007). Excess ROS that are not detoxified by endogenous antioxidant defenses can damage cellular membranes via lipid peroxidation, inactivate structural proteins, enzymes, and ion pumps, and damage nuclear DNA. A "hierarchical stress response" to oxidant-induced injury has been proposed by Nel et al. (2006). Low levels of ROS can activate transcription factors leading to upregulated expression of antioxidant defense-related genes. The second tier of response to ROS is activation of redox-sensitive transcription factors that upregulate genes involved in inflammation, pro-inflammatory cytokines, and chemokines (Albrecht et al., 2004). Finally, high or sustained production of ROS can lead to irreversible mitochondrial injury and cell death by apoptosis or necrosis (Nel et al., 2006).

Endocytosis and phagocytosis of NPs by target cells usually results in fusion with and sequestration in membrane-bound lysosomes (Russell et al., 2009; Chithrani, 2010). Uptake of quantum dots, CNTs containing metal catalyst residues, or metallic and metal oxide NPs provides a pathway for intracellular delivery of toxic metal ions by a "Trojan horse mechanism" (Limbach et al., 2005). In the acidic environment of lysosomes, cadmium ions can be released from quantum dots, redox-active iron from iron oxide NPs, and other toxic metal ions including nickel from CNTs or metallic and metal oxide NPs (Liu et al., 2008; Pietruska et al., 2011). This mechanism may also be exploited to release drug cargoes into the cytoplasm following NP-mediated delivery (De Jong and Borm, 2008).

Cellular uptake of long, rigid, high aspect ratio nanomaterials including CNTs, metallic nanowires, and nanorods is especially hazardous for target cells in the lungs (Donaldson et al., 2010). Macrophages are the initial cells to phagocytize inhaled particulates deposited in the airways or alveoli (Geiser and Kreyling, 2010). Rigid, high aspect ratio ENMs show similar interactions with macrophages as amphibole asbestos fibers. If they are longer than the diameter of macrophages (~10 μm), rigid elongated nanostructures show incomplete uptake or phagocytosis with prolonged generation of ROS by the respiratory burst mechanism of phagocytes

and extracellular release of damaging lysosomal enzymes. Rigid, high aspect ratio nanomaterials also cause permeabilization of the lysosomal membrane, resulting in the release of cathepsins into the cytoplasm and activation of the inflammasome (Hamilton et al., 2009). Cathepsins are proteases active at neutral pH and can cleave precursors of pro-inflammatory cytokines that can initiate inflammatory reactions in the lungs (Biswas et al., 2011) as well as activate procaspases leading to death by apoptosis (Boya and Kroemer, 2008). Activation of the inflammasome, the cytoplasmic protein complex where this proteolytic cleavage and activation occurs, is also triggered by uptake of crystalline minerals including silica and asbestos fibers (Dostert et al., 2008).

Incomplete sequestration of long, rigid, high aspect ratio NPs in lysosomes may also promote their release into the cytoplasm, resulting in physical interference with cytoskeletal function. Cytoskeletal disruption can cause impaired cell motility; for example, macrophage-mediated clearance of asbestos fibers and high aspect ratio NPs is impaired, resulting in sustained lung injury, inflammation, and fibrosis (Donaldson et al., 2010) as discussed below. In other organs, NP-mediated interference with cytoskeletal-mediated transport may occur, for example, impaired bile transport and secretion by hepatocytes (Johnston et al., 2010a). In dividing cells, high aspect ratio NPs may physically interfere with the mitotic apparatus, resulting in chromosomal mis-segregation and polyploidy (Sargent et al., 2010).

Finally, NPs may translocate into the nucleus by diffusion through nuclear pores or via nucleocytoplasmic transport (Unfried et al., 2007). Quantum dots (Hoshino et al., 2004), silicon dioxide NPs (Chen and von Mikecz, 2005), and gold NPs (Pante and Kann, 2002) have been detected in the nucleus. Small gold NPs may intercalate into DNA (Schmid, 2008) and generate ROS that may induce oxidative DNA damage (Kang et al., 2010). Silicon dioxide NPs have been shown to induce intranuclear protein aggregates of histones, topoisomerase I, and fibrillarin, although the functional consequences of this intranuclear protein aggregation are unknown (Unfried et al., 2007).

CAVEATS IN NANOTOXICOLOGY ASSAYS

The unique physical and chemical properties of solid materials at the nanoscale contribute to serious technical limitations and potentially misleading results using conventional toxicology assays (Petersen et al., 2014; Rosslein et al., 2015). Due to their high surface area and hydrophobicity, NPs can adsorb vital dyes, cell culture micronutrients, or released cytokines (Monteiro-Riviere and Inman, 2006; Casey et al., 2007, 2008; Guo et al., 2008; Stone et al., 2009). High surface area carbon nanomaterials such as SWCNTs and graphene have the greatest potential to adsorb aromatic amino acids, vitamins, and fluorescent dyes by π–π interactions with their hydrophobic graphenic surfaces (Guo et al., 2008). Adsorption of bacterial lipopolysaccharide or endotoxin (a component of the cell wall of gram-negative bacteria) is a major concern because even low levels of endotoxin can activate macrophages and dendritic cells (Vallhov et al., 2006). Genotoxicity endpoints are especially sensitive to NP-induced artifacts; for example, particle agglomerates interfere with the comet assay, used to detect DNA breaks, and cytochalasin B used in the cytokinesis-arrested micronucleus assay can interfere with NP uptake (Doak et al., 2009; Stone et al., 2009; Magdolenova et al., 2014; Evans et al., 2017). NP agglomerates in physiological saline may also induce greater cytotoxicity than well-dispersed NPs; however, controls must be included to rule out toxicity of the dispersant (Wick et al., 2007). In all toxicological studies, well-characterized positive and negative reference samples should be included over a range of doses and exposure times using standardized methodology (Pal et al., 2015).

ENMs are highly complex and have unique chemical and physical properties relative to bulk materials of the same chemical composition. Complete materials characterization is required for adequate interpretation of toxicological studies (Warheit, 2008; Stone et al., 2009; Azhdarzadeh et al., 2015). Nanotoxicologists must collaborate with chemists, engineers, and materials scientists in an interdisciplinary research team in order to understand the chemical, physical, and structural properties responsible for biological interactions and potential toxicity of nanomaterials. The relevance of in vivo extrapolation from high-dose, short-term, in vitro toxicity assays is discussed in more detail below.

SAFETY CONSIDERATIONS IN NANOMATERIAL DESIGN

In principle, it should be possible to engineer NPs with desirable surface properties for commercial or biomedical applications, for example, to control their size and geometry in order to prevent indiscriminate entry into cells and frustrated phagocytosis. Capping or coating of NPs using antioxidants such as tocopheryl-polyethyleneglycol-succinate (Yan et al., 2007) may decrease toxicity. Release of toxic metal ions from quantum dots and iron oxide NPs can be minimized using inorganic shells or biocompatible polymers such as chitosan or alginate (De Jong and Borm, 2008). CNTs, fullerenes, and graphene are easily surface functionalized; for example, covalent functionalization with carboxyl groups has been reported to decrease toxicity of these pristine carbon nanomaterials (Sayes et al., 2005, 2006; Sasidharan et al., 2011). There is evidence that CNTs are less pathogenic if short in length, or if entangled to hide their fibrous nature (Poland et al., 2008). A long-term goal of mechanistic nanotoxicology is to reveal structure–activity relations that may allow the design of safe nanomaterials through re-engineering or reformulation (Hutchison, 2008).

CASE STUDY: DESIGNING SAFER SUNSCREENS

Zinc oxide and TiO_2 NPs are widely used in sunscreens because they show less scattering of visible light and appear transparent, while effectively absorbing UV light in both the UVB and UVA wavelengths (Wang and Tooley, 2011). Due to its rapid dissolution in water releasing Zn^{2+} ions, zinc oxide NPs are considered as potential environmental toxicants (Kahru and Dubourguier, 2010). Xia et al. (2011) deliberately doped zinc oxide NPs with iron (1% to 10% by weight) that significantly retarded release of Zn^{2+} ions in water and physiological saline solutions. Zn^{2+} ions inhibit hatching of zebrafish embryos. Pristine zinc oxide NPs also inhibited hatching; however, iron-doped NPs were less inhibitory in this assay. Similarly, iron-doped zinc NPs induced significantly less lung injury and inflammation than pristine zinc oxide NPs following intratracheal instillation in rodents. This study provides proof-of-principle for safe design of ENPs to minimize potential environmental toxicity and adverse health effects.

TiO_2 NPs are used commercially in sunscreens, cosmetics, catalysts, and photocatalytic coatings for buildings and windows. Nanosized TiO_2 is found in small concentrations (typically $<30\%$ number and less than 1% mass) in food stuffs and personal care products (Weir et al., 2012; Warheit et al., 2015). In 2010, 5000 metric tons of TiO_2 NPs were produced (Landsiedel et al., 2010) and have been detected in effluents from waste water treatment plants where they may enter surface water (Westerhoff et al., 2011).

The potential of zinc oxide and TiO_2 NPs to induce phototoxicity and penetrate into the dermis has been a concern for human safety of sunscreens (Wang and Tooley, 2011). These mineral-based sunscreens cause less skin irritation and produce fewer allergic reactions than chemical-based sunscreens. The pristine NPs used in sunscreens form aggregates 30 to 150 nm in diameter; in this size range, these NPs are photoreactive and may generate ROS. Although uncoated TiO_2 NPs exhibit phototoxicity, following coating with hydrophobic stabilizers used in sunscreen formulations, no phototoxicity was detected in a cell culture assay system (Schilling et al., 2010). A series of skin penetration studies using both ex vivo and in vivo models also showed that these NPs do not penetrate deeper than the outer most layer or stratum corneum of intact skin (Schilling et al., 2010). However, TiO_2 NPs suspended in cyclopentasiloxane fluid used in cosmetics did penetrate into hair follicles of shaved skin of micropigs (Senzui et al., 2010). Finally, young pigs were exposed to UVB light producing sunburn and an in vitro skin perfusion system was used to assess penetration of sunscreens containing zinc oxide or TiO_2 NPs. Only superficial penetration into the upper layers of the epidermis was observed and no zinc or titanium above untreated control levels was observed in the perfusate (Monteiro-Riviere et al., 2011). Finally, 36 weekly applications of sunscreens containing TiO_2 or zinc oxide nanoparticles plus ultraviolet radiation applied to hairless mice did not induce any adverse systemic effects or skin tumors in comparison to mice exposed only to ultraviolet radiation (Osmond-McLeod et al., 2016). Collectively, these in vitro and in vivo toxicology assays suggest that the benefits of protection against carcinogenic UV light radiation provided by sunscreens formulated with zinc oxide or TiO_2 NPs outweigh the minimal risks associated with phototoxicity, DNA damage, and skin penetration into shaved or sunburned skin.

MAMMALIAN TOXICOLOGY

Introduction

Nanotoxicology, the science of effects of ENMs on living organisms, is a rapidly growing discipline aimed at identifying and characterizing nanomaterial toxicity that will serve—in combination with exposure data—the ultimate goal of performing a meaningful risk assessment (National Academy of Sciences, 1983). Many ENMs exhibit unique and desirable catalytic, optical, structural, or electronic properties that make them attractive in diverse technological areas, including multiple manufacturing industries and environmental and medical applications. At the same time, concerns have been raised regarding potential acute and chronic adverse effects due to their physicochemical properties in combination with nanostructures, for example, an elongated fiber-like shape of CNTs (Johnston et al., 2010b).

CNTs are a prime example of the two opposing faces of nanomaterials: Many highly desirable properties that are suitable for numerous beneficial applications contrast with reports of serious adverse effects in experimental animals. For example, the excitement about future uses of CNTs in biomedical applications for delivery of drugs, genes, and biosensors or as tissue engineering scaffolds (Liu et al., 2008, 2010b; Liang and Chen, 2010) is dampened by reports of inflammatory fibrogenic and even mesotheliogenic effects in laboratory rodents (Shvedova et al., 2005; Takagi et al., 2008; Jacobsen et al., 2008; Poland et al., 2008; Sakamoto et al., 2009). Results of a number of in vitro studies in different cell systems also revealed an oxidative stress-inducing pro-inflammatory potential of CNTs (Monteiro-Riviere et al., 2005; Pacurari et al., 2008; Hirano et al., 2010; Vankoningsloo et al., 2010; Sanchez et al., 2011); however, other in vitro studies did not confirm such effects (Fenoglio et al., 2006; Flahaut et al., 2006; De Nicola et al., 2007; Pulskamp et al., 2007).

As discussed earlier, a common problem with in vitro studies is that very high doses/concentrations in culture media are used that have no relevance for realistic in vivo conditions. Moreover, results of in vitro assays can be misleading due to interference of the nanomaterials to be tested with the testing reagents, adsorption of induced mediators (Worle-Knirsch et al., 2006; Han et al., 2011), or interference with optical measurements (Pfaller et al., 2010); general problems related to sensitivity and reliability of in vitro systems have also been discussed (Jones and Grainger, 2009). A most recent example of reliability problems of in vitro assays in evaluating the toxicity of CNTs is described by Haniu et al. (2011) who reported contradictory results of increased as well as decreased cell viability as well as either no or high cytokine release when the same CNT and the same cell type were dosed using different dispersants for pretreatment.

Concepts of Nanotoxicology

The previous section discussed the importance of physicochemical properties of ENM. Fig. 29-8 summarizes several of these properties that are known to affect biological/toxicological effects. The size and size distribution are important determinants—aerodynamic—for the deposition efficiency of inhaled materials throughout the respiratory tract (see Fig. 29-8). The quality of dispersion in liquid media can be determined by measuring the hydrodynamic diameter, which is important information for in vitro studies and the ENM behavior in blood and lymph circulation. The shape of ENM (tubes, fibers, planes) influences their aerodynamic and hydrodynamic behavior and is of special significance for cellular uptake, for example, a fiber shape of straight nanotubes with a length exceeding the diameter of alveolar macrophages so that they cannot be completely phagocytosed (frustrated or incomplete phagocytosis) resulting in potent activation and release of inflammatory mediators (Kane and Hurt, 2008). Individual NPs tend to agglomerate (held together by relatively weak forces, e.g., Van der Waals forces) or they are aggregated when produced (sintered together or firmly bundled in the case of fibers) (ISO/TS 27687, 2008; Teleki et al., 2008; Taurozzi et al., 2011). Oftentimes, larger structures in the form of agglomerated aggregates are formed (Fig. 29-8), both in the airborne state and as liquid suspensions.

The terms "agglomeration" and "aggregation" are often used interchangeably, yet ISO/DIS (2011) defines them as based on the forces that keep the primary particles together (agglomerates: weakly bound with external surface area similar to the sum of surface areas of individual components; aggregates: strongly bonded or fused with the external surface significantly smaller than the sum of calculated surface areas of individual components). Both agglomerates and aggregates are also termed "secondary particles" (ISO/DIS, 2011).

The formation of agglomerates of aerosols (coagulation) depends on their number concentration in the airborne state and is a function of time (Hinds, 1982). The effect of such time-dependent agglomeration on decreasing the number of airborne particle concentration and on increasing the overall particle size is illustrated in Table 29-4 assuming simple monodisperse coagulation in still, undisturbed air. For example, it takes only 20 minutes for a very high number concentration of 10^{14} particles/cm^3 to decrease by half, whereas it takes 231 days if the initial concentration is only 100 particles/cm^3. Similarly, in order to double the initial particle size because of a resulting increase in the agglomerate particle size,

Table 29-4

Time for Number Concentration to Halve and Particle Size to Double by Simple Monodisperse Coagulation

STARTING AIRBORNE CONCENTRATION (number/cm³)	TIME TO REACH HALF THE CONC. (½ N_s)	TIME TO DOUBLE PARTICLE SIZE ($N_D = 0.125\ N_s$)
10^{14}	20 μs	140 μs
10^{12}	2 ms	14 ms
10^{10}	0.2 s	1.4 s
10^{8}	20 s	140 s
10^{6}	33 min	4 h
10^{4}	55 h	16 days
10^{2}	231 days	4 year

SOURCE: Data from Hinds (1982).

it takes 140 μm for the initially very high concentration, but 4 years for the low concentration. This dramatic shift in particle size distribution needs to be considered when high concentrations of airborne NPs are measured close to their source.

The agglomeration and the aggregation state of ENM will significantly influence their bulk and apparent density. For example, the specific density of TiO_2 is ~4 g/cm³, whereas the bulk density (density of a powdered material including interparticle spaces) of nano-TiO_2 depending on primary particle size ranges from 0.13 to 0.54 g/cm³. Bulk density—interchangeably also described as apparent or packing density—can be measured by pouring the ENM powder into a fixed volume of a graduated cylinder (freely settled). The volume of the measured mass/cm³ includes the particle volume, the interparticle void volume, and any internal pore volume. Using multiwalled carbon nanotubes (MWCNTs) as an example, the specific density of the base material, carbon, is ~2 g/cm³, but the bulk density of MWCNT was reported as ranging between 0.043 and 0.31 g/cm³ (Ma-Hock et al., 2009; Pauluhn, 2010). The density achieved by packing of individual agglomerate structures of different MWCNTs obviously plays an important role for differences of the bulk or packing density, as do the dimensions of length and diameter, the straightness of the tubes, and the form and size of tangled structures. Knowledge of bulk density is important for understanding the aerodynamic behavior of ENM and their deposition in the respiratory tract. In simple general terms, the aerodynamic diameter (D_{ae}) of an aerosol is correlated with the physical geometric particle diameter (D_{geom}) as $D_{ae} = D_{geom} \times \sqrt{\rho}$ (ρ = material density in g/cm³). For particles below 1 μm, a slip-correction factor needs to be added. For highly agglomerated ENM, it is preferable to replace the true material density with the value of bulk or apparent density in order to more accurately estimate the aerodynamic diameter.

A low bulk density obviously increases the volume of ENM after uptake into cells relative to the volume occupied by the same mass of non-agglomerated material. A volumetric overload concept for alveolar macrophages has been proposed by Morrow (1988), who suggested that when a phagocytized volume of 60% of the normal macrophage volume is reached, movement and clearance function of macrophages is completely abolished, and that a retardation of the clearance function of macrophages starts when ~6% of the cell's volume is exceeded by phagocytized particles. Pauluhn (2010) applied this concept of volumetric overload to results of his subchronic inhalation study with MWCNT in rats for estimating an occupational exposure level (OEL). However, a recent review of this concept by Borm et al. (2015) concluded that a volumetric overload of macrophages does not apply to ultrafine poorly soluble particles. Instead, the particle surface area correlates best with the induced alveolar inflammation and retarded macrophage clearance function.

Surface characteristics of ENM are a critical determinant for interpreting dose–response relationships (surface area as dosemetric), and for nanomaterial cell interactions. Uptake into cells is influenced by their surface charge, surface reactivity, the chemistry of surface coatings (functional groups, polymeric coatings), and also surface defects to the material as-synthesized, or introduced during surface functionalization or processing such as acid purification or ultrasonication for dispersion.

Solubility and solubilization rate are of interest because the shortened biopersistence of NP in the organism terminates any NP-size-related effects. Zinc oxide is an example of an NP that undergoes rapid dissolution, thereby giving rise to high ionic zinc concentrations and induction of inflammation and oxidative stress (Xia et al., 2008b) in contrast to poorly soluble CeO_2 or TiO_2 NPs. Many ENPs are insoluble in the as-produced form (e.g., metallic Ag^+ and Ni^{2+} metallic NPs), and do not undergo simple dissolution, but can undergo chemical oxidation, a kind of nano-corrosion, in solution, in tissue, or in the environment to produce soluble species (e.g., Ag^+ and Ni^{2+}) in a process that gradually degrades and eliminates the particle state (Liu and Hurt, 2010; Liu et al., 2010a; Pietruska et al., 2011). Such NPs can act via a "Trojan Horse" mechanism in that they are taken up into cells and subsequently dissolve, thereby creating a very high intracellular ionic metal concentration that is cytotoxic (Limbach et al., 2007). The same ionic concentration outside the cell will elicit no or only a modest response because of selective cell barriers preventing metal ions from entering the cell. The results by Xia et al. (2008b) and Limbach et al. (2007) are based on in vitro studies. Donaldson et al. (2009) have cautioned that great care should be taken in interpreting that oxidative stress induction by particles observed in vitro will also occur in vivo. This is not to say that the "Trojan Horse" effect does not occur in vivo; it has been described and is being used in designing drug delivery via NP for therapeutic applications (Kreuter, 2004; Iezzi et al., 2012). The cautionary note is more generally directed toward the extrapolation of in vitro-based oxidative stress results to in vivo conditions.

It is a major challenge for nanotoxicological studies that many of these properties, once they have been determined for a given ENM, are not stable but can change when prepared for toxicological testing or during storage or testing. The fact that alterations can occur through oxidation or dissolution (above), or through adsorption of proteins and lipids once they have been taken up into different compartments of the organism, has become an important area of research (Cedervall et al., 2007a, 2007b; Lundqvist et al., 2008; Monopoli et al., 2011), yet methods to study these changes on the surface of ENMs in vivo still need to be developed.

Dosemetrics

Given the importance of physicochemical properties of ENM as determinants of their biological/toxicological properties, the question of the proper dosemetrics has been raised. Toxicologists are used to thinking in units of mass or molarity when characterizing a dose or concentration. However, several in vitro and in vivo studies with NP have shown that expressing dose–response relationships on a convenient mass basis may not be as informative as the surface area of the NP (Oberdörster et al., 1992; Donaldson et al., 1996;

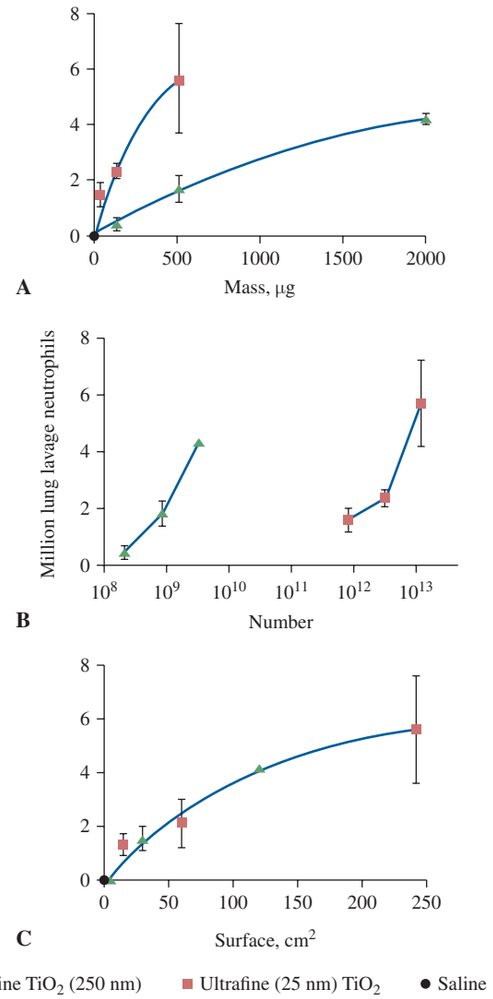


Figure 29-10. *Inflammatory cell response (neutrophil number in lung lavage of rats 24 hours after intratracheal instillation of two sizes of TiO_2 particles expressed by different dosemetrics.* Particle-mass (**A**); -number (**B**); -surface area (**C**). (Reproduced with permission from Oberdörster et al. [2007a].)

Tran et al., 2000). Fig. 29-10 shows the pulmonary inflammatory dose–response relationships of two sizes of TiO_2 particles induced by intratracheal instillation in rats. A significantly greater influx of inflammatory neutrophils into the lung was induced by 25 nm TiO_2 per unit mass than by 250 nm TiO_2. The result is a very steep dose–response for the nanosized TiO_2 and a flatter dose–response for the larger TiO_2. Likewise, there was a clear separation of the dose–response when based on the number as dosemetric; however, when the same data were expressed based on particle surface area, a common dose–response relationship emerged (Fig. 29-10).

Although the concept of particle surface area as dosemetric is biologically plausible because it is the surface of particles that interacts with cell structures, the total surface area may only be a surrogate of the actual biologically relevant surface. As was discussed above, there are a number of surface properties (Fig. 29-8) that affect interaction with cells. Thus, identifying a biologically available surface area will be of great value for defining a proper dosemetric. At present, the specific surface area of ENMs (m^2/g) is most commonly measured by vapor adsorption, in which the number of vapor molecules (usually nitrogen) adsorbed on solid surfaces is quantified over a range of standard conditions and the surface area estimated by application of the BET theory (Brunauer et al., 1938). Both the external and existing inner surface (porosity) in pores larger than the nitrogen molecule are determined by this technique. There is a need for standardization of the method with regard to the use of nitrogen or other gases (Kr; Ar) because different molecule sizes will "see" and report different surface areas in molecular-sized pores (<0.4 nm). Measurements of BET surface area can also contribute to differentiation between agglomerated and aggregated structures; for example, agglomerated NPs have essentially the same specific surface area as the same mass of individual primary particles, whereas aggregated particles have a somewhat lower specific surface area than the same mass of primary particles (ISO/TS 27687, 2008). The measured surface area can then be converted to a BET equivalent particle size for nonporous spherical particles (Teleki et al., 2008).

In addition to mass, number, and surface area as dimensions for a dosemetric, the volume of NPs has been suggested as another dosemetric. This suggestion is based on the interpretation of earlier long-term inhalation studies in rats in which very high concentrations of different poorly soluble particles of low cytotoxicity had resulted in highly overloaded alveolar macrophages so that their normal clearance function was severely impaired or had come to a standstill. Based on the results of these studies, Morrow (1988) formulated a "particle overload" hypothesis: When the volume of phagocytized particles in alveolar macrophages exceeds 6% of the normal macrophage volume, their physiological clearance function becomes impaired; if the phagocytized volume reaches 60%, the clearance function ceases completely. For certain ENMs the volume that they occupy can be very high, for example, larger agglomerates of MWCNT, forming a loosely packed (low specific density) structure that takes up a large volume when taken up by alveolar macrophages in the lung. Pauluhn (2010) has applied this volumetric overload concept to results of a well-performed subchronic rat inhalation study for estimation of a human occupational exposure limit.

Follow-up studies to the mechanistic basis of lung particle overload points to particle surface area of agglomerated phagocytized nano and larger particles as dosemetric, which appears to correlate better with the impaired clearance function of alveolar macrophages rather than the phagocytized volume (Oberdörster et al., 1994; Tran et al., 2000). Borm et al. (2015) pointed out in a recent update that a volumetric overloading of alveolar macrophages following repeated inhalation by rats of high concentrations of poorly soluble ultrafine particles (TiO_2) does not correlate with the induced alveolar inflammation or the impaired alveolar macrophage clearance function. Rather, the surface area of the retained phagocytized particles should be considered the appropriate dosemetric for UFP. Thus, deriving OELs for ENMs from results of chronic rodent inhalation studies based on the volumetric lung particle overload concept is scientifically not justified. Yet—as pointed out before—it is important to remember that not only physical—volume, surface area—but also chemical—surface associated—properties of NPs are critical determinants of effects resulting from NP–cell interactions (Fig. 29-8).

Among the different dosemetrics discussed in this section, particle number is of particular importance with respect to characterizing exposure. Exposure concentrations in the air are expected to be very low, which makes it difficult to determine the precise concentration based on measuring mass. However, a low mass of NP consists of a huge number of particles that can reliably be measured in the airborne state using scanning mobility particle sizers or concentration particle counters. Thus, NP number as an exposure-metric

Table 29-5

Mass/Number/Surface Area Correlations for Selected NPs

PARTICLE TYPE	DIAMETER (nm)	DENSITY (g/cm^3)	SPC. SRF. AREA (m^2/g)	SPC. NUMBER (#/g)	AIRBORNE CONC. OF 100 $\mu g/m^3$ SURFACE (cm^2/m^3)	AIRBORNE CONC. OF 100 $\mu g/m^3$ NUMBER (#/m^3)
Pt	50	21.09	5.69	7.24×10^{14}	5.69	7.24×10^{10}
Gold	50	19.3	6.22	7.91×10^{14}	6.22	7.92×10^{10}
Ag	50	10.5	11.43	1.46×10^{15}	11.43	1.46×10^{11}
Cu	50	8.9	13.48	1.72×10^{15}	13.48	1.72×10^{11}
Al	50	2.7	44.44	5.65×10^{15}	44.44	5.65×10^{11}
TiO_2(R)	50	4.23	28.37	3.61×10^{15}	28.37	3.61×10^{11}
TiO_2(A)	50	3.9	30.77	3.92×10^{15}	30.77	3.92×10^{11}
C	50	2.26	53.10	6.76×10^{15}	53.10	6.76×10^{11}
Polystyrene	50	1.05	114.3	1.46×10^{16}	114.3	1.46×10^{12}
	5		1143.0	1.46×10^{19}	1143.0	1.46×10^{15}
	10		571.4	1.82×10^{18}	571.4	1.82×10^{14}
	100		57.14	1.82×10^{15}	57.14	1.82×10^{11}
	1000		5.71	1.82×10^{12}	5.71	1.82×10^{8}

for mainly isometric-type NP is a valuable component for exposure characterization.

Table 29-5 shows the correlation among the different dosemetrics of different 50-nm-sized isometric NPs, with specific material densities ranging from 21 to 1 g/cm^3. Volume is not listed in this table because it depends on the bulk or packing density, which is different from the material density because of additional void spaces between the packed NP. Surface area and number per unit mass and per 100 $\mu g/m^3$ air volume vary greatly. The table also shows that a 200-fold change of particle size from 5 nm to 1000 nm results in a more than six orders of magnitude change in number concentration with only a 20-fold change in surface area for all NP (shown for polystyrene in Table 29-5). However, although these correlations can readily be established for dry powders, even when they consist of polydispersed particle sizes, these correlations will change upon inhalation and disposition in the body because of different deposition and biodistribution efficiencies in the lungs and other organs.

Portals of Entry

The respiratory tract, the gastrointestinal (GI) tract, and skin are the main organs of direct exposure to ENM. For medical application, injection (intravenous, intramuscular, subcutaneous, and other) will also be an important entry route into the organism. Intake via the respiratory tract is the most prevalent exposure route for occupational exposures; additives of ENM to food (TiO_2; SiO_2) and potential contamination of food from nanoenabled packaging materials result in exposure via GI tract (Jani et al., 1990, 1994; Hoet et al., 2004; FAO/WHO, 2009; Loeschner et al., 2011). Based on available data, translocation of nanomaterials in vivo across GI-tract epithelial cells seems to be limited. Trouiller et al. (2009), however, described DNA damage in bone marrow cells following very high gavage dosing of rats. Skin exposure via cosmetics and skin-care products occurs, although penetration of healthy skin by NP has not been demonstrated (Schneider et al., 2009; Monteiro-Riviere et al., 2011; Prow et al., 2011). Some penetration of quantum dots (~30 nm) applied to the skin of mice after UV radiation-induced sunburn was found (Mortensen et al., 2008). Because the respiratory tract is a major route for humans to exposure of nanomaterials, the following sections will focus on this portal of entry to discuss biokinetics, effects, and concepts for in vivo and in vitro toxicity testing.

Dosing of the Respiratory Tract

The following sections focus on the respiratory tract as portal-of-entry of airborne ENM.

Dosing of the respiratory tract of laboratory rodents as the portal-of-entry for airborne ENMs is mostly and conveniently done by intratracheal instillation (rats) or oropharyngeal aspiration and intranasal instillation (mice), which administer materials as a bolus in a second or less. However, inhalation is the only physiological method and should be considered the gold standard for exposure to airborne materials. Obvious major differences between bolus-type and inhalation exposures relate to the dose rate (see discussion later), use of anesthesia, the distribution of administered material within the respiratory tract, and the need for special expertise and equipment required for inhalation but not bolus-type exposures. Although most of the differences between inhalation- and instillation-type delivery are well known and have been discussed in a Society of Toxicology (SOT)-sponsored White Paper (Driscoll et al., 2000), the limitations of using and interpreting results from bolus-type dosing are most often not considered (Table 29-6). This white paper concluded with several recommendations when intratracheal instillation can be applied. These included evaluation of comparative toxicity involving screening, ranking, range finding of doses, comparison to reference material; also, evaluation of material not respirable by rodents. In order to minimize interference of clumping and localized inflammation, only <~100 μg/rat should be used. The authors also mention situations when instillation should

Table 29-6

Instillation versus Inhalation

ADVANTAGES	DISADVANTAGES
• Low cost	• Non-physiological
• Simple technique	• Invasive delivery
• Less material needed	• Very high dose rate
• Delivered dose is known	• Usually also high dose, overwhelming lung defenses
• Ranking of toxicities	• Bypassing upper respiratory tract
• Avoiding fur contamination (*handling hazard*)	• Different distribution in lower resp. tract: more central, more heterogeneous, less uniform
• Lower risk to investigators	• Focal high lung burdens
• Nonrodent-respirable aerosol can be delivered (*human health relevant*)	• Potential impact of vehicle on distribution, effects, material to be tested
• Specific areas of lung can be dozed (*larger animals*)	• Potential confounder of anesthesia
• Screening for deciding concentr. for inhalation	• Multiple instillations to reduce heterogeneity
• Identifying toxic components of mixture	

Note: Qualitatively, both methods yield similar results for variety of biologic endpoints (pulmonary inflammation; fibrosis; susceptibility to infection, allergic sensitization, PSP lung tumors), but requires thorough understanding of differences between both methods to avoid misinterpretation of instillation results.

SOURCE: Data from Driscoll et al. (2000).

not be considered: for determining deposition pattern in lung to mimic inhalation; toxic effects in the upper respiratory tract; not to use for materials that react with vehicle; they also cautioned that short-term clearance (mucociliary) is not reflected accurately.

Of special importance is the tremendous difference in the delivered dose rate: Bolus-type delivery occurs within a fraction of a second, whereas inhalation at realistic concentrations takes hours to months of exposure in order to deposit the same dose in the lung. Treating a dose delivered by bolus to be the same as a dose that has accumulated in the lung over a life-long exposure is scientifically not justifiable and needs to be discouraged (Oberdörster, 2012). Inundating cells abruptly with an extraordinarily high dose does overwhelm the cell's defense mechanisms and leaves no time for developing adaptive responses. Consequently, underlying mechanisms of effects induced by such unrealistic high doses are different from those induced by relevant doses and dose rates (Slikker et al., 2004, see Fig. 29-3). With respect to the administered dose rate, Fig. 29-11 (Baisch et al., 2014) illustrates a tremendous difference of inducing a pulmonary inflammation by either intratracheally instilling (~0.5 second duration) 200 µg of TiO_2 NP versus depositing the same dose by inhalation over a period of 4 hours or 4 days (4 × 4 hours). The difference in dose rate is four to five orders of magnitude, with a strong inflammatory response at the highest dose rate (instillation) and no response at the lowest dose rate (inhalation).

Evidence that extremely high doses acutely administered within a very short time overwhelm the organism's or the cell's defenses so an adaptive response cannot be mounted was presented in a rat inhalation study with nanosized particles using exposure to UFPs (~18 nm count median diameter) of polytetrafluoroethylene (PTFE) fumes (Johnston et al., 2000). PTFE fumes, similar to metal fumes, when freshly generated consist of nanosized particles, which are highly reactive and are known to give rise to polymer fume and metal fume fever in occupationally exposed workers (Drinker et al., 1927a, 1927b; Drinker and Drinker,1928; Lee and Seidel, 1991; Seidel et al., 1991). When groups of rats were exposed for 5 minutes on each of 3 days to either filtered air or to a concentration (~50 µm³) of these PTFE fumes, followed on day 4 by a 15-minute exposure of both groups to the same concentration of PTFE fumes, the pre-exposed rats survived without any significant

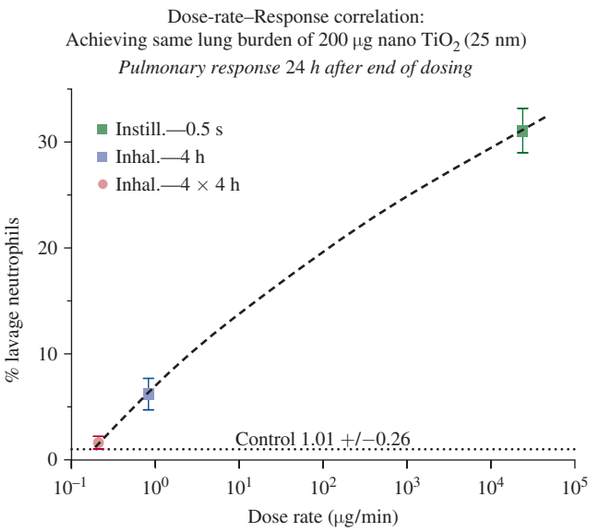


Figure 29-11. *Dose-rate–response correlation.* Deposition of 200 µg nano-TiO_2 in the lungs of rats either by instillation (high dose rate) or by inhalation (low dose rate) induces widely differing pulmonary inflammatory responses as determined by the appearance of inflammatory neutrophils in lung lavage. (Figure provided by G. Oberdörster based on Baisch et al, 2014.)

adverse pulmonary responses (not significantly different from rats that were sham exposed on all days), whereas the filtered air pre-exposed rats died within 3 hours after the 15-minute PTFE exposure (Fig. 29-12A). Estimated deposited dose of PTFE in the lung was only ~60 ng. Further lung tissue analyses revealed that the PTFE pre-exposed rats had mounted a strong "adaptive" response (increased antioxidant and chemokine mRNA), whereas nonadapted rats were unable to induce such a response (Fig. 29-12B). Evidence for such adaptation following inhalation exposure to nanosized particles in humans was already reported eight decades ago when Drinker et al. (1927b) described that repeat exposure to zinc oxide fumes induced "resistance" in human subjects. Adaptive responses are important physiological protective mechanisms, which need to be considered

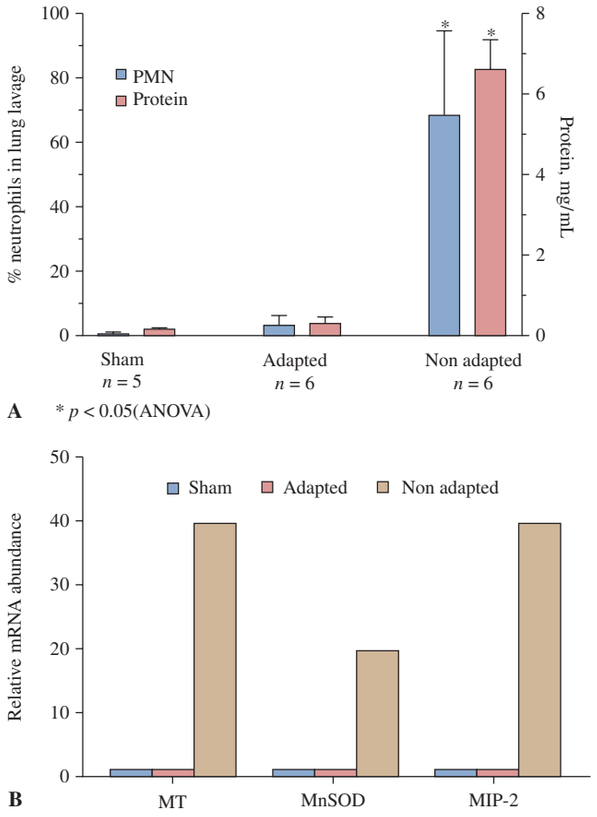


Figure 29-12. *Preventing acute pulmonary injury in rats from inhalation of polymer fume (PTFE, nanosized particles, ~18 nm count median diameter by inducing adaptation with repeated 5-minute PTFE pre-exposures on each of 3 days prior to 15 minutes to exposure on day 4).* (**A**) Adapted rats show no inflammatory response assessed by neutrophil cells in lung lavage; nonadapted rats develop severe lung injury after 15-minute PTFE exposure. Control rats were sham-exposed on all 4 days. (**B**) Change in relative abundance of lung mRNA in PTFE adapted and nonadapted and sham-exposed control rats (Johnston et al., 2000).

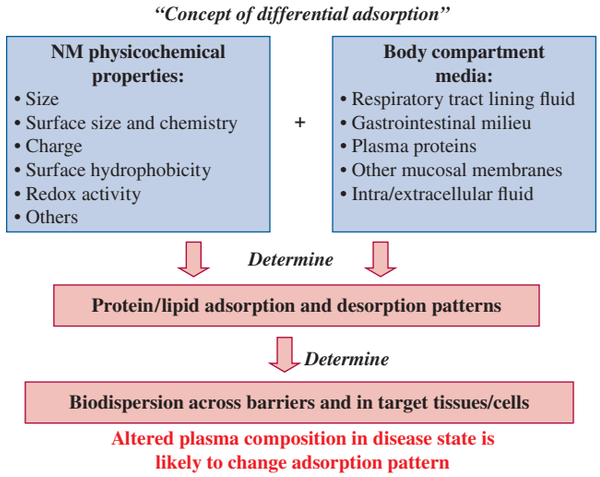


Figure 29-13. *Physicochemical properties of nanomaterials influence the adsorption of proteins and lipids from different organs, which affect the biodistribution of nanomaterials.* (Used with permission from Müller RH, Keck CM. Drug delivery to the brain realization by novel drug carriers. *J Nanosci Nanotechnol.* 2004;May:4(5):471–83.)

when interpreting results of nanomaterial toxicity testing. However, the organism's capacity to adapt may not only be compromised by sudden very high acute exposures of short duration (high dose and high dose rate) but may also be impaired in susceptible parts of the population.

Despite the limitations of bolus-type delivery, they may be viewed as "proof-of-principle" or "hypothesis-forming studies," with the findings to be confirmed by subsequent inhalation studies. Although results from bolus-type studies are useful for toxicity ranking against known positive or negative control ENMs, they cannot be used for quantitative risk assessment. Other in vivo bolus-type deliveries involve intravenous, intraperitoneal, or intrapleural injections. If used for safety assessment of medicinal applications of ENMs, including CNTs, an appropriate range of doses should be applied. However, if intravenous or intracavitary injections are used to simulate doses resulting from translocation of inhaled CNTs after deposition in the lung, results have to be viewed with caution. The amount of NPs translocating from the lung to the blood circulation and subsequently to secondary organs is very low, on the order of 1% to 3% (Kreyling et al., 2002, 2009). Furthermore, the translocation rate from the lung is very low; it occurs over hours and days, which contrasts with the immediate very high dose rate of injection. Bolus-associated problems have been discussed above, and confirmation of results from such "proof-of-principle" studies by follow-up inhalation studies should be mandatory.

The pretreatment of ENMs in preparation of bolus-delivery dosing is an additional difference compared to inhalation and may raise some concern: The recommendation to use dispersants in order to administer the materials in a well-dispersed mode (Fubini et al., 2010; Taurozzi et al., 2011) results in the coating of the treated nanomaterials that changes their surface properties and renders them different relative to their pristine state when inhaled under real-world exposure conditions. Although inhaled ENMs will also be coated by lipids and proteins of the lung lining fluid once deposited, such protein corona is likely to change upon translocation to interstitial and blood compartments based on the "concept of differential adsorption" (Müller and Heinemann, 1989). This concept states that the physicochemical properties of nanomaterials, such as size, surface properties, shape, dissolution, and others when in contact with media in the different body compartments determine protein and lipid adsorption and desorption patterns and thereby influence biodistribution across barriers and in target tissues and cells (Fig. 29-13). This concept has been further developed more recently by Dawson's group (Cedervall et al., 2007a; Lundqvist et al., 2008; Walczyk et al., 2010) who describe the formation of hard and soft protein coronas around NPs upon incubation with blood plasma. Future studies have to show how pretreatment of ENMs for bolus-type in vivo administration will affect both biodistribution and effects. In vitro experiments with various cell types have already demonstrated that the use of different dispersants with MWCNTs resulted in contradictory results (Haniu et al., 2011).

The three different methods of bolus-type dosing of the respiratory tract—intranasal instillation; intratracheal instillation; oropharyngeal aspiration—represent a convenient way to obtain first or preliminary data about effects in the respiratory tract. The limitations and caveats of using these modes of administration—as pointed out in the preceding paragraphs—should be taken into account when interpreting the results. Immediate short-term inflammatory responses will be induced, and these early bolus-associated effects are likely to also influence long-term effects. For example,

when Li et al. (2007) compared pulmonary responses to inhaled and intratracheally instilled MWCNTs in rats, they observed a much lower response in terms of pulmonary pathology after inhalation despite a higher lung burden from inhalation compared to instillation. It is of interest, though, that in another study similar inflammatory, granulomatous, and fibrotic responses were observed in mice following inhalation and oropharyngeal aspiration of SWCNTs. In fact, inhalation exposure was more potent than aspiration of an equivalent mass (estimated) of SWCNTs 1 to 28 days after inhalation exposure to a high concentration (5 mg/m^3, 4 days @ 5 h/day) or oropharyngeal aspiration of 5 and 10 μg (Shvedova et al., 2008). The authors suggested that the similarity of responses between inhalation and aspiration exposure to SWCNTs in mice may be due to smaller SWCNT structures by inhalation of a dry aerosol versus aspiration of a suspension containing micrometer-sized agglomerates. However, both the Li et al. (2007) and Shvedova et al. (2008) studies only estimated the doses deposited in the lung upon inhalation based on inhaled concentration and size distribution and retained doses were not confirmed by actual measurements. In addition, the inhalation exposure by Li et al. was carried out with a static system; that is, MWCNTs were introduced into the exposure chamber as an aerosol and kept there for 90 minutes to slowly settle down while the rats were exposed. This makes it very difficult to determine the actual exposure concentration because it continually decreased, and changes in particle size due to rapid settling of the aerodynamically larger particles will occur. A dynamic exposure system with continuously generated aerosol is the standard and preferred method for inhalation exposures.

Respiratory Tract Deposition

Inhalation by humans of airborne ENM at the workplace or when handling powders of ENM results in significant deposition in the three compartments of the respiratory tract: the nasopharyngeal region from nose/mouth to the larynx; the tracheobronchial region from larynx to terminal bronchioles; and the alveolar region from the first generation of respiratory bronchioles (bronchioles with some alveoli) to the last generation of alveolar ducts. The deposition efficiency of inhaled particles depends on several particle characteristics, the anatomical structure of the airways, and breathing parameters. Particle size, size distribution, density, and shape are most important for their aerodynamic and thermodynamic diameter, which govern deposition in the respiratory tract by inertial impact, gravitational settling, and displacement by diffusion. In addition, interception (in particular for elongated structures or fibers) and electrostatic image forces (positive charge on particles attracted by negative epithelial surface) play a role in certain conditions (see Chap. 15).

Because in vivo toxicological studies are mostly performed in experimental animals, and because of anatomical and some physiological differences in the respiratory system, knowledge about variations in the behavior of inhaled particles is essential for interpreting results of animal inhalation studies with respect to dosimetric *extrapolation* to humans. Several particle deposition models have been developed based on mathematical modeling and supported by results of numerous human studies using inhalation of benign particles of different sizes. The two most frequently used models are the extensive international commission on radiation protection (ICRP) (1994) model for humans, and a user-friendly (computer) Multiple Path Particle Dosimetry (MPPD) model for rats and humans (Asgharian et al., 1999) (available at: MPPD, 2018). Each of these models is based on results of an extensive database of particle deposition studies in humans (ICR Panel MPPD) and in rats (MPPD).

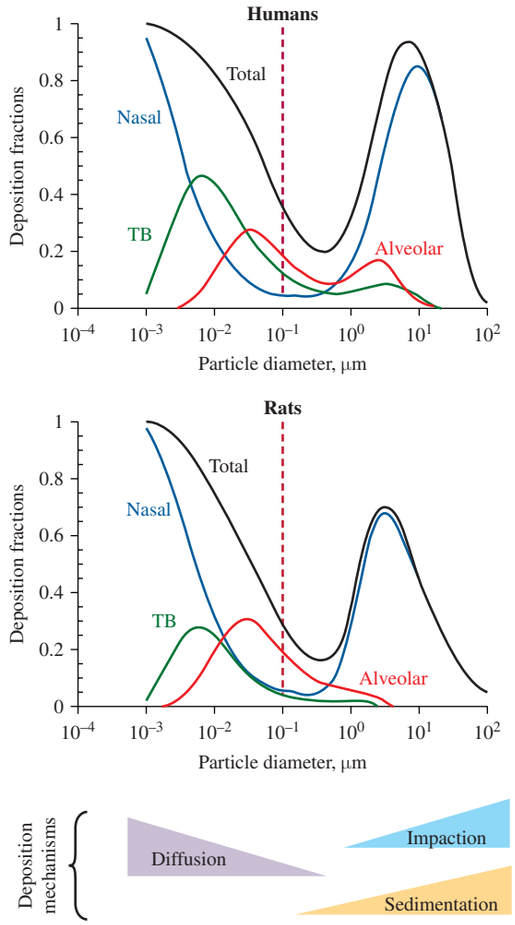


Figure 29-14. *Model predictions of deposition fractions for different particle sizes in humans and rats.* Nanosized particles are left of dashed line (<100 nm); their deposition is governed by diffusion (Brownian motion of air molecules). (Figure provided by B. Asgharian.)

These data have been incorporated into mathematical equations describing airborne particle behavior due to impaction, sedimentation, and diffusion for different airway geometrics in humans and rats. There is good general agreement between results of the two models, although some quantitative differences exist. It should be kept in mind that these are still models to estimate the deposition in the respiratory tract, depending on particle parameters, airway geometrics, and breathing modes. Interindividual time values can be very different from the averages that are calculated by the models.

Fig. 29-14 compares the deposition fractions for monodispersed particles of sizes from about 1 nm to 20 μm diameter between humans and rats based on the MPPD model, assuming unit density material. For both species nasal breathing is assumed, which is the obligatory mode for rats. For NPs below 100 nm (in the thermodynamic size range, less than ~0.5 μm), there is only a small difference in deposition between nasal- and oral-breathing mode, whereas these differences become more pronounced for larger particles in the aerodynamic size range (starting at ~0.5 μm). The mechanisms by which inhaled particles are depositing changes from diffusion (due to bombardment of particles by air molecules) as exclusive mechanism for airborne single NPs to gravitational sedimentation and inertial impaction for the larger particles. Comparing deposition efficiencies between rats and humans, not

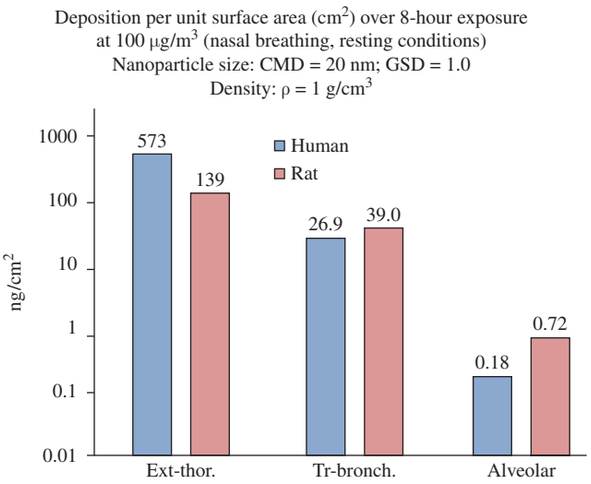


Figure 29-15. *Deposited amount of inhaled nanoparticles of 20 nm diameter (density 1 g/cm³) in the three regions of the respiratory tract of humans and rats, calculated with the Multiple Path Particle Dosimetry model (see text).* Assumed is an inhaled concentration of 100 μg/m³ over an 8-hour exposure with nasal breathing under resting conditions. Deposition is normalized per cm² of surface area of a specific airway region.

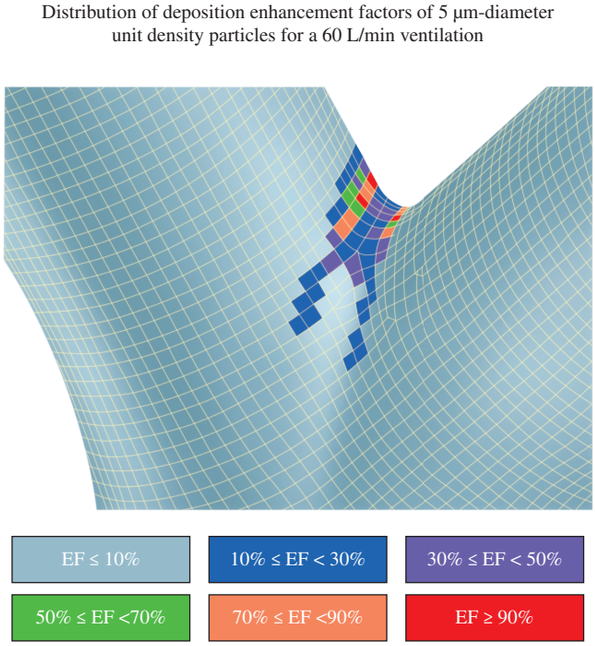


Figure 29-16. *Distribution of hotspots of deposition on carinal ridges of the bronchial region in the human respiratory tract.* The enhancement factor is largest for aerodynamically large particles, but is very low for airborne nanosized particles. (From Balásházy et al. [2003].)

only similarities but also differences become obvious: Qualitatively, size-dependent deposition fractions are similar; for example, there is a lowest point of deposition for inhaled particles in both species around the size of 0.5 μm, which is due to the fact that at this size the combined impact of diffusional displacement and gravitational settling is minimal, so they are most persistent in the air. Of interest is that—contrary to a general misconception—the smallest inhaled particles (<5 nm) are not depositing in the most distal areas of the lung, but in the upper extrathoracic region. Thus, the nasal filtering capacity is effective for both the smallest and largest particles.

Obvious differences between rats and humans are the maximum size of particles that are respirable, that is, will reach the alveolar region. In rats, this is about 5 μm aerodynamic size, in humans about 15 μm. Although these sizes are outside the range of single NP, airborne NPs occur for the most part as agglomerates, which can reach μm size equivalent aerodynamic diameters. The concept of rat versus human respirability also applies to smaller μm size particles. Even for submicron-sized particles, rat/human deposition fractions are not necessarily the same. Thus, if a rat inhalation study is designed to simulate a human exposure scenario with an aerodynamic median particle size of 5 μm, not much of the aerosol will reach the rat's alveolar region. Instead, attempts should be made to streamline the particle size for the rat study such that the deposited dose in the alveolar region is similar between the two species relative to alveolar surface area.

The deposition curves in Fig. 29-14 imply that inhaled particles of ~20 nm have similar deposition efficiencies in all three regions of the respiratory tract. However, a more judicious analysis reveals that the alveolar region actually receives the least amount of these NPs when the deposited dose is expressed per unit surface area (Fig. 29-15). The data in this figure are derived from the MPPD model, assuming an 8-hour exposure to 20 nm monodispersed TiO_2 NPs under nasal-breathing conditions for rats and humans. As can be seen, the deposited dose normalized for surface area is less than 1 ng/cm² in the alveolar region, and is highest for the extrathoracic nasopharyngeal region. This result should also serve as a reminder that realistic in vivo doses to cells of the respiratory tract are mostly orders of magnitude lower than doses that are typically applied in vitro to lung epithelial cell cultures. Moreover, the deposited dose in Fig. 29-15 had accumulated over an 8-hour exposure, which is different from a high in vitro dose rate, similar to dose rate differences between in vivo instillation and inhalation (see Fig. 29-10).

The development of deposition hotspots in the respiratory tract during particle inhalation is often cited as justification for using high doses in vitro studies (Phalen et al., 2006). These hotspots occur at the bifurcations (carinal ridges) of the upper generations of the tracheobronchial region where due to impaction more particles per unit surface will deposit compared to the average deposition per unit surface over the length of the airways (Fig. 29-16). Concentrations up to several 100-fold higher than on average in the airway may be accumulated for larger particles. However, as Balásházy et al. (2003) pointed out using computer modeling, the size of the area receiving the highest concentration is the size of square (patch) with a sidelength of only 0.1 nm, and airborne NPs are contributing to these hotspots to a lesser degree only: for a patch size of 1 mm the enhancement factor is about 30-fold for 1 to 100 nm particles. In the alveolar region where the airflow is very low, no deposition hotspots for NPs exist (Balásházy et al., 2008). This non-homogeneous deposition and formation of hotspots seems to correlate with predilection sites for bronchial carcinoma (Schlesinger and Lippmann, 1978; Balásházy et al., 2003), which is further enhanced by less effective mucociliary clearance at carinal ridges.

Respiratory Tract Clearance and Disposition of NP: Nanomaterials

Once NP are deposited in the respiratory tract, they will encounter physiological clearance mechanisms in the nasal–oropharyngeal, tracheobronchial, and alveolar region as described under "Particle

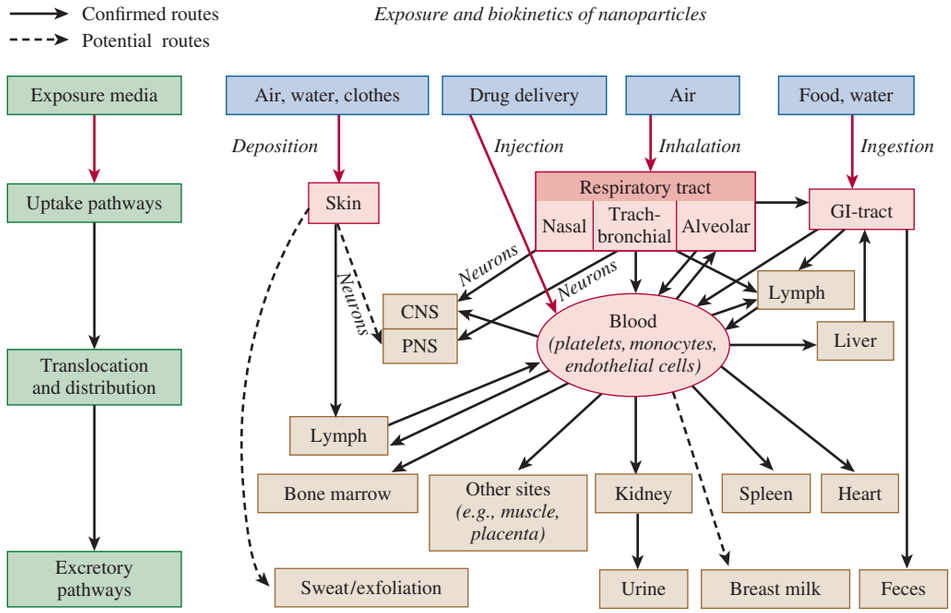


Figure 29-17. *Exposure and biokinetics of nanoparticles' routes of exposure and biokinetics (uptake, distribution, elimination) of nanomaterials.* Translocation rates in general are very low (see text). (Modified from Oberdörster et al. [2005].)

Clearance" in Chap. 15. However, there are several differences that separate NP from larger particles, as indicated in Table 29-2. Alveolar macrophages generally are attracted to deposited particles by chemotactic signals generated at the site of deposition, for example, C5 via activation of complement by particles (Warheit et al., 1988a, 1988b). Nanosized particles may be too small to generate such signal; studies in rodents with inhaled particles ranging from nano- to micro-size have shown that in vivo macrophage surveillance in terms of phagocytosis and clearance of nanosized particles is rather poor (Oberdörster et al., 2005; Semmler-Behnke et al., 2007; Geiser et al., 2008), so that deposited NP will interact with epithelial cells. Uptake into the pulmonary interstitium occurs, and there is evidence that some of the interstitialized NP reenter the airways, possibly the smaller conducting airways, from where they are cleared via the mucociliary escalator (Semmler-Behnke et al., 2007).

Translocation into the interstitium and subsequently into blood and lymph circulation distinguishes nanoparticles from microparticles. Fig. 29-17 depicts the blood compartment as a plenum from which any tissue/organ can be reached by circulating NP. However, the amount of NP translocating from the lung to the blood circulation and accumulation in secondary organs is very low, as results from rat inhalation studies with NP have shown, about 1% to 2% of the lung deposit (Kreyling et al., 2002, 2009; Semmler-Behnke et al., 2007). Long-term retention studies with radioactive iridium NPs (15 to 20 nm) have shown that clearance of NP in extrapulmonary organs following the initial accumulation is very efficient so that after 6 months, with the exception of liver and spleen, only minor amounts—below the level of detection—were still present. Liver and spleen also had decreased sharply to levels of less than 0.05% of the initial lung burden. In general, the overall pattern of long-term clearance from the lungs of rats was similar to that of microparticles with retention halftimes of 70 to 90 days. Elimination of NP from the body occurred via feces (Semmler et al., 2004) and via urine for smaller NPs (see below). Despite the low translocation rates of NP from the portal of entry to secondary organs and efficient clearance, it has to be considered that a continuous exposure may result in significant accumulation in some secondary organs. Significant efforts should, therefore, be made to design studies to assess the biokinetic behavior of ENM following relevant exposures.

Biokinetic studies have shown that organs with major reticuloendothelial system (RES) functions, such as liver and spleen, take up most circulating NP. Coating NP with PEG prevents this uptake by phagocytes and thereby the circulation time is increased, which may be desirable for medical diagnostic or therapeutic applications (Ballou et al., 2004). Other RES organs for accumulation of nanomaterials include the bone marrow and the developing organism in utero. Like the liver and spleen, the bone marrow can receive similarly high or even higher doses of blood-borne NPs (Rinderknecht et al., 2007). As a major RES organ, studies need to be designed to determine responses of stem cells in bone marrow upon interaction with ENM in vivo. A recent study in mice after administration of very high doses in drinking water of TiO_2 NP (~25 nm) for 5 days reported induction of DNA double strand breaks in bone marrow cells (Trouiller et al., 2009). The authors interpreted this finding as secondary to a systemic inflammatory response; multiple other organs also showed oxidative stress responses and genotoxicity. The authors did not attempt to determine the amount of TiO_2 in different organs, which would have strengthened the study.

Organs with tight endothelial junctions, in particular the central nervous system, will not likely accumulate blood-borne NPs, unless the tight blood–brain barrier is damaged or NP surface has been specially modified (Kreuter, 2004). For example, coating NP with the surfactant polysorbate 80 made it possible to deliver a drug loaded into NP across the blood–brain barrier (BBB), to the brain. Translocation across the BBB was facilitated via the endothelial low-density lipoprotein (LDL) receptor, and coating of the NP with ApoE-lipoprotein achieved the same result. The drug was either released to diffuse into the brain after NP uptake into the endothelial cells or via NP transport into the brain (Kreuter, 2004). In either case, the mechanism for drug delivery may be based on a "Trojan Horse" effect in which the coated NP mimic lipoprotein particles.

This underlines again the importance of protein adsorption (corona formation) for the biodistribution of NP following uptake into the organism.

Nanomaterials and the Brain

Fig. 29-18 depicts four basic translocation pathways of inhaled NP from the upper and lower respiratory tract into the CNS. In addition to the blood circulation, which does not efficiently contribute to CNS translocation because of the very tight blood–brain barrier, there is uptake into the lymphatic system—again feeding back into the blood circulation—and transport in or next to sensory neurons from the upper respiratory tract. Perineural translocation along the olfactory nerve will deliver NP into the cerebrospinal fluid (CSF), as reported by Czerniawska (1970) and Zhang et al. (2006). To what degree transport into brain tissue may be inhibited due to a cerebrospinal brain barrier needs to be assessed in further studies. The most efficient pathway of NP translocation to the CNS appears to be via olfactory sensory neurons from the nasal olfactory mucosa directly to the olfactory bulb (Oberdörster et al., 2009). The discovery of this nose to brain connection as an NP passageway goes back to early studies demonstrating this pathway for polio virus ~30 nm transport from the nose to the brain in nonhuman primates following intranasal instillation (Brodie and Elvidge, 1934; Bodian and Howe, 1941), and subsequently visualized by transmission electron microscopy (TEM), with silver-coated gold NP (50 nm) instilled intranasally into squirrel monkeys (De Lorenzo, 1970). More recent inhalation studies with insoluble carbon NP (~36 nm) and poorly soluble Mn-oxide NP (~30 nm) confirmed the olfactory nerve system as an efficient mechanism for airborne NP to reach the CNS (Oberdörster et al., 2004; Elder et al., 2006). Inflammatory responses in the olfactory bulb and several brain regions were induced by 12 days of manganese oxide exposure at concentrations that are lower than those experienced by arc welders after exposure to a mixture of nanosized particles—including manganese oxides. Other nasal instillation studies in mice, applying enormous high doses, reported oxidative stress and inflammatory responses (Wang et al., 2008a, 2008b). Results of epidemiological studies of impaired cognitive function and of neurodegenerative brain pathology associated with exposure to traffic-related particles raised the question as to whether ambient UFPs as constituents of urban air pollution may be etiologically involved (Calderon-Garciduenas et al., 2011; Weuve et al., 2012). Whether such etiological link exists is still to be investigated. The plausibility of a respective hypothesis is supported by findings that loss of olfactory function has been described as a common characteristic of neurodegenerative diseases, that is, Parkinson, Alzheimer, and Huntington disease (Moberg and Doty, 1997; Kovács, 2004; Barrios et al., 2007; Doty, 2008).

In the same study by Trouiller et al. (2009) mentioned before, the authors had also exposed pregnant mice to TiO_2 NP in drinking water for 10 days. The offspring had a higher frequency of DNA deletions in the retina when examined at 20 days of age. Due to the extraordinarily high dose of 500 mg/kg and the lack of verifying TiO_2 in fetal tissues, these results have to be interpreted with caution. Other studies evaluated exposure of the fetus to NP size-dependent translocation across the placenta in mice, with smaller quantum dots transferring more readily than larger ones (Chu et al., 2010). Takeda et al. (2009) injected TiO_2 NP subcutaneously in pregnant mice (100 µg/mouse on each of 4 days). They identified TiO_2 NP in testis and brain of the six-week-old male offspring. The authors also observed reduced daily sperm production and apoptotic cells in the olfactory bulb of these offspring. Results of this bolus-type injection study may be viewed as a proof-of-principle study for demonstrating placenta penetration because of the non-physiological mode of delivery of a high dose and high dose rate; however, the same group had reported in a mouse inhalation study with diesel exhaust the presence of UFPs in the brain of offspring and increased apoptosis of Purkinje cells in the cerebellum (Sugamata et al., 2006).

A more recent proof of principle inhalation study using concentrated ambient UFP for postnatal (day 4 to 13) exposure of mice pups found striking inflammatory effects and microglial activation and persistent male-specific enlargement of lateral ventricles (ventriculomegaly), associated with repetitive and impulsive behavioral effects (Allen et al., 2017). The authors concluded that these results indicate risk factors for autism spectrum disorder and also for other neurodevelopmental disorders.

Elimination of Nanomaterials

Elimination pathways for ENM from the body (Fig. 29-17) include mainly feces and urine. Urinary excretion is restricted to nanostructures <5.5 nm in size for metal-based NP (Choi et al., 2007), limited by the hydrodynamic diameter of the particle that may

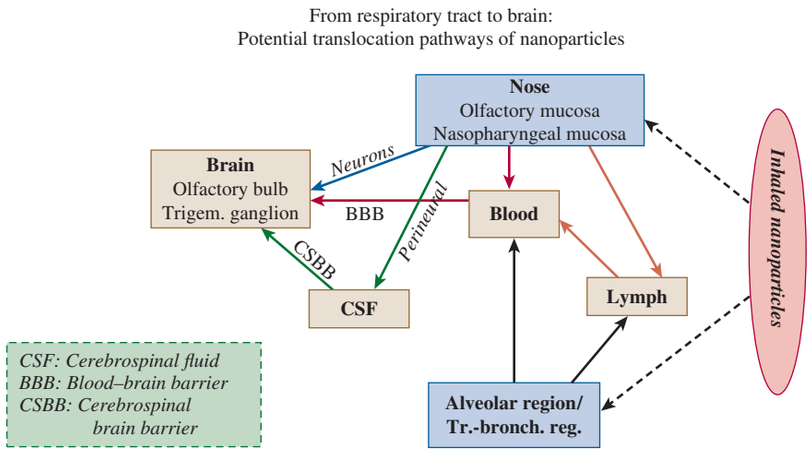


Figure 29-18. *From the respiratory tract to the brain: Potential translocation pathways of inhaled nanomaterials from the upper and lower respiratory tract.* Note that the BBB is very tight and that neuronal pathway circumvent this barrier. (Used with permission from Oberdorster, G., A. Elder, et al. Nanoparticles and the Brain: Cause for Concern? *J Nanosci Nanotechnol*. 2009;Aug:9(8):4996–5007.)

change in circulation due to protein adsorption. This general principle appears to be different for circulating fibrous structures of ENM. Singh et al. (2006) and Lacerda et al. (2008) reported that even 20 to 30 nm diameter MWCNTs with length between 0.5 and 2 μm were collected in urine of rats following intravenous application. They explain this phenomenon by a hydrodynamic lining up of nanotubes so they will pass through glomerular pores. The fecal excretory clearance pathways consist of several inputs: One is mucociliary clearance of deposited particles from the airways into the GI tract; another is via hepatobiliary clearance of blood-borne ENM via liver and bile into the small intestine. This elimination pathway is suggested by results from Semmler-Behnke et al. (2007), and it is also a well-known excretory path for heavy metals in the blood (Clarkson et al., 1988).

With regard to the CNS, no data on ENM elimination are available yet. It is conceivable that the CSF via its connections to the nasal lymphatic system (Czerniawska, 1970) and to the blood circulation could be an excretory pathway for the brain, which needs to be investigated in future studies. Indeed, Segal (2000) concluded from their review on CSF barriers that the CSF may both act as a compartment for distribution of substances to many brain regions, but also can act as an elimination route for waste products into the blood circulation because the brain is considered to have no lymphatics.

However, recent studies in mice reported the discovery of a new cleansing system in the CNS, consisting of lymph-like para-arterial and para-venous vessels in the CNS involving glia cells (Iliff et al., 2012). The authors coined the term "glymphatic system" for this clearance pathway in the brain, connecting to the CSF and clearing interstitial solutes and extra-cellular proteins and peptides such as amyloid beta. This newly discovered system, acting both as delivery (para-arterial) and also as clearance (para-venous) pathway, plays an essential role in maintaining normal CNS physiology (Achariyar et al., 2016). It may well represent also the elusive clearance route for NPs in the CNS.

Another clearance pathway of deposited ENM in the lung involves translocation via interstitium or lymph to the pleura and subsequent elimination via lymphatic openings (stomata) on the parietal pleura to mediastinal lymph nodes from where NP may enter the blood circulation via the right and left thoracic ducts. This pathway is of particular importance for fiber-shaped ENM because the size of the parietal stomata prevents efficient clearance of structures >10 μm in length. Indeed, collection of lymph from the right thoracic duct of dogs that had been dosed intrabronchially with amosite asbestos showed only fibers <9 μm in length appearing in the collected lymph (Oberdörster et al., 1988). As a consequence, the interaction of the retained longer fibers in the pleural cavity with mesothelial cells induce inflammatory and granulomatous responses and in long-term potentially mesothelioma (Donaldson et al., 2010). The following section discusses experimental in vivo data from bolus-type and of the toxicology of CNT that confirm the possibility of a mesotheliogenic response, but also caution to extrapolate directly to realistic inhalation exposures without further verification. A critical appraisal of the available in vivo study concludes this section.

CASE STUDY: MWCNT

Bolus-type Exposures

As described in the previous paragraphs, bolus-type delivery has been the preferred method of dosing to identify as a first step of the potential hazard of ENM. The advantages and disadvantages need to be considered when interpreting the results, in particular the rationale for the administered dose levels causing potential bolus-associated responses should be critically considered. Several doses covering a reasonable range, also including a positive or negative control material for ranking purposes, should be part of a well-designed study. Knowledge about actual—if available—or anticipated exposure levels should be used to justify a selected dose range. Key is a careful physicochemical characterization of CNTs with respect to their original specification (pristine state) as well as any modification due to treatment (dispersants, sonification) prior to administration. Specifically, the presence of impurities (e.g., metals, organics, and amorphous carbon) needs to be considered.

Early intratracheal instillation studies in rats and mice with SWCNTs were performed with very high doses (up to 1.25 mg/rat and 0.5 mg/mouse), causing severe lung injury with rapid persistent granuloma formation including mortality (Lam et al., 2004; Warheit et al., 2004). Doses were so high to induce even physical blockage of conducting airways. Pharyngeal aspiration of lower doses with purified SWCNTs in mice at lower doses caused interstitial fibrosis and granulomatous lesions, possibly due to the existence of large agglomerates in the administered suspension (Shvedova et al., 2005). Subsequent aspiration studies in mice confirmed these findings in mice, showing also that the responses were not affected by the metal content; however, the degree of dispersion influenced responses such that administration of the same mass dose of well-dispersed SWCNTs caused greater and more persistent responses than poorly dispersed SWCNTs (Shvedova et al., 2008; Mercer et al., 2008).

MWCNTs delivered to rats by intratracheal instillation (0.5 to 5 mg/rat) also induced inflammatory, granulomatous, and fibrotic responses; these responses were lower when the material was ground such that the length of the MWCNTs was reduced from 6 to 0.7 μm (Muller et al., 2005). Another study compared pulmonary responses to purified MWCNTs in mice when given either by instillation (50 μg/mouse) or inhalation (32.6 mg/m^3, 6 h/day, up to 15 days, estimated to result in 70 to 210 μg in the lung) (Li et al., 2007). Despite the higher lung dose following inhalation, only moderate effects were induced, whereas instillation resulted in severe inflammation and alveolar wall destruction, demonstrating a stark difference between responses induced by the two modes of dosing with the physiological inhalation exposure avoiding the high bolus-related response. As already mentioned before, the results of both the Li et al. and Shvedova et al. studies have to be interpreted with caution because of the uncertainty of the deposited doses in the lung following inhalation.

Other studies with instilled high doses (up to 1.75 mg/rat) of MWCNTs in rats reported transient inflammatory responses with persistent alveolar wall thickening (Liu et al., 2008). Transient inflammation and granulomatous responses in rats were also reported by Kobayashi et al. (2010) at lower instilled MWCNT doses, whereas Porter et al. (2010) observed a rapid and persistent fibrotic response in mice following aspiration of purified MWCNTs in mice (10 to 80 μg/mouse) which was confirmed by morphometric (lung tissue) analysis (Mercer et al. 2011). Additional evaluations of MWCNT responses in rats and mice confirmed inflammatory, granulomatous, and fibrotic responses following instillation or aspiration dosing (Han et al., 2010; Porter et al., 2010; Aiso et al., 2011).

Translocation of aspirated MWCNT to the pleura following aspiration in mice was also reported (Porter et al., 2010), including penetration into the intrapleural space of mice following aspiration (Mercer et al., 2010). A 6-hour inhalation exposure of mice to a very high concentration of 30 mg/m^3 confirmed that inhaled MWCNTs reaching the subpleural wall (space between peripheral

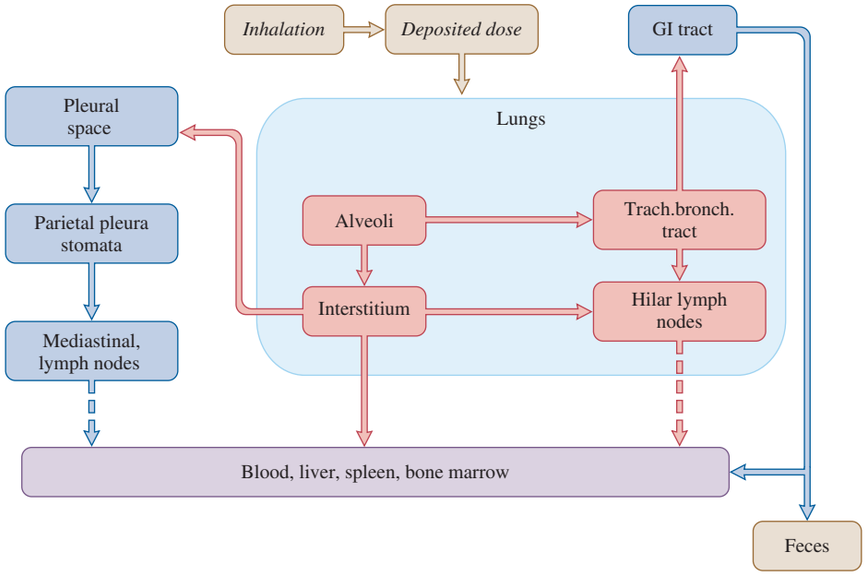


Figure 29-19. *Important translocation pathways of deposited particles in the lung that reach the interstitium are via lymphatic channels to hilar lymph nodes; or migration toward the pleura with subsequent uptake into lymphatic openings (stomata) and clearance toward mediastinal lymph nodes.* Both hilar and mediastinal lymph nodes drain into the right lymphatic duct to reach the jugular vein in the neck area.

alveoli and visceral pleura) are localized within subpleural macrophages (Ryman-Rasmussen et al., 2009a). In summary, bolus-type delivery of CNTs to the respiratory tract of rats and mice revealed induction of dose-dependent significant inflammatory, granulomatous, and fibrogenic responses; they showed also that MWCNTs can reach subpleural and intrapleural sites.

Other bolus-type studies delivered MWCNTs into the peritoneal cavity (intraperitoneal injection, or i.p.)—as a surrogate for the mesothelial lining of the pleural cavity—in order to expose mesothelial cells directly for assessing a mesotheliogenic response. Takagi et al. (2008) found, indeed, mesothelioma induction 1 year following very high intraperitoneal doses in p53 heterozygous mice, which was similar to the positive crocidolite asbestos control. A follow-up study with lower i.p. injection of 50 μg/mouse confirmed this finding (Kanno et al., 2010). Similarly, Sakamoto et al. (2009) induced peritoneal mesothelioma in rats, following high-dose MWCNT injection intrascrotally. Poland et al. (2008) also suggested an asbestos-like response after inducing mesothelial granuloma with amosite asbestos and with long, straight but not with short MWCNT 2 weeks after i.p. injection in mice. Follow-up i.p. studies showed the importance of lymphatic (Fig. 29-19) clearance of fiber structures from the pleural cavity (Murphy et al., 2011): Lymphatic stomata (Wang, 1975) in the parietal pleura with diameters of 3 to 10 μm in rodents (Shinohara, 1997) act as an effective clearance pathway for shorter fibers, whereas longer ones will be retained and induce inflammatory, oxidative stress responses progressing to mesothelioma. Such length-dependent pleural retention and lymphatic clearance was demonstrated by Murphy et al. (2011) after using i.p. injection of different types of MWCNTs and other nanotubes. Only long but not short or entangled MWCNTs caused significant mesothelial cell proliferation and granuloma formation and fibrosis. Long, rigid MWCNTs also induced malignant mesotheliomas in rats following i.p. injection: thin curved or flexible MWCNTs were less effective and the latent period for tumor induction was significantly prolonged (Rittinghausen et al., 2014).

These i.p. injection studies in rodents clearly show the potential of CNTs, specifically MWCNTs, to induce severe adverse length-dependent effects at mesothelial sites once they reach the pleural cavity. Appropriate inhalation studies in rodents need to be designed to determine the translocation of inhaled, generally highly agglomerated airborne CNTs from deposition sites in the lung to the pleura and to confirm the findings from bolus aspiration studies with well-dispersed CNTs.

Inhalation Studies

Several rat inhalation studies with CNTs in rodents have been reported (Table 29-7). Some of these were of short duration (hours to a few days), and only three were designed as subchronic 13-week inhalation studies (Ma-Hock et al., 2009; Pauluhn, 2010; Kasai et al., 2015) and two as 4-week inhalation studies (Oyabu et al., 2011; Morimoto et al., 2012). In addition, a 13-week inhalation study with carbon nanofibers has been reported (DeLorme et al., 2012). A detailed critical analysis of these four subchronic inhalation studies has been published by Oberdörster et al. (2015). All four studies were designed using three or four concentrations, with the higher concentrations causing significant inflammatory and fibrotic responses.

The authors conclude with a list of research needs to fill data gaps for improving design, analysis, interpretation, and extrapolation to humans of results of rodent inhalation studies to refine meaningful risk assessments. One data gap identified is the lack of a 2-year inhalation study. Indeed, a follow-up study by Kasai et al. (2016), designed as a 2-year multidose whole-body inhalation study with MITSUI-7 MWCNT showed the induction of lung carcinomas at concentrations of 0.2 and 2 mg/m^3 in male rats and at 2 mg/m^3 in females. No mesotheliomas were induced at any exposure concentration, and no lung tumors at the lowest concentration 0.02 mg/m^3. This well-designed and executed study clearly showing a carcinogenic response of this specific MWCNT is further discussed below.

Table 29-7

Carbon Nanotube Inhalation Studies in Rodents

AUTHOR AND YEAR	CNT	SPECIES (EXPOSURE)	AEROSOL	CONCENTRATION (mg/m^3)	DURATION	FINDINGS
Kasai et al. (2016)	MWCNT	Rats (whole-body)	MMAD 1.2–1.4 μm cyclone sieve method; dry aerosol generation	0.02; 0.2; 2.0	6 h/day; 5 days/week for 104 weeks	Significant dose-dependent increase in lung adenomas and carcinomas in male rats at 0.2 and 2 mg/m^3 No malignant mesotheliomas in males or females; ~1×10^3 nanotubes in pleural lavage at 2 mg/m^3 significant mesothelial hyperplasia and focal fibrosis of the diaphragm in male rats at 2 mg/m^3 Aggregates of MWCNTs in lungs: lung burdens were 10 μg at 0.02 mg/m^3; 152 μg at 0.2 mg/m^3; 1798 μg at 2 mg/m^3 in male rats
Li et al. (2007, 2009)	MWCNT	Mouse (whole-body)	Powder; no info. static system	32.6 (average) decreasing from 80 mg/m^3 to 1 mg/m^3 in 90 minutes	6 h/day (4 × 90 min) 8; 16; 24 day (once every other day)	Moderate proliferation and thickening of alveolar wall Lung lavage increase of protein, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and acid phosphatase (ACP)
Mitchell et al. (2007)	MWCNT	Mouse (whole-body)	Powder; mass median aerodynamic diameter (MMAD) 0.7–1 μm and 1.8 μm	0.3; 1; 5	6/day 7; 14 days	No lung inflammation or tissue damage; transient systemic immunosuppression; cytokine upregulation and oxidative stress in spleen
Sargent et al. (2014)	MWCNT alone methylcholanthrene (10 μg/g BWip alone); methylcholanthrene followed by MWCNT inhalation after 1 week	Mouse (whole-body)	MMAD 1.59 μm	4.6–4.7; lung burden = 31.2 μg/mouse	5 h/day; 15 days; follow-up for 17 months postexposure	14% of mice exposed to MWCNTs alone had lung adenocarcinomas; 22% of mice exposed to filtered air had lung adenocarcinomas; 62% of mice exposed to methylcholanthrene followed by MWCNTs had lung adenocarcinomas; 1.6% of mice exposed to methylcholanthrene alone and 9% of mice exposed to methylcholanthrene followed by MWCNTs had sarcomatous mesotheliomas
Shvedova et al. (2008)	SWCNT	Mouse (whole-body)	Powder; mass mode aerodynamic diameter 4.2 μm	5.5	5 h/day 4 days	Multifocal granulomatous pneumonia, interst. fibrosis; oxidative stress; K-ras mutation lung
Ryman-Rasmussen et al. (2009b)	MWCNT	Mouse (whole-body)	Collision jet nebulizer from suspension with pluronic F-68, MMAD ~0.16–0.21 μm	1; 30	6 hours	CNT embedded in subpleural wall and in subpleural macrophages; subpleural fibrosis; no pleural fibrosis or granuloma up to 154 weeks postinhalation
Ryman-Rasmussen et al. (2009b)	MWCNT	Mouse, OVA allergic model (whole-body)	Collision jet nebulizer from suspension with pluronic F-68; MMAD 0.714 μm	100	6 hours	MWCNT throughout lung in macrophages and epithelial cells; fibrosis in OVA sensitized mice, but not in normal mice; elevated platelet-derived growth (PDG) factor in nonsensitized mice; OVA and MWCNT act synergistically

(*continued*)

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Table 29-7
(Continued)

AUTHOR AND YEAR	CNT	SPECIES (EXPOSURE)	AEROSOL	CONCENTRATION (mg/m^3)	DURATION	FINDINGS
Ma-Hock et al. (2009)	MWCNT	Rat (nose-only)	Powder MMAD 0.7–2.0 geometric standard deviation (GSD) 2.1–3.6	0.1; 0.5; 2.5	6 h/day 5 day/week 13 weeks	Increased lung weights granulomatous inflammation fibrotic changes no lung lavage data LOAEL 0.1 mg/m^3
Pauluhn (2010)	MWCNT	rat (nose-only)	Powder MMAD 2.74–3.41 GSD 1.98–2.14	0.1; 0.4; 1.5; 6.0	6 h/day 5 day/week 13 weeks	Increased lung weights neutrophils, increased in lung lavage; granulomatous inflammation; fibrosis Pleural thickening (high conc.) NOAEL 0.1 mg/m^3
Pothmann et al. (2015)	MWCNT	rat (nose-only)	Ball-milling and aerosol generation; MMAD 1.62 and 2.3 μm; outer mean diameter = 11.8 and 12.1 nm; mean length=1.05 and 1.07 μm; median agglomerate size=376 and 418 nm	0.066; 0.26; 1.30 0.06; 0.28; 4.84	5 days 90 days	Lung inflammation 90 days after exposure at 5.0 mg/m^3 consistent with overload; clearance and recovery after exposure at 0.25 mg/m^3 No genotoxicity detected in lungs, bone marrow, liver, kidney
Oyabu et al. (2011); Morimoto et al. (2012)	MWCNT (short)	Rat (whole-body)	Jet nebulizer, aqueous suspension with Triton-X: GMD 63 nm GSD = 1.5 Geometric mean length (GML) = 1.1 μm GSD = 2.7 70% single fibers	0.37	6 h/day 5 day/week 4 weeks	~70 μg lung deposition measured; retention $T½$ = 54 days; slight increase in lung weight; increase of chemokines and myeloperoxidase in lung lavage
Morimoto et al. (2012)	SWCNT	Rat (whole-body)	Jet nebulizer, aqueous suspension, Tween 80 pretreated; bundles: (ropes): 0.2 × 0.7 μm 90% well dispersed	0.03; 0.13	6 h/day 5 day/week 4 weeks	Calculated lung burden: 1.8 and 8.8 μg; no significant changes in lung lavage neutrophils or protein, (ALP), chemokines, (CINCs), HO-1 level; no lung pathology

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The value of short-term inhalation studies for risk characterization can be questioned because such exposures to CNTs at realistic low concentrations are not likely to reveal certain chronic effects if long latency periods are involved. Although unreasonable, very high concentrations with short exposures may be helpful for purposes of hazard identification and toxicity ranking—which could be achieved at less cost and effort by bolus-type studies—they are not useful for deriving a no observed adverse effect level (NOAEL) or for quantitative risk assessment. Thus, beside a very costly 2-year inhalation study, most meaningful and best justified for the risk assessment process would be a subchronic multiconcentration (minimum three concentrations) study with sufficient postexposure observation. However, short-term, even one-term, exposure to a relevant concentration is very useful for dosimetric purposes when determining the biodistribution from deposition sites in the lung to secondary organs. Of course, a prerequisite is the availability of sensitive detection methods, such as radioactive labeling (Kreyling et al., 2009) or use of a nonleachable catalyst firmly attached to or embedded within the CNTs.

The choice of the mode of inhalation exposure—whole-body or nose-only—is up to the investigator. Generally, for long-term chronic exposures, whole-body is preferred because the tight confinement of rodents during nose-only exposures induces additional stress, despite careful adaptation prior to starting exposures (Pare and Glavin, 1986; Sistonen et al., 1992; Udelsman et al., 1993; Fawcett et al., 1994; Rothenberg et al., 2000). The disadvantage of whole-body exposure is the contamination of the animals' fur with aerosolized CNTs, which may lead to additional oral intake due to preening. However, the additional dose is very low, and GI-tract exposure is part of any inhalation exposure due to bronchial clearance of the deposited material.

Most of the CNT inhalation studies were performed with MWCNTs; only two used SWCNTs. Exposure concentrations ranged from 0.1 to 32.6 mg/m^3, either inhaled as one concentration only, or up to four concentrations; duration was from 6 hours to 13 weeks. Outcomes (Table 29-7) range from no significant effects to severe pulmonary inflammation/oxidative stress responses; one study reported systemic effects including cytokine and oxidative stress responses in the spleen (Mitchell et al., 2007).

In an initiation-promotion study in mice, exposure to a single injection of methylcholanthrene i.p. followed by inhalation of long, rigid MWCNTs for 15 days at 5 mg/m^3, 62% of the mice developed lung adenocarcinomas after 17 months. Only 13% of mice exposed to filtered air, 22% exposed to methylcholanthrene alone, or 14% exposed to MWCNTs alone developed adenocarcinomas (Sargent et al., 2014). Sarcomatous peritoneal mesotheliomas were also found in 9% of the mice exposed to methylcholanthrene and MWCNTs. On the basis of this initiation-promotion inhalation study, together with mesothelioma induction following i.p. (Nagai et al., 2011; Takagi et al., 2012) or intrascrotal injection (Sakamoto et al., 2009), the International Agency for Research on Cancer concluded that there was sufficient evidence for carcinogenicity of this type of long, rigid MWCNT (Grosse et al., 2014). After consideration of additional mechanistic evidence (reviewed subsequently in Kuempel et al., 2017), this type of MWCNT was classified in Group 2B as possibly carcinogenic to humans. More recently, a similar type of MWCNT was evaluated for carcinogenicity following transtracheal intrapulmonary spraying at a 1mg/rat dose for 2 weeks. After 109 weeks, lung adenomas and carcinomas and malignant mesotheliomas were induced in the pleural or pericardial linings (Suzui et al., 2016). Lung carcinogenicity of long, rigid MWCNTs was also confirmed following inhalation in rats for 104 weeks at three concentrations. The lowest concentration of 0.02 mg/m^3 did not induce a statistically significant increase in lung tumors compared to 13% at a dose of 0.2 mg/m^3 and 16% at 2 mg/m^3 in male rats (Kasai et al., 2016). The authors calculate that 1×10^9 nanotubes need to be deposited in the lungs to induce lung tumors; no tumors were induced at the lowest dose of 0.02 mg/m^3 in male rates. The total lung burdens in male rats exposed to 0.2 or 2 mg/m^3 of MWCNTs that significantly induced lung tumors were 0.15 or 1.8 mg, respectively. These authors calculate that a worker exposed to 8.5 mg/m^3 of MWCNTs for 45 years would have a lung burden of ~38 mg, which is equivalent to a lung burden of 150 μg in these male rats (Kasai et al., 2016). No mesothelioma were induced in this inhalation study. NIOSH has recommended prevention strategies including engineering controls, worker training, and use of personal protective equipment to minimize workplace exposure to CNTs (Castranova et al., 2013).

Although bolus-type delivery did result in pleural effects and translocation of MWCNT toward pleural sites as discussed before, few pleural effects were seen except pleural thickening at the two highest exposure concentrations in the 13-week inhalation studies (Pauluhn, 2010). Following inhalation of long, rigid MWCNTs for 104 weeks, the highest dose of 2 mg/m^3 induced parietal pleural mesothelial hyperplasia and focal fibrosis of the diaphragm in rats (Kasai et al., 2016). Although lung tumors were induced after inhalation of 0.2 or 2 mg/m^3, no malignant mesotheliomas were found. Only 1000 nanotubes were recovered in pleural lavage fluid and this dose was most likely not sufficient to induce mesotheliomas (Kasai et al., 2016). Movement of inhaled MWCNT to subpleural sites was observed by Ryman-Rasmussen et al. (2009a) when individual fibers and very fine agglomerates (160 to 210 nm) were generated from a liquid suspension following a single 6-hour very high concentration exposure of 30 mg/m^3. However, pleural fibrosis or granuloma did not develop up to 22 weeks postexposure. The small size of the aerosol as well as an extremely high dose may have been a factor in a deeper peripheral lung deposition. A follow-up 6-hour study by Ryman-Rasmussen et al. (2009b) with inhaled MWCNT in an ovalbumin (OVA), allergic mouse model found a synergistic effect; MWCNT alone did not induce fibrosis in nonsensitized mice. This result is in contrast to their earlier study where a 6-hour exposure to a threefold lower concentration did induce subpleural fibrosis in mice (Ryman-Rasmussen et al., 2009a). The larger aerosol size in the second study may have prevented significant deposition in the peripheral lung.

Regarding a comparison between MWCNT- and SWCNT-induced pulmonary or secondary organ effects, the only medium-term (4 weeks) SWCNT inhalation study in rats (Morimoto et al., 2012) did not observe any effect; however, estimated lung burdens were just 1.8 and 8.8 μg, about the same as the lowest concentration in the Pauluhn (2010) study with MWCNTs that also did not show any effect (NOAEL).

Critical Appraisal of CNT In Vivo Studies

Several intrapleural injection studies with MWCNTs in mice have indicated that CNTs have a mesotheliogenic potential, discussed in the previous section. In addition, intratracheal instillation studies in rats and oropharyngeal aspiration studies with MWCNTs and SWCNTs have confirmed a pulmonary inflammatory and oxidative stress-inducing potential. These results have raised concern that CNTs may represent and give rise to another case of asbestos fiber toxicity and pathology, in particular insidious because of the decades-long latency period from exposure to manifestation of mesothelioma. Controversy exists regarding the applicability of results of intracavitary injection studies for regulatory purposes. European regulations for synthetic mineral fibers exonerate those from being classified as

a carcinogen if it can be shown that the material fulfills one of the following conditions (EC Directive 97/69/EC, Nota Q, 1997):

- a short-term biopersistence test by inhalation has shown that the fibers longer than 20 μm have a weighted half-time less than 20 days, or
- a short-term biopersistence test by intratracheal instillation has shown that the fibers longer than 20 μm have a weighted half-time less than 40 days, or
- an appropriate intraperitoneal test has shown no evidence of excess carcinogenicity, or
- absence of relevant pathogenicity or neoplastic changes in a suitable long-term inhalation test.

This regulation is based on a number of experimental studies going back to Stanton et al. (1981) and Pott et al. (1987) that showed induction of tumors when different types of asbestos and persistent synthetic mineral fibers were directly injected into the pleural or peritoneal cavity. However, use of this test for regulatory purposes has not been established in the United States; the test has been criticized because of the massive doses being used leading to fibrotic reactions thereby interfering with an overwhelming physiological defense mechanisms. As to whether present European regulations will be applied to CNTs is questionable because they have been issued for man-made vitreous (silicate) fibers and the high bolus doses and associated caveats should be considered.

Still, results of CNT intracavitary injection, trans-tracheal spraying, and inhalation studies summarized in this section have defined the potential of CNTs to induce mesothelioma, depending on their dimensions, rigidity, or flexibility, and their agglomeration state. These appear to be in agreement with results from intraperitoneal injection studies, which did not show an adverse effect of short, straight MWCNTs because of effective removal by lymphatic clearance.

Obviously, given the importance of the physicochemical properties of CNTs for inducing adverse effects, it is of utmost importance to determine these properties, in particular as they appear in the airborne state at sites of human exposures, at occupational sites, or for the consumer. Adding dispersants for testing purposes will change surface properties; conceptually, inhalation studies in experimental animals for purposes of hazard identification should mimic human exposure conditions with regard to airborne size distribution. Of course, differences in respirability between humans and rats/mice have to be considered, and—if necessary—adjustments be made without use of surface altering dispersants or harsh physical treatment.

Appropriately designed multidose subchronic inhalation studies, including a longer recovery period, are essential for deriving NOAELs; results can be used as basis for deriving OELs by applying rodent/human dosimetric adjustments (Fig. 29-20). Using results from bolus-type studies is difficult and raises questions, although the national institute for occupational safety and health (NIOSH, 2011) has combined results of fibrotic responses from diverse bolus-type and inhalation studies to derive a provisional recommended exposure level (REL) of 7 μg/m^3. This REL is based on dose–response data from the available studies with bolus-type and short-term inhalation exposures and a subchronic inhalation study. Differences in lengths, use of dispersants, and impact of hugely different dose rates between the studies were not considered. NIOSH (2011) suggested, though, that the mass-based REL may not be sufficiently sensitive for detecting CNTs by air sampling and calls for research to determine the most sensitive dosemetrics.

Collectively, the available in vivo toxicological database of MWCNTs has identified a fibrogenic and carcinogenic effect

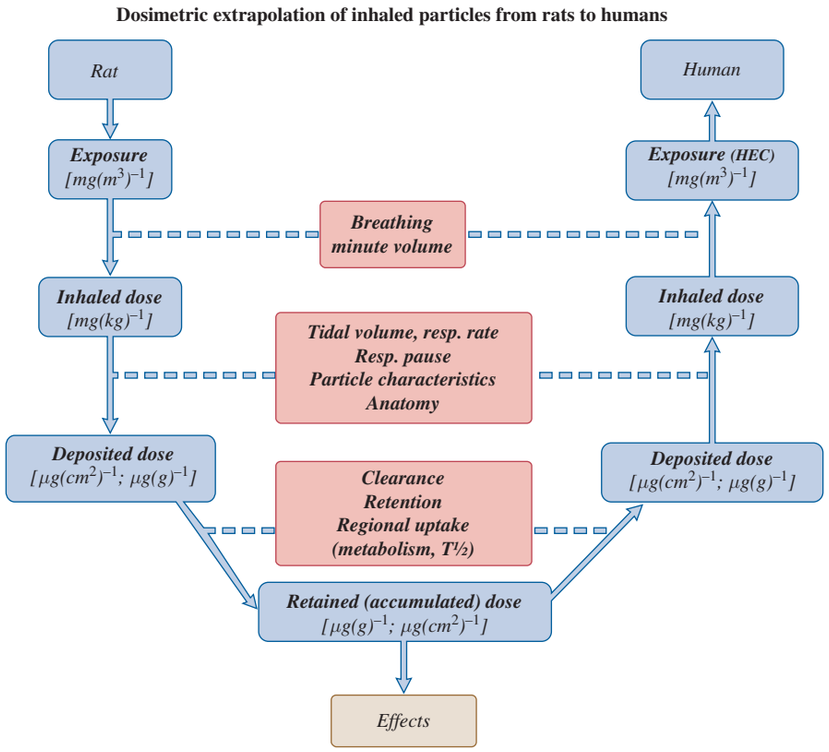


Figure 29-20. *Dosimetric extrapolation of deposition and retention in the respiratory tract from rats to humans: The concept is to achieve the same retained dose in the lungs of humans as has been found at the end of a long-term chronic inhalation study in rats.* According to the scheme above, use of a dosimetric extrapolation model for rats and humans (see text) allows to estimate the respective human equivalent concentration (HEC). (From Oberdörster [1989].)

(hazard identification). SWCNTs also appear to be fibrogenic in the lungs. The old paradigm of dose–dimension–durability for fiber carcinogenicity may also apply to CNTs: they are quite durable (though some studies have now reported degradation of carboxylated SWCNT by peroxidases in vitro, see below), and their dimensions in terms of length, diameter, and agglomeration/entanglement appear to be important for pulmonary effects. Of course, other properties (chemistry, surface reactivity) may play a role as well. There is an obvious and urgent need to perform additional long-term (subchronic to chronic) inhalation studies to assess the carcinogenic potential of multiple types of CNTs and carbon nanofibers and derive protective OELs (Kuempel et al., 2017).

Biological Degradation of Carbon Nanomaterials CNTs have been generally regarded as stable nondegradable materials, which has important implications for long-term health effects following inhalation into the lungs (Donaldson et al., 2006). Recently, however, SWCNT degradation has been observed in acellular assays that simulate the phagolysosome of macrophages, but only if the tubes have been surface carboxylated, which introduces collateral defects in the side walls (Liu et al., 2010c). Carboxylated tubes are also susceptible to biodegradation by exposure to hydrogen peroxide and horseradish peroxidase (Allen et al., 2008, 2009; Kagan et al., 2010) or hypochlorite and the mammalian enzyme myeloperoxidase (Kagan et al., 2010). Kotchey et al. (2011) also reported that graphene oxide, but not reduced graphene oxide, is susceptible to oxidative attack by hydrogen peroxide and horseradish peroxidase. These observations may enable design of safer carbon materials (Yan et al., 2011) that are potentially biodegradable in order to minimize adverse environmental and human health impacts. However, degradation of CNTs in vivo is still to be confirmed.

TOXICITY TESTING

Although numerous in vitro and in vivo studies have reported toxic effects of different ENMs, results are often conflicting and hazards are overstated due to poor understanding and knowledge about the appropriateness of applied doses and concentrations and their relevance to realistic exposures. This issue will be further discussed later. A conceptual testing paradigm is shown in Fig. 29-21. In order to perform risk assessment or to derive OELs, exposure and hazard data are required. To identify and characterize a hazard, in vitro and in vivo studies will be useful, and results should be derived via well-designed dose–response relationships. Key considerations for designing such studies include careful physicochemical characterization of the ENM to be tested, justification of the method(s) of dosing, selection of target cells, tissues or animal species, and appropriate endpoints. Reference materials may also be included in order to rank unknown ENMs against a known positive and/or negative control. Whereas in vitro data alone permit toxicity ranking, appropriately performed in vivo studies (using inhalation when dealing with airborne materials) will allow a full risk assessment because exposure and dosimetric extrapolation to humans can be performed. In the long term, in silico studies may be developed to assess a hazard, and in even a more distant future to predict human risk. Ideally, knowledge about anticipated human exposure would be necessary to inform both animal exposure studies as well as in vitro dosing.

In vivo animal studies obviously do not generally predict human exposures: the lower loops in Fig. 29-21 refer to dosimetric correlations between in vitro/in vivo and in vivo animals/in vivo humans, going in both directions with the goal to (a) inform the design of in vivo animal studies using available human exposure data, and (b) use exposure–dose–response information from animal studies to compare with human data. The upper one-directional animal-to-human loop refers to extrapolating effects and mechanisms from relevant (based on dosimetric relevancy) animal studies to humans, with the goal to be predictive for deriving "safe" exposure levels or OELs; thus, an animal study can be used to predict human equivalent exposure concentrations or OELs. An example for this approach is the recent subchronic rat inhalation study with MWCNT by Pauluhn (2010).

The following paragraphs focus on concepts and results of in vivo experiments as basis for use in the risk assessment process using as example CNTs. However, a need for in vitro studies should be stressed as far as uncovering underlying mechanisms of effects

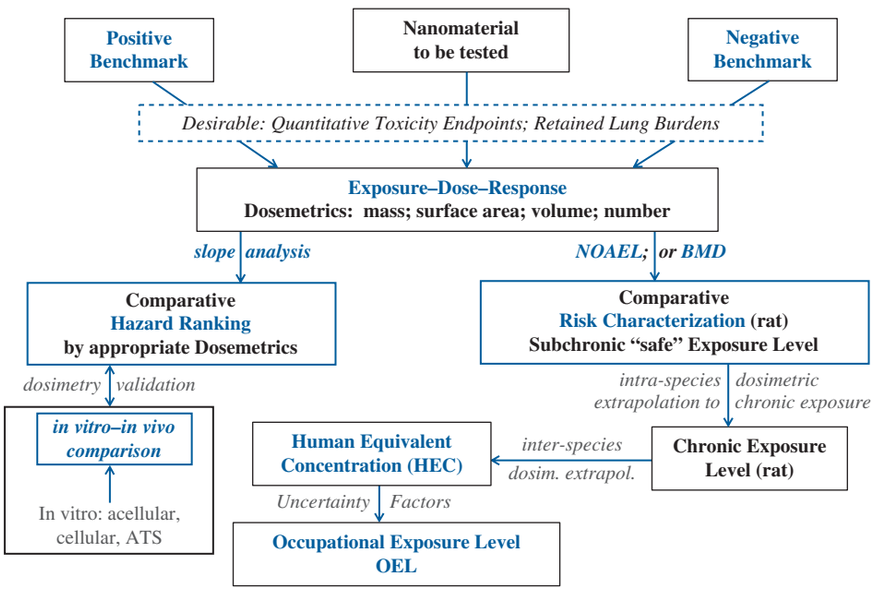


Figure 29-21. *Approach for comparative hazard and risk characterization of inhaled nanoparticles based on subchronic (3 months) rat inhalation studies.* NOAEL: no observed adverse effect level; BMD: benchmark dose; OEL: occupational exposure level; ATS: alternative testing strategy. (From Oberdörster and Kuhlbusch [2018]).

are concerned, provided that the caveats pointed out above are considered. In addition, despite these limitations, in vitro studies are useful for toxicity ranking of nanomaterials for the purpose of hazard identification (Rushton et al., 2010). In contrast, the design of in vivo studies allows the full evaluation of exposure–dose–response relationships, which is necessary for the process of risk assessment (Fig. 29-21).

Some important points should be considered when designing exposure–dose–response studies with CNTs in experimental animals. The major exposure route for CNTs to be expected is via inhalation at manufacturing sites and during distribution and usage (handling, refilling, disposal), although ingestion and dermal exposures also occur but have not received as much attention (Fig. 29-17). For medicinal applications, injection is an important route of exposure requiring specific awareness with respect to assuring desired beneficial (pharmacological) outcomes yet avoiding undesirable (toxicological) responses (Kolosnjaj-Tabi et al., 2010). For example, Yang et al. (2010) concluded from their studies with SWCNT that the desired pharmacological target organelle for drug delivery by SWCNT are the cell lysosomes, whereas the mitochondria are the target organelles for SWCNT toxicity. These aspects will not be discussed further.

In Vitro Dosimetry

The importance of dose rate for bolus-type delivery of ENM in vivo vis-à-vis inhalation has been stressed in previous sections. As most ENM toxicity studies are performed using in vitro assays, which are generally short term, dosing-related questions are highly relevant. Teeguarden et al. (2007) pointed out that the dose received by the cell is a function of colloidal dynamics or "particokinetics" in the culture medium governed by diffusion and settling phenomena, which in turn is governed by particle and media properties that include particle size, agglomeration/aggregation state, shape, density, and charge. This is very different from chemicals dissolved in culture medium and requires a thorough evaluation of particle dosimetry in vivo. At equal mass concentrations in the medium, the magnitude of the cellular dose of ENM will differ significantly from those implied by the media concentration. This can give rise to significant misinterpretation of responses and is likely to contribute to differences in results between different laboratories. For example, particles of the same material but of different sizes will settle onto cells in culture at different rates; and particles of the same size but different densities will also settle differently so that the time for particles to reach the cell layer at the bottom of a dish can vary widely. Similar to the aerodynamic behavior of airborne particles, the hydrodynamic behavior in culture medium expressed as their transport velocity shows a minimum of movement for intermediate sizes where the combination of gravitational settling and diffusional movement is lowest.

Fig. 29-22 illustrates the different mechanisms that determine the in vitro particle kinetics for particle sizes from 1 nm to 1000 nm (Hinderliter et al., 2010). A typical U-shaped form depicting faster transport at both ends of the range of sizes and a slowest transport for particles around 20 to 60 nm is the result of the competing forces and particle and media characteristics. Temperature, mixing, and advection also add to the complexity of particle movement in the media. Teeguarden's group developed an In Vitro Sedimentation Diffusion and Dosimetry (ISDD) model for predicting the in vitro behavior and cell doses of particles. Fig. 29-23 shows the results of example calculations from this model. It indicates that for large TiO_2 particles (500 nm), the dose received by the cells in a dish with 3.1 mm media height is very fast. In contrast, 30 and 50 nm particles have a long deposition rate; only 50% of the media dose has reached the cells by

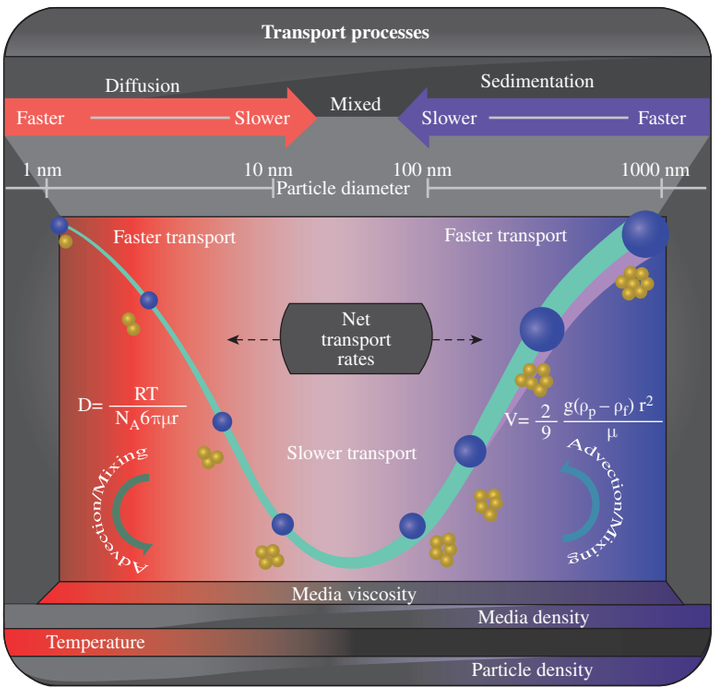


Figure 29-22. *In vitro sedimentation, diffusion, and dosimetry (ISDD) model: particle transport/deposition processes for in vitro systems.* (See text.) (Reproduced with permission from Hinderliter et al. [2010].)

24 hours. TiO_2 NP sizes in between still have not reached the full dose by 24 hours, whereas 200 nm TiO_2 NP are fully settled by 10 hours.

Further advances in in vitro dosimetry have been reported by DeLoid et al. (2015, 2017), using computational fluid dynamics and their new distorted grid (DG) model to estimate delivered dose-metrics. The authors state that these advanced models provide practical and robust tools for obtaining accurate dosemetrics and concentration profiles across the well, and that the DG model allows rapid modeling.

Results of the ISDD shown in Fig. 29-23 and DG prediction have to be verified by experiments. The value of these models lies in the clear separation between exposure (concentration in the cell medium), the deposited dose on the cell surface, and—depending on cellular uptake—the cellular dose. These doses can be expressed by different metrics: mass, number, or surface area of the nanomaterial. Knowledge about the time to deposit a certain dose allows to consider dose rate as a determinant for responses, as was discussed for in vivo exposures (Fig. 29-10). In vitro dosing is often perceived as a bolus-type delivery, similar to in vivo instillation or aspiration studies. However, as the results in Fig. 29-23 show, in vitro dosing with NP is not necessarily equivalent to an instantaneous bolus. The ISDD model allows to express the deposited dose per unit cell surface area of cultured epithelial cells of the respiratory tract and thereby (*i*) compare responses to the same in vivo dose per unit cell surface (based on MPPD model, Fig. 29-15), (*ii*) use in vivo derived doses from a given exposure concentration to design in vitro studies that include realistic doses, and (*iii*) use in vitro dosimetry results and response to verify in vivo. Likewise, doses applied in vitro to cells from secondary organs (e.g., CNS and bone marrow) can be estimated by the ISDD and DG model and better justified by results from in vivo biokinetic studies with respect to doses distributed to these secondary organs. An important difference, however, between in vivo inhalation and in vitro cell exposure is the pristine state of inhaled nanomaterials, whereas for in vitro exposures nanomaterials are either treated with dispersants to separate the particles and achieve spatially uniform dosing, or else culture media constituents (serum proteins) will adsorb to the NP surface to aid in their dispersion. Surface modification is known to affect responses of ENM (Haniu et al., 2011), and this possibility needs to be considered when designing and interpreting in vitro results.

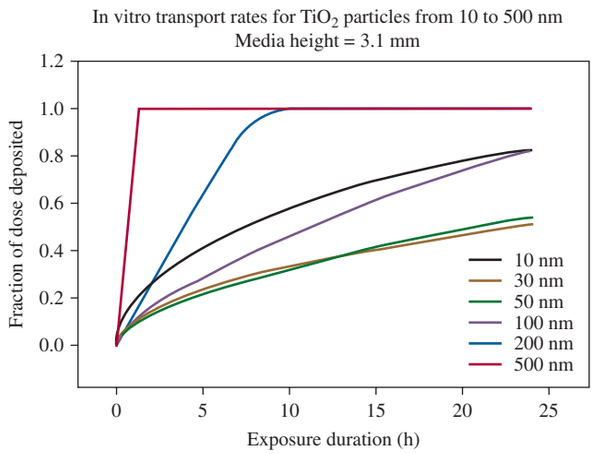


Figure 29-23. *In vitro sedimentation, diffusion, and dosimetry (ISDD) model: prediction for in vitro transport rates for TiO_2 particles from 10 to 500 nm, assuming a media height in the culture dish of 3.1 mm.* (Reproduced with permission from Hinderliter et al. [2010].)

An alternative in vitro methodology that avoids pretreatment of NP with surfactants or proteins prior to testing is exposure via an air-liquid interface (ALI) system (Holder et al., 2008; Savi et al., 2008; de Bruijne et al., 2009; Lenz et al., 2009; Volckens et al., 2009; Tang et al., 2011; Xie et al., 2012). Respiratory tract epithelial cells are grown on porous culture cell inserts and exposed to NP generated the same way as done for in vivo inhalation studies. Before exposure, culture medium is withdrawn so that only the basal cell surface is in contact with the medium, and deposition of the aerosolized NP on the apical cell surface simulates realistic in vivo inhalation exposure. Various nano aerosol chamber devices have been developed for short or longer, repeated exposures of lung target cells at the ALI (Loret et al., 2016; Geiser et al., 2017). Comparative results between conventional in vitro and ALI exposure seem to confirm the apparent advantage and greater sensitivity of the ALI system (Holder et al., 2008; Volckens et al., 2009). However, transition of cell cultures from growing in monolayer to the ALI system for exposure is likely to cause changes in gene expression, which needs to be considered using appropriate control groups (Ross et al., 2007). Other alternative exposure models for inhalation nanotoxicology include precision-cut rat lung slices (Sauer et al., 2014) and acellular human three-dimensional lung scaffolds (Wagner et al., 2014). Additional non-animal epithelial barrier models are being developed for evaluation of lung, intestinal, and skin toxicity (Gordon et al., 2015) as static or microfluidic platforms (Blume et al., 2015; Mahto et al., 2015).

Predictive Toxicology

The NNI developed a comprehensive Environmental, Health, and Safety Research Strategy (NanoEHS research; NNI, 2011) in which urgent research needs for nanomaterial measurements, human exposure assessment, human health, environmental health, risk assessment and risk management methods, and for the input of informatics and modeling for NanoEHS research are discussed. This initiative of the U.S. Government's interagency program for coordinating research and development and for enhancing communication and collaborative activities in nanoscale science, engineering, and technology emphasizes the NAS risk assessment paradigm (1983) as the basis for NanoEHS research. As illustrated in Fig. 29-21, data about hazard and exposure are key for the risk assessment process. Critical elements for hazard identification based on toxicity testing of ENM are detailed information about their physicochemical properties prior to any experiments, the selection of appropriate target cells, validation of in vitro assays in terms of correlation and relevancy to in vivo results, the inclusion of biokinetics in the design of in vivo studies, and the inclusion of realistic doses in the design of dose–response in vitro and in vivo studies. Biokinetic information is crucial for identifying potential secondary target organs based on significant accumulation of ENM. In order to determine relevant doses, information about exposures occurring at anticipated real-world exposure scenarios, either measured or estimated, is essential. With this information, exposure–dose–response (in vivo, rodents) and dose–response (in vitro, target cells) relationships can be established to both characterize a hazard and assess a risk (see below). In terms of toxicity ranking, it is desirable to include reference materials in the study design so that a hazard can be expressed relative to a positive (high hazard) or negative (low hazard) control ENM. Mechanistic information discovered through in vitro assays will further aid in the characterization of hazard, provided that the mechanism is operative at relevant doses/concentrations. High-dose and high-dose rate-induced mechanisms may not be considered relevant and may give rise to improper classification. An awareness of dosimetry-related aspects

is of highest importance (Fig. 29-21) for the risk assessment process, which often gets lost or is ignored because of a misconception of risk being analogous to hazard. Risk is a function of hazard and exposure, and neither aspect alone can determine risk.

The immediate goal of ENM toxicity testing is hazard characterization and the establishment of respective predictive tests. These should ideally be based on validated in vitro studies, for example high throughput in vitro assays, so that ethically controversial animal studies can be replaced if they only serve the purpose of hazard identification. A distant goal is the use eventually of in silico models. International workshops are trying to build a consensus among multiple stakeholders for alternative testing strategies for ENMs (Nel et al., 2013; Stone et al., 2014). However, at present, animal studies are still indispensable for obtaining crucial information about long-term effects of ENM and obtaining results for risk assessment. For example, available data from subchronic (3 months) inhalation studies (Ma-Hock et al., 2009; Pauluhn, 2010; DeLorme et al., 2012; Kasai et al., 2015) provide crucial information for deriving a NOAEL or lowest observed adverse effect level (LOAEL) that can be used for dosimetric extrapolation of OELs for humans.

Fig. 29-20 depicts a conceptual approach for deriving a human equivalent concentration (HEC) from results of a long-term rat inhalation study. The HEC is based solely on the equivalency of the retained dose, expressed per unit mass, or epithelial surface area of the lung. The retained dose can be expressed by different metrics, for example, rather than using the mass of ENM, as is conventional; the surface area or even the number of particles might be used. Pauluhn (2010) used the metric of particle volume retained in alveolar macrophages to estimate an OEL from the experimentally found NOAEL in a 3-month rat inhalation study with MWCNT. As was done in that study, it is highly desirable that the design of a rat study includes the measurement of the retained lung burden; if this is not available, the retained dose can be estimated using a particle deposition and retention model with inputs of rat-specific breathing and airway parameters and the aerosol characteristics of the ENM used in the rat study. The same aerosol characteristics and human-specific airway and breathing parameters as inputs to a human model are then used to determine the HEC. It should be noted that this dosimetric extrapolation should not necessarily imply that the response in humans is the same as in rats. Differences in species sensitivity may require the use of an additional safety factor. The advantage of using the retained dose as the basis is that other particle size distributions specific to human exposures at a workplace can be used instead of the rat aerosol (Oller and Oberdörster, 2010).

Development of a Set of Screening Tools that Reflect Important Characteristics or Toxicity Pathways of the Complex Systems Described Above In 2013, a committee of the National Academy of Sciences undertook an assessment of research progress on environmental health and safety aspects of ENMs (National Research Council, 2013). A major focus of the committee's report was to provide general guidance on research strategies for implementing hazard identification methods and studies for a variety of ENMs. This includes a critical need for developing reliable and validated tools to identify and confirm toxicity pathways of complex systems and address mechanistic issues. This is important because of the necessity for developing a set of reliable and validated screening tools which is paramount for reproducible testing of diverse nanoparticle types. In addition, toxicity results obtained from a variety of in vivo studies often have limited value for determining relevant health hazards, in large part because toxicological studies with ENMs are conducted at much higher doses than might be present from real-world exposures; or at best, they might reflect a short-term toxicological response. Time-course experiments would better simulate potential effects following exposures to nanomaterial types.

Alternatively, given the limitations of animal testing going forward—and with a greater resistance to conducting in vivo experiments, the applications of a spectrum of in vitro investigations could provide some useful mechanistic insights into toxicity pathways; however, several shortcomings to in vitro methodologies need to be addressed. Optimizing the relevance of in vitro studies to real-world toxicity considerations should require the utilization of experimental designs that necessitate dose–response behavior over a full range of doses, and should also require the duration of exposure using relevant cell-types (focusing on the route of exposure regimens) including time-course evaluations, concomitant with comparisons and validation with corresponding in vivo systems.

To summarize the suggested framework and research strategy, implementation of the following considerations would significantly improve the methodology for development of reliable and validated screening tools:

1. Rigorous physicochemical characterization of nanoparticle types and behavior through the life cycle
2. Dose–response characterization and careful attention to reliable dosimetry at relevant human exposure levels
3. Particularly for in vitro studies selection of relevant cell-types and cell models that reflect the route of human exposures
4. Time-course assessments that span acute to chronic exposure durations
5. Application of proper benchmark controls to improve interpretation of toxicological outcomes (National Research Council, 2013; Hussain et al., 2015)

DESIGNING ALTERNATIVE IN VITRO METHODS FOR SCREENING PULMONARY TOXICITY OF FINE AND NANOSCALE PARTICLES

The development of reliable and predictive aerosol-based in vitro lung toxicity assays for screening particulate/nanoparticulate materials might (in the future) limit the need for animal testing, particularly during the early phases of hazard evaluations. Additional benefits would include an enhanced capacity to screen several compounds during the early phases of product development using cost-effective and validated in vitro assays. In addition, reliable hazard data developed from these tests could serve as a bridge to the subsequent implementation of longer-term particle inhalation studies, thereby obviating the need for conducting acute (in vivo) toxicity studies, and significantly reducing the numbers of test animals. These goals are also consistent with the visions of the National Academy of Sciences—Toxicity in the 21st Century Committee which advocated a transformative paradigm shift in implementing safety studies by providing greater abilities to screen chemical hazards at various life stages; to reduce testing costs, and use fewer animals, and to develop a robust scientific basis for assessing health effects of environmental agents.

As described earlier, the paradigm for these studies must include the following aspects: (1) dose–response studies—particularly to determine the slope of the toxicity curve; (2) address the shortcomings/problems of static conditions of in vitro models vs. the dynamic conditions of in vivo effects; (3) the need for longer-term in vitro models—in order to assess a time-course response; (4) the questions

of dose equivalency for estimating the effects of in vitro vs. in vivo exposures; and (5) the need for positive and negative benchmark materials—in order to adequately interpret the results of a particular study—and for purposes of ranking/grouping. At the present time, it seems that only hazard characterization (as the first step in the Risk Assessment Paradigm) can be adequately achieved utilizing current in vitro methodologies.

Unfortunately, the accuracy of predictive results from currently conducted in vitro lung toxicity screening studies has generally been unreliable (Sayes et al., 2007). Previously published results of comparative lung toxicity studies with identical particle-types comparing submerged in vitro cell culture systems vs. in vivo (inhalation and instillation) techniques has demonstrated a lack of convergence between in vivo and in vitro effects using these cell culture methodologies. The toxicity studies used as barometers focused on similar endpoints (e.g., inflammation and cytotoxicity biomarkers) as representative indices for comparative purposes.

The lung is a complex organ system comprising many different cell-types (i.e., types I and II alveolar epithelial cells, macrophages, interstitial cells, vascular cells, etc.) (see Chap. 15); thus, the pulmonary microenvironment and the complex interactions in the lung which occur following inhalation and subsequent deposition of particles are difficult to simulate when employing in vitro techniques. In addition, numerous studies have reported in vitro toxicity results using only single cell-types (e.g., A549 lung epithelial cells—*or* lung or peritoneal macrophages) (National Research Council, 2013). However, these efforts generally represent simplified models and ignore the complex interactions in the lung microenvironment that occur following exposure to aerosols of particles, bacteria, etc. Recently, more complex in vitro systems are being developed, which can, in part, better simulate particle phagocytosis, an important aspect in the simulation of lung defense responses to particles (Hussain et al., 2015). The development of such in vitro techniques will better facilitate (and hopefully expedite) the transition from the current animal-based inhalation testing system to one that is based primarily on human cell lines and in vitro assays. Such reproducible, accurate, and validated cell-based in vitro screening assays for assessing pulmonary and genetic toxicity will have important benefits (i.e., screening more compounds in a faster, more reliable, and less expensive manner) and may provide experimental designs to address mechanistic questions.

In an attempt to improve the efficacy of an in vitro methodological approach, a number of steps have been designed to better simulate the physiology of the distal lung microenvironment, concomitant with the implementation of an aerosol exposure system. Accordingly, this transition from a traditional "submerged" cell culture system to a more physiologically relevant ALI cell culture system, using Transwell® Permeable Support devices (microporous membranes), is useful for providing and maintaining pulmonary cell co-cultures (of rat lung epithelial cells and alveolar macrophages) and exposing the co-cultures to particulate aerosols in a humidified atmosphere.

Many fundamental issues require consideration for optimization when facilitating a transition from an animal inhalation toxicity set-up to an in vitro aerosol pulmonary toxicity study. These considerations include, but are not limited to, the following actions:

- Determining the cell-types to be used in a co-culture system—to better simulate the lung microenvironment
- Transitioning from primary cell types collected from animals to immortalized cells derived from cell lines and tested for biological functionality
- Determining the number or ratio of lung epithelial cells to alveolar macrophages in a co-culture plate
- Determining the aerosol generation method for particles/nanoparticles and appropriate and reproducible and quantifiable dosemetrics to be utilized for particle inhalation deposition assessments and for comparisons of results of one study to another
- Demonstrating reproducibility for particle generation studies by measuring particle deposition on filters—and choosing appropriate filter-types and measurement methods to ensure validation of results
- Deciding on the appropriate endpoints for assessing lung toxicity parameters, which can be benchmarked against in vivo results on the same particle-types
- Validating effects, doses, and mechanism with in vivo test!

Three additional key experimental design features of a newly developed in vitro system should include:

- Time-course protocol to assess the sustainability of any measured response
- Exposure-dose–response assessments
- Utilization of benchmark particulate control test materials, in order to develop a frame of reference for better interpretation of results

Based upon these challenges, one might be able to successfully transition from a submerged cell culture system to an ALI system, for providing co-cultures (of rat lung epithelial L2 cells and NR8383 alveolar macrophages) applicable to in vitro cell exposures and toxicity assays. This more complex in vitro system can, in part, simulate particle phagocytosis and inflammation, important aspects in the assessments of lung defense responses to particles. A temperature- and humidity-controlled in vitro cell exposure chamber—the Nano-Aerosol Chamber for In Vitro Toxicology (NACIVT) system—has been optimized to host and expose the epithelial–macrophage co-cultures (n=24 samples) to aerosolized particulate atmospheres at the ALI. Particle atmospheres are generated from a source chamber prior to delivery to the NACIVT system at approximately 0.6 L/min (i.e., to each cell sample at ~25 mL/min). Particle concentration (mass/volume) and aerosol size (MMAD) are sampled gravimetrically from the source chamber while particle deposition is measured by gravimetric analyses collected on filters in the NACIVT system. Cell metabolism/cytotoxicity assays (e.g., XTT/Lactate dehydrogenase [LDH) assays) and cytokine (e.g., IL-6) release assay have been developed and modified for use to assess toxicity/inflammatory endpoints. The XTT assay is a colorimetric assay designed to assess cell viability as a function of cell number based on metabolic activity.

Read Across—Nanogrouping

As more new nanomaterial types are being produced and entering commerce, the importance of adequately testing and assessing the hazards and potential risks of these particulates becomes critical. Of course, there is insufficient time and adequate funding to thoroughly evaluate the hazards of many of these new materials in vivo. Moreover, the trend away from animal testing makes the necessity for developing a new strategy for hazard evaluation critically important. One recently proposed practical strategy for assessing potential hazards of nanoparticles involves the grouping of similar particle-types, to streamline testing for regulatory purposes. The conceptual design for this strategy is to group nanomaterials for assessment that are similar or complementary in physicochemical characteristics. Recently, the European Centre for Ecotoxicology and Toxicology of Chemicals Task Force (ECETOC) has proposed a methodology for the critical appraisal of existing concepts for the groupings of nanomaterials. These would include the properties

and biophysical interactions of nanomaterials, specific types of use and exposure, uptake and kinetics, as well as possible early and apical biological effects (Arts et al., 2014). Subsequently, the group proposed a decision-making framework for the grouping and testing of nanomaterials (Arts et al., 2015). This process was designed to cover all relevant aspects of a nanomaterial's life cycle and biological pathways. The authors developed four (general) main nanogroups of materials for testing considerations. These included the following groups: (1) soluble nanomaterials; (2) biopersistent high aspect ratio nanomaterials; (3) passive nanomaterials; and (4) active nanomaterials. The proposed grouping was designed to provide a hazard and risk assessment tool and strategy that conceivably could be tested and ultimately contribute to the sustainable development of nanotechnological products.

In a subsequent publication, this proposed methodology was implemented by developing case studies for putting into practice, the decision-making framework for the grouping and testing of nanomaterial groups (Arts et al., 2016). Per the authors, case studies covering the following nanomaterials were implemented: carbonaceous nanomaterials, metal oxide and metal sulfate particulates, amorphous silica and organic pigments were subjected (with limitation) to the decision-making framework for the potential grouping and testing of nanomaterials. The authors initially outlined two (physicochemical) tiers that relied exclusively on non-animal testing methods, followed by a third testing tier, from which data from rat short-term inhalation studies were evaluated per the four groups outlined in the previous paragraph. To summarize, the authors concluded that the utility of this grouping strategy for nanomaterial hazard assessment was confirmed (with limitation). Of course, this methodological strategy, as well as hazard and risk results for nanomaterial-types, will require confirmation and substantiation by independent investigators and research groups, but this testable concept represents an idea for organizing the testing and evaluation of new or untested nanomaterial-types, without the painstaking, time-consuming, and expensive route of individual testing of new and current products in commerce, to which consumers will be exposed.

Transition, Human—Eco-nanotoxicology

The goals of nanotoxicology are to identify and characterize a hazard of ENMs for purposes of risk assessment for humans and the environment, and this requires a highly multidisciplinary team approach, covering expertise in toxicology, biology, chemistry, physics, material science, geology, exposure assessment, PBPK and fate and transport modeling, and medicine is necessary to develop: testing strategies, establish toxicity ranking, determine "safe" exposure levels, and derive preventive exposure guidelines. As depicted in Fig. 29-24, exposures throughout the life cycle of ENMs, from their source to their disposal, have to be considered. Dispersion and intermediate transformations in air, water, food, and soil are important modifiers of ENM–receptor interactions. Identifying underlying mechanisms of such interactions will aid in assessing risk and establishing preventive measures. Developing predictive models is a distant goal for both human and eco-nanotoxicology.

ECOTOXICOLOGY OF ENMS

Environmental Uses and Exposures to Nanomaterials

As with many chemicals in the marketplace, it is estimated that a portion of the nanomaterials used in industry and consumer products will enter the greater environment during some part of their life cycle, either through waste during production or through product use. The exponential increase in use of ENMs in a multitude of industries and consumer products has been documented by the Woodrow Wilson Center's project on Emerging Technologies, which as of March 2011 estimated that self-identified nanomaterial products had grown from 54 to over 1300 in 5 years. This list likely underestimates the number of uses of nanomaterials as it only includes products easily identified as containing nanomaterials and does not include other known uses of nanomaterials, including industrial catalysts, wastewater treatment, in addition to environmental cleanup technologies and pesticides, which would directly place nanomaterials into the environment. Most of the uses identified are in cosmetics, clothing (where nanomaterials are often added for antimicrobial and antiodor functions), personal care products, and sporting goods. The most common nanomaterials included in these products are silver and carbon nanomaterials. Other chemicals used in personal care products, cosmetics, and clothing have been found to wash into the wastewater treatment system and end up in the aquatic environment either directly from the treatment facilities or through land applications of biosolids from wastewater treatment plants. It is anticipated that some of the nanomaterial components may have the same fate, and indeed a few documented cases indicate this may be possible (Wang et al., 2012) but a majority of the estimates of potential environmental release of these chemicals is from modeling based on data from other compounds without regard to specific properties of nanomaterials compared to their bulk counterparts (Mueller and Nowack, 2008; Gottschalk et al., 2009, 2010). These studies estimate that concentrations in the environment can range from ng/L to μg/L, with many agglomerating into larger than nanosized units and much of the NP mass binding to solids and removed into biosolid waste. However, despite these removal potentials, it has been shown that NPs can be emitted through the wastewater process in the NP size range in significant quantities (Westerhoff et al., 2011).

Nanomaterials directly applied to a particular ecosystem, such as those for cleanup of environmental toxicants or as part of a pesticide formulation may also lead to exposures. Nanoscale zero-valent iron and titanium dioxide are currently being evaluated as a removal technology to treat groundwater systems and some surface water or soil systems for metals, arsenic, organic chemicals such as chlorinated solvents, polychlorinated biphenyls (PCBs), and benzenes (Elliott and Zhang, 2001; Chen and von Mikecz, 2005; Quinn et al., 2005). Poly(ethylene) glycol–modified urethane has been proposed for the sequestration and enhanced bioavailability for degradation of organic contaminants (Tungittiplakorn et al., 2005). Pesticide formulations containing carbon-based nanomaterials and others have been sent to the U.S. Environmental Protection Agency (EPA) for evaluation and are assumed to be in development.

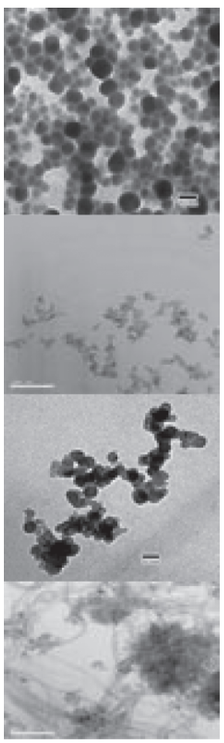
Individual organism measure	Population measure	Community measure	Ecosystem measure
Development Behavior Stress Growth Tumor incidence Egg production Survival	Reproduction LD50, LC50 Genetic diversity Mutation rate Change in recruitment	Bacterial diversity Presence of sensitive species	Nutrient cycling Degradation of habitat Missing food chain links Presence/ absence of functional groups

Figure 29-24. *Measures of ecological effect at the different levels.*

Ecological Risk Assessment of Manufactured Nanomaterials

One of the unusual aspects of the field of nanotechnology is the way in which the science of the risk of these materials to the environment has been instigated relatively early in the adoption of the technology. For most other contaminants, the risk assessment has largely been retrospective often decades after the chemical has been in the marketplace and is now distributed across the globe into many environmental compartments. The goal of this science to date has been to determine how a particulate form of a chemical in a nanosize range influences its toxicity versus its larger bulk counterparts. A further goal is to determine how small changes in surface chemistries, size, shape, and structure affect the interaction with organisms in the environment with the goal of informing industry on safe development. However, there are issues in assessing the risk of nanomaterials that are unique to this class of potential contaminant, and therefore the process of assessing risk also has unique considerations (some that have been mentioned in dealing with human health considerations). The assessment of ecological risk is also more complicated than human health assessments, as there are more species involved and higher level impacts on populations, communities of organisms, and ecosystem function need to be considered. The methods to evaluate the potential impacts of nanomaterials at higher levels of organization (community, ecosystem) have not been developed as is the case with many other chemicals.

The basic framework used for ecological risk assessment (Fig. 29-25) (EPA EPA/630/R-95/002F) involves problem formulation, analysis, and risk characterization. Problem formulation involves selecting endpoints of concern and developing a plan for determining impacts on important endpoints (such as a species decline and ecosystem changes). Analysis involves evaluating the potential for exposure to stressors and potential effects at those exposure levels. Risk characterization involves taking the information on important endpoints and the analysis of exposure and effects data to determine the importance of a stressor. For nanomaterials, most of the ecological risk assessment research has been conducted as the analysis of effects of a limited number of commercially available materials, using traditional acute single-organism mortality endpoints of a few select species and with little information regarding sublethal types of effects or other endpoints of concern at the community or ecosystem level. In addition, the concentrations of exposures are much higher than what is considered to be a probable environmental level. As such it is difficult to draw conclusions of the actual risk of many of even the most common materials and the conclusion is often that these particles may not cause any impact as no acute toxicity is seen. Nanomaterials may also be transformed within the environment, and therefore the toxicity of the initial nanomaterial may not provide a complete idea of the toxicity over the lifetime of the material (Nowack et al., 2012). Part of this is due to the relative newness of this field, the particulate nature of the potential toxicants, and other challenges surrounding the methods for determining toxicity, methods for measuring potential exposures, and uncertainty in estimates of how the materials will be used in the future marketplace. Agencies involved in protecting public and environmental health in individual countries as well as international agencies are grappling with methods to develop a structure to assess risk for all nanomaterials and make predictions regarding new ones as they come into the marketplace. This assessment may take time as methods' development needs to progress with the field



Nanoparticle type	Hydrodynamic diameter
nC60	637.7 nm
C60-OH	171.1 nm
C60-BCD	103.7 nm
SWCNT-low func	1.4 nm in one dimension, μM in total

Figure 29-25. *Example of the differences in larger particle agglomerates with nanomaterial.* All three of these are fullerene particle; however, the unfunctionalized particles are two to six times larger agglomerates in a pure water suspension compared with the functionalized counterparts. The stability in suspension also differs with functionalization (represented by the zeta potential).

of nanotechnology as new materials are being developed. The current state of the field is represented below, but the reader should keep in mind that it is rapidly changing.

Toxicity of Manufactured Nanomaterials

Complications of Assays Traditionally, ecotoxicology assays follow standard protocols and involve a group of species that has been selected to be representative of various organisms in the environment, including Daphnia, bacteria, fish, birds, and insects. The most standard assays involve 24- to 48-hour exposures and mortality endpoints such as LD50. Other standard assays for these species include month-long trials assessing growth and reproduction, tumor development, and other endpoints. Some issues arise in conducting these assays for nanomaterials, which is one of the reasons the current toxicological information regarding nanomaterials is variable. Some of the major issues in toxicology assays include delivery of nanomaterials in media and approximating environmental conditions, characterizing exposures, maintaining exposures throughout an assay, and determining the state of exposure throughout an assay. For example, many nanomaterials are not easily dispersible and aggregate substantially when introduced into common exposure media. A hydrophobic particle will aggregate with other particles in water and to an even greater extent when salts are introduced into the media as is the case with many aquatic systems. This causes several issues when determining toxicity. First, the nanomaterial aggregate may no longer be in the nanosize range (hydrodynamic diameter >100 nm). In some cases, this larger aggregate is nevertheless more toxic than its bulk chemical counterpart. This has not been shown with all nanomaterials, however, and is controversial. Second, the nanomaterials as they aggregate settle out of suspension, so depending on the organism involved the actual exposure may change over time as particles are effectively removed from suspension, or they may increase as some organisms (Daphnia) collect particles not only from the water column but from the bottom of the vessel. Some researchers have attempted to suspend the nanomaterials using solvents or detergents for better distribution. Studies have shown that the solvent or detergent when not removed from the nanomaterials sufficiently can cause toxicity (Lovern and Klaper, 2006; Henry et al., 2007; Kovochich et al., 2009), and in others the data indicate that this smaller aggregate size created by the additive changes the toxicity (e.g., Lovern and Klaper, 2006). Researchers have attempted to circumvent this issue by either changing the surface chemistry of the nanomaterial to make it more readily suspendable by adding a hydrophilic functional group or by altering the exposure conditions to include organic material that assists in suspension. Each of these approaches raises its own issues for toxicology. Changing the surface chemistry of a nanomaterial can also change its toxicity (Klaper et al., 2010). The relationship between surface chemistry and toxicity is important to understand because surface functionalization is an important aspect of nanomaterial formulation for industrial applications. So determining the impact of different surface chemistry changes is a necessary part of not only ecotoxicology but nanotoxicology in general. A bigger issue is that many coatings can cause toxicity on their own regardless of whether it is attached to the nanomaterial, and has also shown to be removed in certain cases by the organisms in the test media, or the environment the organism inhabits, which dramatically changes the properties of the nanomaterial through the experiment (Roberts et al., 2007). Is the toxicity then due to the original particle or now to the altered form of the nanomaterial?

As part of determining the dose an organism actually encounters is determining how much of any nanomaterial actually reaches the organism and is taken up into the organism and its tissues. There are several difficulties in quantifying uptake, including identifying the nanomaterial within the matrix of the organism versus inside the organism, for some nanomaterials the issue is distinguishing the nanomaterial from the matrix of the organism or environment either due to their small size relative to other aggregates in a sample, or because of the chemical similarity of the nanomaterial with the matrix (carbon-based nanomaterials in tissue, for example). Bulk extractions of these materials have provided some indication of potential concentrations (Westerhoff et al., 2011), but missing is the finer detail regarding location and structure of nanomaterials within a matrix, which is necessary to determine that the nanomaterial itself is causing an effect and not its byproduct or a secondary physiological response. Another issue in conducting a normal ecotoxicological assay using nanomaterials is the issue of calculating the dose as a mass versus surface area as reported above. Most nanomaterials when reaching the environment are not in a spherical uniform form, which complicates any calculations of dose based on surface area.

The real adverse impacts of nanomaterials may not be due to ambient environmental concentrations that arise but may be due to some subset of materials that are persistent and biomagnify in the environment. Research indicates that various nanomaterials are taken up by organisms in the environment and much is excreted by the organism within hours (Lovern et al., 2008; Petersen et al., 2009). In addition, gold nanomaterials and cadmium selenide quantum dots have been shown to accumulate in tissues of organisms and are able to pass from one level to another in simple food chain experiments (Judy et al., 2011; Werlin et al., 2011). These include microbial food chains and those involving consumption of algae or bacteria into protozoa, herbivores, or detritivores. However, nanomaterials passing from one level to the next do not guarantee biomagnification up the entire food chain as seen in quantum dot studies using transfers from Daphnia to zebrafish or where protozoa take up quantum dots and accumulate them to a greater extent than the media concentration but rotifers feeding on the protozoa do not show further increases in concentrations of nanomaterials (Holbrook et al., 2008; Lewinski et al., 2011).

Ecotoxicity of Nanomaterials

The studies on the toxicity of ENMs to date have largely focused on simple structures of fullerenes, CNTs, metal oxides, and gold and silver particles with a few examples of the impacts of nanomaterials with varying surface chemistries or functional groups. The major conclusion from these data is that toxicity varies with the type of nanomaterial and is not universal across materials. There are varying degrees of toxicity depending on the type of nanomaterial, organism studied, and the co-occurrence of other environmental factors such as UV light, organic carbon, or low pH. The most common endpoints monitored in nanoecotoxicology are acute mortality measures with traditional toxicological models species such as Daphnia, zebrafish, trout, and Arabidopsis. Most studies find some degree of toxicity, but the concentrations of most nanomaterials that are needed to kill half of the sample population are in the mg/L range, which is far above the estimates of potential exposures to any nanomaterial. Nanomaterials may enter other organisms just as they do humans and other mammals, through external contact with outer tissues and breathing structures (gill, skin), and through ingestion. Nanomaterials have been shown to be ingested with and without food present in many circumstances, thus leading to contact with internal structures. The greatest damage measured has been to gill structures in fish and in digestive tissues in other species (Zhu et al., 2008; Bilberg et al., 2010). In terrestrial studies,

plants have been shown to accumulate nanomaterials in root tissues and it is assumed that inhalation is possible in certain terrestrial situations but impacts on birds and mammals other than rats have not been demonstrated.

Fullerenes and CNTs were some of the first nanomaterials investigated for their potential environmental impact. Fullerenes have been shown to be lethal to Daphnia and microbial organisms (e.g., Lovern and Klaper, 2006; Zhu et al., 2006). However, ecotoxicity studies have indicated that the damaging impacts of these nanomaterials can be dramatically impacted by the method of introduction into exposure media. This includes introduction by solvent carriers or by altering the materials with surface chemistries that make them more water soluble. Unmodified fullerene nanomaterials may only be toxic at very high doses of mg/L and mg/kg of soil (Zhu et al., 2006; Li and Alvarez, 2011; Mouchet et al., 2011) and have been shown to have little impact on bacterial communities and protozoa (Johansen et al., 2008). However, increasing their solubility through either solvent or detergent introduction or by changing surface chemistry can also increase their toxicity to aquatic species (Klaper et al., 2010).

Silver nanomaterials are some of the most widely used materials and appear to demonstrate the greatest toxicity of materials investigated in the literature. Silver, in particular, is toxic at μg/L doses to a variety of organisms (George et al., 2011; Bilberg et al., 2012). However, silver and other metal nanomaterials are subject to dissolution even with various coatings and their entire toxicity may be due to the ionic component of an exposure rather than the nanomaterial form (Kennedy et al., 2010). These metal ions may accumulate in the body of organisms after ingestion of nanomaterials and contribute to toxicity (Asharani et al., 2008; Li et al., 2011). Rather than creating a free radical in media, the impacts of metal nanomaterials may be due to metal imbalance in cells after uptake and accumulation, leading to apoptosis and cellular disregulation (Kao et al., 2012). However, it has been suggested that the dissolution and ionic form of the metals is not the sole reason for nanometal toxicities (Griffitt et al., 2007). Nanosilver and possibly other nanomaterials based on soft metals may also react with environmental sulfides to produce silver sulfide nanomaterials, in which the silver bioavailability and toxicity is much reduced (Kim et al., 2010; Liu and Hurt, 2010).

Mechanisms of Toxicity

As in mammalian toxicology studies, oxidative stress has been implicated as a major way in which nanomaterials exert toxicity either by generating free radicals within the suspension media or by changing the chemistry of the cells in which they come in contact. Metal oxide nanomaterials, in particular, have been found to generate oxidative stress. Hydroxyl radicals and oxidative stress have been found to increase over time in the presence of metal oxides and UV light (Yu et al., 2011). As in human tissues, the stimulation of oxidative stress pathways alone is not an indication of harm and may be countered by adaptation by the organism to exposure. Mortimer et al. (2010) found that despite oxidative stress in initial assays, toxicity decreased over time in protozoa after exposure to metal oxide nanomaterials. However, Klaper et al. (2009) found that acute assays of oxidative stress do provide an early indicator of mortality of Daphnia in chronic assays for a variety of nanomaterial types. Metal oxide nanomaterials appear to have greater toxicity than their bulk counterparts. Titanium dioxide nanomaterials generate more ROS per gram than similar macroparticulates (Xiong et al., 2011). However, they do not differ from larger aggregates in their toxicity to developing fish (Zhu et al., 2008). Most studies have found a greater difference among nanomaterials composed of different metal oxides than the difference between the bulk and nanomaterial form of these compounds.

Metal nanomaterials have been found to cause a suite of effects, which in fish include negative impacts on respiration, oxidative stress, and development (Zhu et al., 2008; Shaw and Handy, 2011), in *Caenorhabditis elegans* increased mortality and decreased reproduction (Wang et al., 2012) and inhibit algal growth (Peng et al., 2011). Differences among metal oxides may be a result of differential solubilities in media.

The toxicity of nanomaterials to aquatic organisms can be greatly dependent upon the interaction of nanomaterials with the media to which they are introduced (Figs. 29-26 to 29-28). For example, dissolution of metal oxides and metal nanomaterials is greatly impacted by the characteristics of the media such as pH and salt content. Li et al. (2011) found that zinc oxide nanomaterials were most toxic in ultrapure water followed by salt, lithium borate (LB), and phosphate buffer saline (PBS) buffers. In the environment, toxicity may differ with the presence of organic matter, and the specific interaction of organic matter with a nanomaterial will differ depending on its composition. The degree of interaction of nanomaterial and organic matter has been shown to impact toxicity (Gao et al., 2009). Toxicity of particles sorbed to natural organic matter can be significantly greater (Edgington et al., 2010) or diminished (Blinova et al., 2010). Toxicity of nanomaterials can be significantly impacted by the surface chemistry, capping agents, and coatings of the particles. Additions of functional groups have been shown to increase toxicity in some cases (Klaper et al., 2009, 2010) and decrease toxicity in others (Allen et al., 2009). A few studies have also examined the influence of aging of nanomaterials in the environment on their toxicity.

Nanomaterials may also impact the bioavailability and toxicity of other contaminants in the environment. Fullerene and CNT particles, in particular, have been shown to bind to various organic contaminants with the strength of the association dependent upon

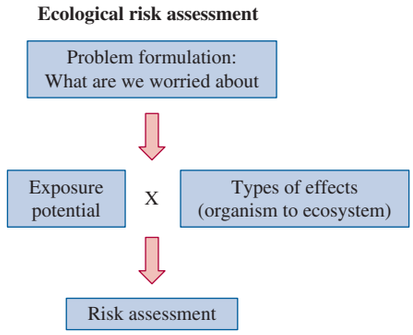


Figure 29-26. *Ecological risk assessment involves determining the types of concerns we have regarding a particular stressor and its impacts, determining the potential for exposure to environmental compartments, the types of potential impacts a stressor (in this case a nanomaterial) may have at those exposures, and using those two factors together to determine the risk to the environment.* With most nanomaterials we are relatively certain that environments such as freshwater systems that are receivers of wastewater will be exposed and most likely agricultural areas that receive solid wastes. The level of exposure is still in question and we have only begun to examine the types of effects. We have some information on model species such as Daphnia and trout as to the chance of mortality and some physiological effects in the acute exposure but little information is available on chronic low-level exposures. As such, it is currently premature to determine the actual risk to the environment.

Figure 29-27. *Suspension characteristics can have a significant impact on toxicity.* In this case single-walled nanotubes that have been functionalized with polyethylene glycol remain in suspension (on right) where their unfunctionalized counterparts do not (on left).

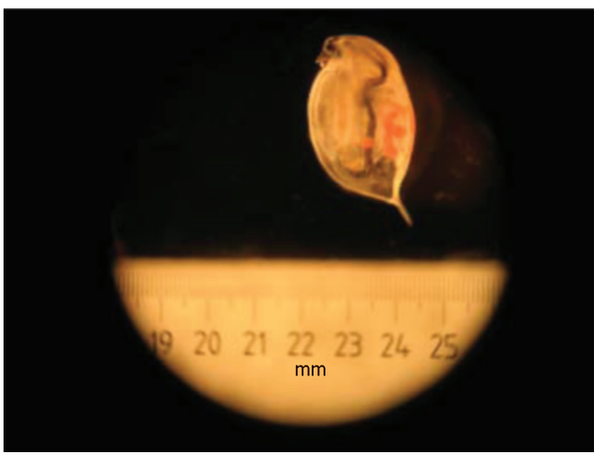


Figure 29-28. *Aquatic organisms, such as Daphnia magna, readily take up nanomaterials from the environment and their guts are rapidly filled with nanomaterials.* Some of these materials have been found to cross the gut barrier and translocate into other tissues within the organism.

the compound. Sorption to these nanomaterials can increase concentrations of bound organics by up to 60%, which if ingested could increase their toxicity (Baun et al., 2008).

As many nanomaterial exposures in the environment will occur at relatively low levels (μg to ng/L exposures), it is important to use assays other than LD50 to indicate the most relevant environmental effect. Including sublethal assays of physiology, assays of impacts of chronic low-level exposures, monitoring of alternative endpoints such as reproduction, growth, and at a higher level changes in community structure and ecosystem function, are essential. Nanomaterials cause a shift in overall behavior in Daphnia, which can indicate a greater susceptibility to predators and a greater energy cost leading to decreased reproduction (Lovern et al., 2007; Brausch et al., 2011). Various nanomaterials have been shown to impact development and physiology of fish species causing greater apoptosis in tissues, stress, and other impacts including structural effects (Chen et al., 2011; Shaw and Handy, 2011; Xiong et al., 2011; Truong et al., 2012). As the immune response is the first interaction of foreign substances with an organism, it has been shown that nanomaterials are stimulatory to the immune system of fish in particular and have an effect that is equal to the response to bacterial cell components, which may indicate an eventual cost to the organism (Klaper et al., 2010; Jovanović et al., 2011). Nanomaterials also have the potential to be mutagenic and in flies cause mutations that alter the phenotype significantly into the second generation (Vecchio et al., 2012).

Measurements of impacts at the community and ecosystem level are uncommon for nanomaterials, as they are for chemical toxicants with a much longer toxicology history. The existing studies indicate that community structure is impacted by the same nanomaterials that show toxicity and developmental impacts such as metals and metal oxides. Cerium dioxide and silver nanomaterials have a significant impact on marine microbial communities and microbial communities in wastewater—decrease in degradation potential and productivity (Fabrega et al., 2011; García et al., 2012). They also cause shifts in decomposition communities in freshwater streams (Pradhan et al., 2011). Nanomaterials such as unfunctionalized fullerenes that have been shown to be less toxic exhibit no impact on soil community structure (Tong et al., 2007), macrophyte structure (Velzeboer et al., 2011), or mutagenesis across generations (Nyberg et al., 2008) but do cause shifts in community composition of soil bacteria (Johansen et al., 2008). The overall composition may also be altered as microbial communities on mucus secretions of carp are different in those that have been exposed to fullerenes (Letts et al., 2011). The overall community impact is greatly affected by complexity in environmental samples (Kang et al., 2009).

Ecosystem impacts may be impacted by these community effects as bacterial community function has a great impact on nutrient cycling. CNTs have been shown to impact microbial activity (Chung et al., 2011), and fullerenes, silver, and CdSe quantum dots impact sediment bacterial oxidation of organics (Gao et al., 2009).

REFERENCES

Achariyar TM, Li B, Peng W, et al. Glymphatic distribution of CSF-derived apoE into brain is isoform specific and suppressed during sleep deprivation. *Mol Neurodegener*. 2016;11(1):74.

Aiso S, Yamazaki K, Umeda Y, et al. Pulmonary toxicity of intratracheally instilled carbon nanotubes in male Fischer 344 rats. *Ind Health*. 2011;48(6):783–795.

Akhavan O, Ghaderi E. Toxicity of graphene and graphene oxide nanowalls against bacteria. *ACS Nano*. 2010;4:5731–5736.

Albrecht C, Borm PJ, Unfried K. Signal transduction pathways relevant for neoplastic effects of fibrous and non-fibrous particles. *Mutat Res*. 2004;553:23–35.

Allen BL, Kichambare PD, Gou P, et al. Biodegradation of single-walled carbon nanotubes through enzymatic catalysis. *Nano Lett*. 2008;8:3899–3903.

Allen BL, Kotchey GP, Chen Y, et al. Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes. *J Am Chem Soc*. 2009;131:17194–17205.

Allen JL, Oberdorster G, Morris-Schaffer K, et al. Developmental neurotoxicity of inhaled ambient ultrafine particle air pollution: parallels with

neuropathological and behavioral features of autism and other neurodevelopmental disorders. *Neurotoxicology*. 2017;59:140–154.
Amenta V, Aschberger K, Boix SA, Calzolai L, Emons H, et al. Towards a review of the EC Recommendation for a definition of the term "nanomaterial" Part 2: Assessment of collected information concerning the experience with the definition. Publications Office of the European Union. 2014 JRC91377. doi:10.2787/97286. http://publications.jrc.ec.europa.eu/repository/handle/JRC91377.
Arts JH, Hadi M, Irfan MA, et al. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). *Regul Toxicol Pharmacol*. 2015;71(2 suppl):S1–S27.
Arts JH, Hadi M, Keene AM, et al. A critical appraisal of existing concepts for the grouping of nanomaterials. *Regul Toxicol Pharmacol*. 2014;70(2):492–506.
Arts JH, Irfan MA, Keene AM, et al. Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. *Regul Toxicol Pharmacol*. 2016;76:234–261.
Asgharian B, Miller FJ, Subramaniam RP. Dosimetry software to predict particle deposition in humans and rats. *CIIT Activities*. 1999;19(3):1–6.
Asharani PV, Lian Wu Y, Gong Z, Valiyaveettil S. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*. 2008;19(25):255102.
Azhdarzadeh M, Saei AA, Sharifi S, Hajipour MJ, et al. Nanotoxicology: advances and pitfalls in research methodology. *Nanomedicine*. 2015;10(18):2931–2952.
Bainbridge W. Public attitudes towards nanotechnology. *J Nanopart Res*. 2002;4:461–470.
Baisch BL, Corson NM, Wade-Mercer P, et al. Equivalent titanium dioxide nanoparticle deposition by intratracheal instillation and whole body inhalation: the effect of dose rate on acute respiratory tract inflammation. *Part Fibre Toxicol*. 2014;11(1):5.
Balásházy I, Hofmann W, Helstracher T. Local particle deposition patterns may play a key role in the development of lung cancer. *J Appl Physiol*. 2003;94:1719–1725.
Balásházy IW, Hofmann W, Farkas F, Madas BG. Three-dimensional model for aerosol transport and deposition in expanding and contracting alveoli. *Inhal Toxicol*. 2008;20(6):611–621.
Ballou B, Lagerholm BC, Ernst LA, Bruchez MP, Waggoner AS. Noninvasive imaging of quantum dots in mice. *Bioconjug Chem*. 2004;15:79–86.
Barrios FA, Gonzalez L, Favila R, et al. Olfaction and neurodegeneration in HD. *Neuroreport*. 2007;18:73.
Baun A, Sørensen SN, Rasmussen RF, Hartmann NB, Koch CB. Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C(60). *Aquat Toxicol*. 2008;86(3):379–387.
Bernstein D, Castranova V, Donaldson K, et al. Testing of fibrous particles: short-term assays and strategies. *Inhal Toxicol*. 2005;17:497–537.
Bianco A, Kostarelos K, Prato M. Making carbon nanotubes biocompatible and biodegradable. *Chem Commun (Camb)*. 2011;47:10182–10188.
Bilberg K, Hovgaard MB, Besenbacher F, Baatrup E. In vivo toxicity of silver nanoparticles and silver ions in Zebrafish (*Danio rerio*). *J Toxicol*. 2012;2012:293784.
Bilberg K, Malte H, Wang T, Baatrup E. Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquat Toxicol*. 2010;96(2):159–165.
Biswas R, Bunderson-Schelvan M, Holian A. Potential role of the inflammasome-derived inflammatory cytokines in pulmonary fibrosis. *Pulmon Med*. 2011;2011:105707.
Blinova I, Ivask A, Heinlaan M, Mortimer M, Kahru A. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environ Pollut*. 2010;158(1):41–47.
Blume C, Reale R, Held M, et al. Temporal monitoring of differentiated human airway epithelial cells using microfluidics. *PLoS One*. 2015;10(10):e0139872.
Boczkowski J, Hoet P. What's new in nanotoxicology? Implications for public health from a brief review of the 2008 literature. *Nanotoxicology*. 2009.
Bodian D, Howe HA. Experimental studies on intraneural spread of poliomyelitis virus. In: Bordley JI, ed. *Bulletin of the Johns Hopkins Hospital*. Baltimore, MD: The Johns Hopkins Press; 1941:LXVIII248–267.
Borm P, Cassee FR, Oberdorster G. Lung particle overload: old school-new insights? *Part Fibre Toxicol*. 2015;12:10.
Borm PJA, Robbins D, Haubold S, et al. The potential risks of nanomaterials: a review carried out for ECETOC. *Part Fibre Toxicol*. 2006;3(11).
Boya P, Kroemer G. Lysosomal membrane permeabilization in cell death. *Oncogene*. 2008;27:6434–6451.
Brausch KA, Anderson TA, Smith PN, Maul JD. The effect of fullerenes and functionalized fullerenes on Daphnia magna phototaxis and swimming behavior. *Environ Toxicol Chem*. 2011;30(4):878–884.
Brodie M, Elvidge AR. The portal of entry and transmission of the virus of poliomyelitis. *Science*. 1934;79(2045):235–236.
Brown SC, Boyko V, Meyers G, Voetz M, Wohlleben W. Toward advancing nano-object count metrology: a best practice framework. *Environ Health Perspect*. 2013;121(11–12):1282–1291.
Brunauer S, Emmett PH, Teller E. Adsorption of gases in multimolecular layers. *J Am Chem Soc*. 1938;60:309–319.
Calderon-Garciduenas L, Engle R, Mora-Tiscareno A, et al. Exposure to severe urban air pollution influences cognitive outcomes, brain volume and systemic inflammation in clinically healthy children. *Brain Cogn*. 2011;77:345–355.
Casey A, Herzog E, Davoren M, et al. Spectroscopic analysis confirms the interactions between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity. *Carbon*. 2007;45:1425–1432.
Casey A, Herzog E, Lyng FM, et al. Single walled carbon nanotubes induce indirect cytotoxicity by medium depletion in A549 lung cells. *Toxicol Lett*. 2008;179:78–84.
Cassee FR, van Balen EC, Singh C, et al. Exposure, health and ecological effects: review of engineered nanoscale cerium and cerium oxide associated with its use as a fuel additive. *Crit Rev Toxicol*. 2011;41:213–229.
Castranova V, Schulte PA, Zumwalde RD. Occupational nanosafety considerations for carbon nanotubes and carbon nanofibers. *Acc Chem Res*. 2013;46(3):642–649.
Cedervall T, Lynch I, Foy M, et al. Detailed identification of plasma proteins adsorbed on copolymer nanoparticles. *Angew Chem Int Ed*. 2007b;46:5754–5756.
Cedervall T, Lynch I, Lindman S, et al. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proc Natl Acad Sci U S A*. 2007a;104:2050–2055.
Chen J, Dong X, Xin Y, Zhao M. Effects of titanium dioxide nano-particles on growth and some histological parameters of zebrafish (*Danio rerio*) after a long-term exposure. *Aquat Toxicol*. 2011;101(3–4):493–499.
Chen M, von Mikecz A. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles. *Exp Cell Res*. 2005;305:51–62.
Chithrani DB. Intracellular uptake, transport, and processing of gold nanostructures. *Mol Membr Biol*. 2010;27:299–311.
Choi HS, Liu W, Misra P, et al. Renal clearance of quantum dots. *Nat Biotechnol*. 2007;25(10):1165–1170.
Chou LY, Ming K, Chan WC. Strategies for the intracellular delivery of nanoparticles. *Chem Soc Rev*. 2011;40:233–245.
Chu M, Wu Q, Yang H, et al. Transfer of quantum dots from pregnant mice to pups across the placental barrier. *Small*. 2010;6(5):670–678.
Chung H, Son Y, Yoon TK, Kim S, Kim W. The effect of multi-walled carbon nanotubes on soil microbial activity. *Ecotoxicol Environ Saf*. 2011;74(4):569–575.
Clarkson TW, Friberg L, Nordberg GF, Sager PR, eds. *Biological Monitoring of Toxic Metals*. New York: Plenum Press; 1988:686.
Cobb MD, Macoubrie J. Public perceptions about nanotechnology: risks, benefits and trust. *J Nanoparticle Res*. 2004;6(4):395–405.
Cole AJ, Yang VC, David AE. Cancer theranostics: the rise of targeted magnetic nanoparticles. *Trends Biotechnol*. 2011;29:323–332.
Colvin VL. The potential environmental impact of engineered nanomaterials. *Nat Biotechnol*. 2003;21:1166–1170.
Cone RA. Barrier properties of mucus. *Adv Drug Deliv Rev*. 2009;61:75–85.
Costa M, Davidson TL, Chen H, et al. Nickel carcinogenesis: epigenetics and hypoxia signaling. *Mutat Res*. 2005;592:79–88.

Czerniawska A. Experimental investigations on the penetration of ^{198}Au from nasal mucous membrane into cerebrospinal fluid. *Acta Otolaryngol*. 1970;70:58–61.

de Bruijne K, Ebersviller S, Sexton KG, et al. Design and testing of electrostatic aerosol in vitro exposure system (EAVES): an alternative exposure system for particles. *Inhal Toxicol*. 2009;21(2):91–101.

De Jong WH, Borm PJ. Drug delivery and nanoparticles: applications and hazards. *Int J Nanomed*. 2008;3:133–149.

De Lorenzo A. The olfactory neuron and the blood-brain barrier. In: Wolstenholme G, Knight J, eds. *Taste and Smell in Vertebrates*. London: J. & A. Churchill; 1970:151–176.

De Nicola M, Gattia D, Bellucci S, et al. Effect of different carbon nanotubes on cell viability and proliferation. *J Phys Condens Matter*. 2007;19(39):395013.

DeLoid GM, Cohen JM, Pyrgiotakis G, et al. Advanced computational modeling for in vitro nanomaterial dosimetry. *Part Fibre Toxicol*. 2015;12:32.

DeLoid GM, Cohen JM, Pyrgiotakis G, Demokritou P. Preparation, characterization, and in vitro dosimetry of dispersed, engineered nanomaterials. *Nat Protoc*. 2017;12(2):355–371.

DeLorme MP, Muro Y, Arai T, et al. Ninety-day inhalation toxicity study with a vapor grown carbon nanofiber in rats. *Toxicol Sci*. 2012;128(2):449–460.

Doak SH, Griffiths SM, Manshian B, et al. Confounding experimental considerations in nanogenotoxicology. *Mutagenesis*. 2009;24:285–293.

Donaldson K, Aitken R, Tran L, et al. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol Sci*. 2006;92:5–22.

Donaldson K, Beswick PH, Gilmour PS. Free radical activity associated with the surface of particles: a unifying factor in determining biological activity? *Toxicol Lett*. 1996;88(1–3):293–298.

Donaldson K, Borm PJ, Castranova V, Gulumian M. The limits of testing particle-mediated oxidative stress in vitro in predicting diverse pathologies: relevance for testing of nanoparticles. *Part Fibre Toxicol*. 2009;6:13.

Donaldson K, Murphy FA, Duffin R, Poland CA. Asbestos, carbon nanotubes and the pleural mesothelium: a review and the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol*. 2010;7(1):5.

Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJA. Nanotoxicology (editorial)—a new frontier in particle toxicology relevant to both the workplace and general environment and to consumer safety. *Occup Environ Med*. 2004;61:727–728.

Dostert C, Petrilli V, Van Bruggen R, et al. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 2008;320:674–677.

Doty RL. The olfactory vector hypothesis of neurodegenerative disease: is it viable? *Ann Neurol*. 2008;63:7.

Dresselhaus MS, Terrones M. Carbon-based nanomaterials from a historical perspective. *Proceedings of the IEEE*. 2013;101(No. 7):1522–1535.

Drinker K, Drinker PRM. Metal fume fever: V. Results of the inhalation by animals of zinc and magnesium oxide fumes. *J Ind Hyg*. 1928;10:56–71.

Drinker P, Finn JL, Thomson RM. Metal fume fever II: resistance acquired by inhalation of zinc oxide on two successive days. *J Ind Hyg*. 1927a;9:98–105.

Drinker P, Thomson RM, Finn JL. Metal fume fever IV: threshold doses of zinc oxide preventive measures and the chronic effects of repeated exposures. *J Ind Hyg*. 1927b;9:331–345.

Driscoll K, Costa DL, Hatch G, et al. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol Sci*. 2000;55:24–35.

Duncan R. Polymer therapeutics as nanomedicines: new perspectives. *Curr Opin Biotechnol*. 2011;22:492–501.

Dutta D, Sundaram SK, Teeguarden JG, et al. Adsorbed proteins influence the biological activity and molecular targeting of nanomaterials. *Toxicol Sci*. 2007;100(1):303–315.

Dvir T, Timko BP, Kohane DS, Langer R. Nanotechnological strategies for engineering complex tissues. *Nat Nanotechnol*. 2011;6:13–22.

EC Directive 97/69/EC, Nota Q. EU Commission, directive dated 5 December 1997. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31997L0069:EN:HTML.

Edgington AJ, Roberts AP, Taylor LM, et al. The influence of natural organic matter on the toxicity of multiwalled carbon nanotubes. *Environ Toxicol Chem*. 2010;29(11):2511–2518.

Elder A, Gelein R, Silva V, et al. Translocation of inhaled ultrafine manganese oxide particles to the central nervous system. *Environ Health Perspect*. 2006;114:1172–1178.

Elliott D, Zhang W. Field assessment of nanoparticles for groundwater treatment. *Environ Sci Technol*. 2001;35:4922–4926.

European Commission. *E.C. Commission Recommendation of . . . . on the Definition of the Term "Nanomaterial"*. Brussels: European Commission; 2011a.

European Commission. E.C. Commission recommendation on the definition of nanomaterial. *Official J European Union*. 2011/696/EU. 2011b (adopted).

Evans SJ, Clift MJ, Singh N, et al. Critical review of the current and future challenges associated with advanced in vitro systems towards the study of nanoparticle (secondary) genotoxicity. *Mutagenesis*. 2017;32(1):233–241.

Fabrega J, Luoma SN, Tyler CR, Galloway TS, Lead JR. Silver nanoparticles: behaviour and effects in the aquatic environment. *Environ Int*. 2011;37(2):517–531.

Fawcett TW, Sylvester SL, Sarge KD, Morimoto RI, Holbrook NJ. Effects of neurohormonal stress and aging on the activation of mammalian heat shock factor 1. *J Biol Chem*. 1994;269(51):32272–32278.

Feng L, Liu Z. Graphene in biomedicine: opportunities and challenges. *Nanomedicine (Lond)*. 2011;6:317–324.

Fenoglio I, Fubini B, Ghibaudi EM, Turci F. Multiple aspects of the interaction of biomacromolecules with inorganic surfaces. *Adv Drug Deliv Rev*. 2011;63:1186–1209.

Fenoglio I, Tomatis M, Lison D, et al. Reactivity of carbon nanotubes: free radical generation or scavenging activity? *Free Radic Biol Med*. 2006;40:1227–1233.

Feynman R. *There's Plenty of Room at the Bottom*. Classic talk given on 12/29/59 at the American Physical Society at Caltech. First published in *Caltech Engineering and Science*, February 1960;23(5):22–36.

Fissan H. Nachhaltige nanotechnologie. In: *Nordrhein-Westfälische Akademie der Wissenschaften*. Herstellung: Ferdinand Schöningh, Paderborn; 2008.

Flahaut E, Durrieu MC, Remy-Zolghadri M, Bareille R, Baquey CH. Investigation of the cytotoxicity of CCVD carbon nanotubes towards human umbilical vein endothelial cells. *Carbon*. 2006;44(6): 1093–1099.

Food and Agriculture Organization of the United Nations/World Health Organization. FAO/WHO Expert Meeting on the Application of Nanotechnologies in the Food and Agriculture Sectors: Potential Food Safety Implications. Meeting Report. Rome; 2009:104.

Fortin JP, Wilhelm C, Christin JS. Size-sorted anionic iron oxide nanomagnets as colloidal mediators for magnetic hyperthermia. *J Am Chem Soc*. 2007;129:2628–2635.

Fubini B, Ghiazza M, Fenoglio I. Physico-chemical features of engineered nanoparticles relevant to their toxicity. *Nanotoxicology*. 2010; 4(4):347–363.

Gao J, Youn S, Hovsepyan A, et al. Dispersion and toxicity of selected manufactured nanomaterials in natural river water samples: effects of water chemical composition. *Environ Sci Technol*. 2009;43(9):3322–3328.

García A, Delgado L, Torà JA, et al. Effect of cerium dioxide, titanium dioxide, silver, and gold nanoparticles on the activity of microbial communities intended in wastewater treatment. *J Hazard Mater*. 2012;199–200:64–72.

Geiser M, Casaulta M, Kupferschmid B, et al. The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles. *Am J Respir Cell Mol Biol*. 2008;38:371–376.

Geiser M, Jeannet N, Fierz M, Burtscher H. Evaluating adverse effects of inhaled nanoparticles by realistic in vitro technology. *Nanomaterials (Basel)*. 2017;7(2):49.

Geiser M, Kreyling WG. Deposition and biokinetics of inhaled nanoparticles. *Part Fibre Toxicol*. 2010;7:2.

Geiser M, Rothen-Rutishauser B, Kapp N, et al. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environ Health Perspect*. 2005;113:1555–1560.

George S, Xia T, Rallo R, et al. Use of a high-throughput screening approach coupled with in vivo zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. *ACS Nano*. 2011;5(3):1805–1817.

Glover RD, Miller JM, Hutchison JE. Generation of metal nanoparticles from silver and copper objects: nanoparticle dynamics on surfaces and potential sources of nanoparticles in the environment. *ACS Nano*. 2011;5(11):8950–8957.

Gordon S, Daneshian M, Bouwstra J, et al. Non-animal models of epithelial barriers (skin, intestine and lung) in research, industrial applications and regulatory toxicology. *ALTEX*. 2015;32(4): 327-78.

Gottschalk F, Sonderer T, Scholz RW, Nowack B. Modeled environmental concentrations of engineered nanomaterials (TiO(2), ZnO, Ag, CNT, Fullerenes) for different regions. *Environ Sci Technol*. 2009;43(24):9216–9222.

Gottschalk F, Sonderer T, Scholz RW, Nowack B. Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. *Environ Toxicol Chem*. 2010;29(5):1036–1048.

Graham UM, Jacobs G, Yokel RA, et al. From dose to response: In vivo nanoparticle processing and potential toxicity. *Adv Exp Med Biol*. 2017;947:71–100.

Graham UM, Tseng MT, Jasinski JB, et al. In vivo processing of ceria nanoparticles inside liver: impact on free-radical scavenging activity and oxidative stress. *Chempluschem*. 2014;79(8):1083–1088.

Griffitt RJ, Weil R, Hyndman KA, et al. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). *Environ Sci Technol*. 2007;41(23):8178–8186.

Grosse Y, Loomis D, Guyton KZ, et al. Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. *Lancet Oncol*. 2014;15(13):1427–1428.

Guo L, Liu X, Vaslet C, Hurt RH, Kane AB. Iron bioavailability and redox activity in diverse carbon nanotube samples. *Chem Mater*. 2007;19:3472–3478.

Guo L, Von Dem Bussche A, Buechner M, et al. Adsorption of essential micronutrients by carbon nanotubes and the implications for nanotoxicity testing. *Small*. 2008;4:721–727.

Hallock MF, Greenlay P, DiBerardinis L, Kallin D. Potential risks of nanomaterials and how to safely handle materials of uncertain toxicity. *J Chem Health Saf*. 2009;16:16–23.

Hamilton RF, Wu N, Porter D, et al. Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity. *Part Fibre Toxicol*. 2009;6:35.

Han GH, Andrews R, Gairola CG. Acute pulmonary response of mice to multi-wall carbon nanotubes. *Inhal Toxicol.* 2010;22:340–347.

Han X, Gelein R, Corson N. Validation of an LDH assay for assessing nanoparticle toxicity. *Toxicology*. 2011;287:99–104.

Haniu H, Saito N, Matsuda Y, et al. Effect of dispersants of multi-walled carbon nanotubes on cellular uptake and biological responses. *Intl J Nanomed*. 2011;6:3295–3307.

Henry TB, Menn FM, Fleming JT, et al. Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. *Environ Health Perspect*. 2007;115(7):1059–1065.

Hinderliter PM, Minard KR, Orr G, et al. ISDD: a computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. *Part Fibre Toxicol*. 2010;7:36.

Hinds WC. Time for number concentration to halve and particle size to double by simple monodisperse coagulation. In: *Aerosol Technology*. New York: John-Wiley; 1982:235–239.

Hirano S, Fujitani Y, Furuyama A, Kanno S. Uptake and cytotoxic effects of multi-walled carbon nanotubes in human bronchial epithelial cells. *Toxicol Appl Pharmacol*. 2010;249(1):8–15.

Hirano S, Kanno S, Furuyama A. Multi-walled carbon nanotubes injure the plasma membrane of macrophages. *Toxicol Appl Pharmacol*. 2008;232(2):244–251.

Hoet PHM, Bruske-Hohlfeld I, Salata OV. Nanoparticles: known and unknown health risks. *J Nanobiotechnol*. 2004;2(12):12–27.

Holbrook RD, Murphy KE, Morrow JB, Cole KD. Trophic transfer of nanoparticles in a simplified invertebrate food web. *Nat Nanotechnol*. 2008;3(6):352–355.

Holder AL, Lucas D, Goth-Goldstein R, Koshland CP. Cellular response to diesel exhaust particles strongly depends on the exposure method. *Toxicol Sci*. 2008;103(1):108–115.

Hoshino A, Fujioka K, Oku T, et al. Physicochemical properties and cellular toxicity of nanocrystal quantum dots depend on their surface modification. *Nano Lett*. 2004;4(11):2163–2169.

Hunter DD, Undem BJ. Identification and substance P content of vagal afferent neurons innervating the epithelium of the guinea pig trachea. *Am J Respir Crit Care Med*. 1999;159:1943–1948.

Hussain SM, Warheit DB, Ng SP, et al. At the crossroads of nanotoxicology in vitro: past achievements and current challenges. *Toxicol Sci*. 2015;147(1):5–16.

Hutchison JE. Greener nanoscience: a proactive approach to advancing applications and reducing implications of nanotechnology. *ACS Nano*. 2008;2:395–402.

ICRP. *Annals of the ICRP, Human Respiratory Tract Model for Radiological Protection*. ICRP Publication 66, Pergamon; 1994;24:1–3.

Iezzi R, Guru B, Glybina IV, et al. Dendrimer-based targeted intravitreal therapy for sustained attenuation of neuroinflammation in retinal degeneration. *Biomaterials*. 2012;33:979–988.

Iliff JJ, Wang M, Liao Y, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Sci Transl Med*. 2012;4(147):147ra111.

ISO/DIS 26824. *Particle characterization of particulate systems—Vocabulary*. ISO/TS; 2011.

ISO/TC 229. Nanotechnologies—Vocabulary—Part 1: Core Terms. ISO/TS 80004-1:2015 (en). Available at: https://www.iso.org/obp/ui#iso:std:iso:ts:80004:-1:ed-2:v1:en. 2015.

ISO/TS 27687. Nanotechnologies—Terminology and definitions for nano-objects—Nanoparticle, nanofibre and nanoplate. ISO/TS; 2008.

Jacobsen NR, Pojana G, White P, et al. Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C(60) fullerenes in the FE1-Mutatrade mark-Mouse lung epithelial cells. *Environ Mol Mutagen*. 2008;49(6):476–487.

Jakubek LM, Marangoudakis S, Raingo J, Liu X, Lipscombe D, Hurt RH. The inhibition of neuronal calcium ion channels by trace levels of yttrium released from carbon nanotubes. *Biomaterials*. 2009;30:6351–6357.

Jani PU, Halbert GW, Langridge J, Florence AT. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. *J Pharm Pharmacol*. 1990;42:821–826.

Jani PU, McCarthy DE, Florence AT. Titanium dioxide (rutile) particle uptake from the rat GI tract and translocation to systemic organs after oral administration. *Int J Pharm*. 1994;105:157–168.

Jiang J, Oberdörster G, Biswas P. Characterization of size surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *J Nanopart Res*. 2009;11:77–89.

Johansen A, Pedersen AL, Jensen KA, et al. Effects of C60 fullerene nanoparticles on soil bacteria and protozoans. *Environ Toxicol Chem*. 2008;27(9):1895–1903.

Johnston CJ, Finkelstein JN, Mercer P, et al. Pulmonary effects induced by ultrafine PTFE particles. *Toxicol Appl Pharmacol*. 2000;168:208–215.

Johnston HJ, Hutchison GR, Christensen FM, et al. A critical review of the biological mechanisms underlying the *in vivo* and *in vitro* toxicity of carbon nanotubes: the contribution of physico-chemical characteristics. *Nanotoxicology*. 2010a;4(2):207–246.

Johnston HJ, Semmler-Behnke M, Brown DM, et al. Evaluating the uptake and intracellular fate of polystyrene nanoparticles by primary and hepatocyte cell lines in vitro. *Toxicol Appl Pharmacol*. 2010b;242:66–78.

Jones CF, Grainger DW. *In vitro* assessments of nanomaterial toxicity. *Adv Drug Deliv Rev*. 2009;61(6):438–456.

Jovanović B, Anastasova L, Rowe EW, et al. Effects of nanosized titanium dioxide on innate immune system of fathead minnow (*Pimephales promelas* Rafinesque, 1820). *Ecotoxicol Environ Saf*. 2011;74(4):675–683.

Judy JD, Unrine JM, Bertsch PM. Evidence for biomagnification of gold nanoparticles within a terrestrial food chain. *Environ Sci Technol*. 2011;45(2):776–781. [Epub December 3, 2010].

Kagan VE, Konduru NV, Feng W. Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. *Nat Nanotechnol*. 2010;5:354–359.

Kagan VE, Tyurina YY, Tyurin VA, et al. Direct and indirect effects of single walled carbon nanotubes on RAW 264.7 macrophages: role of iron. *Toxicol Lett*. 2006;165:88–100.

Kahru A, Dubourguier HC. From ecotoxicology to nanoecotoxicology. *Toxicology*. 2010;269:105–119.

Kane AB, Hurt RH. Nanotoxicology: the asbestos analogy revisited. *Nat Nanotechnol*. 2008;3(7):378–379.

Kang B, Mackey MA, El-Sayed MA. Nuclear targeting of gold nanoparticles in cancer cells induces DNA damage, causing cytokinesis arrest and apoptosis. *J Am Chem Soc*. 2010;132(5):1517–1519.

Kang S, Herzberg M, Rodrigues DF, Elimelech M. Antibacterial effects of carbon nanotubes: size does matter! *Langmuir*. 2008;24:6409–6413.

Kang S, Mauter MS, Elimelech M. Microbial cytotoxicity of carbon-based nanomaterials: implications for river water and wastewater effluent. *Environ Sci Technol*. 2009;43(7):2648–2653.

Kang S, Pinault M, Pfefferle LD, Elimelech M. Single-walled carbon nanotubes exhibit strong antimicrobial activity. *Langmuir*. 2007;23: 8670–8673.

Kanno J, Takagi A, Nishimura T, Hirose A. Mesothelioma induction by micrometer-sized multi-walled carbon nanotube intraperitoneally injected to p53 heterozygous mice. *Toxicologist*. 2010;114:A1397.

Kanno S, Furuyama A, Hirano S. A murine scavenger receptor MARCO recognizes polystyrene nanoparticles. *Toxicol Sci*. 2007;97:398–406.

Kao YY, Chen YC, Cheng TJ, Chiung YM, Liu PS. Zinc oxide nanoparticles interfere with zinc ion homeostasis to cause cytotoxicity. *Toxicol Sci*. 2012;125(2):462–472.

Karmali PP, Simberg D. Interactions of nanoparticles with plasma proteins: implication on clearance and toxicity of drug delivery systems. *Expert Opin Drug Deliv*. 2011;8:343–357.

Kasai T, Umeda Y, Ohnishi M, et al. Thirteen-week study of toxicity of fiber-like multi-walled carbon nanotubes with whole-body inhalation exposure in rats. *Nanotoxicology*. 2015;9:413–422.

Kasai T, Umeda Y, Ohnishi M, et al. Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. *Part Fibre Toxicol*. 2016;13:53.

Kennedy AJ, Hull MS, Bednar AJ, et al. Fractionating nanosilver: importance for determining toxicity to aquatic test organisms. *Environ Sci Technol*. 2010;44(24):9571–9577.

Khandoga A, Stoeger T, Khandoga AG, et al. Platelet adhesion and fibrinogen deposition in murine microvessels upon inhalation of nanosized carbon particles. *J Thromb Haemost*. 2010;8:1632–1640.

Kim BY, Rutka JT, Chan WC. Nanomedicine. *N Engl J Med*. 2010;363:2434–2443.

Klaine SJ, Alvarez PJ, Batley GE, et al. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environ Toxicol Chem*. 2008;27:1825–1851.

Klaper R, Arndt D, Setyowati K, Chen J, Goetz F. Functionalization impacts the effects of carbon nanotubes on the immune system of rainbow trout, *Oncorhynchus mykiss*. *Aquat Toxicol*. 2010;100(2):211–217.

Klaper R, Crago J, Barr J, Arndt D, Setyowati K, Chen J. Toxicity biomarker expression in daphnids exposed to manufactured nanoparticles: changes in toxicity with functionalization. *Environ Pollut*. 2009;157(4): 1152–1156.

Kobayashi N, Naya M, Ema M, et al. Biological response and morphological assessment of individually dispersed multi-wall carbon nanotubes in the lung after intratracheal instillation in rats. *Toxicology*. 2010;276(3):143–153.

Kolosnjaj-Tabi J, Hartman KB, Boudjemaa S, et al. *In vivo* behavior of large doses of ultrashort and full-length single-walled carbon nanotubes after oral and intraperitoneal administration to Swiss mice. *ACS Nano*. 2010;4(3):1481–1492.

Kotchey GP, Allen BL, Vedala H, et al. The enzymatic oxidation of graphene oxide. *ACS Nano*. 2011;5:2098–2108.

Kovács T. Mechanisms of olfactory dysfunction in aging and neurodegenerative disorders. *Ageing Res Rev*. 2004;3:215.

Kovochich M, Espinasse B, Auffan M, et al. Comparative toxicity of C60 aggregates toward mammalian cells: role of tetrahydrofuran (THF) decomposition. *Environ Sci Technol*. 2009;43(16):6378–6384.

Kreuter J. Influence of the surface properties on nanoparticle-mediated transport of drugs to the brain. *J. Nanosci Nanotechnol*. 2004;4:484–488.

Kreyling WG, Semmler M, Erbe F, et al. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A*. 2002;65(20):1513–1530.

Kreyling WG, Semmler-Behnke M, Seitz J, et al. Size dependence of the translocation of the inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. *Inhal Toxicol*. 2009;21(S1):55–60.

Krug HF. Nanosafety research—are we on the right track? *Angew Chem Int Ed*. 2014;53:12304–12319. doi:10.1002/anie.201403367.

Krug HF, Wick P. Nanotoxicology: an interdisciplinary challenge. *Angew Chem Int Ed*. 2011;50:1260–1278.

Kuempel ED, Jaurand MC, Møller P, et al. Evaluating the mechanistic evidence and key data gaps in assessing the potential carcinogenicity of carbon nanotubes and nanofibers in humans. *Crit Rev Toxicol*. 2017;47(1):1–58.

Kunzmann A, Andersson B, Thurnherr T, et al. Toxicology of engineered nanomaterials: focus on biocompatibility, biodistribution and biodegradation. *Biochim Biophys Acta*. 2011;1810:361–373.

Lacerda L, Soundararajan A, Singh R, et al. Dynamic imaging of functionalized multi-walled carbon nanotube systemic circulation and urinary excretion. *Adv Mater*. 2008;20:225–230.

Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev*. 2009;61:158–171.

Lajoie P, Nabi IR. Lipid rafts, caveolae, and their endocytosis. *Int Rev Cell Mol Biol*. 2010;282:135–163.

Lam GW, James JT, McCluskey R, Hunter RL. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol Sci*. 2004;77:125–134.

Landsiedel R, Ma-Hock L, Kroll A, et al. Testing metal-oxide nanomaterials for human safety. *Adv Mater*. 2010;22:2601–2627.

Lee KP, Seidel WC. Pulmonary response of rats exposed to polytetrafluoroethylene and tetrafluoroethylene hexafluoropropylene copolymer fume and isolated particles. *Inhal Toxicol*. 1991;3:237–264.

Lenz AG, Karg E, Lentner B, et al. A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. *Part Fibre Toxicol*. 2009;6:32.

Lesniak W, Bielinska AU, Sun K, et al. Silver/dendrimer nanocomposites as biomarkers: fabrication, characterization, in vitro toxicity, and intracellular detection. *Nano Lett*. 2005;5:2123–2130.

Letts RE, Pereira TC, Bogo MR, Monserrat JM. Biologic responses of bacteria communities living at the mucus secretion of common carp (*Cyprinus carpio*) after exposure to the carbon nanomaterial fullerene (C60). *Arch Environ Contam Toxicol*. 2011;61(2):311–317.

Lewinski NA, Zhu H, Ouyang CR, et al. Trophic transfer of amphiphilic polymer coated CdSe/ZnS quantum dots to *Danio rerio*. *Nanoscale*. 2011;3(8):3080–3083.

Li D, Alvarez PJ. Avoidance, weight loss, and cocoon production assessment for *Eisenia fetida* exposed to C_{60} in soil. *Environ Toxicol Chem*. 2011;30(11):2542–2545.

Li JG, Li QN, Xu JY, et al. The pulmonary toxicity of multi-wall carbon nanotubes in mice 30 and 60 days after inhalation exposure. *J Nanosci Nanotechnol*. 2009;9:1384–1387.

Li JG, Li WX, Xu JY, et al. Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation. *Environ Toxicol*. 2007;22:415–421.

Li M, Zhu L, Lin D. Toxicity of ZnO nanoparticles to *Escherichia coli*: mechanism and the influence of medium components. *Environ Sci Technol*. 2011;45(5):1977–1983.

Li N, Sioutas C, Cho A, et al. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environ Health Perspect*. 2003;111:455–460.

Li X, Fan Y, Watari F. Current investigations into carbon nanotubes for biomedical application. *Biomed Mater*. 2010;5(2):22001.

Li Y, Yuan H, von dem Bussche A, et al. Graphene microsheets enter cells through spontaneous membrane penetration at edge asperities and corner sites. *Proc Natl Acad Sci U S A*. 2013;110:12295–12300.

Liang F, Chen B. A review of biomedical applications of single-walled carbon nanotubes. *Curr Med Chem*. 2010;17:10–24.

Limbach LK, Li Y, Grass RN, et al. Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. *Environ Sci Technol*. 2005;39:9370–9376.

Limbach LK, Wick P, Manser P, et al. Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. *Environ Sci Technol*. 2007;41(11):4158–4163.

Linse S, Cabaleiro-Lago C, Xue WF, et al. Nucleation of protein fibrillation by nanoparticles. *Proc Natl Acad Sci U S A*. 2007;104:8691–8696.

Linsinger T, Roebben G, Gilliand D, et al. Requirements on measurements for the implementation of the European Commission definition of the term "nanomaterial". Publications Office of the European Union. 2012 JRC73260. doi:10.2787/63490. http://publications.jrc.ed.europa.eu/repository/handel/JRC73260.

Liu J, Hurt RH. Ion release kinetics and particle persistence in aqueous nano-silver colloids. *Environ Sci Technol*. 2010;44(6):2169–2175.

Liu J, Sonshine D, Shervani S, Hurt RH. Controlled release of biologically active silver from nanosilver surfaces. *ACS Nano*. 2010a;4(11):6903–6913.

Liu J, Wang Z, Kane A, Hurt RH. Chemical transformations of nanosilver in biological environments. *ACS Nano*. 2012;6(11):9887–9899.

Liu S, Ng AK, Xu R, et al. Antibacterial action of dispersed single-walled carbon nanotubes on *Escherichia coli* and *Bacillus subtilis* investigated by atomic force microscopy. *Nanoscale*. 2010b;2:2744–2750.

Liu S, Wei L, Hao L, et al. Sharper and faster "nano darts" kill more bacteria: a study of antibacterial activity of individually dispersed pristine single-walled carbon nanotube. *ACS Nano*. 2009;3:3891–3902.

Liu X, Guo L, Morris D, Kane AB, Hurt RH. Targeted removal of bioavailable metal as a detoxification strategy for carbon nanotubes. *Carbon*. 2008;46:489–500.

Liu X, Hurt RH, Kane AB. Biodurability of single-walled carbon nanotubes depends on surface functionalization. *Carbon*. 2010c;48:1961–1969.

Liu Y, He L, Mustapha A, Li H, Hu ZQ, Lin M. Antibacterial activities of zinc oxide nanoparticles against *Escherichia coli* O157:H7. *J Appl Microbiol*. 2009;107:1193–1201.

Liu Z, Chen K, Davis C, et al. Drug delivery with carbon nanotubes for *in vivo* cancer treatment. *Cancer Res*. 2008;68(16):6652–6660.

Liu Z, Sun X, Nakayama-Ratchford N, Dai H. Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery. *ACS Nano*. 2007;1:50–56.

Loeschner K, Hadrup N, Ovortrup K, et al. Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. *Part Fibre Toxicol*. 2011;8:18.

Loret T, Peyret E, Dubreuil M, et al. Air-liquid interface exposure to aerosols of poorly soluble nanomaterials induces different biological activation levels compared to exposure to suspensions. *Part Fibre Toxicol*. 2016;13(1):58.

Lovern SB, Klaper R. Daphnia magna mortality when exposed to titanium dioxide and fullerene nanoparticles. *Environ Toxicol Chem*. 2006;25(4):1132–1137.

Lovern SB, Owen H, Klaper R. Electron microscopy of gold nanoparticle intake in the gut of *Daphnia magna*. *Nanotoxicology*. 2008;2(1):43–48.

Lovern SB, Strickler JR, Klaper R. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C_{60}, and $C_{60}HxC_{70}Hx$). *Environ Sci Technol*. 2007;41(12):4465–4470.

Lowry GV, Gregory KB, Apte SC, Lead JR. Transformations of nanomaterials in the environment. *Environ Sci Technol*. 2012;46(13):6893–6899.

Lu CH, Zhu CL, Li J, et al. Using graphene to protect DNA from cleavage during cellular delivery. *Chem Commun (Camb)*. 2010;46:3116–3118.

Lu S, Duffin R, Poland C, et al. Efficacy of simple short-term in vitro assays for predicting the potential of metal oxide nanoparticles to cause pulmonary inflammation. *Environ Health Perspect*. 2009;117:241–247.

Lundqvist M, Stigler J, Elia G, et al. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proc Natl Acad Sci U S A*. 2008;105(38):14265–14270.

Lynch I, Cedervall T, Lundqvist M, et al. The nanoparticle-protein complex as a biological entity: a complex fluids and surface science challenge for the 21st century. *Adv Colloid Interface Sci*. 2007;134–135:167–174.

Ma-Hock L, Trenmann S, Strauss V, et al. Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months. *Toxicol Sci*. 2009;112:468–481.

Magdolenova Z, Collins A, Kumar A, et al. Mechanisms of genotoxicity: a review of in vitro and in vivo studies with engineered nanoparticles. *Nanotoxicology*. 2014;8(3):233–278.

Mahto SK, Charwat V, Ertl P, et al. Microfluidic platforms for advanced risk assessments of nanomaterials. *Nanotoxicology*. 2015;9(3):381–395.

Maynard AD, Warheit DB, Philbert MA. The new toxicology of sophisticated materials: nanotoxicology and beyond. *Toxicol Sci*. 2011;120(suppl 1):S109–S129.

Meng H, Xia T, George S, Nel AE. A predictive toxicological paradigm for the safety assessment of nanomaterials. *ACS Nano*. 2009;3:1620–1627.

Mercer RR, Hubbs AF, Scabilloni JF, et al. Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes. *Particle Fibre Toxicol*. 2010;7:28.

Mercer RR, Hubbs AF, Scabilloni JF, et al. Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes. *Particle Fibre Toxicol*. 2011;8:21.

Mercer RR, Scabilloni J, Wang L, et al. Alteration of deposition pattern and pulmonary response as a result of improved dispersion of aspirated single walled carbon nanotubes in a mouse model. *Am J Physiol Lung Cell Mol Physiol*. 2008;294:L87–L97.

Mitchell LA, Gao J, Wal RV, et al. Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. *Toxicol Sci*. 2007;100(1):203–214.

Moberg PJ, Doty RL. Olfactory function in Huntington's disease patients and at-risk offspring. *Int J Neurosci*. 1997;89:133.

Monopoli MP, Bombelli FB, Dawson KA. Nanobiotechnology: nanoparticle coronas take shape. *Nat Nanotechnol*. 2011;6(1):11–12.

Monteiro-Riviere NA, Inman AO. Challenges for assessing carbon nanomaterial toxicity to the skin. *Carbon*. 2006;44:1070–1078.

Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YY, Riviere JE. Multi-walled carbon nanotube interactions with human epidermal keratinocytes. *Toxicol Lett*. 2005;155(3):377–384.

Monteiro-Riviere NA, Wiench K, Landsiedel R, et al. Safety evaluation of sunscreen formulations containing titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: an in vitro and in vivo study. *Toxicol Sci*. 2011;123:264–280.

Morimoto Y, Hirohashi M, Ogami A, et al. Pulmonary toxicity of well-dispersed multi-call carbon nanotubes following inhalation and intratracheal instillation. *Nanotoxicology*. 2012;6(6):587–599.

Morrow PE. Possible mechanisms to explain dust overloading of the lungs. *Fundam Appl Toxicol*. 1988;10:369–384.

Mortensen LJ, Oberdörster G, Pentland AP, DeLouise LA. In vivo skin penetration of quantum dot nanoparticles in the murine model: the effect of UVR. *Nano Lett*. 2008. Available at: http://pubs.acs.org/journals/nalefd/index.html.

Mortimer M, Kasemets K, Kahru A. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. *Toxicology*. 2010;269(2–3):182–189.

Mouchet F, Landois P, Datsyuk V, et al. International amphibian micronucleus standardized procedure (ISO 21427-1) for in vivo evaluation of double-walled carbon nanotubes toxicity and genotoxicity in water. *Environ Toxicol*. 2011;26(2):136–145.

Mueller NC, Nowack B. Exposure modeling of engineered nanoparticles in the environment. *Environ Sci Technol*. 2008;42:4447–4453.

Muller J, Huaux F, Moreau N. Respiratory toxicity of multi-wall carbon nanotubes. *Toxicol Appl Pharmacol*. 2005;207:221–231.

Müller RH, Heinemann S. In: Gurny R, Junginger HE, eds. *Bioadhesion–Possibilities and Future Trends*. Stuttgart: Wissenschaftliche Verlagsgesellschaft; 1989:202–214.

Murphy FA, Poland CA, Duffin R, et al. Length-dependent retention of carbon nanotubes in the pleural space of mice initiates sustained inflammation and progressive fibrosis on the parietal pleura. *Am J Pathol*. 2011;178:2587–2600.

MPPD. 2018. Available at: https://www.ara.com/products/multiple-path-particle-dosimetry-model-mppd-v-304.

Nagai H, Okazaki Y, Chew SH, Misawa N, et al. Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial

injury and carcinogenesis. *Proc Natl Acad Sci U S A*. 2011;108(49): E1330–E1338.
National Academy of Sciences. *Risk Assessment in the Federal Government: Managing the Process*. Working Papers. Washington, DC: The National Academies Press. 1983.
National Nanotechnology Initiative (NNI). *NNI Environmental, Health, and Safety Research Strategy*. National Science and Technology Council Committee on Technology, Subcommittee on Nanoscale Science, Engineering and Technology; 2011.
National Research Council. *Research Progress on Environmental, Health, and Safety Aspects of Engineered Nanomaterials*. Washington, DC: National Academies Press; 2013.
Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science*. 2006;311:622–627.
Nel AE, Madler L, Velegol D, et al. Understanding biophysicochemical interactions at the nano-bio interface. *Nat Mater*. 2009;8:543–557.
Nel AE, Nasser E, Godwin H, et al. A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment. *ACS Nano*. 2013;7(8):6422–6433.
NIOSH. Current Intelligence Bulletin. *Occupational Exposure to Carbon Nanotubes*. 2011. Available at: www.cdc.gov/niosh/docket/review/docket161A/.
Nowack B, Ranville JF, Diamond S, et al. Potential scenarios for nanomaterial release and subsequent alteration in the environment. *Environ Toxicol Chem*. 2012;31(1):50–59.
Nyberg L, Turco RF, Nies L. Assessing the impact of nanomaterials on anaerobic microbial communities. *Environ Sci Technol*. 2008; 42(6):1938–1943.
Oberdörster G. Dosimetric principles for extrapolating results of rat inhalation studies to humans, using nickel as an example. *Health Phys*. 1989; 57(suppl 1):213–220.
Oberdörster G. Nanotoxicology: in vitro–in vivo dosimetry [letter to editor]. *Environ Health Perspect*. 2012;120:a13–a13.
Oberdörster G, Castranova V, Asgharian B, Sayre P. Inhalation exposure to carbon nanotubes (CNT) and carbon nanofibers (CNF): methodology and dosimetry. *J Toxicol Environ Health B Crit Rev*. 2015;18(3-4):121–212.
Oberdörster G, Elder A, Rinderknecht A. Nanoparticles and the brain: cause for concern? *J Nanosci Nanotechnol*. 2009;9:4996–5007.
Oberdörster G, Ferin J. Metal compounds used in new technologies: metal oxides of ultrafine particles have increased pulmonary toxicity. In: Merian E, Haerdi W, eds. *Metal Compounds in Environment & Life*. Northwood, UR: Science & Technology Ltrs. and Science Reviews, Inc; 1992:443–450.
Oberdörster G, Ferin J, Gelein R, et al. Role of the alveolar macrophage in lung injury: studies with ultrafine particles. *Environ Health Perspect*. 1992;97:193–197.
Oberdörster G, Ferin J, Lehnert BE. Correlation between particle size, in vivo particle persistence, and lung injury. *Environ Health Perspect*. 1994;102(suppl 5):173–179.
Oberdörster G, Kuhlbusch TAJ. In vivo effects: Methodologies and biokinetics of inhaled nanomaterials. *NanoImpact*. 2018;10:38–60.
Oberdörster G, Morrow PE, Spurny K. Size dependent lymphatic short term clearance of amosite fibers in the lung. *Ann Occup Hyg*. 1988;32(suppl, inhaled particles VI):149–156.
Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect*. 2005;113:823–839.
Oberdörster G, Oberdörster E, Oberdörster J. Correspondence: concepts of nanoparticle dose metric and response metric. *Environ Health Perspect*. 2007a;115(6):A290.
Oberdörster G, Sharp Z, Atudorei V, et al. Translocation of inhaled ultrafine particles to the brain. *Inhal Toxicol*. 2004;16(6–7):437–445.
Oberdörster G, Stone V, Donaldson K. Toxicology of nanoparticles: a historical perspective. *Nanotoxicology*. 2007b;1(1):2–25.
OECD Chemicals Committee. Recommendation of the Council on the Safety Testing and Assessment of Manufactured Nanomaterials. September 19, 2013. Available at: http://acts.oecd.org/Instruments/ShowInstrumentView.aspx?InstrumentID=298&Lang=en&Book=False.
Oh YK, Swanson JA. Different fates of phagocytosed particles after delivery into macrophage lysosomes. *J Cell Biol*. 1996;132:585–593.
Oller A, Oberdörster G. Incorporation of particle size differences between animal studies and human workplace aerosols for deriving exposure limit values. *Regul Toxicol Pharmacol*. 2010;57:181–194.
Osmond-McLeod MJ, Oytam Y, Rowe A, et al. Long-term exposure to commercially available sunscreens containing nanoparticles of TiO_2 and ZnO revealed no biological impact in a hairless mouse model. *Part Fibre Toxicol*. 2016;13:44.
Oyabu T, Myojo T, Morimoto Y, et al. Biopersistence of inhaled MWCNT in rat lungs in a 4-week well-characterized exposure. *Inhal Toxicol*. 2011;23(13):784–791.
Pacurari M, Yin XJ, Ding M, et al. Oxidative and molecular interactions of multi-wall carbon nanotubes (MWCNT) in normal and malignant human mesothelial cells. *Nanotoxicology*. 2008;2(3):155–170.
Pal AK, Bello D, Cohen J, Demokritou P. Implications of in-vitro dosimetry on toxicological ranking of low aspect ratio engineered nanomaterials. *Nanotoxicology*. 2015;9(7):871–85.
Palomaki J, Valimaki E, Sund J, et al. Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. *ACS Nano*. 2011;5:6861–6870.
Pante N, Kann M. Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. *Mol Biol Cell*. 2002;13:425–434.
Pare WP, Glavin GB. Restraint stress in biomedical research: a review. *Neurosci Biobehav Rev*. 1986;10:339–370.
Pauluhn J. Subchronic 13—week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structure. *Toxicol Sci*. 2010;113:226–242.
Peng X, Palma S, Fisher NS, Wong SS. Effect of morphology of ZnO nanostructures on their toxicity to marine algae. *Aquat Toxicol*. 2011;102(3–4):186–196.
Petersen EJ, Akkanen J, Kukkonen JVK, Weber WJ Jr. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. *Environ Sci Technol*. 2009;43(8):2969–2975.
Petersen EJ, Henry TB, Zhao J, et al. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environ Sci Technol*. 2014;48(8):4226–4246.
Pfaller TR, Colognato R, Nelissen I, et al. The suitability of different cellular in vitro immunotoxicity and genotoxicity methods for the analysis of nanoparticle-induced events. *Nanotoxicology*. 2010;4(1):52–72.
Phalen RF, Oldham MJ, Nel AE. Tracheobronchial particle dose considerations for in vitro toxicology studies. *Toxicol Sci*. 2006;92(1):126–132.
Pietruska JR, Liu X, Smith A, et al. Bioavailability, intracellular mobilization of nickel, and HIF-1α activation in human lung epithelial cells exposed to metallic nickel and nickel oxide nanoparticles. *Toxicol Sci*. 2011;124(1):138–148.
Poland C, Duffin R, Kinloch I, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat Nanotechnol*. 2008:423–428.
Pope CAI, Dockery DW. Health effects of fine particulate air pollution: lines that connect. *J Air Waste Manage Assoc*. 2006;56:709–742.
Porter D, Sriram I, Wolfarth M, et al. A biocompatible medium for nanoparticle dispersion. *Nanotoxicology*. 2008;2:144–154.
Porter DW, Hubbs AF, Mercer RR, et al. Mouse pulmonary dose- and time course-responses induced by exposure to multi-walled carbon nanotubes. *Toxicology*. 2010;269(2–3):136–147.
Pothmann D, Simar S, Schuler D, et al. Lung inflammation and lack of genotoxicity in the comet and micronucleus assays of industrial multi-walled carbon nanotubes Graphistrength((c)) C100 after a 90-day nose-only inhalation exposure of rats. *Part Fibre Toxicol*. 2015;12:21.
Pott F, Ziem U, Reiffer F-J, et al. Carcinogenicity studies on fibres, metal compounds and some other dusts in rats. *Exp Pathol*. 1987;32:129–152.
Pradhan A, Seena S, Pascoal C, Cássio F. Can metal nanoparticles be a threat to microbial decomposers of plant litter in streams? *Microb Ecol*. 2011;62(1):58–68.
Prow TW, Grice JE, Lin LL, et al. Nanoparticles and microparticles for skin drug delivery. *Adv Drug Deliv Rev*. 2011;63(6):470–491.
Pulskamp K, Diabaté S, Krug HF. Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. *Toxicol Lett*. 2007;168(1):58–74.
Quinn J, Geiger C, Clausen C, et al. Field demonstration of DNAPL dehalogenation using emulsified zero-valent iron. *Environ Sci Technol*. 2005;39(5):1309–1318.

Rauscher H, Roebben G, Amenta V, et al. Towards a review of the EC Recommendation for a definition of the term "nanomaterial"; Part 1: Compilation of information concerning the experience with the definition. Publications Office of the European Union. 2014 JRC89369. doi:10.2788/36237. Available at: http://publications.jrc.ec.europa.eu/repository/handle/JRC89369.

Rauscher H, Roebben G, Boix SA, et al. Towards a review of the EC Recommendation for a definition of the term "nanomaterial"; Part 3: Scientific-technical evaluation of options to clarify the definition and to facilitate its implementation. Publications Office of the European Union. 2015 JRC95675. doi:10.2788/678452. Available at: http://publications.jrc.ec.europa.eu/repository/handle/JRC95675.

Ren H, Wang C, Zhang J, et al. DNA cleavage system of nanosized graphene oxide sheets and copper ions. *ACS Nano*. 2010;4:7169–7174.

Rinderknecht A, Elder A, Prud'homme R, Gindy M, Harkema J, Oberdörster G. Surface functionalization affects the role of nanoparticle disposition. *Am J Respir Crit Care Med*. 2007;175:A246.

Rittinghausen S, Hackbarth A, Creutzenberg O, et al. The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. *Part Fibre Toxicol*. 2014;11:59.

Roberts AP, Mount AS, Seda B, et al. In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. *Environ Sci Technol*. 2007;41(8):3025–3029.

Roberts JR, Mercer RR, Stefaniak AB. Evaluation of pulmonary and systemic toxicity following lung exposure to graphite nanoplates: a member of the graphene-based nanomaterial family. *Part Fibre Toxicol*. 2016;13:34.

Roco MC. Environmentally responsible development of nanotechnology. *Environ Sci Technol*. 2005;39:106A–112A.

Rodrigues DF, Elimelech M. Toxic effects of single-walled carbon nanotubes in the development of *E. coli* biofilm. *Environ Sci Technol*. 2010;44:4583–4589.

Rosenthal SJ, Chang JC, Kovtun O, McBride JR, Tomlinson ID. Biocompatible quantum dots for biological applications. *Chem Biol*. 2011;18:10–24.

Ross AJ, Dailey LA, Brighton LE, Devlin RB. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. *Am J Respir Cell Mol Biol*. 2007;37:169–185.

Rosslein M, Elliott JT, Salit M, et al. Use of cause-and-effect analysis to design a high-quality nanocytotoxicology assay. *Chem Res Toxicol*. 2015;28(1):21-30.

Rothenberg SJ, Parker RM, York RG, et al. Lack of effects of nose-only inhalation exposure on testicular toxicity in male rats. *Toxicol Sci*. 2000;53:127–134.

Rushton EK, Jiang J, Leonard SS, et al. Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. *J Toxicol Environ Health A*. 2010;73(5):445–461.

Russell DG, Vanderven BC, Glennie S, Mwandumba H, Heyderman RS. The macrophage marches on its phagosome: dynamic assays of phagosome function. *Nat Rev Immunol*. 2009;9:594–600.

Ryman-Rasmussen JP, Cesta MF, Brody AR, et al. Inhaled carbon nanotubes reach the subpleural tissue in mice. *Nat Nanotechnol*. 2009a;4:747–751.

Ryman-Rasmussen JP, Tewksbury EW, Moss OR, et al. Inhaled multiwalled carbon nanotubes potentiate airway fibrosis in murine allergic asthma. *Am J Respir Cell Mol Biol*. 2009b;40(3):349–358.

Sager TM, Porter DW, Robinson VA, et al. Improved method to disperse nanoparticles for in vitro and in vivo investigation of toxicity. *Nanotoxicology*. 2007;1:118–129.

Sakamoto Y, Nakae D, Fukumori N, et al. Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. *J Toxicol Sci*. 2009;34(1):65–76.

Salas EC, Sun Z, Luttge A, Tour JM. Reduction of graphene oxide via bacterial respiration. *ACS Nano*. 2010;4:4852–4856.

Sanchez V, Jachak A, Hurt RH, Kane AB. Biological interactions of graphene-family nanomaterials: an interdisciplinary review. *Chem Res Toxicol*. 2012;25(1):15–34.

Sanchez VC, Pietruska JR, Miselis NR, Hurt RH, Kane AB. Biopersistence and potential adverse health impacts of fibrous nanomaterials: what have we learned from asbestos? *Wiley Interdiscip Rev Nanomed Nanobiotechnol*. 2009;1:511–529.

Sanchez VC, Weston P, Yan A, Hurt RH, Kane AB. A 3-dimensional *in vitro* model of epithelioid granulomas induced by high aspect ratio nanomaterials. *Part Fibre Toxicol*. 2011;8(1):17.

Sargent LM, Porter DW, Staska LM, et al. Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes. *Part Fibre Toxicol*. 2014;11:3.

Sargent LM, Reynolds SH, Castranova V. Potential pulmonary effects of engineered carbon nanotubes: in vitro genotoxic effects. *Nanotoxicology*. 2010;4:396–408.

Sasidharan A, Panchakarla LS, Chandran P, et al. Differential nano-bio interactions and toxicity effects of pristine versus functionalized graphene. *Nanoscale*. 2011;3:2461–2464.

Sauer UG, Vogel S, Aumann A, et al. Applicability of rat precision-cut lung slices in evaluating nanomaterial cytotoxicity, apoptosis, oxidative stress, and inflammation. *Toxicol Appl Pharmacol*. 2014;276(1):1–20.

Savi M, Kalberer M, Lang D, et al. A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures. *Environ Sci Technol*. 2008;42(15):5667–5674.

Sayes CM, Gobin AM, Ausman KD, et al. Nano-C60 cytotoxicity is due to lipid peroxidation. *Biomaterials*. 2005;26:7587–7595.

Sayes CM, Liang F, Hudson JL, et al. Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro. *Toxicol Lett*. 2006;161:135–142.

Sayes CM, Reed KL, Warheit DB. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. *Toxicol Sci*. 2007;97(1):163-80.

Schilling K, Bradford B, Castelli D, et al. Human safety review of "nano" titanium dioxide and zinc oxide. Photochemical & photobiological sciences. *Photochem Photobiol Sci*. 2010;9:495–509.

Schlesinger RB, Lippmann M. Selective particle deposition and bronchogenic carcinoma. *Environ Res*. 1978;15:424–431.

Schmid G. The relevance of shape and size of Au^{55} clusters. *Chem Soc Rev*. 2008;37:1909–1930.

Schneider M, Stracke F, Hansen S, Schaefer UF. Nanoparticles and their interactions with the dermal barrier. *Dermato-Endocrinology*. 2009;1(4):197–206.

Scown TM, van Aerle R, Tyler CR. Review: do engineered nanoparticles pose a significant threat to the aquatic environment? *Crit Rev Toxicol*. 2010;40:653–670.

Segal MB. The choroid plexuses and the barriers between the blood and the cerebrospinal fluid. *Cell Mol Neurobiol*. 2000;20:183.

Seidel WC, Scherer KV Jr, Cline D Jr, et al. Chemical, physical, and toxicological characterization of fumes produced by heating tetrafluoroethene homopolymer and its copolymers with hexafluoropropene and perfluoro(propyl vinyl ether). *Chem Res Toxicol*. 1991;4:229–236.

Semmler M, Seitz J, Erbe F, et al. Long-term clearance kinetics of inhaled ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs. *Inhal Toxicol*. 2004;16(6/7):453–459.

Semmler-Behnke M, Takenaka S, Feretsch S, et al. Efficient elimination of inhaled nanoparticles from the alveolar region: evidence for interstitial uptake and subsequent re-entrainment onto airways epithelia. *Environ Health Perspect*. 2007;115(5):728–733.

Senzui M, Tamura T, Miura K, et al. Study on penetration of titanium dioxide (TiO(2)) nanoparticles into intact and damaged skin in vitro. *J Toxicol Sci*. 2010;35:107–113.

Seong SY, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol*. 2004;4:469–478.

Serda RE, Mack A, van de Ven AL, et al. Logic-embedded vectors for intracellular partitioning, endosomal escape, and exocytosis of nanoparticles. *Small*. 2010;6:2691–2700.

Shaw BJ, Handy RD. Physiological effects of nanoparticles on fish: a comparison of nanometals versus metal ions. *Environ Int*. 2011;37(6):1083–1097.

Shi X, von dem Bussche A, Hurt RH, Kane AB, Gao H. Cell entry of one-dimensional nanomaterials occurs by tip recognition and rotation. *Nat Nanotechnol*. 2011;6:714–719.

Shinohara H. Distribution of lymphatic stomata on the pleural surface of the thoracic cavity and the surface topography of the pleural mesothelium in the golden hamster. *Anat Rec*. 1997;249:16–23.

Shvedova AA, Kisin ER, Mercer R, et al. Unusual inflammatory and fibrogenic pulmonary responses to single walled carbon nanotubes in mice. *Am J Physiol Lung Cell Mol Physiol*. 2005;289:698–708.

Shvedova AA, Kisin ER, Murray AR, et al. Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis. *Am J Physiol Lung Cell Mol Physiol*. 2008;295(4):L552–L565.

Shvedova AA, Sager T, Murray AR, et al. Critical issues in the evaluation of possible adverse pulmonary effects from airborne nanoparticles. *Nanotoxicology, Characterization, Dosing and Health Effects*. New York and London: Informa Healthcare, USA Inc; 2007.

Silbergeld EK, Contreras EQ, Hartung T, et al. Nanotoxicology: "the end of the beginning"—signs on the roadmap to a strategy for assuring the safe application and use of nanomaterials. *Altex*. 2011;28:236–241.

Simmons SO, Fan CY, Ramabhadran R. Cellular stress response pathway system as a sentinel ensemble in toxicological screening. *Toxicol Sci*. 2009;111:202–225.

Singh R, Pantarotto D, Lacerda L, et al. Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers. *Proc Natl Acad Sci U S A*. 2006;103(9):3357–3362.

Sistonen L, Sarge KD, Phillips B, et al. *Mol Cell Biol*. 1992;12:4104–4111.

Slikker W Jr, Andersen ME, Bogdanffy MS, et al. Dose-dependent transitions in mechanisms of toxicity. *Toxicol Appl Pharmacol*. 2004;201(3):203–225.

Slowing II, Vivero-Escoto JL, Zhao Y, et al. Exocytosis of mesoporous silica nanoparticles from mammalian cells: from asymmetric cell-to-cell transfer to protein harvesting. *Small*. 2011;7:1526–1532.

Stanton MF, Layard M, Tegeris A, et al. Relation of particle dimension to carcinogenicity in Amphibole asbestoses and other fibrous minerals. *JNCL*. 1981;67:965–975.

Stone V, Johnston H, Schins RP. Development of in vitro systems for nanotoxicology: methodological considerations. *Crit Rev Toxicol*. 2009;39:613–626.

Stone V, Pozzi-Mucelli S, Tran L, et al. ITS-NANO: prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. *Part Fibre Toxicol*. 2014;11:9.

Stone V, Miller MR, Clift MJD, et al. Nanomaterials versus ambient ultrafine particles: an opportunity to exchange toxicology knowledge. *Environ Health Perspect*. 2017;125(10):106002.

Sugamata M, Uhara T, Takano H, Oshio S, Takeda K. Maternal diesel exhaust exposure damages newborn murine brains. *J Health Sci*. 2006;52(1):82–84.

Suzui M, Futakuchi M, Fukamachi K, et al. Multiwalled carbon nanotubes intratracheally instilled into the rat lung induce development of pleural malignant mesothelioma and lung tumors. *Cancer Sci*. 2016;107(7):924–935.

Takagi A, Hirose A, Futakuchi M, Tsuda H, Kanno J. Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice. *Cancer Sci*. 2012; 103(8):1440–1444.

Takagi A, Hirose A, Nishimura T, et al. Induction of mesothelioma in p53+/− mouse by intraperitoneal application of multi-wall carbon nanotube. *J Toxicol Sci*. 2008;33(1):105–116.

Takeda K, Suzuki K-i, Ishihra A, et al. Nanoparticles transferred from pregnant mice to their offspring can damage the genital and cranial nerve systems. *J Health Sci*. 2009;55(1):95–102.

Tang T, Gminski R, Könczöl M, Modest C, Armbruster B, Mersch-Sundermann V. Investigations on cytotoxic and genotoxic effects of laser printer emissions in human epithelial A549 lung cells using an air/liquid exposure system. *Environ Mol Mutagen*. 2011.

Taurozzi JS, Hackley VA, Wiesner MR. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment—issues and recommendations. *Nanotoxicology*. 2011;5:711–729.

Teeguarden JG, Hinderliter PM, Orr G, Thrall BD, Pounds JG. Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. *Toxicol Sci*. 2007;95(2):300–312.

Teleki A, Wengeler R, Wengeler L, Nirschl H, Pratsinis SE. Distinguishing between aggregates and agglomerates of flame-made TiO_2 by high-pressure dispersion. *Powder Technol*. 2008;181:292–300.

Titov AV, Kral P, Pearson R. Sandwiched graphene—membrane superstructures. *ACS Nano*. 2010;4:229–234.

Tong Z, Bischoff M, Nies L, Applegate B, Turco RF. Impact of fullerene (C60) on a soil microbial community. *Environ Sci Technol*. 2007; 41(8):2985–2991.

Tran CL, Buchanan D, Cullen RT, Searl A, Jones AD, Donaldson K. Inhalation of poorly soluble particles. II. Influence of particle surface area on inflammation and clearance. *Inhal Toxicol*. 2000;12:1113–1126.

Tran TH, Nguyen TD. Controlled growth of uniform noble metal nanocrystals: aqueous-based synthesis and some applications in biomedicine. *Colloids Surf B Biointerf*. 2011;88:1–22.

Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer Res*. 2009;69(22):8784–8789.

Truong L, Saili KS, Miller JM, Hutchison JE, Tanguay RL. Persistent adult zebrafish behavioral deficits results from acute embryonic exposure to gold nanoparticles. *Comp Biochem Physiol C Toxicol Pharmacol*. 2012;155(2):269–274.

Tu X, Manohar S, Jagota A, Zheng M. DNA sequence motifs for structure-specific recognition and separation of carbon nanotubes. *Nature*. 2009;460:250–253.

Tungittiplakorn W, Cohen C, Lion LW. Engineered polymeric nanoparticles for bioremediation of hydrophobic contaminants. *Environ Sci Technol*. 2005;39(5):1354–1358.

Udelsman R, Blake MJ, Stagg CA, et al. Vascular heat shock protein expression in response to stress. *J Clin Invest*. 1993;91:465–473.

Unfried K, Albrecht C, et al. Cellular responses to nanoparticles: target structures and mechanisms. *Nanotoxicology*. 2007;1:52–71.

USEPA Chemical Assistance Manual for Premanufacture Notification Submitters. 1997 (OPPT United States Environmental Protection Agency, ed.), Vol. EPA 744-R-97-003, pp. 1–138. Available at: https://www.epa.gov/reviewing-new-chemicals-under-toxic-substances-control-act-tsca/chemistry-assistance-manual.

Vallhov H, Qin J, Johansson SM, et al. The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. *Nano Lett*. 2006;6:1682–1686.

Vankoningsloo S, Piret J-P, Saout C, et al. Cytotoxicity of multi-walled carbon nanotubes in three skin cellular models: effects of sonication, dispersive agents and corneous layer of reconstructed epidermis. *Nanotoxicology*. 2010;4(1):84–97.

Vecchio G, Galeone A, Brunetti V, et al. Mutagenic effects of gold nanoparticles induce aberrant phenotypes in *Drosophila melanogaster*. *Nanomedicine*. 2012;8(1):1–7.

Velzeboer I, Kupryianchyk D, Peeters ET, Koelmans AA. Community effects of carbon nanotubes in aquatic sediments. *Environ Int*. 2011;37(6): 1126–1130.

Volckens J, Dailey L, Walters G, Devlin RB. Direct particle-to-cell deposition of coarse ambient particulate matter increases the production of inflammatory mediators from cultured human airway epithelial cells. *Environ Sci Technol*. 2009;43(12):4595–4599.

Wagner DE, Bonenfant NR, Sokocevic D, et al. Three-dimensional scaffolds of acellular human and porcine lungs for high throughput studies of lung disease and regeneration. *Biomaterials.* 2014;35(9):2664–2679.

Walczyk D, Bombelli FB, Monopoli MP, et al. What the cell "sees" in bionanoscience. *J Am Chem Soc*. 2010;132:5761–5768.

Walker NJ, Bucher JR. A 21st century paradigm for evaluating the health hazards of nanoscale materials? *Toxicol Sci*. 2009;110:251–254.

Wang C, Hou Y, Kim J, Sun S. A general strategy for synthesizing FePt nanowires and nanorods. *Angew Chem Int Ed Engl*. 2007;46:6333–6335.

Wang J, Chen C, Liu Y, et al. Potential neurological lesion after nasal instillation of TiO(2) nanoparticles in the anatase and rutile crystal phases. *Toxicol Lett*. 2008a;183(1–3):72–80.

Wang J, Liu Y, Jiao F, et al. Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO(2) nanoparticles. *Toxicology*. 2008b;254(1–2):82–90.

Wang NS. The preformed stomatas connecting the pleural cavity and the lymphatics in the parietal pleura. *Am Rev Resp Dis*. 1975;111:12–20.

Wang SQ, Tooley IR. Photoprotection in the era of nanotechnology. *Semin Cutan Med Surg*. 2011;30:210–213.

Wang X, Xia T, Ntim SA, et al. Dispersal state of multiwalled carbon nanotubes elicits profibrogenic cellular responses that correlate with fibrogenesis biomarkers and fibrosis in the murine lung. *ACS Nano*. 2011a;5:9772–9787.

Wang Y, Westerhoff P, Hristovski KD. Fate and biological effects of silver, titanium dioxide, and C_{60} (fullerene) nanomaterials during simulated

wastewater treatment processes. *J Hazard Mater*. 2012;201–202:16–22. [Epub November 7, 2011]. PubMed PMID: 22154869.

Wang YY, Lai SK, So C, et al. Mucoadhesive nanoparticles may disrupt the protective human mucus barrier by altering its microstructure. *PLoS One*. 2011b;6:e21547.

Warheit DB. How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? *Toxicol Sci*. 2008;101:183–185.

Warheit DB, Brown SC, Donner EM. Acute and subchronic oral toxicity studies in rats with nanoscale and pigment grade titanium dioxide particles. *Food Chem Toxicol*. 2015;84:208–224.

Warheit DB, Donner EM. How meaningful are risk determinations in the absence of a complete dataset? Making the case for publishing standardized test guideline and "no effect" studies for evaluating the safety of nanoparticulates versus spurious "high effect" results from single investigative studies. *Sci Technol Adv Mater*. 2015;16(3):034603.

Warheit DB, Hartsky MA, Stefaniak MS. Comparative physiology of rodent pulmonary macrophages: in vitro functional responses. *J Appl Physiol*. 1988a;64(5):1953–1959.

Warheit DB, Laurence BR, Reed KL, Rouch DH, Reynolds GAM, Webb TR. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. *Toxicol Sci*. 2004;77:117–125.

Warheit DB, Overby LH, George G, Brody AR. Pulmonary macrophages are attracted to inhaled particles on alveolar surfaces. *Exp Lung Res*. 1988b;14:51–66.

Weir A, Westerhoff P, Fabricius L, Hristovski K, von Goetz N. Titanium dioxide nanoparticles in food and personal care products. *Environ Sci Technol*. 2012;46(4):2242–2250.

Werlin R, Priester JH, Mielke RE, et al. Biomagnification of cadmium selenide quantum dots in a simple experimental microbial food chain. *Nat Nanotechnol*. 2011;6(1):65–71.

Westerhoff P, Song G, Hristovski K, Kiser MA. Occurrence and removal of titanium at full scale wastewater treatment plants: implications for TiO_2 nanomaterials. *J Environ Monit*. 2011;13:1195–1203.

Weuve J, Puett RC, Schwartz J, et al. Exposure to particulate air pollution and cognitive decline in older women. *Arch Intern Med*. 2012;172(3):219–227.

Wick P, Manser P, Limbach LK, et al. The degree and kind of agglomeration affect carbon nanotube cytotoxicity. *Toxicol Lett*. 2007;168:121–131.

Woodrow Wilson Center. 2012. Available at: http://www.nanotechproject.org/inventories/consumer/analysis_draft/.

Worle-Knirsch JM, Pulskamp K, Krug HF. Oops they did it again! Carbon nanotubes hoax scientists in viability assays. *Nano Lett*. 2006;6(6):1261–1268.

Xia T, Kovochich M, Brant J, et al. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano Lett*. 2006;6:1794–1807.

Xia T, Kovochich M, Liong M, et al. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano*. 2008a;2:2121–2134.

Xia T, Kovochich M, Liong M, Zink JI, Nel AE. Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways. *ACS Nano*. 2008b;2:85–96.

Xia T, Zhao Y, Sager T, et al. Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in the rodent lung and zebrafish embryos. *ACS Nano*. 2011;5:1223–1235.

Xia XR, Monteiro-Riviere NA, Riviere JE. An index for characterization of nanomaterials in biological systems. *Nat Nanotechnol*. 2010;5:671–675.

Xie Y, Williams NG, Tolic A, et al. Aerosolized ZnO nanoparticles induce toxicity in alveolar type ii epithelial cells at the air-liquid interface. *Toxicol Sci*. 2012;125(2):450–461.

Xiong D, Fang T, Yu L, Sima X, Zhu W. Effects of nano-scale TiO_2, ZnO and their bulk counterparts on zebrafish: acute toxicity, oxidative stress and oxidative damage. *Sci Total Environ*. 2011;409(8):1444–1452.

Xu C, Xie J, Ho D, et al. Au-Fe3O4 dumbbell nanoparticles as dual-functional probes. *Angew Chem Int Ed Engl*. 2008;47:173–176.

Yan A, Von Dem Bussche A, Kane AB, Hurt RH. Tocopheryl polyethylene glycol succinate as a safe, antioxidant surfactant for processing carbon nanotubes and fullerenes. *Carbon*. 2007;45:2463–2470.

Yan L, Zhao F, Li S, Hu Z, Zhao Y. Low-toxic and safe nanomaterials by surface-chemical design, carbon nanotubes, fullerenes, metallofullerenes, and graphenes. *Nanoscale*. 2011;3:362–382.

Yang K, Zhu L, Xing B. Adsorption of polycyclic aromatic hydrocarbons by carbon nanomaterials. *Environ Sci Technol*. 2006;40:1855–1861.

Yang Z, Zhang Y, Yang Y, et al. Pharmacological and toxicological target organelles and safe use of single-walled carbon nanotubes as drug carriers in treating Alzheimer disease. *Nanomedicine*. 2010;6(3):427–441.

Yu LP, Fang T, Xiong DW, Zhu WT, Sima XF. Comparative toxicity of nano-ZnO and bulk ZnO suspensions to zebrafish and the effects of sedimentation, $^{\bullet}OH$ production and particle dissolution in distilled water. *J Environ Monit*. 2011;13(7):1975–1982.

Zhang LW, Monteiro-Riviere NA. Lectins modulate multi-walled carbon nanotubes cellular uptake in human epidermal keratinocytes. *Toxicol In Vitro*. 2010;24:546–551.

Zhang Q, Huang JQ, Zhao MQ, Qian WZ, Wei F. Carbon nanotube mass production: principles and processes. *ChemSusChem*. 2011;4:864–889.

Zhang QZ, Zha L-S, Zhang Y, et al. The brain targeting efficiency following nasally applied MPEG-PLA nanoparticles in rats. *J Drug Target*. 2006;14(5):281–290.

Zhao F, Zhao Y, Liu Y, Chang X, Chen C, Zhao Y. Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. *Small*. 2011;7:1322–1337.

Zhao Y, Allen BL, Star A. Enzymatic degradation of multiwalled carbon nanotubes. *J Phys Chem A*. 2011;115:9536–9544.

Zhu S, Oberdörster E, Haasch ML. Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow. *Mar Environ Res*. 2006;62(suppl):S5–S9.

Zhu X, Zhu L, Lang Y, Chen Y. Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates. *Environ Toxicol Chem*. 2008;27(9):1979–1985.

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Unit VI

Environmental Toxicology

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chapter 30

Ecotoxicology

Richard T. Di Giulio and Michael C. Newman

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Some Distinct Aspects of Exposure

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Molecular and Biochemical Effects

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Acknowledgments

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INTRODUCTION

Ecotoxicology is the science of contaminants in the biosphere and their effects on constituents of the biosphere (Newman, 2015). It follows from this definition that ecotoxicologists examine large-scale ecological phenomenon (Preston, 2002) in addition to those normally addressed in toxicology: ecotoxicology has an overarching goal of explaining and predicting effect or exposure phenomena at several levels of biological organization (Fig. 30-1). Essential explanations and models include those applied in conventional toxicology and a range of environmental sciences.

Although Truhaut's original definition of this new science encompassed effects to humans (Truhaut, 1977), most recent definitions of ecotoxicology do not. Relevant effects to nonhuman targets range from biomolecular to global. Taking on the classic toxicology vantage initially, suborganismal and organismal effects were emphasized during ecotoxicology's nascent stage; however, studies of higher level effects and interactions are becoming increasingly commonplace as the science matures. Such indirect effects[1] were initially considered problematic and reluctantly relegated to secondary importance (Fleeger et al., 2003) relative to direct effects to individuals. Indirect effects are now known to be as or more important as direct effects to nonhuman targets (Fleeger et al., 2003; Chapman, 2004). As the need to predict major effects to populations, communities, ecosystems, and other higher level entities has become increasingly apparent, more cause–effect models relevant to these higher levels of biological organization are added to the conventional set of toxicology models applied by pioneering ecotoxicologists.

Contaminant chemical form, phase association, and movement among components of the biosphere are also central issues in ecotoxicology because they determine exposure, bioavailability, and realized dose. The context of these biogeochemical studies has expanded in the last several decades to encompass issues of larger scale such as global movement and fractionation of persistent organic pollutants (POPs) (Wania and Mackay, 1996).

From a practical vantage, ecotoxicology informs decision makers about ecological risks associated with contamination. Risk to ecological entities is estimated or predicted by combining exposure and effect information. Risk might involve diminished fitness of individuals, increased risk of local population extinction, a drop in species diversity, or reduced nutrient cycling or primary productivity. Because potential ecological endpoints are so diverse, the ecological risk framework tends to be more flexible than that of the conventional human health risk assessment (Fig. 30-2). This important role of ecotoxicology in ecological risk assessment (ERA) is discussed in more detail in this chapter.

SOME DISTINCT ASPECTS OF EXPOSURE

Predicting exposure and effect is difficult for all relevant ecological entities. In contrast to human toxicology in which information about a few species might be used to predict harm to one (humans), ecotoxicology commonly uses sparse information for a few species to predict effects to many species and their interactions. Identifying exposure pathways, bioavailability, bioaccumulation, and toxicant transfer for all at-risk ecological receptors requires considerable effort.

[1]Indirect effects are effects of toxicants mediated by another ecosystem component (Krivtsov, 2004), such as those that might occur to a flowering plant if a pesticide were to eliminate its primary insect pollinator.

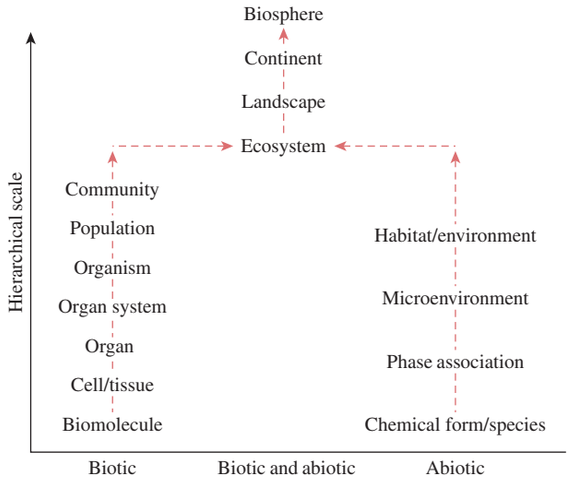

Figure 30-1. *Ecological scales relevant to ecotoxicology.* Solely biological scales relevant to ecotoxicology range from the molecular to the community levels: solely abiotic scales range from the chemical to the entire habitat. Biotic and abiotic components are usually combined at levels above the ecological community and habitat. The ecological community and physicochemical habitat combined to form the ecosystem. Ecological systems can be considered at the landscape scale, such as the combination of marine, freshwater, and terrestrial systems at a river's mouth. Recently, the continental and biospheric scales have become relevant as in the cases of ozone depletion, acid precipitation, and global warming.

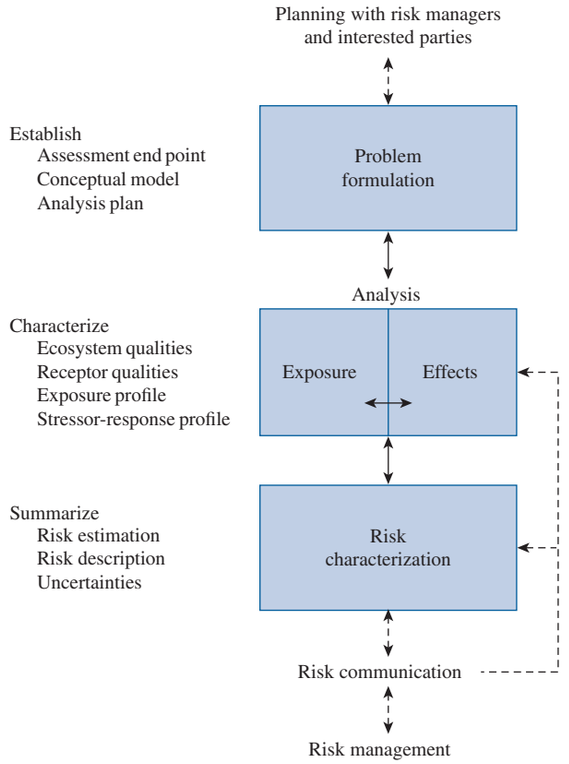

Figure 30-2. *The general form of an ecological risk assessment including problem formulation, analysis, and risk characterization stages.* Problem formulation is done in dialog with risk managers and stakeholders, and involves a clear statement of the ecological entity to be assessed, a conceptual model for the process, and a plan for conducting the assessment. The analysis stage involves exposure and effects characterizations. Using the context developed during problem formulation and information organized together in the analysis stage, a statement of risk and associated uncertainties are made in the risk characterization stage.

Relevant exposure routes are the conventional ingestion, inhalation, and dermal absorption. But unique features of exposure pathways must be accommodated for species that ingest a wide range of materials using distinct feeding mechanisms, breathe gaseous or liquid media using extremely different structures, and come into dermal contact with a variety of gaseous, liquid, and solid media.

Prediction of oral exposure can be compromised because species feed on different materials; however, conventional principles regarding oral bioavailability remain relevant. As an example, some birds are uniquely at high risk of lead poisoning because they ingest and then use lead shot as grit. Shot are ground together in their gizzards under acidic conditions, releasing significant amounts of dissolved lead (Kendall et al., 1996). As true with humans (i.e., the ionic hypothesis of Mathews [1904]), the dissolved form of lead is more available to do harm than solid lead shot. Similar high risk of lead poisoning is present for some raptors feeding on game birds whose tissues can contain lead shot (Wayland and Bollinger, 1999). As another example, complex sorting of filtered materials on the gills of bivalve molluscs strongly influences the metal content and bioavailability of the material that eventually passes into their guts (Allison et al., 1998). Some invertebrate species have elaborate feeding structures that are also involved in respiration (e.g., lugworms and bivalve molluscs) or locomotion (copepods and other zooplankton species). Some zooplankton species feed and digest algal cells in such a way that only metals soluble in the algal cytosol are readily bioavailable (Reinfelder and Fisher, 1991). Unlike most mammalian species, many invertebrate species are capable of sequestering large amounts of metals in intracellular granules (Mason and Nott, 1981). Incorporation of metals into granules by prey species reduces metal bioavailability to predators (Nott and Nicolaidou, 1993). Just as noted with 5-fluoruracil administration or chronic ethanol consumption, some pollutant exposures cause malabsorption by damaging the intestine wall. A relevant situation is intestinal damage to otters caused by ingestion during grooming of oiled fur (Lipscomb et al., 1996; Ormseth and Ben-David, 2000). The principles remain the same in all of these cases but critical exposure pathway details are different.

Many techniques applied to determining human oral bioavailability are available to the ecotoxicologist; however, as just illustrated, modifications are needed for the many pathways for nonhuman species exposure. Typical are those associated with estimating contaminant bioavailability in aquatic environments. As an example, biomimetic and related extraction methods used to predict human oral bioavailability can be modified to predict sediment-bound contaminant bioavailabilities. Sediments are placed into contact with biomimetic solutions (Chen and Mayer, 1998; Leslie et al., 2002) or digestive fluids taken from organisms (Mayer et al., 1996; Weston and Maruya, 2002), and the amount of extracted contaminant used to estimate bioavailability.

Estimation of chemical speciation is central to predicting bioavailability of water-associated contaminants. Speciation can determine the bioavailability of dissolved metals. Movements of nonionic and ionizable organic compounds across the gut or gills are strongly influenced by lipid solubility and the pH-partition theory, respectively. Consequently, determination of a compound's lipophilicity or calculation of pH- and pK_a-dependent ionization facilitates some predictive capability for bioavailability (see Chap. 5, "Absorption, Distribution, and Excretion of Toxicants"). A common application of this approach would be estimation of water pH effects on

ammonia toxicity as the consequence of the ease with which unionized ammonia passes through gills relative to ionized ammonia (Lloyd and Herbert, 1960). The free ion activity model (FIAM) and biotic ligand model (BLM) state that uptake and toxicity of cationic trace metals are best predicted from the interaction of the free ion with ligands on biological surface (Campbell and Tessier, 1996; Erickson, 2013), although exceptions exist to this extension of the ionic hypothesis. Consequently, dissolved metal exposure assessments often begin by estimating the amount of a metal present as the free ion. Normally, such calculations require only thermodynamic modeling based on measured concentrations of dissolved cations and anions. Lipid partitioning is often used to predict dissolved, nonionizing organic compound accumulation in and effects to aquatic biota. The propensity for an organic compound to accumulate in aquatic organisms increases with lipid solubility as often described with a simple quantitative structure–activity relationship (QSAR) (Neely et al., 1974; Mackay, 1982; Chiou, 1985; Connell, 1990). The log of the octanol–water partition coefficient (log K_{ow}) is used to predict measures such as the bioconcentration factor (BCF; the quotient of the concentration in the organism and that in the water from which the organic compound is being accumulated):

$$\mathrm{BCF} = \frac{C_{\mathrm{Organism}}}{C_{\mathrm{Water}}}.$$

Bioavailability, bioaccumulation, or exposure concentrations for sediment-associated toxicants are also approached by considering chemical speciation and phase partitioning. Metals in sediments are either incorporated into one of many solid phases or dissolved in the interstitial waters surrounding the sediment particles. Bioavailabilities of metals in these different forms are difficult to predict (Luoma, 1989) but, nonetheless, various schemes have been applied to that end. Bioavailable metals have been estimated by normalizing sediment metal concentrations to easily extracted iron and manganese concentrations because solid iron and manganese oxides sequester metals in poorly bioavailable solid forms (Luoma and Bryan, 1978). Other chemical extraction methods have been applied with some success (Tessier et al., 1984). A pragmatic method for predicting sediment metal bioavailability has emerged that is based on the assumption that the sediment metal form of most concern is the dissolved metal. Further, for many metals and sediments, the dissolved interstitial metal concentrations are determined by equilibrium between solid (iron and manganese) sulfides and the interstitial water:

$$\mathrm{Cd}^{2+} + \mathrm{FeS}_{\mathrm{Solid}} \leftrightarrow \mathrm{CdS}_{\mathrm{Solid}} + \mathrm{Fe}^{2+}.$$

Because the equilibrium so favors formation of metal sulfide ($\mathrm{CdS}_{\mathrm{Solid}}$ in this case) at the expense of $\mathrm{FeS}_{\mathrm{Solid}}$, insignificant amounts of dissolved metal will be present in the interstitial waters if enough $\mathrm{FeS}_{\mathrm{Solid}}$ is present. This premise has given rise to a standard technique for determining whether sediments might contain enough metal to warrant concern (Di Toro et al., 1990). First, a sediment aliquot is extracted with cold hydrochloric acid. Then the amounts of sulfide (acid volatile sulfides [AVS]) and simultaneously extracted metals (SEM) are quantified in that extract. The difference between the SEM and AVS suggests whether or not enough metal will remain dissolved in the interstitial waters to warrant concern. This method has enjoyed wide application and was subsequently refined by Di Toro et al. (2005) by including metal partitioning to sediment organic matter. Further refinement remains to be done because metal exposure of organisms that ingest sediment particulates is not fully defined by interstitial water concentrations alone (Lee et al., 2000).

Bioavailability and accumulation of sediment-associated organic compounds are predicted with tools similar to those described for waters. The bioavailability of ionizable organic compounds can be approximated with the pH-partition hypothesis that relates the availability of an ionizable compound to the diffusion of its unionized form through membranes as determined by pH and pK_a. Availability of nonionizing organic compounds for accumulation can often be estimated with its log K_{ow} and equilibrium partitioning theory as described already for accumulation from waters. The challenge with nonionizing organic compounds becomes adequately defining the phases between which the compound is partitioning. This might be done by estimating the partitioning of the compound between sediment solid phases and the interstitial water as done by Di Toro et al. (1991). Descriptions of nonionizing organic compound bioaccumulation from sediments can also entail normalization of concentrations to phases thought to be dictating partitioning:

$$\text{Biota sediment accumulation factor (BSAF)} = \frac{\mu\text{g/kg lipid}}{\mu\text{g/kg organic carbon}},$$

where the mass of compound in the organisms is divided by kilograms of organism-associated lipid and the mass of compound in the sediment is divided by kilograms of sediment-associated organic carbon.

Another issue of importance to the ecotoxicologist is the possibility of biomagnification, the increase in contaminant concentration as it moves through a food web. Biomagnification occurs for many persistent organic pollutants (POPs) and some organometals such as methylmercury. MacDonald et al. (2002) conceptualize biomagnification as environmental amplification involving sequential steps of solvent switching followed by solvent depletion. Solvent switching might involve partitioning of the compound from water into the lipids in prey tissues. Next, after the predator ingests a prey, digestion of the prey lipids leads to POP partitioning into lipids of the predator tissues. The result of solvent switching and then depletion is a net increase in POP concentration in the predator relative to the prey. As will be described in the Section "Community," biomagnification can result in harmful exposures to species situated high in the food web such as birds of prey.

TOXICANT EFFECTS

While determining *exposure* comprises one half of the risk assessment paradigm, the other half, understanding chemical effects, lies at the heart of toxicology and, hence, comprises the focus of this chapter. The effects, or deleterious consequences of chemical exposures, can be enormously diverse as demonstrated by previous chapters, and investigated by numerous techniques. One approach to this complex topic of ecotoxicological effects, which we employ here, is to organize effects according to biological levels of organization. Thus, one may consider effects, in ascending order, at the subcellular (molecular and biochemical), cellular, organismal, population, community, ecosystem, or higher levels of organization. As noted earlier, an important distinction between traditional biomedical, or human health-oriented, toxicology and ecotoxicology is the emphasis by the latter on higher levels of biological organization, specifically populations, communities, and ecosystems, while biomedical toxicology focuses on lower levels, from organismal and below. This difference arises from the focus of biomedical toxicology on one species and concerns for protecting the health of individuals of that species. Ecotoxicology, in contrast, deals with,

theoretically at least, all species, and in line with other aspects of natural resource management, the primary concern is one of sustainability. That is, policies and regulations surrounding chemical effects in natural ecosystems are designed to protect ecological features such as population dynamics, community structures, and ecosystem functions. In this light, the individual organism is essentially viewed as expendable, as long as these higher level variables are protected. An exception here is that of endangered or threatened species, where the loss of an individual may have unacceptable legal or ecological consequences.

While higher levels of organization comprise the ultimate focus of those concerned with chemical pollution of natural systems, the science of ecotoxicology includes studies across the entire hierarchy. Studies at lower levels (cellular and below) provide insights into mechanisms of toxicity that can be valuable for making predictions among related compounds or species, and establishing cause–effect relationships in field studies, for generating useful "biomarkers" of chemical exposure and effect, and for providing insights into higher level, often population level, effects. Studies at the organism level have historically played a dominant role in regulatory ecotoxicology; many traditional bioassays, described later, can be viewed as organismal-level queries. Again, understanding effects at the population and higher levels can be viewed as the ultimate goal of ecotoxicology. However, because these effects can require a relatively long time beyond the initial exposure of a system to emerge, their quantification is often difficult, and they generally do not serve to identify the nature of the stressor. Thus, the elucidation of effects of chemical pollutants, as well as other stressors, in natural systems draws on multiple approaches and conclusions are generally based on the weight of evidence available. Adams (2000) discusses examples of effects, or endpoints, that can be measured spanning levels of organization including their relative sensitivities and ecological relevancies, and the importance of integrating studies across levels of organization and mathematical approaches for accomplishing.

An important paradigm that has emerged recently for elucidating linkages across levels of organization is the "Adverse Outcome Pathway" (AOP) concept first described by Ankley et al. (2000). They define the AOP as "a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating effect (e.g., a molecular interaction between a xenobiotic and a specific biomolecule) and an adverse outcome at a biological level of organization relevant to risk assessment." A generalized AOP is presented in Fig. 30-3. In the realm of ecotoxicology and ecological risk assessment, the population is the level generally of greatest relevance. Subsequent papers have addressed the development of the AOP concept, for example, in terms of underlying principles (Villeneuve et al., 2014a), best practices for international harmonization of approaches and databases (Villeneuve et al., 2014b), and the integration of computational and modeling approaches (Perkins et al., 2015). The AOP concept provides perhaps an unusual example of an approach originally developed by ecotoxicologists that has been adopted by the biomedical field (e.g., see Edwards et al., 2016).

In the following sections, we describe important chemical effects that have been addressed at different levels of biological organization in ecotoxicological contexts, including illustrative examples. It is beyond the scope of this chapter to provide discussions of all chemicals that have received ecotoxicological attention. Other chapters in this text, particularly those in Unit V, provide detailed information for most classes of chemicals of concern as pollutants of natural systems, albeit in a primarily mammalian context. Many of the effects described are relevant to other animals, and of course mammals do occur in natural systems! Some plant-specific effects will be addressed herein. It should be noted that, while we have employed a biological level of organization approach as a meaningful way to organize and convey a complex array of information, the phenomena we have categorized into various levels are ultimately interwoven, as will become apparent.

Molecular and Biochemical Effects

This lowest level of organization includes fundamental processes associated with the regulation of gene transcription and translation, biotransformation of xenobiotics, and the deleterious biochemical effects of xenobiotics on cellular constituents including proteins, lipids, and DNA. These effects have been described elsewhere throughout this book in various contexts related to human health. Here we will highlight some aspects of subcellular effects that have received particular attention in the context of ecotoxicology.

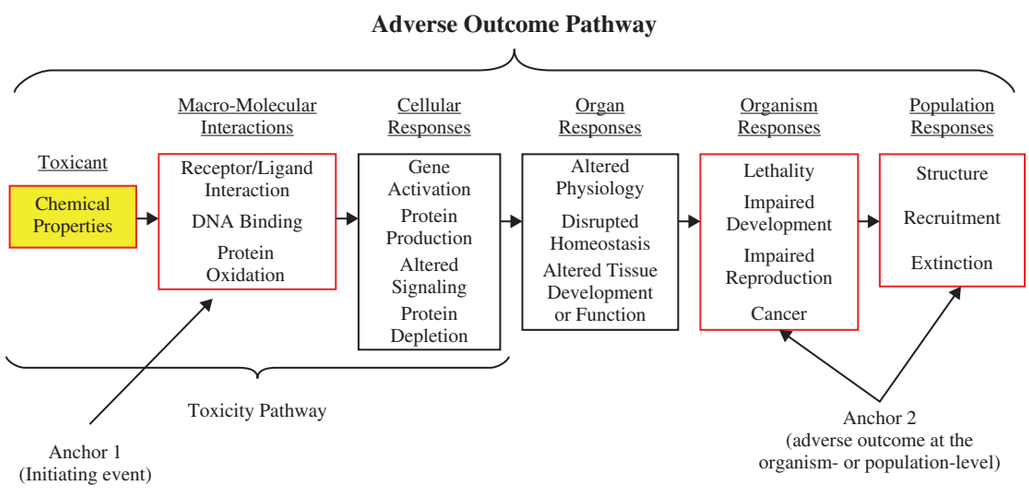

Figure 30-3. *An adverse outcome pathway (AOP) is a conceptual framework that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a level of biological organization relevant to risk assessment.* (From Ankley et al. [2010]; used with permission from Wiley.)

Research in this area has been performed for a variety of reasons, including the elucidation of mechanisms of adaptation and toxicity, understanding species similarities and differences (e.g., to compare selected wildlife species with standard mammalian models, and to identify particularly sensitive species), and developing useful biomarkers of chemical exposure and toxicity for environmental assessments. More in-depth discussions are provided in monographs such as Hoffman et al. (2003), Newman (2015), Mommsen and Moon (2005), Di Giulio and Hinton (2008), and Walker et al. (2012).

Gene Expression and Ecotoxicogenomics

A long-standing mechanistic issue in toxicology concerns chemical effects on gene and protein expression. Xenobiotics can affect gene transcription through interactions with transcription factors and/or the promoter regions of genes that bind transcription factors in the process of activating transcription. In the context of environmental toxicology, perhaps the most studied xenobiotic effects involve ligand-activated transcription factors. These intracellular receptor proteins recognize and bind specific compounds, thus forming a complex that binds to specific promoter regions of genes, thereby activating transcription of mRNAs, and ultimately translation of the associated protein. Two examples of substantial importance in ecotoxicology that illustrate these interactions involve the estrogen receptor (ER) and the aryl hydrocarbon receptor (AhR).

Estrogen Receptor A number of chemicals have been shown to perturb various components of the endocrine system, and the identification and elucidation of "endocrine disruptors" have been a subject of much research and regulatory action in recent years, in the contexts of both human and wildlife health (see Chap. 10, "Developmental Toxicology," Chap. 20, "Toxic Responses of the Endocrine System," and Chap. 21, "Toxic Responses of the Reproductive System"; Rotchell and Ostrander, 2003). Perhaps the most studied component of the vertebrate endocrine system in this context is the ER, particularly ER-α, and responses associated with it. The dominant natural ligand for this nuclear receptor is estradiol (E2); binding of E2 with ER produces a complex that can then bind to estrogen response elements (ERE) of specific genes that contain one or more EREs, thereby causing gene transcription (see Fig. 30-4). Genes regulated in this manner by E2–ER play various important roles in, for example, sexual organ development, behavior, fertility, and bone integrity (Deroo and Korach, 2006).

A number of chemicals including certain drugs and environmental pollutants can serve as ligands for ER; in most cases these "xenoestrogens" activate gene transcription, that is, similar to E2, acting as receptor agonists. The first xenoestrogen identified was diethylstilbestrol (DES), a drug used to prevent miscarriage in the 1940s to the 1970s until it was discovered to have profound developmental effects in some offspring of women receiving the treatment (Trimble, 2001). Since then, a number of environmental pollutants with estrogenic activity have been identified, including certain chlorinated hydrocarbon insecticides (e.g., DDT, methoxychlor, and endosulfan), surfactants (nonylphenol), some polychlorinated biphenyls (PCBs), bisphenol A (used in plastic manufacturing), and ethinyl estradiol, a synthetic estrogen used in birth control pills and observed in municipal effluents and surface waters (Shelby et al., 1996; Larsson et al., 1999; van der Oost et al., 2003) (see Fig. 30-5).

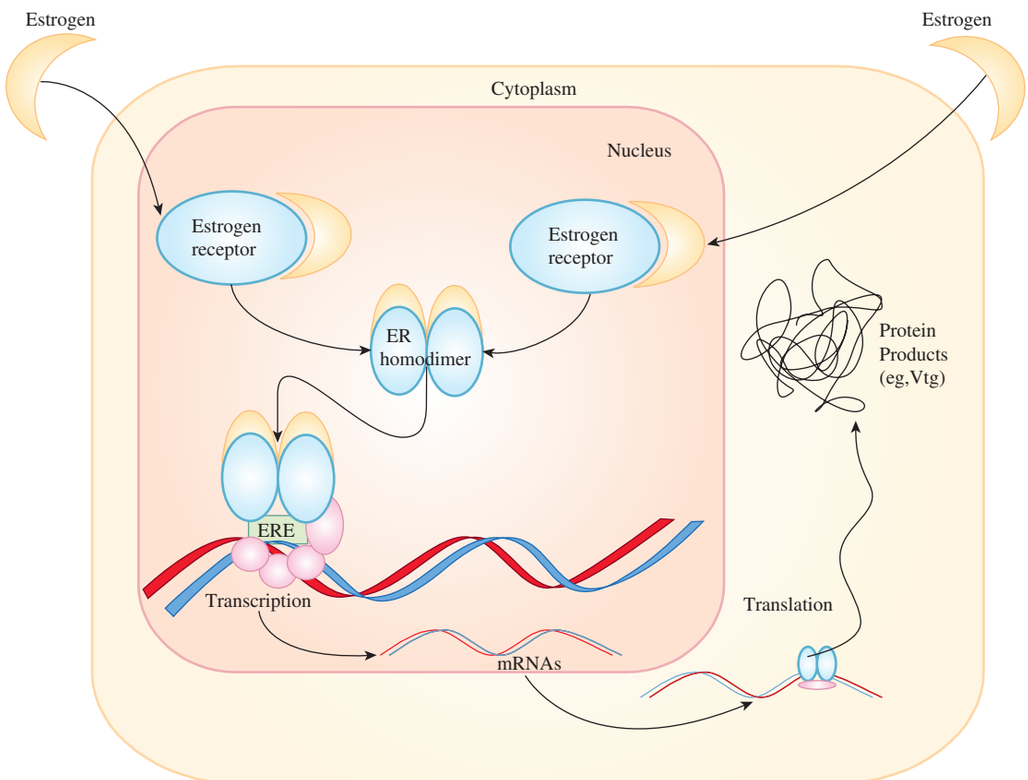

Figure 30-4. *A simplified model of estrogen receptor signaling including the estrogen receptor (ER), which on binding to native hormone ligand (estrogen) or xenoestrogen (see Fig. 30-5) forms the transcriptionally active ER homodimer complex that binds to genes containing estrogen response elements (ERE) and thereby upregulates transcription and translation of genes/proteins regulated by the system, such as vitellogenin (Vtg).* (Figure kindly provided by Carla Rosenfeld, Duke University.)

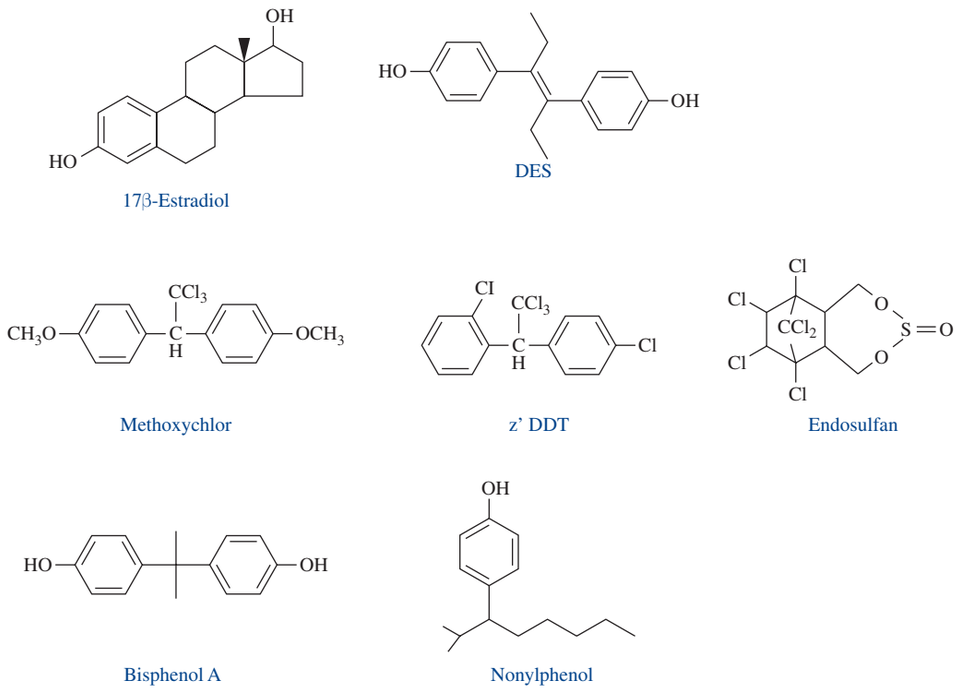

Figure 30-5. *Estrogen receptor agonists, including a native estrogen (17β-estradiol), the drug diethylstilbestrol (DES), a surfactant component (nonylphenol), an industrial intermediate (bisphenol A), and several pesticides (remainder).*

With the exception of ethinyl estradiol, these pollutants exhibit relatively low binding affinities to ER, as compared with E2 or DES (Shelby et al., 1996); however, environmental exposures may be sufficient to perturb reproduction or development.

Evidence for such "endocrine disruptions" by environmental xenoestrogens appears to be overall stronger for wildlife than for humans, likely due to instances of elevated exposures that are less prone to confounding factors than is typically the case for human exposures. Also, egg-laying vertebrates provide a unique biomarker of estrogen exposure that has contributed to ecotoxicological studies in this area. Vitellogenin (Vtg) is a protein that is normally produced by the liver of females and transported via the bloodstream to the ovary where, as a key component of yolk, it provides nourishment to the developing embryo. The production of Vtg is regulated by the estrogen–ER system. Interestingly, males of egg-laying vertebrate species contain the molecular machinery to produce Vtg, but production and circulating levels are normally very low, due to low titers of estrogen. However, exposures of males to estrogen and xenoestrogens upregulate Vtg production, which can be readily measured in blood samples. Consequently, elevated Vtg in males of these species is a useful biomarker of estrogenic chemical exposures (Sumpter and Jobling, 1995; see the section "Biomarkers"). Examples include rainbow trout (*Oncorhynchus mykiss*) caged in surface waters below industrial or municipal effluent sources enriched in alkylphenolic surfactants in the United Kingdom (Harries et al., 1997) or natural and synthetic estrogens in Sweden (Larsson et al., 1999), whitefish (*Coregonus lavaretus*) caged near paper mill effluents in Finland (Mellanen et al., 1999), and in tropical fish inhabiting a wastewater-impacted river in Brazil (Pimentel et al., 2016). While the bulk of research related to estrogenic compounds in natural systems has focused on fish, this approach has merit for other egg-laying vertebrates (Lorenzen et al., 2003; Huang et al., 2005), and for some invertebrates that produce Vtg-like proteins (Porte et al., 2006).

Aryl Hydrocarbon Receptor The AhR is a member of the basic helix–loop–helix Per ARNT Sim (bHLH-PAS) family of receptors/transcription factors that play roles in development, as sensors of the internal and external environment in order to maintain homeostasis, and in establishment and maintenance of circadian clocks (Denison and Nagy, 2003; Hahn et al., 2005). The AhR is among the most intensively studied receptors in toxicology due to its role in regulating a number of genes coding for proteins involved in xenobiotic metabolism, and its responsiveness to a number of widespread environmental contaminants, as well as some drugs and endogenous compounds. The AhR is a ligand-activated cytosolic receptor that on binding to a ligand traverses to the nucleus where it complexes with another transcription factor, the AhR nuclear translocator (ARNT) protein (Fig. 30-6), to form a transcriptionally active dimer. This AhR–ARNT complex binds to promoter sequences of genes regulated by the AHR system; these promoters are most often referred to as "xenobiotic response elements" (XRE) or "dioxin response elements" (DRE).

Characterized genes that are upregulated by the AhR system in large part code for enzymes involved in the metabolism of lipophilic chemicals, including organic xenobiotics and some endogenous substrates such as steroid hormones. These enzymes include specific cytochrome P450s (mammalian CYP1A1, 1A2, and 1B1 and their counterparts in other vertebrates), a glutathione transferase (GST), a glucuronosyl transferase (UDPGT), an alcohol dehydrogenase (ALD), and a quinone oxidoreductase (NQO). In addition to these enzymes, AhR–ARNT upregulates the AhR repressor protein (AhRR), which then competes with AhR for ARNT. This results in a transcriptionally inactive complex and effectively provides a negative feedback loop for controlling AhR-mediated gene transcription (Hahn et al., 2005). The biotransformation enzymes regulated by the AhR system are described in detail in Chap. 6, "Biotransformation of Xenobiotics." Briefly, they play key roles for transforming many lipophilic chemicals, including numerous

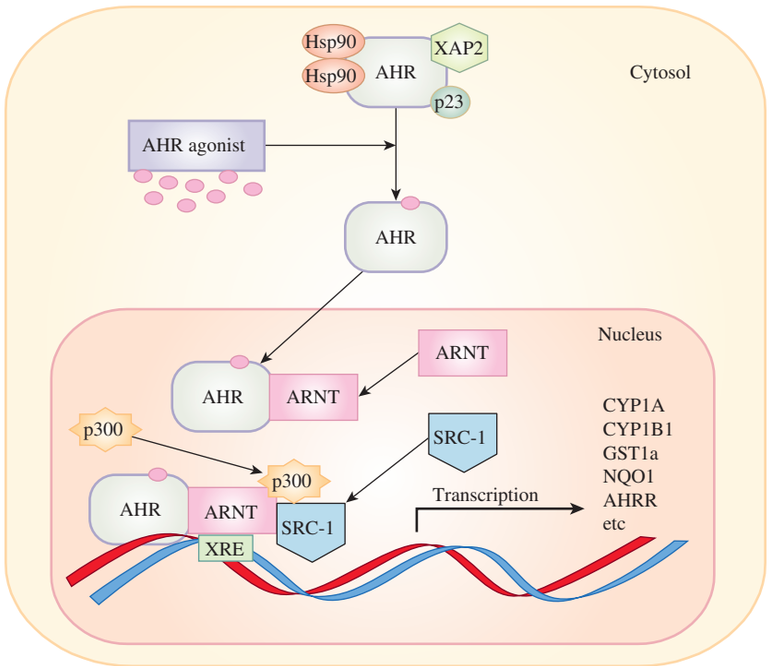

Figure 30-6. *A simplified model of the aryl hydrocarbon receptor (AHR) including activation by AHR ligands (see Fig. 30-7) which allows for dimerization with the AHR nuclear transporter (ARNT) that forms the transcriptionally active complex which binds to xenobiotic response elements (XRE) and thereby upregulates a number of genes, including several involved in biotransformation, indicated here, as well as the AHR repressor (AHRR) that provides negative feedback control of the system.* Hsp90, XAP2, and p23 are chaperone proteins; SRC-1 and p300 are examples of coregulator proteins involved in transcription. (Figure kindly provided by Carrie Fleming and Carla Rosenfeld, Duke University.)

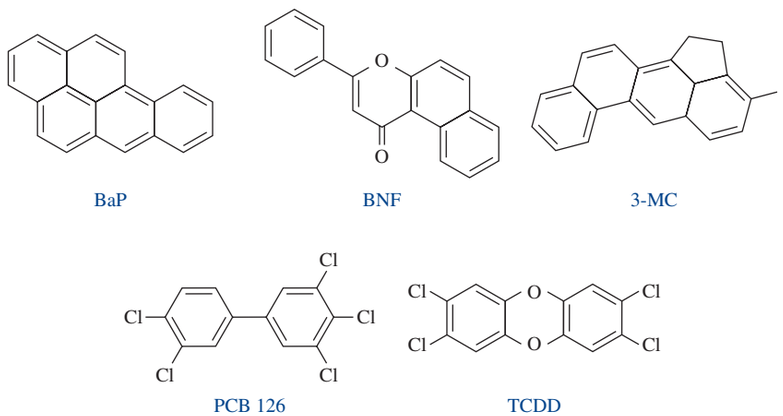

Figure 30-7. *Representative ligands of the aryl hydrocarbon receptor, including 2,3,7,8-tetrachlorodibenzodioxin (TCDD), a coplanar polychlorinated biphenyl (PCB 126), two polycyclic aromatic hydrocarbons (benzo(a)pyrene [BaP] and 3-methylcholanthrene [3-MC]), and a flavone (β-naphthoflavone [BNF]).*

common organic contaminants, into more water-soluble, and hence excretable, products. However, this biotransformation activity can result in production of highly reactive products that are more toxic than their parent compounds, that is, "activation."

Of particular relevance to the present discussion, however, is the ability of some ubiquitous pollutants to act as AhR ligands and markedly upregulate gene transcription via the AhR–ARNT signaling pathway described above (Denison and Nagy, 2003). In some cases, this can be interpreted as an adaptive response—the organism is reacting to exposure to a lipophilic xenobiotic in order to enhance its elimination. However, as noted above, biotransformation can also lead to enhanced toxicity of some substrates. The two major classes of pollutants that have members that act as ligands for the AhR and upregulate gene expression (and thereby "induce" biotransformation enzymes) and have received the greatest attention in ecotoxicology are the polycyclic aromatic hydrocarbons (PAHs) and the polyhalogenated aromatic hydrocarbons (pHAHs); examples of both are provided in Fig. 30-7. The most studied pHAHs are particular "coplanar" PCBs and chlorinated dioxins. While some PAHs and pHAHs share the ability to activate the AhR, important differences between these classes exist. PAHs, whether they are ligands for the AhR or not, are overall very good substrates for the biotransformation systems upregulated via the AhR, which can act to both detoxify and enhance the toxicity of some PAHs (discussed in the section "Cancer" later in this chapter). In general, pHAH-type AhR ligands are more potent AhR ligands and enzyme inducers than

PAHs, but due to extensive halogenation are much poorer substrates for biotransformation. One of the most potent ligands for the AhR is the dioxin, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), which is highly recalcitrant to biotransformation (Denison and Nagy, 2003).

In similarity with xenoestrogen-mediated inductions of Vtg via the ER, the inducibility of biotransformation enzymes via the AhR by xenobiotics has been used for biomonitoring. In this regard, enzymatic activities associated with the CYP1As have been the most widely used AhR-related biomarker, particularly an activity that appears highly specific for these CYPs, ethoxyresorufin *O*-deethylase (EROD), which is most often quantified in liver tissue of vertebrates. Elevated activities of EROD in various vertebrates have been associated with exposures to PCBs, dioxins, PAHs, and complex mixtures of these associated with, for example, harbor sediments, municipal effluents, paper mill effluents, refinery effluents, and crude oil spills (Custer et al., 2001; van der Oost et al., 2003; Miller et al., 2005). Invertebrate AhR homologs examined do not bind to ligands similarly to vertebrate AhRs and do not demonstrate protein inductions analogous to those observed in vertebrates (Butler et al., 2001; Hahn, 2002; Chaty et al., 2004). PAH and pHAH toxicities and the potential roles played by their interactions with the AhR will be discussed at various points in subsequent sections of this chapter, and related discussions from a human health standpoint appear in other chapters.

Genomics and Ecotoxicogenomics Advances in "omics"-level technologies for comprehensive interrogation of biological structure, function, and system dynamics continue to revolutionize molecular biology. These advances have permeated many areas of biological research, including toxicology and environmental science. We will briefly describe many of the techniques that are commonly used in these fields, since several omics technologies other than genomics are now in wide use.

Underlying *genomics* advances are very large projects to sequence the entire genomes of various species, such as the Human Genome Project that was completed in 2003 (Little, 2005). Other species that have been completely or largely sequenced include the mouse, rat, cow, dog, chimpanzee, chicken, zebrafish (*Danio rerio*), puffer fish (*Fugu rubripes*), medaka (*Oryzias latipes*), fruit fly (*Drosophila melanogaster*), a sea urchin (*Strongylocentrotus purpuratus*), a soil nematode (*Caenorhabditis elegans*), a yeast (*Saccharomyces cerevisiae*), and rice (*Oryza sativa*). The number of species with fully sequenced and annotated reference genomes continues to expand, as well as the tools available for genomic research (Kim et al., 2013; Louis et al., 2015; various Web sites, including https://www.ncbi.nlm.nih.gov/pubmed/24124405). Although microarray-based platforms to detect single nucleotide polymorphisms (SNP chips) are still commonly used in large epidemiologic cohorts (Ha et al., 2014), most experimental toxicology and environmental science laboratories are using more quantitative, high-resolution sequencing techniques. New genomics technologies offer significantly higher resolution as compared to previous sequencing techniques, which enables increased detection of rare sequence (Cao et al., 2013) and structural variants (Hanscom and Talkowski, 2014) in somatic or germ cells, somatic rearrangements common in cancer (Quek et al., 2014), or previously unrecognized somatic mosaicism (Gajecka, 2016). Collectively, these new sequencing approaches are often referred to as "next-generation sequencing (NGS)," "massively parallel sequencing," or "high-throughput sequencing" (Koboldt et al., 2013, Muzzey et al., 2015). The same sequencing technology employed in whole genome sequencing can be used to sequence short stretches of sequence at high resolution ("amplicon sequencing") (Andersen et al., 2014), or a defined subset of the genome, such as coding regions in "whole exome sequencing" (Ku et al., 2012), or mitochondrial genomes in "mtGenome sequencing" (Ye et al., 2014; Peck et al., 2016). Sequencing technologies are evolving rapidly, leading to reduced costs and effort and making their application to nontraditional models more practical (see review by Goodwin et al., 2016).

In addition to significant advances in genomics, available technology to interrogate the epigenome has rapidly expanded. The epigenome includes mitotically heritable changes in gene function that occur without changes in the DNA sequence (Jaenisch and Bird, 2003). Structural components of the epigenome that control compaction and accessibility of genomic regions include covalent modification of DNA, commonly methylation of the fifth position of cytosines ("DNA methylation"), posttranslational modifications to histone proteins contained within nucleosomes, and chromatin remodeling proteins that alter the three-dimensional structure of chromatin (Callinan and Feinberg, 2006). Although previously measured by sequencing a small number of cloned alleles (Huang et al., 2013), now DNA methylation is more commonly measured in one of two ways (Yong et al., 2016): either in a subset of the genome using DNA methylation microarray techniques, or with next-generation bisulfite sequencing, of individual amplicons, a subset of the genome enriched for methylated DNA ("reduced-representation bisulfite sequencing"), or whole-genome bisulfite sequencing, all of which require prior modification of the DNA with sodium bisulfite. Sodium bisulfite treatment deaminates unmethylated, but not methylated, cytosines to uracils, which are then amplified as thymines as PCR and are detected as sequence changes when compared to the reference sequence (Huang et al., 2013). Posttranslational modifications (PTMs) to histone proteins can be measured locally using chromatin immunoprecipitation (ChIP), which uses antibodies to specific PTMs to enrich the sample for DNA associated with these marks (Rodriguez-Ubreva and Ballestar, 2014), and subsequent PCR of the region of interest; the same approach can be scaled to assess a subset of the genome using microarray technology (ChIP-chip) or genome-wide with next-generation sequencing (ChIP-seq) (Schulz and Haussler, 2104). Newer peptide arrays enable discovery of novel proteins or protein complexes that are recruited to or interact with individual or combinations of histone PTMs (Fuchs et al., 2011; Rothbart et al., 2012).

Genome sequencing set the stage for genome-wide analysis of gene expression ("transcriptomics" to measure gene transcription and "proteomics" to measure gene translation). The platform most commonly used in ecotoxicology is the expression microarray (Neumann and Galvez, 2002; Jyasundara et al., 2015). cDNAs for known genes can be spotted on glass slides, or chips, resulting in microarrays that can be employed to quantify relative levels, that is, expression, of mRNAs for those genes in samples of interest. In recent years, the microarray approach has been largely supplanted by the transcriptomics technique RNA-seq, which is more quantitative and higher resolution, as well as significantly less expensive, laborious, and error-prone than microarrays (Wang et al., 2010). Induced transcription can be distinguished from constitutive transcription in real-time using new techniques that employ the labeled nucleotide bromouridine (Roberts et al., 2015). Also advancing rapidly are related analyses of global changes in proteins (the translation products of mRNAs), referred to as "proteomics," and resulting metabolite profiles (amounts of sugars, lipids, amino acids, etc. in various tissues that are controlled in part by enzyme activities), referred to as "metabolomics." These approaches often employ sophisticated mass-spectrometry techniques (Altelaar et al., 2013). Translation can be measured in real-time, too, using a technique called "ribosome profiling," which employs capture and

sequencing of transcripts undergoing active translation (Brar and Weissman, 2015).

A major complexity in all of these global analyses is the extremely large data sets that arise, for example, when one examines the responses of thousands of genes from organisms exposed to one or more concentrations of a chemical at one or more time points. This has led to the development and rapid expansion of the field of "bioinformatics" that includes the application of sophisticated statistical and computing approaches for revealing biologically meaningful patterns of gene expression, such as relationships to cellular signaling pathways (Del Chierico et al., 2015).

Omics have spread into the science and applications of ecotoxicology, collectively termed ecotoxicogenomics. As is the case for human health–oriented toxicogenomics, ecotoxicogenomics has great potential for elucidating impacts of chemicals of ecological concern and ultimately for playing an important role in Ecological Risk Assessments (ERAs) and regulatory ecotoxicology (Snape et al., 2004; Ankley et al., 2006; Watanabe and Iguchi, 2006). Specific areas to which this emerging field can contribute include prioritization of chemicals investigated in ERAs, identification of modes of action of pollutants, identification of particularly sensitive species and populations, and effect prediction at higher levels of organization. A recent study employed whole-genome sequencing, RNA-seq, and advanced bioinformatics analyses to elucidate molecular mechanisms underlying the rapid evolutionary adaptation of Atlantic killifish (*Fundulus heteroclitus*) to severe pollution (Reid et al., 2016). Epigenome-wide analyses have also permeated ecotoxicology (Vandegehuchte and Janssen et al., 2011; Suarez-Ulloa et al., 2015). As in other areas of ecotoxicology, a major complexity faced by scientists applying omics techniques is the vast array of species of potential concern. This is a particularly problematic issue in ecotoxicogenomics that requires substantial species-specific molecular information. However, as mentioned earlier, the number of ecologically relevant species for which this information is becoming available is expanding rapidly, and is likely to accelerate as more genomes are fully sequenced and tools are refined. Moreover, as information grows, genomics and related approaches hold great promise for identifying appropriate surrogate species for laboratory studies used in basic ecotoxicological research and in support of regulatory ecotoxicology (Benson and Di Giulio, 2006; Van Aggelen et al., 2010).

Protein Damage The study of chemical effects on proteins, particularly enzymes, has a long history in toxicology. Of particular interest within ecotoxicology are the inhibitions of acetylcholinesterase (AChE) by certain pesticides and of delta-aminolevulinic acid dehydratase (ALAD) by lead. AChE degrades the neurotransmitter acetylcholine, and in so doing controls nerve transmission in cholinergic nerve tracts. The widely used organophosphate and carbamate classes of insecticides kill by inhibiting AChE, and this mechanism is operative for "nontarget" organisms including invertebrates, wildlife, and humans. (See Chap. 22, "Toxic Effects of Pesticides," for a detailed discussion of these pesticides and AChE inhibition.) Of particular ecological concern have been the ingestions of AChE-inhibiting insecticides with food items or granular formulations (mistaken as seed or grit) by birds and exposures to aquatic animals from agricultural runoff (Mineau, 1991; Carr et al., 1997; Wilson et al., 2001). In many field studies, effects of these insecticides on mortality, and relationships between AChE inhibition and mortality, have been of primary concern. However, relationships between AChE inhibition and important sublethal impacts such as behavior have also been observed (Sandahl et al., 2005).

ALAD catalyzes the rate-limiting step of heme synthesis, a key component of cytochromes, hemoglobin, and myoglobin, and ALAD activity is very sensitive to inhibition by lead (ATSDR, 1999). This sensitivity has been exploited widely as a biomarker for lead exposure in humans and wildlife. In wildlife, concerns for lead exposure have included ingestion by birds of spent lead shot used in hunting (Kendall et al., 1996), accumulation of lead by wildlife living near highways (Birdsall et al., 1986), and aquatic organisms inhabiting surface waters contaminated by lead from mine runoff and other industrial activities (Schmitt et al., 2005). Many other enzymes have been observed to be inhibited by numerous pollutants. In addition to enzyme inhibition, chemicals can damage proteins in other ways, including oxidative damage as described in section Oxidative Stress, and by forming stable adducts similar to those formed with DNA, described in section DNA Damage.

Oxidative Stress The classic depiction of aerobic respiration shows molecular oxygen (O_2) as the terminal electron acceptor, with its reduction resulting ultimately in water as high-energy intermediates (NADH, $FADH_2$) are oxidized and cellular energy is captured as ATP. This reduction of O_2 to H_2O requires four electrons that are sequentially added; this process is tightly coupled so that the one-, two-, and three-electron intermediates are released at low amounts (less than 0.1% of O_2 inspired; Fridovich, 2004). These intermediates are, in sequence, the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$). This tight coupling is fortunate because these intermediates, produced during aerobic respiration and other O_2-consuming or O_2-producing processes (such as photosynthesis and CYP-mediated biotransformations), are potentially deleterious products that can indiscriminately damage cellular components; hence, they are referred to as "reactive oxygen species" (ROS); see Chap. 3, "Mechanisms of Toxicity." Some ROS, including $O_2^{\bullet-}$ and $^{\bullet}OH$, are free radicals, that is, they possess an unpaired electron; among ROS, $^{\bullet}OH$ is particularly reactive and toxic. In order to defend themselves from the damaging effects of ROS, all aerobic organisms have evolved complex antioxidant defense systems that include enzymatic and nonenzymatic components (Halliwell and Gutteridge, 1999). Antioxidant enzymes include superoxide dismutases that convert $O_2^{\bullet-}$ to H_2O_2, catalases and peroxidases that detoxify peroxides including H_2O_2, and enzymes involved in the production and maintenance of reduced glutathione (GSH) such as glutamate–cysteine ligase (GCL) and glutathione reductase (GR). Low-molecular-weight, nonenzymatic antioxidants include vitamins A, C, and E, and GSH.

Oxidative stress has been defined as "a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage" (Sies and Cadenas, 1985)—that is, the point at which the production of ROS exceeds the capacity of antioxidants to prevent damage. Numerous environmental contaminants can act as prooxidants and enhance the production of ROS. The resulting oxidative damage can account wholly or partially for toxicity (Halliwell and Gutteridge, 1999). Mechanisms by which chemicals can enhance ROS production include redox cycling, interactions with electron transport chains (notably in mitochondria, microsomes, or chloroplasts), and photosensitization.

Redox cycling is perhaps the most common mechanism by which a diverse array of chemicals including many environmental pollutants generates intracellular ROS. Redox cycling chemicals include diphenols and quinones, nitroaromatics and azo compounds, aromatic hydroxylamines, bipyridyliums, and certain metal chelates, particularly of copper and iron (Di Giulio et al., 1989; Halliwell and Gutteridge, 1999). These include compounds of broad industrial use, many pesticides, ubiquitous elements, and metabolic products of numerous pollutants. In the redox cycle,

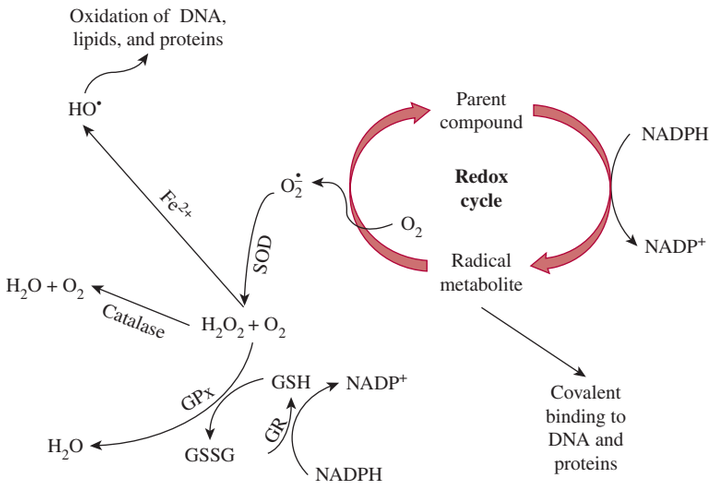

Figure 30-8. *Overview of oxidative stress, including reactive oxygen species stimulation initially by redox cycling, key antioxidant defenses, and potential deleterious biochemical effects.* "Parent compound" in redox cycle could include a number of chemicals such as quinones, nitroaromatics, azo dyes, paraquat and diquat, and transition metal chelates.

the parent compound accepts an electron from a reduced cofactor, such as NADH or NADPH; this reaction is typically catalyzed by a reductase such as xanthine oxidase or cytochrome P450 reductase (Kappus, 1986). In the presence of O_2, the unpaired electron of the radical metabolite is donated to O_2, yielding $O_2^{\bullet}$ and regenerating the parent compound; importantly, the parent compound can repeat this cycle until it is cleared or metabolized to an inactive product. In the course of each redox cycle, two potentially deleterious events occur—a high-energy reducing equivalent is expended (e.g., the oxidation of NADPH to $NADP^+$) and an oxygen radical is produced. A generalized redox cycle that includes associations with cellular toxicities and antioxidant defenses comprises Fig. 30-8.

Additionally, pHAH AhR ligands such as coplanar PCBs and TCDD can enhance ROS production, possibly by inducing CYP1A activity and concomitantly interfering with electron flow mediated by these enzymes (Schlezinger and Stegeman, 2001). PAHs can also upregulate CYP1A and can enhance the production of ROS-generating redox-active quinone metabolites, in contrast to uncoupling mechanisms proposed for pHAHs; Nebert et al. (2000) discuss potential mechanisms by which AhR agonists can produce oxidative stress. The herbicide paraquat is phytotoxic due to interference with chloroplast electron transport. Interestingly, it is a very potent lung toxicant because of its specific uptake by this tissue and subsequent redox cycling (Halliwell and Gutteridge, 1999). Another important mechanism particularly significant in aquatic systems is photosensitization. Ultraviolet (UV) radiation (specifically UVB and UVA) can penetrate surface waters to depths dependent on the wavelength of the radiation and the clarity of the water. The UV radiation generates ROS and other free radicals via excitation of photosensitizing chemicals, including common pollutants of aquatic systems (Larson and Weber, 1994). For example, due to photosensitization, many PAHs are orders of magnitude more acutely toxic to aquatic organisms in the presence of UV radiation than in its absence (Arfsten et al., 1996; Ankley et al., 1997). The ecological relevance of photosensitization, however, is controversial (McDonald and Chapman, 2002).

As noted, ROS are generally indiscriminant molecules and can potentially damage any cellular component. Well-characterized biochemical impacts of ROS include oxidations of unsaturated lipid components of membranes ("lipid peroxidation"), oxidations of amino acids and proteins (resulting in, e.g., the addition of carbonyl groups), and DNA oxidations resulting in products such as 8-hydroxy-guanosine and thyme glycol (Halliwell and Gutteridge, 1999). Another important impact is perturbed redox status (Schafer and Buettner, 2001). Healthy cells typically maintain high ratios of cofactors in their reduced, high-energy state relative to their oxidized state (e.g., NADH/NAD^+, NADPH/$NADP^+$, and GSH/GSSG), as the reduced forms are those most employed, for example, for energy production, biosynthesis, and antioxidant defense. The ROS can drive redox status to a more oxidized state by several direct and indirect mechanisms, potentially reducing cell viability. These ROS-mediated impacts and others have been associated with a number of human diseases including atherosclerosis, arthritis, cancer, and neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (Halliwell and Gutteridge, 1999). With the exception of cancer (see Cancer section), the role of ROS in specific diseases in wildlife has received little attention. However, numerous studies have documented oxidative stress–mediated biochemical and cellular effects in wildlife associated with environmental contamination (Winston and Di Giulio, 1991; Bainy et al., 1996; Livingstone, 2001; van der Oost et al., 2003; Dorval et al., 2005; Birnie-Gauvin et al., 2017). As with humans and various animal models for human disease, it is reasonable to assume that oxidative stress comprises an important mechanism accounting in part for the toxicity of diverse pollutants to free-living organisms. Also, oxidative stress is involved in the effects of air pollutants on plants and likely plays a role in forest diebacks observed downwind of industrialized areas (Richardson et al., 1989; Hippeli and Elstner, 1996).

DNA Damage The importance of DNA as a molecular target in toxicology is indicated by the devotion of Chap. 9, "Genetic Toxicology," of this text to genetic toxicology. As indicated, perhaps the most pressing human health issue associated with xenobiotic–DNA interactions is cancer. Cancer is also an important health outcome associated with chemical exposures in wildlife, particularly for bottom-dwelling fish, as discussed in the section "Cancer" later in this chapter. Of greater concern in ecotoxicology versus human health are other, multigenerational effects of pollutants on genetic structures and resulting phenotypes of populations and communities, through both direct effects on DNA (mutations)

and indirect effects (selection); this topic is discussed in the section "Toxicant Effects: Population."

Chemical contaminants can damage DNA through several mechanisms, including by the formation of DNA–xenobiotic adducts, by causing strand breaks, and by oxidations of DNA bases. In the context of ecotoxicology, the most widely studied form of damage has been the formation of stable DNA adducts, particularly by PAHs. In order to form these adducts, PAHs must first be activated to reactive metabolites by enzyme systems such as the cytochrome P450s. The bulk of PAHs metabolized to various oxidized products (such as phenols, diols, and epoxides) is subsequently conjugated by phase II enzymes (such as GSH, sulfate, and glucuronosyl transferases; see Chap. 6, "Biotransformation of Xenobiotics"); however, some fraction can react with DNA, mainly through covalent bonding with DNA bases. The most studied example of this is benzo(*a*)pyrene (BaP), which can be metabolized to the highly reactive benzo(*a*)pyrene diol epoxide (BPDE) that can bond to DNA. In field studies, the resulting large adducts of DNA with BPDE and other activated PAHs and related compounds can be measured with the highly sensitive ^{32}P-postlabeling assay (Phillips, 1997). This technique has been used extensively to monitor DNA adducts in benthic fish and bivalves inhabiting systems contaminated with hydrocarbons, particularly PAHs (Maccubbin, 1994; Reichert et al., 1998; Shugart, 2000; Amat et al., 2004). Other forms of DNA damage that have been investigated in ecotoxicological studies include DNA strand breaks and oxidized DNA bases (Shugart, 2000; Malins et al., 2006).

Once DNA damage has occurred, whether from chemical exposures or other causes (respiration, UV radiation, viral interactions, normal wear and tear), several subsequent outcomes can occur, including the following: the damage can be properly repaired, the damage can lead to cell death, or a resulting change in DNA structure (base sequence) can become fixed and passed on to daughter cells, that is, mutation occurs. Complex DNA repair systems have been elucidated in prokaryotic and eukaryotic organisms (see Chap. 8, "Chemical Carcinogenesis," and Chap. 9, "Genetic Toxicology"), and while these systems have received relatively little attention in species of ecological relevance, it is a safe assumption that these conserved systems are qualitatively similar across diverse phyla. Overall, these systems exhibit a remarkable capacity for surveying the cellular genome, detecting damage such as oxidations, adducts, and strand breaks, and repairing the damage by, for example, removing a damaged base and replacing it with the correct base. However, misrepair does sometime occur, with the result that an incorrect base is incorporated. Depending on the gene involved and the site within the gene, this change may lead to cell death, or may result in a mutation that may have no effect (occurs at noncritical base sequence) or one that leads to functional change in the protein coded by the gene. Some chemicals cause cancer by mutating genes that play pivotal roles in cellular growth and differentiation, particularly oncogenes and tumor suppressor genes. Examples of discoveries of activated genes (in liver tumors) in field studies include the *K-ras* oncogene in tomcod (*Microgadus tomcod*) from the Hudson River, New York (Wirgin et al., 1989), and in winter flounder (*Pseudopleuronectes americanus*) collected from Boston Harbor (McMahon et al., 1990), and the retinoblastoma (*Rb*) tumor suppressor gene in the marine flatfish, dab (*Limanda limanda*), from the United Kingdom (du Corbier et al., 2005).

Cellular, Tissue, and Organ Effects

Cells Cellular organelles that have received attention as targets in species of ecological interest include mitochondria, lysosomes, and nuclei. Most free-living organisms routinely experience energy deficits. For example, food resources are often highly depleted during the winter for many animals, which adapt by conserving energy (by hibernating or lowering metabolism) or by storing energy beforehand (as the case for many migratory birds). Thus, effects of pollutants on mitochondrial energy metabolism can be of particular importance to wildlife. For example, Sokolova and coworkers (Sokolova, 2004; Cherkasov et al., 2006) have elegantly described the effects of cadmium on several aspects of mitochondrial function in isolated gill and hepatopancreas cells from the eastern oyster (*Crassostrea virginica*), and noted a marked synergy between the metal and increasing environmental temperatures. Lysosomes are involved in the degradation of damaged organelles and proteins, and also sequester a wide variety of environmental contaminants, including metals, PAHs, and nanoparticles (Moore, 2006). The accumulation of xenobiotics by lysosomes can elicit membrane damage, or "membrane instability," which has been used as an early warning measure of pathological chemical effects in both invertebrates and vertebrates (Hwang et al., 2002; Kohler et al., 2002; Moore et al., 2006).

In addition to specific damage to DNA bases described above, chemical effects on nuclei have been examined in ecological contexts with additional techniques. Micronuclei are chromosomal fragments that are not incorporated into the nucleus at cell division, and chemical exposures can markedly increase their frequency. Elevated micronuclei numbers have been observed, for example, in fish erythrocytes from polluted coastal sites in California (Hose et al., 1987) and in hemocytes in clams from a PCB-polluted harbor in Massachusetts (Dopp et al., 1996). Also, a standardized higher plant (*Tradescantia*) assay for micronuclei has been used for monitoring air pollution (Solenska et al., 2006). A cell-based assay that has been used widely in environmental applications is the comet assay. In this assay, cells are imbedded in agarose, lysed, and subjected to gel electrophoresis, and the features of the resulting "comet's tail" on the gel used to assess DNA damage. With appropriate manipulations, the comet assay can be employed to detect and distinguish among a variety of genotoxicities including strand breaks, oxidative damage, and adducts (Moller, 2006). It has been used in a variety of field applications, particularly with bivalves (Steinert, 1999; Nigro et al., 2006), fish (Klobicar et al., 2010), and mice (Husby and McBee, 1999).

Histopathology The detailed microscopic analysis of the structure of cells and tissues can provide important links among chemical exposures, cellular targets and mechanisms, and effects at the organismal level (Hinton, 1994). Moreover, the determination that tissue damage has occurred as demonstrated by histopathological analysis is extremely useful for inferring that a significant deleterious effect has occurred. However, the substantial expertise required for proper histopathological analyses of this nature and the oftentimes time- and labor-intensive nature of these analyses has perhaps limited the application of this powerful approach in ecotoxicological contexts. Nevertheless, histopathological analysis has played an important role in confirming chemically mediated tissue damage in numerous laboratory and field studies. For example, Pacheco and Santos (2002) integrated histopathological analysis with biochemical studies of the effects of various environmental contaminants on the European eel (*Anguilla anguilla*), and Devlin (2006) similarly incorporated this approach in studies of the effects of methylmercury in fathead minnows (*Pimephales promelas*). Handy et al. (2002) relied on histopathology in a study of fish health in rivers in southern England. Wester et al. (2002) reviewed the application and potential contributions of histopathology in aquatic toxicology,

particularly in the context of small fish models. In subsequent sections concerning organismal-level impacts, other examples of the use of histopathology will be provided.

Target Organs Descriptions of chemical impacts on all organ systems of the myriad species relevant to ecotoxicology are beyond the scope of this chapter. Other chapters in this volume address key target organs in the mammalian context, and much of this is relevant to other vertebrates. Target organ toxicology is also the subject of comprehensive reviews by Schlenk and Benson (2001) concerning marine and freshwater fish, and by Gardner and Oberdorster (2005) concerning reptiles. Relevant information in marine mammals was reviewed in Vos et al. (2002). Although we are unaware of similar reviews for birds or invertebrates, understanding target organ toxicity can be crucial for these taxa as in the recent case of widespread renal poisoning of vultures exposed to the veterinary drug diclofenac during scavenging on cattle carcasses (Oaks et al., 2004). Further, the unique properties of the avian respiratory system and its utility for investigating respiratory system toxicity and air pollution were reviewed by Brown et al. (1997).

Another important target organ in ecotoxicology that is not covered elsewhere in this text is the respiratory organ of nonmammalian aquatic vertebrates and many invertebrates, the gill; gills of fish have received the most attention as targets of toxicants. The gill epithelium is the major site of gas exchange, ionic regulation, acid–base balance, and nitrogenous waste excretions for fish and other aquatic animals (Evans, 1987). Gills are immersed in a major exposure medium for these animals (surface water), so metabolically active epithelial cells are in direct contact with this medium. They also receive blood supply directly from the heart, through the ventral aorta. Thus, it is not surprising that gills comprise a very important target for many environmental pollutants, due to their critical physiological functions, central position in blood circulation pathways, and intimate relationship with the environment. The basic structure of fish gills is composed of branchial arches from which extend numerous filaments; from the filaments extend the lamellae (Wendelaar Bonga and Lock, 2008). The lamellae are covered by a layer of epithelial cells that function in gas exchange, whereas the filament epithelium is dominated by other cell types, including pavement cells, mucous cells, and chloride cells; chloride cells are the primary location for ATPase activity and ion channels involved in ion transport.

Common structural lesions in gills caused by a diverse array of chemicals include cell death (via necrosis and apoptosis), rupture of the epithelium, hyperplasia and hypertrophy of various cell populations that can lead to lamellar fusion, epithelial swelling, and lifting of the respiratory epithelium from the underlying tissue (Wendelaar Bonga and Lock, 2008). Chloride cells have received particular attention due to their key role in ionic homeostasis. For example, metals such as cadmium, copper, lead, silver, and zinc have been shown to interfere with their function in ion transport. In some cases, this may be due to inhibition of ATPase activities and/or increased membrane permeability (Spry and Wood, 1985; Wendelaar Bonga and Lock, 1992; Li et al., 1998; Rogers et al., 2003; Bury, 2005). The stress response, which results in elevated blood concentrations of epinephrine and cortisol, and associated responses such as increased cardiac output and elevated blood pressure, can also perturb ionic balance by promoting passive loss of ions such as Na^+ and Cl^- (Wendelaar Bonga and Lock, 2008). A variety of contaminants have been shown to evoke the stress response in fish, sometimes concomitantly with perturbations in ionic balance (Hontela, 1997; Webb and Wood, 1998; Chowdhury et al., 2004). Also, gill damage appears to be the primary cause of the acute toxicity of PAH-mediated phototoxicity in fish (see the section "Oxidative Stress"). Weinstein et al. (1997) reported histopathological impacts of UV + fluoranthene in gills of fathead minnows including severe damage to mucosal cells, inflammation, and apparent accumulation of lipid peroxidation products; these effects likely resulted in respiratory stress and lethality. This study elegantly demonstrates a progression from biochemical mechanism (oxidative stress) to target organ damage (gill respiration) to an important organismal impact (death). Mortality and important sublethal organismal impacts that have received substantial attention among ecotoxicologists are covered in the following section.

Organismal Effects

Mortality In similarity with impacts on human health, chemical pollution of the environment does not in most cases attain levels sufficient to outrightly kill wildlife. Concern in the ecotoxicological context is overall more for long-term, chronic impacts on organismal variables such as survival, reproduction and development, behavior, and disease susceptibility, and how such impacts translate to impacts at population and higher levels of organization. However, numerous cases of wildlife mortalities (particularly birds and fish) due to exposures to chemical pollution have been observed, including cases associated with chronic oil discharges (Wiese and Robertson, 2004) and major oil releases from events such as the Exxon Valdez tanker wreck in Alaska (Peterson et al., 2003) and the 1991 Gulf War (Evans et al., 1993), lead from spent shot (Clark and Scheuhammer, 2003) and mines (Henny, 2003), and pesticide exposures (Mineau et al., 1999).The ingestion of lead ammunition fragments when scavenging on carcasses results in fatal poisonings to endangered California condors (Wiemeyer et al., 1988; Cade, 2007). In this instance, death of an endangered individual condor was relevant. Similarly, oriental white-backed vultures scavenging on cattle carcasses in South Asia consume enough of the veterinary anti-inflammatory drug, diclofenac, to cause fatal kidney failure (Oaks et al., 2004; Balmford, 2013). While not a direct toxic chemical effect, hypoxia can be an important cause of fish and invertebrate mortality in aquatic systems; anthropogenic inputs of nutrients associated with sewage or fertilizers that enhance the growth of phytoplankton can cause or exacerbate hypoxia (Paerl et al., 1999; Wu, 2002). While direct mortality may not be a commonplace effect of toxic chemicals in natural systems, mortality comprises a major endpoint in toxicity testing, discussed in section "Population."

Reproduction and Development Impacts on reproduction and development comprise perhaps the greatest concern among potential sublethal effects of xenobiotics on animals inhabiting natural systems. This is due to sensitivities of the physiological processes involved that have been described for a number of pollutants, and the importance of reproduction and development to population dynamics, a key ecological concern. Moreover, the discovery of the effects of some organochlorine insecticides on avian reproduction (particularly eggshell thinning) and resulting population crashes of several predatory bird species, and the public's awareness through the publication of Carson's *Silent Spring* in 1962, can be associated with the birth of ecotoxicology. Concern for reproductive and developmental effects has increased in recent years, with the widespread detection of endocrine disruptors in the environment and the recognition of the inherent sensitivity of the developing organism.

A variety of environmental contaminants have been associated with reproductive and/or developmental effects in wildlife populations, with this association supported by controlled laboratory studies. Chlorinated hydrocarbons have continued to generate concerns, although many (DDT and other insecticides, and PCBs) have had

their production and use sharply curtailed. For example, DDT, its major metabolite in birds (DDE), and PCBs have been associated with reproductive and developmental impacts in bird populations in the Great Lakes, southern California, the Puget Sound, and the Arctic (Fry, 1995; Custer et al., 1999; Bustnes et al., 2005). Also, alligators (*Alligator mississippiensis*) inhabiting a DDT-polluted lake in Florida have exhibited reproductive and developmental perturbations (Guillette et al., 2000). Evidence also indicates that PCBs impact marine mammal reproduction, including that of bottlenose dolphins (*Tursiops truncatus*) (Schwacke et al., 2002; Wells et al., 2005).

Deleterious impacts on fish reproduction have been associated with environmental exposures to a number of contaminants. Case studies include PAHs (and other chemicals) accumulated in sediments in urban areas and harbors in the Puget Sound (Johnson et al., 1993) and northeastern United States (Johnson et al., 1994) or associated with oil spills such as the Exxon Valdez (Sol et al., 2000) and the Deepwater Horizon spill (Xu et al., 2016). Other examples include effluents from bleached paper mills in various locations, including Canada (Munkittrick et al., 1991), and selenium, for example, emanating from coal-fired power plant fly ash stored near freshwater lakes (Lemly, 2002) or in streams due to coal mine runoff (Holm et al., 2005). Notably, selenium produced severe developmental effects in water birds feeding in a created wetland in central California (Kesterson National Wildlife Refuge) that concentrated naturally occurring selenium (Ohlendorf, 2002). Additionally, severe developmental anomalies have also been observed in natural populations of marine gastropods exposed to tributyltin (TBT), which has been used extensively as an antifouling paint on ship hulls (Ruiz et al., 2005).

The developmental effects of dioxins (TCDD) and coplanar PCBs on vertebrate development have received substantial attention. To some extent, this work was motivated by analyses that indicated that these compounds were responsible for population crashes of Great Lakes' fisheries (particularly lake trout, *Salvelinus namaycush*, in Lake Ontario) in the 1950s and 1960s, as well as for developmental impacts on other wildlife, particularly piscivorous birds and mammals in the region (Gilbertson et al., 1991; Cook et al., 2003). Laboratory investigations, largely with fish and bird models, have shown that embryo development is very sensitive to these compounds, and such effects likely underlaid the population crashes (Fairbrother et al., 1999; Cook et al., 2003). These investigations have included elegant mechanistic studies that revealed cardiac development was particularly sensitive to these chemicals, and concluded that developmental perturbations are largely receptor mediated, that is, they are dependent on binding of the chemical (such as TCDD) with the AhR described earlier in this chapter (Hankinson, 1995; Heid et al., 2001; Tanguay et al., 2003; Antkiewicz et al., 2005). Much of the more recent work in this area has been done with zebrafish (*D. rerio*), a powerful model for molecular and developmental toxicology due to ease of visually examining development through a clear chorion, rapid development (approximately 4 days from fertilization to hatch), and abundant genetic information, including gene sequences (Carney et al., 2006). For example, with a known gene sequence, one can design morpholinos to block translation of specific mRNAs; morpholinos are oligonucleotides with a modified "backbone" that renders them stable (resistant to DNA/RNAase activities) and thus able to transiently block translation of specific protein targets. Carney et al. (2004) employed morpholinos to knock down AhR translation in zebrafish embryos, which greatly reduced the developmental toxicity of TCDD, confirming the role of the AhR in dioxin toxicity. Morpholinos were also employed to investigate the role of CYP1A; for example, its upregulation via TCDD activation of the AhR could enhance oxidative stress. Teraoka et al. (2003) observed marked reductions of TCDD toxicity with either AhR or CYP1A morpholinos, while Carney et al. (2004) observed protection with the AhR but not the CYP1A morpholino. While the role of CYP1A in dioxin effects on development remains unclear, pathways downstream of the AhR other than CYP1A are likely involved.

Similar concerns have emerged for the developmental effects of PAHs, particularly in fish. Hydrocarbons, in large part PAHs, associated with oil spills, contaminated sediments, paper mill effluents, and creosote used for wood treatment have profound developmental effects in fish embryos (Billiard et al., 1999; Carls et al., 1999; Meyer et al., 2002). In many cases, the effects observed visually appear similar to those observed in fish embryos exposed to dioxins and coplanar PCBs, and include malformed hearts ("tube heart"), craniofacial deformities, hemorrhaging, and edema of the pericardium and yolk sac, the latter resulting in a distended, faintly blue yolk sac and hence a name given to this syndrome—"blue sac disease" (Spitsbergen et al., 1991). The mechanisms by which PAHs produce this effect are unresolved, and likely include more than a single mechanism—not surprising in light of the myriad of chemicals comprising hydrocarbon/PAH mixtures in the environment. In some cases, effects appear to be AHR-independent. Incardona et al. (2005) concluded in studies with zebrafish and employing morpholinos that in weathered crude oil, tricyclic PAHs (such as phenanthrene and dibenzothiophene [DBT], the latter a sulfur-substituted PAH) accounted for the bulk of cardiovascular teratogenesis, and rather than mediating toxicity, the AHR–CYP1A pathway afforded some protection. Wassenberg and Di Giulio (2004a) and Wassenberg et al. (2005) observed marked synergies in the developmental toxicity to killifish (*Fundulus heteroclitus*) embryos between higher molecular weight PAHs that are AHR agonists (BaP and BNF) and PAHs that inhibit CYP1A (α-naphthoflavone [ANF], fluoranthene, DBT, and carbazole—a nitrogen-substituted PAH). Also, ANF enhanced the toxicity of a water-based extract of sediments contaminated with weathered creosote (Wassenberg and Di Giulio, 2004b). In subsequent studies with zebrafish embryos investigating the synergistic toxicity of BNF and ANF, the AHR morpholino provided protection, while the CYP1A morpholino enhanced toxicity (Billiard et al., 2006). Collectively, these PAH studies suggest that in the context of embryo toxicity, CYP1A plays a protective role, presumably by mediating metabolism and clearance of these metabolically labile compounds. This is in contrast to the metabolism-resistant dioxin-like compounds, where CYP1A either appears to play a role in mediating toxicity or has no effect. For some PAHs, such as lower molecular weight (tricyclic) PAHs that have little or no activity as AHR ligands, developmental toxicity appears AHR-independent, while the developmental toxicity of some higher molecular weight PAHs that are AHR agonists appears in part AHR-mediated. In addition, oxidative stress may play a role in the developmental toxicity of some PAHs to fish embryos (Bauder et al., 2005). PAHs comprise an important class of contaminants whose widespread occurrence is broadly associated with urbanization, population growth, and use of fossil fuels (Van Metre and Mahler, 2005).

Developmental abnormalities arising from toxicant exposure are also of concern for invertebrate species. As an important example already mentioned, antifouling agents in paints applied to ship hulls leach into coastal waters, causing widespread imposex in many marine snails (Leung et al., 2006). Acting as endocrine disrupters, organotin compounds cause male characteristics such as a penis or vas deferens to develop in females. Often this imposition of male characteristics on females results in an individual snail's

inability to reproduce and eventual collapse of impacted snail populations (Saavedra Alvarez and Ellis, 1990; Leung et al., 2006).

Contaminant effects on development are often difficult to discern in field studies due to the small size of embryos and the fact that developmental impact generally is lethal or greatly reduced survival. However, early life stages of most organisms are generally more sensitive to xenobiotics than other life stages; thus, developmental impacts merit careful attention by ecotoxicologists.

Disease Susceptibility Disease plays an important role in regulating and sometimes seriously impacting populations of free-living organisms. Of great concern are interactions between disease organisms and environmental contaminants, particularly potential impacts of chemicals on immune systems that render organisms more susceptible to disease. The question is often raised about how chemical pollution elevates the role of disease in population viability and dynamics.

Both field observational and laboratory experimental studies motivate this concern. For example, forensic evidence suggested that pHAHs such as dioxins and PCBs may have played a role in mass mortalities of seals and other marine mammals in the Baltic Sea that were directly attributed to viral infections (Ross et al., 1996). Captive harbor seals (*Phoca vitulina*) fed fish from the Baltic Sea displayed a number of immune system deficits relative to seals fed fish from uncontaminated Atlantic Ocean sites, including impaired natural killer (NK) cell activity, in vitro T-lymphocyte function, antigen-specific in vitro lymphocyte proliferative responses, and in vivo delayed-type hypersensitivity and antibody responses to ovalbumin. These effects were correlated with greater concentrations of TCDD equivalents in fish from the Baltic Sea. In a case–control study using long-term data from studies of marine mammal strandings in the United Kingdom, Hall et al. (2006) concluded that each 1 mg/kg increase in total PCB concentrations in blubber resulted in an average increase in mortality due to infectious disease of 2% in harbor porpoises (*Phocoena phocoena*). In a study of free-ranging logger-head sea turtles (*Caretta caretta*) collected in North Carolina, Keller et al. (2006) observed significant correlations between selected immune responses (lysozyme activity and lymphocyte proliferation) and concentrations of PCBs and chlorinated insecticides (DDE and chlordanes); these correlations were supported by in vitro studies with these chemicals in isolated turtle leukocytes. Similarly, Auffret et al. (2006) observed responses associated with immunosuppression in mussels (*Mytilus galloprovincialis*) that generally tracked chemical pollution gradients in the western Mediterranean Sea. Using available laboratory and field data, Loge et al. (2005) developed a model to assess the effects of environmental stressors, including chemicals, on disease susceptibility in migrant juvenile salmon in the Columbia River Basin, Washington. They concluded that chemical and nonchemical stressors contributed equally to disease-induced mortalities that were predicted to range from 3% to 18% of the population, depending on residence time.

Numerous laboratory studies have demonstrated chemical impacts on immune systems in animals of ecological relevance. These include effects of pesticides on amphibians (Albert et al., 2007), PCBs on channel catfish (*Ictalurus punctatus*) (Rice and Schlenk, 1995), heavy metals on rainbow trout (Sanchez-Dardon et al., 1999), PAHs on bivalves (Wootton et al., 2003), and flame retardants (polybrominated diphenyl ethers [PBDEs]) on American kestrels (*Falco sparverius*) (Fernie et al., 2005). Fairbrother et al. (2004) reviewed the literature concerning effects of chemicals on immune systems of birds, emphasizing potential impacts on wildlife species, and Carlson and Zelikoff (2008) performed a similar review for fish. The potential effects of chemicals on immune function and disease susceptibility in wildlife is clearly a very important subject in ecotoxicology and one likely to see significant advances in the near future as powerful genomic tools become more available for representative species.

Behavior The impacts of chemicals on animal behavior have received significant attention among ecotoxicologists. Relatively subtle effects on behaviors associated with, for example, mating and reproduction, foraging, predator–prey interactions, preference/avoidance of contaminated areas, and migration have potentially important ramifications for population dynamics. However, difficulties in objective quantifications of behaviors and laboratory to field extrapolations appear to have limited the application of this area to ecological risk assessments (ERAs), and by extension, perhaps to funds available for basic research. In some cases, however, biochemical mechanisms underlying behavioral effects have been elucidated that may assist with these issues and provide useful biomarkers for behavioral toxicants in field studies.

As noted by Rand (1985), chemicals causing behavioral effects in wildlife are often known from mammalian studies to be neurotoxicants. For example, in an early study, Grue et al. (1982) noted reduced nest attentiveness in female starlings dosed with the AChE-inhibiting organophosphate insecticide dicrotophos; this study took advantage of the relative ease of attracting wild starlings to artificial nest boxes that is advantageous for detailed studies, a phenomenon that has been employed in subsequent avian ecotoxicological studies (Parker and Goldstein, 2000). Grue et al. (1997) and Walker (2003) reviewed the behavioral effects in birds of these and other neurotoxic insecticides.

Behavioral effects of insecticides have also been observed in fish. For example, Scholz et al. (2000) reported adverse impacts of the organophosphate diazinon on olfactory-mediated behaviors such as the alarm response and homing in the Chinook salmon (*Oncorhynchus tshawytscha*), and Sandahl et al. (2005) observed similar thresholds for the effects of another organophosphate (chlorpyrifos) on swimming and feeding behaviors and on AChE inhibition in coho salmon (*O. kistich*). The effects of pollutants, including pesticides, on fish behavior were reviewed by Scott and Sloman (2004).

Mercury, particularly as methylmercury, comprises another potent neurotoxicant that has been shown to perturb behavior in wildlife. For example, golden shiners (*Notemigonus crysoleucas*) fed diets containing methylmercury that resulted in tissue mercury concentrations consistent with those observed in this species in northern U.S. lakes exhibited perturbed predator avoidance behaviors (Webber and Haines, 2003). In a study employing fish captured in the field and brought into the laboratory for behavioral analysis, Smith and Weis (1997) observed that killifish captured from a mercury-polluted tidal creek in New Jersey exhibited reduced feeding activity and greater mortality due to predation than killifish from an uncontaminated site. Using mercury concentrations in feathers as a marker for exposure, Heath and Frederick (2005) observed a negative correlation between mercury exposure and nesting activity among White Ibises (*Eudocimus albus*) in the Florida Everglades that may be related to behavioral effects. In studies with wild mink (*Mustela vison*) collected in Canada, Basu et al. (2005) observed significant correlations between mercury concentrations in brains and densities of neurochemical receptors (cholinergic and dopaminergic) associated with animal behavior. The effects of mercury on wildlife, including behavioral impacts, were reviewed by Wolfe et al. (1998).

Environmental contaminants not generally thought of as neurotoxicants have also been shown to perturb behavior. For example,

cadmium and copper have been shown to impact olfactory neurons and associated behaviors (preference/avoidance to chemicals, including pheromones) in several fish species (Saucier et al., 1991; Baker and Montgomery, 2001; Baldwin et al., 2003; Williams et al., 2016). Copper exposure in zebrafish also led to loss of neurons in the peripheral mechanosensory system ("lateral line"), which could lead to altered behaviors associated with schooling, predator avoidance, and rheotaxis (physical alignment of fish in a current) (Linbo et al., 2006). Carvalho and Tillitt (2004) reported loss of retinal ganglion cells in rainbow trout exposed to TCDD; these cells link the eye with the brain, and in this study deficits in visual acuity and prey capture rates were noted in TCDD-exposed fish. Clearly, numerous mechanisms of chemical toxicity can result in behavioral impacts, including direct toxicity to neurons, alterations in hormones that modulate behaviors, and impaired energy metabolism. In some cases, impaired behavior may comprise a sublethal impact with substantive ecological consequence (Scott and Sloman, 2004).

Cancer Beginning in the 1960s, numerous cases of cancer epizootics in wildlife that are associated with chemical pollution, particularly in specific fish populations, have been reported in North America and northern Europe (Harshbarger and Clark, 1990; Vethaak, 1992). As in humans, cancer in these animals occurs largely in relatively older age classes and therefore is oftentimes considered a disease unlikely to directly impact population dynamics or other ecological parameters. However, this may not always be the case, particularly in species that require many years to attain sexual maturity and/or have low reproductive rates. In any event, the occurrence of high incidences of cancer in wildlife populations raises serious concerns for environmental quality at those locations experiencing these epizootics. For these reasons, as well as for concerns for human health in these areas, and the advantages of alternative models such as fish for understanding chemical carcinogenesis, these epizootics have motivated substantial research in several areas relevant to human health and ecotoxicology.

In field studies of cancer outbreaks in aquatic and marine systems, typically only selected species exhibit elevated cancer rates associated with chemical contamination. A major contributor to this differential cancer susceptibility in wild fish populations is clearly lifestyle; benthic (bottom-dwelling) species such as brown bullhead (*Ameriurus nebulosus*) and white sucker (*Catostomus commersoni*) in freshwater systems, and English sole (*Parophrys vetulus*) and winter flounder (*P. americanus*) in marine systems generally exhibit the highest cancer rates in polluted systems (Baumann, 1998). The bulk of chemicals in these systems associated with cancer epizootics, such as PAHs, PCBs, and other halogenated compounds, reside in sediments; benthic fish live in contact with these sediments and prey in large measure on other benthic organisms. Thus, benthic fish experience greater exposures to carcinogens than other species in these systems. Inherent biological differences may also play a role in species susceptibilities to chemical carcinogenesis; for example, laboratory studies have revealed marked differences among fish species in their abilities to activate PAH procarcinogens to DNA adduct-forming metabolite as well as to detoxify them through phase II metabolism (Collier et al., 1992; Hasspieler et al., 1994; Ploch et al., 1998).

In their analysis of cancer epizootics in fish, Harshbarger and Clark (1990) concluded that cancers of the liver (hepatocellular neoplasms) had the strongest associations with chemical pollution, although cancers have been observed in other tissues in wild fish as well (Ostrander and Rotchell, 2005). PAHs appear to be the most implicated class of carcinogens associated with liver neoplasms in fish cancer epizootics. Studies implicating a key role for PAHs (and key PAH sources) include English sole in the Puget Sound (various urban and industrial sources; Malins et al., 1987), brown bullhead in the Black River, Ohio (a coal-coking facility; Baumann and Harshbarger, 1998), and in the Potomac River watershed near Washington, DC (various point and nonpoint discharges; Pinkney et al., 2001), and killifish in the Elizabeth River, Virginia (a wood treatment plant using creosote; Vogelbein et al., 1990). As stated earlier, PAHs appear to comprise a class of contaminant generally increasing in the environment. The metabolism of PAHs such as BaP to reactive metabolites that form DNA adducts that initiates carcinogenesis, or conversely to excretable conjugates, has been described earlier. It is noteworthy that the molecular and biochemical pathways underlying chemical carcinogenesis, such as PAH metabolism, DNA damage, and effects on oncogenes, are qualitatively similar between most fish and mammalian species examined.

This recognition of shared pathways has in part contributed to the use of various fish models for studying chemical carcinogenesis from a human health as well as from a broader environmental standpoint. An important historical event was the identification in Italy, France, and the United States during the 1950s and 1960s of aflatoxin as a potent liver carcinogen in farm-raised rainbow trout (Sinnhuber et al., 1977). Subsequently aflatoxin, a fungal toxin produced by *Aspergillus flavus* that is of concern where grains and nuts are stored in wet conditions, was found to be carcinogenic to mammals including humans. Thus, the rainbow trout observations led to the discovery of a new and important class of chemical carcinogens, and the recognition that fish can be very sensitive to chemical carcinogenesis. Since that time, other fish species have been employed for laboratory studies related to chemical carcinogenesis, particularly medaka (*O. latipes*) and platyfish/swordtails hybrids (*Xiphophorus* spp.); zebrafish also show promise as a laboratory model (Ostrander and Rotchell, 2005). Compared with rodent models, fish models have advantages of reduced costs for propagation and housing, briefer time intervals between exposures and the expression of tissue changes indicative of carcinogenesis, and greater feasibility of performing large-scale studies with many animals to quantify dose–response relationships.

It is noteworthy that the great bulk of reports of elevated cancer rates in free-living animals occur in fish, with few reports of potentially chemically related cancers to our knowledge in other vertebrates. California sea lions (*Zalophus californianus*) stranded along the central California coast were found to have elevated cancer rates (18%), and concentrations of DDT and PCBs were greater in animals with cancer versus those determined to die of other causes (Ylitalo et al., 2005). Martineau et al. (2002) reported elevated cancer rates (also 18%) in carcasses of beluga whales (*Delphinapterus leucas*) stranded along the shores of the St. Lawrence River estuary in Quebec, a system with elevated levels of PAHs. The authors noted that beluga was the only species of marine mammal among 20 inhabiting this system that exhibited elevated cancer rates, and that cancers are rare worldwide in marine mammals. It is likely that elevated exposures play an important role in the relatively high frequency of reports of cancers in benthic fish; relative inherent sensitivities among mammals, birds, reptiles, amphibians, and fish are unclear.

Population

A population is a collection of individuals of the same species that occupy the same space and within which genetic information can be exchanged. The study of populations is a central theme in ecological sciences and ecotoxicology is no exception. Assessment of toxicant effects on populations has been important in ecotoxicology

since its inception (Newman, 2001). A well-known, early instance is the sharp drop and then slow recovery of coastal populations of osprey (Spitzer et al., 1978) and brown pelican (Anderson et al., 1975) that occurred as a consequence of widespread DDT and DDE spraying and eventual banning. More recently, populations of Asian vultures scavenging on dead cattle dropped severely due to fatal poisoning by the veterinary drug diclofenac (Oaks et al., 2004; Balmford, 2013). Another was the enhanced, genetically based tolerance of pest insect populations chronically sprayed with pesticides (Mallet, 1989). Industrial melanism, the premier example in biology textbooks of natural selection in populations, is another example of population ecotoxicology. Populations of the peppered moth (*Biston betularia*) in industrial regions shifted from a predominance of light colored to melanic individuals as surfaces became progressively darkened due to soot deposition. Predation by visual hunters, especially birds, had exerted strong selective pressure on a single gene locus coding for coloration (Grant, 2004; Van't Hof et al., 2011).

Population ecotoxicology covers a wide range of topics with core research themes being (1) epidemiology of chemical-related disease, (2) effects on general population qualities including demographics and persistence, and (3) population genetics.

The level of belief warranted for possible contaminant-related effects in nonhuman populations is assessed by applying routine epidemiological methods. Many methods described in epidemiology textbooks (Anders, 1993; Woodward, 2005) are applied to nonhuman populations although with a slightly different balance because much more experimental exposure data are potentially available for nonhuman populations than for human populations. Rules of thumb for gauging the level of belief warranted by evidence that emerged from human epidemiology are also applied in population ecotoxicology. Hill's nine aspects of human disease association (Hill, 1965; see Chap. 2, "Principles of Toxicology") might be used directly or after minor modification. As an example, Fox (1991) (Table 30-1) modified such rules of thumb to accommodate slight differences in the subject matter and approaches in population ecotoxicology. Conventional epidemiological descriptors and models are also applied. For example, Horness et al. (1998) quantified prevalence and relative risks for neoplastic liver lesions in English sole from areas with different sediment concentrations of PAHs. Logistic regression models were also used to identify relationships between these lesions and chemical and biological risk factors (Myers et al., 1994).

Defining and predicting alterations in population size, dynamics, and demographic composition due to toxicant exposure have always been central in ecotoxicology and have become increasingly so in the last 20 years as regulatory agencies such as the U.S. Environmental Protection Agency clearly reinforced their long-standing commitment to understanding chemical exposure effects on natural population viability.

> *Protecting populations is an explicitly stated goal of several Congressional and Agency mandates and regulations. Thus it is important that ecological risk assessment guidelines focus upon protection and management at the population, community, and ecosystem levels . . . .*
>
> Environmental Protection Agency (1991)

Ecological theory and research (e.g., Forbes and Calow, 1999) also indicate that metrics of effect to individuals are not especially reliable metrics of toxicant exposure effects to populations.

Models of exposed population dynamics suggest that reductions on population densities are not the only important changes brought about by chemical exposure. Some species populations fluctuate within a range of densities. These fluctuations are characteristic of the species strategy for maintaining itself in various types of habitats and toxicant exposure could potentially change this range (Simkiss et al., 1993). Combined with decreases in population densities driven by external forces such as weather events, these toxicant-induced modifications of the average population densities and dynamics can increase the risk of a population's density falling so low that local extinction occurs (Newman, 1995).

Demographic qualities can change with toxicant exposure in ways that influence the risk of local population extinction. Toxicants can change a species population's vital rates, that is, age- and sex-dependent death, birth, maturation, and migration rates, in complex ways. These changes in combination determine the population density and distribution of individuals among ages and sexes during exposure. The population's ability to resist external forces that reduce its size is determined by these demographic features (Gard,

Table 30-1

A Summary of One Popular Set of Rules of Thumb (Data from Fox [1991]) for Assessing Plausibility of a Causal Association in an Ecological Epidemiology

RULE	DESCRIPTION
1. Strength of association	How strong the association is between the possible cause and the effect, for example, a very large relative risk
2. Consistency of association	How consistently is there an association between the possible cause and the effect, for example, consistent among several studies with different circumstances
3. Predictive performance	How good is the prediction of effect made from the presence/level of the possible cause
4. Monotonic trend	How consistent is the association between possible cause and effect to a monotonic trend (i.e., either a consistent increase or decrease in effect level/prevalence with an increase in exposure)
5. Inconsistent temporal sequence	The effect, or elevated level of effect, occurs before exposure to the hypothesized cause
6. Factual implausibility	The hypothesized association is implausible given existing knowledge
7. Inconsistency with replication	Very poor reproducibility of association during repeated field assessments encompassing different circumstances or repeated formal laboratory testing

NOTE : According to Fox (1991), the first four rules are most useful in supporting a causal hypothesis if found to be true (i.e., very strong, consistent, predictive, or monotonic association). The others are most useful for lessening belief in the causal hypothesis if true.

1992; Sherratt et al., 1999; Kammenga and Laskowski, 2000; Aubone, 2004). Consequently, considerable research effort is being spent on demographic methods for predicting exposed population changes and risks of extinction.

Demography explores vital rates of populations composed of individuals that differ in age and sex. Individuals in field populations can also differ in their spatial distribution and this influences the impact of toxicants (Newman, 2001). Individuals of the same species often are grouped into subpopulations within a habitat and all of these subpopulations together comprise a metapopulation (Fig. 30-9). Subpopulations in the metapopulation have different levels of exchange and different vital rates that depend on the nature of their habitat. Spatial distances and obstacles or corridors for migration influence migration among patches; habitat quality determines vital rates. An inferior habitat, such as a grossly contaminated one, can act as a sink into which individuals migrate from nearby superior (source) habitat. Migration can rescue a subpopulation or reduce its risk of local extinction. An individual migrating from the contaminated habitat to an uncontaminated one can express an adverse effect despite its present distance from the contamination, that is, the action-at-distance hypothesis of ecotoxicology (Spromberg et al., 1998). The viability of the metapopulation can also be as strongly influenced by maintaining important migration corridors among subpopulations and protecting high-quality habitats (i.e., keystone habitats) as by the general level of contamination within the metapopulation's habitat (Mauer and Holt, 1996; O'Connor, 1996; Spromberg et al., 1998; Newman, 2001).

The genetics of exposed populations is studied to understand changes in tolerance to toxicants and to document toxicant influence on field populations. The capacity of some populations to become more tolerant of toxicants via selection is well documented. A few examples include increased tolerance of pine mice to endrin (Webb and Horsfall, 1967) and rats to warfarin (Partridge, 1979) after years of application of these chemicals for rodent control. More recently, Ownby et al. (2002) documented enhanced tolerance in populations of an estuarine fish chronically exposed to PAHs. Although genetically based increases in tolerance are well documented in wild populations, many exposed populations probably find themselves in situations in which they cannot adapt adequately because their genetic resources, nature of the toxicant, or spatial/temporal context within which the enhanced tolerance must evolve are inadequate. Also, in an ecotoxicological sense, all cases of increased tolerance do not fit the conventional context manifested in the examples just given. Some do not involve suborganismal changes to biochemical or anatomical features resulting in enhanced tolerance. For example, industrial melanism increases a peppered moth's fitness in the presence of soot by reducing its likelihood of being taken by a predator. The dominant light form of this moth has a lowered fitness in the presence of dark soot, and genetically based changes in color increase its fitness. The selective mechanism here is ecological, that is, fitness relative to avoiding visual predators.

Genetic qualities are also used to infer past toxicant influence in an exposed population. For example, Mulvey et al. (2002, 2003) showed distinct genetic qualities in estuarine fish populations exposed to high concentrations of PAHs. Another piece of evidence demonstrating past toxicant influence on populations can be a change in genetic diversity. A drop in genetic diversity in populations is thought to be an adverse effect because genetic diversity is required in populations to evolutionarily adapt to environmental changes. Toxicants can influence genetic diversity by purely stochastic means. Genes can be lost in the population if the population is so drastically reduced in size that the chance of a rare gene being lost between generations becomes very high. Also, the average rate at which the frequency of a rare gene decreases through time due to genetic drift increases as the effective population size decreases. The effective population is the number of individuals contributing genes to the next generation so toxicant-related changes in demographic qualities can also accelerate genetic drift.

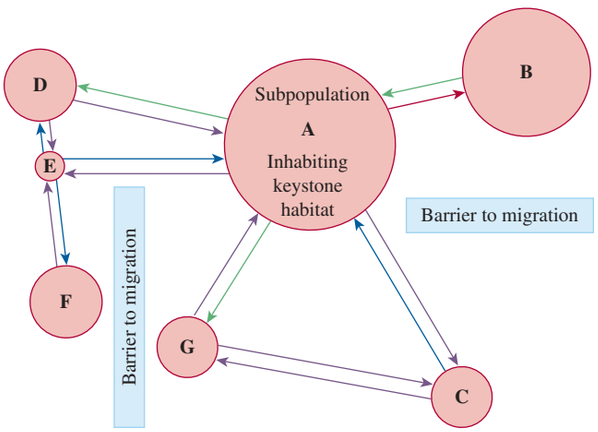

Figure 30-9. *Metapopulations are composed of subpopulations that differ in their vital rates and tendency to exchange individuals.* In this illustration, subpopulation A occupies a keystone habitat. The loss of subpopulation A would devastate the metapopulation. Also, loss of the migration corridor between subpopulations A, B, and D would devastate the metapopulation. In contrast, the loss of subpopulation F would not influence the metapopulation to the same degree.

Community

An ecological community is an interacting assemblage of species populations occupying a defined habitat at a particular time. Populations in a community interact in many ways and, because these many interactions are complex, a community has properties that are not predictable from those of its component populations. Some species have such a crucial role (keystone species) or numerical dominance (dominants) that they are essential to maintaining community structure.[2] Other species contribute to the nature of the community in more subtle ways. Community qualities like the diversity of species are influenced by contaminants. These effects are of concern because a decrease in diversity can diminish a community's ability to retain vital nutrients or resist stress such as that associated with drought (Tilman et al., 2006).

Ambiguity exists about the importance of all the species in a community relative to maintaining overall structure and balancing essential functions such as nutrient cycling, primary productivity, community respiration, and detritus processing. The redundant species hypothesis suggests that species function redundantly: if a species were lost, another with a similar function would increase in numbers to compensate. Only certain critical species such as dominant or keystone species are essential to the community. The rivet popper hypothesis suggests otherwise. Each species in a community is similar to one of the many rivets holding an airplane

[2]In its most rudimentary context, community structure refers to the number of species present and the number of individuals present in each of these species. It can also refer to the distribution of species among different functional groups such as decomposers, detritivores, primary producers, primary consumers such as herbivores, and secondary consumers such as carnivores that consume herbivores.

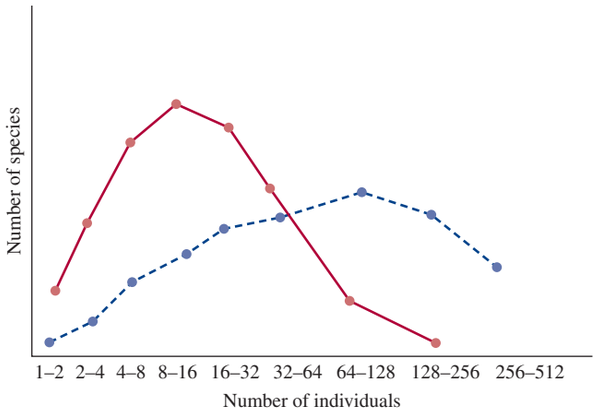

Figure 30-10. *Log-normal model of species abundance for an unexposed (solid line and red points) and toxicant-exposed community (dashed line and blue points).* Communities have distinct structure as shown here with the typical log-normal species abundance model. As first described by data from Patrick (1973), ecotoxicants tend to lower the mode of the species abundance curve and stretch the right tail outward. Ecotoxicants result in fewer intermediate abundance species and more extremely abundant species.

fuselage together. Each lost rivet contributes to a gradual weakening of the fuselage that will lead eventually to a failure in function. By analogy, each species disappearance diminishes a community's functioning.

Ecotoxicologists remain divided about which hypothesis is most relevant. Pratt and Cairns (1996) argue from evidence and a conservative stance that the rivet popper is the most appropriate. Ecotoxicologists and regulators who pragmatically set standards based on concentrations that will not harm more than a specified percentage of species in a community (e.g., Stephan et al., 1985) assume that the redundant species hypothesis is more pertinent. Although the redundant species hypothesis is assumed to be correct in many ERAs (Solomon and Sibley, 2002), recent theory (Loreau, 2004), modeling (Naeem et al., 1994), and experimental evidence (Tilman, 1996; Tilman et al., 1996; Salminen et al., 2001) seem to support the rivet popper hypothesis. Biodiversity tends to foster community stability and function.

Communities take on characteristic structures as predicted by the Law of Frequencies: the number of individual organisms in a community is related by some function to the number of species in the community (Fig. 30-10). Ecotoxicants[3] can alter the resulting community structure in predictable ways by either directly impacting the fitness of individuals in populations that make up the community or altering population interactions. Community ecotoxicologists spend considerable effort trying to understand and predict ecotoxicant influences on community structure and essential functions.

Direct effects involve removal of a population or metapopulation from the community by reducing the Darwinian fitness of individuals enough that the population falls below some critical minimum size. Indirect effects can involve interference with interspecies competition, predator–prey interactions, host–disease/parasite interactions, or symbiotic relationships such as pollination. The simplest competition model (Lotka–Volterra model) can be used to illustrate the potential for both direct and indirect effects on populations:

$$\frac{dN_1}{dt} = r_1 N_1 \left[1 - \frac{N_1}{K_1} - \frac{\alpha_{12} N_2}{K_1}\right],$$

$$\frac{dN_2}{dt} = r_2 N_2 \left[1 - \frac{N_2}{K_2} - \frac{\alpha_{21} N_1}{K_2}\right],$$

where N_1 and N_2 are the population sizes of competitors 1 and 2, K_1 and K_2 the carrying capacities of the environment for competitors 1 and 2, r_1 and r_2 the intrinsic rate of population increase (i.e., birth rate − death rate) for competitors 1 and 2, α_{12} the competition coefficient quantifying the impact of the presence of competitor 2 on competitor 1, and α_{21} the impact of competitor 1 on competitor 2. Not only can exposure directly impact birth rates, death rates, and carrying capacity of each species, but it can also influence species persistence by shifting competition coefficients in favor of another species. Mathematically, it can be shown that the two competitors depicted in the Lotka–Volterra model can coexist only if two conditions are met, $K_1 < K_2/\alpha_{21}$ and $K_2 < K_1/\alpha_{12}$. So, a population can be lost from a community as readily by changing its competitive interactions as by directly changing its death and reproductive rates. Similar statements can be made about changes in predator–prey, host–disease, and various symbiotic interactions. As an example, concern expressed recently about unintended pesticide reductions in the number and diversity of pollinators in European farmlands (Newman et al., 2006) could be partially responsible for the recently reported decline in insect-pollinated plant species in Britain and the Netherlands (Biesmeijer et al., 2006). In another instance, reduced habitat cover and insect densities in European farmlands has had a significant impact on gray partridge populations (Rands, 1985; Chiverton, 1999). As another and final example involving predator–prey interactions, amphibian tadpole exposure to endosulfan, a chlorinated insecticide, increases the risk of predation by dragonfly larvae (Broomhall, 2002). None of these examples involves a direct poisoning by a toxicant, but instead, involves an ecotoxicant that adversely modifies species interactions.

Structural changes to communities can be detected in species abundance plots (see Fig. 30-10) or shifts in conventional community metrics calculated from community samples taken in the proximity of contaminated sites. Common metrics for species richness, diversity, and evenness are used to express changes in biodiversity. Richness is simply the number of species in the sampled community, or if a relative number of species in different communities is all that is needed, the number of species expected in a specified sample size such as a rarefaction richness estimate of 12 species in a sample of 100 individuals from a community. Evenness is a measure of how equitably the individuals in a community are spread among the species. Finally, diversity (heterogeneity) indices combine the elements of richness and evenness into one number. Generally, but not always, ecotoxicants lower species richness, evenness, and overall diversity. The regulatory premise is that these changes reflect a diminished community.

Structural and functional qualities in communities have been combined to generate multimetric indices such as the Index of Biotic Integrity (IBI) (Karr, 1991). Ecological insight is used to select and then numerically combine community qualities such as species richness, health of individual animals in a sample, and the

[3]The conventional context for the term "toxicant" becomes difficult to retain without some qualification when dealing with populations, communities, and other higher level entities because an agent does not necessarily have to directly interact with the individual in order to harm it. As an example, an agent might eliminate a prey species, leading indirectly to the disappearance of a predator species that depended on it for sustenance. The "toxicant" did not poison the predator, yet it caused its demise nonetheless. A distinct term "ecotoxicant" is often applied to avoid confusion in such cases.

number of individuals in a sample belonging to a particular functional group, such as number of piscivorous fish. The IBI score for a study site is calculated and compared with that expected for an unimpacted site in order to estimate its biological integrity.

Another central theme in community ecotoxicology is toxicant transfer during trophic interactions. Toxicant concentrations can decrease (biodiminution), remain constant, or increase (biomagnification) with each trophic transfer within a food web. Persistent organic pollutants (POPs) with moderately high lipid solubility ($5 < \log K_{ow} < 7$ or 8; Thomann, 1989; Connell, 1990) and minimal metabolic breakdown in an organism can biomagnify to harmful concentrations. Metals that biomagnify are mercury and the alkali metals, cesium and rubidium. Zinc, an essential metal that is actively regulated in individuals, can exhibit biomagnification or biominification depending on whether ambient levels are below or above those required by the organism to function properly. Biominification is facilitated in a marine food web after sequestration in intracellular phosphate granules of molluscan prey species (Nott and Nicolaidou, 1993) and biomagnification by active regulation in zinc-deficient terrestrial communities (Beyer, 1986). The biomagnification of mercury is enhanced by its microbial transformation to methylmercury. Biomagnification of the potassium analogs, cesium and rubidium, is facilitated by the differences in their influxes and effluxes that favor retention in organisms (Rowan and Rasmussen, 1994; Campbell et al., 2005).

Quantifying the trophic position of a species in a community is essential to modeling biomagnification. Most trophic systems are not simple "food chains." Most individuals in a community can feed on different species depending on their life stage, seasons, and relative abundances of prey species. These trophic interactions are best described as occurring in a trophic web, not a trophic chain.

Conveniently, trophic position of an individual within a complex food web can be quantified with nitrogen isotopes. Generally, ^{14}N passes through biochemical pathways faster than ^{15}N, resulting in excretion of waste with a slightly higher $^{14}N/^{15}N$ ratio than in ingested food. The relative amounts of ^{14}N and ^{15}N will be slightly biased toward the heavy isotope in tissues of a species relative to those of its food source(s). This discrimination between the heavy and light N isotopes continues through food webs, allowing the trophic position of each participating species to be estimated. The metric used for this purpose, the $\delta^{15}N$, expresses the quotient of these two isotopes in the biological tissue of interest relative to the quotient expected in the atmosphere:

$$\delta^{15}\mathrm{N}=1000\left[\frac{\left[{}^{15}\mathrm{N}_{\mathrm{Tissue}}\right]/\left[{}^{14}\mathrm{N}_{\mathrm{Tissue}}\right]}{\left[{}^{15}\mathrm{N}_{\mathrm{Air}}\right]/\left[{}^{14}\mathrm{N}_{\mathrm{Air}}\right]}-1\right].$$

The change in toxicant concentrations within food webs is modeled using the $\delta^{15}N$, which quantifies trophic position of the species from which the tissue sample was taken (Fig. 30-11). Linear and exponential models are commonly applied:

$$\text{Concentration} = a + b\delta^{15}\mathrm{N},$$

$$\text{Concentration} = 10^{a+b\delta^{15}\mathrm{N}} \text{ or } e^{a+b\delta^{15}\mathrm{N}}.$$

Ecosystem to Biosphere

Ecosystems are the functional unit of ecology composed of the ecological community and its abiotic habitat. Systems ecologists try to describe and predict energy and mass cycling in and flow from ecosystems. The ecotoxicologist's interest in ecosystems includes understanding how toxicants diminish an ecosystem's capacity to perform essential functions and to understand toxicant movement enough to assess exposure within different ecosystem components.

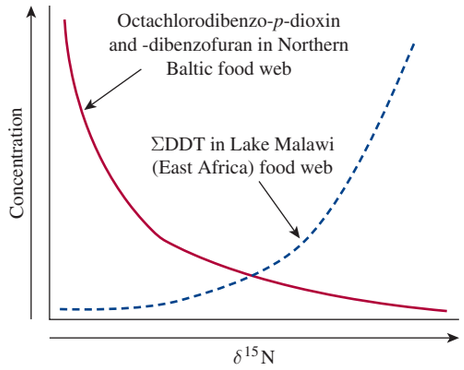

Figure 30-11. *Modeling ecotoxicant concentration versus trophic position as quantified with $\delta^{15}N$.* Power models were applied to octachlorodibenzo-*p*-dioxin/dibenzofuran concentrations in a North Baltic food chain (data from Broman et al. [1992]) and the ΣDDT (sum of primarily the *p,p′* isomers of DDD, DDE, and DDT) in food webs of Lake Malawi (data from Kidd et al. [2001]). Biomagnification and minification were evident for ΣDDT and octachlorodibenzo-*p*-dioxin/dibenzofuran, respectively.

Many of the effects described above for exposed communities are relevant here. As an example, Allred and Giesy (1988) demonstrated that elevating cadmium concentrations in an artificial stream reduced decomposition rates of dead leaves. Odum (1985) suggested that other changes to be expected with increased ecosystem stress include an increased loss of nutrients, increased community respiration, and an imbalance of primary production and respiration.

Studies of toxicant movement within ecological systems are conducted at extremely different scales (Fig. 30-12). Conventional ecosystem studies involve descriptions of contaminant concentrations and movements in easily defined ecosystems such as lakes, forests, or fields. Some toxicants, especially those subject to wide dispersal by air or water, cannot be completely understood in this framework so a landscape scale might be chosen instead. As an example, acid precipitation might be examined in the context of an entire watershed, mountain range, or even a continental region. As another example (see Fig. 30-12, top right panel), the fish tissue concentrations of a PCB congener and other persistent organic pollutants (POPs) were measured in high mountain region lakes of Europe. Concentrations for several were related to the altitude at which a lake sat. Called mountain cold-trapping, the relationship between altitude and concentrations of the various POPs is determined by atmospheric movement of the POPs and each POP's propensity to either volatilize or condense at a particular altitude-dependent temperature regime (Blais et al, 2003; Wania and Westgate, 2008; Westgate and Wania, 2013). Still other ecotoxicants require a global context to fully understand their movements and accumulation. As an example, hexachlorobenzene concentration in tree bark collected worldwide showed a clear latitudinal gradient. Its global distribution and those of other sampled POPs were a function of their relative volatilities. The volatile hexachlorobenzene moved more readily toward the poles than less volatile POPs such as endosulfan and DDT. The differential global movement of POPs due to differences in volatility and partitioning behavior was called global distillation and fractionation (Simonich and Hites, 1995; Wania and Mackay, 1996). The balance between a POP's tendency to condense or evaporate at different latitude-related temperatures

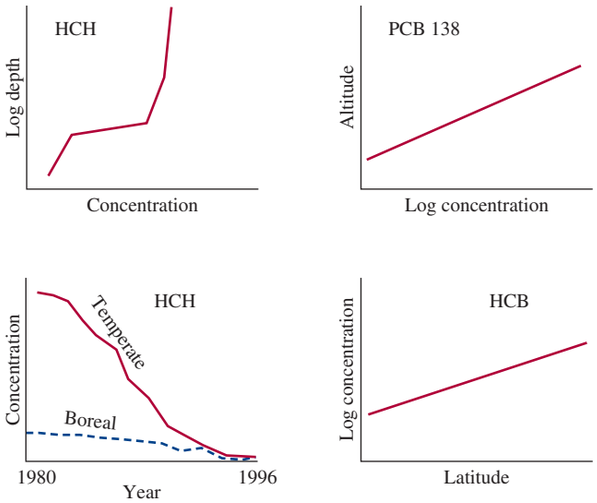

Figure 30-12. *Examples of scales relevant to assessments of ecotoxicant distributions.* The smallest scale example (*top left*) is the vertical distribution of α-hexachlorocyclohexane (HCH) in a stratified region of the Sea of Japan (data from Chernyak et al. [1995]). A slightly larger scale is reflected in the change in the polychlorinated biphenyl congener PCB 138 in tissues of fish inhabiting European lakes at different altitudes. (*Top right panel*, data from Fernandez and Grimait [2003].) Representing a subcontinental scale study is the temporal change in atmospheric α-hexachlorocyclohexane concentrations in temperate and boreal regions of central northern Europe. (*Bottom left panel*, data from Wania et al. [1999].) The largest scale encompasses the entire Earth, showing the influence of latitude on hexachlorobenzene (HCB) concentration in tree bark. (*Bottom right panel*, data from Simonich and Hites [1995].)

determines its atmospheric mobility and its ultimate global deposition pattern (Wania and Mackay, 1996).

APPROACHES

Many approaches have been developed to detect and quantify contaminant effects. These span the levels of organization described above. Approaches widely applied in ecotoxicology include standardized toxicity tests deigned to meet regulatory needs and biomarkers for organismal exposure and effects. For higher levels, a range of ecological methods exists for population, community, and ecosystem effects. Other techniques such as geographic information system (GIS) analysis of impacts allow the ecotoxicologist to assess impact encompassing large spatial scales. These approaches and examples of their applications are described in this section.

Toxicity Tests

Toxicity testing encompassing representative animals and plants at different levels of organization offers a practical approach to characterize chemical effects on biological systems. While it is widely known that toxicity tests cannot mimic the complex interactions and variable conditions of natural ecosystems, they address the potential direct effects of toxic substances on individual ecosystem components in a controlled and reproducible manner. A number of testing guidelines have been put forth by regulatory bodies and organizations worldwide to meet requirements for chemical registration or authorization (OECD, 1981; MAFF, 1985; U.S. EPA OPPTS, 1996a; ASTM International, 2006), with numerous subsequent revisions. Different sets of guidelines apply to specific countries, regions, or products, and can differ significantly in their requirements. The harmonized guidelines put forth by the U.S. EPA Office of Prevention, Pesticides and Toxic Substances (U.S. EPA OPPTS, 1996a) were created in an attempt to lessen variations in testing requirements, and bring together requirements from the Organisation for Economic Co-Operation and Development (OECD), the U.S. EPA Office of Pollution Prevention and Toxics (OPPT), and the Office of Pesticide Programs (OPP).

Ecotoxicology tests feature a wide variety of aquatic (including algae, invertebrates, tadpoles, bivalves, shrimp, fish), avian (quail, duck), and terrestrial species (soil microorganisms, crops, honey bees, earthworms, wild mammals). Species are selected based not only on their traditional use as laboratory animals, but also on ecological relevance, which further complicates global harmonization of ecological testing. In addition, special considerations apply to testing of aquatic species due to the unmistakable differences in the way aquatic species are exposed to toxicants (U.S. EPA OPPTS, 1996b). For instance, water quality monitoring and investigation of the solubility and stability of the test substance under the conditions of testing, along with determination of nominal versus measured concentrations, are common practices in aquatic toxicology. Testing can be conducted in aqueous systems without renewal of the test substance (static), renewal at predetermined time intervals (static renewal), or continuous flow of test substance through the test compartment (flow-through).

Acute toxicity testing consists of single species exposed to various concentrations of the test substance. The most common endpoint in acute tests is death, although abnormal behavioral or other gross observations are commonly noted, and nonlethal endpoints occasionally apply (e.g., immobilization for daphnids and shell deposition in oysters). Variations in acute toxicity studies comprise testing of different species (such as fresh vs. saltwater fish, bobwhite quail vs. mallard duck), life stages (embryo, larva, juvenile), environmental influences (e.g., presence of organic material), or sediment exposures. Data from various test concentrations and time points are used to derive concentration–response curves and predicted values such as the LC_{50} (median lethal concentration), EC_{50} (median effective concentration), or IC_{50} (median inhibition concentration). The LC_{50} represents the concentration of test substance killing 50% of the tested animals and EC_{50} the concentration of test substance affecting 50% of the test population during a specified period of time, such as growth; the IC_{50} is the concentration causing a 50% reduction in a nonquantal measurement (such as movement) for the test population. More quantitative values derived from acute tests are the lowest observed effect concentration (LOEC), that is, the lowest concentration where an effect is observed, and the no observed effect concentration (NOEC), the highest concentration resulting in no adverse effects.

Short-term laboratory studies conducted with single species are useful for rapid screening, provide information on thresholds for effects and selective and comparative toxicity, and can be used as range finders to guide subsequent, often more involved studies. Long-term and reproductive studies evaluate the effects of substances on organisms over extended periods of time and/or sequential generations (chronic toxicity, life cycle, reproduction). Endpoints include both quantal (such as mortality) and nonquantal (reproduction, growth) measurements, and can be used to derive additional values, other than previously mentioned in acute toxicity tests. These include the calculation of threshold values, such as the maximum acceptable toxicant concentration (MATC), which is the maximum chemical concentration not toxic to test organisms, and the BCF, which estimates the tissue concentration in relation to the average exposure concentration in the test medium (e.g., water).

Unique to ecotoxicology are the more elaborate microcosm, mesocosm, and field studies. Microcosms are representative aquatic

or terrestrial ecosystems created under laboratory conditions that include a number of relevant species (such as protozoa, plankton, algae, plants, and invertebrates). Simulated field studies or mesocosms can be created in the laboratory or in the field (e.g., artificial streams, ponds) or consist of enclosures of existing habitats, containing representative soil, water, and biota. Lastly, full-scale field studies (aquatic organisms, terrestrial wildlife, pollinators) evaluate the effects of a substance on wildlife under real-life scenarios of actual use conditions of a product (e.g., pesticide field usage rate), and thus, are more complicated, subject to considerable variability, and require extensive background knowledge of the local population and community dynamics.

As a final point, plant studies are a significant component of ecological toxicity testing, particularly for pesticide registration, and involve tiered testing of both target area and nontarget terrestrial and aquatic plants. Target area plants are those that are present in the area where the substance will be routinely used (application area), but which are not anticipated to be affected. Nontarget plants are those outside of the intended use area. Endpoints of phytotoxicity include seedling emergence and growth, vegetative vigor, and rhizobium–legume toxicity, among others, and central to the toxicity testing with plants are the substrate and environmental conditions, which greatly influence plant health.

Biomarkers

The Nuclear Regulatory Commission (NRC, 1989) defined a biomarker as "a xenobiotically induced variation in cellular or biochemical components or processes, structures, or function that is measurable in a biological system or sample." In the context of ecotoxicology, this definition has been modified slightly to refer to biochemical, physiological, or histological indicators of either exposure to or effects of xenobiotic chemicals at the suborganismal or organismal level (Huggett et al., 1992). The term is most often employed to refer to molecular, physiological, and organismal responses to contaminant exposure that can be quantified in organisms inhabiting or captured from natural systems. A response that is limited to laboratory studies falls outside the generally held concept of a biomarker.

By definition, biomarkers do not directly provide information concerning impacts on the higher levels of organization that ecotoxicology ultimately endeavors to discern. Nevertheless, biomarkers often provide important ancillary tools for discerning contaminant exposures and potential impacts of ecological importance. The development and use of biomarkers in ecotoxicology is motivated by several factors. These include the inherent instabilities of many contaminants (such as PAHs and many pesticides) that make measures of exposure by direct tissue residue analysis difficult, the relative biological sensitivity of many biomarkers, the chemical specificity of some biomarkers that serve to contribute to the identification of chemicals having biological effects, and relatedly, the linkage of some biomarkers to underlying mechanisms of toxic action. Additionally, while populations and higher level effects are of greatest concern, variables associated with these levels are oftentimes relatively insensitive to chemicals and other stressors, take long periods of time to become manifest, and/or have difficult or imprecise methods for their analysis. Thus, biomarkers can provide sensitive early warning signals of incipient ecological damage (van der Oost et al., 2003), in essence an ecological counterpart to the "canary in the coal mine" approach for preventing harm to coal miners. However, biomarkers do not provide adequate stand-alone data in the context of ecological assessments of contaminant effects. At this time and for the foreseeable future, such assessments generally involve a "weight of evidence approach," coalescing information obtained from chemical analyses, toxicity tests, biomarkers, and ecological indicators (sometimes referred to as "bioindicators"; see Adams et al., 1989). The AOP concept described earlier provides a more formal approach for linking data across levels of organization.

In earlier discussions, a number of contaminant effects at the organismal level and below that have been utilized as biomarkers were pointed out. These include effects with some degree of chemical specificity and relationship to a mechanism of toxicity (AChE inhibition by organophosphate and carbamate insecticides, ALAD inhibition by lead, DNA–PAH adducts), responses associated with exposures to chemicals acting through a common receptor (Vtg induction by ER agonists such as natural and synthetic estrogens, some surfactants, plasticizers, and pesticides; CYP1A induction by AhR agonists such as certain pHAHs and PAHs), and broader indices of cellular stress or tissue damage, such as markers of oxidative stress, lysosomal membrane stability, and histopathology. Numerous other identified mechanisms of toxicity, indices of chemical exposure, and cellular and organismal impacts have been exploited, with varying degree of success, as biomarkers (see reviews by Huggett et al., 1992; Peakall, 1992; Adams, 2000; van der Oost et al., 2003; Connon et al., 2012). In addition, new biomarkers continue to emerge; for example, considerable attention is now being given to biomarkers arising from advances in genomic technologies, discussed in Section "Genomics and Ecotoxicogenomics."

In considering the development or use of a selected biomarker, several issues and limitations warrant consideration. For example, while sensitivity is overall an advantage of many biomarkers, it can sometimes raise important questions surrounding interpretation. For example, some molecular and biochemical measures are very sensitive to chemical exposures, but their ramifications for organismal health are unclear. For this reason, some distinguish between biomarkers of exposure and biomarkers of effect (see reviews cited earlier in this chapter). However, this distinction is often blurred and is subject to an individual's view of what constitutes a significant biological chemical effect; some may say the formation of DNA adduct is a significant effect, while others will argue that such adducts only indicate exposure and will require tumor formation to occur before denoting an effect. Certainly most would agree that the tumor is a clearer marker of effect than the adducts, and something more readily grasped by policy makers and the general public. On the other hand, tumors are far less sensitive as a biomarker, a key *raison d'etre*; such trade-offs merit consideration.

Chemical specificity among biomarkers is also highly variable and is imbued with trade-offs. In some cases, such as where one has a good idea of the nature of contaminants likely to occur at a site, chemical-specific biomarkers will likely be most informative. In contrast, if such information is lacking, or mixtures encompassing several classes of chemicals likely occur, nonspecific markers may be superior. In most cases, suites of biomarkers prove to be most effective, although the larger the suite, the more time-intensive and costly the analysis will be—another trade-off. Another important consideration is the influence on the biomarker of variables other than those of concern (chemical contamination). Effects of environmental variables such as temperature, time of day or year, salinity and dissolved oxygen, and physiological variables such as sex, age, reproductive status, and nutritional status need to be controlled for or at least understood and accounted for. Many biomarkers are invasive and require sacrifice of the organism in order to obtain needed tissues. This can be problematic, particularly in cases involving rare species or charismatic species such as marine mammals. In such cases, and in others where feasible, the use of noninvasive biomarkers is either preferred or required (Fossi and Marsili,

1997). In summary, biomarkers can provide powerful tools as early warning signals of ecological damage to assist in assessments of environmental contamination and in determining the effectiveness of various environmental management decisions such as cleanups. However, careful case-specific thought must go into the selection of biomarkers, and they rarely are efficacious alone.

Population

Population-level effects are quantified with both field and laboratory approaches (see Newman, 2001, 2013). Population density is the most common of field population qualities measured in surveys of contaminated habitats. Quadrat, mark-recapture, and removal-based methods are applied. The density of individuals in a series of random quadrats within the area of interest is used to estimate densities in quadrat methods. The total population size can be estimated with knowledge of the total number of quadrats in the area of interest. In cases in which individuals are mobile and capable of avoiding being counted in a quadrat, a mark-recapture method might be applied instead. This involves marking a subset of individuals from the population, allowing them to randomly mix back into the population, and resampling the population. The number of marked and unmarked individuals taken, and the total number originally marked, can be used to estimate population size. Removal-based methods involve repeated sampling of the population without replacement, noting how the number collected per unit of effort declines through the sequence of samplings, and extrapolating this trend down to the point (total number caught previous to a sampling) at which no more individuals will be taken. This point is an estimate of the population size. Obviously, this approach is useful only if sampling decreases the catch noticeably between sampling episodes.

As noted earlier in discussions of metapopulations, the spatial distribution of individuals in a habitat is important to understand. Fortunately, well-established methods are available for this task. Methods vary depending on whether the sampling units are discrete or arbitrary. An arbitrary unit might be the number of razor clams per square meter of beach or number of a zooplankton species per cubic meter of water. A discrete sampling unit might be the number of mallard ducks per pond or squirrels per oak tree. Some methods associated with discrete sampling units attempt to fit the spatial pattern to a specific distribution. Methods for arbitrary sampling units include quadrat-based or distance-to-nearest-neighbor approaches as described by Krebs (1998).

Demographic surveys or experiments can be conducted for exposed populations. Some studies explore age-specific vital rates but others are designed to explore vital rates for different life ages such as nestling, fledgling, juvenile, and adult. Most result in data sets that can be analyzed profitably using either a simple life table or more involved matrix analysis. The matrix method allows one to describe the population state and also to understand the sensitivity of the population to effects occurring to vital rates for various ages or stages (Caswell, 2001). The value of such studies lies in the ability to integrate effects to several effects into a projection of population consequences. Demographic studies are becoming more common in ecotoxicology, especially with species amenable to laboratory manipulation (Jansen et al., 2001; Tanaka and Nakanishi, 2001; Chandler et al., 2004).

Conventional studies of increased tolerance after generations of exposure and molecular genetic surveys of exposed populations are the primary approaches by which genetic consequences are assessed. Increased tolerance is usually detected by subjecting individuals from the chronically exposed population and a naive population to toxicant challenge and formally testing for tolerance differences. A recent example is the study by Ownby et al. (2002) of enhanced tolerance for a PAH-exposed population of killifish from Elizabeth River (Virginia). Alternatively, a change associated with a tolerance mechanism might be examined in chronically exposed and naive populations. As an example, Meyer et al. (2003) found upregulated antioxidant defenses in the same populations of exposed Elizabeth River killifish studied by Ownby et al. (2002). Close examinations of population genetics associated with contaminated habitats are also used to infer consequences of multigenerational exposure. Continuing with the Elizabeth River killifish example, Mulvey et al. (2002, 2003) examined the genetic qualities of fish sampled within the Elizabeth River estuary using allozymes and mDNA. Clear evidence was found using both tools for the influence of contamination on the population genetics of killifish subpopulations within the estuary.

Community and Ecosystem

Most community and ecosystem effects studies by ecotoxicologists use modified methods developed in community and systems ecology (see Magurran [1988] for method descriptions). Recent books, for example, by Newman (2015) and Newman and Clements (2008) provide some details of ecotoxicological applications of these methods. Several general approaches are taken. The approach affording the most control and ability to replicate treatments involves laboratory microcosms. A microcosm is a simplified system that is thought to possess the community or ecosystem qualities of interest. The experimental control and reproducibility associated with microcosms come at the cost of losing ecological realism. Is the laboratory microcosm actually responding in a way that provides insight about how the actual community or ecosystem would respond? Microcosm studies are so common throughout the ecotoxicological literature that standard methods have been proposed for their execution (Taub, 1997).

As a microcosm example, Clarke (1999) established invertebrate communities in the laboratory to determine the influence of oil drilling muds on offshore benthic communities. Relative to the issue of community redundancy discussed in the section "Community," zinc-amended soil microcosms were used in another case by Salminen et al. (2001) and provided minimal evidence to support the current reliance on the redundant species theory by ecological risk assessors.

Gaining back some realism by giving up some degree of tractability, outdoor mesocosms are also applied to community and ecosystem ecotoxicology. Mesocosms are larger experimental systems, usually constructed outdoors that also attempt to simulate some aspect of an ecosystem such as community species composition. Often, terrestrial ecotoxicologists apply the term enclosure instead of mesocosm for such experimental units. Aquatic mesocosms can be artificial ponds such as those developed by Woin (1998), streams such as those used by Kreutzweiser et al. (2000), or river segments such as those used by Culp et al. (2000). Terrestrial mesocosms can be pens, enclosures, or large soil plots depending on the effects being quantified. An example of a terrestrial mesocosm study is that conducted by Korthals et al. (1996) of the effects of long-term copper exposure to soil nematode communities. The Canadian Experimental Lakes program is a unique series of whole-lake studies in which lakes are dosed with various agents and whole ecosystem fate and effects studied. Agents studied in this manner include radionuclides, acid rain, cadmium, climate warming, mercury, 17α-ethynlestradiol, excess nutrients, and PCBs (Blanchfield et al., 2009; Schindler, 2009).

Field studies are the third means of exploring effects at the community or ecosystem level. The high realism of associated

findings from field studies is balanced against the difficulty of achieving true replication and sufficient control of other factors influencing the system's response. Field studies can involve manipulations such as introducing toxicant into replicate water bodies; however, the majority of field studies involve biomonitoring of an existing, notionally impacted, community or ecosystem. This might involve close examination of species composition and comparison to that expected or measured in a similar, but uncontaminated, system. Most biomonitoring efforts focus on community structure instead of function because it is generally thought that changes in community structure will be seen before those in functions. As examples, metal effects on invertebrate and plant community structure were studied by Peeters et al. (2000) and Strandberg et al. (2006), respectively. Despite the tendency to study community structure, study of functions can provide valuable insights as in the case of Day (1993), who found changes in photosynthesis in periphytic algae in response to herbicide exposure. Because mesocosm and field studies involve data generation in the presence of many uncontrolled variables and poor replication or pseudoreplication, multivariate statistical techniques for recognizing patterns among locations or through time are commonly applied (e.g., Landis et al., 1997; Kedwards et al., 1999).

Landscape to Biosphere

The creation and eventual convergence of several key technologies facilitate ecotoxicological study at the landscape to biosphere vantages. These same technologies have also allowed the emergence of large context, environmental disciplines such as landscape[4] ecology (Forman and Godron, 1986), global ecology (Rambler et al., 1989), and global biogeochemistry (Butcher et al., 1992) that contribute concepts to large-scale ecotoxicology efforts.

Technologies for acquiring, processing, and analyzing large amounts of information have been essential. Archived and new imageries from satellites and high-altitude platforms are now integrated with off-the-shelf geographic information system (GIS) software with affordable computers. Much of this imagery is gathered with remote sensing technologies, that is, technologies that do not require physical contact with the feature being measured. However, arrays of sensors are rapidly coming together such as the network coastal observing systems that are quickly linking to form a readily accessible real-time data stream for all of our oceans. Remote sensing data from satellites or aircraft provide information for wide spatial areas and the rapidly emerging, ground- or water-based observing system networks have begun to produce extremely rich data streams. Such technologies facilitate ecotoxicological explorations at spatial scales that were impossible to consider only a few decades ago.

ECOLOGICAL RISK ASSESSMENT

Ecological risk assessment (ERA) applies ecotoxicological knowledge in support of environmental decision making. The ERA approach is an adaptation of human risk assessment methods, notably those articulated in the National Academy of Sciences paradigm (National Research Council, 1983). Adaptations are needed to accommodate differences in exposure pathways and the entities for which risk is to be estimated. Risk might be to an endangered or threatened species, or to a damaged natural resource for which remuneration might be required from a responsible party. In such cases, the ERA might estimate risk to individuals. Alternatively, as emphasized in the above EPA quote, the risk might be to a local species population or to the integrity of an ecological community. A widely dispersed ecotoxicant such as acid precipitation or widely used product such as the herbicide, atrazine, might require assessment of risk at a landscape or subcontinental scale. A recent example of such a risk assessment is that for atrazine, a herbicide used throughout North America (Solomon et al., 1996). Ecotoxicants requiring a global ERA might include greenhouse gases contributing to global warming, hydrofluorocarbons depleting the ozone layer, and persistent organic pollutants (POPs) that accumulate to harmful concentrations in polar regions far from their point of release at highly industrialized latitudes.

Adaptations are based on the context of an ERA. Some ERAs address existing situations. Considerable field information might be available for such a retroactive ERA and epidemiological methods might be applied advantageously. In contrast, predictive ERAs assess possible risk associated with a future or proposed toxicant exposure. In this case, the ERA might rely more heavily on exposure modeling and laboratory-derived effects data. A special case of predictive risk assessment is a life cycle assessment in which "cradle-to-grave" predictions are done for a product that includes all aspects of its raw material extraction, manufacture, distribution, use, and final disposal. Finally, an ERA will be structured slightly differently if it compares the ecological risk of one or more options. An example would be the comparative risk associated with a spill of bunker oil versus Orimulsion® (a bitumen-based fossil fuel). Such a comparative risk assessment might draw insight and data from existing spill sites, laboratory tests, and exposure models. Despite adaptations and differing contexts, most ERAs have the same general form (see Fig. 30-2).

Risk assessors, risk managers, and key stakeholders engage in initial planning together with the intention of formulating a clear statement of the problem. What valued ecological entity or quality is being assessed (assessment endpoint) is defined. A conceptual model is created that links the assessment endpoint and the toxicant, including descriptions of exposure pathways and possible effects. A clear statement of possible or predicted effects (risk hypothesis) is formulated. A clear formulation of the problem with concurrence of key stakeholders is critical to the ERA because of the diversity of possible assessment endpoints and exposure pathways.

Exposure characterization describes or predicts contact between the toxicant and the assessment endpoint. Depending on the ERA context, this could involve a simple calculation of average exposure, or a temporally and spatially explicit description of amounts present in relevant media. Toxicant sources, transport pathways, kinds of contact, and potential costressors are also defined.

Ecological effects characterization describes the qualities of any potential effects of concern, describes the connection between the potential effects and the assessment endpoint, and describes how changes in the level of exposure might influence the effects manifesting in the assessment endpoint. Normally, a statement about the strength of evidence associated with the descriptions is presented in the ecological effects characterization. As a common example of evidentiary uncertainty often requiring explanation is the measurement endpoint. It is not always desirable to derive effects information directly from an assessment endpoint such as an endangered species so uncertainty is introduced by gauging effects to a surrogate (measurement endpoint). Ecological effects characterizations must describe the justifiable confidence in extrapolating

[4]A landscape is formally defined by Forman and Godron (1986) as a heterogeneous landscape that is "composed of a cluster of interacting ecosystems that is repeated in a similar form throughout." Each ecosystem is a part of a whole landscape much as a tessera is part of a mosaic or a subpopulation is part of a metapopulation.

from measurement to assessment endpoints. As discussed earlier, the AOP approach is gaining traction as a powerful approach for ecological (and human health) risk assessments, particularly for effects characterization.

Risk characterization uses the analysis of exposure and ecological effects to address the risk question(s) posed in the problem formulation. This can involve an explicit statement of risk, that is, the probability of a specified intensity of an adverse effect occurring to the assessment endpoint. Often, the information needed to make such an explicit statement is absent and a qualitative statement of the likelihood of an adverse effect is made instead. Regardless of whether a quantitative or qualitative statement of risk is produced, the risk characterization must provide details surrounding the statement, including important uncertainties.

INTERCONNECTIONS BETWEEN ECOSYSTEM INTEGRITY AND HUMAN HEALTH

As noted at the beginning of this chapter, while the original definition of ecotoxicology included effects on humans, most subsequent treatments exclude discussions of humans except as a source of contaminants, with some notable exceptions (e.g., Newman, 2015). This ecotoxicology chapter, imbedded in a book focused on human or biomedical toxicology, describes the younger science of elucidating chemical effects in natural systems. While ecotoxicology has features distinct from biomedical toxicology, it is important to consider parallelisms in the two fields and, more broadly, interconnections between human health and ecological integrity, or health. While obviously related, biomedical and ecological toxicology have historically exhibited relatively little coordination or collaboration among scientists across these fields. This is likely due to a number of reasons, including the different levels of biological organization considered, as well as different academic cultures populating the two fields. However, it is questionable if this gulf has been in the best interest of understanding chemical effects, and ultimately protecting both human and ecological health. This concern has prompted several broad discussions intended to bridge this divide and enhance interdisciplinary natural and social scientific research in these areas (see reviews by Costanza et al. [1992], Di Giulio and Monosson [1996], and Di Giulio and Benson [2002]).

This gulf has resulted in two fields that, while largely disconnected, parallel one another and share common paradigms such as dose–response, toxicokinetics, mechanisms of action, and risk assessment frameworks. However, by generally ignoring how chemicals and other anthropogenic stressors that degrade ecosystems can ultimately impact human health and well-being, and vice versa, an opportunity to holistically understand the results of environmental contamination is lost. Miranda et al. (2002) developed a conceptual model for elucidating the interconnections paradigm that links natural and social systems in a circular manner with continuous feedbacks, as opposed to parallel linear models (exposure to response, in either humans or ecosystems) that dominate toxicology currently. In this conceptual model, the natural system produces both positive outputs (such as natural resources and raw materials) and negative outputs (e.g., hurricanes and disease vectors) to the social system. The culture and institution of the social system in turn transforms the natural system outputs in various ways and subsequently delivers various positive outputs (consumer goods, conservation efforts) and negative outputs (pollution, deforestation) to the natural system. These outputs influence the quantity and quality of life (human and nonhuman) of the natural system, and the circular flow of resources continually creates conditions that influence the well-being of individuals, societies, and ecosystems, now and in the future.

This rather abstract model formalizes the interconnections between human and ecological health that most of us intuitively sense. Some of these connections, in the context of environmental pollution, are obvious. Chemical contamination of seafoods valued by humans is one example. Others are less clear but potentially very significant, such as human impacts on aquatic systems that foster the propagation of human disease vectors, or human impacts on global climate that may concomitantly impact humans and ecosystems in varied and complex ways. Also important to consider is the matter of human perceptions of their environment; people's sense of the health of the environment in which they live (whether their perception is correct or not) can have substantial impacts on their mental and physical health (O'Keefe and Baum, 1996). As noted by Kendall et al. (2001), "the indirect effects of environmental pollution may, in the end, be more important than the direct effects for human health."

There are significant indications that these fields are converging and meaningful interactions are increasing. The concept of "One Health" has gained traction in recent years and is very relevant to this discussion (see Zinsstag et al. [2015]). This concept originally emphasized the role of animals in disease transmission to humans (zoonoses), but broadly recognizes the relationship between ecosystem health and human health. The *EcoHealth Journal* is devoted to this topic. The inclusion of an ecotoxicology chapter in this text is also favorable evidence. This trend is motivated in part by the genomics revolution that provides powerful methods for evaluating fundamental biological similarities across species, including those employed in biomedical and ecotoxicological research. Research in this area has revealed genetic similarities, or conservation, in many genes and the proteins they code for that are important to organismal adaptations and impacts due to environmental stressors, including chemicals (Eaton et al., 2006). Certainly many important species differences also exist that contribute to the great complexity of understanding human–ecological interconnections, but as pointed out by Winston et al. (2002), "in the final analysis, the biological similarities across living systems are probably more impressive than the differences." Discussions among diverse scientists of the promises, limitations, and potential applications of genomics for elucidating cross-species extrapolations are provided by Benson and Di Giulio (2006). Such cross-fertilizations among biomedical and environmental scientists, as well as social scientists and policy makers, are likely to enhance all areas, and catalyze the integrated protection of human and ecosystem health.

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REFERENCES

Adams SM. Evaluating effects of contaminants on fish health at multiple levels of biological organization: extrapolating from lower to higher levels. *Hum Ecol Risk Assess*. 2000;6:15–27.

Adams SM, Shepard KL, Greeley MS, et al. The use of bioindicators for assessing the effects of pollutant stress on fish. *Mar Environ Res*. 1989;28:459–464.

Albert A, Drouillard K, Haffner GD, Dixon B. Dietary exposure to low pesticide doses causes long-term immunosuppression in the leopard frog (*Rana pipiens*). *Environ Toxicol Chem*. 2007;26:1179–1185.

Allison N, Millward GE, Jones MB. Particle processing by *Mytilus edulis*: effects on bioavailability of metals. *J Exp Mar Biol Ecol*. 1998;22:149.

Allred PM, Giesy JP. Use of in situ microcosms to study mass loss and chemical composition of leaf litter being processed in a blackwater stream. *Arch Hydrobiol.* 1988;114:231.

Altelaar AF, Munoz J, Heck AJ. Next-generation proteomics: towards an integrative view of proteome dynamics. *Nat Rev Genet*. 2013;14: 35-48.

Amat A, Pfol-Leskowicz A, Burgeot T, Castegnaro M. DNA adducts as a biomarker of pollution: field study on the genotoxic impact evolution of the *Erika* oil spills on mussels (*Mytilus edulis*) over a period of eleven months. *Polycyclic Aromatic Compounds*. 2004;24:713–732.

Anders A. *Biostatistics for Epidemiologists*. Boca Raton, FL: CRC Press; 1993.

Andersen JD, Pereira V, Pietroni C, et al. Next-generation sequencing of multiple individuals per barcoded library by deconvolution of sequenced amplicons using endonuclease fragment analysis. *Biotechniques*. 2014;57:91–94.

Anderson DW, Jehl JR, Risebrough RW, et al. Brown pelicans: improved reproduction off the Southern California Coast. *Science*. 1975;190:806.

Ankley GT, Bennett RS, Erickson RJ, et al. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem*. 2010;29:730–741.

Ankley GT, Daston GP, Degitz SJ, et al. Toxicogenomics in regulatory ecotoxicology. *Environ Sci Technol*. 2006;40:4055–4065.

Ankley GT, Erickson RJ, Sheedy BR, et al. Evaluation of models for predicting the phototoxic potency of polycyclic aromatic hydrocarbons. *Aquat Toxicol*. 1997;37:37–50.

Antkiewicz DS, Burns CG, Carney SA, Peterson RE, Heideman W. Heart malformation is an early response to TCDD in embryonic zebrafish. *Toxicol Sci*. 2005;84:368–377.

Arfsten DP, Schaeffer DJ, Mulveny DC. The effects of near ultraviolet radiation on the toxic effects of polycyclic aromatic hydrocarbons in animals and plants: a review. *Ecotoxicol Environ Saf*. 1996;33:1–24.

ASTM International. *Annual Book of ASTM Standards, Vol 11.05: Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Responses*. West Conshohocken, PA: ASTM International; 2006.

ATSDR. *Toxicological Profile for Lead*. Washington, DC: U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry; 1999.

Aubone A. Loss of stability owing to a stable age structure skewed toward juveniles. *Ecol Model*. 2004;175:55.

Auffret M, Rousseau S, Boutet I, et al. A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranean. *Ecotoxicol Environ Saf*. 2006;63:393–405.

Bainy ACD, Saito E, Carvalho PSM, Junqueira VBC. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. *Aquat Toxicol*. 1996;34:151–162.

Baker CF, Montgomery JC. Sensory deficits induced by cadmium in banded kokopu, *Galaxias fasciatus*, juveniles. *Environ Biol Fishes*. 2001;62:455–464.

Baldwin DH, Sandahl JF, Labenia JS, Scholz NL. Sublethal effects of copper on coho salmon: impacts on nonoverlapping receptor pathways in the peripheral olfactory nervous system. *Environ Toxicol Chem*. 2003;22:2266–2274.

Balmford A. Pollution, politics, and vultures. *Science*. 2013;339:653–654.

Basu N, Klenavic K, Gamberg M, et al. Effects of mercury on neurochemical receptor-binding characteristics in wild mink. *Environ Toxicol Chem*. 2005;24:1444–1450.

Bauder MB, Palace VP, Hodson PV. Is oxidative stress the mechanism of blue sac disease in retene-exposed trout larvae? *Environ Toxicol Chem*. 2005;24:694–702.

Baumann PC. Epizootics of cancer in fish associated with genotoxins in sediment and water. *Mutat Res Rev Mutat Res*. 1998;411:227–233.

Baumann PC, Harshbarger JC. Long term trends in liver neoplasm epizootics of brown bullhead in the Black River, Ohio. *Environ Monit Assess*. 1998;53:213–223.

Benson WH, Di Giulio RT, eds. *Emerging Molecular and Computational Approaches for Cross-Species Extrapolations*. New York: Taylor & Francis; 2006.

Beyer WN. A reexamination of biomagnifications of metals in terrestrial food chains. *Environ Toxicol Chem*. 1986;5:863–864.

Biesmeijer JC, Roberts SPM, Reemer M, et al. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*. 2006;313:351.

Billiard SM, Querbach K, Hodson PV. Toxicity of retene to early life stages of two freshwater fish species. *Environ Toxicol Chem*. 1999;18:2070–2077.

Billiard SM, Timme-Laragy AR, Wassenberg DM, Cockman C, Di Giulio RT. The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. *Toxicol Sci*. 2006;92:526–536.

Birdsall CW, Grue CE, Anderson A. Lead concentrations in bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans*) tadpoles inhabiting highway drainages. *Environ Pollut*. 1986;40A:233–247.

Birnie-Gauvin K, Costantini D, Cooke SJ, Willmore WG. A comparative and evolutionary approach to oxidative stress in fish: a review. *Fish Fisheries*. 2017;1–15.

Blais JM, Wilhelm F, Kidd KA, et al. Concentrations of organochlorine pesticides and polychlorinated biphenyls in amphopods (*Gammarus lacustris*) along an elevation gradient in mountain lakes of western Canada. *Environ Toxicol Chem*. 2003;22:2605–2613.

Blanchfield PJ, Paterson MJ, Shearer JA, Schindler DW. Johnson and Vallentyne's legacy: 40 years of aquatic research at the Experimental Lakes Area. *Can Fish Aquat Sci*. 2009;66:1831–1836.

Brar GA, Weissman JS. Ribosome profiling reveals the what, when, where and how of protein synthesis. *Nat Rev Mol Cell Biol*. 2015;16: 651–664.

Broman D, Naf C, Rolff C, et al. Using ratios of stable nitrogen isotopes to estimate bioaccumulation and flux of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in two food chains from the Northern Baltic. *Environ Toxicol Chem*. 1992;11:331.

Broomhall S. The effects of endosulfan and variable water temperature on survivorship and subsequent vulnerability to predation in *Litoria citropa* tadpoles. *Aquat Toxicol*. 2002;61:243.

Brown RE, Brain JD, Wang N. The avian respiratory system: a unique model for studies of respiratory toxicosis and for monitoring air quality. *Environ Health Perspect*. 1997;105:188–200.

Bury NR. The changes to apical silver membrane uptake, and basolateral membrane silver export in the gills of rainbow trout (*Oncorhynchus mykiss*) on exposure to sublethal silver concentrations. *Aquat Toxicol*. 2005;72:135–145.

Bustnes JO, Miland O, Fjeld M, et al. Relationships between ecological variables and four organochlorine pollutants in an artic glaucous gull (*Larus hyperboreus*) population. *Environ Pollut*. 2005;136:175–185.

Butcher SS, Charlson RJ, Orians GH, Wolfe GV. *Global Biogeochemical Cycles*. London: Academic Press; 1992.

Butler RA, Kelley ML, Powell WH, Hahn ME, Van Beneden RJ. An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, *Mya arenaria*: evidence that invertebrate AHR homologues lack 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and beta-naphthoflavone binding. *Gene*. 2001;278:223–234.

Cade TJ. Exposure of California condors to lead from spent ammunition. *J Wildl Manage*. 2007;71:2125–2133.

Callinan PA, Feinberg AP. The emerging science of epigenomics. *Hum Mol Genet*. 2006;15:R95–101.

Campbell LM, Fask AT, Wang X, Kock G, Muir DCG. Evidence for biomagnifications of rubidium in freshwater and marine food webs. *Can J Fish Aquat Sci*. 2005;62:1161–1167.

Campbell PGC, Tessier A. Ecotoxicology of metals in the aquatic environment: geochemical aspects. In: Newman MC, Jagoe CH, eds. *Ecotoxicology: A Hierarchical Treatment*. Boca Raton, FL: CRC Press; 1996:11–58.

Cao CC, Li C, Huang Z, et al. Identifying rare variants with optimal depth of coverage and cost-effective overlapping pool sequencing. *Genet Epidemiol*. 2013;37:820–830.

Carls MG, Rice SD, Hose JE. Sensitivity of fish embryos to weathered crude oil: part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasi*). *Environ Toxicol Chem*. 1999;18:481–493.

Carlson E, Zelikoff JT. The immune system of fish: a target organ of toxicity. In: Di Giuio RT, Hinton DE, eds. *The Toxicology of Fishes*. Boca Raton, FL: CRC Press; 2008:489–529.

Carney SA, Peterson RE, Heideman W. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin activation of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator pathway causes developmental toxicity through a CYP1A-independent mechanism in zebrafish. *Mol Pharmacol*. 2004;66:512–521.

Carney SA, Prasch AL, Heideman W, Peterson RE. Understanding dioxin developmental toxicity using the zebrafish model. *Birth Defects Res A*. 2006;76:7–18.

Carr RL, Ho LL, Chambers JE. Selective toxicity of chlorpyrifos to several species of fish during an environmental exposure: biochemical mechanisms. *Environ Toxicol Chem*. 1997;16:2369–2374.

Carson R. *Silent Spring*. Boston, MA: Houghton Mifflin; 1962.

Carvalho PS, Tillitt DE. 2,3,7,8-TCDD effects on visual structure and function in swim-up rainbow trout. *Environ Sci Technol*. 2004;38:6300–6306.

Caswell H. *Matrix Population Models. Construction, Analysis, and Interpretation*. Sunderland, MA: Sinauer Associates; 2001.

Chandler GT, Cary TL, Bejarano AC, Pender J, Ferry JL. Population consequences of fipronil and degradates to copepods at field concentrations: an integration of life cycle testing with Leslie matrix population modeling. *Environ Sci Technol*. 2004;38:6407.

Chapman PM. Indirect effects of contaminants. *Mar Pollut Bull*. 2004;48:411.

Chaty S, Rodius F, Vasseur P. A comparative study of the expression of CYP1A and CYP4 genes in aquatic invertebrate (freshwater mussel, *Unio tumidus*) and vertebrate (rainbow trout, *Oncorhynchus mykiss*). *Aquat Toxicol*. 2004;69:81–93.

Chen Z, Mayer LM. Mechanisms of Cu solubilization during deposit feeding. *Environ Sci Technol*. 1998;32:770.

Cherkasov AS, Biswas PK, Ridings DM, Ringwood AH, Sokolova IM. Effects of acclimation temperature and cadmium exposure on cellular energy budgets in the marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses. *J Exp Biol*. 2006;209:1274–1284.

Chernyak SM, McConnell LL, Rice CP. Fate of some chlorinated hydrocarbons in arctic and far eastern ecosystems in the Russian Federation. *Sci Total Environ*. 1995;75:160–161.

Chiou CT. Partition coefficients of organic compounds in lipid–water systems and correlations with fish bioconcentration factor. *Environ Sci Technol*. 1985;19:57.

Chiverton PA. The benefits of unsprayed cereal crop margins to grey partridge *Perdix perdix* and pheasants *Phasianu colchicus* in Sweden. *Wildl Biol*. 1999;5:8.

Chowdhury MJ, Pane EF, Wood CM. Physiological effects of dietary cadmium acclimation and waterborne cadmium challenge in rainbow trout: respiratory, ionoregulatory, and stress parameters. *Comp Biochem Physiol*. 2004;139C:163–173.

Clark AJ, Scheuhammer AM. Lead poisoning in upland-foraging birds of prey in Canada. *Ecotoxicology*. 2003;12:23–30.

Clarke KR. Nonmetric multivariate analysis in community-level ecotoxicology. *Environ Toxicol Chem*. 1999;18:118–127.

Collier TK, Singh SV, Awasthi YC, Varanasi U. Hepatic xenobiotic metabolizing enzymes in 2 species of benthic fish showing different prevalences of contaminant-associated liver neoplasms. *Toxicol Appl Pharmacol*. 1992;113:319–324.

Connell DW. *Bioaccumulation of Xenobiotic Compounds*. Boca Raton, FL: CRC Press; 1990.

Connon RE, Geist J, Werner I. Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. *Sensors*. 2012;12:12741–12771.

Cook PM, Robbins JA, Endicott DD, et al. Effects of aryl hydrocarbon receptor-mediated early life stage toxicity on lake trout populations in Lake Ontario during the 20th century. *Environ Sci Technol*. 2003;37:3864–3877.

Costanza R, Norton BG, Haskell BD, eds. *Ecosystem Health. New Goals for Environmental Management*. Washington, DC: Island Press; 1992.

Crollius HR, Weissenbach J. Fish genomics and biology. *Genome Res*. 2005;15:1675–1682.

Culp JM, Lowell RB, Cash KJ. Integrating mesocosm experiments with field and laboratory studies to generate weight-of-evidence risk assessments for large rivers. *Environ Toxicol Chem*. 2000;19:1167.

Custer TW, Custer CM, Dickerson K, et al. Polycyclic aromatic hydrocarbons, aliphatic hydrocarbons, trace elements, and monooxygenase activity in birds nesting on the North Platte River, Casper, Wyoming, USA. *Environ Toxicol Chem*. 2001;20:624–631.

Custer TW, Custer CM, Hines RK, et al. Organochlorine contaminants and reproductive success of double-crested cormorants from Green Bay, Wisconsin, USA. *Environ Toxicol Chem*. 1999;18:1209–1217.

da Fonseca RR, Albrechtsen A, Themudo GE, et al. Next-generation biology: sequencing and data analysis approaches for non-model organisms. *Mar. Genom*. 2016;30:3013.

Day KE. Short-term effects of herbicides on primary productivity of periphyton in lotic environments. *Ecotoxicology*. 1993;2:123.

Del Chierico F, Ancora M, Marcacci M, et al. Choice of next-generation sequencing pipelines. *Methods Mol Biol*. 2015;1231:31–47.

Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol*. 2003;43:309–334.

Deroo BJ, Korach KS. Estrogen receptors and human disease. *J Clin Invest*. 2006;116:561–570.

Devlin EW. Acute toxicity, uptake and histopathology of aqueous methyl mercury to fathead minnow embryos. *Ecotoxicology*. 2006;15:97–110.

Di Giulio RT, Benson WE. *Interconnections Between Human Health and Ecological Integrity*. Pensacola, FL: SETAC Press; 2002.

Di Giulio RT, Hinton DE, eds. *The Toxicology of Fishes*. Boca Raton, FL: CRC Press. 2008.

Di Giulio RT, Monosson E, eds. *Interconnections between Human and Ecosystem Health*. London: Chapman & Hall; 1996.

Di Giulio RT, Washburn PC, Wenning RJ, Winston GW, Jewell CS. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environ Toxicol Chem*. 1989;8:1103–1123.

Di Toro DM, Mahony JD, Hansen DJ, et al. Toxicity of cadmium in sediments: the role of acid volatile sulfide. *Environ Toxicol Chem*. 1990;9:1487.

Di Toro DM, McGrath JA, Hansen DJ, et al. Predicting sediment metal toxicity using a sediment biotic ligand model: methodology and initial application. *Environ Toxicol Chem*. 2005;24:2410–2427.

Di Toro DM, Zarba CS, Hanson DJ, et al. Technical basis for establishing sediment quality criteria for nonionic organic chemicals using equilibrium partitioning. *Environ Toxicol Chem*. 1991;10:1541–1583.

Dopp E, Barker CM, Schiffmann D, Reinisch CL. Detection of micronuclei in hemocytes of *Mya arenaria*: association with leukemia and induction with an alkylating agent. *Aquat Toxicol*. 1996;34:31–45.

Dorval J, Leblond V, Deblois C, Hontela A. Oxidative stress and endocrine endpoints in white sucker (*Catostomus commersoni*) from a river impacted by agricultural chemicals. *Environ Toxicol Chem*. 2005;24:1273–1280.

du Corbier FA, Lyons BP, Stentiford GA, Rotchell JM. The role of the retinoblastoma gene in liver tumor development in fish. *Environ Sci Technol*. 2005;39:9785–9790.

Eaton D, Gallagher E, Hooper M, et al. Species differences in response to toxic substances: shared pathways of toxicity—values and limitations of omic technologies to elucidate mechanism or mode of action. In: Benson WH, Di Giulio RT, eds. *Emerging Molecular and Computational Approaches for Cross-Species Extrapolations*. New York: Taylor & Francis; 2006.

Edwards SW, Tan YM, Villeneuve DL, Meek ME, McQueen CA. Adverse outcome pathways—organizing toxicological information to improve decision making. *J Pharmacol Exp Therapeut*. 2016;336:170–181.

Environmental Protection Agency. *Summary Report on Issues in Ecological Risk Assessment*. EPA/625/3-91/018. Springfield, VA: NTIS; 1991.

Erickson RJ. The biotic ligand model approach for addressing effects of exposure water chemistry on aquatic toxicity of metals: genesis and challenges. *Environ Toxicol Chem*. 2013;32:1212–1214.

Evans DH. The fish gill: site of action and model for toxic effects of environmental pollutants. *Environ Health Perspect*. 1987;71:47–58.

Evans MI, Syemens P, Pilcher CWT. Short-term damage to coastal bird populations in Saudi-Arabia and Kuwait following the 1991 Gulf War marine pollution. *Mar Pollut Bull*. 1993;27:157–161.

Fairbrother A, Ankley GT, Birnbaum LS, et al. Reproductive and developmental toxicology of contaminants in oviparous animals. In: Di Giulio RT, Tillitt DE, eds. *Reproductive and Developmental Effects of Contaminants in Oviparous Vertebrates*. Pensacola, FL: SETAC Press; 1999:283–361.

Fairbrother A, Smits J, Grasman KA. Avian immunotoxicology. *J Toxicol Environ Health Crit Rev*. 2004;7B:105–137.

Fernandez P, Grimait JO. On the global distribution of persistent organic pollutants. *CHIMIA*. 2003;57:514.

Fernie KJ, Mayne G, Shutt JL, et al. Evidence of immunomodulation in nestling American kestrels (*Falco sparverius*) exposed to environmentally relevant PBDEs. *Environ Pollut*. 2005;138:485–493.

Fleeger JW, Carman KR, Nisbet RM. Indirect effects of contaminants in aquatic ecosystems. *Sci Total Environ*. 2003;317:207.

Forbes VE, Calow P. Is per capita rate of increase a good measure of population-level effects in ecotoxicology? *Environ Toxicol Chem*. 1999;18:1544.

Forman RTT, Godron M. *Landscape Ecology*. New York: Wiley; 1986.

Fossi MC, Marsili L. The use of non-destructive biomarkers in the study of marine mammals. *Biomarkers*. 1997;2:205–216.

Fox G. Practical causal inference for ecoepidemiologists. *J Toxicol Environ Health*. 1991;33:359.

Fridovich I. Mitochondria: are they the seat of senescence? *Aging Cell*. 2004;3:13–16.

Fry DM. Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ Health Perspect*. 1995;103(suppl 7):165–171.

Fuchs SM, Krajewski K, Baker RW, et al. Influence of combinatorial histone modifications on antibody and effector protein recognition. *Curr Biol*. 2011;21:53–58.

Gajecka M. Unrevealed mosaicism in the next-generation sequencing era. *Mol Genet Genomics*. 2016;291:513–530.

Gard TC. Stochastic models for toxicant-stressed populations. *Bull Math Biol*. 1992;54:827–837.

Gardner SC, Oberdorster E. *Toxicology of Reptiles*. Boca Raton, FL: Taylor & Francis; 2005.

Gilbertson M, Kubiak T, Ludwig J, et al. Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds—similarity to chick edema disease. *J Toxicol Environ Health*. 1991;33: 455–520.

Grant BS. Allelic melanism in American and British peppered moths. *J Hered*. 2004;95:97–102.

Grue CE, Gilbert PL, Seeley ME. Neurophysiological and behavioral changes in non-target wildlife exposed to organophosphate and carbamate pesticides: thermoregulation, food consumption, and reproduction. *Am Zool*. 1997;37:369–388.

Grue CE, Powell GVN, McChesney MJ. Care of nestlings by wild female starlings exposed to an organophosphate pesticide. *J Appl Ecol*. 1982;19:327–335.

Guillette LJ, Crain DA, Gunderson MP, et al. Alligators and endocrine disrupting contaminants: a current perspective. *Am Zool*. 2000;40: 438–452.

Ha NT, Freytag S, Bickeboeller H. Coverage and efficiency in current SNP chips. *Eur J Hum Genet*. 2014;9:1124–1130.

Hahn ME. Aryl hydrocarbon receptors: diversity and evolution. *Chem Biol Interact*. 2002;141:131–160.

Hahn ME, Merson RR, Karchner SI. Xenobiotic receptors in fish: structural and functional diversity and evolutionary insights. In: Mommsen TP, Moon TW, eds. *Environmental Toxicology, Vol 6: Biochemistry and Molecular Biology of Fishes*. Amsterdam, the Netherlands: Elsevier; 2005.

Hall AJ, Hugunin K, Deaville R, et al. The risk of infection from polychlorinated biphenyl exposure in the *harbor porpoise* (*Phocoena phocoena*): a case–control approach. *Environ Health Perspect*. 2006;114:704–711.

Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*. 3rd ed. Oxford, UK: Oxford University Press; 1999.

Handy RD, Runnalls T, Russell PM. Histopathologic biomarkers in three spined sticklebacks, *Gasterosteus aculeatus*, from several rivers in Southern England that meet the freshwater fisheries directive. *Ecotoxicology*. 2002;11:467–479.

Hankinson O. The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol*. 1995;35:307–340.

Hanscom C, Talkowski M. Design of large-insert jumping libraries for structural variant detection using Illumina sequencing. *Curr Protoc Hum Genet*. 2014;80:1–9.

Harries JE, Sheahan DA, Jobling S, et al. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem*. 1997;16:534–543.

Harshbarger JC, Clark JB. Epizootiology of neoplasms of bony fish of North America. *Sci Total Environ*. 1990;94:1–32.

Hasspieler BM, Behar JV, Di Giulio RT. Glutathione-dependent defense in channel catfish (*Ictalurus punctatus*) and brown bullhead (*Ameriurus nebulosus*). *Ecotoxicol Environ Saf*. 1994;28:82–90.

Heath JA, Frederick PC. Relationships among mercury concentrations, hormones, and nesting effort of White Ibises (*Eudocimus albus*) in the Florida Everglades. *Auk*. 2005;122:255–267.

Heid SE, Walker MK, Swanson HI. Correlation of cardiotoxicity mediated by halogenated aromatic hydrocarbons to aryl hydrocarbon receptor activation. *Toxicol Sci*. 2001;61:187–196.

Henny CJ. Effects of mining lead on birds: a case history at Coeur d'Alene Basin, Idaho. In: Hoffman DJ, Rattner BA, Burton GA Jr, Cairns J Jr, eds. *Handbook of Ecotoxicology*. 2nd ed. Boca Raton, FL: Lewis; 2003.

Hill AB. The environment and disease: association or causation? *Proc R Soc Med*. 1965;59:295.

Hinton DE. Cells, cellular responses, and their markers in chronic toxicity. In: Malins DC, Ostrander GK, eds. *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*. Boca Raton, FL: Lewis; 1994: 207–239.

Hippeli S, Elstner EF. Mechanisms of oxygen activation during plant stress: biochemical effects of air pollutants. *J Plant Physiol*. 1996;148:249–257.

Hoffman DJ, Rattner BA, Burton GA, Cairns J, eds. *Handbook of Ecotoxicology*. Boca Raton, FL: Lewis Publishers; 2003.

Holm J, Palace V, Siwik P, et al. Developmental effects of bioaccumulated selenium in eggs and larvae of two salmonid species. *Environ Toxicol Chem*. 2005;24:2373–2381.

Hontela A. Endocrine and physiological responses to xenobiotics in fish: role of glucocorticoid hormones. *Rev Toxicol*. 1997;1:1–46.

Horness BH, Lomax DP, Johnson LL, et al. Sediment quality thresholds: estimates from hockey stick regression of liver prevalence in English sole (*Pleuronectes vetulus*). *Environ Toxicol Chem*. 1998;17:872.

Hose JE, Cross JN, Smith SG, Diehl DL. Elevated circulating erythrocyte micronuclei in fishes from contaminated sites off southern California. *Mar Environ Res*. 1987;22:167–176.

Huang YW, Matthews JB, Fertuck KC, Zacharewski TR. Use of *Xenopus laevis* as a model for investigating in vitro and in vivo endocrine disruption in amphibians. *Environ Toxicol Chem*. 2005;24:2002–2009.

Huang Z, Bassil CF, Murphy SK. Bisulfite sequencing of cloned alleles. *Methods Mol Biol*. 2013;1049:83–94.

Huggett RJ, Kimerle RA, Mehrle PM Jr, Bergman HL, eds. *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*. Boca Raton, FL: Lewis Publishers; 1992.

Husby MP, McBee K. Nuclear DNA content variation and double-strand DNA breakage in white-footed mice (*Peromyscus leucopus*) collected from abandoned strip mines, Oklahoma, USA. *Environ Toxicol Chem*. 1999;18:926–931.

Hwang HM, Wade TL, Sericano JL. Relationship between lysosomal membrane destabilization and chemical body burden in eastern oysters

(*Crassostrea virginica*) from Galveston Bay, Texas, USA. *Environ Toxicol Chem*. 2002;21:1268–1271.
Incardona JP, Carls MG, Teraoka H, et al. Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. *Environ Health Perspect*. 2005;113:1755–1762.
Jansen A, Forbes VE, Parker ED Jr. Variation in cadmium uptake, feeding rate, and life-history effects in the gastropod *Potamopyrgus antipodarum*: linking toxicant effects on individuals to the population level. *Environ Toxicol Chem*. 2001;20:2503.
Johnson L, Casillas E, Sol S, et al. Contaminant effects on reproductive success in selected benthic fish. *Mar Environ Res*. 1993;35:165–170.
Johnson LL, Stein JE, Collier TK, Casillas E, Varanasi U. Indicators of reproductive development in prespawning female winter flounder (*Pleuronectes americanus*) from urban and nonurban estuaries in the northeast United States. *Sci Total Environ*. 1994;141:241–260.
Kammenga J, Laskowski R. *Demography in Ecotoxicology*. Chichester, UK: Wiley; 2000.
Kappus H. Overview of enzyme systems involved in bio-reduction of drugs and in redox cycling. *Biochem Pharmacol*. 1986;35:1–6.
Karr JR. Biological integrity: a long-neglected aspect of water resource management. *Ecol Appl*. 1991;1:66.
Kedwards TJ, Maund SJ, Chapman PF. Community level analysis of ecotoxicological field studies: I. Biological monitoring. *Environ Toxicol Chem*. 1999;18:149.
Keller JM, McClellan-Green PD, Kucklick JR, Keil DE, Peden-Adams MM. Effects of organochlorine contaminants on loggerhead sea turtle immunity: comparison of a correlative field study and in vitro exposure experiments. *Environ Health Perspect*. 2006;114:70–76.
Kendall RJ, Anderson TA, Baker RJ, et al. Ecotoxicology. In: Klaassen CD, ed. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. 6th ed. New York: McGraw-Hill; 2001.
Kendall RJ, Lacher TE Jr, Bunck C, et al. An ecological risk assessment of lead shot exposure in non-waterfowl avian species: upland game birds and raptors. *Environ Toxicol Chem*. 1996;15:4–20.
Kidd KA, Bootsma HA, Hesslein RH, Muir DCG, Hecky RE. Biomagnification of DDT through the benthic and pelagic food webs of Lake Malawi, East Africa: importance of trophic level and carbon source. *Environ Sci Technol*. 2001;35:14–20.
Kim M, Lee KH, Yoon SW, et al. Analytical tools and databases for metagenomics in the next-generation sequencing era. *Genomics Inform*. 2013;11:102–113.
Klobicar GI, Stambuk A, Pavlica M, Sertic Peric M, Kutuzovic Hackenberger B, Hylland K. Genotoxicity monitoring of freshwater environments using caged carp (*Cyprinus carpio*). *Ecotoxicology*. 2010;19:77–84.
Koboldt DC, Steinberg KM, Larson DE, et al. The next-generation sequencing revolution and its impact on genomics. *Cell*. 2013;155:27–38.
Kohler A, Wahl E, Soffker K. Functional and morphological changes of lysosomes as prognostic biomarkers of toxic liver injury in a marine flatfish (*Platichthys flesus* (L.)). *Environ Toxicol Chem*. 2002;21:2434–2444.
Korthals GW, Alexiev AD, Lexmond TM, Kammenga JE, Bongers T. Long-term effects of copper and pH on the nematode community in an agroecosystem. *Environ Toxicol Chem*. 1996;15:979.
Krebs CJ. *Ecological Methodology*. 2nd ed. New York: Benjamin Cummings; 1998.
Kreutzweiser DP, Capell SS, Scarr TA. Community-level responses by stream insects to NEEM products containing azadirachtin. *Environ Toxicol Chem*. 2000;19:855.
Krivtsov V. Investigations of indirect relationships in ecology and environmental science: a review and the implications for comparative theoretical ecosystem analysis. *Ecol Model*. 2004;174:37.
Ku CS, Wu M, Cooper DN, et al. Exome versus transcriptome sequencing in identifying coding region variants. *Expert Rev Mol Diagn*. 2012;12:241–251.
Landis WG, Matthews RA, Matthews GB. Design and analysis of multispecies toxicity tests for pesticide registration. *Ecol Appl*. 1997;7:1111.
Larson RA, Weber EJ. *Reaction Mechanisms in Environmental Organic Chemistry*. Boca Raton, FL: Lewis Publishers; 1994.
Larsson DGJ, Adolfsson-Erici M, Parkkonen J, et al. Ethinyloestradiol—an undesired fish contraceptive? *Aquat Toxicol*. 1999;45:91–97.
Lee B-G, Griscom SB, Lee J-K, et al. Influences of dietary uptake and reactive sulfides on metal bioavailability from aquatic sediments. *Science*. 2000;287:282.
Lemly AD. Symptoms and implications of selenium toxicity in fish: the Belews Lake case example. *Aquat Toxicol*. 2002;57:39–49.
Leslie HA, Oosthoek AJP, Busser FJM, Kraak MHS, Hermens JLM. Biomimetic solid-phase microextraction to predict body residues and toxicity of chemicals that act by narcosis. *Environ Toxicol Chem*. 2002;21:229.
Leung KMY, Kwong RPY, Ng WC, et al. Ecological risk assessments of endocrine disrupting organotin compounds using neogastropods in Hong Kong. *Chemosphere*. 2006;65:922–938.
Li J, Quabius ES, Wendelaar Bonga SE, Flik G, Lock RAC. Effects of water-borne copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). *Aquat Toxicol*. 1998;43:1–11.
Linbo TL, Stehr CM, Incardona JP, Scholz NL. Dissolved copper triggers cell death in the peripheral mechanosensory system of larval fish. *Environ Toxicol Chem*. 2006;25:597–603.
Lipscomb TP, Harris RK, Rebar AH, Ballachey BE, Haebler RJ. *Exxon Valdez Oil Spill State/Federal Natural Resource Damage Assessment, Final Report. Pathological Studies of Sea Otters (Marine Mammal Study 6–11)*. Anchorage, AK: U.S. FWS; 1996.
Little PFR. Structure and function of the human genome. *Genome Res*. 2005;15:1759–1766.
Livingstone DR. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar Pollut Bull*. 2001;42:656–666.
Lloyd R, Herbert DWM. The influence of carbon dioxide on the toxicity of unionized ammonia to rainbow trout (*Salmo gairdneri* Richardson). *Ann Appl Biol*. 1960;48:399.
Loge FJ, Arkoosh MR, Ginn TR, Johnson LL, Collier TK. Impact of environmental stressors on the dynamics of disease transmission. *Environ Sci Technol*. 2005;39:7329–7336.
Loreau M. Does functional redundancy exist? *Oikos*. 2004;104:606.
Lorenzen A, Williams KL, Moon TW. Determination of the estrogenic and antiestrogenic effects of environmental contaminants in chicken embryo hepatocyte cultures by quantitative-polymerase chain reaction. *Environ Toxicol Chem*. 2003;22:2329–2336.
Louis A, Nguyen NTT, Muffato M, Crollius HR. Genomics update 2015: KaryoView and MatrixView provide a genome-wide perspective to multispecies comparative genomics. *Nucleic Acids Res*. 2015;43:D682–D689.
Luoma SN. Can we determine the biological availability of sediment-bound trace elements? *Hydrobiologia*. 1989;379:176–177.
Luoma SN, Bryan GW. Factors controlling the availability of sediment-bound lead to the estuarine bivalve *Scrobicularia plana*. *J Mar Biol Assoc UK*. 1978;58:793.
Maccubbin AE. DNA adduct analysis in fish: laboratory and field studies. In: Malins DC, Ostrander GK, eds. *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*. Boca Raton, FL: Lewis Publishers; 1994:267–294.
Mackay D. Correlation of bioconcentration factors. *Environ Sci Technol*. 1982;16:274.
MAFF (Ministry of Agriculture, Forestry and Fisheries). *Guidance on Toxicological Study Data for Application of Pesticide Registration*. No. 59-Nousan-4200. Japan: Ministry of Agriculture, Forestry and Fisheries; 1985.
Magurran AE. *Ecological Diversity and its Measurement*. Princeton, NJ: Princeton University Press; 1988.
Malins DC, Anderson KM, Stegeman JJ, et al. Biomarkers signal contaminant effects on the organs of English sole (*Parophrys vetulus*) from Puget Sound. *Environ Health Perspect*. 2006;114:823–829.
Malins DC, Krahn MM, Brown DW, et al. Toxic chemicals in marine sediment and biota from Mukilteo, Washington: relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). *J Natl Cancer Inst Monogr*. 1987;74:487–494.
Mallet J. The evolution of insecticide resistance: have the insects won? *Trends Ecol Evol*. 1989;14:336.
Martineau D, Lemberger K, Dallaire A, et al. Cancer in wildlife, a case study: beluga from the St. Lawrence estuary, Quebec, Canada. *Environ Health Perspect*. 2002;110:285–292.

Mason AZ, Nott JA. The role of intracellular biomineralized granules in the regulation and detoxification of metals in gastropods with special reference to the marine prosobranch *Littorina littorea*. *Aquat Toxicol*. 1981;1:239.

Mathews AP. The relation between solution tension, atomic volume, and the physiological action of elements. *Am J Physiol*. 1904;10:290.

Mauer BA, Holt RD. Effects of chronic pesticide stress on wildlife populations in complex landscapes: processes at multiple scales. *Environ Toxicol Chem*. 1996;15:420.

Mayer LM, Chen Z, Findlay RH, et al. Bioavailability of sedimentary contaminants subject to deposit-feeder digestion. *Environ Sci Technol*. 1996;30:2641.

MacDonald R, Mackay D, Hickie B. Contaminant amplification in the environment. *Environ Sci Technol*. 2002;36:457A–462A.

McDonald BG, Chapman PM. PAH phototoxicity—an environmentally irrelevant phenomenon? *Mar Pollut Bull*. 2002;44:1321–1326.

McMahon G, Huber LJ, Moore MJ, Stegeman JJ, Wogan GN. Mutations in c-Ki-ras oncogenes in diseased livers of winter flounder from Boston Harbor. *Proc Natl Acad Sci U S A*. 1990;87:841–845.

Mellanen P, Soimasuo M, Holmbloom B, Oikari A, Santti R. Expression of the Vitellogenin gene in the liver of juvenile whitefish (*Coregonus lavaletus* L. s.l.) exposed to effluents from pulp and paper mills. *Ecotoxicol Environ Saf*. 1999;43:133–137.

Meyer JN, Nacci D, Di Giulio RT. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. *Toxicol Sci*. 2002;68:69–81.

Meyer JN, Smith JD, Winston GW, Di Guilio RT. Antioxidant defenses in killifish (*Fundulus heteroclitus*) exposed to contaminated sediments and model prooxidants: short-term and heritable responses. *Aquat Toxicol*. 2003;65:377.

Miller KA, Assuncao MGL, Dangerfield NJ, Bandiera SM, Ross PS. Assessment of cytochrome P450 1A in harbour seals (*Phoca vitulina*) using a minimally-invasive biopsy approach. *Mar Environ Res*. 2005;60:153–169.

Mineau P, ed. *Cholinesterase-Inhibiting Insecticides: Their Impact on Wildlife and the Environment*. Amsterdam, the Netherlands: Elsevier; 1991.

Mineau P, Fletcher MR, Glaser LC, et al. Poisoning of raptors with organophosphorus and carbamate pesticides with emphasis on Canada, US and UK. *J Raptor Res*. 1999;33:1–37.

Miranda ML, Mohai P, Bus J, et al. Policy concepts and applications. In: Di Giulio RT, Benson WH, eds. *Interconnections between Human Health and Ecological Integrity*. Pensacola, FL: SETAC Press; 2002.

Moller P. The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. *Basic Clin Pharmacol Toxicol*. 2006;98:336–345.

Mommsen TP, Moon TW. *Environmental Toxicology. Biochemistry and Molecular Biology of Fishes*. Vol 6. Amsterdam, the Netherlands: Elsevier; 2005.

Moore MN. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ Int*. 2006;32:967–976.

Moore MN, Allen JI, McVeigh A. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. *Mar Environ Res*. 2006;61:278–304.

Mulvey M, Newman MC, Vogelbein W, Unger MA. Genetic structure of *Fundulus heteroclitus* from PAH-contaminated and neighboring sites in the Elizabeth and York Rivers. *Aquat Toxicol*. 2002;61:195.

Mulvey M, Newman MC, Vogelbein W, Unger MA, Ownby DR. Genetic structure and mtDNA diversity of *Fundulus heteroclitus* populations from PAH-contaminated and neighboring sites. *Environ Toxicol Chem*. 2003;22:671.

Munkittrick KR, Portt CB, van der Kraak GJ, et al. Impact of bleached kraft mill effluent on population characteristics, liver MFO activity, and serum steroid levels of a Lake Superior white sucker (*Catostomus commersoni*) population. *Can J Fish Aquat Sci*. 1991;48:1371–1380.

Muzzey D, Evans EA, Lieber C. Understanding the basics of NGS: from mechanism to variant calling. *Curr Genet Med Rep*. 2015;3:158–165.

Myers MS, Stehr CM, Olson OP, et al. Relationships between toxicopathic hepatic lesions and exposure to chemical contaminants in English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and white croaker (*Genyonemus lineatus*) from selected marine sites on the Pacific coast, USA. *Environ Health Perspect*. 1994;102:200.

Naeem S, Thompson LJ, Lawler SP, Lawton JH, Woodfin RM. Declining biodiversity can alter the performance of ecosystems. *Nature*. 1994;6473:734–737.

National Research Council. *Risk Assessment in the Federal Government: Managing the Process*. Washington, DC: National Academy of Sciences; 1983.

Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP. Role of the aromatic hydrocarbon receptor and [*Ah*] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem Pharmacol*. 2000;59:65–85.

Neely WK, Branson DR, Blau GE. Partition coefficient to measure bioconcentration potential of organic chemicals in fish. *Environ Sci Technol*. 1974;8:1113.

Newman MC. *Population Ecotoxicology*. Chichester, UK: Wiley; 2001.

Newman MC. *Quantitative Ecotoxicology*. Boca Raton, FL: CRC Press; 2013.

Newman MC. *Fundamentals of Ecotoxicology: The Science of Pollution*. 4th ed. Boca Raton, FL: Lewis Publishers; 2015.

Newman MC, Clements WH. *Ecotoxicology: A Comprehensive Treatment*. Boca Raton, FL: CRC Press; 2008.

Newman MC, Crane M, Holloway G. Does pesticide risk assessment in the European Union assess long-term effects? *Rev Environ Toxicol*. 2006;187:1.

Nigro M, Falleni A, Del Barga I, et al. Cellular biomarkers for monitoring estuarine environments: transplanted versus native mussels. *Aquat Toxicol*. 2006;77:339–347.

Nott JA, Nicolaidou A. Bioreduction of zinc and manganese along a molluscan food chain. *Comp Biochem Physiol*. 1993;104A:235.

NRC (Nuclear Regulatory Commission). *Biologic Markers in Reproductive Toxicology*. Washington, DC: National Academy Press; 1989.

Oaks JL, Gilbert M, Virani MZ, et al. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*. 2004;427:630–633.

O'Connor RJ. Toward the incorporation of spatiotemporal dynamics into ecotoxicology. In: Rhodes OE Jr, Chesser RK, Smith MH, eds. *Population Dynamics in Ecological Space and Time*. Chicago, IL: University of Chicago Press; 1996:281–317.

Odum EP. Trends expected in stressed ecosystems. *Bioscience*. 1985;35:419–422.

OECD (Organisation for Economic Co-Operation and Development). *OECD Guidelines for the Testing of Chemicals*. Paris: OECD; 1981.

Ohlendorf HM. The birds of Kesterson Reservoir: a historical perspective. *Aquat Toxicol*. 2002;57:1–10.

O'Keefe MK, Baum A. Perceptions of ecosystem health, stress and human well-being. In: Di Giulio RT, Monosson E, eds. *Interconnections between Human and Ecosystem Health*. London: Chapman & Hall; 1996.

Ormseth OA, Ben-David M. Ingestion of crude oil: effects on digesta retention times and nutrient uptake in captive river otters. *J Comp Physiol B*. 2000;170:419.

Ostrander GK, Rotchell JM. Fish models of carcinogenesis. In: Mommsen TP, Moon TW, eds. *Environmental Toxicology, Biochemistry and Molecular Biology of Fishes*. Vol 6. Amsterdam, the Netherlands: Elsevier; 2005.

Ownby D, Newman MC, Mulvey M, Unger M, Vogelbein W. Fish (*Fundulus heteroclitus*) populations with different exposure histories differ in tolerance of creosote-contaminated sediments. *Environ Toxicol Chem*. 2002;21:1897.

Pacheco M, Santos MA. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). *Ecotoxicol Environ Saf*. 2002;53:331–347.

Paerl HW, Pinckney JL, Fear JM, Peierls BL. Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse river estuary, North Carolina, USA. *Mar Ecol Prog Ser*. 1999;166:17–25.

Parker ML, Goldstein MI. Differential toxicities of organophosphate and carbamate insecticides in the nestling European starling (*Sturnus vulgaris*). *Arch Environ Contam Toxicol*. 2000;39:233–242.

Partridge GG. Relative fitness of genotypes in a population of *Rattus norvegicus* polymorphic for warfarin resistance. *Heredity*. 1979;43:239.
Patrick R. Use of algae, especially diatoms in the assessment of water quality. *ASTM Spec Tech Bull*. 1973;528:76.
Peakall D. *Animal Biomarkers as Pollution Indicators*. New York: Chapman & Hall; 1992.
Peck MA, Brandhagen MD, Marshall C, et al. Concordance and reproducibility of a next generation mtGenome sequencing method for high-quality samples using the Illumina MiSeq. *Forensic Sci Int Genet*. 2016;24:103–111.
Peeters ETHM, Gardeniers JJP, Koelmans AA. Contribution of trace metals in structuring *in situ* macroinvertebrate community composition along a salinity gradient. *Environ Toxicol Chem*. 2000;19:1002.
Perkins E, Garcia-Reyero N, Edwards S, et al. The adverse outcome pathway: a conceptual framework to support toxicity testing in the twenty-first century. In: Hoenig J, Peitsch MC. *Computational Systems Toxicology*. New York: Springer. 2015:1–26.
Peterson CH, Rice SD, Short JW, et al. Long-term ecosystem response to the Exxon Valdez oil spill. *Science*. 2003;302:2082–2086.
Phillips DH. Detection of DNA modifications by the ^{32}P-postlabelling assay. *Mutat Res.* 1997;178:1–12.
Pimentel MF, Damasceno EP, Jimenez PC, et al. Endocrine disruption in *Sphoeroides testudineus* tissues and sediments highlights contamination in a northeastern Brazilian estuary. *Environ Monitor Assess*. 2016;188:298.
Pinkney AE, Harshbarger JC, May EB, Melancon MJ. Tumor prevalence and biomarkers of exposure in brown bullheads (*Ameiurus nebulosus*) from the tidal Potomac River, USA, watershed. *Environ Toxicol Chem*. 2001;20:1196–1205.
Ploch SA, King LC, Kohan MJ, Di Giulio RT. Comparative *in vitro* and *in vivo* benzo(*a*)pyrene–DNA adduct formation and its relationship to CYP1A activity in two species of Ictalurid catfish. *Toxicol Appl Pharmacol*. 1998;149:90–98.
Porte C, Janer G, Lorusso LC, et al. Endocrine disruptors in marine organisms: approaches and perspectives. *Comp Biochem Physiol*. 2006;143C:303–315.
Pratt JR, Cairns J Jr. Ecotoxicology and the redundancy problem: understanding effects on community structure and function. In: Newman MC, Jagoe CH, eds. *Ecotoxicology. A Hierarchical Treatment*. Boca Raton, FL: CRC Press; 1996:347.
Preston BL. Indirect effects in aquatic ecotoxicology: implications for ecological risk assessment. *Environ Manage*. 2002;29:311.
Quek K, Nones K, Patch AM, et al. A workflow to increase verification rate of chromosomal structural rearrangements using high-throughput next-generation sequencing. *Biotechniques*. 2014;57:31–38.
Rambler MB, Margulis L, Fester R. *Global Ecology: Towards a Science of the Biosphere*. Boston, MA: Academic Press; 1989.
Rand GM. Behavior. In: Rand GM, Petrocelli AR, eds. *Fundamentals of Aquatic Toxicology*. Washington, DC: Hemisphere; 1985:221–263.
Rands MRW. Pesticide use on cereals and the survival of grey partridge *Perdix perdix* chicks in a field experiment. *J Appl Ecol*. 1985;22:49.
Reichert WL, Myers MS, Peck-Miller K, et al. Molecular epizootiology of genotoxic events in marine fish: linking contaminant exposure, DNA damage, and tissue-level alterations. *Mutat Res Rev Mutat Res*. 1998;411:215–225.
Reid NM, Proestou DA, Clark BW, et al. The genomic landscape of rapid repeated evolutionary adaptation to toxic pollution in wild fish. *Science*. 2016;354:1305–1308.
Reinfelder JR, Fisher NS. The assimilation of elements ingested by marine copepods. *Science*. 1991;251:794.
Rice CD, Schlenk D. Immune function and cytochrome P4501A activity after acute exposure to 3,3,4,4,5-pentachlorobiphenyl (PCB 126) in channel catfish. *J Aquat Anim Health*. 1995;7:195–204.
Richardson CJ, Di Giulio RT, Tandy NE. *Free Radical Mediated Processes as Markers of Air Pollution Stress in Trees. Biologic Markers of Air-Pollution Stress and Damage in Forests*. Washington, DC: National Academy Press; 1989:251–260.
Roberts TC, Hart JR, Kaikkonen MU, et al. Quantification of nascent transcription by bromouridine immunocapture nuclear run-on RT-qPCR. *Nat Protoc*. 2015;10-1198-211.
Rodriguez-Ubreva J, Ballestar E. Chromatin immunoprecipitation. *Methods Mol Biol*. 2014;1094:309–318.
Rogers JT, Richards JG, Wood CM. Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol*. 2003;64:215–234.
Ross P, De Swart R, Addison R, et al. Contaminant-induced immunotoxicity in harbour seals: wildlife at risk? *Toxicology*. 1996;112:157–169.
Rotchell JM, Ostrander GK. Molecular markers of endocrine disruption in aquatic organisms. *J Toxicol Environ Health B*. 2003;6:453–495.
Rothbart SB, Krajewski K, Strahl BD, et al. Peptide microarrays to interrogate the "histone code." *Methods Enzymol*. 2012;512:107–135.
Rowan DJ, Rasmussen JB. Bioaccumulation of radioceasium by fish: the influence of physicochemical factors and trophic structure. *Can J Fish Aquat Sci*. 1994;51:2388–2410.
Ruiz JM, Barreiro R, Gonzalez JJ. Biomonitoring organotin pollution with gastropods and mussels. *Mar Ecol Prog Ser*. 2005;287:169–176.
Saavedra Alvarez MM, Ellis DV. Widespread neogastropod imposex in the Northeastern Pacific: implications for TBT contamination surveys. *Mar Pollut Bull*. 1990;21:244–247.
Salminen J, Van Gestel CAM, Oksanen J. Pollution-induced community tolerance and functional redundancy in a decomposer food web in metal-stressed soil. *Environ Toxicol Chem*. 2001;20:2287.
Sanchez-Dardon J, Voccia I, Hontela A, et al. Immunomodulation by heavy metals tested individually or in mixtures in rainbow trout (*Oncorhynchus mykiss*) exposed *in vivo*. *Environ Toxicol Chem*. 1999;18:1492–1497.
Sandahl JF, Baldwin DH, Jenkins JJ, Scholtz NL. Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in coho salmon exposed to chlorpyrifos. *Environ Toxicol Chem*. 2005;24:136–145.
Saucier D, Astic L, Rioux P. The effects of early chronic exposure to sublethal copper on the olfactory discrimination of rainbow trout, *Oncorhynchus mykiss*. *Environ Biol Fishes*. 1991;30:345–351.
Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med*. 2001;30:1191–1212.
Schindler DW. A personal history of the Experimental Lakes Project. *Can J Fish Aquat Sci*. 2009; 66: 1837–1847.
Schlenk D, Benson W. *Target Organ Toxicity in Marine and Freshwater Teleosts*. New York: Taylor & Francis; 2001.
Schlezinger JJ, Stegeman JJ. Induction and suppression of cytochrome P450 1A by 3,3,4,4,5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (*Stenotomus chrysops*). *Aquat Toxicol*. 2001;52:101–115.
Schmidt CW. Toxicogenomics. *Environ Health Perspect*. 2002;110:A750–A755.
Schmitt CJ, Whyte JJ, Brumbaugh WG, Tillitt DE. Biochemical effects of lead, zinc, and cadmium from mining on fish in the Tri-States district of northeastern Oklahoma, USA. *Environ Toxicol Chem*. 2005;24:1483–1495.
Scholz NL, Truelove NK, French BL, et al. Diazinon disrupts antipredator and homing behaviors in Chinook salmon (*Oncorhynchus tshawytscha*). *Can J Fish Aquat Sci*. 2000;57:1911–1918.
Schwacke LH, Voit EO, Hansen LJ, et al. Probabilistic risk assessment of reproductive effects of polychlorinated biphenyls on bottlenose dolphins (*Tursiops truncatus*) from the southeast United States coast. *Environ Toxicol Chem*. 2002;21:2752–2764.
Schulz S, Haussler S. Chromatin immunoprecipitation for ChIP-chip and ChIP-seq. *Methods Mol Biol*. 2104;1149:591–605.
Scott GR, Sloman KA. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquat Toxicol*. 2004;68:369–392.
Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL. Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. *Environ Health Perspect*. 1996;104:1296–1300.
Sherratt TN, Roberts G, Williams P, et al. A life-history approach to predicting the recovery of aquatic invertebrate populations after exposure to xenobiotic chemical. *Environ Toxicol Chem*. 1999;18:2512.
Shugart L. DNA damage as a biomarker of exposure. *Ecotoxicology*. 2000;9:329–340.
Sies H, Cadenas E. Oxidative stress: damage to intact cells and organs. *Philos Trans R Soc Lond B Biol Sci*. 1985;311:617–631.

Simkiss K, Daniels S, Smith RH. Effects of population density and cadmium toxicity on growth and survival of blowflies. *Environ Pollut*. 1993;81:41.
Simonich SL, Hites RA. Global distribution of persistent organochlorine compounds. *Science*. 1995;269:1861.
Sinnhuber RO, Hendricks JD, Wales JH, Putnam GB. Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann N Y Acad Sci*. 1977;298:389–408.
Smith GM, Weis JS. Predator–prey relationships in mummichogs (*Fundulus heteroclitus* (L.)): effects of living in a polluted environment. *J Exp Mar Biol Ecol*. 1997;209:75–87.
Snape JR, Maund SJ, Pickford DB, Hutchinson TH. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquat Toxicol*. 2004;67:143–154.
Sokolova IM. Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *J Exp Biol*. 2004;207:2639–2684.
Sol SY, Johnson LL, Horness BH, Collier TK. Relationship between oil exposure and reproductive parameters in fish collected following the Exxon Valdez oil spill. *Mar Pollut Bull*. 2000;40:1139–1147.
Solenska M, Micieta K, Misik M. Plant bioassays for an in situ monitoring of air near an industrial area and a municipal solid waste—Zilina (Slovakia). *Environ Monit Assess*. 2006;115:499–508.
Solomon KR, Baker DB, Richards RP, et al. Ecological risk assessment of atrazine in North America surface waters. *Environ Toxicol Chem*. 1996;15:31–76.
Solomon KR, Sibley P. New concepts in ecological risk assessment: where do we go from here? *Mar Pollut Bull*. 2002;44:279.
Spitsbergen JM, Walker MK, Olson JR, Peterson RE. Pathological alterations in early life stages of lake trout, *Salvelinus namaycush*, exposed to 2,3,7,8-tetrachloro-*para*-dioxin as fertilized eggs. *Aquat Toxicol*. 1991;19:41–71.
Spitzer PR, Risebrough RW, Walker W II, et al. Productivity of ospreys in Connecticut–Long Island increases as DDE residues decline. *Science*. 1978;202:333.
Spromberg JA, John BM, Landis WG. Metapopulation dynamics: indirect effects and multiple distinct outcomes in ecological risk assessment. *Environ Toxicol Chem*. 1998;17:1640.
Spry DJ, Wood CM. Ion flux rates, acid–base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft water. *Can J Fish Aquat Sci*. 1985;42:1332–1341.
Steinert SA. DNA damage as a bivalve biomarker. *Biomarkers*. 1999;4:492–496.
Stephan CE, Mount DI, Hansen DJ, Gentile JH, Chapman GA, Brungs WA. *Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and their Uses*. Duluth, MN: U.S. EPA; 1985.
Strandberg B, Axelsen JA, Pedersen MB, Jensen J, Attrill MJ. Effect of a copper gradient on plant community structure. *Environ Toxicol Chem*. 2006;25:743.
Sumpter JP, Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ Health Perspect*. 1995;103(suppl 7):173–178.
Tanaka Y, Nakanishi J. Life history elasticity and the population-level effect of *p*-nonylphenol on *Daphnia galeata*. *Ecol Res*. 2001;16:41.
Tanguay RL, Andreasen EA, Walker MK, Peterson RE. Dioxin toxicity and aryl hydrocarbon receptor signaling in fish. In: Schecter A, Gasiewicz TA, eds. *Dioxins and Health*. Hoboken, NJ: Wiley; 2003:603–628.
Taub FB. Unique information contributed by multispecies systems: examples from the standardized aquatic microcosm. *Ecol Appl*. 1997;7:1103–1110.
Teraoka H, Dong W, Tsujimoto Y, et al. Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in zebrafish. *Biochem Biophys Res Commun*. 2003;304:223–228.
Tessier A, Campbell PGC, Auclair JC, Bisson M. Relationships between partitioning of trace metals in sediments and their accumulation in the tissues of the freshwater mollusc *Elliptio complanata* in a mining area. *Can J Fish Aquat Sci*. 1984;41:1463.
Thomann RV. Bioaccumulation model of organic chemical distribution in aquatic food chains. *Environ Sci Technol*. 1989;23:699.
Tilman D. Biodiversity: population versus ecosystem stability. *Ecology*. 1996;77:350.
Tilman D, Reich PB, Knops JMH. Biodiversity and ecosystem stability in a decade-long grassland experiment. *Nature*. 2006;441:629–632.
Tilman D, Wedin D, Knops J. Productivity and sustainability influenced by biodiversity in grassland ecosystems. *Nature*. 1996;379:718.
Trimble EL. A guest editorial: update on diethylstilbestrol. *Obstet Gynecol Surv*. 2001;56:187–189.
Truhaut R. Ecotoxicology: objectives, principles and perspectives. *Ecotoxicol Environ Saf*. 1977;1:151.
U.S. EPA OPPTS. *OPPTS Harmonized Guidelines, Series 850—Ecological Effects Test Guidelines*. Washington, DC: Office of Prevention, Pesticides, and Toxic Substances, U.S. Environmental Protection Agency; 1996a.
U.S. EPA OPPTS. *Ecological Effects Test Guidelines, OPPTS 850.1000. Special Considerations for Conducting Aquatic Laboratory Studies*. EPA 712–C–96–113. Washington, DC: Office of Prevention, Pesticides, and Toxic Substances, U.S. Environmental Protection Agency; 1996b.
Van Aggelen G, Ankley GT, Baldwin WS, et al. Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: hurdles, achievements, and future outlook. *Environ Health Perspect*. 2010;118:1–5.
van der Oost R, Beyer J, Vermeulen NPE. Fish bioaccumulation and biomarkers in environmental risk assessment. *Environ Toxicol Pharmacol*. 2003;13:57–149.
Van Metre PC, Mahler BJ. Trends in hydrophobic organic contaminants in urban and reference lake sediments across the United States, 1970–2001. *Environ Sci Technol*. 2005;39:5567–5574.
Van't Hof AE, Edmonds N, Dalikova M, Marec F, Saccheri IJ. Industrial melanism in British peppered moths has a singular and recent mutational origin. *Science*. 2011;332:958–960.
Vethaak ADT. Fish disease as a monitor for marine pollution: the case of the North Sea. *Rev Fish Biol Fish*. 1992;2:1–32.
Villeneuve DL, Crump D, Garcia-Reyero N, et al. Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicol Sci*. 2014a;142:312–320.
Villeneuve DL, Crump D, Garcia-Reyero N, et al. Adverse outcome pathway (AOP) development II: best practices. *Toxicol Sci*. 2014b;142:321–330.
Vogelbein WK, Fournie JW, Van Veld PA, Huggett RJ. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. *Cancer Res*. 1990;50:5978–5986.
Vos JG, Bossart G, Fournier M, O'Shea T. *Toxicology of Marine Mammals*. New York: Taylor & Francis; 2002.
Walker CH. Neurotoxic pesticides and behavioural effects upon birds. *Ecotoxicology*. 2003;12:307–316.
Walker CH, Sibley RM, Hopkin SP, Peakall DB. *Principles of Ecotoxicology*. 4th ed. Boca Raton, FL: CRC Press; 2012.
Wania DM, Mackay D. Tracking the distribution of persistent organic pollutants. *Environ Sci Technol*. 1996;30:390A.
Wania DM, Mckay D, Li Y-F, Bidleman TF, Strand A. Global chemical fate of α-hexachlorocyclohexane. 1. Evaluation of a global distribution model. *Environ Toxicol Chem*. 1999;18:1390.
Wania F, Westgate JN. On the mechanism of mountain cold-trapping of organic chemicals. *Environ Sci Technol*. 2008;42:9092–9098.
Wassenberg D, Nerlinger A, Battle L, Di Giulio R. Effects of the PAH heterocycles, carbazole and dibenzothiophene, on *in vivo* and *in vitro* CYP1A activity and PAH-derived embryotoxicity. *Environ Toxicol Chem*. 2005;24:2526–2532.
Wassenberg DM, Di Giulio RT. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonists with cytochrome P4501A inhibitors in *Fundulus heteroclitus*. *Environ Health Perspect*. 2004a;112:1658–1664.
Wassenberg DM, Di Giulio RT. Teratogenesis in *Fundulus heteroclitus* embryos exposed to a creosote-contaminated sediment extract and CYP1A inhibitors. *Mar Environ Res*. 2004b;58:163–168.
Watanabe H, Iguchi T. Using ecotoxicogenomics to evaluate the impact of chemicals on aquatic organisms. *Mar Biol*. 2006;149:107–115.
Wayland M, Bollinger T. Lead exposure and poisoning in bald eagles and golden eagles in the Canadian prairie provinces. *Environ Pollut*. 1999;104:341.
Webb NA, Wood CM. Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem*. 1998;17:579–588.

Webb RE, Horsfall F Jr. Endrin resistance in pine mouse. *Science*. 1967;156:1762.

Webber HM, Haines TA. Mercury effects on predator avoidance behavior of a forage fish, golden shiner (*Notemigonus crysoleucas*). *Environ Toxicol Chem*. 2003;22:1556–1561.

Weinstein JE, Oris JT, Taylor DH. An ultrastructural examination of the mode of UV-induced toxic action of fluoranthene in the fathead minnow, *Pimephales promelas*. *Aquat Toxicol*. 1997;39:1–22.

Wells RS, Tornero V, Borrell A, et al. Integrating potential life-history and reproductive success data to examine relationships with organochlorine compounds for bottlenose dolphins (*Tursiops truncatus*) in Sarasota Bay, Florida. *Sci Total Environ*. 2005;349:106–119.

Wendelaar Bonga SE, Lock RAC. Toxicants and osmoregulation in fish. *Neth J Zool*. 1992;42:478–493.

Wendelaar Bonga SE, Lock RAC. The osmoregulatory system. In: Di Giulio RT, Hinton DE, eds. *The Toxicology of Fishes*. Boca Raton, FL: CRC Press. 2008.

Wester PW, van der Ven LTM, Vethaak AD, Girnwis GCM, Vos JG. Aquatic toxicology: opportunities for enhancement through histopathology. *Environ Toxicol Pharmacol*. 2002;11:289–295.

Westgate JN, Wania F. Model-based exploration of the drivers of mountain cold-trapping in soil. *Environ Sci: Processes Impacts*. 2013;15:2220–2232.

Weston DP, Maruya KA. Predicting bioavailability and bioaccumulation with in vitro digestive fluid extraction. *Environ Toxicol Chem*. 2002;21:962.

Wiemeyer SN, Scott LM, Anderson MP, Bloom PH, Stafford CJ. Environmental contaminants in California condors. *J Wildl Manage*. 1988;52:238–247.

Wiese FK, Robertson GJ. Assessing seabird mortality from chronic oil discharges at sea. *J Wildl Manage*. 2004;68:627–638.

Williams CR, MacDonald JW, Bammler TK, et al. Cadmium exposure differentially alters odorant-driven behaviors and expression of olfactory receptors in juvenile coho salmon (*Oncorhynchus kisutch*). *Toxicol Sci*. 2016;154:267–277.

Wilson L, Martin PA, Elliot JE, Mineau P, Cheng KM. Exposure of California quail to organophosphorus insecticides in apple orchards in the Okanagan Valley, British Columbia. *Ecotoxicology*. 2001;10:79–90.

Winston GW, Adams SM, Benson WH, et al. Biological bases of similarities and differences. In: Di Giulio RT, Benson WH, eds. *Interconnections Between Human Health and Ecological Integrity*. Pensacola, FL: SETAC Press; 2002.

Winston GW, Di Giulio RT. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat Toxicol*. 1991;19:137–161.

Wirgin I, Currie D, Garte SJ. Activation of the K-ras oncogene in liver tumors of Hudson River tomcod. *Carcinogenesis*. 1989;10:2311–2315.

Woin P. Short- and long-term effects of the pyrethroid insecticide fenvalerate on an invertebrate pond community. *Ecotoxicol Environ Saf*. 1998;41:137.

Wolfe MF, Schwarzbach S, Sulaiman RA. Effects of mercury on wildlife: a comprehensive review. *Environ Toxicol Chem*. 1998;17:146–160.

Woodward M. *Epidemiology: Study Design and Data Analysis*. 2nd ed. Boca Raton, FL: Chapman & Hall; 2005.

Wootton EC, Dyrynda EA, Ratcliffe NA. Comparisons of PAH-induced immunomodulation in three bivalve mollusks. *Aquat Toxicol*. 2003;65:13–25.

Wu RSS. Hypoxia: from molecular responses to ecosystem response. *Mar Pollut Bull*. 2002;45:35–45.

Xu EG, Mager EM, Grosell M, et al. Time- and oil-dependent transcriptomic and physiological responses to Deepwater Horizon oil in mahi-mahi (*Coryphaena hippurus*) embryos and larvae. *Environ Sci Technol*. 2016;50:7842–7851.

Ye F, Samuels DC, Clark T, et al. High-throughput sequencing in mitochondrial DNA research. *Mitochondrion.* 2014;17:157–163.

Ylitalo GM, Sten JE, Hom T, et al. The role of organochlorines in cancer-associated mortality in California sea lions (*Zalophus californianus*). *Mar Pollut Bull*. 2005;50:30–39.

Yong WS, Hsu FM, Chen PY. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

Zinsstag J, Schelling E, Waitner-Toews MW, Tanner M., eds. *One Health: The Theory and Practice of Integrated Health Approaches*. Oxfordshire, UK: CABI; 2015.

chapter 31

Air Pollution*

Daniel L. Costa and Terry Gordon

Air Pollution in Perspective
- A Brief History of Air Pollution and its Regulation

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- Animal-to-Human Extrapolation: Issues and Mitigating Factors

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Acknowledgments

References

AIR POLLUTION IN PERSPECTIVE

The second half of the 20th century was marked by remarkable changes in how the public viewed its relationship to the environment. In the 19th and early 20th centuries, national pride and prosperity in Western countries were often depicted as an expanse of urban factories with smokestacks belching opaque dark clouds of industrial effluent into a neutral blue sky. But the price of that unchecked human progress led to several air pollution catastrophes highlighting the profoundly detrimental impact that reckless prosperity could have on the environment. These images of "modern" life gradually gave rise to public outcry for governmental action to protect air quality and public health—a challenge to industry that had been focused on economic growth alone. The ensuing 50 years of regulatory legislation in the United States and Western Europe along with cost efficient innovations by the private sector have remade this industrial image in most technologically developed nations.

Ironically, as regulatory control measures began to reduce emissions from stationary industrial sources of air pollution, highways to "open spaces" and urban flight took many people to the suburbs with its cleaner air and safe, comfortable lifestyle. Meanwhile, the developing world saw little of this growth and what grew was frequently cast-off old technology and variants of exploitation by the Western corporations attracted by abundant resources, a cheap work force, and fewer regulations or constraints. This situation has persisted in the 21st century but is evolving with broader globalization of improved technology and communication.

The change in land use and demography in the United States in the 1950s and 1960s altered the national character and distribution of air pollution. The commute from suburban home to city workplace back to suburban home led increasingly to congested thoroughfares, whose emissions contributed to a photochemical cauldron of oxidant air pollution around expanding metro-suburban areas. Moreover, postwar population growth and rising expectations

*This chapter has been reviewed by the U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and the policies of the Agency.

for a better (peace-time) standard of living led to unrestrained consumption, including inexpensive gasoline for commuting and recreation. Similar patterns of unrestrained growth are occurring in developing nations throughout the world.

Today, the search is worldwide for cheap oil to fuel transportation and goods movement and international sources are increasingly prominent. For nontransportation sectors of industry and electricity generation, low cost and domestic availability have brought coal to the top of the energy pyramid in many countries. Other energy sources, natural gas and biomass in its varied forms, are being intensively explored to meet the energy demands of modern life. Most notable as a potential major resource is natural gas which is being recovered through less conventional methods (e.g., fracking), which has brought about a revolution in energy resources in the United States. Decades to come will see an evolution in our energy portfolio driven by cost and access, environmental impacts including climate change, and technological innovation. Nevertheless, so long as organically derived fuel is combusted to derive energy, its potential for impact on air quality and on public health and the environment will remain.

While great strides have been made in balancing regulation and technology to reduce emissions from stationary and mobile sources, unsatisfactory air quality continues to impose a risk to public and environmental health in developing countries and even in highly developed countries throughout the world. For example, more than half the U.S. population resides in counties that are not in compliance with current National Ambient Air Quality Standards (NAAQS) (Fig. 31-1). These noncompliant areas correspond well with where people live and work and reflect the redistribution of population growth from major urban and industrial centers. Air pollutants can also be transported across many miles such that previously pristine, rural areas have measurable pollution above their historic background levels. Indeed, as the developing world grows industrially, air pollution now is intercontinental with transport through the atmosphere via pathways close to the Earth's surface as well as the upper atmosphere. Emissions of precursors of ozone can come from hundreds of miles away and contribute to local pollution levels; likewise other emissions such as sand storms in Asia can travel many thousands of miles from the original sources.

While episodes of extreme air pollution are rare in the Western world today, occasionally, unusual meteorological stagnations exaggerate typical air pollution patterns both in the levels of pollutants and their expanse (e.g., 1995 in the United Kingdom and Western Europe; 2017 in Delhi). Likewise, in specific locales prone to stagnations due to topography (e.g., Utah Valley through the 1980s to 1990s) or where there exists a single dominant source (e.g., port areas, transport terminals, or smelters), repeated episodic or even patterned poor air quality can impact public health. From a broader public community perspective, typical exposures are characterized by prolonged periods of relatively low, cyclic levels of complex mixtures of photochemically transformed industrial and mobile emissions with occasional moderate excursions due to weather patterns. As pollution now extends even into remote and wilderness areas, significant damage to flora and crops can also occur.

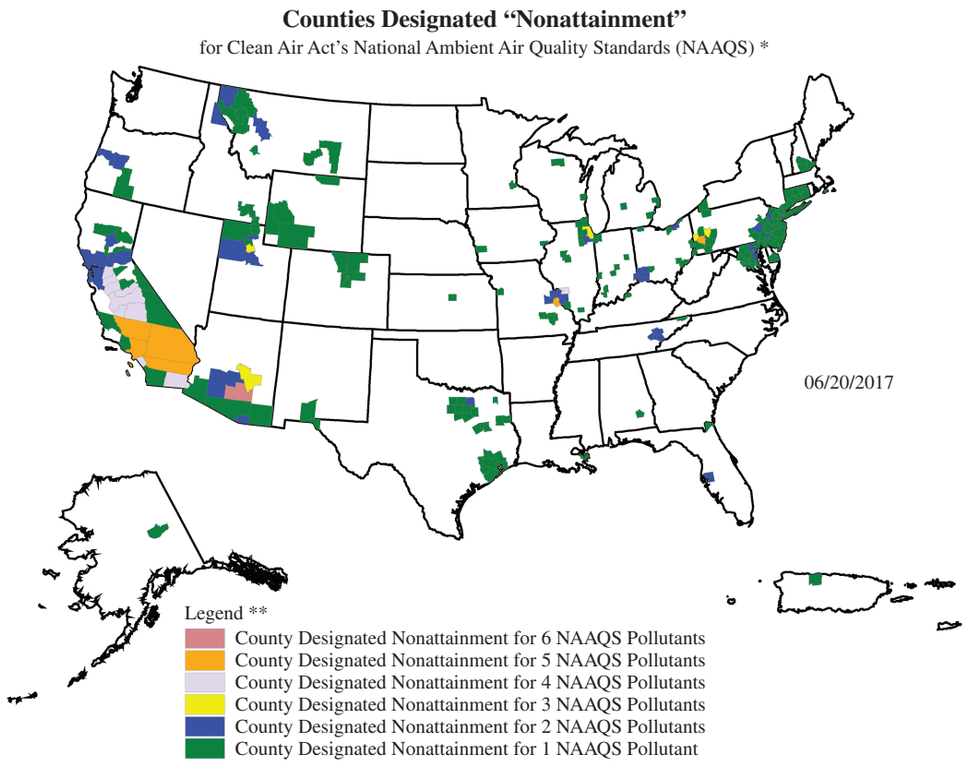


Figure 31-1. *Counties in the United States not in attainment (current as of July 2017) for the NAAQS criteria pollutants O_3, $PM_{2.5}$ and PM_{10}, SO_2, and Pb at their latest promulgated standard* (available at: https://www.epa.gov/green-book). There were no designated nonattainment areas based on the 1971 NAAQS for CO and NO_2. As of 2010 a revised NAAQS for NO_2 was promulgated—designations are not completed. The composite map can be found at: https://www3.epa.gov/airquality/greenbook/mapnpoll.html.

The situation in many developing countries experiencing rapid population growth, industrialization, and economic expansion is reminiscent of the urban, industrial pollution in the United States and Europe more than half a century ago. The lessons of Western industrial nations and the consequences of inadequate air pollution control in some countries have not necessarily outweighed economic drivers or public health concerns. For example, despite being a modern economic center, two marathoners died in Hong Kong in February 2006 while running on a weekend during a protracted period of severe air pollution. Just as in U.S. history, the urban air in rapidly industrialized nations simply reflects poor air quality management and the desire to prosper economically. Air pollutants levels in some of the developing urban centers can run 10 or more times those of the U.S. today, and their expanding worldwide distribution contributes substantially to the global burden of many air pollutants such as mercury, particulate matter, sulfate/SO_2, and major global warming pollutants such as black carbon, CO_2, and methane. One example of episodic air pollution occurs in India when the postharvest burning of agricultural residue and Diwali festival celebrations coincide. A willingness to balance worldwide economic growth and industrialization using lessons learned from the misadventures of the Second Industrial Revolution and the Age of the Automobile will determine the impact of the "new" global economy on the health of both the Earth and its inhabitants.

Over the last several decades, there has been growing awareness and documentation of the disparities in health across the globe. These disparities are linked to poverty, access to clean food and water, as well as health care and education, autocratic governments, wars, and all that is associated with these population deficiencies and stressors. Globally scientists, physicians, and various public health agencies—led by the World Health Organization (WHO)—have taken yearly accounting of disease, deaths, births, and various related data to compile and publish a series of articles in *The Lancet*, every 2 to 3 years, providing a comprehensive assessment of global health that is called the *Global Burden of Disease (GBD)* (Murray, 2016). Air pollution—both indoor and outdoor—and tobacco smoke account for a large fraction of chronic cardiopulmonary disease, the major cause of death in what is estimated as 8 million deaths per year. Importantly, inhalation of toxic inhalants is among the five top killers around the planet.

The problem facing many parts of the developing world ties closely to national domestic culture and economy, as well as to the level of technological sophistication. Prime among these problems is exposure to carbon and soot from combustion of biomass in cooking and heating in domestic stoves. Approximately 3 billion people worldwide use biomass for home cooking in households with little ventilation. Among the 7 to 8 million deaths per year resulting from these exposures are primarily women who are exposed day in and day out over many years, often with their infant children by their sides. Understanding the intersection of technological as well as socioeconomic and political challenges will be at the core of any resolution to these issues.

In the developed world, air pollution has a long history, but in the last century it has been largely tied to technological and energy evolution. Estimates of smoke, which could be seen with the naked eye, could be collected and compiled as general indices of haziness or the darkness of a spot on a filter. Many people experienced irritancy, choking or soot accumulation around their noses which eventually drove interest into learning whether the soot was unhealthy. Then when there were health symptoms, interest in causation over time drove science to focus more on individual pollutants associated with some source or haze to better understand the linkages with health and to aid in mitigation of effects. In turn, this knowledge was used to develop both public health standards and to establish regulatory controls—an evolution in public spirit and perseverance that took decades.

Despite the obvious complexities of urban air pollution, it was felt that single pollutant research and regulations were the best path to understanding what was going on, and indeed, this approach brought great strides in providing the compelling information needed to drive both public and political sentiment. However, research agendas have advanced and have taken the challenge to study multipollutant interactions and transformation of individual pollutant components within complex photochemical, atmospheric systems—a truer reflection of real-world exposure complexities. These multipollutant approaches entail sophisticated statistical models and empirical study designs, all with the goal of a more complete systems-based understanding of air pollution and risk to maximize benefits while minimizing costs and unintended consequences.

This chapter presents an overview of the current state of knowledge regarding air pollution and its ensuing impacts on human health, and the role of toxicology and translational biology in unraveling the complexities to allow for informed assessment of risk. As such, the contextual complexities of regulatory decisions and risk assessment are also addressed. The intent is to provide the reader with both a fundamental knowledge and appreciation of the nature of the problem, how traditional and modern-day approaches aid in this understanding, as well as a sense of the uncertainties in need of future investigation to resolve issues even before they arise.

A Brief History of Air Pollution and its Regulation

For most of history, air pollution has been a problem of microenvironments and domestic congestion. The smoky fires of early cave and hut dwellers choked the air inside their homes, and even when the emissions were vented outdoors, they simply combined with those of the neighbors to settle around the village on damp cold nights. With urbanization and a concomitant decrease in forest wood as a source of fuel to heat and cooking, the need for energy led to the burning of easily accessible, dirty coal and the ambient release of sulfurous, sooty smoke. Industrialization brought kilns to make quicklime for construction and metal smelters needed for the development of progressive "modern" cities, only to push smoke and chemical emissions into the air. Unfortunately, the city dwellers who worked near these industries had to endure the bad air, while those of wealth frequently had country homes to which they could escape. The poor quality of urban air has been captured historically by many writers including Charles Dickens, who noted London's fogs in his writings, all the way back to the ancients like Seneca, the Roman philosopher, who in AD 61 wrote: "As soon as I had gotten out of the *heavy air* of Rome, and from the *stink* of the chimneys thereof, which being stirred, poured forth whatever *pestilential vapors and soot* they had enclosed in them, I felt an *alteration to my disposition*" (emphasis added: Miller and Miller, 1993).

Historically, efforts to regulate air pollution have, like today, competed with concerns for national, regional, and industrial economies, and as a result, they have evolved slowly. Early on, in the time of Greece and Rome, individual civil suits could be levied against local polluters, although these were of marginal success. Beginning in the 13th century, community-based outcries received some recognition by governing officials; one example was the banning of "sea coal" from lime kilns and domestic heaters in London by Edward I. Enforcement, however, was not effective and the populace largely resigned itself to polluted air as part of urban life. By the 17th century, England, in the middle of several decades that some refer to as "the little ice age," depleted its forest wood resources, which only increased reliance on

sea coal for domestic heating. Meanwhile Percival Pott's discovery that chimney soot was related to the incidence of scrotal cancer in chimney sweeps led to some in the health community to recommend: "Fly the city, shun its turbid air; breathe not the chaos of eternal smoke . . ." (Brimblecombe, 1999)—essentially the same advice advanced by Seneca 1600 years earlier. In the late 18th century, the industrial revolution, which was powered by the burning of "cleaner" mined coal, added a second dimension to urban air pollution. These emissions were more acidic and hung in the air longer than the fluffy soot of the cheaper sea coal. Continued soiling of buildings and damage to nearby crops brought community boards to address sanitary reforms to cut the worse of the pollution peaks and episodes, but any gains were soon offset by growth. By the end of the 19th century and into the early 20th century, power plants were being built to provide energy for factories and eventually to light homes in the Western world. Steel mills and other industries proliferated along riverbanks and lakeshores, oil refineries rose in port cities and near oil fields, and smelters roasted and refined metals in areas near large mineral deposits.

By 1925, air pollution was common to all industrialized nations in the Western hemisphere. However, people slowly grew less tolerant of the nuisance of acidic-soot corrosion of all exposed surfaces and the general discomfort that came with smoky air—this acidic, sooty form of air pollution was termed *reducing* air pollution because of its reducing chemical nature. Public surveys were initiated—in Salt Lake City in 1926, New York City in 1937, and Leicester, Great Britain, in 1939—to bring political attention to the problem and promote the implementation of controls (Miller and Miller, 1993). However, it was the cumulative impact of the great air pollution disasters in the Meuse Valley, Belgium, in 1930; Donora, Pennsylvania, in 1948; and the great London fog of 1952 that indicted air pollution as a health risk. In the United States, California was already leading the way with passage of the Air Pollution Control Act of 1947 to regulate the discharge of opaque smokes. Visibility problems in Pittsburgh during the 1940s had also prompted efforts to control smoke from local industries. At the national level, however, it was the initiative of President Truman that provided the federal impetus to deal with air pollution. His early efforts culminated in congressional passage of a series of acts starting with the Air Pollution Control Act of 1955 under the Eisenhower administration.

The prosperity within North America in the late 1950s and the accompanying suburban sprawl introduced the third and perhaps most chemically complex dimension of air pollution. The term *smog,* though originally coined to describe the mixture of smoke and fog that hung over large cities such as London, was curiously adopted for the eye-irritating photochemical reaction products of auto exhaust that blanketed cities like Los Angeles and Mexico City. Early federal legislation addressing stationary sources was soon expanded to include automobile-derived pollutants in the United States (the Clean Air Act of 1963, amended in 1967, and the Motor Vehicle Air Pollution Control Act of 1965). The landmark Clean Air Act Amendments (CAAA) of 1970 evolved from the early legislation, and despite being only an amendment to the CAA, it was revolutionary. It recognized the problem of air pollution as a national issue and set forth a plan to control it. The Act charged the newly instituted U.S. Environmental Protection Agency (U.S. EPA) with the responsibility to protect the public from the hazards of polluted outdoor air. Seven "criteria" air pollutants (ozone (O_3), sulfur dioxide (SO_2), particulate matter (PM), nitrogen dioxide (NO_2), carbon monoxide (CO), lead (Pb), and total hydrocarbons—the last now dropped from the list, leaving six criteria pollutants) were specified as significant health hazards in need of *individual* NAAQS. Sections 108 and 109 of the CAA mandates a process for review of the NAAQS every 5 years as to the adequacy of the existent standard to protect human health (Table 31-1), although adherence to this schedule has generally not been achieved due to the rigor of the process itself (U.S. EPA, 2005). The explosion in the literature databases for the criteria pollutants and the extensive review and commentary process has typically led to delays in completing the process on schedule. In 2006, however, this process was streamlined through the creation of an Integrated Science Assessment (ISA) to replace each Criteria Document where the findings since the last assessment could be integrated with older studies (summarized with the foundation literature confined to tables and an appendix). This ISA is combined with a Risk and Exposure Assessment into a Policy Assessment to develop a range of proposed standards based on risk analyses. As per the CAAA of 1970, the U.S. EPA Administrator considers proposals from both "evidence" and "risk" based assessments to establish policy and set the NAAQS. Regarding the Primary NAAQS, only health criteria can be considered in the development of the standard, including safety considerations for potentially susceptible groups. The Secondary NAAQS consider agricultural and structural welfare. Economic impacts are not to be involved in standard setting process itself—only in assessing the cost of the implementation procedures. Other hazardous air pollutants (HAPs), of which there were 8 listed in 1970, were also to undergo health assessments to establish emission controls—today there are 188 (see the section "Hazardous Air Pollutants"). The CAAA of 1970 was by far the most far-reaching legislation to control air pollution to date.

Disasters involving HAPs have occurred and the accidental release of 30 tons of methyl isocyanate vapor into the air of the shanty village of Bhopal, India, on December 3, 1984, killed an estimated 3000 people within hours of the release, with several thousand delayed deaths and 200,000 injured or permanently impaired. The tragedy shocked the world, and raised the issue of HAPs in the United States to a new level of concern. While such a disaster

Table 31-1

National Ambient Air Quality Standards*

POLLUTANT	PRIMARY/ SECONDARY	AVERAGING	TIME LEVEL
Sulfur dioxide	Primary Secondary	1 hour 3 hours	75 ppb 0.5 ppm
Carbon monoxide	Primary	1 hour 8 hours	35 ppm 9 ppm
Ozone	Primary and secondary	8 hours	0.070 ppm
Nitrogen dioxide	Primary	1 hour Annual	100 ppb 53 ppb
Particulates: PM_{10}	Primary and secondary	24 hours	150 $\mu g/m^3$
$PM_{2.5}$	Primary and secondary	24 hours Annual	35 $\mu g/m^3$ 12 $\mu g/m^3$
Lead	Primary and secondary	Rolling 3-month average	0.15 $\mu g/m^3$

**For detailed information regarding policy and precise statistical and time-based computations to achieve attainment, contact EPA Web site: http://www.epa.gov/air/criteria.html.*

has never struck the United States, accidental industrial releases or spills of toxic chemicals are surprisingly common, with 4375 cases recorded between 1980 and 1987, inflicting 11,341 injuries and 309 deaths (Waxman, 1994). The Toxic Release Inventory (TRI) of 650 chemicals reported annually by industry is required by EPA and suggests a decrease in releases (approximately 9% in 10 years across land, water, and air), but these data are self-reported and do not include most of the 80,000 low-volume chemicals in use today.

The HAPs, which had been the stepsister of the criteria pollutants for more than a decade after the passage of the 1970 CAA, have since garnered more public and policy attention. There is concern not only for the acute effects of accidental releases of fugitive or secondary chemicals—such as phosgene, benzene, butadiene, and dioxin into the air of populated industrial centers—but also for potential chronic health effects, with cancer often being the focus of attention. The slow progress of regulatory decisions on HAPs (only eight between 1970 and 1990) led to a mandated acceleration of the process under the CAA amendment of 1990. Section 112(b) currently lists 188 chemicals or classes of chemicals for which special standards and risk assessments are required. The chemicals listed are those of greatest concern based on toxicity (including cancer) and estimated release volumes. Currently, there is a list of 33 HAPs from the list of 188 that are deemed to be of greatest concern, so-called a list of the "dirty 30." Emissions of HAPs are mandated for control to the maximal achievable control technology (MACT), and any residual health risk after MACT is to be considered in a separate quantitative risk assessment. The database for this process utilizes existing knowledge or, if necessary, mandates further research by the emitter. While many of these chemicals are now better controlled in the United States than in the past, residual risk estimates are yet to be completed for many HAPs. The database from which these assessments are made is called the Integrated Risk Information System (IRIS; U.S. EPA, 2016) and currently contains 754 chemicals which have completed assessments.

Emissions from motor vehicles include several HAPs (e.g., benzene and butadiene) as well as NAAQS pollutants (e.g., CO, NO_x, and PM), but in the United States these are addressed primarily under the CAA Title II, Emission Standards for Mobile Sources. The health data may draw from IRIS but the regulatory aspect is specific to this legislation. The reduction of emissions from mobile sources is complex and involves both fuel formulation and engine/vehicle reengineering. Refinements in combustion engineering are ongoing making use of advanced computerized ignition and timing, but fuel properties have drawn most recent attention for improvement. For example, to reduce wintertime CO, several oxygenates (including ethers and alcohols) have been formulated into fuels both to reduce cold-start emissions and enhance overall combustion. Perhaps the most prominent of the ethers is MTBE (methyl tertiary butyl ether), which became a controversial additive in the early 1990s, arising in part from odor and reports of asthma-like reactions by some individuals during auto refueling. However, the controversy took an unexpected twist; MTBE was removed from fuel, not because health concerns associated with airborne exposure but rather due to leakage from service-station storage tanks. Ironically, this prescribed remedy for an air problem evolved into an unexpected problem: groundwater contamination. This example illustrates the broad complexity of all pollution control measures in that unappreciated factors can transcend engineering or other targeted features if a broader systematic assessment is not undertaken. Nevertheless, fuel additives have been developed to boost octane ratings of fuels and/or improve engine performance and combustion (e.g., organic oxygenates, methylcyclopentadienyl manganese tricarbonyl (MMT), and platinum compounds for diesel). These additives are reviewed under Title II because of concerns regarding the potential changes in combustion product reactivity or the introduction of metals into the environment, reminiscent of use of lead in fuels from the 1930s to the 1970s, when lead fuel additives were banned in the United States.

Internationally, the magnitude and control of air pollution sources vary considerably, especially among developing nations, which often forgo concerns for health and welfare because of cost and the desire to achieve prosperity. Fig. 31-2 illustrates the international variation in $PM_{2.5}$ air pollution, including the world's megacities with

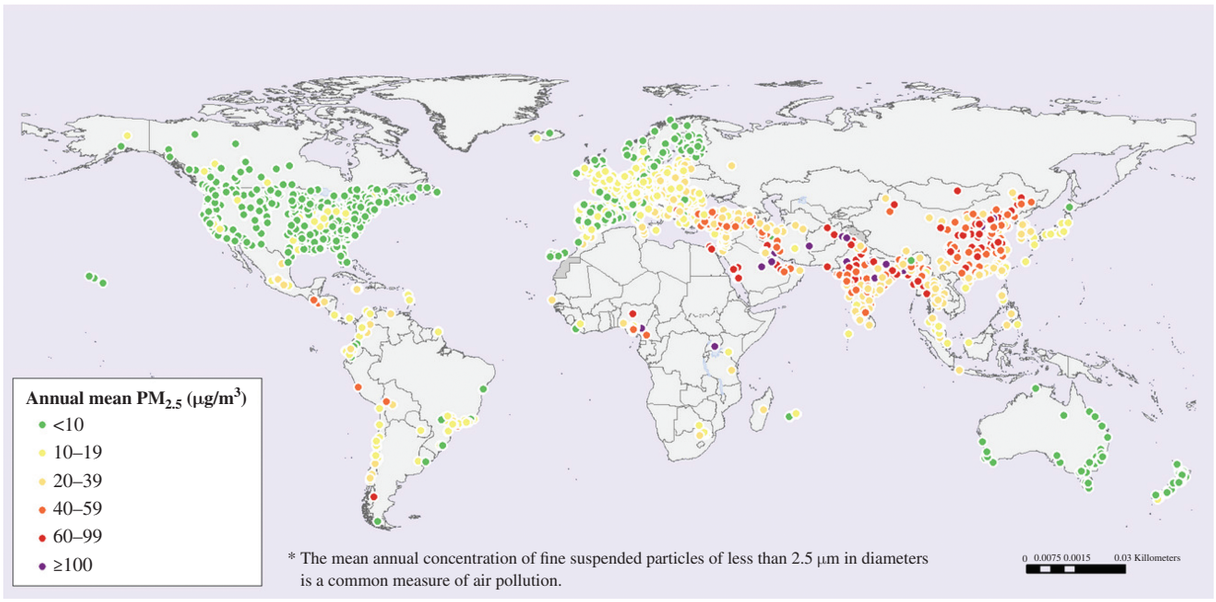


Figure 31-2. *Concentration of $PM_{2.5}$ in nearly 3000 urban areas in the world.* Data are from the World Health Organization's outdoor air pollution database from 2008 to 2015. An interactive map for the WHO database is available at: http://maps.who.int/airpollution/. (From WHO Map Production: Information Evidence and Research [IER]).

major air pollution problems. The political upheaval with the reunification of Eastern Europe revealed the consequences of decades of uncontrolled industrial air pollution, which today are largely rectified. While vast improvements are now evident in this area, as industries are being modernized and emissions controlled, many Asian, African, and South American cities have virtually unchecked air pollution fueled by the desire for economic growth and expansion of low-cost labor. Many nations have adopted WHO guidelines (WHO, 2005) on air pollution as a rational basis for guiding control measures, but the lack of binding regulations and/or unconstrained economic opportunities impede significant controls and improvements.

In addition to local socioeconomic and political concerns, emissions of air pollutants have spawned problems of "international pollution." Long-range transport of polluted air masses from one country to another has been anticipated as a global issue for many years (Wuebbles et al., 2007). This issue was the subject of some controversy between Canada and the United States in the late 1980s and into the 1990s as a result of the air mass transport of acid sulfates from industrial centers of the midwestern United States to southern Ontario. However, reduction in SO_2 emissions somewhat relieved this tension (Fig. 31-3). Improvement in NO_x, also a product of stationary source fossil fuel burning, is also apparent. Long-range transport of air pollution also occurs on a global scale. Importantly, growing prosperity in China has led to enormous growth in its power sector and coal combustion resulting in transoceanic transport of airborne mercury such that transported mercury now accounts for a majority of mercury deposition in waterways and land across the globe (Ci et al., 2012). Similarly, dust or sand storms originating in arid and semi-arid regions of Africa and Asia can also undergo global transport (Fig. 31-4).

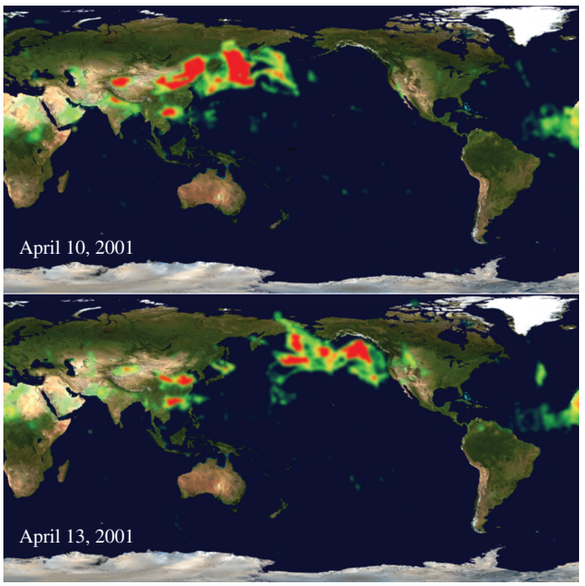


Figure 31-4. *Global images of an Asian dust cloud captured by NASA's Earth-Probe TOMS satellite.* The April 10, 2001 panel shows the dust cloud over China and traveling east and the April 13 panel shows the cloud reaching the west coastline of North America. (Images courtesy of NASA's GSFC/Earth Science Data and Information System [ESDIS].)

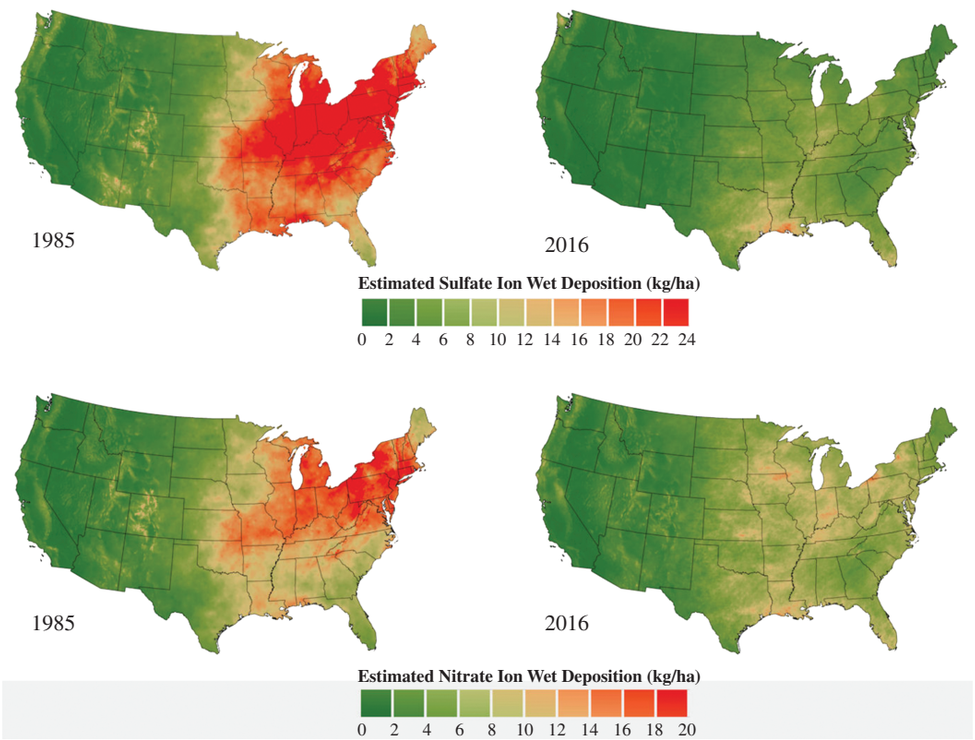


Figure 31-3. *Reduction in ambient particulate sulfate and nitrate concentrations between 1988 and 2008 in the eastern half of the United States (from CASTNet monitoring data).* Sulfates and nitrates arising from industrial centers of the Midwest contribute to acid rain deposition (see Fig. 31-14). Sulfates are readily dispersed by winds toward the eastern half of the country. Nitrates arise from the industrial centers as well as from motor vehicles metropolitan areas and show both a local and dispersed pattern. (Adapted from Explore Nature National Park Service: https://nature.nps.gov/air/monitoring/wetmon.cfm.)

TOOLS TO ASSESS RISKS ASSOCIATED WITH AIR POLLUTION

Risk assessment is a formalized process, originally described in the landmark 1983 National Research Council Report, whereby toxicity, exposure, and dose-dependent outcome data can be systematically integrated to estimate risk to a population. Fig. 31-5 provides a modified version of the paradigm of the NAS (NRC, 2009), incorporating recent interest in providing evidence of "accountability" that the regulations indeed do have impacts—presumably improvement. The health database for any air pollutant may comprise data from animal toxicology, controlled human studies, and/or epidemiology. But, because each of these research approaches has inherent strengths and limitations, an appropriate risk assessment of an air pollutant requires the careful integration and interpretation of data from all three methodologies. Thus, one should be aware of the attributes of each (Table 31-2).

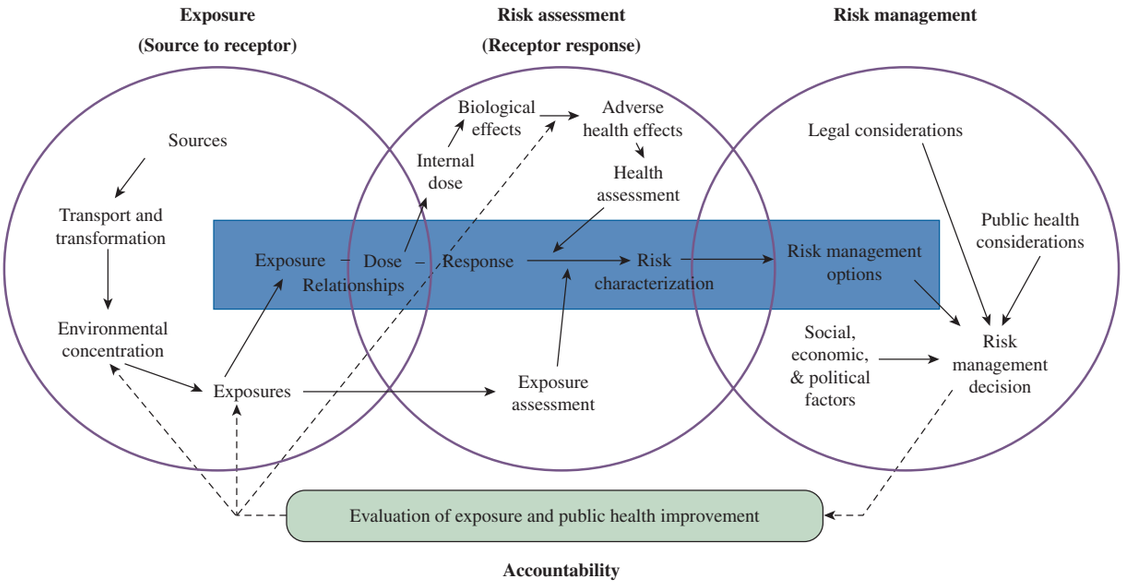


Figure 31-5. *NRC risk assessment paradigm.* Components of *risk assessment* within the left circle provide data to development of *risk management* as depicted in the right circle, modified to include an "accountability" component as a means to address air quality management impacts on the process risk reduction. (From the National Research Council [2009].)

Table 31-2

Strengths and Weaknesses of Disciplinary Approaches for Obtaining Health Information

DISCIPLINE	POPULATION	STRENGTHS	WEAKNESSES
Epidemiology	Communities	Natural exposure	Difficult to quantify exposure Many covariates
	Diseased groups	No extrapolation Isolates susceptibility trait Long-term, low-level effects	Minimal dose–response data Association vs. causation
	Field/Panel groups	Good exposure data Fewer covariates Focus on host traits Utilizes clinical evaluations	Usually short-term Volunteers Expensive
Clinical studies	Experimental Diseased subjects	Controlled exposures Few covariates Isolates susceptibility trait Cause–effect	Artificial exposures Acute effects only Hazards Volunteers
Toxicology	Animals	Maximum control Dose–response data Cause–effect	Human extrapolation Realistic models of human disease?
	In vitro systems	Rapid data acquisition Mechanisms	In vivo extrapolation

SOURCE: Modified from Boubel et al. (1994), with permission.

Epidemiologic studies reveal associations between exposure to a pollutant(s) and the health effect(s) in the *community* or *population* of interest. Because data are garnered directly under real-world exposure conditions and often involve large numbers of people, the data are of direct utility to regulators assessing pollutant impacts. With proper design and analysis, studies can explore either acute or long-term exposures and theoretically can examine spatial and temporal patterns in mortality and morbidity, both acute and chronic, especially if these responses appear disproportionately in population subsets (i.e., sensitive groups). Why, then, is this epidemiological approach to the study of air pollution not the exclusive choice of regulators in decision making? The problem is that it is difficult to control confounding personal variables in the population. Factors such as genetic diversity and lifestyle differences among individuals, and population mobility are difficult to control, but perhaps most problematic is the lack of adequate exposure data—especially on a personal basis. Exposure assessment is often one of the major weaknesses of an epidemiologic study, not only because of the difficulties of assessing exposure (as a measure of dose) to the pollutant of interest, but also because it is difficult to segregate a single pollutant from correlated co-pollutants and other environmental influences, such as meteorology. Thus, only associations, and not causality, can be drawn between the broad-based exposure data and effects observed in epidemiology studies. Causal relationships are sometimes inferred in the presence of strong statistical significance, but such determinations are likely to be criticized. However, advances in exposure estimation and study design and analysis (e.g., time series) have allowed epidemiologists to examine relationships with greater confidence and specificity. These models limit the impact of covariates and longer time-based influences and thus allow epidemiologists to tease out effects of short-term pollution not accessible formerly (Zeger et al., 2006). Similarly, newer approaches that employ field studies—sometimes called *panel studies*—incorporate time-series design and multiple-regression analyses of more focused and complete exposure data (ideally personal) and targeted clinical endpoints in the exposed population under study. The endpoints often derive from empirical human and animal studies and therefore have a priori conceptual ties. The advent of new genetic approaches for characterizing polymorphisms of potentially influential traits opens the genomic door for assessing gene–environment interactions (London and Romieu, 2009). Such studies have focused on individual genetic polymorphisms (Tujague et al., 2006) or utilized "fishing expedition–like" genome-wide approaches to discover polymorphisms associated with oxidative stress (Minelli et al., 2011) or lung cancer (Hosgood et al., 2015). These factors may well underlie a significant portion of the human susceptibility to air pollution. Novel approaches such as this are evident in the most recent studies of PM air pollution (see the section "Susceptibility").

Studies that involve *controlled human exposures* have been used extensively to evaluate the criteria air pollutants regulated by the U.S. EPA. Because most people are exposed to these pollutants in their daily lives, human volunteers can be ethically exposed to them in a highly controlled fashion (except for Pb, which has cumulative and irreversible effects). Exposures are conducted in a controlled environment (usually in a chamber or with a mask or mouthpiece) and are generally of short or limited repeat durations, given assurances that all responses are reversible. Clearly, data of this type are very valuable in assessing potential human risk, since they are derived from the species of concern (i.e., human) and are rooted in well-established clinical knowledge and experience.

Suspected "susceptible or sensitive" individuals representing potential higher-risk groups can also be studied to better understand the breadth of response in the exposed public. However, clinical studies have several practical limitations. Ethical issues are involved in every aspect of a clinical test; potentially irreversible effects and carcinogenicity are always of concern, along with the definition of an acceptable level of hyperresponsiveness in so-called sensitive individuals who volunteered to participate in the study. Likewise for any test subject, there are obvious restrictions on the invasiveness of biological procedures, although sophistication in medical technology has made accessible a large array of molecular biomarkers from peripheral blood and nasal, bronchial, and alveolar lavage fluids as well as biopsied cells from airway segments (Mirowsky and Gordon, 2015). With the advent of cutting-edge genomic and proteomic high-throughput technologies, new tools are emerging to identify responses in humans and the relationships to innate susceptibility. Obviously, the issue of cost, the limited numbers of subjects that can be practically evaluated, and the inability to address chronic exposure issues remain constraints on human clinical testing. Where partnerships with animal toxicology studies have been established, studies in laboratory animal species can sometimes provide ethical justification for at least limited direct human exposure for addressing critical questions. Analogously, in vitro studies in both human and animal cell and tissue systems, often augmented with similar genomic tools, allow the elucidation of mechanisms of toxicity. These basic biological responses inform extrapolation models that link animal data to humans, and they support the relevance of nonhuman studies and preclude risks that may reside in human studies with some toxic air pollutants (see the section "Hazardous Air Pollutants").

Animal toxicology is used to predict or corroborate, through plausible mechanisms, suspected effects in humans. In the absence of human data, animal toxicology constitutes the essential first step of risk assessment: *hazard identification*. Animal toxicology is often required before any controlled human exposure study can be conducted. It is particularly useful in elucidating pathogenic mechanisms involved in toxic injury or disease, providing basic knowledge that is critical to extrapolating databases across species, estimating uncertainties, and determining the relevance of information to humans. Knowledge of the toxic mechanism(s) provides the underpinnings to the "plausibility" of findings in the human context and, under carefully defined and highly controlled circumstances, may allow *quantitative* estimates of risk to human populations. Animal toxicology studies have been used to investigate all of the criteria air pollutants and many of the HAPs as well. The strength of this discipline is that it can involve methodology that is not practical in human studies and can provide more rapid turnaround of essential toxicity data under diverse exposure concentrations and durations. The minimization of uncontrolled variables (e.g., genetic and environmental) may be the greatest strength of the animal bioassay.

Animal-to-Human Extrapolation: Issues and Mitigating Factors

The clear limitation of animal studies in human risk assessment lies in the unknowns of biology and exposure that weaken the extrapolation of findings in animals to the day-to-day human life scenario. Ideally, a test animal is selected with knowledge that it responds in a manner similar to that of the human (*homology*). *Qualitative* extrapolation of homologous effects is not unusual with many toxic inhalants, but *quantitative* extrapolation is frequently clouded by uncertainties of the relative *sensitivity* of the animal or specific target tissue compared with that of the human. Uncertainties about the target tissue dose also loom large, constituting the first obstacle to quantitative extrapolation, see the section "Extrapolation." With

respect to the target tissue dose, however, most animal toxicologists make every effort to keep exposure concentrations at 5- to 10-fold that of the anticipated human exposure until appropriate dosimetric data can be ascertained. An often-overlooked issue is that the dose to the target (lung region) for the test animal is frequently less than that of the human under similar exposure conditions—especially when exposures are conducted during dormancy for the animals (Wichers et al., 2006). Most human inhalation studies involve exercise exposure paradigms where increased ventilation augments dose. Thus, in the absence of exercise, higher doses may be needed to achieve a statistically significant group response among a limited pool of genetically similar animals (typically with an *n* of 6 to 10) to represent a large population effect. However, it must be appreciated that mechanisms may well differ at different dose levels and some responses may be misleading if assessed only at the higher dose levels. Despite these limitations, however, animal studies have provided the largest database on a wide range of air toxicants and have proven utility in predicting adverse human responses to chemicals. New high-throughput technologies offer great promise in shortening the toxicity assessment process and reducing costs of both the test process and the consumption of animals, but it remains to be seen how this new technology aids in the quantitative assessment of toxic inhalants.

To be effective, any health assessment should consider the strengths and weaknesses of the approaches selected to estimate actual toxic risk. In the larger picture, other scientific disciplines can be highly valuable to a more accurate assessment of the impact of air pollution on society. The atmospheric sciences, particularly the chemical and physical sciences, provide insights into exposure by better characterizing what is in the air. Better pollutant characterization, linked to exposure assessment, can only strengthen epidemiological outcome associations. Similarly, better exposure characterization and dose estimates support toxicity evaluations based on biological test systems. In the latter case, the uncertainties of dose relevancy can be reduced and linkages to defined pollutant physicochemical attributes and interactions can be pursued. Recreating realistic exposure environments to the extent possible is invaluable to developing models to estimate human risk.

Lastly, studies of botanical responses to air pollutants are now appreciated more than ever. Not only are commercial and native vegetation affected by pollution but some plant species are being exploited as sensitive "sentinels," warning of the impacts of pollution on both human and environmental receptors. When considered collectively, economists can inform regulators and the public at large of the cumulative impact and adversity of pollution on our quality and standard of living (Martenies et al., 2015). Interestingly, some basic mechanisms (e.g., the involvement of antioxidants) between plants and animals have remarkable parallels.

The value of animal toxicology in inhalation studies is highly dependent on the ability to extrapolate or relate empirical findings to real-world human scenarios. Several factors of study design play into the process of extrapolation (e.g., exposure concentration, duration, and patterns), but most important is the selection of the animal species that will serve as the toxicological model. Therefore, this selection should involve more than considerations of cost and convenience. Whenever possible, effects that are homologous and involve the same mode of action between the study species and the human should guide the decision of the most appropriate test species. For example, if upper airway irritant responses like bronchoconstriction are anticipated (e.g., SO_2 or formaldehyde), then the guinea pig, with its human-like reactive airway reflexes, should be selected over the rat, which is not particularly responsive in this regard. However, if the underlying molecular events in tissue remodeling are of interest, then the rat or mouse might better serve as a model because of cellular mechanistic parallels with human tissue responses. In part, the availability of probes to aid in such studies could factor into the selection of the rodent strain as well. Additionally, as inbred strains of rats and mice differ in their neutrophilic responsiveness to deep lung inhalants (e.g., O_3), contrasting responsiveness or the mode of action may be more revealing and support one strain over another in its selection for study (Costa et al., 1985). Other innate differences in sensitivity among species may also relate to differences in lung structure, regional distribution of cell types having varied metabolism, genetic polymorphisms, or antioxidant defenses. Genetic control of responses such as inflammation have been identified in mice where a specific Quantitative Trait Locus (QTL) on chromosome 17 (*Inf2*) exhibits a more robust neutrophilic response to O_3 (Bauer and Kleeberger, 2010). Thus, when a test animal strain is selected where such nuances are unclear or unknown, the replication of responses in multiple species builds confidence in the finding as being homologous or having species-conserved modes of action that are relevant to the human and bear careful consideration in a risk assessment.

An essential, but often overlooked, part of response extrapolation from species to species is knowledge of the relative dosimetry of the pollutant along the respiratory tract. Significant advances in studies of the distribution of gaseous and particulate pollutants have been made with empirical and mathematical models, the latter of which incorporate parameters of respiratory anatomy and physiology, fluid dynamics, and physical chemistry into predictions of deposition and retention. Empirical models combined with theoretical models aid in relating animal toxicity data to humans and help refine the study of injury mechanisms due to better estimates of the target dose. Fig. 31-6 illustrates the application of such an approach to the reactive gas O_3 and insoluble 0.6-μm spherical particles, as each is distributed along the respiratory tract of humans and rats. Anatomic differences between the species clearly affect the deposition of both gases and particles, but the relative distributions are quite similar. This is not surprising if one argues teleologically that the lungs of each species evolved with similar functional drivers (i.e., O_2–CO_2 exchange and blood acid/base balance), mechanical impediments, and environmental stresses. One needs only a cursory review of the comparative lung physiology literature to appreciate the allometric consistency of the mammalian respiratory tract to meet the challenge of breathing air. This design coherency has provided the fundamental rationale for the use of animal models for the study of air pollutants. With knowledge of ventilation parameters, one should be able to compute nominal tissue doses based on lung surface area using available published data (Anjilvel and Asgharian, 1995).

OVERARCHING CONCEPTS

What Is an Adverse Health Effect?

Establishing criteria to define an "adverse effect" of air pollution is difficult. When relating a health effect to an air pollutant, a response must be appreciated at two levels—that of the individual person and that of the population. Clearly, an effect upon an individual can be beyond an acceptable limit potentially putting that person's overall health in jeopardy; but this response may be lost in an index reflecting a population-based response. The risk to a population reflects the averaging of individual responses or risks and may be measured as a shift in the normal distribution of some index of response for that population. Hence, on average, the entire population may be judged to be at some enhanced risk. These two forms of risk are clearly related, but most often in practice, the population

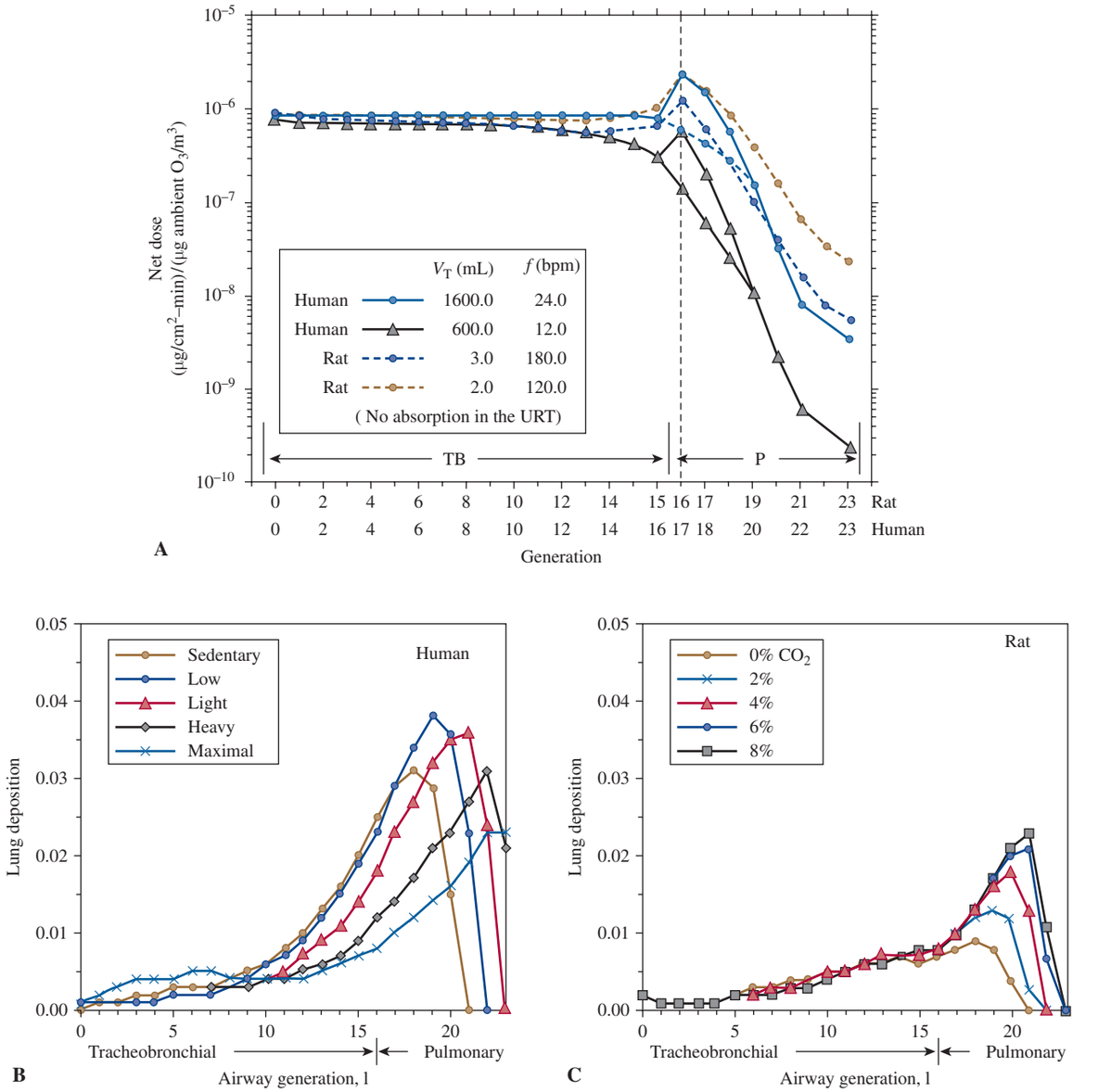


Figure 31-6. *Theoretical (normalized to the concentration in inspired air) uptake curves for the reactive gas ozone in a resting/exercising human and a rat (**A**). Likewise, the percent deposition in the airways of a 0.6-μm insoluble particle in the respiratory tracts of a resting/exercising human (**B**) and rat (**C**).* Eight percent inspired CO_2 in the rat augments ventilation up to threefold. Airway generation refers to that airway branch numbered from the trachea (0). (Panel A is from Overton and Miller [1987], and Panels B and C are from Martonen et al. [1992]. Reproduced with permission.)

risk is considered most appropriate from a public health perspective. Generally, it is also most credibly quantifiable. However, subpopulations that carry some related risk factor may represent a more limited group with unusual risk that too may be quantifiable. Such a group is often called a susceptible subpopulation.

Defining an air pollutant effect as "adverse" within the range of effects that may result from exposure is not always straightforward. Clearly, in humans, some effects would pass uncontested as adverse, for example, death, acute life-threatening dysfunction or disease, irreversible impairments, and pain. In animal models, pathology has traditionally been the hallmark of an adverse effect. In either humans or animals, however, other effects that reflect minor and temporary dysfunctions or discomfort could be argued as not warranting significant or costly concern, especially if the effects are minor and transient with no long-term untoward outcomes. This vein of thought would simply attribute these effects to be within normal physiologic ranges and are readily compensated within functional or biochemical reserve. Thus, if one is to try to assess the impacts of air pollution on health, it is desirable that there exist some objective criteria to define what is indeed adverse based on the nature and the magnitude of the effect under evaluation. Moreover, distinguishing an air pollution effect from other adverse stimuli or disease processes can be complex and fraught with confounding factors, such as smoking and negative lifestyle factors.

Not surprisingly, air pollution impacts have long been focused on the lung as the primary target organ. Regarding acute effects in humans, the American Thoracic Society in 1985 issued a position paper that attempted to define an adverse effect related to air pollution in terms of its impact on lung function (where, e.g., a 10% or more drop in FVC was deemed adverse); at that time, lung function was the primary human health effect that could be studied under controlled inhalation conditions. The definition of "adverse" was revised in 2000, because of the many advances in clinical medicine and empirical health sciences, to include seven broad areas: biomarkers, quality of life, physiologic impacts, symptoms, clinical outcomes, mortality, and population health versus individual risk (ATS, 2000). The summary conclusion states that caution should be exercised in evaluating the many new biomarkers of effect (especially cell and molecular markers), as there is need for validation that *small* changes in these markers represent a progression along a course to disease or permanent impairment. Since that time, further advances in molecular markers and systems assessment of toxic pathways have opened a new dimension of evaluation of health outcomes where their detailed pathogenesis can be examined. Of course, many of the same questions arise as to the criteria used to define when a pathogenic process is qualitatively or quantitatively adverse, but clearly the ability to measure effects and understand their nature and relationship to chronic disease is advancing rapidly. The WHO has also provided a framework to categorize adverse effects in relation to benchmarks. In this framework, a scale is based upon the strength of evidence linking exposure and adverse changes ranging from a posited change to a clear clinical adverse effect. More recently, the European Respiratory Society (ERS) and the ATS wrote a joint statement that addresses adverse effects in additional target organs or systems, such as the reproductive and developmental systems, the brain, and cancer, that are altered by air pollution (Thurston et al., 2017).

Susceptibility

A common thread through these subject areas is the influential role of susceptibility, which can take the form of hyperresponsiveness or loss of reserve. What is a minor reversible effect in the majority of individuals may be a dysfunction that cannot be reversed or compensated in certain individuals (Fig. 31-7). Obvious examples would be cardiopulmonary-compromised individuals who function with little or no reserve. In the end, however, the ATS, ERS, and WHO statements acknowledge the limits of definitions and the importance of value judgment in the final assessment. Implied in this position is that a loss in quality of life due to air pollution as well as enduring its associated effects may also be designated as adverse. As science continues to advance, especially in the realm of molecular biology where small signals can be detected that may forecast an adverse effect or otherwise may identify individuals or groups at risk, the definition of adverse will certainly need reexamination (Thurston et al., 2017). That same sensitivity in measurement that serves to predict an adverse effect must be separated from signals that are essential for homeostasis and the maintenance of life. Clearly, dissecting and defining these phenomena will have implications not only for assessing clinical adversity but also for predictive toxicology.

In actuality, there is no widely accepted definition for a "susceptible" individual and quite frequently the term is used interchangeably with "vulnerable." The U.S. EPA, however, separates the two terms and utilizes "vulnerability" to refer to extrinsic (i.e., nonbiologic) factors (e.g., an increased exposure to ambient air pollutants because one's school is located adjacent to a high traffic volume roadway), whereas "susceptibility" refers to intrinsic biologic factors such as genetics, age, or preexisting disease. Regardless of the precise definition, a susceptible subpopulation encompasses both intrinsic and extrinsic factors that increase the response to air pollution. As demonstrated in both toxicologic and epidemiologic studies, a combination of factors influences the incidence and magnitude of adverse responses of susceptible subpopulations to inhaled pollutants (Table 31-3). For example, over the last 20 years, hundreds of publications have documented an association

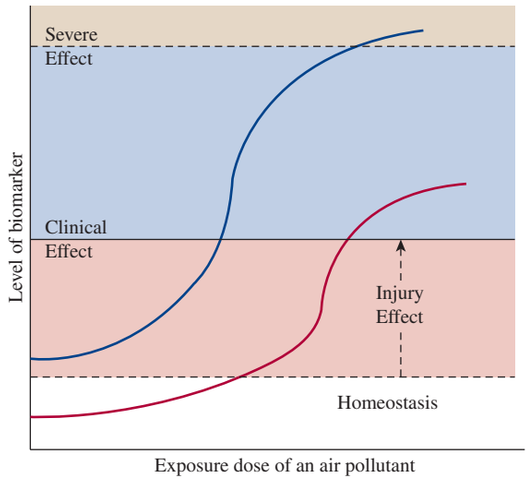


Figure 31-7. *Schematic illustration of the elements of the dose–response to an air pollutant(s) of a susceptible (blue curve) versus a healthy (red curve) individual.* The hypothetical susceptible individual may be more sensitive or may have a *loss of reserve*, either of which results in an *inability to maintain* homeostasis. The *leftward shift or increased slope* in the dose–response curve suggests an increase in responsiveness. Either situation may contribute to sensitivity and the likelihood of enhanced progression from subtle to severe outcomes.

Table 31-3
Factors That Influence the Response of Individuals or Subpopulations to Ambient Air Pollutants

SUSCEPTIBILITY FACTORS	VULNERABILITY/EXPOSURE FACTORS
Preexisting cardiopulmonary disease	Proximity to point source
Genetic factors	Proximity to high traffic volume roadway
Age	Occupation
Gender	Activity level
Race/Ethnicity	Use of air conditioning/building leakiness
Obesity	In utero exposure
Pregnancy	Geographic location (e.g., East vs. West coast of the United States)
Diabetes	Lower social economic status

SOURCE: Adapted from Table 4-1 of SO_2 Integrated Science Assessment , 2010 (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=198843).

between ambient PM levels and increases in cardiopulmonary morbidity and mortality. Age has been consistently shown to influence health outcomes, and the association between ambient air pollution and increases in hospital admissions for asthma is driven by the response seen in children. Conversely, the association between episodic exposure to PM and cardiac mortality is strongest in those over 65 years of age.

Susceptible subpopulations that show exaggerated responsiveness to pollutants merit special mention. The existence of sensitive individuals and groups is well accepted among those who conduct air pollution health assessments, but relatively little is actually known about the host traits that render certain individuals sensitive to inhaled particles and gases. This appreciation for sensitive populations is specifically noted in the CAA, where protection of these groups is mandated in the setting of the NAAQS in the United States. The WHO has a broader definition which encompasses issues that stand out in developing nations as well as those in prosperous countries. They use the term "vulnerability" as the degree to which a population, individual, or organization is unable to anticipate, cope with, resist, and recover from the impacts of disasters (WHO, 2002). There are some definable subgroups that are considered inherently more susceptible or vulnerable to air pollution, including children, the elderly, and those with a preexisting disease (e.g., asthma, cardiovascular disease, and lung disease), but there are other social stressors which also come into play. The importance of susceptibility in air pollutant responses is gaining more and more attention, as test subject responses that were once considered "outliers" in empirical or observational studies may well be evidence of unusual responsiveness.

In some cases, susceptibility may simply reflect differences in dosimetry. Children spend more time outdoors than adults, are more active, and have basal ventilation rates that exceed adults on a volume-to-body weight ratio, and therefore may experience overall greater dose to the lungs. Certainly, rapidly growing tissues may also factor in and may have contributed, perhaps with dosimetry factors, to epidemiology findings showing that polluted urban air retards lung growth (Gauderman et al., 2004). Similarly, adult humans and animals with obstructive airway disease, for example, may have "hotspots" of particle deposition in the airways that exceed normal local tissue doses many-fold (Sweeney et al., 1995; Kim and Kang, 1997). Another often underappreciated aspect of susceptibility relates to the loss of functional reserve or compensation due to age or disease, perhaps altering a response threshold or impairing recovery and the re-establishment of homeostasis. For example, air pollution–induced changes in physiologic measurements such as blood pressure or heart rate may be easily restored in a young healthy individual but not in a more fragile older person with cardiovascular disease.

As already noted, there is also growing interest in gene–environment interactions where genetic differences can influence the responsiveness to environmental stimuli. For example, because the variability in response to ozone was much greater among human subjects than within subjects exposed to ozone in a controlled clinical exposure setting, research in the 1990s suggested that genetic factors influence the response to ozone (McDonnell, 1991). This observation was preceded by an animal study in which significant inter-strain differences were found in inbred mice exposed to high levels of ozone (Goldstein et al., 1973). Since these early studies, human (controlled exposure and epidemiology studies) and animal studies have clearly shown that the response to an inhaled pollutant is dependent on genetics. In humans, the null GSTM1 allele affects about 50% of the population and thus a defect in gene function may undermine antioxidant defenses in those individuals (McCunney, 2005). A number of studies have shown that the null (deletion) GSTM1 genotype, in comparison to the normal wild-type allele, is associated with a greater response to ozone and that the observed pulmonary function changes are even larger if other gene variants involved in oxidative stress are present. Unfortunately, the majority of human studies have examined the contribution of only one or a handful of gene variants at a time. Current state-of-the-art techniques, however, now permit the study of the individual and interactive role of numerous (and someday all) gene polymorphisms in the response to inhaled pollutants. Animal studies have advanced from classical quantitative linkage analysis studies of single sensitive and resistant inbred mouse strains to haplotype mapping studies which take advantage of the complete sequencing or deep single nucleotide polymorphism (SNP) analysis of multiple inbred mouse strains. Similarly, in human studies, we now have the capability to perform genome-wide association (GWA) studies which link up to a million SNPs or haplotypes with a given phenotype/disease in large (i.e., tens of thousands) population studies. Not surprisingly, these GWA studies have determined that individual gene polymorphisms contribute to only a small fraction of the variability in response or disease. Thus, at present, genetic susceptibility, as well as overall susceptibility, to inhaled pollutants is a complex concept that bears significantly on any response. Individual variations in response to ambient air pollution may reflect differences in dose, sensitivity, and/or compensation. As a result, outcomes among individuals may be similar, but the balance of contributing factors that underlie the individual response may be multifactorial.

The study of adverse effects in human volunteers undergoing controlled exposure to air pollutants is ethically limited, but it is even more so for compromised human subjects. Such studies of susceptibility are confined to subjects of only modest suspected risk such as mild asthmatics, healthy older adults, and asymptomatic cardiopulmonary subjects. Inroads have nevertheless been made in recent years because of more thorough prestudy assessments of potential risk factors, allowing researchers to design studies that need not carry undue risk of serious complications. New molecular methods also allow more sensitive assessment of putative biomarkers of response. Additionally, the development of more appropriate animal models of disease or dysfunction (e.g., obese or diabetic mouse models) provides a useful adjunct to explore susceptibility factors more extensively prior to study in humans. Hence, coordinated studies in animals and human subjects are proving to be useful to investigate specific questions such as the roles of pre-existing disease, diet (e.g., antioxidant content), exercise (as it relates to dosimetry), age, gender, and race. The goal of such susceptibility studies is to elucidate patterns, common factors, or pathways that may inform potential intervention or mitigation strategies as well as basic information to reduce the uncertainties regarding risk factors (Kodavanti et al., 1998).

To augment genome studies in humans, recent advances in molecular biology allow the assessment of phenotypic traits in animal models tied to identifiable genes that are homologous to humans. Natural variants in mouse genetics and specially bioengineered transgenic and knockout mouse strains (and in some cases rats) are now widely used to explore the mechanistic basis of phenotypic responses to air pollutants. These new biological tools hold great promise in better understanding responses and establishing gene–environment interactions that may underlie variation in human responsiveness. Transgenic strains can be devised to express desired gene-linked traits using genes inserted from humans as well as other animal models, while knockout models with specific genes removed or silenced can be made devoid of specific traits to isolate responsiveness mechanisms to a toxic challenge. The use of CRISPR/Cas9

technology to target and generate specific mutations in the mouse genome has significantly reduced the time and costs associated with the development of genetically modified mice. These engineered animal models add to the availability of natural mutants that have been inbred historically to "fix" a desired genotype with a specific phenotype expression (Glasser and Nogee, 2006; Shapiro, 2007). Current technology can also target specific genes for isolated expression in the lung (e.g., linked to surfactant protein C), and in some cases controlled by genes which an investigator can switch on or off using a pharmacologic or chemical pre-challenge. These advances allow the dissection of underlying mechanisms under very controlled scenarios and avoid the problems of having a gene inappropriately active or inactive through all life stages or throughout all body tissues.

The use of genetically modified animal models in air pollution research has generally lagged behind that of basic science and toxicology. The reasons for this are likely many, including practicality and expenses, but may relate to the difficulties in incorporating such data into conventional risk-assessment paradigms. However, with recent interest in susceptible groups, there has been a definitive upswing in the use of pharmacologically, naturally altered, or bioengineered animal models to more closely link mechanistic profiles to basic human biology. Ozone has been a common test pollutant in these new studies, because more is known about O_3 and its effects in humans than about any other air pollutant. Frequently, these studies address aspects of inflammation and antioxidant capacity relative to challenge by O_3 and other oxidants (Bauer and Kleeberger, 2010; Groves et al., 2013). But with the current interest in PM health effects, these and other models are being redirected. Examples include strain differences and acid-coated PM (Ohtsuka et al., 2000); atherosclerosis-prone ApoE$^{-/-}$ mice and concentrated ambient PM (Chen et al., 2010). Among rats, the spontaneously hypertensive rat (SHR) and its variants have gained considerable popularity because hypertension is thought to be a human risk factor and because this rat model resembles human hypertension with serum oxidant imbalances, heart disease, as well as its sensitivity to lung injury from inhalants (Kodavanti et al., 2015). The curious are directed to the rapidly evolving literature in this area of research.

EXPOSURE

Air Pollution: Sources and Personal Exposure

Six major air pollutants (PM, O_3, NO_x, SO_2, CO, and Pb) are considered ubiquitous to industrialized communities and are thought to carry the greatest risk to human and environmental health. Except for O_3, these pollutants are emitted by anthropogenic combustion processes along with the myriad of special chemical compounds (mostly volatile organic compound [VOCs]) considered under the category of HAPs. There are many natural sources of air pollutants as well (e.g., volcanoes, wildfires, windblown dust, and natural biogenic vapors), but it is the anthropogenic sources that emit pollutants that concentrate where people live that raise concerns about their potential health impacts. These factors do not dismiss the significance of potential risks posed by the natural emissions but put focus on the potential for human exposure and risk.

Great strides have been made in reducing the emissions of some pollutants. Airborne Pb is down 99% in the United States since it was removed from motor vehicle gasoline in 1973. Similar measures were taken throughout Europe in the 1970s and 1980s. The United Nations Environment Program has been actively advising all countries in the world to use only unleaded gasoline and currently only a small number of countries have not restricted the use of Pb in gasoline. What remains in ambient air emanates from a few isolated stationary sources and re-entrained Pb in road dust. Of the other five criteria pollutants, overall emissions in the United States have been cut 71% since 1970, while at the same time the U.S. Gross Domestic Product (GDP) has increased 246%. Energy consumption has increased 44%, and vehicle miles driven have increased 184% (2014 National Emissions Inventory [NEI] and 2017 Air Pollutant Emissions Trends) (U.S. EPA, 2018). The changing profile of pollutant emissions since 1900 is reflected in Figs. 31-8 and 31-9. From 1980 to 2015, air quality has continued to improve with reductions in ambient SO_2 being largest (−84%) (Table 31-4). O_3, which is formed in the air from emitted precursors of both natural and man-made sources, has changed

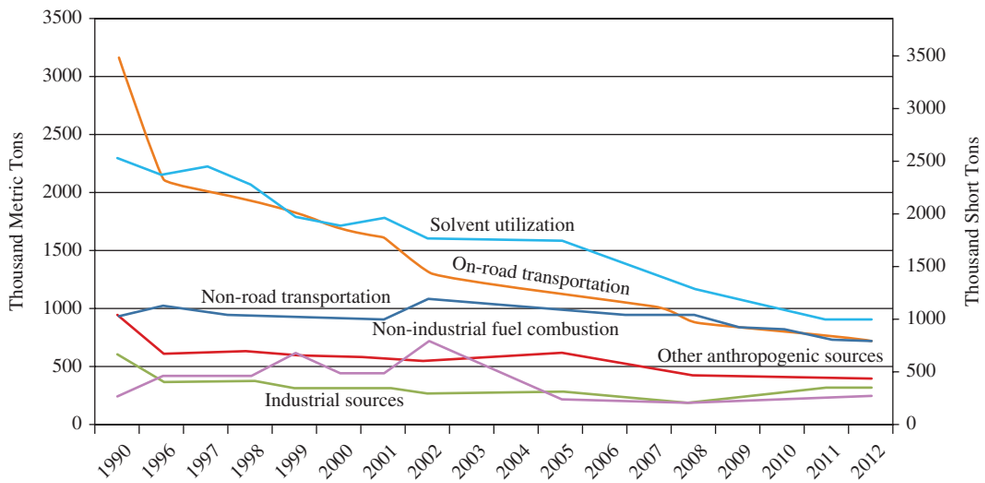


Figure 31-8. *U.S. emission trend for volatile organic compounds (VOC) from 1990 to 2012. From Canada–United States Air Quality Agreement Progress Report 2014.* Modified from Figure 17 of the Report which reports VOC source trends from a defined region in both countries known as the Pollutant Emission Management Area (PEMA), which includes central and southern Ontario, southern Quebec, and 18 U.S. states where emission reductions are most critical for reducing transboundary O_3. Source: Canada–United States, Air Quality Agreement Progress Report 2014. Figure 17.

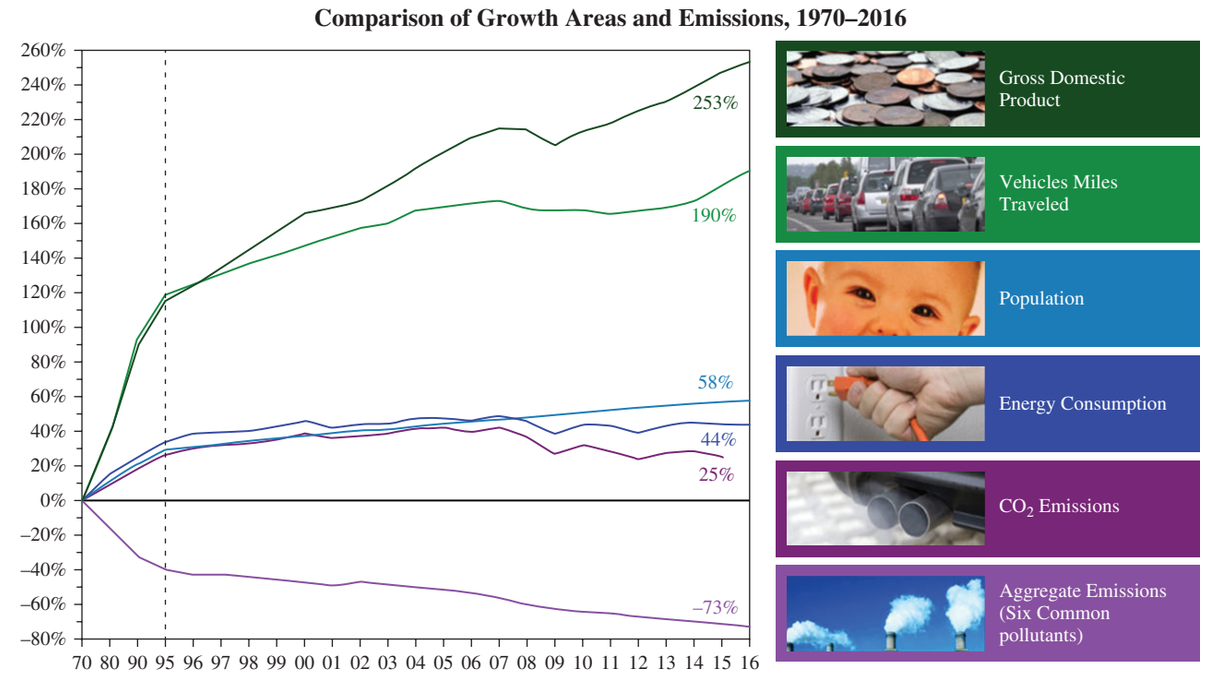


Figure 31-9. *Comparison of growth measures and emissions from 1970 to 2015.* The U.S. population grew by more than 40% during this period. Energy consumption and associated CO_2 levels which historically tracked with population have tailed off in part due to the economic downturn between 2008 and 2012, but these have not rebounded due to conservation activities and alternate energy forces. Meanwhile, miles traveled have doubled and the economy has grown 2.5 times. Yet, the six common criteria air pollutants fell by two-thirds are indicative of great advances in industrial and motor vehicle efficiencies. (From Air Quality—National Summary; U.S. EPA [2016]. Figure used with permission.)

Table 31-4

Air Quality Trends in the United States from 1980 to 2015

POLLUTANT	1980 vs. 2015	1990 vs. 2015	2000 vs. 2015
Carbon monoxide	−84%	−77%	−60%
Lead	−99%	−99%	−91%
Nitrogen dioxide (annual)	−60%	−54%	−45%
Nitrogen dioxide (1-hour)	−59%	−47%	−31%
Ozone (8-hour)	−32%	−22%	−17%
PM_{10} (24-hour)	—	−39%	−36%
$PM_{2.5}$ (annual)	—	—	−37%
$PM_{2.5}$ (24-hour)	—	—	−37%
Sulfur dioxide (1-hour)	−84%	−81%	−69%

Note: The table, listing the % decrease in airborne concentrations of these criteria pollutants across the United States, shows that considerable progress has been made in improving air quality (https://www.epa.gov/air-trends/air-quality-national-summary#Emissions%20Trends).

the least (–32%). Obviously, for any specific locality, air quality can vary depending on the emission profiles of local sources, geographic topography, and meteorology. In the vicinity of a smelter, SO_2, metals, and/or PM, for example, may dominate the pollutant profile, while an oil refinery air shed might be dominated by VOCs and other carbonaceous products. In suburban areas, where the automobile is the main source of pollution, CO, VOCs, and NO_2 would prevail along with their primary photochemical product O_3. NO_x releases (including NO_2) by stationary sources also contribute to the local O_3 levels along with that derived secondarily from auto emissions. Importantly, in 2015 about 121 million people in the United States lived in counties that have violations of the NAAQS designed to minimize risks to the criteria pollutants, whereas in 2014, 92% of the world population resided in places in which WHO air quality guidelines were exceeded.

Assessing exposure to an air pollutant has long rested on observational measures of what is in the air. Indeed, much of epidemiology rests on the principle that what is in the air reflects what an individual is actually exposed to and ultimately inhaled. Exposure science is advancing rapidly and now utilizes approaches that range from novel statistical treatments of traditional exposure metrics to sophisticated models that systematically involve aerodynamic and microenvironmental characteristics to estimate or predict exposures to individuals or populations. The treatment of this subject is well beyond the scope of this chapter (Zou et al., 2009), but the concept is critical to any toxicological evaluation of air pollutants. It also bears heavily on the appropriate approach to conducting inhalation studies in animal models to study air pollutants. Typically, inhalation studies of animals typically involve square-wave exposure patterns, although it is well-appreciated that human exposures vary spatially and temporally. Moreover, animal studies are generally done during the workday (daytime), which is the nocturnal sleep time of most rodent models. In the latter case, one might expect different ventilation patterns in sleeping rodents as compared to humans in their daytime activities and perhaps inverted diurnal

biochemical activities associated with feeding cycles. Such differences may not have significant impacts on animal toxicological results, but the potential for differences needs to be appreciated and respected in translational or interpretational activities.

Indoor versus Outdoor People in the United States (and in most industrialized nations) spend in excess of 80% of their time indoors while at work, school, and home or between these places in an automobile (Robinson and Nelson, 1995). Generally, the time spent indoors is disproportionately higher for adults, who have relatively less time to participate in outdoor activities, especially during the day, when outdoor pollutants are usually at their highest levels. The outdoor activity pattern for children is changing, however, and potentially has consequences such as the increased risk of obesity and other health/growth parameters. Perhaps the most significant risk factor in indoor air for children is the presence of asthma. With an asthma prevalence of nearly 10% in children, the risk of exposure to pollutants and allergens in concentrated form is particularly great.

As to the issue of total exposure, children and outdoor workers are thought to be more likely to encounter outdoor air pollution at its worst; in fact, the relatively high physical activity levels of these subgroups leads to larger doses of any given pollutant being delivered to the lungs. Thus, while it is important to characterize and track pollution levels in outdoor air, a realistic exposure estimate is perhaps best achieved when some indoor/outdoor proportionality can be included as well as some indicator of physical activity. From these data, it would be possible to estimate lung dose, although uncertainties can increase with each increment of personal detail. Defining personal exposure can be extremely difficult, as personal monitoring is tedious, expensive, and can sometimes be confounded by other contributions to the indicator being monitored. Hence, exposure measures are typically drawn from ambient measurements or derived from models developed from studies of groups of people carefully characterized across personal exposure modifiers—exercise, personal lifestyles, etc.

The indoor environment has gained appreciation as a major contributor to total personal exposure. The energy crisis of the 1970s spurred efforts to increase home and building insulation, reduce infiltration of outside air, and minimize energy consumption. At the same time, indoor sources of air contaminants have been on the rise from household products and furnishings, which—when combined with poorly ventilated heating systems and overall reductions in air-exchange rates—give rise to potentially unhealthy indoor air environments. As people begin to notice patterns of odors, microbiologic growth, and even ill health, measures of indoor air become a significant part of environmental risk assessment. Personal exposure has, therefore, come to include the myriad of potential sources, both outdoors and indoors.

It is clear now that indoor air can at times be more complex than outdoor air, a point often raised in challenging the legitimacy of studies that rely solely on outdoor monitoring data. Nevertheless, the national monitoring network for the criteria pollutants has been shown to reflect human exposure reasonably well for some pollutants, especially those that are chemically nonreactive. Indeed, outdoor air permeates the indoor environment despite the reduced air exchange in most buildings. However, many variables determine how well components of the outdoor air infiltrate. The current evidence suggests that the average insulated home has about one air change per hour, resulting in indoor concentrations of pollutants that range from 30% to 80% of that outdoors. For nonreactive gases (e.g., CO), there could likely be nearly a 1:1 indoor/outdoor ratio in the absence of a "sink" for that gas; the ratio for fine PM (less than 2.5 μm) could also be fairly high (approximately 0.4 to 0.7), since these particles can easily penetrate through cracks and open spaces. In contrast, the indoor/outdoor ratio of O_3 is low (less than 0.3) because of its reactivity. Obviously, household differences in the use of window ventilation and air conditioning can also be important variables. Not to be overlooked, of course, are independent indoor sources of contamination where the ratio of an indoor pollutant to that outdoors can even exceed 1 (e.g., NO_2). Particles and gases from tobacco smoke, unvented space heaters, and poorly vented fireplaces and wood stoves as well as fresh paint and cleaning agents can be significant indoor sources. Attention is also being directed toward the many and varied insidious sources of indoor contaminants: certain soils and construction masonry emit radon, gas cooking appliances emit NO_x, sidestream tobacco smoke emits PM, CO, and a host of carcinogenic polyaromatics, and carpets, furnishings, dry-cleaned clothes, and household air fresheners can emit a wide range of VOCs. Responses appear also to be affected by ambient comfort factors such as temperature and humidity. Additionally, some of these chemicals can even interact with one another as has been found to occur with O_3 diffused indoors reacting with VOCs emitted from household cleaners to form small particles. The complexity of these multiple sources underscores the importance of appreciating the total exposure scenario if we are to understand the nature of air pollution and its potential effects on human health (Fig. 31-10).

There remain two broadly defined illnesses that were identified in the 1980s that are largely unique to the indoor building environment (Brooks and Davis, 1992). The first is "Sick-Building Syndrome," which is a collection of ailments defined by a set of persistent symptoms enduring at least 2 weeks (Table 31-5) and appears to occur in at least 20% of those exposed. The pattern is complaints by the building inhabitants, typically of unknown specific etiology sometimes even with ambient measurements, but is relieved sometime after an affected individual leaves the offending building (Hayes et al., 1995). Frequently but not always, this syndrome occurs in new, poorly ventilated, or recently refurbished office buildings. The suspected causes include combustion products, cleaning chemicals, biological emissions from mold, and vapor emissions from furnishings. The perception of irritancy to the eyes, nose, and throat ranks among the predominant symptoms that can become intolerable with repeated exposures. Controlled clinical studies have shown concentration- and duration-dependent worsening of sensory discomfort after exposure to a complex mixture of 22 VOCs commonly found in the indoor environment (Molhave et al., 1986). The many factors contributing to such responses are poorly understood but include various host susceptibility factors such as personal stress and fatigue, diet, and alcohol use. Current biomarkers of response used in the laboratory include sensory irritancy to the eyes in volunteer test subjects and sometimes in animals. However, in general, animal studies using standard measures of sensory irritation or other corporal endpoints have had limited success in assessing sick-building syndromes (SBS) and related syndromes. Human perception factors are believed to be critical to the response patterns.

The second syndrome (building-related illnesses) is a group of illnesses, which in contrast to the SBS, consists of well-documented conditions with defined diagnostic criteria and generally recognizable etiology. These illnesses typically call for conventional medical treatment strategies, since simply exiting the building where the illness was contracted may not readily reverse the symptoms. Several biocontaminant-related illnesses (e.g., Legionnaires disease, hypersensitivity pneumonitis, humidifier fever) fall into this group, as do allergies to animal dander, dust mites, and cockroaches. Medical treatment and mitigation of exposure (source elimination or personal

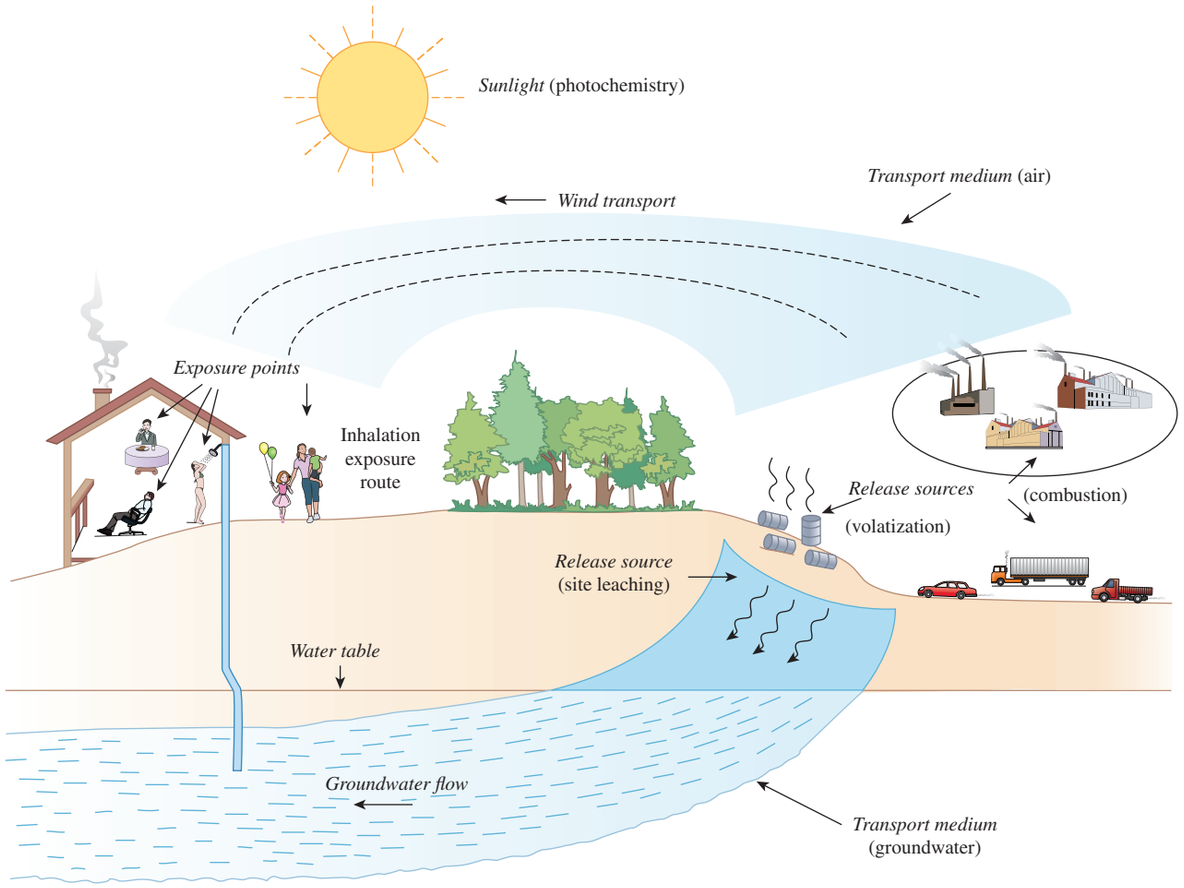


Figure 31-10. *Illustration of contributors to the total personal exposure paradigm showing how these indoor and outdoor factors interact.*

Table 31-5

Symptoms Commonly Associated with the Sick-Building Syndromes

Eyes, nose, and throat irritation
Headaches
Fatigue
Reduced attention span
Irritability
Nasal congestion
Difficulty breathing
Nosebleeds
Dry skin
Nausea

SOURCE: Modified from Brooks and Davis (1992), with permission.

protection) are generally needed to abate symptoms. Some typical outdoor pollutants can also be problematic indoors—most notably, CO from poorly vented heaters. Indoor exposures to NO_2 can pose problems as well, especially in people who may be sensitive—such as asthmatics. At times, however, when the concentrations of CO, NO_2, and many VOCs (passively emitted from new furniture or rugs, or from mold in the ventilation system) result in less discernible or definable signs and symptoms, the responses may be misdiagnosed as SBS, which can complicate assessment of the situation.

Many inhalants, such as NO_2 and trichloroethylene (a VOC common to the indoor air arising from chlorinated water or dry-cleaned clothes) have been shown in animal toxicology studies to suppress immune defenses and allow opportunistic pathogens to proliferate in the lung. The involvement of immunologic suppression is a particularly controversial, yet important attribute of indoor pollution because of its insidious nature and its implications for all building-related illnesses. This problem is further complicated in that complex indoor environments comprising of chemicals and biologicals (dust mites, fungi, molds, etc.) may also lead to unexpected interactions that, to date, remain virtually unstudied and thus are under-appreciated in the assessment of indoor pollution.

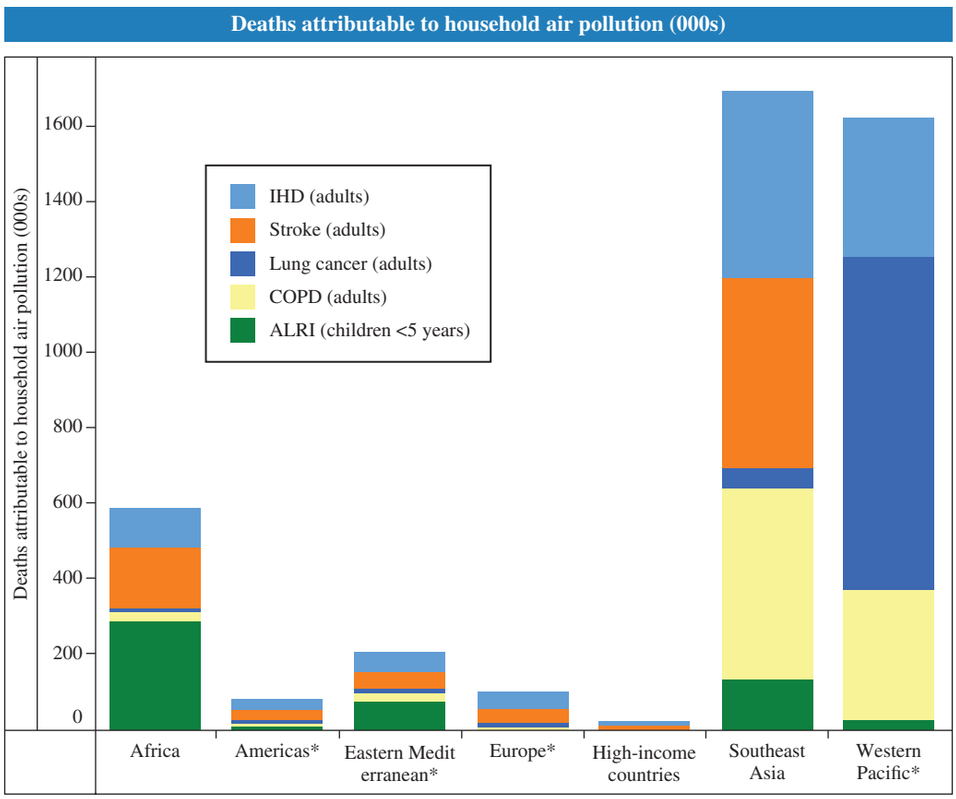
Recently, there have been incidents that highlight indoor air pollution. Post Hurricane Katrina (2005) deployment of trailers where people lived for extended periods resulted in exposure to formaldehyde from urea foam insulation with a myriad of effects on inhabitants—notably children. More recently, wallboard of Chinese origin, which had sulfate and other chemical components on the sealant surface paper that vaporized into the air, resulted in a wide array of symptoms among inhabitants as well as corrosion of some materials. Clearly, the indoor environment is not to be underestimated as to its potential for impact on human health.

Indoor Air in the Developing World Pollution of the indoor environment in the developing world is a major issue; in some parts of the world this issue actually overrides outdoor air pollution. In the more urbanized environments, there is indoor

infiltration of outdoor pollutants unhampered due to poor housing stock. Superimposed on the infiltration of ambient air pollutants are indoor emissions from cooking practices, the cultural use of incense, tobacco, and various other substances, like perfumes. In less developed communities, unvented or poorly vented cookstoves which burn biomass are used much as they have been for centuries. The emissions from these cookstoves, used by approximately 3 billion people worldwide, accumulate indoors exposing families, and especially women and children, to soot and account for 4.3 million premature deaths around the world (Fig. 31-11). Chronic lung diseases, such as bronchitis, emphysema, and cancer, are major killers of exposed women, while children suffer from bronchitis and various other infectious lung diseases. The cookstove issue is the focus of a UN Foundation established in 2010 called the Global Alliance for Clean Cookstoves (UN Foundation, 2010), which is a multinational effort to replace existing dirty stoves with clean culturally practical alternative cooking stoves and devices. The black carbon (soot) emitted from the dirty stoves is also believed to be a major contributor to global climate change.

Evolving Profile of Outdoor Air Pollution Classically, ambient air pollution was distinguished on the basis of the chemical redox nature of its primary components. Dickens's 18th-century "London's particular" referred to a chilled, acrid fog resulting from the accumulation of SO_2 and smoke from incomplete fossil fuel combustion during frequent meteorological inversions. This acidic mix reacts with surfaces, corroding metal, and eroding masonry, characteristic of reductive chemistry. These reducing-type atmospheres have long been associated with smelting and related combustion-based industries (as along the Meuse River in 1930 and Donora, Pennsylvania, in 1948) as well as with large, urban centers (e.g., London in 1952; New York in 1962) burning coal or other dirty fossil fuels for energy. In contrast, Los Angeles has always had a characteristically "oxidant-type" pollution consisting of NO_x and other secondary photochemical oxidants, such as O_3, aldehydes, and electron-hungry hydrocarbon radicals. By virtue of its regional topography and summer meteorology, Los Angeles is particularly prone to trap traffic emissions, while other areas, like Atlanta and regions of India, can be plagued by stagnant, humid summer air.

The classical types of air pollution were implicitly seasonal. Reducing-type air pollution occurred during winter periods of oil and coal combustion used for heating and power coupled with meteorological inversions, while the oxidant atmospheres occurred during the warmer months of spring and summer, when sunlight is most intense and can catalyze reactions among the constituents of auto exhaust. Today the urban distinctions between reducing and oxidant smogs have become largely an academic or historic exercise. Most U.S. urban areas have virtually eliminated smoky, sulfurous emissions (through regulation and a changing national industrial profile) and now with suburban sprawl have a proliferation of automobiles that contribute tons of oxidant precursors into the ambient air. However, in the Midwest there remains still



* Low- and middle-income countries.

Figure 31-11. *Illustration of contribution of burning solid fuels to global mortality by region.* The figure shows considerable region-by-region differences in total deaths that can be attributed to combustion of solid fossil fuels such as wood and other biomass materials. ALRI, acute lower respiratory infection; COPD, chronic obstructive pulmonary disease; IHD, ischemic heart disease. (Courtesy of the World Health Organization.)

relatively heavy industrial activity that continues to emit SO_2, albeit much less than decades ago, but whose emissions are targeted for further reduction. These emissions continue to have important regional implications as they undergo complex cloud chemistry to form sulfate and impact visibility (regional haze) as well as contribute significantly to PM mass, the primary metric linked to human health effects (Zeger et al., 2006). The air above metropolitan areas in the eastern half of the United States is currently comprised of regional reducing pollutants that distribute and blanket a large segment of the country as well as temporally and spatially varied local source pollutants (HAPs) and secondary oxidant pollutants. Increasingly, oxidants and sulfates formed high in the atmosphere are transported across regions to other locales. In the East, sulfates may still predominate over nitrates in the air, in contrast to the Southwest United States, but nitrates occupy a higher ratio of the pollutant mix than ever due in large part to the growth in traffic and, thus, no longer the Northeastern air pollution is simply a sulfur-based problem. Adding further to the complexity of the ambient air profile is that not only has the composition of the urban haze changed, but the ambient chemistry has affected its temporal patterns. Long extended periods of O_3 (8 to 12 hours) now prevail over wide areas such as southern California and the U.S. Southeast and even along the Northeast corridor. The prototypic spike pattern of an hour or two depicted for Los Angeles in the 1970s, and assumed to be the norm for photochemical processes, has changed. In part, the chemical mixes are different as is the distribution of the sources (motor vehicle traffic). Atmospheric conditions today also seem to promote transport across broad areas affecting background levels. As climate change progresses, there is expectation that the underlying chemistry of O_3 will be promoted further altering these patterns because the assumptions and data that led to current O_3 control measures will be undermined.

Outside the United States, many international megacities remain plagued by the classic reducing and oxidant forms of air pollution. For example, uncontrolled industrial and coal-fired power plant emissions surrounding cities such as Beijing and the northern sectors of Mexico City are dominated by sulfurous, particulate emissions, whereas southern Mexico City, Santiago, and Tokyo have substantially (although not exclusively) automobile-associated oxidant smogs. Impacts on health, visibility, and general welfare are clear and are bringing ever increasing public concern in an all too familiar scenario played out in the United States in the mid-to-late 20th century. Urban air pollution is a worldwide problem, with the attributable yearly mortality for people exposed to O_3 is estimated at 700,000 (Anenberg et al., 2010), whereas PM-related mortality is higher at 3.1 million per year (Lim et al., 2012).

EPIDEMIOLOGIC EVIDENCE OF HEALTH EFFECTS

Outdoor Air Pollution

Acute and Episodic Exposures A number of air pollution incidents have been documented where pollutant concentrations rose to levels that are clearly hazardous to human health. Where a single chemical has been accidentally released (e.g., methyl isocyanate in Bhopal, India), establishing the relationship between cause and ill effect is straightforward. However, most air pollution situations involve complex atmospheres, and establishing a specific cause other than the air pollution incident itself can be difficult. Three acute episodes of community air pollution are considered classic (Meuse Valley, Belgium, 1930; Donora, Pennsylvania, 1948; London, United Kingdom, 1952). In each event, community inhabitants were clearly affected adversely; hospitalizations were concomitant with an elevated mortality rate. Likewise, each involved a meteorological inversion (cold air capped above by a blanket of warm air, with little or no vertical air mixing) that prevailed for 3 to 5 days, during which time the concentration of an array of combustion pollutants rose well above the normal levels for these already heavily polluted areas. No actual measurements of pollution were made in the Meuse Valley and Donora, but crude daily averages of smoke and SO_2 of the London smog incident were recorded—estimated at 4.5 mg/m^3 and 1.34 ppm, respectively, on the worst day. Brief (on the order of hours) peaks of these pollutants certainly were likely much higher. During the Meuse Valley episode, 65 people died, while in Donora the number was 20. These deaths are considered "excess" deaths when compared with normal mortality rates for that time of year that were likely already affected by the background air pollution.

The "London smog" of 1952 was a true disaster with an estimated 4000 excess deaths during the event itself with perhaps thousands more in the weeks to follow. Hospital admissions increased dramatically, mainly among the elderly and those with preexisting cardiac and/or respiratory disease. Even otherwise healthy pedestrians, their visibility limited to as little as 3 ft, covered their noses and mouths in an attempt to minimize their exposure to the acrid air. Many reports of sudden death were reported among workers commuting on bicycle or on foot with symptoms described as "choking." People with preexisting cardiac and lung problems were particularly affected. It is ironic that 16 years earlier, shortly after the Meuse Valley episode, Firkert (1936) predicted that 3200 deaths would occur should a Meuse-like smog strike London. Although the London incident brought the issue of air pollution to the public consciousness, additional episodes occurred with the 1956 and 1962 incidents being among the most notable. But none were of the magnitude seen in 1952. Interestingly, in December 1991, London experienced a winter inversion smog-alert, but one with a very different modern pollutant profile: NO_2 at 423 ppb, black smoke at 148 μg/m^3, and SO_2 at 72 ppb (five, four, and two times the seasonal average, respectively). The differences between this episode and previous episodes were that the air pollution was neither of the magnitude of earlier events nor was it completely dominated by coal emissions as traffic was at this point a major source of oxidant emissions (Anderson, 1999). Overall increases in mortality and hospital admissions were not so apparent, but regional comparisons suggested impacts among the elderly and cardiopulmonary-impaired (mortality: ↑14% cardiovascular and ↑22% respiratory; ↑43% for respiratory admissions). Susceptible individuals are always the first to respond to these pollution episodes, but the impacts eventually are widespread.

During the latter half of the 20th century, other cities experienced notable air pollution events; among these were New York City, Steubenville, Ohio; Pittsburgh, Pennsylvania; Athens, Greece, and major regions of Western Europe from the Netherlands to the Ruhr Valley of Germany. Major episodes of anthropogenic air pollution continue to decrease in the developed world, both in frequency and intensity, but "natural" events like the vegetation/forest fires of Indonesia of 1997 and 1998, greater Moscow in 2009, northern California in 2009 and 2010 all saw significant impacts on wide regional air quality and public health. The impacts of these fires, like that of the U.S. Northwest in 2003, were tracked as their plumes dispersed across the country. Today, the particulate matter generated by wild fires contributes a large fraction of PM in North America, Europe, and Southeast Asia (Faustini et al., 2015; Crippa et al., 2016; Liu et al., 2016). It has been estimated that wild fire emissions can make up as high as 37% of ambient PM (U.S. EPA, 2018a; NWCG Smoke Committee, 2014).

So much had the air cleared in much of the United States from the late 1960s into the 1970s that many thought that the problem of industrial pollution was on its way to full resolution. Smoke from factory chimneys was less visible and the air epidemiology of the times showed little health impact. However, in the 1980s, it was realized that smoky skies were largely due to carbonaceous material and that more efficient burning had eliminated much of what could be seen in the air. But the sulfur in fuel emitted as sulfur dioxide reacted in clouds to form acidic sulfate and traveled great distances depositing and acidifying lakes and streams and at the same time painting the leaves and the ground resulting in defoliation of forests (Calvert et al., 1985). There were also new studies showing that acid haze with O_3 was associated with emergency room visits among susceptible populations—for example, asthmatics (Bates and Sizto, 1987). A series of studies showed acute effects of regional summer haze in areas of Central and Northeastern North America. These 2- to 3-day episodes of haze were typified by increases in O_3 and acid and neutralized (ammonium) sulfates, characteristic of the new generation of pollution in many U.S. urban areas. Interestingly, the apparent combined temporal or sequential patterns of O_3 and sulfate were associated with the health effects, and neither constituent seemed to be acting alone. Similar results were reported for the upstate New York area as well (Thurston et al., 1992), but it seemed the acidity as $[H^+]$ of the haze was the active toxicant. Studies of children at summer camps, where children are active and outdoors most of the day, had reported decrements in daily measured pulmonary function on days of haze when both O_3 and acidity levels were elevated. The responses appeared to exceed those predicted by chamber studies of O_3 alone suggesting dose differences or interactions between haze components (Lippmann, 1989). Clinical studies in adolescent asthmatics and animal studies lent further support to the belief that H^+ can affect airway function, particularly in the presence of O_3. Studies in the South and Southwest similarly found effects in young asthmatics and diminished performance in athletes, but these appeared to relate more specifically to O_3, since sulfate was less prominent in the ambient air of these regions. Yet these epidemiological and field studies showing associations with "acid" haze could not be replicated in the laboratory with either animal or human exposure studies. There could be any number of reasons for this lack of coherence but the exposure concentrations were often orders of magnitude more than in the ambient air. When refined acid measurements in ambient air were used, the consistency of the findings could not be reliably replicated. With no *apparent* impact on mortality in epidemiological studies, the focus of air pollution toxicology focused more and more on O_3 where clinical and animal exposure studies easily could measure impacts on lung function. It was felt that O_3 was the likely culprit and its ubiquity in urban air argued for greater and greater attention. Indeed, many health effects could be ascribed to oxidant/irritant properties of O_3.

Of the many air pollution studies over the last 25 years, none have had more impact on the perception of pollutant risk and the direction of research today than a series of epidemiological studies that showed an association between PM mass concentration (as measured by the regulatory monitoring network in the United States) and daily mortality. These studies departed from conventional epidemiological methods used for air pollution studies in the United States and utilized novel time-series analyses that could more easily blunt the impact of weather, smoking, and other variables that might obscure patterns in health variables linked to the air monitoring data. These studies showed significant and consistent associations between health outcomes of ambient PM at levels previously thought to be safe. Prior to this period, measurable effects of PM and SO_2 were not easily detected below the 24-hour means for smoke and SO_2 250 $\mu g/m^3$ and 0.19 ppm, respectively. In fact, the new findings showed effects, as evidenced by increases in mortality and morbidity rates at or below contemporaneous NAAQS levels (1987: 50 $\mu g/m^3$, annual mean; 150 $\mu g/m^3$ daily maximum for PM of diameter less than 10 μm (PM_{10})). Time-series analyses are based on Poisson regression modeling to distinguish changes in daily death counts (or hospital admissions) associated with short-term changes in air pollution. The studies initially found effects with cruder measures of PM—total suspended particulates (TSP), which includes virtually all particles less than 100 μm in mass median aerodynamic diameter (MMAD—a median particle size normalizing the particle to unit density and spherical shape for aerodynamic comparison). These studies were followed with stronger associations with particles considered almost fully inhalable—PM_{10} (an MMAD at which PM is aerodynamically separated at an initial 50% efficiency at 10 μm and increasingly at smaller sizes). Beginning in the mid-1990s, even stronger associations have been found with an analogous but fully and deeply respirable particle—$PM_{2.5}$. As the PM diameter gets smaller, it better represents anthropogenic sources of pollution. The statistical methodology applied in these time-series studies had an advantage over conventional regression analyses in that it could detect short-term trends and minimize the effects of other pollutants and potential confounders with longer time constants (Schwartz, 1991; reviewed by Pope and Dockery [2006]).

In contrast to the three epidemiologic studies used by the U.S. EPA to develop the 1987 PM_{10} NAAQS, there were more than 30 such studies for the 1997 revision and the promulgation of the new index, $PM_{2.5}$ NAAQS (15 $\mu g/m^3$, annual mean; 65 $\mu g/m^3$ daily maximum for PM of diameter less than 2.5 μm ($PM_{2.5}$)). Today, there are hundreds of corroborating epidemiological studies with those looking at long-term outcomes being of greatest importance. Collectively, these studies show a significant health impact of PM, linked to mass and not necessarily sulfate or any other constituent, although there is some suggestion of prime PM properties. The most consistent attribute is the size—$PM_{2.5}$ appears to be the most generalizable feature in health outcomes. In 2006, the PM NAAQS was revised keeping the annual $PM_{2.5}$ NAAQS at 15 $\mu g/m^3$, with a tightening of the daily maximum to 35 $\mu g/m^3$. The NAAQS for PM is reviewed about every 5 years depending on legal or procedural issues as data accrue; an update on the primary (human) standards for $PM_{2.5}$ (fine PM) and $PM_{2.5–10}$ (coarse PM) is expected in the 2018 timeframe. The PM NAAQS is specifically noted because of all the NAAQS, it has greatest impact on human health of any U.S. regulation both in terms of cost or benefit. As a result, the health science (epidemiology, clinical human exposure and animal toxicology studies) is scrutinized more than any other U.S. regulatory decision.

PM stands as the preeminent air pollutant because of its health impact as well as the pollutant that opened the door to unsuspected targets of injury. The major health outcome revealed in the study of PM has been the involvement of the cardiovascular system as a prime target for adverse impact, and not the lung as historically assumed. While the heart, as part of the cardiopulmonary system, has always held an indirect role in health impacts or disease from air pollution, both epidemiological and toxicological studies now point to major cardiac involvement in PM-associated mortality. Current thinking is that cardiac-mediated effects are more germane to the PM-associated mortality findings than pulmonary effects (Brook et al., 2010). Not surprisingly, effects are most apparent in subpopulations already compromised by cardiopulmonary and perhaps vascular diseases (e.g., diabetes). There exists no one generally accepted mechanism or mode of action to account for these findings (Brook et al., 2010; Cascio, 2016). However, several pathways have been proposed that attempt to link exposure and cardiac effects that may

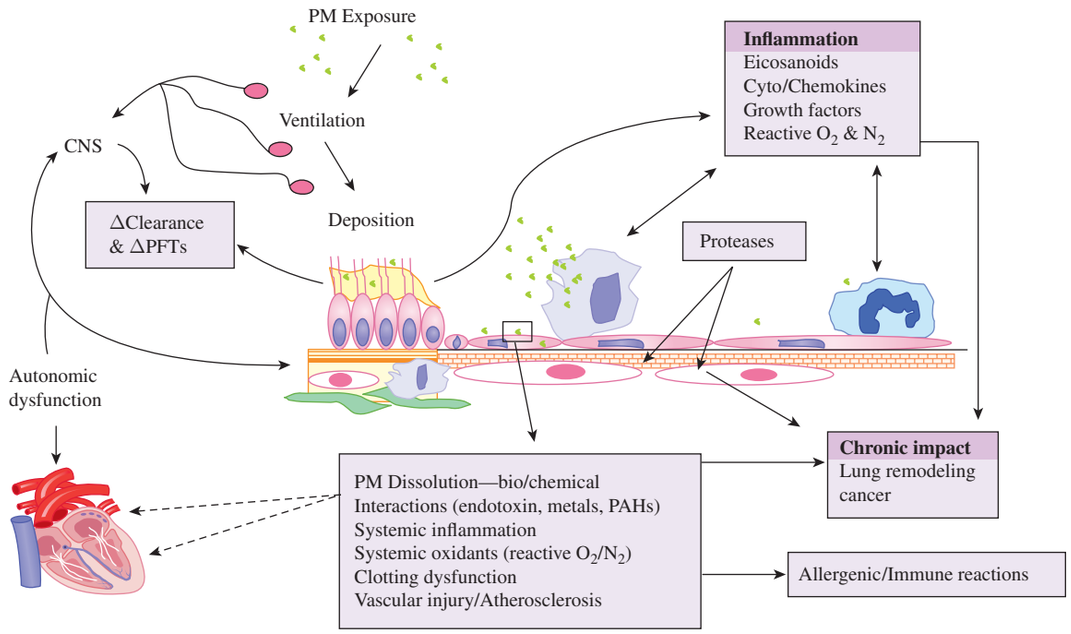


Figure 31-12. *Schematic of the multiple mechanisms thought to function in cardiopulmonary response(s) to air pollutants—derived from current hypothesized mechanisms for particulate matter.*

or may not include pulmonary mediation. These potential mechanisms are illustrated in Fig. 31-12.

From a toxicological perspective, it has been difficult to accept that the association between PM and health outcomes is not somehow linked to particle composition rather than mass alone. The linkage to mass and not composition is counterintuitive, especially in light of the fact that all PM is not constitutively identical. The actual "biochemical lesion" caused by PM is generally thought to involve oxidant mechanisms (generation of reactive oxygen and perhaps nitrogen species) by constituents or attributes (e.g., reactive surface area) of the particles at the cell or molecular level. Initially, there was a credibility hurdle ("biologically plausibility") that day-to-day fluctuations in the mass concentration of about 10 $\mu g/m^3$ airborne PM would result in an increase of about 0.6% to 1% (excess) mortality. However, there now exist several plausible hypotheses that have some degree of empirical support: metals, organics, nanosize with reactive surfaces, and constitutive oxidants to name those most prominent.

The experience with PM has expanded the thinking of the possible extrapulmonary effects of other air pollutants. Potential links to cardiovascular toxicity of several of the NAAQS pollutants have been demonstrated although not with the consistency of PM. Moreover, there are new data showing pollutant-related impacts on birth outcomes, postnatal development, and neurologic health. In the latter case, it is unclear if these effects are related to vascular impacts such as those caused by PM or to other systemic oxidant intermediates.

Long-Term Exposures Epidemiologic studies of the chronic effects of air pollution are difficult to conduct by the very nature of the goal: outcomes associated with long-term exposures. Looking back in time with retrospective, cross-sectional studies was the common approach, but these studies were frequently confounded with unknown variables and inadequate historical exposure data. A good example of the problem of confounding is cigarette smoking. Without extensive information on both active and passive smoking, the ability to discern the impact of an air pollution disease outcome such as chronic bronchitis and emphysema would be greatly impaired. Prospective studies, on the other hand, have the advantage of more precise control of confounding variables, such as the tracking of urinary cotinine as an index of tobacco smoke exposure, but they can be very expensive and require substantial time and dedication on both the part of the investigators as well as the study population. Depending on the study size and design, exposure assessments can be complex, and the loss of subjects due to dropout is sometimes unpredictable.

Despite these deficiencies and problems, there are both retrospective and prospective epidemiology studies that have tackled the issue of long-term air pollution health effects. In general, these studies have suggested a positive association between urban pollution and progressive pulmonary impairments. On the one hand, cross-sectional studies in the Los Angeles Air Basin have found evidence of accelerated "aging-like" loss of lung function in people living for extended periods in regions of high oxidant pollution when compared with areas where sea air circulation lowers overall pollutant concentrations (Detels et al., 1991). Similarly, exposure to SO_2 and PM in the Netherlands over a 12-year period was shown prospectively to gradually impair lung function (Van De Lende et al., 1981). Even rural areas in western Pennsylvania, which are swept by reducing-type pollutants transported from Midwestern industrial centers, have been shown to have a higher incidence of respiratory symptoms as determined from a questionnaire-based design (Schenker et al., 1983). While a negative impact of any specific pollutant followed in these studies is difficult to dissect, the message that long-term exposure to air pollution contributes to deterioration of lung health seems clear.

Among the most detailed prospective epidemiologic studies of chronic health effects across a range of levels of air pollution has been the so-called Harvard Six Cities Study begun in the early 1970s. The cities were chosen to represent a range of air quality based on SO_2 and PM—it was designed as a study of "acid" air

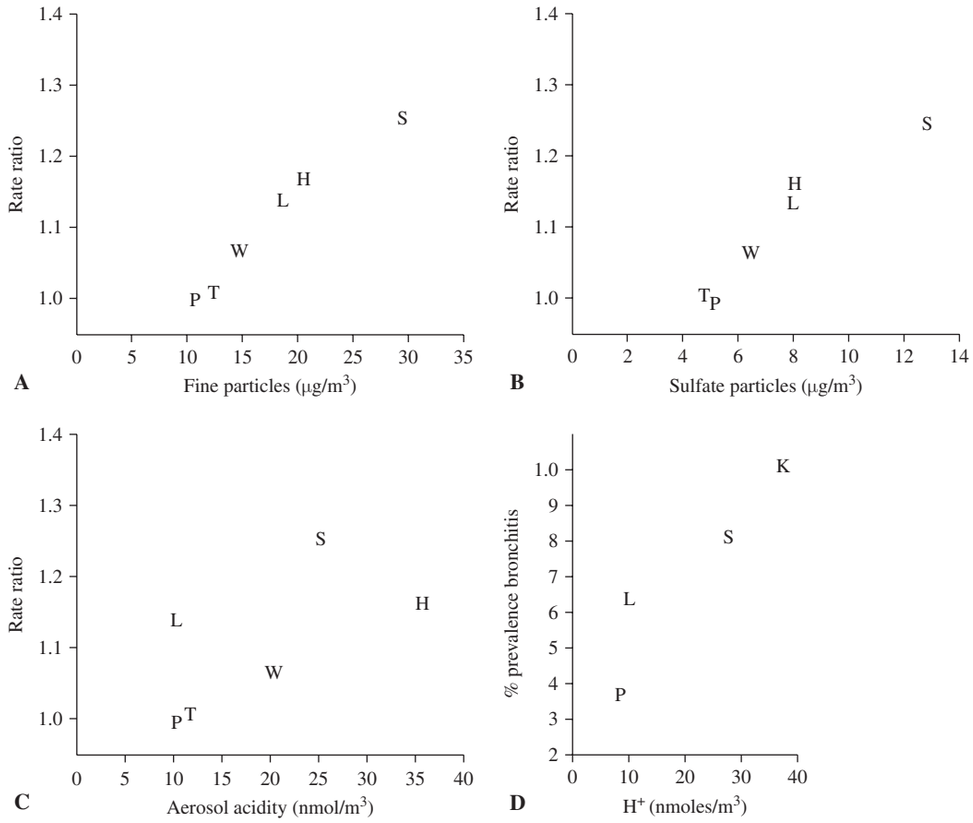


Figure 31-13. *Data from the Harvard Six Cities Studies indicating the superior relationship of PM_{10} and sulfate to mortality rates (**A–C**) in contrast to acidity (**D**), which correlates better with the prevalence of bronchitis in children.* Rate ratio is the mortality rate of each relative to Portage, Wisconsin (P); S = Steubenville, OH; W = Watertown, MA; H = Harriman, TN; L = St. Louis, MO; T = Topeka, KA. (Parts **A** to **C** reproduced with permission from Dockery et al. [1993] and Part **D** reproduced with permission from Speizer [1989].)

pollution. Early on in the study, there was great dependence on routine regional air-monitoring data, but over time the investigators themselves conducted the air analyses of exposure microenvironments (local and indoor). The initial design of these studies included the gathering of parental questionnaire data (including some 20,000 people) about the prevalence of respiratory problems in school children and has continued over 30 years along with periodic assessments of pulmonary function. When compared across cities, $[H^+]$ (measured in four of the six cities) was correlated (Fig. 31-13A) better than was sulfate with the prevalence of bronchitis in children age 10 to 12 (Speizer, 1989). However, as the assessment program evolved, more detailed study revealed mortality associations with PM as represented in Fig. 13B–D. The role of $[H^+]$ in relation with acute mortality was less convincing than that associated with the sulfate or $PM_{2.5}$ (sulfates co-associate with fine PM in the atmosphere) (Dockery et al., 1993). More importantly with regard to long-term health, this study showed very significant effects of PM on the life spans of people living in Steubenville, Ohio—the dirtiest of the industrial centers. Over a 15-year period, the average human life span was reduced by about 2 years due to PM exposure. These findings were corroborated by a prospective cohort-based mortality study using a database collected on over 500,000 people by the American Cancer Society (ACS) across 151 cities from 1982 to 1989 (Pope et al., 1995). The study showed a 15% to 17% increased mortality risk over 7 years due to PM, about equivalent to the risk of smoking over that period. Pope et al. (2002) later reported results of a 10- and again an 18-year follow-up study of the ACS cohort (Pope et al., 2011). Subsequent reports on AARP (Thurston et al., 2016) and large Medicare (Di et al., 2017) cohorts substantiated the earlier findings and demonstrated that cardiovascular effects are observed at very low levels of ambient $PM_{2.5}$. These updated analyses, which included gaseous co-pollutant and new fine-particle measurements, and more comprehensive personal information on the enrollees, confirmed the linkage of health effects, including an increased risk of lung cancer, with $PM_{2.5}$ exposures. These findings reinforce the concerns for potential chronic health impacts of PM and the heightened risk of premature death from lifelong air pollution exposure. Similarly, a study that assessed the health impacts of air pollution in a large multiethnic cohort in North America observed that atherosclerosis and other cardiovascular disease outcomes that might be considered underlying causes of cardiac mortality were significantly associated with long-term exposure to ambient PM (Kaufman et al., 2016).

POLLUTANTS OF OUTDOOR AMBIENT AIR

Classic Reducing-Type Air Pollution

The acute air pollution episodes of the 20th century showed that high concentrations of the reducing-type air pollution, characterized by SO_2 and smoke, are capable of producing dramatic human health effects. Empirical studies in human subjects and animals have long stressed the irritancy of SO_2 and its reaction products in these incidents, while the full potential for interactions among the

co-pollutants in the smoky, sulfurous soup has a mixed record of replication in the human exposure laboratory. Because of its history, SO_2 is often thought of as the prototypic air pollutant of the industrial age, and as such it has a detailed historical literature base. It is an irritant gas that has a toxicology of its own and, through atmospheric reactions, can transform photochemically into sulfites or sulfates as a secondarily irritant particle. The impact of SO_2 emissions on irritancy, the formation of secondary particles that affect visibility, and resultant damaging acidification of the environment have been the drivers of much regulation for coal-fired power plants since 1970. Levels are greatly reduced (more than 80%) in the United States and continue to fall especially after the CAA 1990 and subsequent cross-state regulations.

Sulfur Dioxide

General Toxicology Sulfur dioxide is a water-soluble irritant gas. As such, it is absorbed predominantly in the upper airways. It is a sensory irritant and can stimulate bronchoconstriction and mucus secretion in a number of species, including humans. Early studies with relatively high exposure concentrations of SO_2 showed airway cellular injury and subsequent proliferation of mucus-secreting goblet cells. This attribute of SO_2 led to its use (more than 250 ppm) in the production of laboratory animal models of bronchitis and airway injury (Kodavanti et al., 1998). Chronic exposure studies of experimental animals from mice to monkeys at levels still well-above ambient have yielded qualitative, if variable evidence of airway pathology (Alarie et al., 1970, 1972). Nevertheless, at even lower concentrations (<0.1 ppm), such as might be encountered near an industrial source, long-term residents may experience a higher incidence of bronchitis. Indeed, prior to the breakup of the Soviet Bloc, many eastern European cities were renowned for widespread public affliction with bronchitis; 20 years later, the prevalence of bronchitis was greatly reduced (von Mutius et al., 1994). While other factors (diet, access to health care, other pollutants) may well have been involved in this reversal, reductions in ambient smoke and SO_2 are generally thought to be most important. The concentrations of SO_2 likely to be encountered in the United States continue to decrease and as an annual average are considerably less than 0.01 ppm. As such, not much new SO_2-focused empirical research has been conducted in the past 30 years, but epidemiology has revealed hospitalizations and acute events associated with brief (1 hour) SO_2 spikes (as from stack downdrafts) in exposed sensitive groups.

Contributing to risk is the degree of penetration of SO_2 into the lungs most notable during mouth as compared to nose breathing. Likewise, an increase in the airflow during deep rapid breathing by nose or mouth augments penetration of the gas into the deeper lung. As a result, persons exercising would inhale more SO_2 and, as noted with asthmatics, would be more likely to experience greater irritation. Once deposited along the airway, SO_2 dissolves into surface lining fluid as sulfite or bisulfite and is readily distributed throughout the body and cleared by the kidneys.

Pulmonary Function Effects The basic pulmonary response to inhaled SO_2 is mild bronchoconstriction, which is reflected as a measurable increase in airflow resistance due to narrowing of the airways. Concentration-related increases in resistance have been observed in guinea pigs, dogs, and cats as well as humans consistent with receptor-mediated sensory stimulation. Airflow resistance increased more when the gas was introduced through a tracheal cannula than via the nose, since nasal scrubbing of the water-soluble gas was bypassed. Isolated nasal exposures increased airflow resistance through the nose largely as a result of mucosal swelling, but the irritant effect appeared to signal the more distal airways as well (Frank and Speizer, 1965; Nadel et al., 1965). Intravenous injection of atropine (a parasympathetic receptor blocker) or cooling of the cervical vagal nerves abolishes bronchoconstriction in the cat model; rewarming of the nerve reestablishes the response. The rapidity of the response and its reversal nature emphasize the parasympathetic tonal change in airway smooth muscle. Studies in human subjects have confirmed the predominance of parasympathetic mediation, but histamine from inflammatory cells may play a secondary role in the bronchoconstrictive responses of asthmatics to inhaled SO_2 (Koenig et al., 1981; Sheppard et al., 1981), which raises health concern about this subpopulation when exposure is near point sources.

Chronic Effects Only a few long-term studies have been conducted with SO_2 at levels approaching those found in ambient air and these studies were conducted long ago (Alarie et al., 1970, 1972). Few effects were found other than mild mucosal changes. High levels of SO_2 (200 to 350 ppm for 30 days or more) have altered airway mucus secretion, goblet cell topography, and lung function and have served as a means of developing animal models of bronchitis, but are of little relevance to ambient exposure (Reid, 1963; Lewis et al., 1969).

Sulfuric Acid and Related Sulfates

In the United States, sulfate remains the predominant component of most PM east of the Mississippi River. Its health effects are puzzling in that, as discussed below, alone it seems to have less impact than might be anticipated. However, it may well work in concert with other components of PM to elicit effects; nevertheless, because of its prominence, it merits attention as a classic pollutant that despite all the studies remains a potential contributor to the comprehensive health outcome of exposure to PM.

The conversion of SO_2 to sulfate is favored in the environment with subsequent ammonia neutralization to ammonium sulfate $[(NH_4)_2SO_4]$ or ammonium bisulfate $[NH_4HSO_4]$. During oil and coal combustion or the smelting of metal ores, sulfuric acid condenses downstream of the combustion processes with available metal ions and water vapor or in smoke plumes to form submicron sulfuric acid fume and sulfated fly ash. Further, photochemical reactions also promote acid sulfate formation via both metal-dependent and independent mechanisms in clouds. Although the fine-particle sulfates may exist as sulfuric acid (the primary source of free H^+), partially or fully neutralized forms of sulfate predominate due to the abundance of natural atmospheric ammonia. As fine PM sulfates are transported long distances, they may contribute to regional summer haze and pose a health hazard to certain groups such as asthmatics (Koenig et al., 1989), and may also stress the general environment as acid rain (Calvert et al., 1985) (Fig. 31-14). The reduction of sulfur emissions since the CAA and subsequent regulations (e.g., the 1990 Acid Rain Program and the 2012 Cross State Air Pollution Rule) continue to reduce the environmental acidification and visibility problems associated with transport and deposition of acidic products formed from stack emissions in North America.

General Toxicology Sulfuric acid irritates by virtue of its ability to protonate (H^+) receptor ligands and other biomolecules. This action can either directly damage membranes or activate sensory reflexes that initiate inflammation. Ammonia, which exists in free air at about 25 ppb and in much higher concentrations within the mammalian naso-oropharynx (in the human up to 350 ppm), is capable of neutralizing most of the inhaled irritant acidic sulfates (Utell et al., 1989). Neutralization can also be quite efficient in standard whole-body animal exposure-studies, in which excreta and bacteria in the chambers interact, giving rise to in-chamber

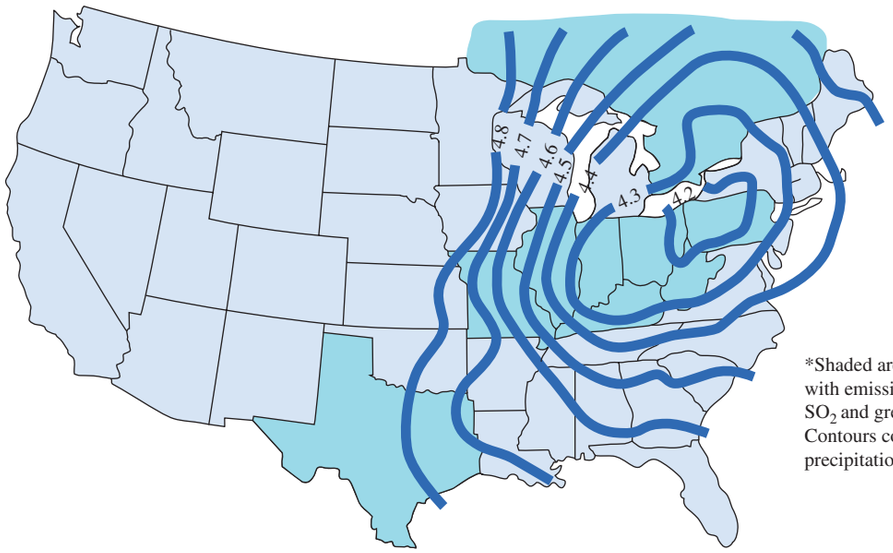


Figure 31-14. *Areas in 1988 where precipitation in the east fell below pH 5: acid rain.* The acidity of the air in the east is thought to result from air mass transport of fine sulfated particulate matter from the industrial centers of the Midwest. (From U.S. EPA [2000].)

ammonia concentrations up to 1100 ppb—more than enough to fully neutralize sulfuric acid droplets up to several mg/m^3 (Higuchi and Davies, 1993).

Interestingly, there is considerable species variability in sensitivity to sulfuric acid, with guinea pigs being quite responsive to acid sulfates, in contrast to rats which seem generally resistant. The reasons for this difference relate to sensory fiber network density in the airways, and probably not on differences in neutralization by ammonia in the airway. The sensitivity of healthy humans appears to fall somewhere in between, with asthmatic humans being perhaps best modeled by the guinea pig. Overall, however, the collective data involving animals and humans are remarkably coherent (Amdur, 1989).

Unlike other irritants, such as O_3, inhaled sulfuric acid does not appear to stimulate a classic neutrophilic lung inflammation. Rather, eicosanoid homeostasis appears to be disturbed resulting in macrophage dysfunction and altered host defense.

Pulmonary Function Effects Sulfuric acid, like SO_2, produces an increase in flow resistance in guinea pigs due to reflex airway narrowing, or bronchoconstriction. Responsiveness to sulfuric acid is both species- (as noted above) and particle size–dependent. In guinea pigs, large particles induce upper airway narrowing and increased resistance. Some human asthmatics can have similar responses in a fog. But typically, only very high "mg" concentrations induce these effects thought largely due to the neutralization of acidity by ammonia. Because of the predominantly neutralized form of sulfate in the atmosphere, sulfates have been largely thought not to be primary or singular bad-actors in air pollution incidents unless at very high concentrations—for example, London Smog (Amdur et al., 1978; Utell et al., 1984; Hackney et al., 1989; Koenig et al., 1989; Costa and Schlegele, 1998). The variability in asthmatics may well relate to differences in the degree of impairment or underlying inflammation in the subjects as has been shown in some animal models, but this hypothesis remains to be confirmed.

Effects on Mucociliary Clearance and Macrophage Function Sulfuric acid does alter the clearance of particles from the lung. Using insoluble, radioactively labeled ferric oxide particles as a probe exposure in donkeys, rabbits, and human subjects show impaired clearance after a single 1-hour exposure (Schlesinger, 1984). Curiously, there appears to be a biphasic response to acid. In general, brief, single exposures of less than 250 μg/m^3 accelerate clearance, while high concentrations of more than 1000 μg/m^3 clearly depress clearance. With repeated daily exposures to low levels, there appears to be a cumulative (concentration times duration) dose-related depression of clearance. Longer-term exposure of rabbits to low-level acid also results in hyperplasia of airway mucosecretory cells (Gearhart and Schlesinger, 1989).

Collectively, there seems to be coherence in the data to rank sulfate irritancy: sulfuric acid > ammonium bisulfate > ammonium sulfate. Acidity [H^+] appears to be the primary driver on most respiratory effects. Nevertheless, in the complexity of summer haze, it remains unclear whether the bioactive form of H^+ is more appropriately assayed as free ion concentration (as pH) or as total available ion concentration (titratable H^+).

Chronic Effects As a fine aerosol, sulfuric acid deposits deeper along the respiratory tract, and its high specific acidity imparts greater effect on various cells (e.g., phagocytes and epithelial cells). As noted earlier, the primary concern with regard to chronic inhalation of acidic aerosols is the potential for bronchitis, because this has been a problem in occupational settings in which employees are exposed to sulfuric acid mists (e.g., battery plants). Studies in several animal models raise these concerns as cigarette smoke shows parallel responses. Related studies suggest that the airways of exposed animals become progressively more sensitive to challenge with acetylcholine, show a progressive decrease in diameter, and experience an increase in the number of secretory cells, especially in the smaller airways (Schlesinger et al., 1992). While highly variable, the possibility that chronic irritancy by inhaled sulfuric acid may elicit bronchitis-like disease in susceptible individuals (perhaps over a lifetime or in children as noted in the Six Cities Study) appears to be reasonable.

Particulate Matter

Particulate matter (PM) was referred to as "soot" in the "reducing-type" air pollution of the classic episodes. The major constituents of this soot consisted of incompletely burned carbonaceous materials,

acid sulfates, various metals, and silicates associated with the solid nature of the fuel. Metal compounds were abundant, with a considerable amount of zinc in the form of zinc sulfate—as reported from post-episode analyses of the Donora PM. Soot is indicative of poorly (inefficiently) combusted fuel. Over time, combustion technology and improved fuels provided gains by increasing efficiency and minimization of gross soot emissions. Improvements in combustion methods simultaneously reduced the size of emitted particles and overall less mass. A side benefit of the smaller particles was the reduction in light diffraction through the emissions and hence a less visible plume. As such, much of the early cleanup was largely achieved through technological improvements. Carbon in fuel that is not fully oxidized to CO_2 persists as black carbon which typically is a mix of the elemental form with variable products of incomplete combustion—organic carbon. Oxidized sulfur becomes sulfate and many of the metals appear as oxides. As noted above, sulfate was long suspected as the essential culprit of most health impacts associated with stationary sources, but this relationship is less discernable in contemporary particle epidemiology and has no good evidence for adverse health effects at reasonable concentrations in either human or animal toxicology studies.

In the last 20 years, PM has reemerged as the dominant societal issue in air pollution, overtaking O_3 as the impact leader in health outcomes. Not that O_3 is of minimal concern, but emergent epidemiology data consistently showed increased mortality, the adverse effect of greatest import having a major societal impact when viewed through cost/benefit analyses. As already noted, the collective studies showed health impacts (both morbidity and mortality) at levels of PM thought to be "safe" with no apparent threshold (reviewed in Pope and Dockery [2006]). Over time, science advances and re-analyses have confirmed the initial mortality findings (U.S. EPA, 2009). In the United States, studies show that even with great reductions in PM, $PM_{2.5}$ still accounts for about 5% of the total mortality burden; indeed, the World Health Organization estimates that PM accounts for up to 7 million deaths a year worldwide. In the latter case, the deaths may be associated with incomplete combustion emissions from domestic burning of biomass.

Several hypotheses lend "biologic plausibility" to the findings and have been linked to an array of effects that have modes of action coherent with potential mortality. The impacts of PM on health have eventually been appreciated to be more systemic than originally thought, beyond the lung as the primary target organ. Significant impacts on the cardiovascular system have emerged and are now generally believed to be the predominant driver of air pollution–related mortality outcomes. Further, there is an ever growing body of data suggesting impacts on neurologic and reproductive health, as well as the more expected impact on the lung—especially in children where chronically PM appears to impact lung growth and development (Gauderman et al., 2015).

Particulate matter in the atmosphere can be solid, liquid, or a combination of both with a mélange of variably combusted or photochemically derived organic compounds coexistent with inorganic species and biological materials or associated compounds. The compositional matrix of PM can vary significantly over time and regionally likely due to varied emission sources and secondary transformations in the atmosphere, which are weather mediated. Long-range transport of emissions or transformation products include sulfates from the oxidation of sulfur in fossil fuel emissions and complex organic compounds called secondary organic aerosols (SOA). Hence, these components comprise the regional matrix of PM, particularly in the case of sulfate in East Coast $PM_{2.5}$. Particles in urban centers, notably in the Western United States, have SOA as the prime component. Particles of larger size (PM_{10}) tend to have more local sources being that they are formed from dispersed dust and attrition of materials. Being of greater mass, the larger particles tend to "fall out" or settle from the air due to gravity (although winds can in fact carry these particles great distances—e.g., Sahara desert particles have been found on the U.S. East Coast). Particles in the range of 10 to 2.5 μm ($PM_{10–2.5}$—coarse PM) are highly inhalable by humans but tend to deposit in the upper airways again due to their mass. In urban settings throughout the world, there is considerable spatial and temporal heterogeneity of coarse PM while $PM_{2.5}$ appears somewhat more homogenous throughout a regional air shed. The size designation of fine and coarse PM is based on their relative respirability—those in the range of PM_{10} are inhalable and deposit in the nasal cavity and larger thoracic airways while the $PM_{2.5}$ is respirable and deposits in the deeper reaches (small airways and gas exchange areas) of the lung (see Chap. 15, "Toxic Responses of the Respiratory System").

The foundational epidemiologic database contending that PM elicits both short- and long-term health effects is largely founded on data from monitoring networks used for PM regulation in North America and Europe. As such, the PM-associated effects appear to be dependent on the gravimetric measures that these networks yield. From this, it might then be argued that the effects are not influenced by particle composition (e.g., inorganic and organic components). Further, because the strength of the impacts increases with decreasing particle size (e.g., total suspended particulate mass [pre-1978 index], PM_{10}, $PM_{2.5}$), it appears that it is the mass concentration of PM, despite compositional complexity and variation, that is the index of choice. However, toxicologists argue that a mass-based relationship contradicts the basic tenets of conventional air pollution toxicology, which is rooted in the concept of chemical-specific toxicities. That is certainly born out with exposures due to proximity to specific sources (e.g., a smelter or coke oven), but oddly data continue to show mass as a primary driver. On the other hand, source apportionment studies strongly suggest, at least epidemiologically, that particular PM sources, such as coal-fired power plants and high volume traffic, contribute to a greater share of the adverse health outcomes and toxicological studies from airsheds of varied compositional character provide refinement in the nature and causation of adversity. The mass driver which encompasses components and interactions among them seems at this point the exposure metric for mortality.

A number of hypotheses that draw upon various physical and chemical attributes of PM have also been offered in search of a "biologically plausible" explanation for the reported epidemiologic observations. However, no one constituent has been identified as singularly determinant of the spectrum of health impacts. The smallest particles, which derive from anthropogenic combustion activities, appear to drive many of the health effects of particles. Prominently included among the causative hypotheses that tie toxicity to particle characteristics are metals, organics, acidity, size distribution (focusing on the potentially unique bioactivity of ultrafine PM—PM less than 0.1 μm), PM oxidant activity or reactivity, and the presence of potentially toxic or allergenic biologicals. However, at present, there remains insufficient understanding of the relative importance of these theories to choose one over another, especially if the spectrum of health effects is included. As a result, there seems to be insufficient reason to unseat the PM mass-based correlation with health outcomes as the driver of regulatory policies. Although the animal and human toxicological database is growing rapidly with regard to the issue of causation, much remains to be learned before new regulatory indices can be considered.

From research directed initially toward potential occupational hazards, it is known that several metals and silicates that make up much of the inorganic phase of PM can be cytotoxic to lung cells. Organic constituents associated with black carbon, as well, can

induce toxicity either directly or via metabolism products—some of which are genotoxic. Other PM attributes may also come into play. Studies focusing on very small, ultrafine particles suggest that although these particles are low in mass, they are high in number and thus provide substantial reactive particle surface to interact with biological substances or that they can more easily penetrate through the lung surface and impart their effects systemically. Less is known about the role of biologically derived ambient materials, such as endotoxin, glucans, plant glycoproteins, and bioallergen fragments, which may elicit rudimentary inflammatory responses in the lung. The involvement of biologicals may be greatest in agricultural and indoor exposure environments. There has been considerable interest in PM–co-pollutant interactions, but our knowledge in this area is somewhat limited and has spurred toxicologists to better access the experience and knowledge of atmospheric scientists as to the atmospheric interactions that are most relevant to biologic outcomes.

Metals There have been many conventional acute and chronic rodent inhalation studies conducted with specific metal compounds, often as oxides, chlorides, or sulfates. The varied toxicological effects of metal compounds are presented in detail in Chap. 23, "Toxic Effects of Metals." However, it should be appreciated that the effects of metals delivered by inhalation may differ from their impacts when administered by other routes and as such inhalation exposures perhaps best relate to occupational exposures. Metals may arise from natural as well as anthropogenic activities, and as a result metals are a common constituent in ambient PM. The PM metal profiles vary geographically and by contributing sources; hence, they can differ appreciably in concentration and type and the size mode in which they occur. Coarse $PM_{10-2.5}$ arises largely from natural sources and thus has prominent earthen metals such as iron, sodium, silica, and magnesium—usually in geologically stable oxide forms. On the other hand, fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) are richer in combustion-derived metals, often reflecting the fuel source and nature of combustion. For example, diesel fuel may have vanadium, nickel, and perhaps zinc and iron, while coal may have zinc and selenium. Their chemical forms vary from water-soluble metal salts and sulfates to oxide and phosphate forms. High temperatures and efficient combustion frequently take metals to their smallest size and most oxidized form—the oxides. Metals are also emitted from vehicles burning fuels to which metal compounds were added to alter functionality (e.g., lead, manganese, and platinum) or as engine wear and catalyst by-products. Similarly, metals may also be derived not from combustion processes but from brake (copper, iron), tire (zinc), and road (earthen silicates or dust re-dispersion) wear—again in the larger coarse mode. Metals have many biologic properties, some essential to life while others can be directly toxic to cells or act indirectly as pro-oxidants. Thus, metals have garnered considerable interest regarding their potential role in PM toxicity (Costa and Dreher, 1997).

Metal compounds can be separated nominally by physicochemical characteristics: those that are essentially water-insoluble (e.g., metal oxides and hydroxides such as those that might be released from high-temperature combustion sources or derived from the geocrustal matrix) and those that are soluble or somewhat soluble in water (often chlorides or sulfates such as those that might form under acidic conditions in a smoke plume or leach from acid-hydrated silicate particles in the atmosphere). Solubility appears to play a role in the toxicity of many inhaled ambient-PM associated metals by enhancing metal bioavailability (e.g., nickel from nickel chloride versus nickel oxide), but insolubility also can be a critical factor in determining toxicity by increasing pulmonary residence time within the lung (e.g., insoluble cadmium oxide versus soluble cadmium chloride). Moreover, some metals, either in their soluble forms or when partially coordinated on the surface of silicate or bioorganic materials, can promote electron transfer to form reactive oxidants (Fenton chemistry). Complexes with particulate organic material in a partially hydrated form (as might be promoted by the presence of sea salt) have been shown to interact with poorly soluble metals to free coordination sites to yield pro-oxidant activity (Kieber et al., 2005). Thus, caution is warranted in assessing inhaled PM-associated metals simply on the basis of their chemical form and concentration, as both their chemical and physical attributes and their potential interactions with co-contaminants in PM may influence their ultimate toxicity. Simply measuring total metal mass to estimate adverse effects in the lung can be misleading.

Perhaps the most compelling evidence for a role of soluble metals in PM toxicity derives from a series of U.S. studies on PM sampled in Utah Valley where a large open hearth steel mill went from full production to a 1 year hiatus due to a labor strike and then resumed its open hearth operations. These studies related the metal content of PM sampled in the air surrounding the plant showing a substantial reduction in total PM as well as a reduction in its usual PM metal content during the strike period. Studies of hospital admissions for a variety of lung ailments and death rates showed reductions during the steel mill hiatus in operations which were mirrored in toxicological studies where PM extractions from the operational and shutdown periods were tested in human and animal lungs, as well as on human lung epithelial cells. All studies showed adverse effect correlations with plant operations and PM metal content (Pope, 1996; Dye et al., 2001; Ghio, 2004).

Gas–Particle Interactions As already noted, gas–particle interactions can be extremely complex involving multiple components of the particles, gases/vapors, and sunlight. While most of this chemistry occurs in the air, more than 50 years ago, generic binary interactions between particles and gases, in the absence of light, were shown to alter the toxicity of either the particle or the gas compared to acting alone. The guinea pig bronchoconstriction model used for many years by Amdur and associates showed early-on that SO_2 can interact with hydrated metal salts to potentiate particle irritancy. The mechanism(s) behind this interaction has yet to be fully discerned, but it appears to involve solubility of SO_2 in a hydrated aerosol and the ability of the metal to catalyze the oxidation of the dissolved SO_2 to sulfate during inhalation. In the case of a sodium chloride aerosol, potentiation appeared to be governed primarily by the solubility of SO_2 in the salt droplet and enhanced respiratory penetration. The metal salts of manganese, iron, and vanadium, however, were shown to rapidly catalyze the formation of acid sulfate that could then be quantified in the aerosol, and mediated the irritancy in the bioassay. Interestingly, similar studies in humans have been less revealing about such interactions and their toxic expression, but this in vivo bioassay database affirms the need to consider the complexity of the atmospheric challenge in estimating biological outcomes.

Complex chemistry also occurs within the effluent of the combustion source. Using a laboratory scaled furnace, the emission mix of sulfuric acid and metal oxide particles common to metal smelting and coal combustion was used to explore potential plume interactions that might impact respiratory irritancy (Amdur et al., 1986). These emitted metal oxide particles, once aged and cooled, were a mixture of respirable singlet and agglomerated ultrafine particles. Exposure of guinea pigs to 30 to 60 $\mu g/m^3$ sulfuric acid combined with ultrafine zinc oxide produced progressive decreases in the diffusing capacity for CO (DL_{CO}), total lung capacity, and vital capacity and increases in cells, protein, and a variety of enzymes in lavage fluid that were not completely resolved 96 hours after exposure (Amdur, 1989). It is unclear whether the acid was on the surface

of the particles or made the metal more soluble, but the combination was clearly more toxic than acid alone. Recently, atmospheric studies have suggested that acid sulfate may contribute to the dissolution of metals from particles and make them bioavailable for effects. This could show a role for sulfuric acid that is beyond its apparent minimal intrinsic impacts on the lung—except perhaps in some asthmatics.

Combustion studies using different coals again emphasized the significance of surface-associated acidic *S*-compounds. Ultrafine combustion particles from Illinois No. 6 coal had a layer of sulfuric acid adsorbed on the surfaces resulting in greater effects in guinea pigs than the more alkaline Montana lignite. Despite the greater sulfur content of the Montana coal, this alkaline ultrafine particle had neutralized the irritating sulfate (Chen et al., 1990). Similar studies using inert carbon black appear consistent with its role as carrier for reactive gases or reactive products of the interactions with pollutants such as O_3 and various aldehydes, perhaps enhancing delivery of toxic materials to the deep lung (Jakab, 1992). The result of the latter study was enhanced infectivity when the carbon + gas pre-exposed test animals were subsequently exposed to pathologic bacteria.

Similar interactions may result from gaseous pollutants that impair the clearance of particles from the lung or otherwise alter their metabolism. Studies by Laskin at New York University in the 1960s showed an intriguing interaction of SO_2 and benzo(*a*)pyrene. It was thought that impaired clearance and greater residence time in the lung led to the enhanced probability of carcinogenic potential of the particle. Similarly, rats exposed to an urban 8-hour daily profile of O_3 for 6 weeks, followed by a single exposure to asbestos, were found to retain three times as many fibers deep in the lung 30 days after exposure. The fibers were deposited in the distal airways and penetrated more deeply into airway tissues making them less accessible to phagocytic removal (Pinkerton et al., 1989). These studies, together with those focusing on irritancy and infectivity, raise the prospect that realistic exposure scenarios of gaseous and particulate pollutants can interact through either chemical or physiologic mechanisms to enhance health risks of complex polluted atmospheres.

Ultrafine Carbonaceous Matter Ultrafine ambient carbon particles (often called black or elemental carbon) are emitted during combustion and can mix with less well combusted carbonaceous material (sometimes called brown carbon or particles of incomplete combustion [PICs]) or may occur in a fairly pure form from high-temperature pyrolysis. Black carbon, an older term, is measured differently from elemental carbon, but both are highly correlated and often the terms are used interchangeably. The size of these particles allows them to slip (Brownian motion) between gas molecules moving primarily by diffusion. Agglomeration among themselves, on surfaces, or with larger particles in the air is their primary mode of dissipation. When concentrations exceed about a million particles per cubic centimeters, they rapidly agglomerate with each other to form larger clumps or chains of ultrafine particles. These agglomerates are typified by diesel exhaust particles in the air. As an air pollutant, therefore, elemental carbon particles generally do not exist as singlets except near their emission points—for example, traffic or other high temperature sources. Fine PM consist in part of agglomerates of these and other carbonaceous organic material (brown carbon or products of photo-oxidation known as secondary transformation), which if partially oxidized are variably (though mostly not) soluble in water. Some organic materials which exist in the vapor form may also condense on ultrafine black carbon (e.g., diesel PM).

Estimates of the carbonaceous (including organic) content of ambient fine PM vary considerably but are nominally considered to be about 10% to 60% of the total PM mass depending on the urban or regional area. The sources of organic carbon are varied and include the combustion products of natural smoke (e.g., forest fires), engine exhaust, and stationary sources (even fast-food exhaust emissions). Where there is a strong solar energy source, transformed condensates from volatile organic compounds (VOCs) in the air also can contribute substantially to PM mass. It has been estimated that diesel exhaust contributes about 7% of the primary fine urban PM load, but estimates in urban canyons vary widely. The higher use of diesel fuel for transportation in Europe and areas of concentrated trucking in the United States have led to estimates as high as 30% of local ambient fine PM mass. Elemental carbon has traditionally been associated with traffic. In the United States, black carbon is considered a better marker of older and off-road vehicles, but as new engine technology has emerged in 2007 and 2010 for diesels to meet new emission standards, black carbon emissions are substantially less (less than 99%). New automobiles also emit less black carbon as technology transfers to these vehicles. The expected result over the next 20 years, with the turnover of diesel-powered truck fleets in particular, is a great reduction in ambient levels of black carbon with California already showing these reductions as early adopters of the new technology.

Diesel exhaust particles vary widely in the ratio of organic and elemental carbonaceous materials, which in empirical studies has been shown to influence toxic outcomes, such as their inflammatory and carcinogenic potential (Singh et al., 2004; HEI, 2015a, 2015b). Some diesel particles also appear to have adjuvant activity when tested with bioallergens in both animals and humans (Diaz-Sanchez et al., 1999). When reacted in vitro with O_3, there appears to be an enhancement of lung inflammation relative to the diesel or O_3 alone (Madden et al., 2000). However, it is important to realize that diesel particles should not be equated with whole diesel exhaust that also contains significant amounts of gaseous pollutants: NO_x, CO, and SO_x as well as various VOCs and carbonyl irritants. Exposure to diluted diesel exhaust in humans reveals that the exhaust mix is inflammogenic in the lung (Salvi et al., 2000) and to a degree cytotoxic to airway cells (Ghio et al., 2012). The use of diesel particles alone in toxicology studies does not seem to display similar toxicity to the total exhaust, thus underscoring the potential importance of interactions among air pollutants as a critical consideration in air pollution toxicity (Mauderly and Samet, 2009).

Recent epidemiological and field studies with PM have focused on black or elemental carbon as an index of traffic and reported strong associations between traffic (through the carbon indicator) and adverse health effects. Ultrafine particles in the near-traffic environment exist in extremely high numbers but contribute negligibly to mass, the metric which has proven to be most credible to date. However, the predominance of cardiovascular associations gives some credence to a hypothesis that ultrafine particles somehow find their way into the systemic circulation and/or trigger systemic inflammation that links to the cardiovascular effects (Peters, 2006; Tong et al., 2010). The ultrafine mode of black carbon has been suggested to be more toxic than fine-mode $PM_{2.5}$, perhaps due to enhanced surface reactivity or co-pollutant carrying capacity as noted above, but tissue penetration (Donaldson et al., 1998; Oberdorster et al., 2002) has emerged as a hypothesis as well. The penetration of ultrafine particles from the lung into the circulation under experimental conditions has been reported, but appears to be composition dependent (Kreyling et al., 2009); this unique potential of ultrafines to act through systemic penetration is intriguing but remains to be shown as a major contributing factor to ambient PM toxicity.

Pure elemental carbon itself is generally considered to be of low toxicity, although long-term, high-concentration exposure conditions in rats can lead to lung "overload" where there is evidence of lung damage and carcinogenicity. Recent commercial introduction

of "engineered" nanoparticles brings many of the same concerns as ultrafines by virtue of their similar sizes. Additionally, being "engineered" particles, they may possess design features that "natural" combustion ultrafine (or nano) particles do not.

Internationally, the impact of black carbon remains complex as many of the older transportation and trucking vehicles being replaced are sold to the developing world so their contribution to global emissions will not fall as rapidly. Moreover, the use of two-cycle engines and the domestic combustion of biomass in the rural developing world will continue to contribute greatly to the global load and health impact of black carbon and PM. Not fully appreciated as an indirect impact of black carbon to air-related adverse health is via the impact of black carbon on climate. Black carbon from developing world, as well as older industrial world, sources undergoes significant intercontinental transport which in the Northern Hemisphere is preferentially north resulting in deposition on northern snow mass. Unlike CO_2 which has long residency in the atmosphere, black carbon ultimately does deposit and hence is called a short-term climate forcer via its northern polar impacts. The impact on climate and secondary impacts on atmospheric chemistry link black carbon to potential worsening air health as climate evolves in the future through its contribution to the formation of other pollutants such as O_3.

Chronic Effects and Cancer In 2013, the WHO's International Agency for Research on Cancer (IARC) examined the published science and concluded that air pollution causes lung cancer (Straif et al., 2013). Moreover, based on published data, the committee concluded that the component of air pollution most likely responsible was PM due to the complexity of carcinogenic components (polycyclic aromatic hydrocarbons [PAHs]) that comprise PM. The cumulative epidemiology literature is largely consistent with this assessment (Hamra et al., 2014). Linked to the PM is its composition of complex organic compounds (e.g., PAHs), which in part are thought to be linked to diesel exhaust, which IARC designated as a primary human carcinogen in 2012 largely based on studies involving occupational exposures to diesels in mines (Silverman et al., 2012). Hence, the carcinogenicity issue appears to be generally accepted by the scientific community. It should be noted, however, that post 2007 heavy duty diesel engines have been re-engineered and most possess filters greatly (more than 99%) reducing PM and its empirical carcinogenicity (HEI, 2015a).

Cigarette smoking (also replete with PAHs) remains a confirmed cause of lung cancer and clearly associated with other systemic cancers. The IARC assessments and U.S. cohort studies, such as that using the American Cancer Society (ACS) cohort (Pope et al., 2011), show a significant linkage between ambient PM and lung cancer in the United States even at the generally lower PM levels compared other countries. Yet, at the same time, it has been difficult to separate PM cancer from those cancers that appear linked to the many hazardous air pollutants (HAPs) that occur in urban air and are also thought to be carcinogenic. Many suspect HAPs, but few (about 10%) of the more than 2800 compounds that have been identified in the air have been assayed for carcinogenic potency. Fig. 31-15 gives estimates of the relative contributions of various chemicals to the lung cancer risk for outdoor and indoor air (Loh

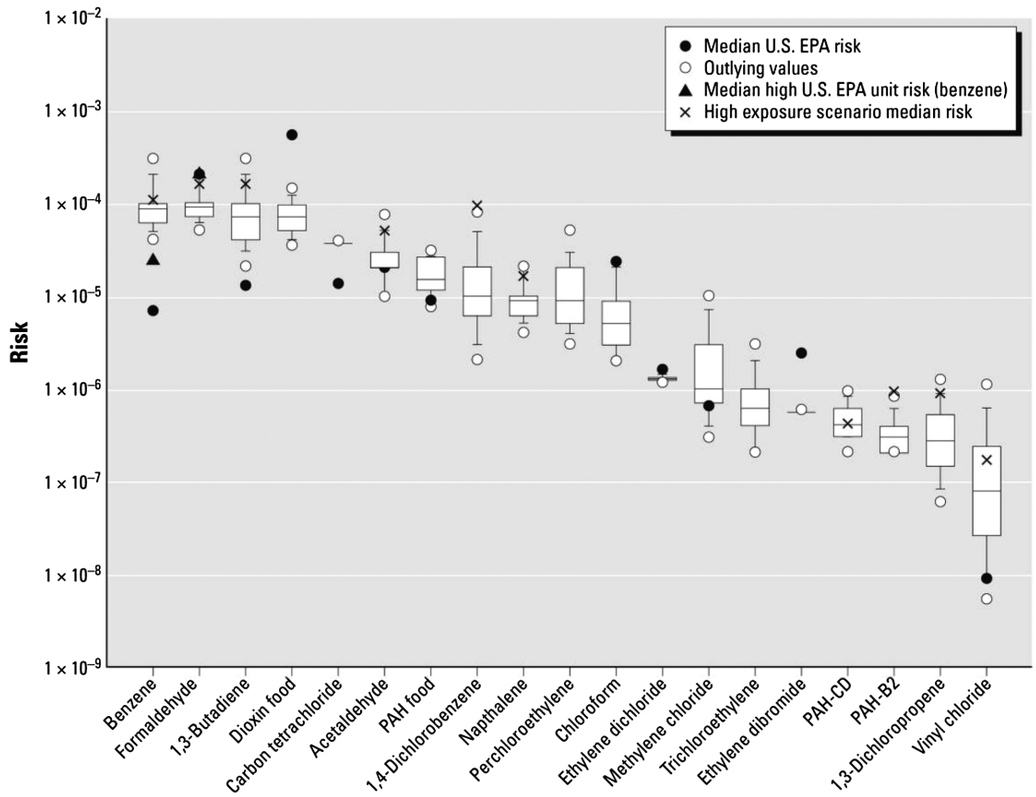


Figure 31-15. *Relative contribution of individual airborne hazardous pollutants to lung cancer rates using California Office of Environmental Health and Hazard Assessment (OEHHA) and U.S. EPA unit risks (Loh et al., 2007).* The symbols reflect unit risks for the inhalation route of exposure (except as noted for food) at home and outdoors. Bars represent 5% and 95% and boxes represent 25%, 50%, and 75%. PAH = polyaromatic hydrocarbons grouped into B2 (evidence of carcinogenicity in animal studies) and CD (less experimental evidence).

et al., 2007). Volatile and related halogenated organic and nitrogen-containing compounds account for most of the compounds that have been studied with animal and genotoxicity bioassays. Most of these compounds are derived from combustion sources ranging from tobacco to power plants to incinerators to motor vehicles. Other potential carcinogens arise from mobile sources as organic emission products of incomplete combustion as well as their even more complex atmospheric transformation products. Fugitive or accidental chemical releases also figure into the many chemicals in ambient and indoor air. The National Scale Air Toxics Assessment (NATA) is an assessment of the cancer and noncancer risks associated with ambient HAPs (U.S. EPA, 2018). The profile of outdoor carcinogens contrasts with that of indoor air, where the sources are thought to derive largely from environmental tobacco smoke and radon, with some contribution from off-gassed organics (e.g., adhesives, carpet polymers, and cleaning chemicals). Thus, human exposure to airborne toxicants is highly complex compositionally as well as in its temporal and spatial heterogeneity.

Despite all the evidence linking air pollution with lung cancer, the risk of any individual is a complex function of the carcinogenic nature of the substance, the amount, and nature of material deposited in the lungs, which is itself a function of the concentration in the ambient air, the physical and chemical properties of the inhalant that may determine deposition efficiency, innate repair mechanisms, and the cumulative volume of air inhaled. A significant body of data suggests that the majority of lung cancer risk from ambient air pollution lies within the PM fraction. Among the many potent chemicals are the polycyclic organic chemicals, along with a group of less volatile organics sometimes referred to as "semi-volatiles" (including nitroaromatics). These persistent organics associate with the PM matrix and thus could have a prolonged residence time at deposition sites within the respiratory tract. Genetic bioassays have revealed the potent mutagenicity, and presumably carcinogenicity, of various chemical fractions of ambient aerosols (Lewtas, 2007). Some of these compounds require metabolic transformation to activate their potency while others may be detoxified by their metabolism. Not to be forgotten are the VOCs noted earlier that are likely carcinogenic (e.g., benzene) and which are inhaled but have target tissues away from the lung—in the case of benzene, bone marrow leukemia.

Not to be undervalued is that the innate susceptibility of the individual (including genotype-driven characteristics and environmental factors such as diet) is also likely to be important. The cells lining the respiratory tract turn over relatively quickly as they interface directly with the ambient environment with every breath. As such, their DNA would be vulnerable to carcinogenic or oxidant-induced replication errors from inhaled substances that, when fixed as mutations, could give rise to tumors. Co-pollutants, such as irritant gases, that initiate inflammation may promote this carcinogenic activity by damaging cells and enhancing their turnover. For example, there is experimental evidence that benzo(*a*)pyrene inhaled by rats whose respiratory tracts have been chronically exposed to SO_2 are prone to bronchogenic carcinomas. Likewise, epidermoid carcinomas were produced in mice that inhaled ozonized gasoline vapors, containing reactive organic products, but this occurred only if the mice had previously been infected with influenza virus and presumably had inflamed lungs. Many believe that the so-called rural–urban gradient of lung cancer, apparent even when corrected for cigarette smoking, is a product of such complex chemical–biological interactions. Thus, while the initiation of environmental lung cancer remains poorly understood, there is general sentiment for the opinion expressed early-on by Kotin and Falk (1963): "Chemical, physical and biological data unite to form a constellation that strongly implicates the atmosphere as one dominant factor in the pathogenesis of lung cancer." At the time of this statement, however, even the role of tobacco smoke in cancer was not widely recognized.

To understand the role of inhaled particles in non-cigarette-smoke-related lung cancer, chronic exposure studies have been conducted with a number of particles ranging from the relatively inert particles of titanium dioxide and carbon to the more complex particles of diesel exhaust and coal fly ash. Of these substances, diesel exhaust has been the most extensively studied (reviewed by Ghio et al. [2012] and HEI [2015b]). The primary concern with diesel has been the long-held suspicion that it can induce lung cancer (as noted now as an IARC—designated human carcinogen). While the assembled epidemiology data on diesel exhaust formed the basis of the IARC designation of diesel exhaust (including the PM and gases) as a primary carcinogen, the empirical studies of carcinogenicity alone has long been suggested by several chronic exposure studies in animals and by in vitro data indicating mutagenicity in *Salmonella* bacteria and enhanced sister chromatid exchange rates in Chinese hamster ovary cells. (These latter genotoxic effects have been linked to the nitroarenes associated with the diesel PM.) Rodent studies, unfortunately, have not fully provided great insights as to the mechanisms involved other than the relatively generic—mutation/inflammation—pathways, and it has only been at very high exposures in which mucociliary clearance has been sufficiently overwhelmed allowing in a progressive buildup of particles in the lungs leading to what has been termed "overload" (see below). By 12 months into exposure, in the rat, clearance has ceased and there is concomitant inflammation, oxidant generation, epithelial hyperplasia, and fibrosis. Particle agglomerates within the alveolar lumen become the focus of inflammation, injury, and the eventual development of adenosarcomas and squamous cell carcinomas. At lower concentrations, where the particle buildup (i.e., overload) does not occur, tumors do not develop.

"Overload" is a common finding at the highest exposure concentrations of chronic particle inhalation bioassays. While this phenomenon is not likely in humans exposed to ambient PM, it is a hypothesis worthy of consideration in a discussion of particle health effects, as it relates to the interpretation of toxicological data relevant to product use and industrial exposures. Several poorly soluble particles (PSPs), such as titanium dioxide and toner carbon, have induced lung tumors in chronic rat bioassays under conditions of overload; tumors have not developed under similar conditions in mice and hamsters. Thus, rats seem to react more to this circumstance. Particles in the ultrafine mode seem particularly potent. In the rat, pathogenesis is qualitatively and temporally the same for all of the particle types tested. In contrast, the degree of active inflammation in the mouse and hamster under similar overload conditions appears less intense, and thus is an important distinction among the species that relates to their relative sensitivities. Such exposures do not typically demonstrate classic genotoxicity, although bulky adducts and other modifications to DNA have been described after in vivo exposure to carbon black and diesel exhaust (reviewed by Schins and Knaapen [2007]). In addition, mutation of the *hprt* (hypoxanthine guanine phosphoribosyltransferase) gene was found in rat epithelial cells cultured with bronchoalveolar lavage from chronic carbon black and titanium dioxide treated rats (Driscoll et al., 1997).

Even though these few studies have demonstrated inert particle-induced modifications to DNA, generalizing this finding to overload cancers, not surprisingly, remains controversial. Typically, the pathways leading to lung cancer by relatively inert and poorly soluble particles (e.g., carbon black and diesel exhaust particles) are thought to be secondary and involve pathways such as oxidative

stress rather than a primary carcinogenesis mechanism such as DNA mutations (Greim et al., 2001). The closest analogy in humans would be coal miners who do not appear to have an enhanced risk of lung cancer except when smoking is not involved.

The issue, then, is whether rat bioassay cancer data under conditions of overload are relevant to risk assessment. A review by an expert panel (Olin, 2000) concluded that rats, while apparently unique in this response, may represent a sensitive subgroup, and that tumorigenesis data from the rat bioassay under conditions of overload cannot be summarily dismissed as not relevant to the consideration of cancer risk in humans. However, the data should be interpreted and weighed in the context of lower concentrations and the tumor incidence and pathology found therein.

Photochemical Air Pollution

Photochemical air pollution (notably O_3) remains the most elusive of the criteria pollutants in ambient air to bring under regulatory control. It is estimated that more than 130 million Americans reside in areas not in compliance with the health-based standard and hence are at some risk of adverse health impacts. Ozone arises secondarily from a series of complex reactions in the troposphere activated by the ultraviolet (UV) spectrum of sunlight. In addition to O_3, it comprises a mixture of nitric oxides (NO_x), aldehydes, peroxyacetyl nitrates (PAN), and a myriad of aromatics and alkenes along with analog reactive radicals and intermediates. If SO_2 is present, sulfate particles may also be formed and, collectively, they yield "summer haze." Likewise, the complex chemistry can generate organic PM, nitric acid vapor, and various condensates. Attempts to mitigate photochemical smog (especially O_3) by controlling hydrocarbon and/or NO_x emissions have proven difficult due to the complex stoichiometry of atmospheric photochemistry. Some progress has been made in controlling peak values in the United States (regulated by the pre-1997 1-hour NAAQS), but the longer time-framed 8-hour NAAQS has seen generally smaller progress. Because of particularly strong regulatory efforts in Southern California, however, the average number of high ozone days per year dropped by 33% between 2000 and 2010 in the Los Angeles–Long Beach–Riverside metropolitan area, the most polluted part of the United States, in terms of ambient ozone levels, and high ozone days continue to decrease (American Lung Association, 2017). In 2016, Los Angeles experienced fewer high ozone days than previously recorded.

From the point of view of the toxicology of photochemical air pollutant gases, O_3 is by far the toxicant of greatest concern. It is highly reactive and more toxic than NO_x, and because its generation is fueled through cyclic hydrocarbon radicals, it reaches greater concentrations than the hydrocarbon radical intermediates. The gaseous hydrocarbons, so integral to the chemistry, are no longer listed collectively as a criteria pollutant in the United States because they do not have a strong health-based driver. In general, the concentrations of the hydrocarbon precursors in ambient air do not reach levels high enough to produce acute toxicity, although some individual compounds fall under the HAPs rule. Rather, the importance of these hydrocarbons stems from their role in the chain of photochemical reactions.

Although O_3 is of toxicological importance in the troposphere, in the stratosphere it plays a critical protective role. About 10 to 50 km above the Earth's surface, UV light directly splits molecular O_2 into atomic $O^{\bullet}$, which then combines with O_2 to form O_3. The O_3 also dissociates back but much more slowly. The result is an accumulation of O_3 to several ppm within a relatively thin strip of the stratosphere forming an effective "permanent" barrier by absorbing the short-wavelength UV in the chemical process. This barrier had in recent years been threatened by various anthropogenic emissions (Cl_2 gas and certain chlorofluorocarbons) that enhance O_3 degradation (creation of an "O_3-hole"), but recent restrictions in the use of these degrading chemicals seem to have been effective in reversing this process, although recently concern is mounting that the hole may be growing again due to the release of other halogenated gases. The benefits of this stratospheric O_3 are the reduction of excess UV light infiltration to the Earth's surface and reduced skin cancer risk.

The impact of O_3 in the troposphere, where its accumulation serves no known purpose, poses a threat to the respiratory tract when inhaled. Near the Earth's surface, NO_2 arising from combustion processes efficiently absorbs the longer wavelength UV light spectrum, from which a free O atom is cleaved, initiating the following simplified series of reactions:

$$NO_2 + h\nu(\text{UV light}) \rightarrow O^{\bullet} + NO^{\bullet}. \quad (31\text{-}1)$$

$$O^{\bullet} + O_2 \rightarrow O_3. \quad (31\text{-}2)$$

$$O_3 + NO^{\bullet} \rightarrow NO_2. \quad (31\text{-}3)$$

This process is inherently cyclic, with NO_2 regenerated by the reaction of the $NO^{\bullet}$ and O_3. In the absence of unsaturated hydrocarbons (olefins and substituted aromatics) arising from fuel vaporization or combustion, as well as biogenic terpenes, this series of reactions would approach a steady state with little buildup of O_3. The free electrons of the double bonds of unsaturated hydrocarbons are attacked by free atomic $O^{\bullet}$, resulting in oxidized compounds and radicals that react further with $NO^{\bullet}$ to produce more NO_2. Thus, the balance of the reactions sequence shown in Eqs. (31-1) to (31-3) is tipped to the right, leading to buildup of O_3. This reaction is particularly favored when the Sun's intensity is greatest at midday, utilizing the NO_2 provided by morning rush-hour traffic. Carbonyl compounds (especially short-chained aldehydes) are also by-products of these reactions. Formaldehyde and acrolein account for about 50% and 5%, respectively, of the total aldehyde content in urban atmospheres. Peroxyacetyl nitrate (CH_3COONO_2), often referred to as PAN, and its homologs also arise in urban air, most likely from the reaction of the peroxyacyl radicals with NO_2. In the evening when the Sun's intensity wanes, the second rush-hour peak of NO_2 shifts the balance back by reacting with O_3 slowly bringing the daytime peak concentration down. In assessing the excess adverse health effects of O_3, it is important to determine the background level of ambient O_3. Interestingly, background levels of ozone in North America are known to peak as high as 0.14 ppm (1 hour), particularly in high elevation regions such as Wyoming, although the contribution of these transient 1 hour levels of ozone to general public health is unclear. This high altitude peak concentration is thought to be due to a combination of high VOC levels, NO_x, and the additional UV light reflected off snow cover. However, other factors such as season, thickness of boundary layers, vertical intrusions from the stratosphere, and long range transport also play roles in determining background levels of O_3 in North America. The recent growth in natural gas fracking operations along the eastern range of the United States has resulted in fugitive losses of methane which may also be contributing to O_3 formation. This issue is highly controversial and shows the tenuous temporality of any intervention to reduce air pollutants formed secondarily due to complex chemistry.

Short-Term Exposures to Smog

During the 1950s to 1960s, early air pollution toxicologists were challenged by the complexity of photochemical air pollution to ascertain its potential both to adversely affect human health and to

determine what about these atmospheres was responsible for the effects. The focus was immediately placed on O_3 because its toxicity was found to be high even at low ppm concentrations. Concerns that the complex atmosphere might be even more hazardous led to a number of studies with actual (outdoor-derived) or synthetic (photolyzed laboratory–prepared) smog to assess the potency of the ambient-like pollution mixtures. When human subjects were exposed to real-world photochemical air pollution (Los Angeles ambient air pumped into a laboratory exposure chamber), they experienced changes in lung function similar to those described in controlled clinical studies of O_3 alone (i.e., reduction in spirometric lung volumes), thus supporting the view that O_3 was the pollutant of primary concern. Acute animal studies using synthetic atmospheres (usually irradiated auto exhaust) provided supportive evidence indicating deep lung damage, primarily within the small airway and proximal alveolar epithelium.

As a result, O_3 was the focus in many of early air pollution studies despite a modicum of evidence that co-pollutants might also be involved in eliciting the effects observed with smog. For example, when guinea pigs were exposed to irradiated auto exhaust, airway resistance increased quickly, which contrasts with the pattern of O_3-related dysfunction that expresses as an increase in breathing rate. Perhaps a more soluble irritant(s) was involved, such as reactive aldehydes, but these pollutants were not monitored. With the potency of O_3 so apparent, attention was drawn to its effects and less to the array of seemingly less dramatic effects of a complex atmosphere. Even today, the focus on smog is almost exclusively centered on O_3 so it is not too surprising that the regulatory perspective is also tied to this single pollutant.

Chronic Exposures to Smog

Epidemiological studies in human populations as well as empirical studies in animals have attempted to link degenerative lung diseases with chronic exposure to photochemical air pollution. Cross-sectional and prospective field studies have suggested an accelerated loss of lung function in adults living in areas of high pollution; however, as with many such studies, there were problems with confounding factors (meteorology, poor exposure assessment, and confounding variables).

Studies conducted in children living in modern-day Mexico City, which has oxidant and PM levels far in excess of any city in the United States, have found abnormalities in the nasal epithelium using it as exposure surrogates for deep lung effects as would be assayed by invasive biopsy and lavage methodologies. Dramatic effects were reported in exposed children, consisting of severe epithelial damage and metaplasia as well as permanent remodeling of the nasal epithelium. When children migrated into Mexico City from cleaner, nonurban regions, even more severe damage was observed, suggesting that the tissue remodeling in the permanent residents imparted some degree of incomplete adaptation. Because the children were of middle-class origin, these observations were less likely confounded by socio-economic variables (Calderon-Garcidueñas et al., 1992). Changes in lung hyperinflation as estimated by x-ray tomography and impaired lung function along with markers of oxidative stress have been reported among similarly exposed children living in Mexico City (Calderon-Garcidueñas et al., 2003; Hernández-Escobar et al., 2009) raising further concerns of long-term effects.

Many studies have focused on children living in variably polluted areas of the Los Angeles basin showing the impact of air pollution—O_3 and PM—on lung growth, lung function, and other growth-related variables from childhood through high school age. Recently, a study examined the improvement of many of these variables as air quality has improved in various communities over a 13-year period. Improved lung function could be attributed to decreases in PM, NO_2, but less so with O_3 which itself changed less than the other primary pollutants (Gauderman et al., 2015).

A now-classic synthetic smog animal study was undertaken at the Cincinnati EPA laboratory in the mid-1960s in an attempt to address the potential for long-term lung disease. Beagle dogs were exposed on a daily basis (16 hours) for nearly 6 years, followed by a clean-air recovery period of about 3 years (Lewis et al., 1974). A series of physiologic measurements were made on the dogs after the exposure, and after their 3-year recovery. They were then moved to the University of California at Davis. The lungs of the dogs then underwent extensive morphologic examination to correlate with the physiology measurements. The dogs had been exposed to non-irradiated auto exhaust, irradiated auto exhaust, SO_2 plus sulfuric acid, the two types of exhaust plus the sulfur mixture, and a high and a low level of NO_x. The atmospheres were quite complex with only the irradiated exhaust mixtures containing O_3 and NO_2 and all contained CO. In short, all the mixture exposure groups had functional abnormalities, most of which persisted or worsened over the 3-year recovery period in clean air with enlargement of airspaces and loss of interalveolar septa consistent with emphysema and chronic obstructive pulmonary disease (COPD) (Hyde et al., 1978). The degenerative and progressive nature of the smog-induced lesions was reminiscent of lung disease with lifelong tobacco smoking.

Long-term "sentinel animal" studies in highly polluted cities have shown similar abnormalities, although some have been confounded by background infection due to the nature of the study. One such study, conducted in rats exposed for 6 months to the air of São Paulo, Brazil, found considerable airway damage, lung function alterations, and altered mucus rheology (Saldiva et al., 1992). This collage of effects is not unlike a composite of injury one might suspect from a mixed atmosphere of oxidants and acid PM in controlled laboratory animal studies. Strangely enough, however, a 7-week study in rats exposed to the polluted Mexico City air, which had induced significant lesions in children, did not reveal nasal or lung histopathology in F-344 rats (Moss et al., 2001). Thus, the evolution of air pollution–induced lung disease has potentially many variables, but overall there is coherence that long-term exposure of children and experimental animals to urban polluted atmospheres has long-term negative consequences on lung health.

Ozone

General Toxicology Ozone is the primary oxidant of concern in photochemical smog because of its inherent bioreactivity and its concentration relative to other reactive species. Approximately 140 million Americans lived in areas not in compliance with the 8-hour NAAQS in 2016. Progress has been made in reducing ambient O_3 levels and Los Angeles, for example, has not exceeded the Stage 1 smog alert (1-hour peak of 0.20 ppm or more) since 1998; it has dropped the number of exceedances of the 0.075 ppm (2006) NAAQS in half from 1990 to 2011. Unlike SO_2 and PM reductions that have largely been done at their source, mitigation strategies for O_3 have been somewhat less successful despite significant reductions in automobile emissions. Reductions in O_3 have been partially offset by population growth, which brings with it additional vehicles and vehicle miles driven. With suburban sprawl and the downwind transport of air masses from populated areas to more rural environments, the geographic distribution of those exposed has also expanded, as has the temporal profile of individual exposure. In other words, ambient O_3 exposures are no longer stereotyped as

brief 1- to 2-hour peaks as in the 1960s and 1970s. Instead, O_3 forms over longer drive periods and transport of pollutants spreads the period of exposure to 6 hours or more at or near the NAAQS level. This important change in the exposure profile to O_3 has given rise to concerns that cumulative impacts occur over an exposure of several hours rather than what one might experience with shorter pulse-like exposures. It is feared that many more people might be at risk than previously thought. With the 1997 revision of the U.S. O_3 NAAQS to include an 8-hour daily average of 0.08 ppm, more cities and suburban areas found themselves in violation of the standard. If indeed the damage to the lung is cumulative over an 8-hour time period, then people in areas of compliance but with exposure levels near the standard may be affected. The most recent revision of the O_3 NAAQS reduced the level to 0.07 ppm (2015). The American Lung Association estimates that nearly 50% of U.S. inhabitants live in counties that are not in O_3 compliance (American Lung Association, 2017).

Ozone induces a variety of effects in humans and experimental animals at concentrations that occur in urban areas (reviewed by Lippmann [1989] and U.S. EPA [2013]). These effects include morphologic, functional, immunologic, and biochemical alterations mostly in the lungs. Because of its low water solubility, a substantial portion of inhaled O_3 penetrates deep into the lung, but its reactivity is such that about 17% and 40% are scrubbed by the nasopharynx of resting rats and humans, respectively (Gerrity et al., 1988; Hatch et al., 1994). The reason for the higher degree of scrubbing in humans is unclear, but the finding is reproducible. Moreover, the mouth appears to scrub as well as the nose. Nevertheless, regardless of species, the region of the lung that is predicted to have the greatest O_3 deposition (dose per surface area) is the centriacinar region, from the terminal bronchioles to the alveolar ducts, also referred to as the proximal alveolar ductal region (Overton and Miller, 1988). Because O_3 penetration increases with increased tidal volume and flow rate, exercise increases the dose to the target area. Using $^{18}O_3$ (a nonradioactive isotope of oxygen), Hatch et al. (1994) have shown that the dose to the distal lung and the degree of damage to the lung as determined by leakage of plasma protein into the alveolar space (as collected by bronchoalveolar lavage) in exercising human subjects are similar to the magnitude of inflammation and protein leak in rats exercising or at rest but exposed to higher ventilation-adjusted concentrations. Thus, it is important to consider the role of exercise-associated dosimetry in a study of O_3 or any inhalant before making cross-study comparisons, especially if that comparison is across species.

Animal studies indicate that the acute morphologic responses to O_3 involves epithelial lining cells along the entire respiratory tract. The pattern of injury parallels the dosimetry profile, with the majority of damage occurring in the distal lung. Along the conducting airways, ciliated cells appear to be most sensitive to O_3, while Clara cells and mucus-secreting cells are the least sensitive. Studies in the rat nose indicate that O_3 also is an effective mucus secretagogue. In the alveolar region, type 1 epithelial cells are very sensitive to O_3 relative to type 2 cells, which have an active metabolic machinery and can also serve as the stem cells for the replacement of damaged type 1 cells. At lower concentrations (less than 0.8 ppm in the rat), a single exposure is followed by recovery with no residual pathology after a few days. In humans, a single exposure to high ambient levels of O_3 results in airways that appear "sunburned" when a bronchoscope is used to peer into the human bronchus. What is uncertain is the impact of repeated "sun-burning" of the airways and lung.

Studies have shown particular sensitivity to O_3-induced inflammation in neonatal rats; effects decrease with age into adulthood. However, enhanced sensitivity to O_3 again appears in older rats as evidenced by interstitial edema that is not seen in younger adult animals. Older rats have lower ascorbate in the lung and this diminishment of antioxidant capacity may be in part responsible. Ascorbate in humans has been found to reduce the lung function effects in controlled exposures and other antioxidant compounds seem to have similar albeit varied impacts (Tashakkor et al., 2011). Elderly humans, however, seem less responsive to the lung function impacts of high ambient O_3. The reasons for this are unclear, but caution is warranted in linking lung function and inflammation in the O_3 response.

The mechanisms by which O_3 causes injury have been studied using cellular as well as cell-free systems. As a powerful oxidant, O_3 seeks to extract electrons from other molecules. The surface fluid lining the respiratory tract and the cell membranes that underlie the lining fluid contain a significant quantity of polyunsaturated fatty acids (PUFA), either free or as part of the lipoprotein structures of the cell. The double bonds within these fatty acids have a labile, unpaired electron that is easily attacked by O_3 to form ozonides that progress through a less stable zwitterion or trioxolane (depending on the presence of water); these ultimately recombine or decompose to lipohydroperoxides, aldehydes, and hydrogen peroxide. These pathways are thought to initiate propagation of lipid radicals and autooxidation of cell membranes and macromolecules (Fig. 31-16).

Evidence of free radical–related damage in vivo includes detection of exhaled breath pentane and ethane and tissue measurements of diene conjugates. Damage to the air–blood interface disrupts its barrier function and promotes inflammation. Inflammatory cytokines (e.g., interleukins 6, 8, and others, and TNF) are released from epithelial cells and macrophages that mediate early responses and initiate repair. This inflammatory process is generally transient, but it may also interact with neural irritant responses to affect lung function acutely. This latter response may have implications for those with preexistent inflammation or disease.

Pulmonary Function Effects Exposure to O_3 produces a variety of pulmonary function changes; unlike with the inflammation effect, humans appear to be more sensitive than most animal models. Although the human studies almost always involve exercise, their deficit in lung function occurs more rapidly and is more dramatic than animals that have been tested similarly. It is noteworthy that in controlled human studies involving O_3, considerable interindividual differences in response have been observed that are linked to individual physical and biological features as well as

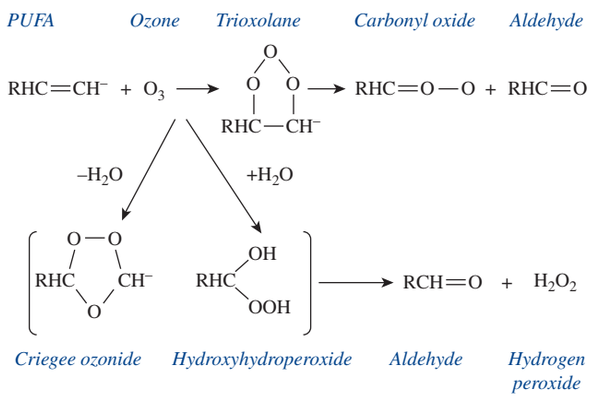


Figure 31-16. *Major reactions pathways of O_3 with lipids in lung lining fluid and cell membranes.* (Adapted with permission from U.S. EPA [2013].)

possibly genetic factors. Animal studies show similar host factors of susceptibility.

It is not entirely clear what mechanisms underlie the altered lung function (in terms of changes in FEV_1) produced by O_3. Chest pain/discomfort on deep breathing is thought to contribute to O_3-induced decreases in an effort-dependent lung function maneuver such as FEV_1. These are thought to be vagally mediated and involve C-fiber stimulation; the responses can be abrogated by analgesics, such as ibuprofen and opiates, which also reduce pain and inflammation (see below). Thus, pain reflexes involving C-fiber networks are thought to be important in the reduction in forced expiratory volumes. Animal studies also show a prominent role for vagal reflexes in altered airway responsiveness and bronchoconstriction. There is also evidence to implicate vagal reflexes in cardiac as well as corporal thermal regulation, at least in rodents, as part of a nonspecific defense mechanism decreasing ventilation.

Airway responsiveness to specific (e.g., allergen) and nonspecific (e.g., cold air and inhaled methacholine) bronchoconstriction is another commonly used test of the pulmonary response to inhaled pollutants such as O_3. These types of tests are very important because airway hyperresponsiveness is a central feature of asthma and asthmatics are a sizeable subpopulation (7% to 9% of the total population in the United States and 1% to 18% worldwide) that may be particularly sensitive to the adverse respiratory effects of inhaled pollutants. A number of animal models have demonstrated that O_3 induces airway hyperresponsiveness and that innate factors such as genetics, diet, and obesity can modify this effect. For the most part, many of these studies have utilized O_3 exposure concentrations that are far above ambient levels. Importantly, controlled human exposure studies have not only verified the findings of the high dose animal studies, but also demonstrated adverse effects at concentrations relevant to ambient O_3 levels. Airway hyperresponsiveness to nonspecific agonists in humans has been reported after acute exposure to as little as 0.08 ppm O_3. It is widely thought that hyperreactive airways may be a marker of predisposition to other pollutants or aeroallergens.

Inflammation of the Lung and Host Defense The mechanism by which O_3 produces decrements in pulmonary function appears to be largely neurologic, but the cross-links with airway inflammation are unclear. For example, O_3-induced lung dysfunction does not appear to be enhanced in asthmatics whose lungs are generally in a state of inflammation, yet asthmatics are more sensitive to the bronchoconstrictive effects of both SO_2 and sulfuric acid aerosols. It may be that inflammation sensitizes nerve endings to external irritants. Age and obesity in animal models suggests these factors may also play a role in the pulmonary response to O_3. These studies have demonstrated that O_3-induced lung injury and inflammation is greater in neonatal compared to adult rabbits, rats, and mice (Vancza et al., 2009). Obesity predisposes mice to O_3-induced inflammation and airway hyperresponsiveness as well (Williams et al., 2015). Because obesity is associated with ongoing systemic inflammation, it suggests that a preexisting physiologic or disease state enhances the adverse effects of O_3.

Both human and animal studies have demonstrated that sensitivity to O_3 appears to have a genetic component as well. Studies in inbred stains of mice have shown that O_3-induced pulmonary neutrophilia, airway hyperresponsiveness, and permeability are significantly affected by SNPs in a single gene linked to the Toll-like receptor 4 (Tlr4) locus that has been associated with endotoxin sensitivity (Kleeberger et al., 2000). Further research has determined that the participation of an innate immunity factor, such as Tlr4, in the multifaceted response to O_3 involves the release of hyaluronan fragments that appear to directly activate the Tlr4 cascade in mice (Li et al., 2011). The role of genetic polymorphisms in the pulmonary response to O_3 is complicated, however, and many other pathways including TNF, IL-1, Nrf-2, and NF-κB can modulate the response to O_3 in animal models (Bauer and Kleeberger, 2010).

The growth of genomic and proteomic technologies has made it possible to begin studies in humans to define genetic linkages to O_3 sensitivity. These studies have typically focused on genes involved in oxidative stress pathways. For example, again using Mexico City as a backdrop, it was found that a polymorphism in glutathione-*S*-transferase Mu 1 (GSTM1) appears to convey O_3 sensitivity (Romieu et al., 2004). Similarly, functional polymorphisms in the catalase and myeloperoxidase genes have been associated with enhanced response to ambient ozone. More detailed, controlled exposure O_3 studies in humans have confirmed that genetic polymorphisms in these and other genes, including NAD quinone oxidoreductase (NQO), GSTP1, heme oxygenase-1, and tumor necrosis factor (TNF), which are associated with responsiveness to O_3 and that gene–gene interactions are involved.

The strong epidemiologic evidence that O_3 can exacerbate asthma (e.g., increased hospital admissions) is strong, but causal ties to exacerbated immune responses have depended on empirical exposure studies. The potential for O_3 to influence allergic sensitization and/or challenge-responses has received a significant amount of investigation in both human and animal studies. Controlled human exposure studies have shown that asthmatics respond in a manner not overly dissimilar from normal, but because asthmatics have less functional reserve, lung function deficits are more evident (Kehrl et al., 1999). There is also evidence of heightened airway responsiveness to nonspecific airway challenges—pharmacologic agents, cold air, exercise, etc. Exaggerated responsiveness to allergens in allergic subjects has also been reported but seems less consistent or antigen-dependent, with variable evidence of lung function impacts yet more demonstrable enhancement of inflammation with changes in immune cell phenotypes and oxidative stress (Alexis and Carlsten, 2014). Likewise, eosinophils and IL-4, central components of allergic asthma, have also been reported to be increased in some but not all human chamber studies of O_3 exposure (U.S. EPA, 2013). Diary studies of asthmatic nurses, which involve a broader range of susceptibility, however, report worsened allergy symptoms, as well as durations thereof, at concentrations of O_3 near the NAAQS (Schwartz, 1992); similar results have reported in children in Southern California (Malig et al., 2016).

Enhanced response to allergen challenge has also been noted in a number of animal species exposed to O_3. Biochemical, cellular, and pulmonary function parameters of allergic response have all been observed to increase in sensitized animals exposed to O_3 prior to antigen challenge. Ozone's effect on allergic asthma also appears to occur at the sensitization stage in which repeated exposure to O_3 before or during the sensitization process exacerbates a range of allergic endpoints. Thus, in general, animal studies have shown the ability of O_3 to enhance both attributes of the allergic process, while the evidence in humans lies only in the exacerbation of response and this attribute itself.

Regarding other immune responses, early research showed that exposure to O_3 before a challenge with aerosols of infectious agents produced a higher incidence of infection than is seen in control animals (Coffin and Blommer, 1967). Studies show that this type of effect can be a direct result of altered phagocytosis by macrophages in O_3-exposed mice previously infected with an aerosol of *Streptococcus* (group C) bacteria (Gilmour et al., 1993). The host resistance model has shown responsiveness to an exposure as low as 0.08 ppm for 3 hours—perhaps the most sensitive

model that exists for O_3. The susceptibility of mice and hamsters to *Klebsiella pneumoniae* aerosol is also increased by prior exposure to O_3. In the rat, altered microbe-killing may relate to membrane or receptor damage in macrophages, thus impairing the production of bactericidal agents such as superoxide anions. Overall, the rat appears less susceptible than mice because it has a more vigorous polymorphonuclear leukocyte (PMN) response to bacteria than do mice, which seems to compensate for macrophage impairments. This is yet another example of where susceptibility lies more in the inability to compensate than in the initial responsiveness to a given challenge. It is not clear whether O_3's impairment of bacterial infections in the lung also extends to viral infections. While ambient O_3 has been associated with increased hospital admissions for viral and bacterial infections, animal studies show mixed results for O_3's effect on viral infections, although some of the contradictory findings may be a result of experimental design differences (e.g., O_3 exposure occurring before or after viral infection).

Chronic Effects Morphometric studies of the centriacinar region of rat lungs exposed for 12 hours per day for 6 weeks to 0.12 or 0.25 ppm O_3 show hyperplasia and hypertrophy of type 1 alveolar cells (smaller and thicker cells) coupled with damage and alterations in ciliated and Clara cell populations in upstream small airways (Barry et al., 1988). A collective cross-protocol analysis of type 1 cell hypertrophy was conducted, and type 1 cell thickness appeared to be linearly related to the O_3 $C \times T$ (Chang et al., 1991, 1992). This finding suggested that over a season, the impact of O_3 in the distal lung may be cumulative and perhaps more importantly may be without threshold. The biological significance of this change is unclear—it may be part of a compensatory response to "thicken" that part of the alveolar-duct junction that receives the greatest dose and is most affected. This response may thus protect because the thickened cells are smaller, offering less exposure-surface for the incoming O_3 and therefore limiting membrane damage. When returned to clean air, most of this epithelial pathology regressed, but there was nonetheless evidence of residual interstitial remodeling beneath the epithelium in the alveolar-duct region.

A long series of studies at the University of California, Davis has examined the structural and functional changes produced in nonhuman primates exposed chronically to O_3. Because of the greater similarity of monkey respiratory tract to that of humans, the burden of extrapolation to humans is lessened and has proven important in establishing causality for analogous epidemiology studies. Daily 8-hour exposures to 0.2 ppm O_3 for 90 days produced cellular changes throughout the respiratory tract, including the nose. In the second phase of these studies, infant rhesus monkeys were cyclically exposed to 0.5 ppm O_3 for 8 h/day for 5 days followed by several days of exposure to air. While the study design didn't permit a direct comparison of the responsiveness of infant versus adult monkeys, the infant monkeys showed evidence of structural changes that parallel epidemiology studies in children. The infant monkeys also showed a significant interaction between sensitization to house dust mite antigen and ozone. While antigen or O_3 alone produced small changes in baseline airway resistance and airway responsiveness, antigen plus O_3 produced more than additive effects on both parameters.

Studies involving episodic exposures of rats and monkeys using a pattern of alternating months of O_3 (0.25 ppm) for 18 months indicated a range of effects, notably thickening of interstitial fibrous matrix (Tyler et al., 1988), suggesting loss of lung compliance. These interstitial changes were quantitatively similar for the continuous and intermittent exposure profile despite the twofold difference in the cumulative exposure dose (i.e., $C \times T$). This would imply that a pattern of exposure resembling seasonal O_3 patterns, with real-world relevance, might result in more serious lesions than predicted by dose alone—perhaps even more than would have occurred had the exposure been continuous. Hence, the concept of "more dose . . . more effect" may not hold in chronic episodic scenarios for O_3, as it appears with uninterrupted exposures. The number of episodes experienced may well be more significant to long-term outcomes than total dose—a phenomenon not unlike that of repeated sun-burning and deterioration of the skin.

Studies of lung function in rodents exposed chronically to O_3 have yielded mixed results at relevant exposure concentrations. Generally, the observed dysfunction is reflective of stiffened or fibrotic lungs, particularly at higher concentrations. There have been two prominent chronic O_3 studies—an EPA 18-month chronic study fashioned after a realistic urban exposure profile with a peak exposure of 0.25 ppm (Chang et al., 1992; Costa et al., 1995) and the National Toxicology Program (NTP)—Health Effects Institute (HEI, 1994–1995) study of 0.125 to 1 ppm for 20 months (square wave; 6 h/day; 5 days/week). From an environmental relevance perspective, the $C \times T$ doses for these studies were similar, but the urban profile study produced evidence for centriacinar interstitial fibrosis consistent with earlier evidence of the influence of exposure pattern. There was no general biochemical evidence for fibrosis, as reported in monkey and rat studies that had been conducted at higher O_3 concentrations, perhaps due to the focal nature of the pathology. If one attempts to compare these results with the classic Cincinnati beagle study, one finds that the synthetic smog atmosphere showed degenerative and not fibrotic lung lesions. However, it should be noted that the air pollutant mixture in the beagle study involved more than O_3, was more complex, and had pollutants at significantly higher levels.

Paradoxically, O_3 has the ability to induce tolerance to itself and is a curious phenomenon in light of pathologies seen in episodic and chronic exposures. Classic O_3 tolerance takes the form of protection against a high or even lethal dose in rodents that has a pre-exposure to a very low initial challenge(s) several days before. This term, *tolerance*, is sometimes used to describe "adaptation" or acclimatization with repeated exposures to near-ambient levels of O_3, and as such, has led to some confusion. However, with regard to "adaptation" to O_3, the process begins during and immediately after the initial exposure and is fully expressed in 2 to 4 days. This adaptive phenomenon has been substantiated in humans with regard to diminished lung function and correlative inflammatory endpoints (Devlin et al., 1993). Lavage lactate dehydrogenase (LDH; a marker of cell injury) and elastase (enzymatically active against lung matrix material), interestingly, did not appear to adapt in one study carried out over 2 weeks in humans. An analogous pattern of adaptation of functional and biochemical endpoints (including LDH and elastase) also takes place in rodents with repeated exposures up to a week. But to date, the linkages between acute, adaptive, and long-term processes remain unclear, because both pathology and lung dysfunction do develop over longer periods of exposure in animals. The precise mechanism for O_3 adaptation is not known and several theories abound, including changes in cell profiles, lung surface fluids, and induced antioxidants. Few studies have tackled the problem but in rats the adaptation of the neutrophilic response appears to be related to the induction of an endogenous acute-phase response (McKinney et al., 1998). However, adaptation to lung function changes in rats after chronic exposure appears linked to lung antioxidants such as ascorbic acid (Wiester et al., 2000). The human relevance of this finding is uncertain because ascorbic acid is not endogenously synthesized as it is in the rat. However, self-administration of ascorbate has been shown to reduce O_3-induced lung function decrements in adults (Mudway et al., 1999), while in

runners it did not enhance performance (Gomes et al., 2011). Yet in children, supplementation with ascorbate and α-tocopherol lessened nasal inflammatory responses to O_3 in Mexico City (Sienra-Monge et al., 2004). Despite these interesting findings, it remains unclear whether antioxidant supplements can protect humans from long-term O_3 consequences given the many mechanisms that may be involved in the various responses. How supplementation may interplay with long-term adaptation and the likelihood of degenerative disease prevention is unclear.

Ozone Interactions with Co-pollutants The effects of O_3 may be altered by co-pollutants in the mixture or may otherwise interact to yield a novel composite outcome. How such interactions can be studied is often debated. Smog exposures are often difficult to deductively disassemble into a comprehensible configuration. However, one approach simplifying the complexity of smog, yet addressing the issue of pollutant interactions involves a bottom-up assembly of likely interacting pollutants. The exposures of animals or humans may be to binary or "assembled" synthetic mixtures of pollutants that occur together in ambient air. As such, the most frequent combinations involve interactions of O_3 and NO_2 or O_3 and PM (e.g., ambient, sulfuric acid, or diesel particles). Not surprisingly, study designs involving temporal or spatial exposures add a level of complexity in interpretation that may or may not support either augmentation or antagonism of lung function impairment, lung pathology, or a host of other indices of injury. This complexity only emphasizes the need to carefully consider the myriad of factors that might affect outcomes as well as how the design compares to real-world exposures of concern.

Studies of O_3 mixed with PM also have shown either enhanced or antagonistic responses that varied with exposure design. Studies of human volunteers exposed acutely with exercise to O_3 and diesel exhaust particles showed enhanced deficits in lung function (Madden et al., 2014). Similarly, as noted above, field studies of children in camps and studies of asthma admissions in the Northeastern United States and in Canada suggested an interaction of acid aerosols and O_3 resulting in augmented functional deficits to a composite ambient summer haze. Allergic mice, however, showed reduced lung infiltration of inflammatory cells to the O_3 and diesel particle exposure, with no change in airways resistance; yet, airway hyperreactivity was increased (Farraj et al., 2010). Likewise, rabbits exposed intermittently for 1 year to an O_3 (0.1 ppm) and sulfuric acid (125 $\mu g/m^3$) mixture had a temporal shift in secretory cell number, increasing midway through the study and then decreasing—with no evidence of mucociliary clearance dysfunction (Schlesinger et al., 1992). Studies of highly complex atmospheres such as one study involving acid-coated carbon combined with O_3 at near-ambient levels similarly show varied evidence of interaction on lung function and macrophage receptor activity (Kleinman et al., 1999). Thus, as can be gleaned, the interpretability of any multicomponent study is dependent on its statistical design and the ability to either separate or determine the nature of the interacting variables. The bottom line is that interactions are highly dependent on the conditions of exposure, endpoints selected for study, and a host of other factors. As the number of interacting variables increases, so does the difficulty in interpretation. Given the challenge that it is indeed the complex mixture to which people are exposed that we wish to evaluate, creative toxicological approaches to understanding mixture responses will demand new systems perspectives if real progress is to be achieved (Mauderly, 2006).

Nitrogen Dioxide

General Toxicology Nitrogen dioxide, like O_3, is a deep lung irritant that can produce pulmonary edema if it is inhaled at high concentrations. It is a much less potent irritant and oxidant than O_3, but NO_2 can pose clear toxicological problems. Potential life-threatening exposure is a real-world problem for farmers, as near-lethal high levels of NO_2 can be liberated from fermenting fresh silage. Being heavier than air, the generated NO_2 and CO_2 displace air and oxygen at the base of silo and diffuse into closed spaces where workers can inadvertently get exposed to very high concentrations perhaps with depleted oxygen. Typically, shortness of breath rapidly ensues with exposures nearing 75 to 100 ppm NO_2, with delayed edema and symptoms of pulmonary damage. Not surprisingly, the symptoms are collectively termed "silo-filler's disease." NO_2 is also an important indoor pollutant, especially in homes with unventilated gas stoves or kerosene heaters (Spengler and Sexton, 1983) or, in developing countries, with the unvented burning of biomass fuels (Kumie et al., 2009). Under such circumstances, very young children and their mothers who spend considerable time indoors may be especially at risk. In general, indoor environments with NO_2 sources achieve concentrations far in excess of those observed outdoors at central monitoring sites. Peak levels of NO_2 near high traffic volume roadways can be several fold higher than away from roadways and are thus similar to peak levels observed indoors with unvented stoves (i.e., 0.2 to 0.5 ppm). Interestingly, protocols that simulate an urban (rush-hour) or household (cooking) patterns of 2 daily peak exposures superimposed on a low continuous background concentration have elicited effects in experimental animals that continuous exposure to NO_2 did not evoke, suggesting an important dependency on exposure profile. Among the common air pollutants, empirical studies of NO_2 have frequently shown greater effects with higher peak concentrations at equivalent $C \times T$ steady-state concentrations.

Although the distal lung lesions produced by acute NO_2 are similar among species, there exist differences in species sensitivity. Where direct comparison is possible, guinea pigs, hamsters, and monkeys appear more sensitive than rats, although comparative dosimetry information might explain some of this difference. As in the case of O_3, theoretical dosimetry studies indicate that NO_2 is deposited along the length of the respiratory tree, with preferential deposition in the distal airways. Not surprisingly, the pattern of damage to the respiratory tract reflects this profile: damage is most apparent in the terminal bronchioles, just a bit more proximal in the airway than is seen with O_3. At high concentrations, the alveolar ducts and alveoli are also affected, with type 1 cells again showing their sensitivity to oxidant challenge. In the airways of these animals, there is also damage to epithelial cells in the bronchioles, notably with loss of ciliated cells, as well as a loss of secretory granules in Clara cells. Thus, the pattern of injury of NO_2 is quite similar to that of O_3, although its potency is about an order of magnitude lower.

Pulmonary Function Effects Exposure of normal human subjects to concentrations of less than or equal to 4 ppm NO_2 for up to 3 hours produces no consistent effects on spirometry. However, a study has shown slightly enhanced airway reactivity with 1.5 to 2.0 ppm. Interestingly, ascorbic acid pretreatment of human subjects appeared to protect them from this hyperreactivity (Mohsenin, 1987). Whether asthmatics have a particular sensitivity to NO_2 is a controversial issue. A number of factors appear to be involved (e.g., exercise, inherent sensitivity of the asthmatic subject, and exposure method). Some studies have reported effects in some individuals at 0.2 ppm, which approximates an indoor level in a household with an unvented gas stove. Meta-analyses, which have incorporated the findings of many studies to achieve a weight-of-evidence perspective, support a heightened effect of NO_2 on asthmatics, but it's not clear if the observed small but statistically significant changes in

airway hyperresponsiveness in asthmatics exposed to NO_2 below 0.6 ppm are adverse. As for an appropriate animal model, only very high concentrations (10 ppm NO_2) invoke an irritancy response in guinea pigs (tachypnea); these levels are well above those a person probably would encounter in everyday life. However, NO_2 has been found to be associated with mortality in some time-series studies of air pollution attempting to tease out specific pollutant effects (focusing mainly on PM) (Gold et al., 2000; Samoli et al., 2006). These epidemiology studies have demonstrated that each 20 to 30 ppb increase in NO_2 results in a small but significant increase in acute cardiovascular and respiratory mortality. Similarly, NO_2 has been associated with increased hospital admissions and emergency room visits, but these epidemiology studies are confounded by the co-exposure to multiple pollutants. Thus, as stressed elsewhere, it is important that epidemiology studies results be interpreted with caution when considering causality and that controlled human exposure and animal studies be utilized, within ethical limits, to confirm the biologic plausibility of the effect of NO_2, or any other pollutant, on the cardiopulmonary system. This caution obviously extends to many recent studies that have consistently linked adverse health outcomes with living near high traffic volume roadways. Because of the many constituents present in the exhaust of both gas and diesel combustion engines, as well as their ever changing chemistry, it is unclear from these studies whether NO_2 is acting as a marker or surrogate for vehicular traffic rather than as an indication of a specific NO_2 effect. These studies have found correlates with cardiovascular deaths, which have raised new questions of the mechanisms by which pollutants might affect health in susceptible subgroups (Rosenlund et al., 2006). Nevertheless, NO_2 does appear as a reasonable proxy for traffic for both cancer and noncancer outcomes in epidemiological studies, although the true nature of the NO_2 role remains unclear (Faustini et al., 2014; Hamra et al., 2015).

Inflammation of the Lung and Host Defense Unlike O_3, NO_2 does not induce significant neutrophilic inflammation in humans at exposure concentrations encountered in the ambient outdoor environment. There is some evidence for bronchial inflammation after 4 to 6 hours at 2.0 ppm, which approximates the highest transient peak indoor levels of this oxidant. Exposures at 2.0 to 5.0 ppm have been shown to affect T lymphocytes, particularly $CD8^+$ cells and natural killer cells that function in host defenses against viruses. Although these concentrations may be high, epidemiologic studies variably show effects of NO_2 on respiratory infection rates in children, especially in indoor environments. Animal models, by contrast, have for years shown associations between NO_2 and bacterial infection (Gardner, 1984). As noted for other effects, the incidence of infection in exposed models appears to be governed more by the peak exposure concentration than by exposure duration. The effects are ascribed to suppression of macrophage function and clearance from the lung, in the form of suppressed bactericidal and/or motility functions of macrophages from rabbits exposed to 0.3 ppm for 3 days (Schlesinger, 1987). Similar effects have been reported in humans exposed to 0.10 ppm for 6.6 hours (Devlin et al., 1991).

Toxicological studies of the interaction of NO_2 with viruses also suggest enhanced infectivity. Squirrel monkeys infected with nonlethal levels of A/PR-8 influenza virus and then exposed continuously to 5 or 10 ppm NO_2 suffered high mortality rates (Henry et al., 1970). Other experiments suggest that exposure of squirrel monkeys for 5 months to 5 ppm NO_2 depresses the formation of protective antibodies against the A/PR-8 influenza virus. Controlled human studies with virus challenges, however, have been inconclusive, perhaps because of low subject numbers. One study showed decreased virus inactivation by alveolar macrophages recovered from four of nine subjects when cultured and exposed for 3.5 hours to 0.6 ppm NO_2 in vitro (Frampton et al., 1989). Thus, the potential for augmented risk of viral infection associated with NO_2 exposure remains unclear and suggests a role for underlying host susceptibility. This concern would be greatest for children, especially during seasonal use of unvented gas-heaters, who have less mature pulmonary immune function.

Chronic Effects Concern about the chronic effects of NO_2 stem from early observations that a continuous 30-day, 30-ppm exposure produces emphysema in hamsters. Whether the result of this continuous exposure scenario relates to cyclic human exposures at 1/100th that level is unclear. On the one hand, an 18-month study in rats exposed to an urban pattern of NO_2 in which a daily background of 0.5 ppm peaked at 1.5 ppm for 4 hours each day showed little ultrastructural damage to the distal lung (Chang et al., 1988). On the other hand, mice exposed for a year to a base level of 0.2 ppm NO_2 with a 1-hour spike of 0.8 ppm twice a day 5 days per week (Miller et al., 1987) displayed effects that differed between the base-only and peak-only exposure groups. The base level produced no effects, while the overlaid peaks induced slight functional impairment and augmented susceptibility to bacterial infection. Early studies (Ehrlich and Henry, 1968) showed that clearance of bacteria from the lungs is suppressed with 0.5 ppm NO_2 through 12 months of exposure. Interestingly, studies with a similar double diurnal peak design for NO_2, with NO used as a negative control, showed more pronounced effects of NO on alveolar septal remodeling than did NO_2 (Mercer et al., 1995). These and similar studies utilizing varied peak-plus-baseline NO_x exposures indicate the importance of exposure profile for at least this pollutant.

Other Oxidants

While a number of other reactive oxidants have been identified in photochemical smog, most are short-lived because of their reaction with co-pollutants. Peroxyacetyl nitrate (PAN), which is thought to be responsible for much of the eye-stinging activity of smog, is known to exist in smog situations. It is more soluble and reactive than O_3, and hence rapidly decomposes in mucous membranes before it can penetrate into the respiratory tract. The cornea is a sensitive target and is prominent in the burning/stinging discomfort often associated with oxidant smogs. A few studies with high levels of PAN have shown that it can cause lung damage and have mutagenic activity in bacteria, but it is not likely that these scenarios are relevant to ambient levels of PAN.

Carbon Monoxide

Carbon monoxide is classed toxicologically as a chemical asphyxiant because its toxic action stems from its formation of carboxyhemoglobin, preventing oxygenation of the blood for systemic transport. The fundamental toxicology of CO and the physiologic factors that determine the level of carboxyhemoglobin attained in the blood at various atmospheric concentrations of carbon monoxide are detailed in Chap. 11, "Toxic Responses of the Blood."

The normal concentration of carboxyhemoglobin (COHb) in the blood of nonsmokers is about 0.5%. This is attributed to endogenous production of CO from heme catabolism, yet higher levels can be found in nonsmokers who are regularly exposed to secondhand smoke from cigarettes. Blood COHb is a function of the concentration in air, the length of exposure, and the ventilation of the exposed individual. Uptake is said to be ventilation-limited, implying that virtually all the CO inspired in a breath is absorbed and bound to the available hemoglobin. Thus, continuous exposure of human subjects to 30 ppm CO leads to an equilibrium value of 5% COHb.

The Haldane equation is used to compute the COHb equilibrium under a given exposure situation. The equilibrium values generally are reached after 8 hours or more of exposure, but the time required to reach equilibrium can be shortened by physical activity.

Carbon monoxide emissions from automobiles have progressively decreased since the 1970s. Increased combustion efficiency and catalytic convertors have reduced emissions at the tailpipe by more than 98%. However, motor vehicles can still account for two-thirds of urban CO. Depending on the location in a community, traffic density, vehicle types and age, and the urban structure, CO concentrations can vary widely. Concentrations predicted inside passenger compartments of motor vehicles in downtown traffic were almost three times those for central urban areas and five times those expected in residential areas. Occupants of vehicles traveling on expressways had CO exposures somewhere between those in central urban areas and those in downtown traffic, whereas concentrations in underground garages, tunnels, and buildings directly over highways can still reach high levels but substantially less and slower to accumulate. But it must be kept in mind that there are many sources of CO—anywhere combustion is ongoing. Certainly both main and side-stream tobacco smoke are a source. Faulty home heating systems and mobile auxiliary heating sources emit CO which when used inappropriately (i.e., unventilated) can create life-threatening circumstances, and nowadays, these indoor exposures are the most significant CO threat to health with about 430 deaths a year in the United States.

No overt clinical human health effects have been demonstrated for COHb levels below 2%, while levels above 40% cause fatal asphyxiation. However, there is evidence that in some people COHb in the range of 2.0% to 2.4% can elicit acute cardiovascular effects. A 90-minute exposure to about 50 ppm CO would likely result in COHb levels of about 2.5%, with a resultant impairment of time-interval discrimination even in healthy subjects; at approximately 5% COHb, there is an impairment of other psychomotor faculties. At 5% COHb in nonsmokers (the median COHb value for smokers is about 5%), however, maximal exercise duration and maximal oxygen consumption are reduced (Aronow, 1981). Clear cardiovascular changes also may be produced by exposures sufficient to yield COHb in excess of 5%. These include increased cardiac output, arteriovenous oxygen difference, and coronary blood flow in patients without coronary disease. Decreased coronary sinus blood PO_2 occurs in patients with coronary heart disease, and this would impair oxidative metabolism of the myocardium. In the early 1990s, a series of studies in subjects with cardiovascular disease were conducted in several laboratories under the sponsorship of the Health Effects Institute (HEI) to determine the potential for angina pectoris when they exercised moderately with COHb levels in the range of 2% to 6% (Allred et al., 1989). The results of these studies indicate that premature angina can occur under these conditions but that the potential for the induction of ventricular arrhythmias remains uncertain.

Carbon monoxide has been implicated as a potentially important co-pollutant of PM with potential human health effects at low ambient concentrations. Independent CO effects have been reported in some epidemiological studies of ambient air pollution, but its singular role versus indicator role (for traffic) continues to be debated. The fact that effects noted in these studies are cardiovascular and involve angina and effects on EKG patterns lend credence to a role for CO in epidemiologic health outcomes. Other data from epidemiologic studies have shown a linkage between CO and some general CNS indices as well as birth outcomes. The sensitivity of newer analysis methods for these population studies are revealing previously unappreciated health effects. Among these are preterm births, cardiac birth defects, and infant mortality in the postnatal period. Moreover, toxicological studies with animals also suggest potential developmental risks.

Reducing CO emissions from motor vehicles is a major goal because of its high hazard—a colorless and odorless non-irritant—so technical combustion engineering advances are being pursued as well as changes in fuel formulations for specific seasonal conditions. For example, the introduction of gasoline oxygenates such as MTBE in the 1990s and other ether derivatives constitute attempts to enhance fuel combustion and reduce CO emissions. The experience with MTBE (as noted earlier) has led to such gasoline reformulations being approached with some caution with greater attention being placed on assessments of potential unexpected consequences. Biofuels, comprised primarily of ethanol at varied percentages, in gasoline or diesel fuel, are part of an effort to reduce fossil fuel consumption and enhance renewables. Another goal is reduction in emissions that deteriorate the atmosphere directly or indirectly but the data supporting this outcome remains inconclusive. Regardless, CO remains a toxic air pollutant both indoors (e.g., secondhand smoke and cook stoves) and outdoors (combustion engines), which merits considerable attention wherever there is combustion.

Hazardous Air Pollutants

Hazardous air pollutants (so-called air toxics or HAPs) represent an inclusive classification for air pollutants of anthropogenic origin that are generally of measurable quantity in the air, and are not covered in the so-called Criteria Pollutant list of the Clean Air Act (CAA) of 1970. They are covered under Section 112 of the CAA. For a variety of reasons, HAPs are regulated in the United States not by ambient concentration (due to their generally focal nature near sources or uneven national distribution, complexity of measurement) but rather by application of best available technology to emission reduction, followed with an assessment of residual risk with that which is released. As such, most exposure estimates for these pollutants are derived from emission inventories that are modeled into the National Air Toxics Assessment (U.S. EPA, 2016). The most recent NATA assessment (2015) used 2011 data on 180 of the 187 HAPs, plus diesel PM. The diverse nature of even 30 of the 187 HAPs (the so-called "dirty 30"—Table 31-6) complicates a general discussion of their toxicology because the group

Table 31-6

List of 30 Urban Air Toxics ("Dirty 30")

ACETALDEHYDE	DIOXIN	MERCURY COMPOUNDS
Acrolein	Propylene dichloride	Methylene chloride (dichloromethane)
Acrylonitrile	1,3-Dichloropropene	Nickel compounds
Arsenic compounds	Ethylene dichloride (1,2-dichloroethane)	Polychlorinated biphenyls (PCBs)
Benzene	Ethylene oxide	Polycyclic organic matter (POM)
Beryllium compounds	Formaldehyde	Quinoline
1,3-Butadiene	Hexachlorobenzene	1,1,2,2-Tetrachloroethane
Cadmium compounds	Hydrazine	Tetrachloroethylene (perchloroethylene)
Chloroform	Lead compounds	Trichloroethylene
Chromium compounds	Manganese compounds	Vinyl chloride

SOURCE: From U.S. EPA (2016).

includes various classes of organic chemicals (by structure, e.g., acrolein, benzene), minerals (e.g., asbestos), polycyclic hydrocarbon particulate material (e.g., benzo(*a*)pyrene), and various metals and metal compounds (e.g., mercury and beryllium compounds) and pesticides (e.g., carbaryl and parathion).

The focus to date on the HAPs has been on their potential carcinogenicity, as shown in chronic bioassays, mutagenicity tests in bacterial systems, structure–activity relationships, or—in a few special cases (e.g., benzene and asbestos)—their known carcinogenicity in humans. Hazardous air pollutant cancers need not be, and generally are not, simply pulmonary in origin. Details on the systemic carcinogenicity of many of these chemicals can be found elsewhere in this text or in the literature (Loh et al., 2007). However, noncancer issues frequently relate to direct lung toxicants that, upon accidental release or fugitive emission, might put at risk those susceptible (e.g., children) or who have preexisting disease (e.g., asthma or other chronic lung or cardiac disease). The assessment of noncancer risk to a given air toxic whether to the lung or other organ system is determined via the computation of long-term risk reference exposure concentrations (RfCs). This RfC is that concentration to which an individual may be exposed over a lifetime without adverse, irreversible injury. The official computational process to HAP assessment is discussed in detail at www.epa.gov/risk/review-reference-dose-and-reference-concentration-processes-document. This process remains hotly debated but attempts to use the latest and best data available to address potential risks to the diverse public. A short-term RfC method has been developed for exposures up to 30 days that is sometimes used to assess noncancer applications. In the NATA assessment of toxic inhalants, acrolein ranks as the top risk based on emission inventories, the potential for exposure, and its inherent irritancy. Discussion of this topic and an approach to mixed exposures to noncancer HAPs has been reviewed (Costa, 2003).

Accidental and "Fence-Line" Exposures

The relationship between the effects associated with an accidental release of a large quantity of a volatile chemical into the air from a point source such as a chemical plant and the effects associated with a chronic low-level exposure over many years or a lifetime is not clear. Regarding cancer risk, which typically defaults to a linearized model of dose and effect, the issue is fairly straightforward. Exposures must be minimized if not eliminated if cancer risk is to be kept as close to zero as possible. With noncancer risks, the roles of nonspecific or specific host defenses, thresholds of response, and repair and recovery after exposure complicate the assessment of risk. In large part, the issue here relates to a cumulative index, often a $C \times T$. Can we better relate disease or injury to a *cumulative dose* or a *peak* concentration for any given or a protracted exposure? Is there an exposure peak beyond which a cumulative approach fails (i.e., the effect is concentration driven), or is concentration always the dominant determinant? Many of these questions have yet to be answered, not to mention their specificity with regard to individual compounds and tissues affected.

Methyl isocyanate provides a contrast between the catastrophic effects of a large accidental release versus long-term effects linked to cyclic or continuous fugitive releases of small amounts of chemical vapor. The reactive nature of methyl isocyanate with aqueous environments is of such magnitude that upon inspiration, almost immediate mucous tissue corrosion can be perceived. The vapor undergoes hydrolysis within the mucous lining of the airways to generate hydrocyanic acid, which destroys the airway epithelium and causes acute bronchoconstriction and edema. The damage is immediately life-threatening at concentrations above 50 ppm; at 10 ppm, it is damaging in minutes. These concentrations are in the range of the dense vapor cloud that for several hours in 1984 enshrouded the village of Bhopal (India) bordering a Union Carbide pesticide plant. Studies in guinea pigs have shown the immediate irritancy of this isocyanate, which in just a few minutes also results in significant pathology (Alarie et al., 1987). Rats exposed to 10 or 30 ppm for 2 hours also showed severe airway and parenchymal damage, which did not resolve in surviving rats; transient effects were seen at 3 ppm. Even 6 months after exposure, the airway and lung damage remained, having evolved into patchy, mostly peribronchial fibrosis with associated functional impairments (Stevens et al., 1987). There was also cardiac involvement secondary to the damage to the pulmonary parenchyma and arterial bed (i.e., pulmonary hypertension and right-sided heart hypertrophy). This same spectrum of health effects has resulted in disability and the deaths of thousands of initial survivors since the incident.

In the United States, methyl isocyanate has been measured in the ambient air in Katawba Valley, Texas, as a result of small but virtually continual fugitive releases of the vapor into the community air ("fence-line") from an adjoining region with several chemical plants. While these levels of methyl isocyanate are not sufficient to cause the damage seen in Bhopal, there is concern that low-level exposure over many years may have more diffuse, chronic effects. Residents complain of odors and a higher frequency of respiratory disorders, but clear evidence of injury or disease is lacking. Fence-line exposures to a variety of industrial chemicals remain a significant potential risk for many communities—often low-income or worker communities near one or more industrial sources. Silent continuous or periodic releases from pipelines or transfer points within facilities can disperse through communities unbeknownst to anyone. The advent of newer sensor technologies and better conduit systems for process operations is slowly reducing risks in more advanced nations but the exposure risks remain high elsewhere.

Phosgene is best known for its use as a war gas, but it is also a common intermediate reactant used in the chemical industry, particularly in pesticide formulation, and subject to accidental or low-level fugitive releases. Because of its direct pulmonary reactivity, it lends itself to use as a model pulmonary toxicant for studies addressing $C \times T$ relationships that can inform assessment of risk under varied exposure patterns. These studies suggest that there may be a threshold below which compensatory and other bodily defenses (e.g., antioxidants) may be able to cope perhaps with long-term low-level tolerance, although as with O_3 there is evidence of mild interstitial fibrosis. In rat studies, this threshold for acute toxicity for phosgene appears to be at or below the current threshold limit value of 0.1 ppm for 8 hours, which suggests little or no margin of safety for workers. At higher concentrations, however, peaks in concentration (C) appear to be the primary determinants of injury triggering disease with duration of exposure being less important to the injury process. There can be some adaptation with time, but there is not sufficient recovery and hence the lesion appears to be a concentration-driven response exceeding that predicted arithmetically by $C \times T$. This relationship appears to be different from that of acute exposures to O_3 at ambient levels, which can be approximated by the $C \times T$ paradigm. Hence, the $C \times T$ concept for lung injury appears to function best at low ambient levels and less well for event exposures such as in accidents.

Aldehydes

Among the HAPs that have broader exposure potential for the public, two aldehydes capture attention for their toxicity/irritancy and their ubiquity. These two aldehydes are formaldehyde (HCHO) and

acrolein ($H_2C{=}CHCHO$). As carbonyl compounds, they are short-chained (2 to 4 C) and are generated as photo-oxidation products of unsaturated hydrocarbons in the outdoor air, and as common intermediates in chemical reactions, they can arise in the indoor environment. Formaldehyde accounts for about 50% of the estimated total aldehydes in polluted air, while acrolein, the more irritating of the two, accounts for about 5% of the total. Other aldehydes, such as acetaldehyde (C_3HCHO) and many other longer-chain aldehydes, make up the remainder, but they are not as intrinsically irritating, exist at low concentrations, and have less solubility in airway fluids. Formaldehyde and particularly acrolein are also found in mainstream tobacco smoke (about 90 and about 8 ppm, respectively, per puff) and side-stream smoke as well. Formaldehyde is also an important indoor air pollutant and can often achieve higher concentrations indoors than outdoors due to off-gassing by new upholstery or other furnishings. In the United States, the formaldehyde off-gassing issue was highlighted in the controversy surrounding the trailer homes provided to hurricane-displaced home owners post-hurricane Katrina.

Empirical studies have shown that formaldehyde and acrolein are competitive agonists for similar irritant receptors in the airways. Thus, irritation may be related not to "total aldehyde" concentration but to specific ratios of acrolein and formaldehyde. Their relative difference in solubility, with formaldehyde being somewhat more water-soluble and thus having more nasopharyngeal uptake, may distort this relationship under certain exposure conditions (e.g., exercise). However, acrolein is very reactive and may interact easily with many tissue macromolecules and, for example, can form DNA adducts.

Formaldehyde

Formaldehyde is a primary sensory irritant. Because it is very soluble in water, it is absorbed in mucous membranes in the nose, upper respiratory tract, and eyes. The concentration–response curve for formaldehyde is steep: 0.5 to 1 ppm yields a detectable odor; 2 to 3 ppm produces mild irritation; and 4 to 5 ppm is intolerable to most people. Formaldehyde is thought to act via sensory C-fibers that signal locally as well as through the trigeminal nerve to reflexively induce bronchoconstriction through the vagus nerve. In guinea pigs, a 1-hour exposure to about 0.3 ppm of formaldehyde induces an increase in airflow resistance accompanied by a decrease in compliance (Amdur, 1960). The no observed effect level (NOEL) using these lung function criteria is about 0.05 ppm. The general pattern of the irritant response and its rapid recovery is similar to that produced by higher concentrations of the irritant, SO_2.

Two aspects of formaldehyde toxicology have brought it from relative obscurity to the forefront of attention in recent years. One is its near ubiquitous presence in indoor atmospheres as an off-gassed product of construction materials such as plywood, furniture, or improperly polymerized urea–formaldehyde foam insulation. In studies relating household formaldehyde levels to chronic effects, children were found to have significantly lower peak expiratory flow rates (about 22% in homes with 60 ppb) than did unexposed children, and asthmatic children were affected below 50 ppb. Also, there is epidemiologic evidence that links formaldehyde to asthma (McGwin et al., 2010) and an increase in lower respiratory tract infections in children (Roda et al., 2011).

A longtime concern regarding formaldehyde has been its potential carcinogenicity. In 2004, IARC concluded that formaldehyde was a "probable human carcinogen" based on evidence of nasopharyngeal cancer and possibly leukemia—based on animal studies (Cogliano, 2005). Nasal cancer had been induced empirically with formaldehyde vapor in a 2-year study where rats were exposed to 2, 6, or 14 ppm 6 hours per day, 5 days per week. Mice were much less sensitive than rats. The detection of DNA adducts in the noses of the two species paralleled the difference in the incidence of tumors. However, the risk associated with exposure to formaldehyde remains controversial because of its widespread industrial use. The reader is encouraged to sample the recent literature (e.g., Nielsen et al., 2017).

Acrolein

Because acrolein is an unsaturated aldehyde, it is more reactive than formaldehyde. It penetrates a bit deeper into the airways and may not have the same degree of sensory irritancy but it may cause more cellular damage. Concentrations below 1 ppm cause irritation of the eyes and the mucous membranes of the respiratory tract. Exposure of guinea pigs to more than or equal to 0.6 ppm reversibly increased pulmonary flow resistance and tidal volume and decreased respiratory frequency (Murphy et al., 1963). The mechanism of increased resistance appears to be mediated through a both local C-fiber and centrally mediated cholinergic reflexes (Bessac and Jordt, 2010).

Exposures of rats to 0.4, 1.4, or 4.0 ppm acrolein for 6 hours per day, 5 days per week for 13 weeks resulted in paradoxical effects on lung function (Costa et al., 1986). The lowest concentration resulted in hyperinflation of the lung with an apparent reduction in small-airway flow resistance, while the highest concentration resulted in airway injury and peribronchial inflammation and fibrosis. The intermediate concentration was functionally not different from the control, although airway pathology was evident. It appears that the high-concentration response reflected the cumulative irritant injury and remodeling as a result of the repeated acrolein, while the low-concentration group had little overt damage and appeared to have slightly stiffened airways, perhaps a result of the protein cross-linking action of acrolein. The pathology in these rats contrasts with that found in formaldehyde studies of similar duration where only upper airway involvement was observed. Ambient exposure to acrolein probably would be about 10% to 20% of the low concentration used in the subchronic study discussed above. Because of a lack of sufficient human data, these animal data were used to perform a risk assessment analysis which showed an excess risk to humans for the adverse effect of ambient levels of acrolein on pulmonary function (Woodruff et al., 2007). Ambient concentrations of acrolein are well below those found in mainstream and secondhand tobacco smoke and occupational exposure levels. As reviewed elsewhere (Bein and Leikauf, 2011; Henning et al., 2017), acrolein produces a wide range of adverse pulmonary effects including lung cancer that may be linked mechanistically to *p53* (a tumor suppressor gene) DNA adducts and mutations, as well as cardiovascular effects.

Thus, as a class the aldehydes can be very irritating and may constitute a significant fraction of the discomfort and sensation experienced during an oxidant pollution episode, especially in mixed atmospheres containing particles.

THE MULTIPOLLUTANT REALITY OF AIR POLLUTION

The pollutant profiles of any community's atmosphere vary considerably in space and time. These pollutants are emitted locally and are mixed with pollutants transported over varied distances while undergoing photochemical transformation—hence the phrase: "urban soup." For decades, these complexities have been daunting for toxicologists, and therefore scientists and risk assessors are

prompted to adopt a reductionist approach—evaluating nominally one pollutant at a time. While the reductionist approach has been very successful in providing data to target pollutants of primary concern and improving public health, it is widely thought that there are likely chemical and physiological interactions between and among pollutants that are of public health consequence, but have not been appreciated. Indeed, interactions are more likely to be apparent as exposure concentrations decrease, and while we may be concerned about synergistic interactions as found with some particles and SO_2, it may be that antagonisms also occur among pollutants that are prevalent. As the science and the statistical methods to look at interactions have improved, there is renewed interest in multipollutant toxicology.

Publications are revisiting this issue (Mauderly and Samet, 2009; Buckley and Farraj, 2015; Davalos et al., 2017), where scientists from all sectors are trying to determine whether there are better approaches to studying interactions (Dominici et al., 2010; Vedal and Kaufman, 2011), synthesizing or modeling findings to extract interactions (Johns et al., 2012), and then translating and implementing the findings to reduce the components or factors central to mixture behavior in terms of health risk (Fann et al., 2011). There is yet to be consensus on approaches and most likely there will be more than one approach; however, the advances in the science and evolution of the air pollution scenario to one of lower background and dominant source contributors suggests that novel thinking is needed on this issue. Early toxicology studies that were focused on complex mixtures, some of which were photochemically altered (discussed earlier), along with epidemiology studies usually came to conclusions relevant to that mixture alone. But the revival of the PM story, and that it is itself a mixture of trace elements and organic and elemental carbon, has raised questions about the mix of key PM attributes that drive outcomes and how co-pollutants interplay in these outcomes. While single pollutant approaches to air pollution research, health assessments, and ambient air quality standard setting have been successful in reducing global air pollution over past decades, there is a clear need for novel ideas to address this next dimension of air quality management via scientific and regulatory/policy innovation.

CONCLUSIONS

In writing this textbook chapter on air pollution toxicology, the authors' goal has been to relate empirical studies in animals as well as controlled studies in humans to provide biologic plausibility to health observations revealed or described through epidemiology. The breadth and complexity of the problem of air pollution—from the development of credible databases to utilizing these data sources to inform regulatory action and decision making—have been the theme throughout the chapter. The classical and still most prominent air pollutants provide a foundation for understanding and appreciating the nuances and dissecting outcomes upon which defensible air pollution policy and decision making can be applied to public health. The key role of the toxicologist is to ask questions to provide understanding while developing sensitive and relevant assay methods. An understanding of air pollution exposure as well as basic biology is key to revealing the causality information needed to support decision making hand-in-hand with epidemiology.

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REFERENCES

Alarie Y, Ferguson JS, Stock MF, et al. Sensory and pulmonary irritation of methyl isocyanate in mice and pulmonary irritation and possible cyanide-like effects of methyl isocyanate in guinea pigs. *Environ Health Perspect*. 1987;72:159–168.

Alarie YC, Ulrich CE, Busey WM, et al. Long-term continuous exposure of guinea pigs to sulfur dioxide. *Arch Environ Health*. 1970;21:769–777.

Alarie YC, Ulrich CE, Busey WM, et al. Long-term continuous exposure to sulfur dioxide in cynomolgus monkeys. *Arch Environ Health*. 1972;24:115–128.

Alexis NE, Carlsten C. Interplay of air pollution and asthma immunopathogenesis: a focused review of diesel exhaust and ozone. *Int Immunopharmacol*. 2014;23:347–355.

Allred EN, Bleeker ER, Chaitman BR, et al. Short-term effects of carbon monoxide exposure on individuals with coronary heart disease. *N Engl J Med*. 1989;321:1426–1432.

Amdur MO, Dubriel M, Creasia DA. Respiratory response of guinea pigs to low levels of sulfuric acid. *Environ Res*. 1978;15:418–423.

Amdur MO, Sarofim AF, Neville M, et al. Coal combustion aerosols and SO_2: an interdisciplinary analysis. *Environ Sci Tech*. 1986;20:139–145.

Amdur MO. The response of guinea pigs to inhalation of formaldehyde and formic acid alone and with a sodium chloride aerosol. *Int J Air Pollut*. 1960;3:201–220.

Amdur MO. Sulfuric acid: the animals tried to tell us: 1989 Herbert E. Stokinger lecture. *Appl Ind Hyg Assoc J*. 1989;4:189–197.

American Lung Association. *State of the Air: Ozone Pollution*. 2017. Available at: http://www.lung.org/our-initiatives/healthy-air/sota/key-findings/ozone-pollution.html.

American Thoracic Society (ATS). What constitutes an adverse health effect of air pollution? *Am J Respir Crit Care Med*. 2000;161:665–673.

Anderson HR. Health effects of air pollution episodes. In: Holgate ST, Samet JM, Koren H, Maynard RL, eds. *Air Pollution and Health*. London: Academic Press; 1999:461–484.

Anenberg SC, Horowitz LW, Tong DQ, West JJ. An estimate of the global burden of anthropogenic ozone and fine particulate matter on premature human mortality using atmospheric modeling. *Environ Health Perspect*. 2010;118:1189–1195.

Anjilvel S, Asgharian B. A multiple-path model of particle deposition in the rat lung. *Fundam Appl Toxicol*. 1995;28:41–50.

Aronow WS. Aggravation of angina pectoris by two percent carboxyhemoglobin. *Am Heart J*. 1981;101:154–157.

Barry BE, Mercer RR, Miller FJ, et al. Effects of inhalation of 0.25 ppm O_3 on the terminal bronchioles of juvenile and adult rats. *Exp Lung Res*. 1988;14:225–245.

Bates DV, Sizto R. Air pollution and hospital admissions in southern Ontario: the acid summer haze effect. *Environ Res*. 1987;65:172–194.

Bauer AK, Kleeberger SR. Genetic mechanisms of susceptibility to ozone-induced lung disease. *Ann N Y Acad Sci*. 2010;1203:113–119.

Bein K, Leikauf GD. Acrolein—a pulmonary hazard. *Mol Nutr Food Res*. 2011;55:1342–1360.

Bessac BF, Jordt SE. Sensory detection and responses to toxic gases: mechanisms, health effects, and countermeasures. *Proc Am Thorac Soc*. 2010;7:269–277.

Boubel RW, Vallero D, Fox DL, Turner B, Stern AC. *Fundamentals of Air Pollution*. London, UK: Elsevier; 1994:107.

Brimblecombe P. Air pollution and health history. In: Holgate ST, Samet JM, Koren H, Maynard RL, eds. *Air Pollution and Health*. London: Academic Press; 1999:10.

Brook RD, Rajagopalan S, Pope CA III, et al. Particulate matter air pollution and cardiovascular disease: an update to the scientific statement from the American Heart Association. *Circulation*. 2010;121:2331–2378.

Brooks BO, Davis WF. *Understanding Indoor Air Quality*. Boca Raton, FL: CRC Press; 1992.

Buckley B, Farraj A. Conceptual model for assessing criteria air pollutants in a multipollutant context: a modified adverse outcome pathway approach. *Toxicology*. 2015;335:85–94.

Calderon-Garcidueñas L, Mora-Tiscareno A, Fordham LA, et al. Respiratory damage in children exposed to urban pollution. *Pediatr Pulmonol*. 2003;36(2):148–161.

Calderon-Garcidueñas L, Osorno-Velazquez A, Bravo-Alvarez H, et al. Histopathological changes of the nasal mucosa in southwest metropolitan Mexico City inhabitants. *Am J Pathol*. 1992;140:225–232.

Calvert JG, Lazrus A, Kok GL, et al. Chemical mechanisms of acid generation in the troposphere. *Nature*. 1985;317:27–35.

Cascio WE. Proposed pathophysiologic framework to explain some excess cardiovascular death associated with ambient air particle pollution: insights for public health translation. *Biochim Biophys Acta*. 2016;1860:2869–2879.

Chang L, Huang Y, Stockstill BL, et al. Epithelial injury and interstitial fibrosis in the proximal alveolar regions of rats chronically exposed to a simulated pattern of urban ambient ozone. *Toxicol Appl Pharmacol*. 1992;115:241–252.

Chang L, Miller FJ, Ultman J, et al. Alveolar epithelial cell injuries by subacute exposure to low concentrations of ozone correlate with cumulative exposure. *Toxicol Appl Pharmacol*. 1991;109:219–234.

Chang LY, Mercer RR, Stockstill BL, et al. Effects of low levels of NO_2 on terminal bronchiolar cells and its relative toxicity compared to O_3. *Toxicol Appl Pharmacol*. 1988;96:451–464.

Chen LC, Lam HF, Kim EJ, et al. Pulmonary effects of ultrafine coal fly ash inhaled by guinea pigs. *J Toxicol Environ Health*. 1990;29:169–184.

Chen LC, Quan C, Hwang JS, Jin X, et al. Atherosclerosis lesion progression during inhalation exposure to environmental tobacco smoke: a comparison to concentrated ambient air fine particles exposure. *Inhal Toxicol*. 2010;22(6):449–459.

Ci Z, Zhang X, Wang Z. Enhancing atmospheric mercury research in China to improve the current understanding of the global mercury cycle: the need for urgent and closely coordinated efforts. *Environ Sci Technol*. 2012;46:5636–5642.

Coffin DL, Blommer EJ. Acute toxicity of irradiated auto exhaust: its indication by enhancement of mortality from streptococcal pneumonia. *Arch Environ Health*. 1967;15:36–38.

Cogliano VJ. *IARC Monographs*, leukemia, nasopharyngeal cancer, sinonasal cancer. *Environ Health Perspect*. 2005;113:1205–1208.

Costa DL. Issues that must be addressed for risk assessment of mixed exposures: the EPA experience with air quality. *J Toxicol Environ Health*. 2003;67:195–207.

Costa DL, Dreher KL. Bioavailable transition metals in particulate matter mediate cardiopulmonary injury in healthy and compromised animal models. *Environ Health Perspect*. 1997;105(suppl 5):1053–1060.

Costa DL, Kutzman RS, Lehmann JR, et al. Altered lung function and structure in the rat after subchronic exposure to acrolein. *Am Rev Respir Dis*. 1986;133:286–291.

Costa DL, Schafrank SN, Wehner RW, et al. Alveolar permeability to protein in rats differentially susceptible to ozone. *J Appl Toxicol*. 1985;5(3):182–186.

Costa DL, Schlegele E. Irritant air pollutants. In: Swift DL, Foster WM, eds. *Air Pollutants and the Respiratory Tract*. New York: Marcel Dekker; 1998:119–145.

Costa DL, Tepper JS, Stevens MA, et al. Restrictive lung disease in rats chronically exposed to an urban profile of ozone. *Am J Respir Crit Care Med*. 1995;151:1512–1518.

Crippa P, Castruccio S, Archer-Nicholls S, et al. Population exposure to hazardous air quality due to the 2015 fires in Equatorial Asia. *Sci Rep*. 2016;6:37074.

Davalos AD, Luben TJ, Herring AH, Sacks JD. Current approaches used in epidemiologic studies to examine short-term multipollutant air pollution exposures. *Ann Epidemiol*. 2017;27:145-153.

Detels R, Tashkin DP, Sayre JW, et al. The UCLA population studies of CORD: X. A cohort study of changes in respiratory function associated with chronic exposure to SO_x, NO_x, and hydrocarbons. *Am J Public Health*. 1991;81:350–359.

Devlin RB, Folinsbee LJ, Biscardi F, et al. Attenuation of cellular and biochemical changes in the lungs of humans exposed to ozone for five consecutive days. *Am Rev Respir Dis*. 1993;147:A71.

Devlin RB, McDonnell WF, Mann R, et al. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am J Respir Cell Mol Biol*. 1991;4:72–81.

Di Q, Wang Y, Zanobetti A, Wang Y, et al. Air pollution and mortality in the medicare population. *N Engl J Med*. 2017;376:2513–2522.

Diaz-Sanchez D, Garcia MP, Wang M, et al. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J Allergy Clin Immunol*. 1999;104(6):1183–1188.

Dockery DW, Pope CA, Xu X, et al. An association between air pollution and mortality in six U.S. cities. *N Engl J Med*. 1993;329(24):1753–1759.

Dominici F, Peng RD, Barr CD, Bell ML. Protecting human health from air pollution: shifting from a single-pollutant to a multipollutant approach. *Epidemiology*. 2010;21:187–194.

Donaldson K, Li XY, MacNee W. Ultrafine (nanometre) particle mediated lung injury. *J Aerosol Sci*. 1998;29:553–560.

Driscoll KE, Deyo LC, Carter JM, et al. Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells. *Carcinogenesis*. 1997;18(2):423–430.

Dye JA, Lehmann JR, McGee JK, et al. Acute pulmonary toxicity of particulate matter filter extracts in rats: coherence with epidemiologic studies in Utah Valley residents. *Environ Health Perspect*. 2001;109(suppl 3):395–403.

Ehrlich R, Henry MC. Chronic toxicity of nitrogen dioxide: I. Effect on resistance to bacterial pneumonia. *Arch Environ Health*. 1968;17:860–865.

Fann N, Roman HA, Fulcher CM, et al. Maximizing health benefits and minimizing inequality: incorporating local-scale data in the design and evaluation of air quality policies. *Risk Anal*. 2011;31:908–922.

Farraj AK, Boykin E, Ledbetter A, Andrews D, Gavett SH. Increased lung resistance after diesel particulate and ozone co-exposure not associated with enhanced lung inflammation in allergic mice. *Inhal Toxicol*. 2010;22:33–41.

Faustini A, Alessandrini ER, Pey J, et al. Short-term effects of particulate matter on mortality during forest fires in Southern Europe: results of the MED-PARTICLES Project. *Occup Environ Med*. 2015;72(5):323–329.

Faustini A, Rapp R, Forastiere F. Nitrogen dioxide and mortality: review and meta-analysis of long-term studies. *Eur Respir J*. 2014;44:744–753.

Firkert M. Fog along the Meuse Valley. *Trans Faraday Soc*. 1936;32:1192–1197.

Frampton MW, Smeglin AM, Roberts NJJ, et al. Nitrogen dioxide exposure *in vivo* and human macrophage inactivation of influenza virus *in vitro*. *Environ Res*. 1989;48:179–192.

Frank NR, Speizer FE. SO_2 effects on the respiratory system in dogs: changes in mechanical behavior at different levels of the respiratory system during acute exposure to the gas. *Arch Environ Health*. 1965;11:624–634.

Gardner DE. Oxidant induced enhanced sensitivity to infection in animal models and their extrapolation to man. *J Toxicol Environ Health*. 1984;13:423–439.

Gauderman WJ, Avol E, Gilliland F, et al. The effect of air pollution on lung development from 10 to 18 years of age. *N Engl J Med*. 2004;351(11):1057–1067.

Gauderman WJ, Urman R, Avol SE, et al. Association of improved air quality with lung development in children. *N Engl J Med*. 2015;372:905–913.

Gearhart JM, Schlesinger RB. Sulfuric acid–induced changes in the physiology and structure of the tracheobronchial airways. *Environ Health Perspect*. 1989;79:127–136.

Gerrity TR, Weaver RA, Bernsten J, et al. Extrathoracic and intrathoracic removal of ozone in tidal breathing humans. *J Appl Physiol*. 1988;65:393–400.

Ghio AJ. Biological effects of Utah Valley ambient air particles in humans: a review. *J Aerosol Med*. 2004;17:157–164.

Ghio AJ, Smith CB, Madden MC. Diesel exhaust particles and airway inflammation. *Curr Opin Pulm Med*. 2012;18:144–150.

Gilmour MI, Park P, Doerfler D, Selgrade MK. Ozone-enhanced pulmonary infection with *Streptococcus zooepidemicus* in mice: the role of alveolar macrophage function and capsular virulence factors. *Am Rev Respir Dis*. 1993;147:753–760.

Glasser SW, Nogee LM. Genetically engineered mice in understanding the basis of neonatal lung disease. *Semin Perinatol*. 2006;30:341–349.

Gold DR, Litonjua A, Schwartz J, et al. Ambient pollution and heart rate variability. *Circulation*. 2000;101(11):1267–1273.

Goldstein BD, Lai LY, Ross SR, Cuzzi-Spada R. Susceptibility of inbred mouse strains to ozone. *Arch Environ Health*. 1973;27:412–413.

Gomes EC, Allgrove JE, Florida-James G, Stone V. Effect of vitamin supplementation on lung injury and running performance in a hot, humid, and ozone-polluted environment. *Scand J Med Sci Sports*. 2011;21:452–460.

Government of Canada. 2014. Available at: http://www.ec.gc.ca/air/default.asp?lang=En&n=D560EA62-1&offset=2&toc=hide.

Greim H, Borm PJA, Schins RPF, et al. Toxicity of fibers and particles. Report of the workshop held in Munich, Germany. *Inhal Toxicol*. 2001;13:101–119.

Groves AM, Gow AJ, Massa CB, et al. Age-related increases in ozone-induced injury and altered pulmonary mechanics in mice with progressive lung inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2013;305:L555–L568.

Hackney JD, Linn WS, Avol EL. Acid fog: effects on respiratory function and symptoms in healthy asthmatic volunteers. *Environ Health Perspect*. 1989;79:159–162.

Hamra GB, Guh N, Cohen A, et al. Outdoor particulate matter exposure and lung cancer: a systematic review and meta-analysis. *Environ Health Perspect*. 2014;122:906–911.

Hamra GB, Laden F, Cohen AJ, et al. Lung cancer and exposure to nitrogen dioxide and traffic: a systematic review and meta-analysis. *Environ Health Perspect*. 2015;123(11):1107–1112.

Hatch GE, Slade R, Harris LP, et al. Ozone dose and effect in humans and rats: a comparison using oxygen-18 labeling and bronchoalveolar lavage. *Am J Respir Crit Care Med*. 1994;150:676–683.

Hayes SM, Gobbell RV, Ganick NR, eds. *Indoor Air Quality: Solutions and Strategies*. New York: McGraw-Hill; 1995.

Health Effects Institute (HEI). *Consequences of Prolonged Inhalation of Ozone on F344 Rats: Collaborative Studies*. Studies I-XIII, Report #65. Boston, MA: Health Effects Institute; 1994–1995.

Health Effects Institute (HEI). *Diesel Emissions and Lung Cancer: An Evaluation of Recent Epidemiological Evidence for Quantitative Risk Assessment*. 2015a. Available at: https://www.healtheffects.org/system/files/SR19-Diesel-Epidemiology-2015_0.pdf.

Health Effects Institute (HEI). *Diesel Exhaust: Executive Summary. The Advanced Collaborative Emissions Study (ACES) Communication—ACES*. 2015b. Available at: https://www.healtheffects.org/publications/air-pollution/diesel-exhaust/content.

Henning RJ, Johnson GT, Coyle JP, Harbison RD. Acrolein can cause cardiovascular disease: a review. *Cardiovasc Toxicol*. 2017;17:227–236.

Henry MC, Findlay J, Spengler J, Ehrlich R. Chronic toxicity of NO_2 in squirrel monkeys: III. Effect on resistance to bacterial and viral infection. *Am Ind Hyg Assoc J*. 1970;20:566–570.

Hernández-Escobar SA, Avila-Casado MC, Soto-Abraham V, et al. Cytological damage of nasal epithelium associated with decreased glutathione peroxidase in residents from a heavily polluted city. *Int Arch Occup Environ Health*. 2009;82:603–612.

Higuchi MA, Davies DW. An ammonia abatement system for whole-body animal inhalation exposures to acid aerosols. *Inhal Toxicol*. 1993;5:323–333.

Hosgood HD III, Song M, Hsiung CA, et al. Interactions between household air pollution and GWAS-identified lung cancer susceptibility markers in the Female Lung Cancer Consortium in Asia (FLCCA). *Hum Genet*. 2015;134(3):333–341.

Hyde D, Orthoefer J, Dungworth D, et al. Morphometric and morphologic pulmonary lesions in beagle dogs chronically exposed to high ambient levels of air pollutants. *Lab Invest*. 1978;38(4):455–469.

Olin SS. The relevance of the rat lung response to particle overload for human risk assessment: A workshop consensus report. *Inhal Toxicol*. 2000;12: 1–17.

Jakab GJ. Relationship between carbon black particulate-bound formaldehyde, pulmonary antibacterial defenses and alveolar macrophage phagocytosis. *Inhal Toxicol*. 1992;4:325–342.

Johns DO, Stanek L, Walker K, et al. Practical advancement of multipollutant scientific and risk assessment approaches for ambient air pollution. *Environ Health Perspect*. 2012;120(9):1238–1242.

Kaufman JD, Adar SD, Barr RG, et al. Association between air pollution and coronary artery calcification within six metropolitan areas in the USA (the Multi-Ethnic Study of Atherosclerosis and Air Pollution): a longitudinal cohort study. *Lancet*. 2016;388(10045):696–704.

Kehrl HR, Peden DB, Ball B, Folinsbee LJ, Horstman DJ. Increased specific airway reactivity of persons with mild allergic asthma after 7.6 hours of exposure to 0.16 ppm ozone. *Allergy Clin Immunol*. 1999;104:1198–204.

Kieber RJ, Skrabal SA, Smith BJ, Willey JD. Organic complexation of Fe(II) and its impact on the redox cycling of iron in rain. *Environ Sci Technol*. 2005;39:1576–1583.

Kim CS, Kang TC. Comparative measurement of lung deposition of inhaled fine particles in normal subjects and patients with obstructive airway disease. *Am J Respir Crit Care Med*. 1997;155(3):899–905.

Kleeberger SR, Reddy S, Zhang LY, et al. Genetic susceptibility to ozone-induced lung hyperpermeability. Role of toll-like receptor 4. *Am J Respir Cell Mol Biol*. 2000;22(5):620–627.

Kleinman MT, Mautz WJ, Bjarnason S. Adaptive and non-adaptive responses in rats exposed to ozone, alone and in mixtures, with acidic aerosols. *Inhal Toxicol*. 1999;11(3):249–264.

Kodavanti UP, Costa DL, Bromberg P. Rodent models of cardiopulmonary disease: their potential applicability in studies of air pollutant susceptibility. *Environ Health Perspect*. 1998;106(suppl 1):111–130.

Kodavanti UP, Russell JC, Costa DL. Rat models of cardiometabolic diseases: baseline clinical chemistries, and rationale for their use in examining air pollution health effects. *Inhal Toxicol*. 2015;27(suppl 1):2–13.

Koenig JQ, Covert DS, Pierson WE. Effects of inhalation of acidic compounds on pulmonary function in allergic adolescent subjects. *Environ Health Perspect*. 1989;79: 173–178.

Koenig JQ, Pierson WE, Horike M, Frank R. Effects of SO_2 plus NaCl aerosol combined with moderate exercise on pulmonary function in asthmatic adolescents. *Environ Res*. 1981;25:340–348.

Kotin P, Falk HF. Atmospheric factors in pathogenesis of lung cancer. *Cancer Res*. 1963;7:475–514.

Kreyling WG, Semmler-Behnke M, Seitz J, et al. Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. *Inhal Toxicol*. 2009;21(suppl 1):55–60.

Kumie A, Emmelin A, Wahlberg S, et al. Magnitude of indoor NO_2 from biomass fuels in rural settings of Ethiopia. *Indoor Air*. 2009;19:14–21.

Lewis TR, Campbell KI, Vaughan TR Jr. Effects on canine pulmonary function: via induced NO_2 impairment, particulate interaction, and subsequent SO_x. *Arch Environ Health*. 1969;18:596–601.

Lewis TR, Moorman WJ, Yang YY, Stara JF. Long-term exposure to auto exhaust and other pollutant mixtures. *Arch Environ Health*. 1974;21:102–106.

Lewtas J. Air pollution combustion emissions: characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. *Mutat Res*. 2007;636:95–133.

Li Z, Potts-Kant EN, Garantziotis S, Foster WM, Hollingsworth JW. Hyaluronan signaling during ozone-induced lung injury requires TLR4, MyD88, and TIRAP. *PLoS One*. 2011;6(11):e27137.

Lim SS, Vos T, Flaxman AD, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012;380(9859):2224–2260.

Lippmann M. Health effects of ozone: a critical review. *J Air Pollut Control Assoc*. 1989;39:67–96.

Liu JC, Mickley LJ, Sulprizio MP, et al. Particulate air pollution from wildfires in the western US under climate change. *Clim Change*. 2016;138(3):655–666.

London SJ, Romieu I. Gene by environment interaction in asthma. *Annu Rev Public Health*. 2009;30:55–80.

Loh MM, Levy JI, Spengler JD, Houseman EA, Bennett DH. Research ranking cancer risks of organic hazardous air pollutants in the United States. *Environ Health Perspect*. 2007;115:1160–1168.

Madden MC, Richards J, Daily LA, et al. Effect of ozone on diesel exhaust particle toxicity in the rat lung. *Toxicol Appl Pharmacol*. 2000;168:140–148.

Madden MC, Stevens T, Case M, et al. Diesel exhaust modulates ozone-induced lung function decrements in healthy human volunteers. *Part Fibre Toxicol*. 2014;11:37–47.

Malig BJ, Pearson DL, Chang YB, et al. A time-stratified case-crossover study of ambient ozone exposure and emergency department visits for specific respiratory diagnoses in California (2005–2008). *Environ Health Perspect*. 2016;124:745–753.

Martenies SE, Wilkins D, Batterman SA. Health impact metrics for air pollution management strategies. *Environ Int*. 2015;85:84–95.

Martonen TB, Zhang Z, Yang Y. Interspecies modeling of inhaled particle deposition patterns. *J Aerosol Sci*. 1992;23(4):389–406.

Mauderly JL. Health hazards of complex environmental exposures: a difficult challenge to inhalation toxicology. *Inhal Toxicol*. 2006;18:137–141.

Mauderly JL, Samet JM. Is there evidence for synergy among air pollutants in causing health effects? *Environ Health Perspect*. 2009;117:1–6.

McCunney RJ. Asthma, genes, and air pollution. *J Occup Environ Med*. 2005;47(12):1285–1291.

McDonnell WF. Intersubject variability in human acute ozone responsiveness. *Pharmacogenetics*. 1991;1:110–113.

McGwin G, Lienert J, Kennedy JI. Formaldehyde exposure and asthma in children: a systematic review. *Environ Health Perspect*. 2010;118: 313–317.

McKinney WJ, Jaskot RH, Richards JH, et al. Cytokine mediation of ozone-induced pulmonary adaptation. *Am J Respir Cell Mol Biol*. 1998;18:696–705.

Mercer RR, Costa DL, Crapo JD. Effects of prolonged exposure to low doses of nitric oxide on the alveolar septa of the adult rat lung. *Lab Invest*. 1995;73(1):1–9.

Miller FJ, Graham JA, Raub JA, et al. Evaluating the toxicity of urban patterns of oxidant gases: II. Effects in mice from chronic exposure to nitrogen dioxide. *J Toxicol Environ Health*. 1987;21:99–112.

Miller FW, Miller RM. *Environmental Hazards: Air Pollution—A Reference Handbook*. Contemporary World Issues. Santa Barbara, CA: ABC-CLIO; 1993:1–18.

Minelli C, Wei I, Sagoo G, et al. Interactive effects of antioxidant genes and air pollution on respiratory function and airway disease: a HuGE review. *Am J Epidemiol*. 2011;173:603–620.

Mirowsky J, Gordon T. Noninvasive effects measurements for air pollution human studies: methods, analysis, and implications. *J Expo Sci Environ Epidemiol*. 2015;25:354–380.

Mohsenin V. Effect of vitamin C on NO_2-induced airway hyperresponsiveness in normal subjects. *Am Rev Respir Dis*. 1987;136:1408–1411.

Molhave LB, Bach B, Pederson O. Human reactions to low concentrations of volatile organic compounds. *Environ Int*. 1986;12:165–167.

Moss OR, Gross EA, James RA, et al. Respiratory tract toxicity in rats exposed to Mexico City air. *Res Rep Health Effects Inst*. 2001;(100):1–24.

Mudway IS, Krishna MT, Frew AJ, et al. Compromised concentrations of ascorbate in fluid lining the respiratory tract in human subjects after exposure to ozone. *Occup Environ Med* 1999;56(7):473–481.

Murphy SD, Klingshirn DA, Ulrich CE. Respiratory response of guinea pigs during acrolein inhalation and its modification by drugs. *J Pharmacol Exp Ther*. 1963;141:79–83.

Murray C, ed. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet*. 2016;388:1459–544.

Nadel JA, Salem H, Tamplin B, Tokiwa Y. Mechanism of bronchoconstriction during inhalation of sulfur dioxide. *J Appl Physiol*. 1965;20:164–167.

National Research Council (NRC). *Science and Decisions. Advancing Risk Assessment*. Washington, DC: National Academies of Science; 2009.

Nielsen GD, Larsen ST, Wolkoff P. Re-evaluation of the WHO (2010) formaldehyde indoor air quality guideline for cancer risk assessment. *Arch Toxicol*. 2017;91:35–61.

NWCG Smoke Committee. *2014 EPA National Emission Inventory for Wildland Fire*. 2014. Available at: https://www.frames.gov/files/5214/1030/1118/SmoC_BP_2014_EPA_NEI_3-13-14.pdf.

Oberdorster G, Sharp Z, Atudorei V, et al. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *J Toxicol Environ Health A*. 2002;65(20):1531–1543.

Ohtsuka Y, Clarke RW, Mitzner W, et al. Interstrain variation in murine susceptibility to inhaled acid-coated particles. *Am J Physiol Lung Cell Mol Physiol*. 2000;278(3):L469–L476.

Overton JH, Miller FJ. Modeling ozone absorption in the respiratory tract. 80th Annual Meeting of the Air Pollution Control Association, paper no. 87–99.4; 1987.

Overton JH, Miller FJ. Dosimetry modeling of inhaled toxic reactive gases. In: Watson AY, Bates RR, Kennedy D, eds. *Air Pollution and Public Health*. Washington, DC: National Academies Press; 1988.

Peters A. Commentary: inflamed about ultrafine particles? *Int J Epidemiol*. 2006;35:1355–1356.

Pinkerton KE, Brody AR, Miller FJ, Crapo JD. Exposure to low levels of ozone results in enhanced pulmonary retention of inhaled asbestos fibers. *Am Rev Respir Dis*. 1989;140(4):1075–1081.

Pope CA III. Particulate pollution and health: a review of the Utah valley experience. *J Expo Anal Environ Epidemiol*. 1996;6:23–34.

Pope CA III, Burnett RT, Thun MJ, et al. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. *JAMA*. 2002;287(9):1132–1141.

Pope CA III, Burnett RT, Turner MC, et al. Lung cancer and cardiovascular disease mortality associated with ambient air pollution and cigarette smoke: shape of the exposure-response relationships. *Environ Health Perspect*. 2011;119:1616–1621.

Pope CA III, Dockery DW. Health Effects of Fine Particulate Air Pollution: Lines that Connect. 2006 Critical Review. *J Air Waste Manag Assoc*. 2006;56:709–742.

Pope CA III, Thun MJ, Namboodiri MM, et al. Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. *Am J Respir Crit Care Med*. 1995;151:669–674.

Reid L. An experimental study of hypersecretion of mucus in the bronchial tree. *Br J Exp Pathol*. 1963;44:437–440.

Robinson J, Nelson WC. *National Human Activity Pattern Survey Data Base*. Research Triangle Park, NC: U.S. Environmental Protection Agency; 1995.

Roda C, Kousignian I, Guihenneuc-Jouyaux C, et al. Formaldehyde exposure and lower respiratory infections in infants: findings from the PARIS cohort study. *Environ Health Perspect*. 2011;119:1653–1658.

Romieu I, Sienra-Monge JJ, Ramirez-Aguilar M, et al. Genetic polymorphism of GSTM1 and antioxidant supplementation influence lung function in relation to ozone exposure in asthmatic children in Mexico City. *Thorax*. 2004;59(1):8–10.

Rosenlund M, Berglind N, Pershagen G, et al. Long-term exposure to urban air pollution and myocardial infarction. *Epidemiology*. 2006;17(4):383–390.

Saldiva PHN, King M, Delmonte VLC, et al. Respiratory alterations due to urban air pollution: an experimental study in rats. *Environ Res*. 1992;57:19–33.

Salvi SS, Nordenhall C, Blomberg A, et al. Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways. *Am J Respir Crit Care Med*. 2000;161(2 pt 1):550–557.

Samoli E, Aga E, Touloumi G, et al. Short-term effects of nitrogen dioxide on mortality: an analysis within the APHEA project. *Eur Respir J*. 2006;27(6):1129–1138.

Schenker MB, Samet JM, Speizer FE, et al. Health effects of air pollution due to coal combustion in the Chestnut Ridge region of Pennsylvania: results of cross-sectional analysis in adults. *Arch Environ Health*. 1983;38:325–333.

Schins RP, Knaapen AM. Genotoxicity of poorly soluble particles. *Inhal Toxicol*. 2007;19(suppl 1):189–198.

Schlesinger RB. Comparative irritant potency of inhaled sulfate aerosol: effects on bronchial mucociliary clearance. *Environ Res*. 1984;34:268–279.

Schlesinger RB. Intermittent inhalation of nitrogen dioxide: effects on rabbit alveolar macrophages. *J Toxicol Environ Health*. 1987;21:127–139.

Schlesinger RB, Gorczynski JE, Dennison J, et al. Long-term intermittent exposure to sulfuric acid aerosol, ozone, and their combination: alterations in tracheobronchial mucociliary clearance and epithelial secretory cells. *Exp Lung Res*. 1992;18:505–534.

Schwartz J. Particulate air pollution and daily mortality in Detroit. *Environ Res*. 1991;56:204–213.

Schwartz J. Air pollution and the duration of acute respiratory symptoms. *Arch Environ Health*. 1992;47(2):116–122.

Shapiro SD. Transgenic and gene-targeted mice as models for chronic obstructive pulmonary disease. *Eur Respir J*. 2007;29:375–378.

Sheppard DA, Saisho A, Nadel JA, Boushey HA. Exercise increases sulfur dioxide induced bronchoconstriction in asthmatic subjects. *Am Rev Respir Dis*. 1981;123:486–491.

Sienra-Monge JJ, Ramirez-Aguilar M, Moreno-Macias H. Antioxidant supplementation and nasal inflammatory responses among young asthmatics exposed to high levels of ozone. *Clin Exp Immunol*. 2004;138(2):317–322.

Silverman DT, Samanic CM, Lumin JH, et al. The Diesel Exhaust in Miners study: a nested case–control study of lung cancer and diesel exhaust. *J Nat Cancer Inst.* 2012;104:855–868.

Singh P, DeMarini DM, Dick CA, et al. Sample characterization of automobile and forklift diesel exhaust particles and comparative pulmonary toxicity in mice. *Environ Health Perspect*. 2004;112(8):820–825.

Speizer FE. Studies of acid aerosols in six cities and in a new multi-city investigation: design issues. *Environ Health Perspect*. 1989;79:61–68.

Spengler JD, Sexton K. Indoor air pollution: a public health perspective. *Science*. 1983;221:9–17.

Stevens MA, Fitzgerald S, Menache MG, et al. Functional evidence of persistent airway obstruction in rats following a two-hour inhalation exposure to methyl isocyanate. *Environ Health Perspect*. 1987;72:89–94.

Straif K, Cohen A, Samet J, et al. *Air Pollution and Cancer*. IARC Scientific Publication No. 161. Lyon, France: IARC; 2013.

Sweeney TD, Skornik WA, Brain JD, et al. Chronic bronchitis alters the pattern of aerosol deposition in the lung. *Am J Respir Crit Care Med*. 1995;151(2 pt 1):482–488.

Tashakkor AY, Chow KS, Carlsten C. Modification by antioxidant supplementation of changes in human lung function associated with air pollutant exposure: a systematic review. *BMC Public Health*. 2011;11:532–543.

Thurston GD, Ahn J, Cromar KR, et al. Ambient particulate matter air pollution exposure and mortality in the NIH-AARP diet and health cohort. *Environ Health Perspect*. 2016;124:484–490.

Thurston GD, Ito K, Kinney PL, Lippmann M. A multi-year study of air pollution and respiratory hospital admissions in three New York state metropolitan areas: results for 1988 and 1989 summers. *J Expos Anal Environ Epidemiol*. 1992;2:429–450.

Thurston GD, Kipen H, Annesi-Maesano I, et al. A joint ERS/ATS policy statement: what constitutes an adverse health effect of air pollution? An analytical framework. *Eur Respir J*. 2017;49(1): pii: 1600419.

Tong H, Cheng WY, Samet JM, Gilmour MI, Devlin RB. Differential cardiopulmonary effects of size-fractionated ambient particulate matter in mice. *Cardiovasc Toxicol*. 2010;10:259–267.

Tujague J, Bastaki M, Holland N, et al. Antioxidant intake, GSTM1 polymorphism and pulmonary function in healthy young adults. *Eur Respir J*. 2006;27:282–288.

Tyler WS, Tyler NK, Last JA, et al. Comparison of daily and seasonal exposures of young monkeys to ozone. *Toxicology*. 1988;50:131–144.

UN Foundation. 2010. Available at: http://www.unfoundation.org/assets/pdf/global-alliance-for-clean-cookstoves-factsheet.pdf.

U.S. EPA. *National Air Quality Emissions Trends Report, 1998*. EPA 454/R-00-003. Washington, DC: U.S. Environmental Protection Agency; 2000. Available at: https://nepis.epa.gov/Exe/tiff2png.cgi/000032KL.PNG?-r+75+-g+7+D%3A%5CZYFILES%5CINDEX%20DATA%5C00THRU05%5CTIFF%5C00000006%5C000032KL.TIF.

U.S. EPA. 2005. Available at: https://www.epa.gov/criteria-air-pollutants/process-reviewing-national-ambient-air-quality-standards.

U.S. EPA. *Integrated Science Assessment (ISA) for Particulate Matter*. Final Report, December 2009. EPA/600/R-08/139F. Washington, DC: U.S. Environmental Protection Agency; 2009.

U.S. EPA. *Integrated Science Assessment (ISA) of Ozone and Related Photochemical Oxidants*. Final Report, February 2013. EPA/600/R-10/076F. Washington, DC: U.S. Environmental Protection Agency; 2013.

U.S. EPA. 2016. IRIS. Available at: https://www.epa.gov/iris.

U.S. EPA. 2018a. Available at: https://www.epa.gov/air-emissions-inventories.

U.S. EPA. 2018b. Available at: https://www.epa.gov/national-air-toxics-assessment.

Utell MJ, Mariglio JA, Morrow PE, et al. Effects of inhaled acid aerosols on respiratory function: the role of endogenous ammonia. *J Aerosol Med*. 1989;2:141–147.

Utell MJ, Morrow PE, Hyde RW. Airway reactivity to sulfate and sulfuric acid aerosols in normal and asthmatic subjects. *J Air Pollut Control Assoc*. 1984;34:931–935.

Van De Lende R, Kok TJ, Reig RP, et al. Decreases in VC and FEV_1 with time: indicators for effects of smoking and air pollution. *Bull Eur Physiopathol Respir*. 1981;17:775–792.

Vancza EM, Galdanes K, Gunnison A, Hatch G, Gordon T. Age, strain, and gender as factors for increased sensitivity of the mouse lung to inhaled ozone. *Toxicol Sci*. 2009;107:535–543.

Vedal S, Kaufman JD. What does multi-pollutant air pollution research mean? *Am J Respir Crit Care Med*. 2011;183:4–6.

von Mutius E, Martinez FD, Fritzsch C, et al. Prevalence of asthma and atopy in two areas of West and East Germany. *Am J Respir Crit Care Med*. 1994;149(2 pt 1):358–364.

Waxman HA. Title III of the 1990 Clean Air Act Amendments. In: Patrick DR, ed. *Toxic Air Pollution Handbook*. New York: Van Nostrand Reinhold; 1994:25–32.

WHO. *The World Health Report 2002: Reducing Risks, Promoting Healthy Life*. 2002. Available at: http://www.who.int/whr/2002/en/.

WHO. 2005. Available at: http://www.who.int/phe/health_topics/outdoorair/outdoorair_aqg/en/.

Wichers LB, Rowan WH, Nolan JP, et al. Particle deposition in spontaneously hypertensive rats exposed via whole-body inhalation: measured and estimated dose. *Toxicol Sci*. 2006;93(2):400–410.

Wiester MJ, Winsett DW, Richards JE, et al. Ozone adaptation in mice and its association with ascorbic acid in the lung. *Inhal Toxicol*. 2000;12:577–590.

Williams AS, Mathews JA, Kasahara DI, et al. Innate and ozone-induced airway hyperresponsiveness in obese mice: role of TNF-α. *Am J Physiol Lung Cell Mol Physiol*. 2015;308:L1168–L1177.

Woodruff TJ, Wells EM, Holt EW, Burgin DE, Axelrad DA. Estimating risk from ambient concentrations of acrolein across the United States. *Environ Health Perspect*. 2007;115:410–415.

Wuebbles DJ, Lei H, Lin J. Intercontinental transport of aerosols and photochemical oxidants from Asia and its consequences. *Environ Pollut*. 2007;150:65–84.

Zeger SL, Irizarry R, Peng RD. On time series analysis of public health and biomedical data. *Annu Rev Public Health*. 2006;27:57–79.

Zou B, Wilson JG, Zhan FB, Zeng Y. Air pollution exposure assessment methods utilized in epidemiological studies. *J Environ Monit*. 2009;11:475–490.

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Unit VII

Applications of Toxicology

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chapter 32

Analytical and Forensic Toxicology

Bruce A. Goldberger, Dayong Lee, and Diana G. Wilkins

Analytical Toxicology
Role in General Toxicology
Role in Forensic Toxicology
Toxicological Investigation of a Poison Death
- Case History and Specimens
- Toxicological Analysis

Interpretation of Analytical Results
Criminal Poisoning of the Living
Forensic Drug Testing
Human Performance Testing
Courtroom Testimony

Role in Clinical Toxicology
Role in Therapeutic Monitoring
Summary
Acknowledgments
References

What is there that is not poison? All things are poison and nothing without poison. Solely the dose determines that a thing is not a poison.

—Paracelsus

It is impossible to consider the topic of forensic toxicology without discussing analytical toxicology in detail, as analytical toxicology has its roots in forensic applications. Therefore, it is logical to discuss these mutually dependent areas together. Analytical toxicology involves the application of the tools of analytical chemistry to the qualitative and/or quantitative estimation of chemicals that may exert effects on living organisms. Generally, the chemical that is to be measured (the analyte) is a xenobiotic that may have been altered or transformed by metabolic actions of the organism. Frequently, the specimen that is to be analyzed consists of a matrix composed of body fluids or solid tissues removed from the organism. Both the identity of the analyte and the complexity of the matrix can present formidable problems to an analytical toxicologist.

Forensic toxicology involves the use of toxicology for the purposes of the law (Cravey and Baselt, 1981). Although this broad definition includes a wide range of applications, such as regulatory toxicology and workplace urine testing to detect drug use, by far the most common application is to identify any chemical that may serve as a causative agent in inflicting injury or death on humans, or in causing damage to property. Frequently, as a result of such unfortunate incidents, charges of liability or criminal intent are brought that must be resolved by the judicial system. At times, indirect or circumstantial evidence is presented in an attempt to prove cause and effect. However, there is no substitute for the unequivocal identification of a specific chemical substance that is demonstrated to be present in tissues from the victim at a sufficient concentration to explain the injury with certainty. For this reason, forensic toxicology and analytical toxicology have long shared a mutually supportive partnership.

To aid in deciding whether adverse effects of xenobiotics contribute to death, injury, or other harm to persons or property, great efforts are made to initiate and implement analytical procedures in a forensically credible manner. Laws prescribing punishment to drug-impaired drivers are evidence of attempts to mitigate impaired driving. The measurement of ethanol in blood or breath at specific concentrations is generally required to prove impairment by this agent (Fisher et al., 1968). Similarly, the decade of the 1980s saw a growing response by society to the threat of drug abuse. Identification of drug users through testing urine for the presence of drugs or their metabolites, using methods and safeguards developed by forensic toxicologists, has become required by law (Department of Health and Human Services, 1988).

The diagnosis and treatment of health problems induced by chemical substances and the closely allied field of therapeutic drug monitoring also rely greatly on analytical toxicology. Although the analytes are present in biological matrices similar to those obtained for traditional forensic toxicology, the results must be reported rapidly to be of use to clinicians in treating patients. This requirement of a rapid turnaround time often limits the number of chemicals that can be measured because analytical methods, equipment, and personnel must all be available for a swift response to toxicological emergencies. More recently, analytical toxicology methods have also been employed for the detection of drugs and other chemicals used for the purpose of performance-enhancement, which can also require rapid reporting in selected settings, including athletic events.

Occupational toxicology and regulatory toxicology also require analytical procedures for their implementation or monitoring. In occupational toxicology, the analytical methods used to monitor threshold limit values and other criteria for estimating the exposure of workers to toxic hazards may utilize simple, nonspecific, but economical screening devices. However, to determine the actual exposure of a worker, it is necessary to analyze blood, urine, breath, or another specimen by employing methods similar to those used in clinical or forensic toxicology. For regulatory purposes, a variety of matrices (e.g., food, water, and air) must be examined for

extremely small quantities of analytes. Frequently, this requires the use of sophisticated methodology with extreme sensitivity. Both of these applications of analytical toxicology impinge on forensic toxicology because an injury or occupational disease in a worker can result in a legal proceeding, as well as can occur with a violation of a regulatory law.

Other applications of analytical toxicology occur frequently during the course of experimental studies. Confirmation of the concentration of dosing solutions and monitoring of their stability often can be accomplished with the use of simple analytical techniques. The bioavailability of a dose may vary with the route of administration and the vehicle used. Blood concentrations can be monitored as a means of establishing this important parameter. In addition, an important feature in the study of any toxic substance is the characterization of its metabolites as well as the distribution of the parent drug, together with its metabolites, to various tissues. This requires sensitive, specific, and valid analytical procedures. Similar analytical studies can be conducted within a temporal framework to gain an understanding of the dynamics of the absorption, distribution, metabolism, and excretion of toxic chemicals.

It is evident that analytical toxicology is intimately involved in many aspects of experimental and applied toxicology. Because toxic substances include all chemical types and the measurement of toxic chemicals may require the examination of biological or nonbiological matrices, the scope of analytical toxicology is broad. Nevertheless, a systematic approach and a reliance on the practical experience of generations of forensic toxicologists can be used in conjunction with the sophisticated tools of analytical chemistry to provide the data needed to better understand the hazards of toxic substances.

ANALYTICAL TOXICOLOGY

In light of the statement by Paracelsus five centuries ago, "All substances are poisons: there is none which is not a poison," analytical toxicology potentially encompasses all chemical substances. Forensic toxicologists learned long ago that when the nature of a suspected poison is unknown, a systematic, standardized approach must be used to identify the presence of most common toxic substances. An approach that has stood the test of time was first suggested by Chapuis in 1873 in *Elements de Toxicologie*. It is based on the origin or nature of the toxic agent (Petersen et al., 1923). Such a system can be characterized as follows:

1. Gases
2. Volatile substances
3. Corrosive agents
4. Metals
5. Anions and nonmetals
6. Nonvolatile organic substances
7. Miscellaneous

Closely related to this descriptive classification is the method for separating a toxic agent from the matrix in which it is embedded. The matrix is generally a biological specimen such as a body fluid or tissue. The agent of interest may exist in the matrix in a simple solution or may be bound to protein and other cellular constituents. The challenge is to separate the toxic agent in sufficient purity and quantity to permit it to be characterized and quantified. At times, the parent compound is no longer present in large enough amounts to be separated. In this case, known metabolites may indirectly provide a measure of the parent substance (Hawks and Chiang, 1986). With other substances, interaction of the poison with tissue components may require the isolation or characterization of a protein adduct (San George and Hoberman, 1986; Stockham and Blanke, 1988). Methods for separation have long provided a great challenge to analytical toxicologists. Over the previous 20 years, improved methods have become available that permit direct measurement of some analytes without prior separation from the matrix.

Gases are most simply measured by means of gas chromatography. Some gases are extremely labile, and the specimen must be collected and preserved at temperatures as low as that of liquid nitrogen. Generally, the gas is carefully liberated by incubating the specimen at a predetermined temperature in a sealed container. The gas, freed from the matrix, collects in the empty "headspace" between the specimen and the sealed container, where it can be sampled and injected into the gas chromatograph. Other gases, such as carbon monoxide, interact with proteins. These gases can be carefully released from the protein, or the adduct can be measured independently, as in the case of carboxyhemoglobin.

Volatile substances are generally liquids of a variety of chemical types. The temperature at which they boil is sufficiently low such that older methods of separation employed microdistillation or diffusion techniques. Gas chromatography is the simplest approach for simultaneous separation and quantitation of volatile substances. The simple alcohols (e.g., ethanol) can be measured by injecting a diluted body fluid directly onto the analytical column of the gas chromatograph. A more common approach is to use the headspace technique, as is done for gases, after incubating the specimen in a sealed vial at an elevated temperature.

Corrosives include mineral acids and bases. Many corrosives consist of ions that are normal tissue constituents. Clinical chemical techniques can be applied to detect these ions when they are in great excess over normal concentrations. Because these ions are endogenous constituents, the corrosive effects at the site of contact of the chemical, together with other changes in blood chemistry values, can confirm the ingestion of a corrosive substance.

Metals are encountered frequently as occupational and environmental hazards. Elegant analytical methods are available for most metals even when they are present at extremely low concentrations. Classic separation procedures involve destruction of the organic matrix by chemical or thermal oxidation. This leaves the metal to be identified and quantified in the inorganic residue. Unfortunately, this prevents the determination of the metal in the oxidation state or in combination with other elements, as it existed when the metal compound was absorbed. For example, the toxic effects of metallic mercury, mercurous ion, mercuric ion, and dimethylmercury are all different. Selected analytical methods must be capable of determining the speciation and relative amount of each form present to interpret the degree of toxicity. The analytical difficulty in doing so has resulted in the unfortunate practice of describing the toxicity of metals as if each metal existed as a single entity.

Toxic anions and nonmetals present an analytical challenge. With the exception of phosphorus, these agents are rarely encountered in an uncombined form. Some anions can be trapped in combination with a stable cation, after which the organic matrix can be destroyed, as with metals. Others can be separated from the bulk of the matrix by dialysis, after which they are detected by colorimetric or chromatographic techniques. Still others are detected and measured by ion-specific electrodes.

The *nonvolatile organic substances* constitute the largest group of substances that must be considered by analytical toxicologists. This group includes drugs, both prescribed and illicit, pesticides, natural products, pollutants, and industrial compounds. These substances are solids or liquids with high boiling points. Thus, separation procedures generally rely on differential extractions of biological

fluids and tissues (Fig. 32-1). These extractions often are inefficient, and recovery of the analyte from the matrix may be poor. When the nature of the analyte is known, immunoassay procedures, if available, are useful because they allow a toxicologist to avoid using separation procedures. These compounds can be classified as follows:

1. Organic strong acids
2. Organic weak acids
3. Organic bases
4. Organic neutral compounds
5. Organic amphoteric compounds

Separation of the classes specified above is generally achieved by adjusting the acidity of the aqueous matrix and extracting with a water-immiscible solvent or a solid-phase sorbent material. Finally, a *miscellaneous* category must also be included to cover the large number of compounds that cannot be detected by the routine application of the methods described above. Venoms and other toxic mixtures of proteins, or uncharacterized constituents, fall into this class. Frequently, if antibodies can be produced against the active constituent, an immunoassay may be the most practical means of detecting and measuring these highly potent and difficult-to-isolate substances. Unfortunately, unless highly specific monoclonal antibodies are used, the analytical procedure may not be acceptable for forensic purposes. Most frequently, specific analytical procedures must be developed for each analyte of this type. At times, biological endpoints are utilized to semiquantify the concentration of the isolated product.

ROLE IN GENERAL TOXICOLOGY

In almost all experimental studies in toxicology, an agent, generally a single chemical substance is administered in known amounts to an organism. It is universally acknowledged that the chemical under study must be either pure, or the nature of any contaminant well-characterized, to enable interpretation of the experimental results with validity. However, it is a common practice to proceed with the experimental study without verifying the identity and purity of the compound. Not only does this practice lead to errors in establishing an accurate dose, but also, depending on the nature of the study, other erroneous conclusions may be drawn. For example, the presence of related compounds in the dosage form of a tricyclic antidepressant led to erroneous conclusions about the metabolic products of the drug when it was administered together with the unidentified contaminants (Saady et al., 1981). An even greater error may result when a small amount of a contaminant may be supertoxic. A well-publicized

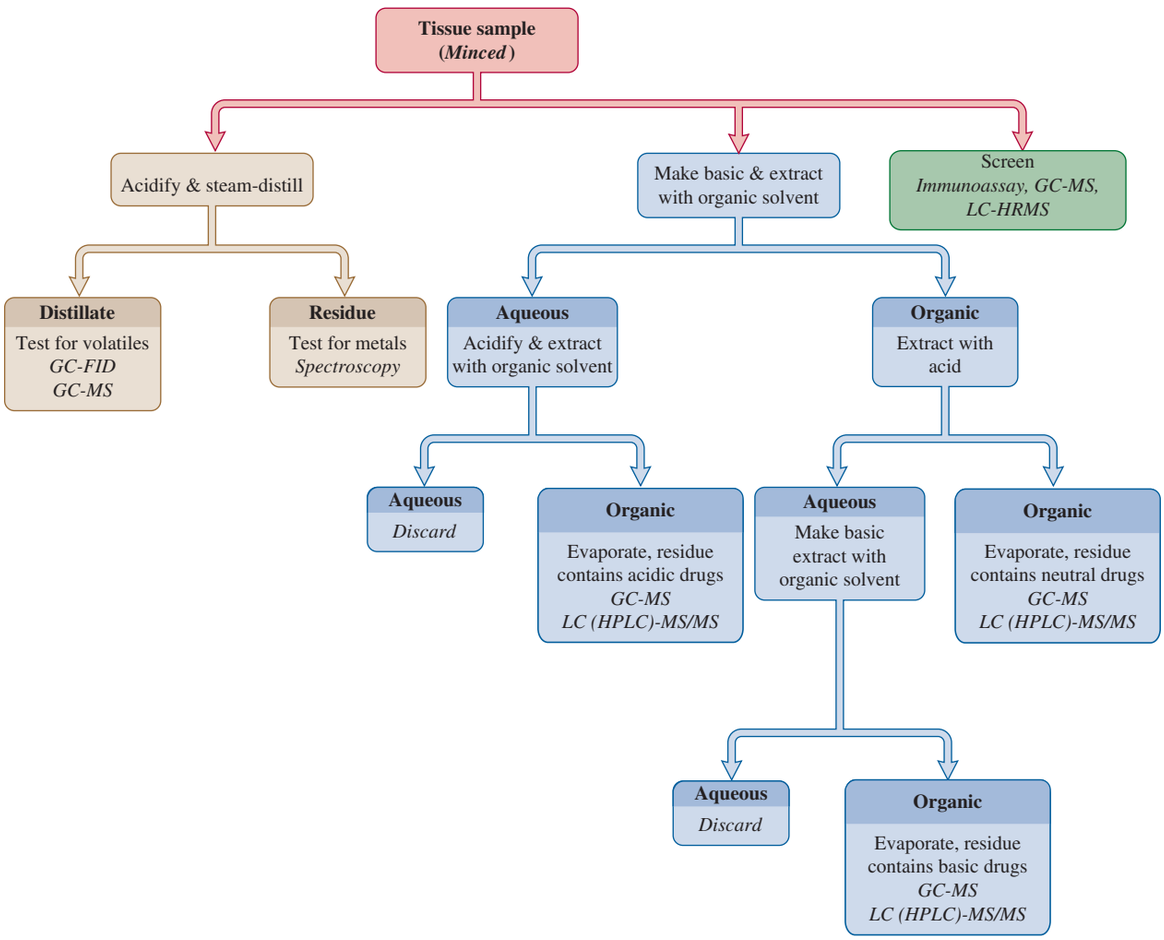

Figure 32-1. *A scheme of separation for poisons from tissues.*

example of this error involved the presence of dioxin in mixtures of the defoliants 2,4-D and 2,4,5-T (Panel on Herbicides, 1971) used during the Vietnam War as Agent Orange. Some of the adverse effects of Agent Orange are considered to be due to the low concentration of dioxin in those mixtures. Other researchers have reported that the toxicity of mixtures of polybrominated biphenyls may be due to the high toxicity of specific components, whereas other brominated biphenyls are relatively nontoxic (Mills et al., 1985).

A related application of analytical toxicology is the monitoring of dosage forms or solutions for stability throughout the course of an experimental study. Chemicals may degrade when in contact with air, by exposure to ultraviolet or other radiation, by interaction with constituents of the vehicle or dosing solution, and by other means. Developing an analytical procedure by which these changes can be recognized and corrected is essential in achieving consistent and reliable results over the course of a study (Blanke, 1987; Peters et al., 2007).

Finally, analytical methods are necessary to determine the bioavailability of a compound that is under study. Some substances with low water solubility are difficult to introduce into an animal, and a variety of vehicles may be investigated. However, a comparison of the blood concentrations for the compound under study provides a simple means of comparing the effectiveness of vehicles. Introducing a compound into the stomach in an oil vehicle may not be the most effective means of enhancing the absorption of that compound (Granger et al., 1987). Rather than observing dose–effect relationships, it may be more accurate to describe blood (or serum) concentration–effect relationships.

ROLE IN FORENSIC TOXICOLOGY

The duties of a forensic toxicologist in postmortem investigations include the qualitative and quantitative analysis of drugs or poisons in biological specimens collected at autopsy and the interpretation of the analytical findings with respect to the physiological and behavioral effects of the detected chemicals on the deceased at the time of injury and/or death.

The complete investigation of the cause or causes of sudden death is an important civic and legal responsibility. The responsibility of establishing the cause of death rests with the medical examiner or coroner, but success in arriving at the correct conclusion often depends on the combined efforts of the pathologist and the toxicologist. The cause of death in cases of poisoning cannot be proved beyond contention without toxicological analysis that confirms the presence of the toxicant in either body fluids or tissues of the deceased.

Many drugs or poisons do not produce characteristic pathological lesions; their presence in the body can be demonstrated only by chemical methods of isolation and identification. If toxicological analyses are limited, deaths resulting from poisoning may be erroneously ascribed to an entirely different cause or poisoning may be designated as the cause of death without empirical proof. Thus, limiting analysis may have significant legal and social consequences.

Additionally, a toxicologist can furnish valuable evidence concerning the circumstances surrounding a death. Such cases commonly involve demonstrating the presence of intoxicating concentrations of ethanol in victims of automotive or industrial accidents, or measurements of concentrations of carbon monoxide in fire victims. Arson may be used to conceal homicide. The degree of carbon monoxide saturation of the blood may indicate whether the deceased died as a result of the fire or was dead before the fire started. Also, licit or illicit psychoactive drugs often play a significant role in the circumstances associated with sudden or violent death. The behavioral toxicity of many illicit drugs may explain the bizarre or "risk-taking" behavior of the deceased that led to his or her demise. At times, a negative toxicological finding is of particular importance in assessing the cause of death. For example, toxicology studies may demonstrate that a person with a seizure disorder was not taking the prescribed medication and that this noncompliance contributed to the fatal event.

Additionally, the results of postmortem toxicological testing provide valuable epidemiological and statistical data. Forensic toxicologists are often among the first to alert the medical community to new epidemics of substance abuse (Poklis, 1982) and the dangers of abusing over-the-counter drugs (Garriott et al., 1985). Similarly, they often determine the chemical identity and toxicity of novel analogs of psychoactive agents that are subject to abuse, including "designer drugs" such as "china white" (methylfentanyl) (Henderson, 1988), "ecstasy" (methylenedioxymethamphetamine) (Dowling et al., 1987), and GHB (gamma-hydroxybutyric acid) (Andresen et al., 2011). Subsequently, several newer groups of designer drugs, including the synthetic cathinones and derivatives such as 4-methylmethcathinone (mephedrone) and methylone (Cawrse et al., 2012; Prosser and Nelson, 2012), 3,4-methylenedioxypyrovalerone and 5,6-methylenedioxy-2-aminoindane (MDAI) (Coppola and Mondola, 2012; Gallagher et al., 2012; Ross et al., 2012), and products containing synthetic cannabinoids, such as "K2" and "Spice" (Seely et al., 2012), among others, have emerged as new trends, particularly among young age groups (Rosenbaum et al., 2012). More recently, the increasing abuse of fentanyl, fentanyl analogs, and other synthetic opioids (Table 32-1) became a serious threat to the public health. Fentanyl is a synthetic opioid 50 to 100 times more potent than morphine, and carfentanil is a fentanyl analog estimated to be 10,000 times more potent morphine. These highly potent synthetic opioids contributed to

Table 32-1

List of Fentanyl Analogs and Other Synthetic Opioids Reported

FENTANYL ANALOGS	OTHER SYNTHETIC OPIOIDS
Acetylfentanyl	AH-7921
Acrylfentanyl	MT-45
4-ANPP	U-47700
Butyrylfentanyl	U-49900
Carfentanil	U-50488
Cyclopropylfentanyl	
ortho-Fluorofentanyl	
para-Fluorobutyryl fentanyl (FIBF)	
para-Fluorofentanyl	
Furanylfentanyl	
Isobutyrylfentanyl	
Methoxyacetylfentanyl	
4-Methoxybutyrylfentanyl	
4-Methylphenethylacetylfentanyl	
3-Methylfentanyl	
Beta-hydroxythiofentanyl	
Valerylfentanyl	

approximately 20,000 overdose deaths in 2016, a fivefold increase from 3105 deaths in 2013 (O'Donnell et al., 2017).

Today, there are numerous specialized areas of study in the field of toxicology; however, it is the forensic toxicologist who is obliged to assist in the determination of the cause of death for a court of law and who has been historically recognized by the title "toxicologist."

Until the 19th century, physicians, lawyers, and law enforcement officials harbored extremely faulty notions about the signs and symptoms of poisoning (Thorwald, 1965). Unless a poisoner was literally caught in the act of the crime, there was no way to establish whether the victim died from poisoning. In the early 18th century, a Dutch physician, Hermann Boerhoave, theorized that various poisons in a hot, vaporous condition yield characteristic odors. He placed substances suspected of containing poisons on hot coals and evaluated their smells. Although Boerhoave was not successful in applying his method, he was the first to suggest a chemical method for proving the presence of poison.

White arsenic (arsenic trioxide) has been widely used with murderous intent for over a thousand years. Therefore, it is not surprising that the first milestones in the chemical isolation and identification of a poison in body tissues and fluids were described for arsenic. In 1775, Karl Wilhelm Scheele, a Swedish chemist, discovered that white arsenic is converted to arsenous acid by chlorine water. The addition of metallic zinc reduced the arsenous acid to poisonous arsine gas. If gently heated, the evolving gas would deposit metallic arsenic on the surface of a cold vessel. In 1821, Serullas utilized the decomposition of arsine for the detection of small quantities of arsenic in stomach contents and urine in poisoning cases. In 1836, James M. Marsh, a chemist at the Royal British Arsenal in Woolwich, applied Serullas' observations in developing the first reliable method to determine the presence of an absorbed poison in body tissues and fluids such as liver, kidney, and blood. After acid digestion of the tissues, Marsh generated arsine gas, which was drawn through a heated capillary tube. The arsine decomposed, leaving a dark deposit of metallic arsenic. Quantitative measures were performed by comparing the length of the deposit from known concentrations of arsenic with those of the test specimens.

The 1800s witnessed the development of forensic toxicology as a scientific discipline. In 1814, Mathieiv J. B. Orfila (1787 to 1853), widely considered the "father of toxicology," published *Traité des Poisons*, the first systematic approach to the study of the chemical and physiological nature of poisons (Gettler, 1977). Orfila's role as an expert witness in many famous murder trials, particularly his application of the Marsh test for arsenic in the trial of the poisoner Marie Lafarge, aroused both popular and scholarly interest in the new science. As dean of the medical faculty at the University of Paris, Orfila trained numerous students in forensic toxicology.

The first successful isolation of an alkaloidal poison was performed in 1850 by Jean Servias Stas, a Belgian chemist, using a solution of acetic acid in warm ethanol to extract nicotine from the tissues of the murdered Gustave Fougnie. As modified by the German chemist Fredrick Otto, the Stas–Otto method was quickly applied to the isolation of numerous alkaloidal poisons, including colchicine, coniine, morphine, narcotine, and strychnine. In the latter half of the 19th century, European toxicologists were in the forefront of the development and application of forensic sciences, providing valuable evidence of poisoning. A number of these trials became "causes célèbres" and the testimony of forensic toxicologists captured the imagination of the public and increased awareness of the development and application of toxicology. It was thought that murderers could no longer poison with impunity.

In the United States, Rudolph A. Witthaus, professor of chemistry at Cornell University Medical School, made many contributions to toxicology and called attention to the new science by performing analyses for the city of New York in several famous morphine poisoning cases, including the murder of Helen Potts by Carlyle Harris and that of Annie Sutherland by Robert W. Buchanan. In 1911, Witthaus and Tracy C. Becker edited a four-volume work on medical jurisprudence, forensic medicine, and toxicology—the first standard forensic textbook published in the United States. In 1918, the city of New York established a medical examiner's system, and the appointment of Alexander O. Gettler as toxicologist marked the beginning of modern forensic toxicology in this country. Although Gettler made numerous contributions to the science, perhaps his greatest was the training and direction he gave to future leaders in forensic toxicology. Many of his associates went on to direct laboratories in medical examiner and coroner systems in major urban centers throughout the country.

In 1949, the American Academy of Forensic Sciences (AAFS) was established to support and further the practice of legal (forensic) medicine in the United States. The members of the Toxicology Section represented the vast majority of forensic toxicologists working in medical examiners' and coroners' offices, as well as numerous referral laboratories. Several other international, national, and local forensic science organizations, such as the Society of Forensic Toxicologists and the California Association of Toxicologists, offer a forum for the exchange of scientific data pertaining to analytical techniques and case reports involving new or infrequently used drugs and poisons. The International Association of Forensic Toxicologists (TIAFT) founded in 1963 with members from all regions of the world facilitates worldwide collaboration in resolving the technical problems confronting toxicology.

In 1975, the American Board of Forensic Toxicology (ABFT) was formed to examine and certify forensic toxicologists. One of the stated objectives of the board is "to make available to the judicial system, and other publics, a practical and equitable system for readily identifying those persons professing to be specialists in forensic toxicology who possess the requisite qualifications and competence." Those certified as Fellows of the Board must have a doctor of philosophy or science degree, have at least 3 years of full-time professional experience, and pass a written examination. Diplomates of the Board must have a bachelor's or master's degree, 3 years of full-time professional experience, and must pass a written examination.

In 1998, the ABFT began an accreditation program for forensic toxicology laboratories. ABFT-accredited laboratories must conform to standards of having qualified, experienced personnel and forensically sound procedures for the handling of evidence, analysis of specimens, and reporting of results. Laboratories must pass biannual on-site inspections, which include a review of laboratory procedures and casework.

TOXICOLOGICAL INVESTIGATION OF A POISON DEATH

The toxicological investigation of a poison death may be divided into three steps: (1) obtaining the case history and suitable specimens, (2) the toxicological analyses, and (3) the interpretation of the analytical findings.

Case History and Specimens

Today, thousands of compounds are readily available that are lethal if ingested, injected, or inhaled. Usually, a limited amount of specimen is available on which to perform analyses; therefore, it is imperative that, before the analyses are initiated, as much

information as possible concerning the facts of the case be collected. The age, sex, weight, medical history, and occupation of the decedent as well as any treatment administered before death, the gross autopsy findings, the drugs available to the decedent, and the interval between the onset of symptoms and death should be noted. In a typical year, a postmortem toxicology laboratory will perform analyses for such diverse poisons as over-the-counter medications (e.g., analgesics and antihistamines), prescription drugs (e.g., benzodiazepines and opioids), drugs of abuse (e.g., cocaine, marijuana, and methamphetamine), and gases (e.g., inhalants and carbon monoxide). Obviously, a thorough investigation of the death scene including a tentative identification of the administered poison is helpful prior to beginning the analysis (Ernst et al., 1982).

The pathologist at autopsy usually performs the collection of postmortem specimens for analysis. Specimens of many different body fluids and organs are necessary, as drugs and poisons display varying affinities for body tissues (Fig. 32-2). Therefore, detection of a poison is more likely in a tissue in which it accumulates. A large quantity of each specimen is needed for thorough toxicological analysis because a procedure that extracts and identifies one compound or class of compounds may be ineffective in extracting and identifying others (Table 32-2). Autolytic and putrefactive changes, however, may reduce specimen quality and therefore alter the selection and utility of individual specimens on a case-by-case basis. Further improvements in our knowledge regarding degradation mechanisms, as well as specimen-handling protocols to increase storage stability, may enable the forensic toxicologist to circumvent possible analytical and interpretive difficulties (Dinis-Oliveira et al., 2010).

When collecting the specimens, the pathologist labels each container with the date and time of autopsy, the name of the decedent, the identity of the sample, an appropriate case identification number, and his or her signature or initials. It is paramount that the handling of all specimens be authenticated and documented. A form developed at the collection site that identifies each specimen is submitted to the laboratory with the specimens. The form is signed and dated by the pathologist, and subsequently by any individual handling, transferring, or transporting the specimens. In legal terms, this form constitutes a "chain-of-custody" of specimens documenting all transfers. The chain-of-custody enables a toxicologist to introduce his or her results into legal proceedings, having established that the specimens analyzed were obtained from the decedent.

Fluids and tissues should be collected before embalming, as this process will dilute or chemically alter the poisons present, rendering their detection difficult or impossible. Conversely, methyl or ethyl alcohol may be a constituent of embalming fluid, thereby affecting interpretation of the analytical findings.

Although forensic toxicology laboratories typically receive blood, urine, liver tissue, and/or stomach contents for identification of xenobiotics, they have been increasingly called upon to meet the analytical challenges of many alternative types of samples (Skopp, 2004; Flanagan et al., 2005; Kronstrand and Druid, 2006; Gallardo and Queiroz, 2008). Nontraditional matrices, such as bone marrow, hair, and nails, among others, may be submitted to the laboratory. For example, on occasion, toxicological analysis is requested for cases of burned, exhumed, putrefied, and skeletal remains. In such instances, it is necessary to analyze unusual specimens such as bone marrow, hair, nails, skeletal muscle, and even insects (Inoue and Seta, 1992). Numerous drugs have been successfully identified in bone marrow and bone washings from skeletal remains even after decomposition and burial (Benko, 1985; Drummer, 2010; Cartiser et al., 2011). Similarly, the vitreous humor of the eye is isolated and sequestered from putrefaction, charring, and trauma; thus, it is a useful specimen for the detection of most drugs, anions, and even volatile poisons such as alcohols, ketones, and glycols (Coe, 1993; Bévalot et al., 2011). Hair analysis is a rapidly growing technique in forensic toxicology and has been used to measure individual exposure to heavy metals, such as arsenic, mercury, and lead, as well as many drugs of abuse and other pharmaceuticals, pesticides, and plastics (Hambidge, 1973; Yamaguichi et al., 1975; McKenzie, 1978; Baumgartner et al., 1981; Puschel et al., 1983; Suzuki et al., 1984; Marigo et al., 1986; Martz, 1988; Harkey and Henderson, 1989; Schroeder and Nason, 1989; Strang et al., 1990; Shen et al., 2002; Boumba et al., 2006). Analysis of hair for selected drugs has been utilized as an adjunct specimen in forensic settings for postmortem examinations for many years (Baumgartner et al., 1979; Couper et al., 1995; Gaillard and Pepin, 1997; Pragst et al., 1997; Rollins et al., 1997; Yegles et al., 1997; Muller et al., 2000; Villain et al., 2005; Pragst and Balikova, 2006; Kintz, 2010; Barroso et al., 2011). Nails, another keratinized matrix, have also been used to determine exposure to selected xenobiotics in both antemortem and postmortem cases (Palmeri et al., 2000; Garside, 2010). Limited data are available to support a direct

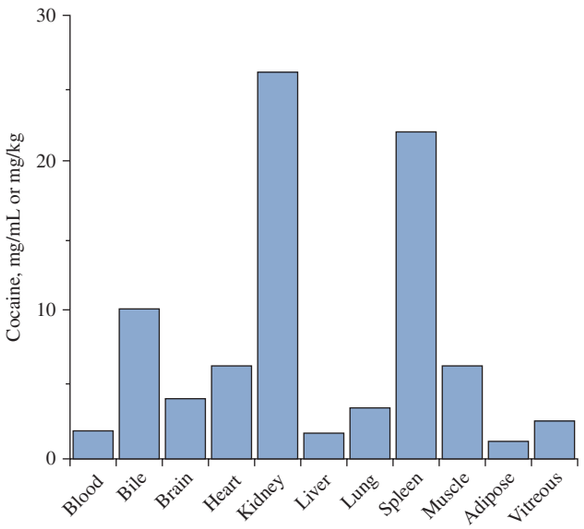

Figure 32-2. *Cocaine tissue distribution in a fatal poisoning.* (Data drawn from Poklis et al. [1985].)

Table 32-2

Suggested List of Specimens and Amounts to Be Obtained at Autopsy

SPECIMEN	QUANTITY
Brain	50 g
Liver	50 g
Kidney	50 g
Heart blood	25 mL
Peripheral blood	10 mL
Vitreous humor	All available
Bile	All available
Urine	All available
Gastric contents	All available

SOURCE: Data from SOFT/AAFS Forensic Toxicology Laboratory Guidelines (2006).

correlation between quantitative hair and nail values and drug doses in forensic cases; however, qualitative results have been accepted as indicators of previous drug or xenobiotic exposure.

Finally, in severely decomposed bodies, the absence of blood and/or the scarcity of solid tissues suitable for analysis have led to the collection and testing of maggots (fly larvae) feeding on the body (Pounder, 1991). The fundamental premise underlying maggot analysis is that if drugs or intoxicants are detected, they could only have originated from the decedent's tissues on which the larvae were feeding. Surprisingly, analysis of maggots is rather straightforward, requiring no special methodology beyond that routinely applied in toxicology laboratories. Case reports have documented the detection of numerous drugs and intoxicants in maggots collected from decomposed bodies. The compounds detected include barbiturates, benzodiazepines, phenothiazines, morphine, and malathion. Controlled studies in which maggots were allowed to feed on tissues to which drugs had been added have demonstrated the accumulation of propoxyphene, amitriptyline and nordiazepam, among others, in the larvae (Goff et al., 1993; Gagliano-Candela and Aventaggiato, 2001; Pien et al., 2004).

Toxicological Analysis

Before the analysis begins, several factors must be considered, including the amount of specimen available, the nature of the poison sought, and the possible biotransformation of the poison. In cases involving oral administration of the poison, the gastrointestinal (GI) contents are analyzed first because large amounts of residual unabsorbed poison may be present. The urine may be analyzed next, as the kidney is the major organ of excretion for most poisons and high concentrations of toxicants and/or their metabolites often are present in urine. After absorption from the GI tract, drugs or poisons are carried to the liver before entering the general systemic circulation; therefore, the first analysis of an internal organ is conducted on the liver. If a specific poison is suspected to have caused or contributed to a death, the toxicologist may first analyze the tissues and fluids in which the poison concentrates.

A thorough knowledge of drug biotransformation is often essential before an analysis is performed. The parent compound and any major pharmacologically active metabolites should be isolated and identified. In some instances, the metabolites provide the only evidence that a drug or poison has been administered. Many screening tests, such as immunoassays, are specifically designed to detect not the parent drug but its major urinary metabolite. An example of the relationship of pharmacokinetic and analytical factors is provided by cocaine. The major metabolites of cocaine biotransformation are benzoylecgonine and ecgonine methyl ester (Fig. 32-3). The co-ingestion of alcohol with cocaine results in the hepatic transesterification of cocaine to form cocaethylene (Hime et al., 1991), a psychoactive metabolite. The disposition of these compounds in various body fluids and tissues (including hair) is shown in Fig. 32-4. Thus,

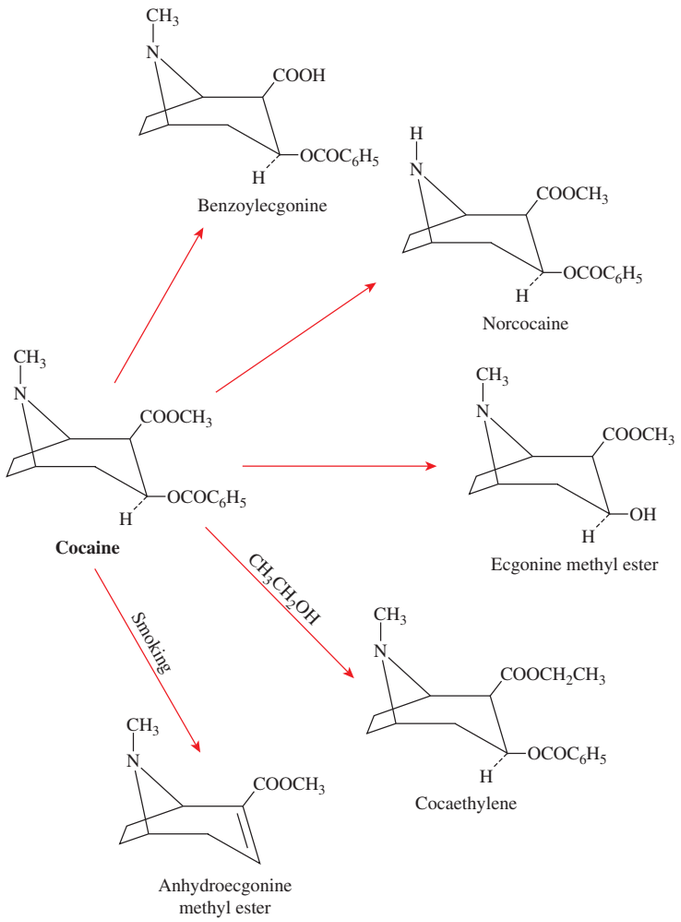

Figure 32-3. *Biotransformation and pyrolysis products of cocaine.*

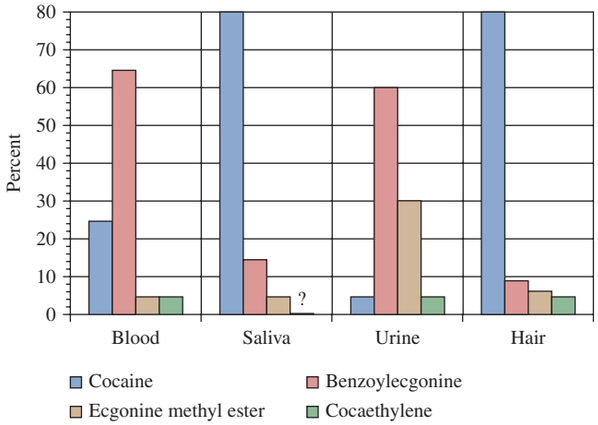

Figure 32-4. *Disposition of cocaine and cocaine metabolites in human fluids and hair.* (Data redrawn from Spiehler [1994].)

the initial testing of urine to determine cocaine use is performed with immunoassays specifically designed to detect the presence of benzoylecgonine, the major urinary metabolite. In contrast, if oral fluid (saliva) or hair is tested, parent cocaine, as well as metabolites, is also sought. To determine a profile of each analyte present in a specimen, chromatographic procedures such as gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) or tandem MS are commonly used, which facilitates the simultaneous separation and quantification of each compound.

Besides GC- and LC-MS targeted analysis, clinical and forensic toxicologists increasingly utilize high-resolution MS technologies including time-of-flight (TOF) and Orbitrap mass analyzers. This analytical advance offers retrospective data interrogation and versatile screening methods where new analytes can be rapidly included in the test panel (Wu et al., 2012; Kronstrand et al., 2014). The more recent generation of MS technologies interfaces quadrupole mass analyzer with TOF allowing higher resolution and greater mass accuracy for simultaneous screening and quantitative analyses of over a hundred drugs and metabolites (Bidny et al., 2017). Such analytical development is imperative to address the growing complexity and transience of the recreational drug market as the popularity of synthetic psychoactive substances known as designer drugs or novel/new psychoactive substances (NPS) has exponentially grown in the past decade (Pasin et al., 2017).

The analysis may be complicated by the normal chemical changes that occur during the decomposition of a cadaver. The autopsy and toxicological analysis should be started as soon after death as possible. The natural enzymatic and nonenzymatic processes of decomposition and microbial metabolism may destroy a poison that was present at death or produce substances or compounds with chemical and physical properties similar to those of commonly encountered poisons. As early as the 1870s, the so-called cadaveric alkaloids isolated from the organs of putrefied bodies were known to produce color test reactions similar to those produced by morphine and other drugs. These cadaveric alkaloids resulted from the bacterial decarboxylation of the amino acids ornithine and lysine, producing putrescine and cadaverine, respectively (Evans, 1963). Similarly, during decomposition, phenylalanine is converted to phenylethylamine, which has chemical and physical properties very similar to those of amphetamine. The hydrolysis, oxidation, or reduction of proteins, nucleic acids, and lipids may generate numerous compounds, such as hydroxylated aliphatic and aromatic carboxylic acids, pyridine and piperidine derivatives, and aromatic heterocyclics such as tryptamine and norharmane (Kaempe, 1969). All these substances may interfere with the isolation, identification, and quantitation of the toxicants being sought. The concentration of cyanide and ethyl alcohol and the carbon monoxide saturation of the blood may be decreased or increased, depending on the degree of putrefaction and microbial activity. However, many poisons—such as arsenic, barbiturates, mercury, and strychnine—are extremely stable and may be detectable many years after death.

Before analysis, the purity of all chemicals used in laboratory procedures should be established. The purity of the primary reference material used to prepare calibrators and controls should be verified, and the salt form or degree of hydration should be determined (Blanke, 1989). All reagents and solvents should be of the highest grade possible and should be free of contaminants that may interfere with or distort analytical findings. For example, the chloroform contaminants phosgene and ethyl chloroformate may react with primary or secondary amine drugs to form carbamyl chloride and ethyl carbamate derivatives (Cone et al., 1982). Specimen containers, lids, and stoppers should be free of contaminants such as plasticizers, which often interfere with chromatographic or GC/LC-MS determinations. Care should be exercised to ensure a clean laboratory environment. This is of particular concern in the analysis of metals, as aluminum, arsenic, lead, and mercury are ubiquitous environmental and reagent contaminants.

Forensic toxicology laboratories analyze specimens by using a variety of analytical procedures. Initially, nonspecific tests designed to determine the presence or absence of a class or group of analytes may be performed directly on the specimens. Examples of tests used to rapidly screen urine are the FPN (ferric chloride, perchloric, and nitric acid) color test for phenothiazine drugs and immunoassays for the detection of amphetamines, benzodiazepines, and opiate derivatives, among others. Positive results obtained with these tests must be confirmed by a second analytical procedure that identifies the particular drug. The detection limit of the confirmatory test should be lower than that of the initial nonspecific test. Some analytical procedures identify specific compounds. Even in such instances, a second test should be performed to identify and confirm the presence of the analyte. The second test should be based on a chemical or physical principle different from that of the first test. Such additional testing is performed to establish an unequivocal identification of the drugs or poisons present. Whenever possible, the most specific test for the compound of interest should be performed. Today, GC-MS and LC-MS are the most widely applied methodology in toxicology and are generally accepted as unequivocal identification for all drugs. Analyte identification is typically based on the retention time in the chromatographic system coupled with the characteristic ion fragmentation spectrum in the mass spectrometer. The analyte mass spectrum is the pattern of mass-to-charge ion fragments and their relative abundance.

Analytical methods must be of sufficient rigor to provide accurate and reliable qualitative and quantitative data. Numerous approaches for the use of quality control have been suggested, and professional organizations have developed recommendations for the implementation of quality control in forensic laboratories (Goldberger et al., 1997; Ferrara et al., 1998; Scientific Working Group for Forensic Toxicology, 2013). The limit of detection, the lowest concentration of analyte reliably identified by the assay, and the specificity of all qualitative methods should be well documented. The laboratory must demonstrate that the assay response of blank or negative calibrators (controls) does not overlap with the response of the lowest-positive calibrator. In certain instances, qualitative identification of a poison or drug is sufficient to resolve forensic

toxicology issues. However, most cases require reliable estimates of poison concentrations for forensic interpretation. For quantitative analysis, the accuracy, precision, linearity, and specificity of the procedure must also be established. Linearity should be determined by using multiple drug-fortified calibrators whose concentrations bracket the anticipated concentrations in the biological specimen. Precision, which statistically demonstrates the variance in the value obtained, is determined by replicate analyses of a specimen of a known concentration. Additional assay parameters, such as analyte stability and recovery from the biological matrix, for example, can also be determined. For a variety of reasons, a quantitative result occasionally will deviate spuriously from the true value. Therefore, replicate quantitative determinations are highly recommended when sufficient specimen volume is available (Blanke, 1987).

When unusual samples such as bone marrow, fingernails, hair, and maggots are analyzed, the extraction efficiency of a procedure may vary greatly, depending on the complexity of the matrix. Therefore, all calibrators and controls should be prepared in the same matrix type as the specimens and analyzed concurrently with the specimens. Often the matrix is "unique" or impossible to match, such as decomposed or embalmed tissue. In these instances, the method of "standard additions" may be used. Known amounts of the analyte of interest are added to specimen aliquots and these are analyzed. The concentration of poison in the test specimen is determined by comparing the proportional response of the "poison fortified" specimens to that of the test specimens.

Interpretation of Analytical Results

Once the analysis of the specimens is complete, the toxicologist must interpret his or her findings with regard to the physiological or behavioral effects of the toxicants on the decedent at the concentrations found. Specific questions may be answered, such as the route of administration, the dose administered, and whether the concentration of the toxicant present was sufficient to cause death or alter the decedent's actions enough to cause his or her death. Assessing the physiological or behavioral meanings of analytical results is often the most challenging aspect confronted by the forensic toxicologist.

In determining the route of administration, the toxicologist notes the results of the analysis of the various specimens. As a general rule, the highest concentrations of a poison are found at the site of administration. Therefore, the presence of large amounts of drugs and/or poisons in the GI tract and liver indicates oral ingestion, while higher concentrations in the lungs than in other visceral organs can indicate inhalation or intravenous injection. The ratio or relative distribution of drugs in different tissues may also differentiate oral from parenteral administration (Fig. 32-5). Drugs may also be detected in the tissue surrounding an injection site following intramuscular or intravenous injection. Smoking is a popular route of administration for abusers of controlled substances (illicit) such as cocaine, heroin, marijuana, and phencyclidine. Pyrolysis of these drugs leads to the inhalation not only of the parent drug, but also of characteristic breakdown products of combustion. For example, a major pyrolysis product of "crack" cocaine smoking is anhydroecgonine methyl ester (Martin et al., 1989) (Fig. 32-3). Thus, identification of relatively high concentrations of this compound along with cocaine or cocaine metabolites in urine or other body fluids or tissues may indicate smoking as the route of cocaine administration (Jacob et al., 1990).

The presence of a toxic material in the GI tract, regardless of the quantity, does not provide sufficient evidence to establish that agent as the cause of death. It is necessary to demonstrate that

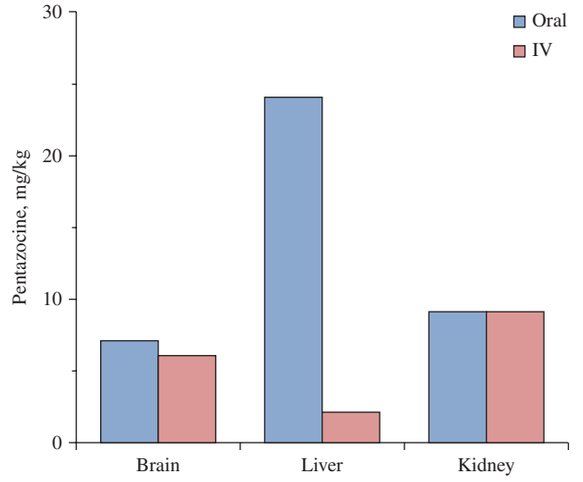

Figure 32-5. *Comparison of pentazocine distribution in fatal poisonings due to intravenous injection and oral administration.* (Data from Baselt [1982]; Poklis and MacKell [1982].)

absorption of the toxicant has occurred and that it has been transported by the general circulation to the target organ in order to exert its lethal effect. This is established by blood and tissue analyses. An exception to the rule is provided by strong, corrosive chemicals such as sulfuric acid, lye, and phenol, which exert their deleterious effects by directly digesting tissue, causing hemorrhage and shock. The results of urinalysis are often of little benefit in determining the physiological effects of a toxic agent. Urine results establish only that the poison was present in the body at some time before death. Correlation of urine values with physiological effects is poor because of various factors that influence the rate of excretion of specific compounds and the urine volume.

The physiological effects of most drugs and poisons are generally correlated with their concentrations in blood or blood fractions such as plasma and serum. Indeed, in living persons, this association is the basis of therapeutic drug monitoring. However, postmortem blood has been described as a fluid resembling blood that is obtained from the vasculature after death. Therefore, interpretation of postmortem blood results requires careful consideration of the case history, the site of collection, and postmortem changes. The survival time between the administration of a poison and death may be sufficiently long to permit biotransformation and excretion of the agent. Blood values may appear to be nontoxic or consistent with therapeutic administration. Death from hepatic failure after an acetaminophen overdose usually occurs at least 3 to 4 days after ingestion. Postmortem acetaminophen concentrations in blood may be consistent with the ingestion of therapeutic doses. Therefore, fatal acetaminophen overdose is determined by case history, central lobular necrosis of the liver, and, if available, analysis of serum specimens collected from the decedent when he or she was admitted to the emergency department (Price et al., 1991). Furthermore, emergency medical treatment—such as the administration of fluids, plasma extenders, bicarbonate, diuretics, and blood transfusions—may dilute, remove, or enhance the elimination of toxic agents. Similarly, prolonged survival on a mechanical respirator, hemodialysis, or hemoperfusion may substantially reduce initially lethal blood concentrations of poisons.

For a long time, it was generally assumed that postmortem blood drug concentrations were more or less uniform throughout the

body. However, in the 1970s, several investigators noted that postmortem concentrations of digoxin in heart blood greatly exceeded those in simultaneously collected femoral blood. They also observed that postmortem blood concentrations, particularly in heart blood, exceeded the expected values at the time of death (Vorpahl and Coe, 1978; Aderjan et al., 1979). This postmortem increase in blood digoxin concentrations was apparently due to release of the drug from tissue stores, particularly the myocardium. Subsequently, other researchers demonstrated that for many drugs, blood concentrations in the same body vary greatly depending on the site from which the specimen is collected—subclavian vein, thoracic aorta, inferior vena cava, femoral vein, and so forth. For example, in a case of fatal multiple drug ingestion, analysis of postmortem blood collected from 10 different sites demonstrated imipramine concentrations that differed by as much as 760% (2.1 to 16.0 mg/L) (Jones and Pounder, 1987). In an extensive investigation, Prouty and Anderson (1990) demonstrated that postmortem blood drug concentrations were not only site-dependent but also increased greatly over the interval between death and specimen collection, particularly in heart blood. This increase over the postmortem interval was most pronounced for basic drugs with large apparent volumes of distribution, such as tricyclic antidepressants.

In an overt drug overdose, postmortem blood concentrations are elevated sufficiently to render an unmistakable interpretation of fatal intoxication. However, in many cases, the postmortem redistribution of drugs may significantly affect the interpretation of analytical findings. For drugs whose volume of distribution, plasma half-life, and renal clearance vary widely from person to person or that undergo postmortem redistribution, tissue concentrations readily distinguish therapeutic administration from drug overdose (Apple, 1989). Therefore, to provide expert opinions with a sound scientific foundation in regard to the role of a drug in the death of an individual, it is recommended that, in addition to heart blood, a peripheral blood specimen and tissues be analyzed.

The analysis of tissue specimens is important for the estimation of a "minimal administered dose" or body burden of a drug or poison. In order to calculate a minimum body burden, it is necessary to analyze as many different body tissues and fluids as possible to determine the concentrations of the drug present. The concentration of drug in each separate specimen is then multiplied by the total weight or volume of that particular tissue or fluid. In this manner, the total amount of drug in each different tissue or fluid is determined. The amounts of drug in each separate tissue and fluid are then added together to give the total body burden or minimal administered dose. This simple approach has often proven extremely effective in resolving legal medical issues. For example, lidocaine is commonly administered in 50- to 100-mg bolus injections as an antiarrhythmic agent for ventricular arrhythmia during resuscitation efforts. Because of poor circulation and tissue perfusion during arrhythmias, lidocaine is not well distributed in the body of the victim of a fatal heart attack. Postmortem bloods often exceed 50 mg/L in such cases, whereas values for effective antiarrhythmic prophylaxis do not exceed 5 mg/L. Therefore, a blood lidocaine value of 50 mg/L may be an artifact of resuscitation efforts or might represent a fatal overdose. Tissue distribution studies have resolved this issue in both accidental and homicidal poisoning with lidocaine (Poklis et al., 1984).

Postmortem toxicology results are often used to corroborate investigative findings. Improved methods for determining drug exposure, with respect to longer timeframes, are advantageous because drug concentrations in plasma, urine, and liver often reflect only the dosage taken within (at most) the last several days prior to sampling. Compared to traditional biological matrices, keratinized tissues such as hair and nails provide a longer window of detection for drugs and other compounds due to their slow growth and possible permanent retention of drugs. The presence of certain xenobiotics and their metabolites has been demonstrated in human hair or nails at times when plasma and urine drug concentrations are not measurable (Rollins et al., 1997; Garside, 2010), which can provide evidence of exposure to these agents weeks, months, or even years prior to death. It has been suggested that for some xenobiotics, hair also serves to characterize the drug exposure over time. For example, the analysis of sequential sections of hair provides a reliable correlation with the pattern of arsenic exposure (Smith, 1964). Significant increases in the arsenic content of the root and the first 5 mm of the hair occur within hours after the ingestion of arsenic. The germinal cells are in relatively close equilibrium with circulating arsenic; thus, as arsenic concentrations in blood rise or fall, so does arsenic deposition in growing hair. Normal arsenic content in hair varies with nutritional, environmental, and physiological factors; however, the maximum upper limit of normal deposition with a 99% confidence limit in persons not exposed to arsenic is 5 mg/kg (Shapiro, 1967). Hair grows at a rate of approximately 1.06 cm per month (LeBeau et al., 2011). Therefore, analysis of 1.0-cm segments provides a monthly pattern of exposure (Fig. 32-6). Such analyses can be performed in cases of homicidal poisoning to demonstrate that increases in arsenic deposition in the victim's hair correlate with times when a poisoner had an opportunity to administer the poison. Continuously elevated hair arsenic values indicate chronic rather than acute poisoning as the cause of death. A similar approach using segmental analysis of hair has been used to determine patterns of exposure for various drugs and their metabolites; however, care must be taken in order to account for the potential effects of haircuts, chemical treatments, and other environmental insults that may have occurred to the hair during the timeframe of interest.

An extension of forensic toxicology is the analysis of impurities of illicit drug synthesis in biological specimens. Many drugs of abuse, particularly methamphetamine, are illicitly manufactured

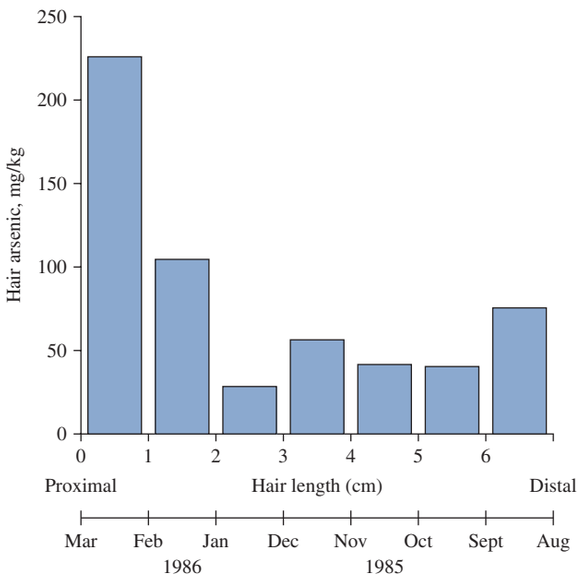

Figure 32-6. *Results of neutron activation analysis for arsenic in sequential sections of hair, demonstrating chronic arsenic poisoning.* Increased exposure in the first two sections is consistent with fatal events. Lower values in section three are consistent with 2 weeks of hospitalization. (Data from Poklis and Saady [1990].)

in clandestine laboratories. There are several popular methods of methamphetamine synthesis; when these are applied in clandestine laboratories, side reactions or incomplete conversion of the reactants yield an impure mixture of methamphetamine and synthetic impurities. These impurities can be characteristic of a particular synthetic method, and their detection in biological specimens can indicate the use of an illicitly produced drug that is not a legal pharmaceutical product, suggest the synthetic method that was used to produce the drug, point to a possible common source of illicit production, and provide a link between manufacturers, dealers, and users. An example of impurity analysis was the detection of metabolites of α-benzyl-*N*-methylphenethylamine (BNMPA) in the urine of abusers of methamphetamine (Moore et al., 1996b) and in a case of fatal drug overdose involving methamphetamine (Moore et al., 1996a). BNMPA is an impurity arising from the synthesis of methamphetamine via the Leukart reaction using phenyl-2-propanone (P2P) synthesized from phenylacetic acid. Clandestine laboratories often must synthesize the P2P starting material, as its sale and distribution is regulated by the U.S. Drug Enforcement Administration.

As discussed above, a wide range of tissues are available for the purpose of identifying xenobiotics in postmortem specimens. In contrast, for many years, traditional specimens used for the detection and quantification of substances in antemortem biological specimens have been limited to whole blood, plasma, serum, and urine, largely due to their ease of collection and accessibility in the living. More recently, the advent of improved analytical techniques with greater sensitivity and selectivity, including GC-MS/MS and LC-MS/MS, have expanded the array of biological specimens in which substances can be detected. These specimens include oral fluid (saliva), sweat, meconium, amniotic fluid, breast milk, and semen among others. In oral fluid, for example, the pharmacokinetics of many drugs and metabolites is closely aligned to that of blood pharmacokinetics, and thus can serve as an alternative matrix for illicit and therapeutic drug detection (Bosker and Huestis, 2009; Spiehler and Cooper, 2010), as well as detection of exposure to environmental toxicants and pesticides (Lanaro et al., 2011; Yang et al., 2011).

CRIMINAL POISONING OF THE LIVING

Over the past few decades, forensic toxicologists have become more involved in the analysis of specimens obtained from living victims of criminal poisonings. Generally, this increase in testing is a result of two types of cases: (1) administration of drugs to incapacitate victims of kidnapping, robbery, or sexual assault and (2) poisoning as a form of child abuse.

For centuries, those severely intoxicated from alcohol often became victims of kidnapping, robbery, or sexual assault. The kidnapping of drunks in seaports was a common way of obtaining sailors for long commercial voyages such as those involved in whaling. Late in the 19th century, the mixing of the powerful hypnotic chloral hydrate with alcohol produced the legendary "Mickey Finn." While alcohol is still often a primary factor in cases of alleged sexual assault, common drugs of abuse or other psychoactive drugs are often involved (Table 32-3). Of particular concern are the many potent inductive agents medically administered prior to general anesthesia. Many of these drugs, such as benzodiazepines and phenothiazines, are available today through illicit sources or legal purchase in foreign countries. When administered surreptitiously, they cause sedation and incapacitate the victim while also producing amnesia in the victim as to the events while drugged, without causing severe central nervous system depression. These cases often present a difficult analytical challenge to the toxicologist. Usually, the victim does not bring forth an allegation of assault until 24 hours to several days after the attack. Thus, the intoxicating drug may have been largely eliminated or extensively metabolized such that extremely low concentrations of drug or metabolites are present in the victim's blood, urine, and/or hair specimens. Sophisticated, highly sensitive analytical methods such as GC-MS or LC-MS may be required to accurately identify drugs in such specimens. To provide guidance in the choice of analytical approaches to such cases, recommendations for the toxicological investigation of drug-facilitated crimes including sexual assaults have been formulated (LeBeau et al., 1999; Society of Forensic Toxicologists).

Poisoning as a form of child abuse involves the deliberate administration of toxic or injurious substances to a child, usually by a parent or other caregiver. The victims of such poisonings range in age from a few months to the teens. Common agents used to intentionally poison children have included syrup of ipecac, table salt, laxatives, diuretics, antidepressants, sedative-hypnotics, and narcotics (Yin, 2010; American Association of Poison Control Centers, 2011; Oral et al., 2011). The motivation for such heinous behavior is in the province of psychiatry, not toxicology. However, toxicologists must have some understanding of the nature of these poisonings to aid in the investigation of such cases. In some instances, it is a form of child battering committed by persons with low tolerance to the child's upsetting behaviors. The poison may be given to an infant to stop its crying or be force-fed to older children as a form of punishment. Such behavior is characteristic of persons with a cultural background where violence toward children is common or who have severe personality disorders or other mental illnesses. "Munchausen syndrome by proxy" is another form of child poisoning (Murray, 1997). The term *Munchausen syndrome* (MS) is used to describe patients who seek admission to hospitals with apparent illness along with plausible, dramatic, albeit fictitious medical histories. Such people seek medical treatment solely to assume the role of a patient and receive the attention derived from this deception. "MS by proxy" refers to the situation in which an individual, usually a parent, presents not himself or herself but a child with

Table 32-3

Distribution of Drugs of Abuse Encountered Urine Specimens in 1179 Cases of Alleged Sexual Assault*

RANK	DRUG/DRUG GROUP	INCIDENCE
1	No drugs found	468
2	Ethanol	451
3	Cannabinoids	218
4	Benzoylecgonine (cocaine metabolite)	97
5	Benzodiazepines	97
6	Amphetamines	51
7	Gamma-hydroxybutyrate (GHB)	48
8	Opiates	25
9	Propoxyphene	17
10	Barbiturates	12

**Thirty-five percent of the drug-positive specimens were positive for more than one drug.*

SOURCE: Data from ElSohly and Salamone (1999).

a fictitious illness often induced by physical or chemical means. The purpose of the poisoning is not to kill the child but to induce signs and symptoms of illness that will assure medical attention. Thereby, the parent as caregiver gains the craved attention. Given a fictitious case history and the obvious illness of the child, these cases are almost always and understandably misdiagnosed. Often, the child may be chronically poisoned at home and in the hospital for as long as a year before suspicion leads to the collection of specimens for extensive toxicological testing. Although the parent may not have intended such an outcome, some children have died from fatal poisoning in these situations. As in the case of sexual assault, sophisticated MS testing methods may be required to detect such chemicals as emetine and cephaeline, the emetic alkaloids in syrup of ipecac. Testing in these cases is best performed in a laboratory with forensic experience, as positive drug findings will usually result in some form of legal proceeding.

FORENSIC DRUG TESTING

Concerns regarding the potentially adverse consequences of substance abuse for the individual, the workplace, and society have led to widespread urine analysis for controlled or illicit drugs (Gust and Walsh, 1989). Currently, such testing is conducted routinely by the military services, regulated transportation and nuclear industries, many federal and state agencies, public utilities, federal and state criminal justice systems, and numerous private businesses and industries. Significant ethical and legal ramifications are associated with such testing. Those having positive test results may not receive employment, be dismissed from a job, be court-martialed, or suffer a damaged reputation.

To assure the integrity of workplace urine testing, two certification programs currently accredit forensic urine-testing laboratories. Laboratories conducting testing of federal employees are required to be certified under the Department of Health and Human Services (HHS) Mandatory Guidelines for Workplace Drug Testing as published in the April 11, 1988, *Federal Register*, which has been periodically revised; the latest revision was published in the January 23, 2017, *Federal Register* where HHS allowed collection of an alternative specimen such as oral fluid (Department of Health and Human Services, 1988, 2017). The College of American Pathologists (CAP) also conducts a certification program for urine drug testing laboratories. The federal program regulates the process from specimen collection through testing to the reporting of results, whereas the CAP program allows flexibility in the construction of programs servicing a broad range of clients. Both programs involve proficiency testing and periodic on-site inspection of laboratories.

Forensic drug testing differs from other areas of forensic toxicology in which urine or an alternative specimen is the only specimen analyzed and testing is performed for a limited number of drugs and metabolites. While urine remains as the most prevalent biological matrix for forensic drug testing, the popularity of oral fluid as an alternative matrix is increasing because the collection is more convenient and less invasive (Tsanaclis et al., 2012). Under the federal certification program, analyses are performed for a limited number of classes or drugs of abuse (Table 32-4). While HHS-certified laboratories may typically analyze hundreds to thousands of urine specimens daily, only a relatively small number of those specimens are positive for drugs. To handle this large workload, initial testing is performed by immunoassays on rapid, high-throughput chemistry analyzers. A confirmation analysis in HHS-certified laboratories is performed by GC-MS and LC-MS/MS.

Proper forensic drug testing is a challenge to good laboratory management. As with all forensic activities, every aspect of the laboratory operation must be thoroughly documented—specimen collection, chain of custody, quality control procedures, method validation, testing, qualifications of personnel, and the reporting of results. The laboratory facility must be constructed and operated to assure total security of specimens and documents. Confidentiality of all testing results is paramount; only specifically authorized persons should receive the results. The presence of a controlled or illicit drug in a single random urine specimen is generally accepted as proof of recent or past substance abuse. However, positive urine drug findings are only evidence that, at some time before the collection of the sample, the individual was administered the drug, self-administered it, or was exposed to it. Positive urine tests do not prove impairment from drug abuse or addiction.

Forensic drug test results are reported only as positive or negative for the drugs sought. Cutoff values are established for both

Table 32-4

Forensic Urine Drug Testing Analytes and Cutoff Concentrations

	CONCENTRATION (ng/mL)	
ANALYTE	INITIAL TEST	CONFIRMATORY TEST
Marijuana metabolites	50	
Δ-9-tetrahydrocannabinol-9-carboxylic acid	–	15
Cocaine metabolite	150	
Benzoylecgonine	–	100
Opiates	2000	
Morphine	–	2000
Codeine	–	2000
Hydrocodone/hydromorphone	300	
Hydrocodone	–	100
Hydromorphone	–	100
Oxycodone/oxymorphone	100	
Oxycodone	–	100
Oxymorphone	–	100
6-Acetylmorphine (heroin metabolite)	10	10
Phencyclidine	25	25
Amphetamines	500	
Amphetamine	–	250
Methamphetamine	–	250
MDMA/MDA	500	
MDMA	–	250
MDA	–	250

SOURCE: Department of Health and Human Services: Mandatory guidelines for federal workplace drug testing programs. Fed Reg. 53(69), 11983, April 11, 1988; revised: Fed Reg. 82(13), 7920, October 1, 2017. https://www.federalregister.gov/documents/2017/01/23/2017-00979/mandatory-guidelines-for-federal-workplace-drug-testing-programs.

the initial and confirmation assays (Table 32-4). The cutoff value is a concentration at or above which the assay is administratively considered positive. Below the cutoff value, the assay is reported to be negative for that drug or drug class. Obviously, drugs may be present below the cutoff concentration. However, the use of cutoff values allows uniformity in the drug testing and reporting of results. All test reports indicate the drug tested and its cutoff value. The testing laboratories must be thoroughly familiar with all regulatory and analytical issues related to urine testing and devise strategies to resolve uncertainties.

Many individuals who are subject to regulated urine testing have devised techniques to mask their drug use either by physiological means such as the ingestion of diuretics or by attempting to adulterate the specimen directly with bleach, vinegar, or other products that interfere with the initial immunoassay tests (Warren, 1989; George and Braithwaite, 1996; Ferslew et al., 2003; Paul, 2004; Burrows et al., 2005). Thus, specimens are routinely tested for adulteration by checking urinary pH, creatinine, and specific gravity, nitrates, chromates, and noting any unusual color or smell. A mini-industry has developed to sell various products that are alleged to "beat the drug test." These products contain chemicals that, when added to a urine specimen, interfere with either the initial or confirmatory drug test (Dasgupta, 2007; Jaffee et al., 2007; Larson et al., 2008). For example, several of these products contain glutaraldehyde, which will react with the nitrogen atoms of the antibody proteins of the immunoassay screening test, thereby cross-linking the antibodies and inactivating the assay. However, this disruption of the test is so complete that the immunoassay analyzer records almost no signal, thus indicating possible adulteration of the specimen. Another adulterant, for the marijuana metabolite urine test, contains sodium nitrite. In acidic urine, the nitrite salt is converted to nitrous acid that then converts the marijuana metabolites to nitroso derivatives, rendering them undetectable by routine GC-MS analysis (Lewis et al., 1999). Often, the pH of the urine is insufficiently acidic for complete nitroso conversion of the tetrahydrocannabinol (THC) metabolite, THC-carboxylic acid. Thus, the urine will screen positive by the initial immunoassay. When urine is acidified to extract the THC acid metabolite for MS confirmation testing, the metabolite is completely oxidized and undetectable. However, the deuterated THC–carboxylic acid added to the sample as the internal standard of the confirmation test is also completely destroyed. Failure to detect the internal standard readily alerts the analyst that an oxidant adulterant had been added to the urine. In such cases, a quantitative test for nitrite is performed. Nitrite may be present in urine from numerous internal and external sources such as foods, drugs, pathological conditions, and infection from nitrate-reducing microorganisms. However, none of these sources produces urinary nitrite concentrations that even begin to approach those obtained by the addition of adulterant amounts of potassium nitrite equal to 1000 mg/L (Urey et al., 1998). Most chemical adulterants can be detected in urine by specific colorimetric tests that can be readily adapted to high-volume auto-analyzers (Table 32-5). Thus, the laboratories now routinely test not only for drugs of abuse but also for a wide variety of chemical adulterants. In most instances, a positive test result for adulteration has a consequence similar to a positive drug test.

There may be valid reasons other than substance abuse for positive drug findings, such as therapeutic use of controlled substances, inadvertent intake of drugs via food, and passive inhalation. For example, the seed of *Papaver somniferum*, poppy seed, is a common ingredient in many pastries and breads. Depending on their botanical source, poppy seeds may contain significant amounts of morphine. Several studies have demonstrated that the ingestion of certain poppy-seed foods results in the urinary excretion of readily detectable concentrations of morphine (ElSohly and Jones, 1989). Morphine is a major urinary metabolite of heroin. Therefore, to readily differentiate heroin abuse from poppy-seed ingestion, analysis may be performed for 6-acetylmorphine, a unique heroin metabolite (Fehn and Megges, 1985).

Even over-the-counter medications may present potential problems for laboratories conducting urine drug testing. Methamphetamine may occur as a racemic mixture of D- and L-optical isomers. D-Methamphetamine, a Schedule II controlled substance, is a potent central nervous system stimulant subject to illicit drug abuse, while L-methamphetamine (L-desoxyephedrine) is an alpha-adrenergic stimulant available in over-the-counter Vicks® inhalers as a nasal decongestant. Cross-reactivity of L-desoxyephedrine with the initial immunoassay screening test may occur after excessive use of the Vicks inhaler (Poklis and Moore, 1995). Additionally, the most popular conformational GC-MS products for amphetamines are achiral. Therefore, if such analyses are performed, a "false-positive" result for D-methamphetamine may be reported. This dilemma is easily resolved if confirmation testing is done with a chiral GC-MS procedure, which can readily resolve the stereoisomers of methamphetamine (Fitzgerald et al., 1988).

HUMAN PERFORMANCE TESTING

Forensic toxicology activities also include the determination of the presence of ethanol and other drugs and chemicals in blood, breath, or other specimens and the evaluation of their role in modifying human performance and behavior. The most common application of human performance testing is to determine impairment while driving under the influence of ethanol (DUI) or drugs (DUID). Although operation of a motor vehicle is a common experience to most people, few appreciate the complexity of mental and physical functioning involved. A driver must simultaneously coordinate fine motor skills in tracking the road course and applying pressure to accelerator or brake—with visual attention immediately in front, to the horizon, and to the periphery of the vehicle—while continuously judging distance, speed, and appropriateness of response to signals, traffic, and unexpected events. The threshold blood alcohol concentration (BAC) for diminished driving performance of these complex functions in many individuals is as low as 0.04 g/dL, the equivalent of ingestion of two beers within an hour's time to

Table 32-5

Urine Adulterants Detected by Chemical Test

ADULTERANT	DETECTION METHOD
Acids, baking soda	pH
Bleach	Smell, color test
Detergents, diuretics, salt	Specific gravity
Diuretics	Creatinine, specific gravity
Glutaraldehyde	Color test, gas chromatography
Nitrite	Color test
Pyridinium chlorochromate	Color test, atomic absorption spectrophotometry (chromate), gas chromatography (pyridine)

an average size person. The statutory definition of DUI in all 50 states is a BAC of 0.08 g/dL; the state of Utah recently passed the law that lowers the legal threshold to 0.05 g/dL, which will take effect in December 2018 (Utah Department of Public Safety, n.d.). These concentrations are consistent with diminished performance of complex driving skills in the vast majority of individuals. Over the past half century, an enormous amount of data has been developed correlating blood ethanol concentrations with intellectual and physiological impairment, particularly of the skills associated with the proper operation of motor vehicles. Numerous studies have demonstrated a direct relationship between an increased BAC in drivers and an increased risk of involvement in vehicular accidents (Council on Scientific Affairs, 1986). For example, alcohol-impaired driving fatalities accounted for 28% of all motor vehicle traffic fatalities in the United States in 2016 (National Highway Traffic Safety Administration, 2017).

During the past decade, there has been growing concern about the deleterious effects of drugs other than ethanol on driving performance. Several studies have demonstrated a relatively high occurrence of drugs in impaired or fatally injured drivers (White et al., 1981; Mason and McBay, 1984; Van der Linden et al., 2013). These studies tend to report that the highest drug-use accident rates are associated with the use of such illicit or controlled drugs as cocaine, benzodiazepines, marijuana, and phencyclidine. However, most studies test for only a few drugs or drug classes, and the repeated reporting of the same drugs may be a function of limited testing. Before "driving under the influence of drugs" testing is as readily accepted by the courts as ethanol testing, many legal and scientific problems concerning drug concentrations and driving impairment must be resolved (Consensus Report, 1985). The ability of analytical methodology to routinely quantify minute concentrations of drug in blood must be established. Also, drug-induced driving impairment at specific blood concentrations in controlled tests and/or actual highway experience must be demonstrated.

COURTROOM TESTIMONY

The forensic toxicologist often is called upon to testify in legal proceedings. As a general rule of evidence, a witness may testify only to facts known to him or her. The witness may offer opinions solely on the basis of what he or she has observed (Moenssens et al., 1973). Such a witness is called a "lay witness." In contrast, the toxicologist is referred to as an "expert witness." A court recognizes a witness as an expert if that witness possesses knowledge or experience in a subject that is beyond the range of ordinary or common knowledge or observation. An expert witness may provide two types of testimony: objective testimony and "opinion." Objective testimony by a toxicologist usually involves a description of his or her analytical methods and findings. When a toxicologist testifies as to the interpretation of his or her analytical results or those of others, that toxicologist is offering an "opinion." Lay witnesses cannot offer such opinion testimony, as it exceeds their ordinary experience.

Before a court permits opinion testimony, the witness must be "qualified" as an expert in his or her particular field. In qualifying someone as an expert witness, the court considers the witness's education, on-the-job training, work experience, teaching or academic appointments, and professional memberships and publications as well as the acceptance of the witness as an expert by other courts. Qualification of a witness takes place in front of the jury members, who consider the expert's qualifications in determining how much weight to give to his or her opinions during their deliberations.

Whether a toxicologist appears in criminal or civil court, workers' compensation, or parole hearings, the procedure for testifying is the same: direct examination, cross-examination, and redirect examination. The attorney who has summoned the witness to testify conducts direct examination. Testimony is presented in a question-and-answer format. The witness is asked a series of questions that allow him or her to present all facts or opinions relevant to the successful presentation of the attorney's case. During direct examination, an expert witness has the opportunity to explain to the jury the scientific bases of his or her opinions. Regardless of which side has called the toxicologist to court, the toxicologist should testify with scientific objectivity. Bias toward his or her client and prejudgments should be avoided. An expert witness is called to provide informed assistance to the jury. The jury, not the expert witness, determines the guilt or innocence of the defendant.

After direct testimony, the opposing attorney questions the expert. During this cross-examination, the witness is challenged as to his or her findings and/or opinions. The toxicologist will be asked to defend his or her analytical methods, results, and opinions. The opposing attorney may imply that the expert's testimony is biased because of financial compensation, association with an agency involved in the litigation, or personal feelings regarding the case. The best way to prepare for such challenges before testimony is to anticipate the questions the opposing attorney may ask.

After cross-examination, the attorney who called the witness may ask additional questions to clarify any issues raised during cross-examination. This allows the expert to explain apparent discrepancies in his or her testimony raised by the opposing attorney. Often, an expert witness is asked to answer a special type of question, the "hypothetical question." A hypothetical question contains only facts that have been presented in evidence. The expert is then asked for his or her conclusion or opinion based solely on this hypothetical situation. This type of question serves as a means by which appropriate facts leading to the expert's opinion are identified. These questions are extremely long and convoluted. The witness should be sure he or she understands all the facts and implications in the question. Like all questions, this type should be answered as objectively as possible.

ROLE IN CLINICAL TOXICOLOGY

Analytical toxicology in a clinical setting plays a role very similar to its role in forensic toxicology. As an aid in the diagnosis and treatment of toxic incidents, as well as in monitoring the effectiveness of treatment regimens, it is useful to clearly identify the nature of the toxic exposure and measure the amount of the toxic substance that has been absorbed. Frequently, this information, together with the clinical state of the patient, permits a clinician to relate the signs and symptoms observed to the anticipated effects of the toxic agent. This may permit a clinical judgment as to whether the treatment must be vigorous and aggressive or whether simple observation and symptomatic treatment of the patient are sufficient.

A cardinal rule in the treatment of poisoning cases is to remove any unabsorbed material, limit the absorption of additional poison, and hasten its elimination. The clinical toxicology laboratory serves an additional purpose in this phase of the treatment by monitoring the amount of the chemical remaining in circulation or measuring what is excreted. In addition, the laboratory can provide the data needed to permit estimations of the total dosage or the effectiveness of treatment by changes in known pharmacokinetic parameters of the drug or other chemical ingested.

Although the instrumentation and the methodology used in a clinical toxicology laboratory are similar to those utilized by a forensic toxicologist, a major difference between these two applications is responsiveness. In emergency toxicology testing, results

must be communicated to the clinician within hours to be meaningful for therapy. A forensic toxicologist may carefully choose the best method for a particular test and conduct replicate procedures to assure maximum accuracy. A clinical laboratory cannot afford this luxury and may sacrifice accuracy for a rapid turnaround time. Additionally, because it is impossible to predict when toxicological emergencies will occur, a clinical laboratory must provide rapid testing 24 hours a day, every day of the year.

Primary examples of the usefulness of emergency toxicology testing are the rapid quantitative determination of acetaminophen, salicylate, alcohols, and glycol serum concentrations in instances of suspected overdose. Acetaminophen serum values related to the time after ingestion (see Chap. 33) not only indicate an overdose, but provide a prognosis for possible delayed hepatotoxicity and the need to continue administration of *N*-acetylcysteine antidote. In addition, continuous monitoring of serum values permits an accurate pharmacokinetic calculation of the ingested dose (Melethil et al., 1981). Similarly, salicylate serum values related to the time after ingestion may indicate an overdose, providing a prognosis for possible delayed severe metabolic acidosis and the need for lifesaving dialysis treatment. Continuous monitoring of serum salicylate values permits an accurate assessment of the efficacy of dialysis.

Ethanol is the most common chemical encountered in emergency toxicology. Although relatively few fatal intoxications occur with ethanol alone, serum values are important in the assessment of behavioral, physiologic, and neurological function, particularly in trauma cases where the patient is unable to communicate and surgery with the administration of anesthetic or analgesic drugs is indicated. Intoxications from accidental or deliberate ingestion of other alcohols or glycols—such as methanol from windshield deicer or paint thinner, isopropanol from rubbing alcohol, and ethylene glycol from antifreeze—are often encountered in emergency departments. Following ingestion of methanol or ethylene glycol, patients often present with similar neurological symptoms and severe metabolic acidosis due to the formation of toxic aldehyde and acid metabolites. A rapid quantitative serum determination for these intoxicants will indicate the severity of intoxication and the possible need for dialysis therapy. Alcohol infusion, in order to saturate the enzyme alcohol dehydrogenase, blocks the conversion of methanol and ethylene glycol to their toxic metabolites. Continuous monitoring of serum values not only permits an assessment of the clearance of the intoxicant by dialysis, but also assures a proper infusion rate of alcohol for effective antidotal concentrations (Fig. 32-7). To provide effective service to the emergency department, laboratories should have available chromatographic methods for the rapid separation and detection of alcohols and glycols (Edinboro et al., 1993).

The utilization of the analytical capabilities of a clinical toxicology laboratory has increased enormously in recent years. Typically, the laboratory performs testing not only for the emergency department but also for a wide variety of other medical departments, as drugs and toxic chemicals may be a consideration in diagnosis. Urine is analyzed from substance abuse treatment facilities to monitor the administration of methadone or other therapeutic agents and/or to assure that patients do not continue to abuse drugs. Similarly, psychiatrists, neurologists, and physicians treating patients for chronic pain need to know whether patients are self-administering drugs before such patients undergo psychiatric or neurological examinations. Analysis for drugs of abuse in meconium and urine obtained from neonates is used to corroborate the diagnosis of withdrawal symptoms in newborns and document fetal exposure to controlled substances. Toxic metal determinations, such as blood lead concentration, are often performed to assess possible toxic metal exposure or severity of toxicity. Analysis of heavy metals in 24-hour urine specimens is often used to rule out toxic metal exposure as a cause of symmetrical peripheral neuropathy prior to the diagnosis of neurological disorders such as Guillian–Barré syndrome. The clinical toxicology laboratory may often perform unique diagnostic tests that require sophisticated analytical capabilities such as GC-MS and LC-MS analysis for organic acids and amino acids to detect inborn errors of metabolism in infants and children. Methods for the analysis of abnormal organic acids will also detect acidic drugs and other intoxicants such as salicylates, ethylene glycol, GHB, and valproic acid. Another such diagnostic test requiring sensitive chromatography is the timed metabolism of lidocaine to its monoethylglycinexylidide (MEGX) metabolite (O'Neal and Poklis, 1996). The rate of this conversion is a sensitive indicator of hepatic dysfunction and is often used to assess hepatic viability in donor livers prior to transplantation. MEGX formation may also be useful in monitoring the severity of the histological condition of patients with chronic hepatitis and cirrhosis (Shiffman et al., 1994).

ROLE IN THERAPEUTIC MONITORING

Historically, the administration of drugs for long-term therapy was based largely on experience. A dosage amount was selected and administered at appropriate intervals based on what the clinician had learned was generally tolerated by most patients. If the drug seemed ineffective, the dose was increased; if toxicity developed, the dose was decreased or the frequency of dosing was altered. At times, a different dosage form might be substituted. Establishing an effective dosage regimen was particularly difficult in children and the elderly.

The factors responsible for individual variability in responses to drug therapy include the rate and extent of drug absorption, distribution, and binding in body tissues and fluids, rate of metabolism and excretion, pathological conditions, and interaction with other drugs (Blaschke et al., 1985). Monitoring of the plasma or serum concentration at regular intervals will detect deviations from the average serum concentration, which, in turn, may suggest that one or more of these variables need to be identified and corrected.

With multiple administrations of a given drug at regular intervals, plasma drug concentrations will gradually increase and eventually reach a plateau over the course of therapy. The plateau is referred to as a steady-state condition, whereas the amount of drug absorbed is in equilibrium to the amount of drug eliminated. Dosage regimes are calculated so that plasma drug concentrations during steady-state conditions are within the therapeutic range, and monitoring such conditions assures that an effective concentration

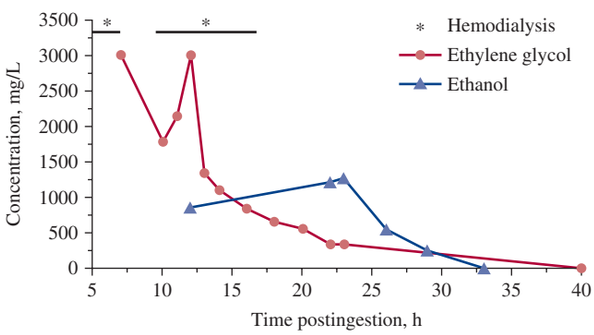

Figure 32-7. *Serum ethylene glycol and ethanol concentrations monitored during dialysis and ethanol infusion therapy.* (Data from the Toxicology Laboratory, Medical College of Virginia Hospital, Richmond, VA.)

Table 32-6

Drugs Commonly Indicated for Therapeutic Monitoring

Antiarrhythmics
Digoxin
Digitoxin
Lidocaine
Procainamide and *N*-acetylprocainamide
Quinidine
Antibiotics
Amikacin
Chloramphenicol
Gentamicin
Tobramycin
Vancomycin
Anticancer
Methotrexate
Anticonvulsants
Carbamazepine
Gabapentin
Lamotrigine
Levetiracetam
Phenobarbital
Phenytoin
Primidone
Topiramate
Valproic acid
Zonisamide
Antidepressants
Amitriptyline and nortriptyline
Doxepin and nordoxepin
Fluoxetine and norfluoxetine
Impramine and desipramine
Paroxetine
Sertraline and desmethylsertraline
Antipsychotics
Clozapine
Pimozide
Bronchodilators
Caffeine
Theophylline
Immunosuppressants
Azathioprine
Cyclosporine
Mycophenolic acid
Sirolimus
Tacrolimus
Mood stabilizing
Lithium

is present. For drugs that have a defined correlation between serum concentrations and undesired toxic effects, the lowest serum concentration immediately prior to dosing (trough) and the highest expected serum concentration (peak) are monitored to assure efficacy and minimize toxicity.

Because the drug being administered is known, qualitative characterization of the analyte generally is not required. Quantitative accuracy is required, however. Frequently, the methodology applied is important, particularly in regard to its selectivity. For example, methods that measure the parent drug and its metabolites are not ideal unless the individual analytes can be quantified separately. Depending on the drug, metabolites may or may not be active to a different degree than the parent drug. The cardiac antiarrhythmic drug procainamide is acetylated during metabolism to form *N*-acetylprocainamide (NAPA). This metabolite has antiarrhythmic activity of almost equal potency to that of the parent drug procainamide. There is bimodal genetic variation in the activity of the *N*-acetyltransferase for procainamide, so that, in "fast acetylators," the concentration of NAPA in the serum may exceed that of the parent drug. For optimal patient management, information should be available about the concentrations of both procainamide and NAPA in serum (Bigger and Hoffman, 1985).

Because absolute characterization of the analyte is not necessary for many drugs, immunoassay procedures are commonly used. This is particularly true of drugs with extremely low serum concentrations, such as cardiac glycosides, and drugs that are difficult to extract because of a high degree of polarity, such as the aminoglycoside antibiotics. In these cases, serum can be conveniently assayed directly by using commercially available kits for immunoassays.

The chromatographic methods in which an appropriate internal standard is added are favored when more than one analyte is to be quantified or if metabolites with structures similar to those of the parent drugs must be distinguished. Because the nature of drugs is varied, many different analytical techniques may be applied, including atomic absorption spectrophotometry for measuring lithium used to treat manic disorders. Virtually all the tools of the analyst may be used for specific applications of analytical toxicology. Drugs that are commonly monitored during therapy are presented in Table 32-6.

SUMMARY

The analytical techniques employed by forensic toxicologists have continued to expand in complexity and improve in reliability and sensitivity. Many new analytical tools have been applied to toxicological problems in almost all areas of the field, and the technology continues to open new areas of research. Forensic toxicologists continue to be concerned about conducting unequivocal identification of toxic substances in such a manner that the results can withstand a legal challenge. The issues of substance abuse, designer drugs, increased potency of therapeutic agents, and widespread concern about pollution, and the safety and health of workers present challenges to the analyst's knowledge, skills, and abilities. As these challenges are met, analytical toxicologists will continue to play a substantial role in the expansion of the discipline of toxicology.

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REFERENCES

Aderjan R, Bahr H, Schmidt G. Investigation of cardiac glycoside levels in human postmortem blood and tissues determined by a special RIA procedure. *Arch Toxicol.* 1979;42:107–114.

American Association of Poison Control Centers. Drugs used in child maltreatment. *Prescrire Int.* 2011;20(120):236–237.

Andresen H, Aydin BE, Mueller A, Iwersen-Bergmann S. An overview of gamma-hydroxybutyric acid: pharmacodynamics, pharmacokinetics,

toxic effects, addiction, analytical method, and interpretation of results. *Drug Test Anal*. 2011;3(9):660–668.
Apple FS. Postmortem tricyclic antidepressant concentrations: assessing cause of death using parent drug to metabolite ratio. *J Anal Toxicol*. 1989;13:197–198.
Barroso M, Gallardo E, Vieira DN, López-Rivadulla M, Queiroz JA. Hair: a complementary source of bioanalytical information in forensic toxicology. *Bioanalysis*. 2011;3(1):67–79.
Baselt RC. *Disposition of Toxic Drugs and Chemicals in Man*. 2nd ed. Davis, CA: Biomedical Publications; 1982:603–606.
Baumgartner A, Jones P, Baumgartner W. Detection of phencyclidine in hair. *J Forensic Sci*. 1981;26:576–581.
Baumgartner W, Jones P, Baumgartner W, Black C. Radioimmunoassay of hair for determining opiate-abuse histories. *J Nucl Med*. 1979;20:748–752.
Benko A. Toxicological analysis of amobarbital and glutethimide from bone tissue. *J Forensic Sci*. 1985;30:708–714.
Bévalot F, Gustin MP, Cartiser N, Le Meur C, Malicier D, Fanton L. Interpretation of drug concentrations in an alternative matrix: the case of meprobamate in vitreous humor. *Int J Legal Med*. 2011;25(3):463–468.
Bidny S, Gago K, Chung P, Albertyn D, Pasin D. Simultaneous screening and quantification of basic, neutral and acidic drugs in blood using UPLC-QTOF-MS. *J Anal Toxicol*. 2017;41:181–195.
Bigger JT Jr, Hoffman BF. Antiarrhythmic drugs. In: Gilman AG, Goodman LS, Rall TW, Murad F, eds. *The Pharmacological Basis of Therapeutics*. 7th ed. New York: Macmillan; 1985:763.
Blanke RV. Quality assurance in drug-use testing. *Clin Chem*. 1987;33:41B–45B.
Blanke RV. *Validation of the Purity of Standards*. Irving, TX: Abbott Laboratories, Diagnostic Division; 1989.
Blaschke TF, Nies AS, Mamelock RD. Principles of therapeutics. In: Gilman AG, Goodman LS, Rall TW, Murad F, eds. *The Pharmacological Basis of Therapeutics*. 7th ed. New York: Macmillan; 1985:52.
Bosker WM, Huestis MA. Oral fluid testing for drugs of abuse. *Clin Chem*. 2009;55(11):1910–1931.
Boumba VA, Ziavrou KS, Vougiouklakis T. Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants. *Int J Toxicol*. 2006;25(3):143–163.
Burrows DL, Nicolaides A, Rice PJ, Dufforc M, Johnson DA, Ferslew KE. Papain: a novel urine adulterant. *J Anal Toxicol*. 2005;29(5):275–295.
Cartiser N, Bévalot F, Fanton L, Gaillard Y, Guitton J. State-of-the-art of bone marrow analysis in forensic toxicology: a review. *Int J Legal Med*. 2011;125(2):181–198.
Cawrse BM, Levine B, Jufer RA, et al. Distribution of methylone in four postmortem cases. *J Anal Toxicol*. 2012;36(6):434–439.
Chapuis E. *Elements de Toxicologie*, 1873, cited in Peterson F, Haines WS, Webster RW. *Legal Medicine and Toxicology*. 2nd ed. Vol 2. Philadelphia, PA: WB Saunders; 1923.
Coe JI. Postmortem chemistry update: emphasis on forensic applications. *Am J Forensic Med Pathol*. 1993;14:91–117.
Cone EJ, Buchwald WF, Darwin WD. Analytical controls in drug metabolism studies: 11. Artifact formation during chloroform extraction of drugs and metabolites with amine substitutes. *Drug Metab Dispos*. 1982;10: 561–567.
Consensus Report. Drug concentrations and driving impairment. *JAMA*. 1985;254:2618–2621.
Coppola M, Mondola R. Synthetic cathinones: chemistry, pharmacology and toxicology of a new class of designer drugs of abuse marketed as "bath salts" or "plant food". *Toxicol Lett*. 2012;211(2):144–149.
Council on Scientific Affairs. Alcohol and the driver. *JAMA*. 1986;255:522–527.
Couper FJ, McIntyre IM, Drummer OH. Detection of antidepressant and antipsychotic drugs in postmortem human scalp hair. *J Forensic Sci*. 1995;40(1):87–90.
Cravey RH, Baselt RC. The science of forensic toxicology. In: Cravey RH, Baselt RC, eds. *Introduction to Forensic Toxicology*. Davis, CA: Biomedical Publications; 1981:3–6.
Dasgupta A. The effects of adulterants and selected ingested compounds on drugs-of-abuse testing in urine. *Am J Clin Pathol*. 2007;128(3):491–503.
Department of Health and Human Services, SAMHSA. Mandatory guidelines for federal workplace drug testing: final guidelines—notice. *Fed Reg*. 1988;53(69):11970–11989.
Department of Health and Human Services, SAMHSA. Mandatory guidelines for federal workplace drug testing programs. *Fed Reg*. 2017;82(13):7920–7970.
Dinis-Oliveira RJ, Carvalho F, Duarte JA, et al. Collection of biological samples in forensic toxicology. *Toxicol Mech Methods*. 2010;20(7):363–414.
Dowling GP, McDonough ET, Bost RO. "Eve" and "ecstasy": a report of five deaths associated with the use of MDEA and MDMA. *JAMA*. 1987;257:1615–1617.
Drummer O. Drugs in bone and bone marrow. In: Jenkins A, ed. *Drug Testing in Alternate Biological Specimens*. Totowa, NJ: Humana Press; 2010:131–136:chap 8.
Edinboro LE, Nanco CR, Soghoian DM, Poklis A. Determination of ethylene glycol in serum utilizing direct injection on a wide-bore capillary column. *Ther Drug Monit*. 1993;15:220–223.
ElSohly MA, Jones AB. Morphine and codeine in biological fluids: approaches to source differentiation. *Forensic Sci Rev*. 1989;1:13–22.
ElSohly MA, Salamone SJ. Prevalence of drugs used in cases of alleged sexual assault. *J Anal Toxicol*. 1999;23(3):141–146.
Ernst MF, Poklis A, Gantner GE. Evaluation of medicolegal investigators' suspicious and positive toxicology findings in 100 drug deaths. *J Forensic Sci*. 1982;27:61–65.
Evans WED. *The Chemistry of Death*. Springfield, IL: Charles C Thomas; 1963.
Fehn J, Megges G. Detection of O6-monoacetylmorphine in urine samples by GC-MS as evidence for heroin use. *J Anal Toxicol*. 1985;9:134–138.
Ferrara DS, Tedeschi L, Frison G, Brusini G. Quality control in toxicological analysis. *J Chromatogr B Biomed Sci Appl*. 1998;713(1):227–243.
Ferslew KE, Nicolaides AN, Robert TA. Determination of chromate adulteration of human urine by automated colorimetric and capillary ion electrophoretic analyses. *J Anal Toxicol*. 2003;27(1):36–39.
Fisher RS, Hine CH, Stetler CJ (Committee on Medicolegal Problems). *Alcohol and the Impaired Driver: A Manual on the Medicolegal Aspects of Chemical Tests for Intoxication*. Chicago, IL: American Medical Association; 1968.
Fitzgerald RL, Ramos JM, Bogema SC, Poklis A. Resolution of methamphetamine stereoisomers in urine drug testing: urinary excretion of R(-)-methamphetamine following use of nasal inhalers. *J Anal Toxicol*. 1988;12:255–259.
Flanagan RJ, Connally G, Evans JM. Analytical toxicology: guidelines for sample collection postmortem. *Toxicol Rev*. 2005;24(1):63–71.
Gagliano-Candela R, Aventaggiato L. The detection of toxic substances in entomological specimens. *Int J Legal Med*. 2001;114(405):19–203.
Gaillard Y, Pepin G. Screening and identification of drugs in human hair by high-performance liquid chromatography-photodiode-array UV detection and mass gas chromatography-mass spectrometry after solid phase extraction. A powerful tool in forensic medicine. *J Chromatogr*. 1997;762:251–267.
Gallagher CT, Assi S, Stair JL, et al. 5,6-Methylenedioxy-2-aminoindane: from laboratory curiosity to "legal high". *Hum Psychopharmacol*. 2012;27(2):106–112.
Gallardo E, Queiroz JA. The role of alternative specimens in toxicological analysis. *Biomed Chromatogr*. 2008;22(8):795–821.
Garriott JS, Simmons LM, Poklis A, Mackell MS. Five cases of fatal overdose from caffeine-containing "look-alike" drugs. *J Anal Toxicol*. 1985;9:141–143.
Garside D. Drugs-of-abuse in nails. In: Jenkins A, ed. *Drug Testing in Alternate Biological Specimens*. Totowa, NJ: Humana Press; 2010:43–63:chap 3.
George S, Braithwaite RA. The effect of glutaraldehyde adulteration of urine specimens on syva EMIT II drugs-of-abuse assays. *J Anal Toxicol*. 1996;20(3):195–196.
Gettler AD. Poisoning and toxicology, forensic aspects: Part 1: historical aspects. *Inform*. 1977;9:3–7.
Goff ML, Brown WA, Omori AI, LaPointe DA. Preliminary observations of the effects of amitriptyline in decomposing tissue on the development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and implications of this effect to estimation of postmortem interval. *J Forensic Sci*. 1993;38:316–322.
Goldberger BA, Huestis MA, Wilkins DG. Commonly practiced quality control and quality assurance procedures for gas chromatography mass spectrometry analysis in forensic urine drug-testing laboratories. *Forensic Sci Rev*. 1997;9(2):59–80.

Granger RH, Condie LW, Borzelleca JF. Effect of vehicle on the relative up-take of haloalkanes administered by gavage. *Toxicologist*. 1987;40:1.

Gust SW, Walsh JM. *Drugs in the Workplace: Research and Evaluation Data*. NIDA Research Monograph 91. Washington, DC: US Government Printing Office; 1989.

Hambidge KM. Increase in hair copper concentration with increasing distance from the scalp. *Am J Clin Nutr*. 1973;26:1212–1215.

Harkey MR, Henderson GL. Hair analysis for drugs of abuse. *Adv Anal Toxicol*. 1989;2:298–329.

Hawks RL, Chiang CN. Examples of specific drug assays. In: Hawks RL, Chiang CN, eds. *Urine Testing for Drugs of Abuse*. NIDA Research Monograph 73. Rockville, MD: US Department Health and Human Services, PHS, ADAMHA; 1986:93.

Henderson GL. Designer drugs: past history and future prospects. *J Forensic Sci*. 1988;33:569–575.

Hime GW, Hearn WL, Rose S, Cofino J. Analysis of cocaine and cocaethylene in blood and tissues by GC-NPD and GC-Ion Trap mass spectrometry. *J Anal Toxicol*. 1991;15:241–245.

Inoue T, Seta S. Analysis of drugs in unconventional samples. *Forensic Sci Rev*. 1992;4:89–107.

Jacob P, Lewis ER, Elias-Baker BA, Jones RT. A pyrolysis product, anhydroecgonine methyl ester (methylecgonidine), is in the urine of cocaine smokers. *J Anal Toxicol*. 1990;14:353–357.

Jaffee WB, Trucco E, Levy S, Weiss RD. Is this urine really negative? A systematic review of tampering methods in urine drug screening and testing. *J Subst Abuse Treat*. 2007;33(1):33–42.

Jones GR, Pounder DJ. Site-dependence of drug concentrations in postmortem blood—a case study. *J Anal Toxicol*. 1987;11:186–190.

Kaempe B. Interfering compounds and artifacts in the identification of drugs in autopsy material. In: Stolman A, ed. *Progress in Chemical Toxicology*. Vol 4. New York: Academic Press; 1969:1–57.

Kintz P. Drug testing in hair. In: Jenkins A, ed. *Drug Testing in Alternate Biological Specimens*. Totowa, NJ: Humana Press; 2010:67–79:chap 4.

Kronstrand R, Brinkhagen L, Birath-Karlsson C, Roman M, Josefsson M. LC-QTOF-MS as a superior strategy to immunoassay for the comprehensive analysis of synthetic cannabinoids in urine. *Anal Bioanal Chem*. 2014;406:3599–3609.

Kronstrand R, Druid H. Hair in postmortem toxicology. In: Kintz P, ed. *Analytical and Practical Aspects of Drug Testing in Hair [International Science and Forensic Investigation]*. Boca Raton, FL: CRC Press; 2006:223–240:chap 10.

Lanaro R, Costa JL, Fernandes LC, Resende RR, Tavares MF. Detection of paraquat in oral fluid, plasma, and urine by capillary electrophoresis for diagnosis of acute poisoning. *J Anal Toxicol*. 2011;35(5):274–279.

Larson SJ, Holler JM, Magluilo J Jr, Dunkley CS, Jacobs A. Papain adulteration in 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid-positive urine samples. *J Anal Toxicol*. 2008;32(6):438–443.

LeBeau M, Andollo W, Hearn WL, et al. Recommendations for toxicological investigation of drug-facilitated sexual assaults. *J Forensic Sci*. 1999;44:227–230.

LeBeau M, Montgomery MA, Brewer JD. The role of variations in growth rate and sample collection on interpreting results of segmental analyses of hair. *Forensic Sci Int*. 2011;210(1–3):110–116.

Lewis SA, Lewis LA, Tuinman A. Potassium nitrite reaction with 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid in urine in relation to drug screening analysis. *J Forensic Sci*. 1999;44:951–955.

Marigo M, Tagliaro F, Poiesi C, Lafisca S, Neri C. Determination of morphine in the hair of heroin addicts by high performance liquid chromatography with fluorometric detection. *J Anal Toxicol*. 1986;10:158–161.

Martin BR, Lue LP, Boni JP. Pyrolysis and volatilization of cocaine. *J Anal Toxicol*. 1989;13:158–162.

Martz R. The identification of cocaine in hair by GC-MS and MS/MS. *Crime Lab Dig*. 1988;15:67–73.

Mason AP, McBay AJ. Ethanol, marijuana, and other drug use in 600 drivers killed in single-vehicle crashes in North Carolina. *J Forensic Sci*. 1984;29:987–1026.

McKenzie JM. Alteration of zinc and copper concentration of hair. *Am J Clin Nutr*. 1978;31:470–476.

Melethil S, Poklis A, Schwartz HS. Estimation of the amount of drug absorbed in acetaminophen poisoning: a case report. *Vet Hum Toxicol*. 1981;23:421–423.

Mills RA, Millis CD, Dannan GA, et al. Studies on the structure-activity relationships for the metabolism of polybrominated biphenyls by rat liver microsomes. *Toxicol Appl Pharmacol*. 1985;78:96–104.

Moenssens AA, Moses RE, Inbau FE. *Scientific Evidence in Criminal Cases*. Mineola, NY: Foundation Press; 1973.

Moore KA, Daniel J, Fierro M, et al. Detection of a metabolite of α-benzyl-*N*-methylphenylamine synthesis in a mixed drug fatality involving methamphetamine. *J Forensic Sci*. 1996a;41:524–526.

Moore KA, Ismaiel A, Poklis A. α-Benzyl-*N*-methylphenethylamine (BNMPA) an impurity of illicit methamphetamine synthesis: III. Detection of BNMPA and metabolites in urine from methamphetamine users. *J Anal Toxicol*. 1996b;20:89–92.

Muller C, Vogt S, Goerke R, Kordon A, Weinmann W. Identification of selected psychopharmaceuticals and their metabolites in hair by LC/ESI-CID/MS and LC/MS/MS. *Forensic Sci Int*. 2000;113(1–3):415–421.

Murray JB. Munchausen syndrome/Munchausen syndrome by proxy. *J Psychol*. 1997;131:343–352.

National Highway Traffic Safety Administration, Traffic safety facts—2016 data: alcohol-impaired driving, DOT HS 812 450, October 2017.

O'Donnell JK, Halpin J., Mattson CL, Goldberger BA, Gladden RM. Deaths involving fentanyl, fentanyl analogs, and U-47700—10 States, July-December 2016. *MMWR Morb Mortal Wkly Rep*. 2017;66:1197–1202.

O'Neal CL, Poklis A. A sensitive HPLC assay for the simultaneous quantitation of lidocaine and its metabolites; monoethylglycinexylididie and glycinexylidide in serum. *Clin Chem*. 1996;42:330–331.

Oral R, Bayman L, Assad A, et al. Illicit drug exposure in patients evaluated for alleged child abuse and neglect. *Pediatr Emerg Care*. 2011;27(6):490–495.

Palmeri A, Pichini S, Pacifici R, Zuccaro P, Lopez A. Drugs in nails: physiology, pharmacokinetics and forensic toxicology. *Clin Pharmacokinet*. 2000;38(2):95–110.

Panel on Herbicides. Report on 2,4,5-T. In: *A Report of the Panel on Herbicides of the President's Science Advisory Committee*. Executive Office of the President, Office of Science and Technology. Washington, DC: US Government Printing Office; 1971.

Pasin D, Cawley A, Bidny S, Fu S. Current applications of high-resolution mass spectrometry for the analysis of new psychoactive substances: a critical review. *Anal Bioanal Chem*. 2017; Epub ahead of print.

Paul BD. Six spectroscopic methods for detection of oxidants in urine: implication in differentiation of normal and adulterated urine. *J Anal Toxicol*. 2004;28(7):599–608.

Peters FT, Drummer OH, Musshoff F. Validation of new methods. *Forensic Sci Int*. 2007;165(2-3):216–224.

Petersen F, Haines WS, Webster RW. *Legal Medicine and Toxicology*. Vol 2. 2nd ed. Philadelphia, PA: WB Saunders; 1923.

Pien K, Laloup M, Pipeleers-Marichal M, et al. Toxicological data and growth characteristics of single post-feeding larvae and puparia of *Calliphora vicina* (Diptera: Calliphoridae) obtained from a controlled nordiazepam study. *Int J Legal Med*. 2004;118(4):190–193.

Poklis A. Pentazocine/tripelennamine (T's and blues) abuse: a five year survey of St. Louis, Missouri. *Drug Alcohol Depend*. 1982;10:257–267.

Poklis A, MacKell MA. Toxicological findings in deaths due to pentazocine: a report of two cases. *Forensic Sci Int*. 1982;20:89–95.

Poklis A, Mackell MA, Graham M. Disposition of cocaine in a fatal poisoning in man. *J Anal Toxicol*. 1985;9:227–229.

Poklis A, Mackell MA, Tucker EF. Tissue distribution of lidocaine after fatal accidental injection. *J Forensic Sci*. 1984;29:1229–1236.

Poklis A, Moore KA. Response of EMIT amphetamine immunoassays to urinary desoxyephedrine following Vicks inhaler use. *Ther Drug Monit*. 1995;17:89–94.

Poklis A, Saady JJ. Arsenic poisoning: acute or chronic? Suicide or murder? *Am J Forensic Med Pathol*. 1990;11:226–232.

Pounder DJ. Forensic entomo-toxicology. *J Forensic Sci Soc*. 1991;31:469–472.

Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. *Clin Chim Acta*. 2006;370(1–2):17–49.

Pragst F, Rothe M, Hunger J, Thor S. Structural and concentration effects on the deposition of tricyclic antidepressants in human hair. *Forensic Sci Int*. 1997;84(1–3):225–236.

Price LM, Poklis A, Johnson DE. Fatal acetaminophen poisoning with evidence of subendocardial necrosis of the heart. *J Forensic Sci*. 1991;36:930–935.

Prosser JM, Nelson LS. The toxicology of bath salts: a review of synthetic cathinones. *J Med Toxicol*. 2012;8(1):33–42.

Prouty BS, Anderson WH. The forensic science implications of site and temporal influences on postmortem blood-drug concentrations. *J Forensic Sci*. 1990;35:243–270.

Puschel K, Thomasch P, Arnold W. Opiate levels in hair. *Forensic Sci Int*. 1983;21:181–186.

Rollins DE, Wilkins DG, Gygi SP, Slawson MH, Nagasawa PR. Testing for drugs of abuse in hair—experimental observations and indications for future research. *Forensic Sci Rev*. 1997;9:23–25.

Rosenbaum CD, Carreiro SP, Babu KM. Here today, gone tomorrow . . . and back again? A review of herbal marijuana alternatives (K2, Spice), synthetic cathinones (bath salts), kratom, *Salvia divinorum*, methoxetamine, and piperazines. *J Med Toxicol*. 2012;8(10):15–32.

Ross EA, Reisfield GM, Watson MC, Chronister CW, Goldberger BA. Psychoactive "bath salts" intoxication with methylenedioxypyrovalerone. *Am J Med*. 2012;125:854–858.

Saady JJ, Narasimhachari N, Friedel RO. Unsuspected impurities in imipramine and desipramine standards and pharmaceutical formulations. *Clin Chem*. 1981;27:343–344.

San George RC, Hoberman RD. Reaction of acetaldehyde with hemoglobin. *J Biol Chem*. 1986;261:6811–6821.

Schroeder HA, Nason AP. Trace metals in human hair. *J Invest Dermatol*. 1989;53:71–78.

Scientific Working Group for Forensic Toxicology. Standard practices for method validation in forensic toxicology. *J Anal Toxicol*. 2013;37:452–474.

Seely KA, Lapoint J, Moran JH, Fattore L. Spice drugs are more than harmless herbal blends: a review of the pharmacology and toxicology of synthetic cannabinoids. *Prog Neuropsychopharmacol Biol Psychiatr*. 2012;39(2):234–243.

Shapiro HA. Arsenic content of human hair and nails: its interpretation. *J Forensic Med*. 1967;14:65–71.

Shen M, Xiang P, Wu H, Shen B, Huang Z. Detection of antidepressant and antipsychotic drugs in human hair. *Forensic Sci Int*. 2002;126(2):153–161.

Shiffman ML, Luketic VA, Sanyal AJ, et al. Hepatic lidocaine metabolism and liver histology in patients with chronic hepatitis and cirrhosis. *Hepatology*. 1994;31:933–940.

Skopp G. Preanalytic aspects in postmortem toxicology. *Forensic Sci Int*. 2004;142(2–3):75–100.

Smith H. The interpretation of the arsenic content of human hair. *J Forensic Sci Soc*. 1964;4:192–199.

Society of Forensic Toxicologists. Available at: www.soft-tox.org/dfcc.

Spiehler V. Society of Forensic Toxicology Conference on Drug Testing in Hair; October 29, 1994; Tampa, FL.

Spiehler V, Cooper G. Drugs-of-abuse testing in saliva and oral fluid. In: Jenkins A, ed. *Drug Testing in Alternate Biological Specimens*. Totowa, NJ: Humana Press; 2010:83–95:chap 5.

Stockham TL, Blanke RV. Investigation of an acetaldehyde-hemoglobin adduct in alcoholics: alcoholism. *Clin Exp Res*. 1988;12:748–754.

Strang J, Marsh A, Desouza N. Hair analysis for drugs of abuse. *Lancet*. 1990;26:740.

Suzuki O, Hattori H, Asano M. Nails and hair as useful material for detection of methamphetamine of amphetamine abuse. *Forensic Sci Int*. 1984;24:9–16.

Thorwald J. *The Century of the Detective*. New York: Harcourt; 1965.

Tsanaclis LM, Wicks JF, Chasin AA. Workplace drug testing, different matrices different objectives. *Drug Test Anal*. 2012;4:83–88.

Urey FM, Komaromy-Hiller G, Staley B, et al. Nitrite adulteration of workplace urine drug-testing specimens: Part 1: sources and associated concentrations of nitrite in urine and distinction between natural sources and adulteration. *J Anal Toxicol*. 1998;22:89–95.

Utah Department of Public Safety. Commissioner Squires releases information on .05 BAC change. n.d. Available at: https://publicsafety.utah.gov/2017/03/31/commissioner-squires-releases-information-on-05-bac-change/.

Van der Linden T, Isalberti C, Silverans P, Legrand SA, Verstraete AG. Comparison of drug concentrations measured in roadside surveys and in seriously injured drivers in Belgium. *Drug Test Anal*. 2013;5:541–548.

Villain M, Concheiro M, Cirimele V, Kintz P. Screening method for benzodiazepines and hypnotics in hair at pg/mg level by liquid chromatography-mass spectrometry/mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci*. 2005;825(1):72–78.

Vorpahl TE, Coe JI. Correlation of antemortem and postmortem digoxin levels. *J Forensic Sci*. 1978;23:329–334.

Warren A. Interference of common household chemicals in immunoassay methods for drugs of abuse. *Clin Chem*. 1989;35:648–651.

White JM, Clardy MS, Groves MH, et al. Testing for sedative-hypnotic drugs in the impaired driver: a survey of 72,000 arrests. *Clin Toxicol*. 1981;18:945–957.

Wu AH, Gerona R, Armenian P, French D, Petrie M, Lynch LK. Role of liquid chromatography-high-resolution mass spectrometry (LC-HR/MS) in clinical toxicology. *Clin Toxicol (Phila)*. 2012;50:733–742.

Yamaguichi S, Matsumoto H, Kaku S, Tateishi M, Shiramizu M. Factors affecting the amount of mercury in human scalp hair. *Am J Pub Health*. 1975;65:484–488.

Yang SH, Morgan AA, Nguyen HP, Moore H, Figard BJ, Schug KA. Quantitative determination of bisphenol A from human saliva using bulk derivatization and trap-and-elute liquid chromatography coupled to electrospray ionization mass spectrometry. *Environ Toxicol Chem*. 2011;30(6):1243–1251.

Yegles M, Mersch F, Wennig R. Detection of benzodiazepines and other psychotropic drugs in human hair in GC-MS. *Forensic Sci Int*. 1997;84(1–3):211–218.

Yin S. Malicious use of pharmaceuticals in children. *J Pediatr*. 2010;157(5):832–836.

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chapter 33

Clinical Toxicology

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History of Clinical Toxicology
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HISTORY OF CLINICAL TOXICOLOGY

Historical Aspects of the Treatment of Poisoning

The history of poisons and poisoners dates back to ancient times. One of the earliest documented uses of poisons was for military use. The use of toxic smoke can be tracked to as early as 2000 BC in ancient India. Formulas for creating poisonous and noxious vapors have also been found from 1000 BC in Chinese writings. The incorporation of fire-generated arsenic-containing smoke from the burning of wood covered with pitch and sulfur containing material was reportedly used by Sparta against Athens around 400 BC (Osius, 1957; Mayor, 2003). Documentation regarding the use of antidotes can be found in the Odyssey and Shastras from approximately 600 BC. What is considered to be the first documented use of a specific antidote may be found in Homer's Odyssey where it is suggested to Ulysses that he take moli to protect himself from poisoning. Moli may actually be *Galanthus nivalis*, a plant-derived cholinesterase inhibitor that might counteract the effects of the anticholinergic plant *Datura stramonium* (Plaitakis and Duvoisin, 1983).

Galen (129 to 200 AD) wrote three books called *De Antidotis I, De Antidotis II,* and *De Theriaca ad Pisonem* that described the development of a universal antidote known as alexipharmic or theriac by King Mithridates VI of Pontus who lived from 132 to 63 BC (Wax, 1997). The antidote reportedly contained 36 or more ingredients that were ingested every day, conferring protection against a broad spectrum of poisons such as venomous stings and bites from vipers, spiders, and scorpions (Jarcho, 1972).

The refinement of theriac (antidote) formulations is documented for nearly 2000 years. Andromachus (1st century AD) was a physician to Nero and improved the Mithridates theriac by modifying the formula to include up to 73 ingredients (Wax, 1997). The use of these ancient antidotes included treatment of acute poisoning and prophylactic treatment to make one "poison proof." The Mithridates theriac with subsequent modifications remained in use until the early 20th century. William Heberden wrote *Antitheriaka: An Essay on Mithridatium and Theriaca* in 1745 in which he questioned the effectiveness of these products (Jarcho, 1972). Despite the skepticism concerning these antidote formulations, their use continued for two millenniums.

One of the earliest writings on the prevention of the gastrointestinal absorption of poisons was by Nicander (Major, 1934). In this ancient writing, the induction of emesis by ingestion of an emetic agent or mechanical stimulation of the hypopharynx was described as a method to prevent poison absorption. It would not be until the 1600s when the use of ipecacuanha for induction of emesis was recommended by William Piso (Reid, 1970).

The use of oral charcoal, now a mainstay in the treatment of many human poisonings, can be dated to early Greek and Roman civilization when wood charcoal was used for the treatment of maladies such as anthrax and epilepsy (Cooney, 1995). The antidotal properties of charcoal were demonstrated in the 1800s by the French with dramatic demonstrations of a reduction in lethality when charcoal was ingested with potentially lethal dosages of arsenic trioxide by Bertrand and strychnine by Touery (Holt and Holz, 1963). One of the earliest reported human studies examining the efficacy of charcoal in poisoning was in 1948 by the American physician Rand (Holt and Holz, 1963). As recently as the early 1960s, poison treatment center employees were known to start their workday by burning toast to create a supply of poison absorbent (charcoal) for the day's

*Deceased

work. The use of superheated steam to treat the charcoal to enhance its absorption capacity was reported by Ostrejko, a Russian scientist in 1900 (Greensher et al., 1987). By the 1960s, the use of activated charcoal became routinely recommended for the treatment of patients poisoned with substances thought to be adsorbed by charcoal.

Modern Development of Effective Antidotes for Poisoning

The search for safe and effective antidotes has been challenging to say the least. From the time of the early, self-experimenting proponents of effective antidotes to as recently as the later part of the last century, discovery, refinement, and determination of conditions to permit optimal use of antidotes has been less than ideal. Much the same could be said for the state of drug development more broadly during the same time period. It was not until the mid-20th century that a more informed scientific and systematic approach to drug development became possible and, in fact, became a requirement by newly created regulatory agencies in charge of controlling the sale and distribution of medicinal products.

The therapeutic area of poison treatments and prophylaxis presents several additional hurdles compared to drug development for other, more prevalent disease categories. These challenges include the inability to perform controlled human studies to evaluate efficacy, in part due to the small numbers of poisoned patients available for enrollment in therapeutic trials and also due to the often-sporadic occurrence of specific poisonings. These realities make it difficult to easily perform acceptable evaluations of poisoning antidotes. In addition, when trying to transition the nonclinical data for poison treatments to humans, there are sometimes significant interspecies differences encountered that make extrapolation of experimental efficacy or safety findings difficult. In recognition of these challenges, in 2002 the Food and Drug Administration enacted the Animal Efficacy Rule, which allows FDA to approve products for serious or life-threatening conditions caused by exposure to lethal biological, chemical, radiological, or nuclear substances without human efficacy testing. The rule allows the sponsor of the product to demonstrate adequate efficacy of the product in a valid animal or in vitro model of the toxicity and not humans, for obvious ethical or in the setting of an infectious outbreak, logistic reasons. The human safety data is then determined by controlled clinical trials that involve administration of therapeutic dosages of the chem/bio/rad treatment agent in the absence of the poisoning to healthy volunteers (Gaffney, 2015).

INTRODUCTION OF THE POISON CONTROL CENTER

Advances in the field of clinical toxicology and improvements in the quality of care of the poisoned patient have paralleled the evolution of poison control centers (PCCs). The introduction of the poison center and subsequent formation of associations involving healthcare professionals who work in these centers brought with it an opportunity to share information through publications and national conferences that ultimately resulted in systematic enhancements in the treatment of the poisoned patient. One of the earliest local efforts to systematically collect, analyze, and distribute to physicians, clinical information about poisoning was led by Jay Arena, MD, a Duke University pediatrician. In 1939, he published a case series detailing the clinical outcome of 50 cases of lye poisoning (Martin and Arena, 1939). Interest in clinical information regarding the treatment of poisoned patients and resultant patient outcomes was also growing in Europe during approximately the same time. During the 1940s, several European communities developed hospital-based treatment facilities for poisoning (Manoguerra and Temple, 1984). This local poison information effort continued for several years as further realization of the growing importance of the problem of poisoning was appreciated. A study by the American Academy of Pediatrics completed in 1952 reported that more than half of childhood accidents involved unintentional poisoning in the United States. Possibly in response to this study, Edward Press, MD, and Louis Gdalman, RPh, started the first United States poison control center (PCC) in Chicago, Illinois in 1953. Mr Gdalman collected toxicological information on more than 9000 commercial and noncommercial products throughout the 1940s and early 1950s. Their Chicago poison center provided telephone advice to healthcare professionals as well as collected poisoning data systematically during the telephone exchange. As more centers were created they grew to become valuable resources for information about product ingredients, potential toxicity, and recommendations for the treatment of poisoned patients. PCCs proliferated over the next two decades and peaked at 661 centers in 1978 (Scherz and Robertson, 1978). At the peak of this proliferation, each state in the United States had at least one poison center; several states had more than 20 poison centers active at the same time, but there was little standardization throughout the specialty. Information resources gradually evolved from collected experiences of the individual poison center to a centralized collection of product contents, toxicities, and recommended treatments provided in a microfiche database to today's Internet-based globally available database that is frequently updated and incorporates consensus recommendations for various treatment protocols for specific poisonings. Through regionalization, consolidation, and certification, the number of PCCs decreased significantly over time to 55 centers in 2015 (Mowry et al., 2016).

Today, poison centers are generally staffed by a medical director (Medical Toxicologist), administrator or managing director, specialists in poison information, and educators for poison prevention programs. The medical toxicologist, managing director, and specialists in poison information are healthcare professionals who are credentialed by their respective boards. The American Board of Medical Subspecialties offers a subspecialty certificate in Medical Toxicology to physicians who successfully complete the certifying examination. The American Board of Applied Toxicology (ABAT) offers a certification examination for nonphysicians and the American Association of Poison Control Center (AAPCC) provides a certification of the specialists in poison information who are usually nurses, pharmacists, or other healthcare personnel. The managing director of the poison center is usually either the same person as the medical director or a nonphysician certified by the ABAT. The nonphysician toxicologists are referred to as clinical toxicologists.

Poison centers provide a significant service to the general public and to practicing healthcare providers. These services include direct information to patients with expert recommendations for needed treatment, critical diagnostic and treatment information for healthcare professionals, education for healthcare personnel, and poison prevention activities through public education. In recognition of this valuable service, the "Poison Control Center Enhancement and Awareness Act" was signed in 2000, which ensured that all citizens had toll-free access to a PCC, and it was subsequently amended to include partial federal funding in 2008 in part to serve as an early-warning system for potential chemical, biological, or radiologic terrorist attacks (Woolf et al., 2011). This funding was

recently extended through 2019 (Rep. Terry, 2014). Central data collection via the National Poison Data System (NPDS) in near real time has demonstrated the capability for the PCC network to serve in the early warning role (Wolkin et al., 2012). Recent reporting from this network shows that poisoning case data from PCCs are uploaded every 8 minutes on average supporting this role on a national level (AAPCC, 2017).

In addition to this public health alert capability, the ability for PCCs to provide data that can lead to the recognition of improper use of medications, emergence of new illicit drug exposures or practice, prescription product diversion, and product tampering events has been demonstrated. Several examples can be found that indicate that public access to a certified PCC is cost effective, in terms of overall healthcare expenditures. One early publication documented the economic consequence of a 1-year forced closure of the Louisiana State Poison Center several years ago. Without a poison center to call, residents of the community were left with the expensive alternative of visiting a nearby hospital's emergency room or doctor's office visit for possible treatment of their exposure. It was estimated that the increased costs to the state for emergency medical services (EMS) were $1.4 million for that year (King and Palmisano, 1991). Since that study, several other studies have shown that for each dollar spent to operate a PCC, a savings of approximately $3 to $7.67 can be realized for the healthcare system (Miller and Lestina, 1997; Blizzard et al., 2008).

In 2004, the Institute of Medicine (IOM) affirmed the value and need for a poison center system by their commissioned in-depth study of poison prevention and control services in the United States. Their report (IOM, 2004) recommended further standardization of the regional poison centers, partial federal funding for the poison center system to stabilize core functions of each center, and integration of PCCs with public health agencies at the federal, state, and local level including integration with the Centers for Disease Control and Prevention for chemical and biological terrorism preparedness. Since that time, other reports have shown the cost-benefit value of the national poison control system for these functions (Lewin Group, 2012).

CLINICAL STRATEGY FOR THE TREATMENT OF THE POISONED PATIENT

The setting for the initial treatment of a seriously poisoned patient is usually in a hospital emergency room but can sometimes be in other, less ideal settings such as the battlefield, workplace, home, or street setting. In treatment environments that permit a complete assessment and initiation of treatments, most clinical toxicologists agree that a methodically executed, stepwise approach for the evaluation and treatment of the poisoned patient is recommended for optimal care (Ellenhorn, 1997a, 1997b; Hoffman, 2015). In this section, an overview of the general approach to the treatment of poisoned patients in the emergency department (ED) or acute intensive care setting of a hospital will be described. Please note that once the patient is relatively stabilized, the early steps listed often are initiated in parallel. In that setting, the following general steps represent important elements of the initial clinical encounter for a poisoned patient:

1. Clinical stabilization of the patient
2. Clinical evaluation (history, physical, laboratory, radiology)
3. Prevention of further toxicant absorption
4. Enhancement of toxicant elimination
5. Administration of antidote (if available)
6. Supportive care, close monitoring, and clinical follow-up

Clinical Stabilization

The first priority in the treatment of poisoning is to stabilize the patient. Assessment of the patient's airway (ability to move air into and out of the lungs), breathing (the presence of spontaneous respirations), and circulation (adequate blood pressure and perfusion of vital organs) is the initial step of emergency treatment. Some poisoned patients, soon after even a potentially lethal exposure of the toxicant has occurred, will not show significant symptoms. Most poisoned patients, with a toxic exposure, will exhibit symptoms early in their presentation. For this reason, the initial triage of a poisoned patient can be difficult. On occasion, the patient is asymptomatic on presentation but by history has had a potentially serious toxic exposure. Clinicians and medical support staff, who may not be knowledgeable about the toxic exposure level of a specific drug or chemical, may mistakenly stratify the patient to a lower treatment priority, which can have disastrous results. The improper stratification sometimes can include a delay in calling for a clinical toxicology consultation from a regional PCC or a regional poison treatment center. Some drugs, such as a benzodiazepine, can cause significant sedation early after exposure but often have a comparatively mild clinical course, whereas other chemicals, such as camphor, show little clinical effects initially but can produce a fatal outcome. Some chemicals and drugs can cause seizures as part of their toxic-effect profile. Control of drug/toxicant-induced seizures can be an important component of the initial stabilization of the poisoned patient. A full discussion of the various methods available to treat abnormal conditions with a patient's airway, breathing, circulation, and control of toxicant-induced seizures is beyond the scope of this chapter. The reader is referred to textbooks of *Emergency Medicine and Critical Care Medicine* for further information on this subject. After the initial stabilization of the patient, the remainder of the assessment and treatment steps can proceed. On occasion, especially in critically ill patients, treatment interventions are initiated before a patient is completely stable.

Clinical History in the Poisoned Patient

The primary goal of obtaining the medical history in poisoned patients is to determine the substance the patient was exposed to and the extent and timing of exposure. Unfortunately, in contrast to most specialties of medicine, the clinical history from the initial clinical encounter with the poisoned patient is sometimes not available because either the patient is unresponsive and unable to provide the history or the history provided is unreliable. The history may be unreliable due to the inability of the patient to recall pertinent facts relating to the ingestion or exposure, or in the setting of an attempted suicide or a patient who has taken illegal substances, the patient often is not willing to provide an accurate history. For these reasons, additional sources for the clinical history are often incorporated to aid the clinical team in determining the exposure history. Examples of additional resources sometimes employed to obtain an informative clinical history include interviewing family members and emergency medical technicians who were at the scene, searching an electronic medical record, or contacting a pharmacist who can sometimes provide a listing of prescriptions recently filled or an employer who can provide a list of chemicals found in the work environment for an occupational exposure. Many times, identification of the exact substance responsible for the observed toxic effects is not known for hours to days after the ingestion, or on occasion detected during autopsy in fatal cases.

When estimating the extent of exposure to the poison, it is generally recommended that one maximize the estimated dose received. That is, one should assume that the entire contents of the

prescription bottle were ingested, that the entire bottle of liquid was consumed, most of the body surface area was exposed to a topical chemical, or that the highest possible concentration of airborne contaminant was present for a patient poisoned by inhalation unless definite evidence exists to the contrary. Maximizing the potential dose or exposure level allows one to prepare adequately for the worst-case scenario, that is, the most clinically severe manifestations of the poisoning rather than underestimating the exposure and therefore the anticipated toxic effects and, as a result, be unprepared for a serious clinical presentation in a poisoned patient.

Once an appropriate estimate of the dose is made, the toxicologist can refer to various information resources to determine what the range of expected clinical effects might be from the estimated exposure. This is one of the most important early functions of the poison center. The estimation of expected toxicity greatly assists with the triage of poisoned patients. Poison information specialists working in the PCCs routinely give telephone recommendations regarding the level of medical care required for a given ingestion based on the expected clinical effects from the reported ingestion. The majority of in-the-home accidental pediatric exposures are treated with at-home observation and occasionally with the additional recommendation for home-administered activated charcoal if readily available. In the hospital setting, ED staff use the estimation of expected clinical effects to effectively triage the poisoned patient. For example, a 3-year-old patient who accidentally ingested 25 children's chewable multivitamins without iron would likely be triaged to a noncritical care area of the ED, whereas another similar patient who ingested a large amount of a potent anticholinergic medication would be triaged to a high-intensity care section of the ED.

Estimating the timing of the exposure to the poison is frequently the most difficult aspect of the clinical history when treating the poisoned patient. Often the toxicologist must turn detective to determine the most likely window of time that the exposure occurred. For example, if a pediatric patient was being watched by a baby-sitter at all times except for a 20-minute period that preceded the onset of symptoms and the discovery of an open prescription bottle for a calcium channel blocking agent is found, then it is straightforward to assume that the ingestion occurred in that 20-minute window. Other situations require estimation of a far broader window for exposure. For a person found down with several empty prescription bottles nearby who was not seen by anyone for the preceding 24 hours, one would not be able to accurately estimate the time of ingestion other than sometime in that 24-hour window. Therefore, in view of these often-encountered limitations, obtaining an accurate history in the poisoned patient can be very challenging and occasionally completely lacking. When the history is unobtainable or unreliable, the treating clinical toxicologist is left in the setting of empirical treatment of an "unknown ingestion" poisoning. This type of treatment will be discussed later in this chapter.

Physical Examination

One of the most important actions performed during the initial clinical encounter in the treatment of the poisoned patient is the physical examination. A thorough examination of the patient is required to assess the patient's condition, determine the patient's mental status, and, if altered, determine possible nontoxicology explanations for the abnormal mental status such as trauma or central nervous system infection. In addition, findings from the physical examination allow one to categorize the patient's physical examination parameters into broad clinical presentation classes referred to as toxic syndromes. These toxic syndromes have been called toxidromes (Mofenson and Greensher, 1970; Holstege and Borek, 2012; Hoffman, 2015). A toxidrome is a constellation of clinical signs and symptoms that, when taken together, are likely associated with exposure from certain toxicological classes of chemicals. The major toxic syndromes include narcotic, cholinergic, sympathomimetic, anticholinergic, sedative-hypnotic, and withdrawal from ethanol/sedative-hypnotics and withdrawal from opioids. Table 33-1 lists the clinical features of these major toxic syndromes.

Table 33-1

Clinical Features of Toxic Syndromes

	BLOOD PRESSURE	PULSE	TEMPERATURE	PUPILS	LUNGS	ABDOMEN	NEUROLOGICAL
Sympathomimetic	Incr.	Incr.	Slight Incr.	Mydriasis	NC	NC	Hyperalert, incr. reflexes
Anticholinergic	Slight incr. or NC	Incr.	Incr.	Mydriasis	NC	Decr. bowel sounds	Altered mental status
Cholinergic	Slight decr. or NC	Decr.	NC	Miosis	Incr. bronchial sounds	Incr. bowel sounds	Altered mental status
Opioid	Decr.	Decr.	Decr.	Miosis	NC or rales (late)	Decr. bowel sounds	Decr. level of consciousness
Ethanol-sedative hypnotics	Decr.	Decr.	Decr.	Variable	NC	Decr. bowel sounds	Agitation; decr. level of consciousness
Withdrawal from ethanol-sedative hypnotics	Incr.	Incr.	Incr.	Mydriasis	NC	Incr. bowel sounds	Agitation; hallucinations; seizures
Withdrawal from opioids	Incr.	Incr.	Variable	Mydriasis	NC	Incr. bowel sounds; vomiting	Anxious, piloerection

In certain treatment settings, the precise agent responsible for the poisoning is not known during the critically important phase of initial treatment of the poisoned patient. When the treating physicians are able to categorize the patient's presentation into a toxic syndrome, it permits initiation of rationale treatment based on the most likely category of toxicant responsible without knowing the specific chemical causing the poisoning. For example, if a patient presents with altered mental status, mydriasis (dilated pupils), mild hypertension, tachycardia, warm skin, dry mucous membranes, and diminished bowel sounds in the abdomen, the clinical toxicologist can characterize the patient's presentation as consistent with the anticholinergic toxic syndrome. The treatment would then be directed at support of respiration and potentially administering a pharmacological antidote like physostigmine as well as additional situation-requiring supportive care.

Occasionally, a characteristic odor can be detected on the poisoned patient's breath or clothing, which may point toward exposure or poisoning by a specific chemical. Table 33-2 lists some of the better-recognized odors and the substance associated with the odor. Detection of one of these odors may provide an important historical clue as to the agent responsible for the poisoning.

Periodic re-examination of the patient is a very important aspect of clinical toxicology treatment procedures. Follow-up clinical examinations can help gauge the progression of the clinical course of poisoning as well as determine the effectiveness of treatment interventions and assess the need for additional treatment procedures.

Laboratory Evaluation

A common misconception concerning the initial treatment of poisoned patients is that a definitive diagnosis of the specific agent or poison responsible for the patient's clinical presentation is frequently made based upon clinical laboratory testing in real time. Unfortunately, the availability of an assay to identify the specific drug or industrial chemical on a rapid turn-around basis (STAT, e.g., within 1 hour) is very limited. Table 33-3 lists drugs or other chemical tests that are typically available for STAT measurement in a medical center-type hospital facility (UCSF, 2017). As one can see, the number of chemicals for which quantitative detection is possible in a rapid turn-around timeline is extremely limited, compared to the large number of possible chemicals that can poison patients. This further emphasizes the importance of recognizing clinical syndromes for poisoning and for the clinical toxicologist to be able to initiate general treatment and supportive care for the patient with poisoning from an unknown substance.

For the relatively few chemicals that can be quantified on a rapid turn-around basis in an ED setting, the quantitative measurement can often provide both prognostic and therapeutic guidance. Several authors have suggested certain "action levels" for specific therapeutic drugs and chemicals, which when met or exceeded in the patient's plasma should trigger specific interventions or predict likely clinical outcomes. In some cases, measurement of an indicator of the biological effect of a poison provides sufficient information to render definitive treatment to the patient. Measurement of methemoglobin concentration in a patient poisoned by one of many chemicals that can cause this chemical transformation of the hemoglobin molecule is sufficient to initiate treatment for methemoglobinemia without identification of the specific toxicant that caused the condition. Similarly, most hospital laboratories have the capability for rapid measurement of carboxyhemoglobin concentrations, which permits treatment of carbon monoxide poisoning based on the laboratory test measuring a surrogate marker for carbon monoxide exposure.

For some commonly ingested drugs, a nomogram has been established to predict the severity of the poisoning and is important in some cases to guide therapeutic intervention based on the measured plasma concentration of the drug and the time elapsed from the expose. Proper use of such nomograms is necessary for the clinical management of poisoning cases where available.

The clinical usefulness of a drug plasma concentration measured by the clinical laboratory was suggested for salicylates approximately 50 years ago. In 1960, Done published a nomogram to predict the clinical outcome from poisoning with salicylates (Done, 1960). After years of routine clinical use, further investigation led to the conclusion that the clinical utility of the Done nomogram was insufficient to recommend continued applicability to human salicylate poisoning. As a result, use of this nomogram in practice is no longer recommended (Dugandzic et al., 1989; American College of Medical Toxicology, 2015).

In 1975, Rumack and Matthew published a nomogram for acetaminophen poisoning shown in Fig. 33-1 (Rumack and Matthew, 1975). This nomogram predicts clinical outcome and has maintained

Table 33-2

Characteristic Odors Associated With Poisonings

ODOR	POTENTIAL POISON
Apple blossoms	Chloroacetophenone (tear gas)
Bitter almonds	Cyanide
Carrots	Cicutotoxin (water hemlock)
Eggs	Hydrogen sulfide, mercaptans
Garlic	As, organophosphates, DMSO, thallium
Mothballs	Naphthalene, camphor
Pear-like	Paraldehyde, chloral hydrate
Rotten eggs	Hydrogen sulfide, mercaptans, *N*-acetylcysteine
Vinyl	Ethchlorvynol
Wintergreen	Methylsalicylate

Abbreviation: DMSO, dimethyl sulfoxide.

Table 33-3

List of Tests that Are Commonly Measured in a Hospital Setting on a STAT Basis

Acetaminophen	Osmolality
Carbamazepine	Phenobarbital
Carboxyhemoglobin	Phenytoin
Digoxin	Procainamide/NAPA
Ethanol	Quinidine
Gentamicin	Salicylates
Iron	Theophylline
Lithium	Tobramycin
Magnesium	Valproic acid
Methemoglobin	Vancomycin

Abbreviation: NAPA, N-acetylprocainamide.

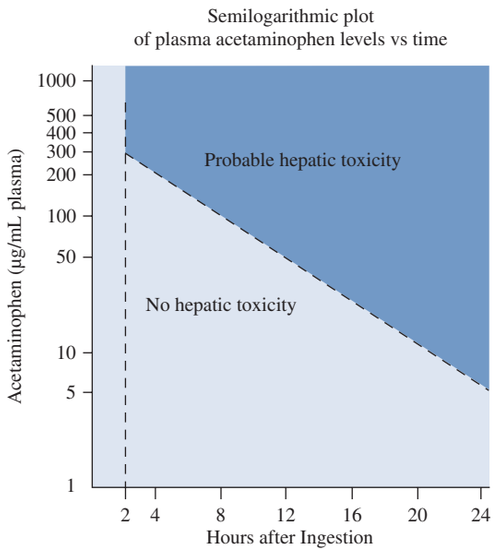

Figure 33-1. *Rumack–Matthew nomogram for acetaminophen poisoning.* (From Rumack and Matthew [1975], with permission. Copyright American Academy of Pediatrics [1975].)

its clinical utility over time as a valuable tool to guide the clinician in the decision as to whether or not to administer *N*-acetylcysteine (NAC), an antidote for significant acetaminophen ingestion. Laboratory evaluation of a patient potentially poisoned with acetaminophen is crucial to assess what hepatic injury may have already occurred and to determine the plasma concentration of acetaminophen for prognostic purposes. Accurate estimation of acetaminophen in the plasma should be done on samples drawn at least 4 hours after ingestion, when or past the time that peak plasma levels can be expected. Once an accurate plasma concentration of acetaminophen has been obtained at least 4 hours post ingestion, it should be plotted on the Rumack–Matthew nomogram (Fig. 33-1) to determine whether NAC therapy is indicated. This nomogram is based on a series of patients with and without hepatotoxicity and their corresponding acetaminophen plasma concentrations at presentation.

In the appropriate patient, the decision to treat an acetaminophen-poisoned patient is based on the plasma concentration of parent acetaminophen plotted on the nomogram. Although some authors recommend that NAC treatment only be administered to a patient with an acetaminophen plasma concentration in the "definite toxic" range, many clinical toxicologists agree that NAC should be given at or above the "probably toxic" action level due to the often present uncertainty in the clinical history with respect to the exact time of the ingestion. It should be noted, however, that proper use of this nomogram is required, namely that certain conditions should be met to apply the nomogram to a specific clinical case. The nomogram was validated for a single acetaminophen ingestion, the time of ingestion is critically important to establish the X-axis coordinate for the data point and the plasma concentration should have been obtained at least 4 hours after ingestion to assure that the peak plasma concentration of parent acetaminophen has occurred.

Similar, though, perhaps less well established, predictive relationships of drug plasma concentration and clinical outcome and/or suggested concentrations that require therapeutic interventions are available for several other drugs including lithium, digoxin, iron, phenobarbital, and theophylline. Some authors have identified "action levels" or toxic threshold values for the measured plasma concentrations of various drugs or other chemicals (Ellenhorn, 1997a, 1997b). Generally, these values represent mean concentrations of the respective substance that have been shown to produce a significant harmful effect during a retrospective analysis of clinical case series. However, the pharmaco- (or toxico-) dynamic variability for a given toxicant or for a combination of toxicants is significant. For example, a patient with a "normal" or "nontoxic" digoxin level may display significant toxic effects and conversely a patient with an elevated or "toxic" plasma concentration of digoxin may not show any sign of harmful effects. The clinical toxicologist is required to critically evaluate the measured drug concentration in context of the clinical presentation of the patient and not react to the measured laboratory value alone. An additional confounding factor is the often-encountered co-ingestion of another substance in the poisoning. The applicability of exposure-response models to predict clinical severity in the presence of a second or multiple toxicants is unknown.

Because of the limited clinical availability of rapidly available "diagnostic" laboratory tests for poisons (Table 33-3), toxicologists can sometimes gain important insight into what potential drugs were ingested by performing simple calculations based on routine clinical laboratory data. Two examples of these laboratory-derived methods to potentially identify what substance was ingested are the anion gap (AG) and the osmol gap calculations. An elevated AG or osmol gap suggests a finite differential diagnosis for significant exposures in a poisoned patient. Both calculations are used as diagnostic aids when the clinical history suggests poisoning and the patient's condition is consistent with exposure to chemicals known to cause elevations of these parameters.

The AG is calculated as the difference between the serum Na ion concentration and the sum of the serum Cl and HCO_3 ion concentrations.

$$\text{AG} = [\text{Na mEq/L} - (\text{Cl mEq/L} + \text{HCO}_3\text{ mEq/L})]$$

$$\text{Normal AG} \leq 12$$

When there is laboratory evidence of metabolic acidosis (low blood pH and low serum HCO_3) in a poisoned patient, the finding of an elevated AG would suggest systemic toxicity from a relatively finite list of chemicals. A popular clinical mnemonic (AT MUD PILES) is commonly employed as a memory aid for this differential diagnosis. Table 33-4 lists the more common chemicals that have metabolic acidosis with an elevated AG as part of the clinical presentation that are included in this pneumonic. Additional substances not included in this table, such as benzyl alcohol, carbon monoxide, hydrogen sulfide, metformin, phenformin, formic acid, and other organic or mineral acids, as well as the anti-seizure medication valproic acid, can also result in elevated AG with systemic metabolic acidosis.

The second calculated parameter from clinical chemistry values is the osmol gap. The osmol gap is calculated as the numerical difference between the measured serum osmolality and the serum osmolarity calculated from the clinical chemistry measurements of the serum sodium ion, glucose, and blood urea nitrogen (BUN) concentrations (Smithline and Gardner, 1976).

$$\text{Osmol gap} = \text{measured serum osmolality (mOsm)} - \text{calculated serum osmolarity (mOsm)}$$

where calculated serum osmolarity = 2 × Na mEq/L + glucose mg/dL/18 + BUN mg/dL/2.8

$$\text{Normal osmol gap} < 10\text{ mOsm}$$

Table 33-4

Differential Diagnosis of Metabolic Acidosis with Elevated Anion Gap: "AT MUD PILES"

A	Alcohol (ethanol ketoacidosis)
T	Toluene
M	Methanol
U	Uremia
D	Diabetic ketoacidosis
P	Paraldehyde
I	Iron, isoniazid
L	Lactic acid
E	Ethylene glycol
S	Salicylate

Table 33-5

Differential Diagnosis of Elevated Osmol Gap

Methanol
Ethanol
Ethylene glycol
Isopropanol
Dimethyl sulfoxide (DMSO)
Mannitol
Propylene glycol

Note that the serum osmolality must be measured by freezing point depression and not boiling point elevation as the latter method can fail to detect the osmolality contribution of volatile, low-molecular-weight substances due to vaporization of the substance during measurement.

An elevated osmol gap in the setting of a poisoned patient suggests the presence of an osmotically active substance in the plasma that is not accounted for by the Na, glucose, or BUN concentrations. Table 33-5 lists several substances that when ingested can be associated with an elevated osmol gap in humans.

Although calculation of both the AG and the osmol gap can provide very useful information from readily available clinical chemistry measurements, these determinations must be interpreted cautiously in certain clinical settings. For example, even though a patient may have ingested a large, significantly toxic amount of methanol, if measured late in the clinical course of the exposure, the osmol gap may not be significantly elevated as most of the osmotically active methanol has left the plasma and has been biotransformed or cleared but is still producing serious clinical effects.

Radiographic Examination

The utility of the plain x-ray radiographic examination to diagnose specific poisonings is relatively limited. This is due primarily to the lack of radiopacity of many oral forms of medication. Sometimes orally ingested toxicants can be visualized radiographically, depending on the specific substance ingested. Early radiographic surveys of the radiopacity of oral medications revealed that the vast majority of commercially available tablets and capsules surveyed were essentially undetectable by a radiograph (Handy, 1971; O'Brien et al., 1986). These and other studies have shown that relatively few formulations of drugs are radiopaque and would likely be detectable by plain x-ray of the abdomen. Generally, plain radiographs can detect a significant amount of ingested oral medication containing ferrous or potassium salts. However, a study of the in vitro and in vivo visualization of chewable oral formulations of iron supplements showed that once the chewable iron was ingested, it was no longer detectable by plain abdominal radiograph (Everson et al., 1989). However, certain formulations that have an enteric coating or certain types of sustained release products are radiopaque and can be visualized (Savitt et al., 1987; Nelson et al., 1993).

The most useful radiographs ordered during the initial management of the poisoned patient include the chest and abdominal radiographs and the computed head tomography study. For example, the abdominal radiograph has been used to detect recent lead paint ingestion in children due to pica for many years. Although the presence of radiographic evidence of lead-based paint chips probably underrepresented the proportion of children with moderate-to-severe lead poisoning in one study, an abdominal radiograph showing pica was associated with significantly elevated blood lead concentrations (McElvaine et al., 1992). Another situation in which an abdominal radiograph may be helpful is the setting of the ingestion of a halogenated hydrocarbon such as carbon tetrachloride or chloroform. If a sufficient amount of either liquid is ingested, it is likely that these organic solvents will be visualized as a radiopaque liquid in the gut lumen on the abdominal film relatively recently after ingestion (Dally et al., 1987). Finally, abdominal plain radiographs have been helpful in the setting where foreign bodies are detected in the gastrointestinal tract. The most common example of this is found when a toddler is being evaluated for potential oral ingestion of a foreign body, such as a watch battery. Early detection by plain radiograph is essential to localize the battery to determine which interventional approach is recommended (Kramer et al., 2015). Another example of an important use of radiography in the acute management of the poisoned patient is during the evaluation of an international traveler to the United States who becomes acutely ill with signs of severe sympathomimetic excess and numerous foreign bodies are visualized throughout the gastrointestinal tract. This type of patient is referred to as a body packer who smuggles illegal substances, such as cocaine (as in this case) or heroin, by swallowing latex or plastic storage vesicles filled with the substance (McCarron and Wood, 1983; Beerman et al., 1986; Sporer and Firestone, 1997). Occasionally, these storage devices rupture and the drug is released into the gastrointestinal tract with serious and sometimes fatal results. Aside from these relatively uncommon situations, the overall clinical utility for detection and diagnosis of poisons by radiography is limited.

In contrast to the limited clinical utility of plain radiography for the identification of a specific poison or to diagnose poisoning, plain radiography and other types of diagnostic imaging in clinical toxicology can be extremely valuable for the diagnosis of toxicant-induced pathology and to aid the clinical toxicologist in the ongoing treatment and patient-management phases of the drug overdose. Detection of drug-induced noncardiac pulmonary edema as seen in some cases of serious intoxication with salicylates and opioid agonists is an example of this occurrence (Stern et al., 1968; Heffner et al., 1990). Plain chest radiography can detect this abnormality, which would likely correlate with the findings observed during physical examination of the lungs. This radiographic finding would increase the severity stratification of the poisoning case and likely alter the planned therapeutic strategy for the patient.

Another example of the use of radiological imaging in clinical toxicology is with computed tomography (CT) of the brain. Significant exposure to carbon monoxide (CO) has been associated with focal CT lesions of the brain consisting of low-density areas,

also referred to as lucencies in the cerebral white matter and in the basal ganglia, especially the globus pallidus. This finding is not specific for CO poisoning and may be seen with other intoxications and disease conditions that cause relative hypoxemia or hypoglycemia. In the setting of CO intoxication however, these CT findings in the brain can be useful for estimating the prognosis for a patient who survives the initial phase of CO poisoning (Miura et al., 1985; Jones et al., 1994). Similar correlations have been shown with magnetic resonance imaging (MRI). Finally, concurrent intracranial pathology, such as trauma or hemorrhage, can be detected by CT or MRI of the brain to aid in the diagnosis of additional injury or pathology previously not appreciated during the early phases of treatment of the poisoned patient.

The initial clinical evaluation of the poisoned patient is a critically important phase of the therapeutic process to treat poisoned individuals. The physical, laboratory, and radiological examination all contribute to the initial diagnostic steps for poison treatment. The physical and laboratory examinations are generally utilized more from a diagnostic and acute management standpoint, whereas the radiological examination, with the exception of visualization of foreign bodies or radiopaque toxicants, tends to be more useful for detection and management of toxicant-induced pathology.

Prevention of Further Poison Absorption

During the early phases of treatment of a poisoned patient who has had a toxic exposure via the oral, inhalation, or the topical route, the opportunity to prevent further absorption of the poison to minimize the total amount of chemical that reaches the systemic circulation may be possible. For chemicals presented by the inhalation route, the main intervention to prevent further absorption is removal of the patient from the environment where the toxicant is found and to provide adequate ventilation and ensure oxygenation for the patient. For topical exposures, patient clothing containing the chemical must be removed and properly disposed in airtight wrappings or containers to ensure that the rescuers and healthcare providers are adequately protected from secondary exposure. Most topical exposures require gentle washing of the skin with water and mild soap taking care not to cause cutaneous abrasions of the skin that may enhance dermal absorption (Burgess et al., 1999; Braue et al., 2008).

The optimal time to intervene to prevent continued absorption of an oral poison is as soon as possible after the ingestion. Four primary methods are currently available for this purpose: induction of emesis with syrup of ipecac, gastric lavage, oral administration of activated charcoal, and whole-bowel irrigation. Historically, induction of emesis was accomplished by a variety of concoctions such as tartar emetic, an antimony salt, mustard mixed in water, concentrated solutions of copper and zinc salts, and various botanical substances. In hospitals, apomorphine injection was given even in the 1980s to cause emesis in patients with a history of potentially toxic ingestion. Since the 1980s, the primary agent used to induce emesis to treat an oral ingestion of a potential toxicant is syrup of ipecac. This product is available as an over-the-counter (OTC) emetic in the United States since 1965 and, although a mainstay for poison center-directed induction of emesis for decades, today the agent is rarely used to treat poisonings. The reason for the decline in use is primarily the recognition of the overall low, and highly variable, effectiveness for syrup of ipecac as an agent for gastric decontamination (Auerbach et al., 1986). In addition, reports of the potential adverse effects associated with ipecac use gained wider recognition, especially in terms of the risk for aspiration of gastric contents. Additionally, there were data to suggest that the administration of syrup of ipecac could result in a delay in the administration of activated charcoal, thought to be a more effective agent for prevention of oral absorption of toxicants, as discussed below. The American Academy of Clinical Toxicology and the European Association of Poisons Centres and Clinical Toxicologists issued a position paper regarding the use of syrup of ipecac (Krenzelok et al., 1997) followed by an additional, similar consensus publication in 2004 (Krenzelok et al., 2004), which stated that the syrup of ipecac should not be used routinely in the management of the poisoned patient and that there was insufficient data to either support or exclude ipecac administration soon after poison ingestion. Many clinical toxicologists believe that there remains a limited role for the clinical use of syrup of ipecac, mainly in rural areas where the length of time before a poisoned patient can reach medical care is significant, especially when the substance ingested is poorly adsorbed to activated charcoal, as discussed below. Examples of substances poorly absorbable to activated charcoal include ethanol and other alcohols, ethylene glycol, potassium, mineral acids, iron and other metals (variable), and halides such as fluoride. The accepted contraindications for use of syrup of ipecac are (1) children less than 6 months of age; (2) in the ingestion of a caustic agent (acid or alkali); (3) in a patient with a depressed level of consciousness or gag reflex or when the toxicant ingested is expected to cause either condition within a short period of time; or (4) when there is a significant risk of aspiration of gastric contents such as for ingestion of a liquid hydrocarbon with high aspiration potential.

The use of gastric lavage, the technique of placing an orogastric tube into the stomach and aspirating fluid then cyclically instilling fluid and aspirating until the effluent is clear, has also diminished in recent years. The reasons for the decline in the use of this technique include a growing appreciation of the risk of aspiration during the lavage procedure and evolving evidence that the overall clinical effectiveness of gastric lavage may be more limited than originally thought. Like induction of emesis, where the efficacy of the procedure declines with increasing time interval between the use of the procedure and the time of ingestion, gastric lavage too appears to have this important limitation (Vale and Kulig, 2004). Many toxicologists agree that if gastric lavage is to be used, to optimize effectiveness, it should be performed within 1 hour of ingestion of the oral poison. It is more likely to be recommended if the patient ingested a highly toxic substance, especially in the cases where it is likely that activated charcoal will not be effective for the substance ingested. Careful attention to the patient's gag reflex prior to initiation of this procedure is important. If the patient does not exhibit a gag reflex, then endotracheal intubation must be performed to adequately protect the airway and prevent aspiration. It is essential that the orogastric tube be of sufficient size (36 to 40 French for an adult; 22 to 28 French for a child) to be useful. Even with a large bore orogastric tube some tablets or capsules may not be able to pass through the tube. In the pediatric patient, there are practical limitations to the use of large bore tubes. In this case, orogastric lavage may only be useful to attempt removal of liquid toxicant or possibly dissolved tablets or capsules.

As described earlier, the medicinal use of charcoal to prevent the absorption of orally ingested toxins dates back more than 150 years. The first reported medicinal use of oral charcoal to adsorb an ingested toxicant is credited to the American physician, Hort, who, in 1834, used large amounts of powdered charcoal to successfully treat a patient poisoned with a chloride salt of mercury. A dramatic demonstration of the adsorptive effect of oral charcoal was that by Tourey, a French pharmacist who reportedly mixed a lethal dose of strychnine with charcoal and consumed the combination before

his colleagues at the French Academy in 1930 (Andersen, 1946). Early in vitro investigations of the adsorptive properties of activated charcoal demonstrated the effect of charcoal on various chemical substances in aqueous solutions (Andersen, 1946), the effect of pH on charcoal adsorption properties (Andersen, 1947), and the effect of activated charcoal on the adsorption of strychnine from gastric contents (Andersen, 1948).

During the last two to three decades, there has been an increasing use of oral charcoal as a therapeutic intervention for oral poisoning. For many years, orally administered activated charcoal has been routinely incorporated into the initial treatment of a patient poisoned by the oral route. The term "activated" refers to the substantially increased adsorptive capacity that results from the processing of charcoal obtained from the burning of carbonaceous substances such as wood pulp, sugars, organic material, and industrial wastes. The processing involves extensive treatment with steam, carbon dioxide, oxygen, zinc chloride, sulfuric acid, or phosphoric acid at temperatures of 500°F to 900°F to "activate" the residue oxidation, which leads to a significant increase in surface area through creation of small pores in the material. Many organic molecules are significantly bound to activated charcoal. Generally, low-molecular-weight and polar compounds such as ethanol tend to be less well bound to activated charcoal. Substances such as lithium, iron, and certain inorganic salts are also not appreciably bound.

In acute oral overdose, activated charcoal is typically administered at a dosage of 1.0 to 1.5 g/kg. When the patient is unable to safely drink, the charcoal is placed into the stomach via an orogastric or nasogastric tube. Proper use of activated charcoal to prevent the absorption of orally administered toxicants requires that the patient's gag reflex be intact and expected to remain so in order to minimize the risk for aspiration of the administered charcoal. Once a recommendation to use a single dose of activated charcoal routinely for oral poisonings, now many are more cautious regarding the use of this moiety for decontamination (Chyka et al., 2005). The main reason cited for the reluctance is lack of definitive evidence of improvement in patient clinical outcomes. Given the obvious limitations to conduct adequately statistically powered, randomized, controlled clinical trials, many toxicologists are more inclined to utilize this method for potentially serious ingestions with appropriate clinical caution (Juurlink, 2016).

The incorporation of the procedure of whole bowel irrigation for the treatment of the poisoned patient has gained acceptance in specific poisoning situations. Whole bowel irrigation is a procedure that cleanses the lumen of the gastrointestinal tract. The procedure is accomplished with a poorly absorbed, osmotically neutral polyethylene glycol electrolyte solution that is administered orally to expel the contents of the intestines via the rectal route. This procedure is similar to that used to prepare a patient for endoscopic medical procedures of the large intestine. A unique use of this procedure in the setting of poisoning is for removal of ingested packets of illegal drugs swallowed by people smuggling the material and hoping to avoid detection by concealing the drugs in their intestines. Sometimes, the containers carried by body packers may leak and begin to cause symptoms that often lead the carrier to detection and treatment. Additional uses for this gastrointestinal decontamination technique are to prevent the ongoing absorption of sustained release formulations of drugs and for the early treatment of ingestions of substantial amounts of iron preparations or other orally ingested metallic poisons (Tenebein et al., 2004).

In summary, many clinical toxicologists agree that, for the prevention of further absorption following oral ingestion of most chemicals, the administration of oral activated charcoal provides an opportunity to lessen the systemic exposure of orally ingested toxicants that are likely to bind to charcoal, and if given in a timely fashion with appropriate clinical airway precautions. The other modalities described above have either a more limited role (whole bowel irrigation and gastric lavage) or a very rare place in gastric decontamination (syrup of ipecac) for use in the treatment of a poisoned patient. Most poison treatment centers favor the use of activated charcoal without lavage for treatment of oral poisoning and drug overdose. Many PCCs recommend home use of activated charcoal for immediate treatment of potentially significant poisonings. The use of ipecac syrup is largely limited to individual patients in whom it is likely to make a difference in outcome, such as the patients in rural environments, hours away from more definitive medical treatment, in whom it appears to be the most effective method available in that particular set of circumstances, or in whom it is the only method available to attempt gastric emptying.

Enhancement of Poison Elimination

There are several methods available to enhance the elimination of specific poisons or drugs once they have been absorbed into the systemic circulation. The primary methods employed for this use today include alkalinization of the urine, hemodialysis, hemoperfusion, hemofiltration, plasma exchange or exchange transfusion, and the administration of oral activated charcoal serially during the treatment time course.

The use of urinary alkalinization results in the enhancement of the renal clearance of certain weak acids. The basic principle applied is to increase urinary filtrate pH to a level sufficient to ionize the weak acid and prevent renal tubule reabsorption of the molecule. This is also referred to as ion trapping. The ion-trapping phenomenon occurs when the pK_a of the chemical is such that after the chemical undergoes glomerular filtration into the renal tubules, alteration of the pH of the urinary filtrate can ionize and "trap" the chemical in the urinary filtrate, lessening the extent to which it is reabsorbed. This increases the amount of toxicant that is eliminated in the urine per unit time, which is an indicator of the renal clearance of the toxicant. A schematic representation of this is shown for salicylate in Fig. 33-2.

Clinical use of this alkalinization procedure requires adequate urine flow and close clinical monitoring including that of the pH of the urine. The procedure is accomplished by adding sterile sodium bicarbonate to sterile water with 5% dextrose for intravenous (IV) infusion and administering the mixture intravenously to titrate the urine pH to 7.5 to 8.5. The drugs for which this procedure has been shown clinically efficacious include salicylate compounds and phenobarbital that have pK_as of 3.2 and 7.4, respectively. The increase in total body clearance for salicylate, for example, by increasing urinary pH from 5.0 to 8.0 can be substantial. The AAPCC and the European Association of Poisons Centres and Clinical Toxicologists 2004 Position Paper on Urinary Alkalinization recommend this procedure for moderately severe salicylate poisoning for a patient not meeting criteria for hemodialysis (Proudfoot et al., 2004).

Theoretically, there could be similar advantages to be gained from acidification of the urine regarding enhancement of clearance of drugs such as amphetamine and phencyclidine; however, there are significant adverse events associated with acidification such as acute renal failure and acidosis and electrolyte disturbances. For this reason, acidification of the urine is not recommended as a therapeutic intervention in the treatment of poisoning.

The dialysis techniques, either hemodialysis, continuous venous filtration, or peritoneal dialysis, rely on passage of the

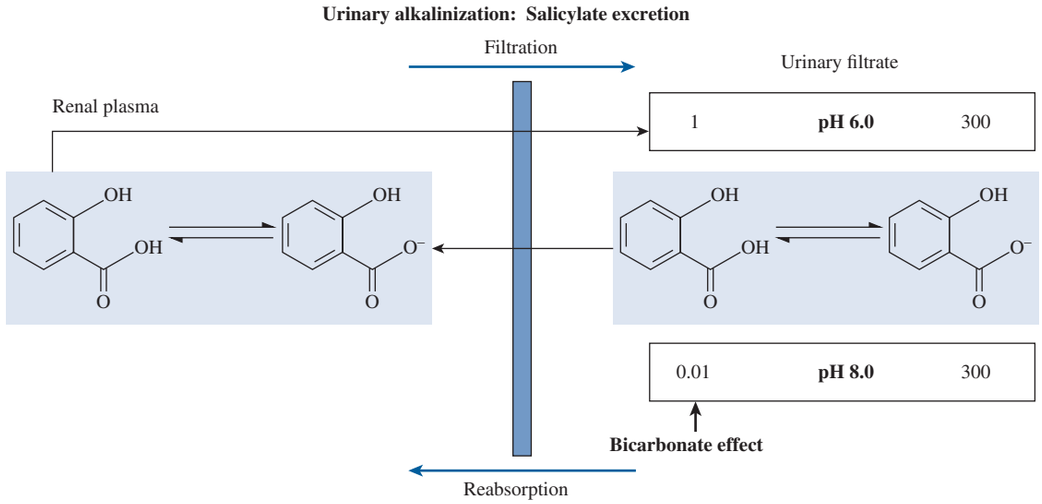

Figure 33-2. *Ion-trapping schematic for salicylate in the renal tubule.*

toxic chemical through a semipermeable dialysis membrane (or the patient's peritoneal membrane for peritoneal dialysis) so that it can equilibrate with the dialysate and subsequently be removed. Hemodialysis incorporates a blood pump to pass blood in close proximity to a dialysis membrane to allow chemicals permeable to the membrane to pass through and reach equilibrium. In order for this method to be clinically beneficial, the chemical must have a relatively low volume of distribution, low protein binding, a relatively high degree of water solubility, and low molecular weight. Use of hemodialysis to attempt to remove a chemical with the latter three characteristics but with a high volume of distribution, such as digoxin, would not be clinically beneficial, because the vast majority of the drug is not in the physiological compartment (blood) accessible to the dialysis membrane. Therefore, despite hemodialysis being able to effectively clear the digoxin in plasma during the dialysis run, most of the body burden of digoxin is located outside of the blood compartment and is not appreciably affected by the procedure. Similarly, if a drug is highly protein-bound, only a small percentage (the free fraction of the circulating toxicant) would be available to pass through the dialysis membrane and be cleared from the body. Some drugs, such as phenobarbital, can readily cross these membranes and go from a high concentration in plasma to a lower concentration in the dialysate. Phenobarbital has a relatively low volume of distribution (0.5 to 0.7 L/kg) and protein binding is also relatively low (30% to 50%), so the characteristics of the drug and its pharmacokinetic properties create appropriate conditions for the drug to be removed from the total body burden to make the technique valuable in serious cases of overdose (Brown et al., 1985; Cutler et al., 1987). Drugs and other chemicals for which hemodialysis has been shown to be clinically effective in the treatment of poisoning by these agents is shown in Table 33-6.

The technique of hemoperfusion is similar to hemodialysis except that there is no dialysis membrane or dialysate involved in the procedure. The patient's blood is pumped through a perfusion cartridge where it is in direct contact with adsorptive material (usually activated charcoal) that has a coating of material such as cellulose or a heparin-containing gel to prevent the adsorptive material from being carried back to the patient's circulation. The principle characteristics for a drug or other chemical to be successfully removed by this technique are low volume of distribution and adsorption by activated charcoal. This method can be used successfully with lipid-soluble compounds and with higher-molecular-weight compounds than for hemodialysis. Protein binding does not significantly interfere with removal by hemoperfusion. Because of the more direct contact of the patient's blood with the adsorptive material, the medical risks of this procedure include thrombocytopenia, hypocalcemia, and leukopenia. This technique is primarily used for the treatment of serious toxic exposures to theophylline, colchicine, paraquat, meprobamate, and possibly amanita phalloides toxin exposure. The technique is not often employed and, perhaps for this reason, not all healthcare facilities actively stock the specialized, sterile hemoperfusion cartridge required for the procedure. This limitation results in the procedure usually only available at major, metropolitan treatment centers.

The use of the technique of hemofiltration for the treatment of poisoning has been suggested as an additional modality for enhancement of chemical elimination. During this procedure, the patient's blood is delivered through hollow fiber tubes and an ultrafiltrate of plasma is removed by hydrostatic pressure from the blood side of the membrane. Different membrane pore sizes are available for use so the size of the filtered molecules can be controlled during the

Table 33-6

Chemicals for Which Hemodialysis Has Been Shown Effective as a Treatment Modality for Poisoning

Alcohols	Lithium
Antibiotics	Meprobamate
Boric acid	Metformin
Bromide	Methotrexate
Caffeine	Paraldehyde
Calcium	Phenobarbital
Chloral hydrate	Potassium
Carbamazepine	Salicylates
Diethylene glycol	Strychnine
Fluorides	Theophylline
Iodides	Thiocynates
Isoniazid	Valproic acid
Isopropanol	

procedure. The perfusion pressure for the technique is either generated by the patient's blood pressure (for arteriovenous hemofiltration) or by a blood pump (for venovenous hemofiltration). Needed fluid and electrolytes removed in the ultrafiltrate are replaced intravenously with sterile solutions. The procedure has the advantage of running continuously as opposed to the 4- to 6-hour limitation for a hemodialysis treatment cycle. One potential advantage of continuous filtration versus intermittent hemodialysis is that the rebound phenomenon does not occur with continuous filtration. Rebound phenomenon is the observed sharp increase in plasma toxicant concentration shortly after hemodialysis is terminated due to redistribution of the toxicant from the nonvascular, tissue, compartment to the vascular space. This rebound is commonly seen during hemodialysis for lithium overdose (Bosinski et al., 1998). Thus far, clinical evidence to support continuous filtration for enhancing total body clearance of toxicants in poisoning cases is insufficient to encourage wide usage of the procedure (Goodman and Goldfarb, 2006).

The use of either plasma exchange or exchange transfusions has been relatively limited in the field of clinical toxicology. Although the techniques afford the potential advantage of being able to remove high-molecular-weight and/or plasma protein–bound toxicants, the clinical utility in poison treatment has been limited. Plasma exchange or plasma pheresis involves removal of plasma and replacement with frozen donor plasma, albumin, or both with IV fluid. The risks and complications of this technique include allergic-type reactions, infectious complications, and hypotension (Mokrzycki and Kaplan, 1994). Exchange transfusion involves replacement of a patient's blood volume with donor blood. The use of this technique in poison treatment is relatively uncommon and mostly confined to the setting of inadvertent drug overdose in a neonate or premature infant in the setting of a neonatal intensive care unit.

Serial oral administration of activated charcoal, also referred to as multiple-dose activated charcoal (MDAC), has been shown to increase the systemic clearance of various drug substances. The mechanism for the observed increase in total body clearance associated with the use of repeated doses of oral charcoal is thought to be translumenal efflux of drug from blood to the charcoal passing through the gastrointestinal tract (Berg et al., 1982). In addition, MDAC is thought to produce a further enhancement in clearance by interrupting the enteroenteric–enterohepatic circulation of drugs that undergo that pharmacological route of elimination and recirculation. After systemic absorption, a drug may reenter the gut lumen by passive diffusion if the intraluminal drug concentration is lower than that in blood. The rate of this passive diffusion depends on the concentration gradient and the intestinal surface area, permeability, and blood flow. The activated charcoal in the gut lumen serves as a "sink" for toxicants. A concentration gradient is maintained and the chemical passes continuously into the gut lumen, where it is adsorbed to charcoal. The characteristics of chemicals that favor enhanced elimination by MDAC include (1) significant enteroenteric–enterohepatic circulation, including the formation of active recirculating metabolites, (2) prolonged plasma half-life after an overdose, (3) small (<1.0 L/kg) volume of distribution, (4) limited (<60%) plasma protein binding, (5) a pK_a that maximizes transport of the drug across cell membranes, (6) sustained-release/resin-form tablets and/or capsules, and (7) onset of organ failure (e.g., kidney) that results in reduced capacity of the major route of elimination of the toxicant (for renally excreted chemicals) so that MDAC may make a considerable contribution to total body clearance.

The technique involves continuing oral administration of activated charcoal beyond the initial dosage (described above) every 2 to 4 hours with approximately one-half the initial dose, or 0.5 g/kg.

Table 33-7

Drugs for Which Multiple Dose Activated Charcoal Has Been Shown Effective as a Treatment Modality for Poisoning

Carbamazepine	Nadolol
Dapsone	Phenobarbital
Digoxin	Salicylates
Digitoxin	Theophylline

The charcoal is generally mixed as an aqueous slurry and a cathartic substance is not incorporated due to the potential for electrolyte abnormalities with repeated administration of cathartic agents. An alternative technique for MDAC is to give the activated charcoal via an orogastric tube or nasogastric tube a loading dose of 1.0 g/kg of an aqueous slurry of activated charcoal, followed by a continuous infusion intragastrically of 0.2 g/kg/h. The duration of gastric infusion depends on the clinical status of the patient and repeated monitoring of plasma drug concentrations where indicated (Pollack et al., 1981; Van de Graaff et al., 1982; Park et al., 1983, 1986; Mofenson et al., 1985; Ohning et al., 1986; Ilkhanipour et al., 1992; Goulbourne et al., 1994; Chyka, 1995; Weisman, 1998).

Studies in animals and human volunteers have shown that MDAC increases drug elimination significantly, but few prospective randomized controlled clinical studies in poisoned patients have been published, demonstrating a significant reduction in patient morbidity or mortality when MDAC is employed. One study, however, does provide a striking example of clinical benefit from the use of MDAC (de Silva et al., 2003). The study showed that patients who intentionally poisoned themselves with yellow oleander seeds (a cardiac poison) and were randomized to MDAC (after all patients received an adequate initial single dose of activated charcoal) had a statistically improved outcome in both mortality and cardiac morbidity. The early use of MDAC is an attractive alternative to more complex methods of enhancing toxicant elimination, such as hemodialysis and hemoperfusion, although only in a relatively small subset of patients. The decision to use MDAC depends on the clinical situation, including the specific chemical involved, the presence of contraindications (e.g., intestinal obstruction) to the use of MDAC, and the likely effectiveness of alternative methods of therapy. A list of chemicals for which MDAC has been shown as an effective means of enhanced body clearance is provided in Table 33-7.

Use of Antidotes in Poisoning

A relatively small number of specific antidotes are available for clinical use in the treatment of poisoning. There are several reasons for the overall paucity of available specific antidotes for poisonings. The two primary reasons are the practical difficulties in performing clinical research in poisoned patients, and the relatively small financial incentives for commercial development of poisoning antidotes. Shortly after the terrorist attack on the United States in 2001, the federal government put in place an FDA authorization to stimulate innovative development of therapeutic agents to treat chemical, biologic, and radioactive attacks. In 2002, the FDA enacted the Animal Efficacy Rule (Animal Rule), a regulation that allows sponsors to attain FDA approval of some products based on safety testing in humans and efficacy testing only in animals (Gaffney, 2015). This rule is intended to allow FDA to approve products intended to treat serious or life-threatening conditions caused by exposure to lethal or permanently disabling toxic biological, chemical, radiological, or

nuclear substances for the indications of treatment of chemical, biologic, or radiologic mass poisonings, such as could be expected in a terrorist attack. For obvious reasons, testing of therapeutic agents for efficacy would not be possible for ethical and/or logistical reasons. The Animal Rule allows the sponsor to establish product efficacy in a validated animal model and then provide preapproval human safety data, in healthy human volunteers. The human safety data would involve administration of therapeutic dosages of the chem/bio/rad treatment agent in the absence of the poisoning. The pharmaceutical company-sponsor would also be expected to perform a field study post-approval, in the event that the indicated exposure occurs naturally for infectious outbreaks or during an attack.

In addition to the Animal Rule Act, the FDA has in place, since 1983, the Orphan Drug Act that provides financial incentives to companies who develop therapies for treatment of rare diseases, defined as affecting less than 200,000 persons in the United States (Orphan Drug Act, 2013). Several treatments for poisoning have been approved under the orphan regulations. Fomepizole (4-methylpyrazole), a chemical inhibitor of alcohol dehydrogenase, was approved as an antidote for ethylene glycol (and later methanol) poisoning via the Orphan Drug pathway in December 1997. Two chelating agents, insoluble Prussian blue and Pentetate Calcium Trisodium, were approved in 2003 and 2004, respectively, for the treatment of exposure to radioactive heavy metals. Another Orphan Drug Act approval was hydroxocobalamin, a cyanide chelating agent. This chemical was approved in the United States in December 2006 after being approved in Europe many years earlier. Despite these early successes, there have not been a large number of antidote products approved to this point in time as a result of this regulatory incentive.

The mechanism of action of various antidotes is quite different. For example, a chelating agent for heavy metal poisoning or Fab fragments specific to digoxin or crotalid venom work by physically binding the toxicant, preventing the chemical from exerting a deleterious effect in vivo and, in some cases, facilitating body clearance of the chemical. In essence, the Fab fragments serve as biologically derived chelating agents to bind and render the specific chemical inactive. Other antidotes pharmacologically antagonize the effects of the toxicant at the endogenous receptor level. Such is the case with atropine, an antimuscarinic, anticholinergic drug used to pharmacologically antagonize at the receptor level, the effects of organophosphate insecticides or acetylcholinesterase-inhibiting nerve gases, which produce cholinergic, muscarinic effects, which if sufficient, can be lethal. Certain chemicals exert their antidote effects by chemically reacting with biological systems to increase the detoxifying capacity for the toxicant. For example, sodium nitrite is given to patients poisoned with cyanide to cause formation of methemoglobin, which serves as an alternative binding site for the cyanide ion, thereby making it less toxic to vital body systems. Other chemicals, such as L-carnitine, are available to mitigate the biochemical toxicity of high exposures to valproic acid (an antiseizure medication) at the level of the mitochondria. Just as in other therapeutic areas of drug development, basic research into fundamental mechanisms can reveal viable drug targets that can be exploited to produce a point of intervention for a drug/antidote to lessen the effects of a toxic exposure.

An example of an efficacious antidote with an uncertain mechanism is intravenous lipid emulsion (ILE) therapy. ILE as a product has been in clinical use for decades as a nutritional supplement for seriously ill patients who were not able to receive nutrition via their gastrointestinal tract. As an antidote, the 20% lipid emulsion solution is most often employed. The discovery of the beneficial effects of ILE comes from the anesthesiology community, through initial basic science work and then experimental clinical use in potentially fatal human accidental exposures. Briefly, the use of regional nerve block local anesthesia has been longstanding. A significant and potentially fatal adverse consequence of this procedure was inadvertent IV injection of a local anesthetic (LA) drug that results in high, likely cardiotoxic concentrations of the LA in the systemic circulation. There were no effective treatments readily available to prevent this toxicity once the misdirected injection had occurred. Because LA drugs are generally highly lipophilic, the anesthesiologists experimented with the immediate use of ILE in animal models and found a beneficial effect on survival (Weinberg et al., 1998). Subsequently, beneficial effects in patients having nerve block procedures, who experienced inadvertent IV administration of the anesthetic drugs, were published as case reports (Rosenblatt et al., 2006).

The current understanding of the mechanism of action is that the ILE serves as a lipid sink in the blood compartment, partitioning the LA predominantly into the lipid phase in blood (ILE), reducing the relative concentration of the cardiotoxic LA in the aqueous phase of the blood. This partitioning then decreases the fraction of the LA that is then available to penetrate target organ tissue and exert a deleterious effect. Other mechanisms proposed as either primary or contributory to the beneficial effect of ILE on LA toxicity include enhancement of cardiac metabolism, that is, fatty acids from the ILE provide an energy source to cardiac myocytes; another possible mechanism is that ILE activates cardiac Ca and Na channels. While there is some evidence to support the energy enhancement and ion channel mechanisms, the partitioning mechanism at this point in time is more strongly supported.

The use of ILE therapy is recommended for treatment of significant systemic toxicity of LAs, including cardiac arrest from inadvertent IV administration of the LA. ILE use for the treatment of non-LA poisoning by lipophilic toxicants is not well supported by evidence (Cao et al., 2015; Levine et al., 2016). Despite the lack of evidence to support the use of ILE non-LA poisonings, many clinical toxicologists will recommend a therapeutic trial of this treatment in the setting of serious cardiovascular effects from lipophilic toxicants such as a calcium channel blocker or beta-blocker not responsive to first or second line treatments. More research is needed to fully understand the optimum use of ILE outside of intravenous poisoning with LA agents.

The time course for antidote onset of action is highly variable across currently available antidotes. Naloxone, given intravenously (IV), can have a dramatic effect on the level of consciousness of an opiate-poisoned patient within minutes of administration. Chelating agents such as desferoxamine may require multiple dosages over many days before a clinically detectable effect is seen.

The skillful therapeutic use of antidotes is essential to optimize the treatment of the poisoned patient. Many antidotes have a relatively narrow safety margin or low therapeutic index. Excessive dosing with an antidote can in some instances be more harmful than the expected effects of the toxicant itself. An example of this is when physostigmine (a cholinergic agent) is given at an excessive dose or dosing rate to a patient with mild-to-moderate anticholinergic poisoning, the antidote can cause a potentially fatal bradycardia that can progress to a fatal cardiac arrest. Some antidotes require an adjustment of their dosage based on a measured blood concentration of the chemical (e.g., digoxin Fab fragments), or based on the clinical assessment of the patient such as with sodium bicarbonate usage in a tricyclic antidepressant overdose. A significant part of the clinical training in the field of medical toxicology is devoted to learning how to use antidotes skillfully.

An important area of research in clinical toxicology has been in the study of prognostic indicators of poisoning severity and predictors for the level of treatment required. For practical reasons

much of this work has been retrospective in nature but has resulted in significant aids to guide the treatment rendered by clinical toxicologists. Several authors have proposed "action levels" that are a threshold for a certain level of clinical intervention based upon a measured plasma concentration of the chemical or a clinical manifestation of the poisoning. One of the best examples of a quantitative relationship between plasma concentrations and prognostic stratification is the previously mentioned nomogram used for acetaminophen concentration and risk of hepatotoxicity (Rumack et al., 1981). As discussed below as a case example, this retrospective analysis of 662 patients with excess acetaminophen exposure was the basis of a nomogram to aid in the risk stratification for significant hepatic toxicity that is still in use today. Similarly, a patient with a measured plasma valproic acid concentration of 900 mcg/mL after a single oral exposure would be expected to exhibit significant toxicity. If the patient's clinical condition correlated with the measured laboratory plasma concentration (i.e., laboratory error was unlikely), the patient would likely require aggressive treatment measures such as hemodialysis instead of only supportive care. Use of a patient-derived prognostic indicator that correlates with clinical outcome can also involve electrocardiographic measurements. An example of this is the case series study considering the publication that described the relationship of the QRS interval on the patient's 12-lead electrocardiogram (ECG) and the severity of poisoning by tricyclic antidepressant drugs (Boehnert and Lovejoy, 1985). This important observational study helped to stratify patients poisoned with an overdose of first-generation tricyclic antidepressants into risk categories based on the drug-induced, lengthening of the QRS on the patient's ECG to predict the eventual development of seizures or cardiac arrhythmias in the course of the poisoning. This, like the Rumack–Matthew nomogram for acetaminophen, is still clinically useful more than 30 years after publication.

Another way that prognostic information is studied is when a constellation of clinical signs and symptoms is proven to correlate with a clinical outcome following exposure to a specific poison. An example of this would be a worker with a significant topical methanol exposure who demonstrates a metabolic acidosis with an elevated AG, an osmol gap, and visual symptoms. Based on clinical signs and symptoms, this patient would likely undergo hemodialysis even in the absence of a confirmatory measurement of the methanol concentration in serum. These relationships, the correlation of serious clinical effects with a valproic acid level above 900 mcg/mL or visual symptoms in a methanol-poisoned patient (Ellenhorn, 1997a, 1997b), have been derived from many years of observational study of poisoning outcomes by investigators in the field of clinical toxicology.

Early on in clinical toxicology, the majority of publications were primarily case reports making it difficult to determine the relative effectiveness of various treatments being assessed. There were also significant discrepancies in the initial management of the patients confounding the ultimate outcomes of the cases. The case series or meta-analysis type of scientific analysis was a helpful step to advance the study of clinical outcomes and assess the quality of treatment provided to poisoned patients. The highest level of evidence for establishing efficacy and safety for therapeutic interventions is the prospective, double blinded, controlled, clinical trial. Whenever possible to successfully execute, these clinical investigations are most likely to result in objective data with the least confounding of results if properly conducted. These advantages better enable clinicians to validate (or dismiss) new therapeutic modalities and treatment strategies. Unfortunately, the ability to perform these trials in the evaluation of new treatments for poisoning is significantly limited due to ethical concerns when considering withholding effective treatment (placebo treatment), or the ability to effectively blind treatment groups in the poison treatment setting. In addition, the heterogeneity between individual poisoning cases would likely introduce potential unbalanced, confounding factors into the study that could lead to inaccurate results.

Supportive Care of the Poisoned Patient

Once the initial treatment phase in the clinical management of the poisoned patient has been successfully completed, the care of the patient is generally shifted to an inpatient hospital setting for those patients who will require admission. This supportive care phase of poison treatment is very important. Poisoned patients who are unstable or at risk for significant clinical instability during the later phases of their poisoning are generally admitted to a critical care unit for close monitoring. In addition, patients who are excessively sedated from their poisoning or those who require mechanical ventilation or invasive hemodynamic monitoring are also candidates for an intensive care stay. Not only are there certain poisonings that have delayed toxicity such as acetaminophen, paraquat, and diphenoxylate, but there are also toxicants that exhibit multiple phases of toxicity that include delayed effects (i.e., ethylene glycol, salicylate, and buspirone).

Similar to other seriously ill, hospitalized patients, patients admitted for continued treatment of poisoning are at risk for nosocomial infections, iatrogenic fluid and electrolyte disturbances, as well as potential harmful effects from the initial therapies that they received for treatment of their poisoning. For example, gastric lavage, or orogastric infusion of activated charcoal can cause aspiration and lead to pneumonitis. Any drug that severely alters a patient's mental status causing obtundation can allow aspiration of gastric contents associated with a loss of the gag reflex. Organ system dysfunction due to profound hypotension caused by a poisoning can result in delayed complications such as acute renal failure, hepatic failure, and permanent brain damage. Close clinical monitoring can detect later phase poisoning complications and allow an opportunity for prompt medical intervention to minimize patient morbidity and mortality. These are but a few of the reasons that close vigilance is a very important component of the support phase of poison treatment.

Another important component of the supportive care phase of poison treatment is the psychiatric assessment. For intentional self-poisonings, a formal psychiatric evaluation of the patient should be performed prior to patient discharge. In many cases, it is not possible to perform a psychiatric interview of the patient during the early phases of treatment and evaluation. Once the patient has been stabilized and is able to effectively communicate, the psychiatric evaluation should be obtained prior to determining the patient's disposition. Generally, a patient who has attempted suicide should be constantly observed until they have been evaluated by the psychiatric consultant and judged to be at low risk for being without constant surveillance.

CASE EXAMPLES OF SPECIFIC POISONINGS

Acetaminophen

A 16-year-old female patient arrives in the ED by ambulance after being found by a parent in what appeared to be an intoxicated state with empty pill bottles scattered about her room. The parent reports the patient was despondent recently after breaking up with her boyfriend. The patient is tearful and reports abdominal pain and admits to drinking alcohol and taking over-the-counter (OTC) pills in an apparent suicide attempt. The estimated time of ingestion is 6 hours prior to arrival in the ED. The patient does not use prescriptions, OTC medications, or dietary supplements on a regular basis and

is not known to have a history of regular consumption of alcoholic beverages or use illicit drugs. In addition, the patient did not have a history of psychiatric disorders or previous suicide attempt.

On physical examination the vital signs were blood pressure 118/80 mm Hg, pulse 88/min and regular, respiratory rate 18/min, and temperature 37.0°C. She was awake and oriented to person, place and time, responded to questions appropriately with slightly slurred speech. Other pertinent findings included normal bowel sounds with mild epigastric tenderness. The neurological examination was only significant for slightly slurred speech.

Routine clinical laboratory studies were ordered STAT (electrolytes, creatinine, BUN, glucose, complete blood count with differential, coagulation studies, urine analysis, and urine toxicology screen) an ethanol level, salicylate level and a plasma acetaminophen level. Chest and abdominal radiography were normal.

The patient was given 1.5 g/kg oral activated charcoal as a slurry in a sorbitol cathartic and placed in the intensive monitoring section of the ED while the laboratory tests were being performed. Forty minutes later, the laboratory results returned and showed a mildly increased white blood cell count, liver transaminase values were elevated at approximately three times the upper limit of normal, and an acetaminophen concentration was 308 μg/mL. Salicylate was not detected; ethanol level was 89 mg/dL (just above the legal threshold for driving while intoxicated in most states). She denied taking any other medications with the acetaminophen and alcohol. Based on the Rumack–Matthew nomogram (Fig. 33-1), a plasma acetaminophen concentration of 308 μg/mL at approximately 6 hours after ingestion is well within the "probable hepatic toxicity" range, and therefore treatment with *N*-acetylcysteine (NAC) was required.

The patient received the first dose of IV NAC in the ED and was admitted to the medical ward to complete the treatment course of IV NAC. Hepatic transaminases remained elevated over the ensuing 2 days of the hospitalization, peaking at five times the upper limit of normal. The patient's bilirubin remained within the normal range as did indices of synthetic hepatic function (coagulation pathway measurements). The patient was seen by the Psychiatry Consultation service, which determined she was not actively suicidal; she was discharged from the hospital 2 days after admission with scheduled psychiatric and medical follow-up appointments.

Acetaminophen has been used as an analgesic and antipyretic since the mid-1950s and has become more prominently recognized as a potential hepatotoxicant in the overdose situation since the original British reports in the late 1960s (Proudfoot and Wright, 1970). Work on the mechanisms of the liver toxicity of this drug has provided a scientific basis for therapy (Mitchell et al., 1973).

The clinical presentation of patients poisoned with acetaminophen is sufficiently confusing in some cases; it is difficult to estimate the time of ingestion. Due to the paucity of clinical symptoms with acute overdose, waiting for the appearance of symptoms is an inadequate strategy for the clinical decision-making process regarding institution of treatment. Most clinicians will request an acetaminophen concentration be measured for any patient suspected of having a toxic exposure to any substance precisely for the reason highlighted above—that is the paucity of signs and symptoms associated with an acetaminophen overdose makes inadvertent missing of a potentially fatal overdose until the window for maximum antidote effectiveness has passed.

Acetaminophen in normal individuals is inactivated by sulfation (approximately 52%) and glucuronide conjugation (42%). About 2% of the drug is excreted unchanged. The remaining 4% is biotransformed by the cytochrome P-450 mixed-function oxidase system. The P-450 isozyme responsible for acetaminophen biotransformation is CYP2E1. Metabolism by CYP2E1 results in a potentially toxic metabolite that is normally detoxified by conjugation with glutathione and excreted as the mercapturate. Evidence extrapolated from animal studies estimates that when 70% of endogenous hepatic glutathione is consumed, the toxic metabolite becomes available for covalent binding to hepatic cellular components. However, patients who are concurrently using, or have recently used, chemicals that induce CYP2E1, such as in the case of chronic ethanol exposure or phenobarbital use, may produce more than 4% of the toxic metabolite. It is therefore important to determine, whenever possible, whether or not the patient's CYP2E1 system may be induced when assessing the risk of hepatic necrosis from acetaminophen overdose in any given individual. When there is evidence (medical history) of concurrent chemicals that induce CYP2E1, the treatment nomogram for acetaminophen does not directly apply, and interpretation by the treating physician must be modified to a lower threshold for treatment with NAC (Rumack et al., 1981).

Follow-up liver biopsy studies of patients who have recovered 3 months to a year after hepatotoxicity have demonstrated no long-term sequelae or chronic toxicity (Clark et al., 1973). A very small percentage (0.25%) of patients in the national multi-clinic study conducted in Denver may progress to hepatic encephalopathy with subsequent death. The clinical nature of the acetaminophen overdose is one of sharp peaks of serum glutamic-oxaloacetic transaminase (SGOT) by day 3, with recovery to less than 100 IU/L by day 7 or 8. Patients with SGOT levels as high as 20,000 IU/L have shown complete recovery and no sequelae 1 week after ingestion (Arena et al., 1978).

Laboratory evaluation of a potentially poisoned patient is crucial in terms of both hepatic measures of toxicity and plasma levels of acetaminophen. Accurate estimation of acetaminophen in the plasma should be done on samples drawn at least 4 hours after ingestion, when peak plasma concentrations are expected.

Once an accurate plasma level has been obtained, it should be plotted on the Rumack–Matthew nomogram to determine whether NAC therapy is indicated (Fig. 33-1). This nomogram is based on a series of patients with and without hepatotoxicity and their corresponding measured plasma acetaminophen concentrations.

Treatment should be instituted in any patient with a plasma concentration in the potentially toxic range. Some clinical toxicologists recommend withholding NAC treatment unless the plasma concentration is at or above the "probably toxic" demarcation, but most often, due to the inherent uncertainty in the history regarding the time of ingestion (the *x*-axis of the Rumack nomogram), the more conservative approach is recommended. Standard gastric decontamination measures with administration of activated charcoal in the case of a relatively recent oral ingestion should accompany the administration of IV NAC. The protective effect of NAC in oral acetaminophen poisoning was demonstrated when contrasted with controls not receiving antidotal therapy (Rumack et al., 1981; Smilkstein et al., 1988). Because NAC is most effective if it is given within 8 to 12 hours of acetaminophen ingestion, patients in whom blood levels cannot be obtained quickly should have NAC treatment instituted and therapy terminated only if levels are nontoxic. The dosing regimen for oral NAC is a loading dose of 140 mg/kg orally, followed by 70 mg/kg orally for 17 additional doses (Peterson and Rumack, 1977). The dosage regimen for IV NAC is 150 mg/kg loading dose over 15 minutes, followed by 50 mg/kg over the next 4 hours then 100 mg/kg over 16 hours for a total dose of 300 mg/kg in 20 hours. Anaphylactoid reactions have been associated with the use of IV NAC; therefore, some authors do not recommend this route of administration for adults with asthma. Children can receive IV NAC but the volume required for the dilution can cause electrolyte abnormalities.

The advantage from using the IV form is that the complete dosage regimen is completed in 20 hours in contrast to the 72-hour oral regimen. Children less than 9 to 12 years of age have a lower incidence of hepatotoxicity after an overdose than do adults, but are still treated with NAC according to the Rumack–Matthew nomogram. Patients with trivial or "nontoxic" acetaminophen ingestions should not be given NAC therapy because the antidote can produce clinical side effects.

Ethylene Glycol

A 37-year-old female was brought to the ED after being found unresponsive in her home. The patient was comatose and unresponsive to pain and without obvious signs of trauma. At the scene, emergency medical personnel administered oxygen and naloxone and performed a finger stick for glucose (standard procedure for encountering a person with altered mental status and suspected toxic ingestion), which showed a normal value of 95 mg/dL. The patient's spouse reported that she had been depressed and despondent with the recent loss of her job. No empty pill bottles or liquid containers were found with her at home.

Upon arrival to the hospital, she remained comatose and had the following vital signs: blood pressure 105/65 mm Hg, pulse 78/min, respiratory rate elevated at 32/min, and her body temperature was normal. The remainder of the physical examination was significant as her pupils were 3 mm and sluggishly reactive to light; the lung and heart examinations were normal; the abdominal examination revealed diminished but present bowel sounds, no tenderness, organomegaly, or masses were detected. The rectal examination was normal; the stool was without detectable gross or occult blood. Neuro examination was nonfocal with a diminished gag reflex.

The patient was placed on a cardiac monitor, an IV line was started, clinical laboratory specimens were obtained, and she was placed on oxygen, given naloxone, thiamine, and one dose of dextrose (50%) intravenously. Similar to the EMSs, these treatments are standard for patients presenting with an altered mental status to an ED. Chest and abdominal radiography was without abnormality. A 12-lead ECG was also normal. Faced with the uncertainty of oral ingestion versus topical and inhalation exposure, a decision was made to proceed with gastric decontamination. Because no direct history from the patient was available, a mixed ingestion could not be ruled out as well. Because of a diminished gag reflex, the patient was endotracheally intubated to protect her airway before an orogastric tube was placed. Gastric lavage was performed and no blood or pill fragments were found. The gastric lavage fluid withdrawn from the stomach was bright yellow in appearance and slightly viscous. When a Wood's lamp illuminated this fluid in a darkened room, fluorescence was observed. This finding suggests the presence of automotive antifreeze that contains ethylene glycol. Activated charcoal (2.0 g/kg) was placed via the orogastric tube into the stomach with a cathartic even though the efficacy for binding ethylene glycol is limited; the use of activated charcoal here was intended for other, potentially unknown coingestants. Clinical laboratory results returned showing the following:

Serum Chemistries

Na = 140 mEq/L	K = 3.1 mEq/L
Cl = 94 mEq/L	HCO_3 = 8 mEq/L
BUN = 12 mg/dL	Glucose = 100 mg/dL

Arterial blood gas:

pH = 7.20; pCO_2 = 20 mm Hg; pO_2 = 98 mm Hg

The complete blood count was normal, the urine analysis was normal, measured serum osmolarity was 330 mOsm/kg, and acetaminophen and salicylate levels were below the limits of detection, and the urine toxicology screen was negative.

The laboratory results were interpreted as follows: a metabolic acidosis with elevated AG (AG = 38; normal < 12) and an elevated osmol gap (40 mOsm; normal < 10 mOsm). These findings are consistent with either methanol or ethylene glycol poisoning (Tables 33-4 and 33-5). A blood sample for measurement of methanol and ethylene glycol was sent for analysis but based on the history and finding of fluorescent, yellow fluid in the stomach and the acid–base disorder detected, the working diagnosis of ethylene glycol poisoning was established. The patient was treated with IV fomepizole (4-methylpyrazole) and transfer to a nearby regional hospital that has hemodialysis capability was requested. Sodium bicarbonate was given intravenously for the profound metabolic acidosis and the patient was successfully transferred to the regional medical center where she underwent hemodialysis shortly after arrival. After 4 hours of hemodialysis, the acid–base and electrolyte abnormalities were corrected but the patient remained comatose. Approximately, 9 hours after the blood specimen was sent from the first hospital, the laboratory reported a "toxic" serum ethylene glycol concentration of 366 mg/dL, a likely fatal exposure to ethylene glycol. The patient underwent a second 4-hour course of hemodialysis 8 hours following the initial hemodialysis treatment to again correct a recurrence, although less severe, of her metabolic acidosis with the appearance of minor renal injury (serum creatinine increased to 1.8 mg/dL). She regained normal consciousness approximately 18 hours after arrival at the second hospital; her renal function recovered completely within 5 days. Subsequently, the patient admitted that she intentionally drank more than half a container of antifreeze with the intent of harming herself. She was evaluated by the psychiatry consultation service and transferred to their service for further care.

This case demonstrates the importance of utilizing the anion and osmol gap calculations in overdose patients as well as all available diagnostic tools (e.g., the Wood lamp) during the initial evaluation of a potentially poisoned patient. It also highlights the life-saving features of rapidly and competently applied therapeutic modalities in poisoning treatment. Without a readily available drug such as fomepizole (or sterile IV ethanol a substitute for fomepizole that also inhibits the alcohol dehydrogenase enzyme) and the ability to rapidly transfer to a facility with definitive care capability (hemodialysis), this patient would have likely succumbed to her poisoning. Initiation of antidote therapy early and providing hemodialysis to increase removal of the poison prior to receipt of the confirmatory laboratory test (measured serum concentration of the specific poison—ethylene glycol) was also required in this case. Although not considered diagnostic (Walker et al., 1986), the presence of both a metabolic acidosis with an AG and an osmol gap is highly suggestive of either methanol or ethylene glycol given the patient's presentation despite a scant history. It is commonplace for hospital clinical laboratories to have to "send out" blood specimens for ethylene glycol and methanol analysis as these are not routinely performed on-site. A turn-around time of 6 to 12 hours for this test result to be available is typical in most settings.

Ethylene glycol exerts its primary toxicity after undergoing biotransformation by alcohol dehydrogenase to glycolic acid and then to glyoxylic and oxalic acid by the action of aldehyde dehydrogenase. Fig. 33-3 contains a schematic representation of the metabolism of ethylene glycol to its toxic metabolites. The latter two acid metabolites are thought to be responsible for both the renal and the acid–base toxicity observed during poisoning by ethylene glycol (Swartz et al., 1981). If untreated or treated too late, ethylene glycol poisoning can

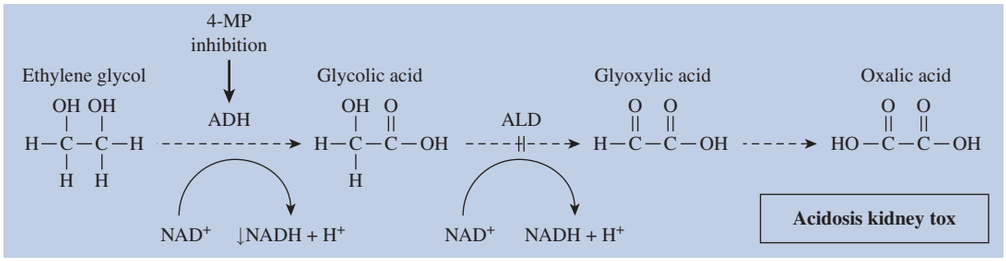

Figure 33-3. *Metabolic pathway for the metabolism of ethylene glycol.*

result in fatal cerebral edema with seizures as well as irreversible renal damage. Hemodialysis can remove the unmetabolized ethylene glycol and toxic, more polar metabolites, eliminating the substrate for production of these harmful metabolites and the damaging metabolites themselves. Administration of an inhibitor or competing substrate for alcohol dehydrogenase can be instituted before and during transport of the patient to a healthcare facility where hemodialysis is available. Fomepizole (4-methylperazole) is a commercially available inhibitor of alcohol dehydrogenase that is FDA approved for the treatment of methanol and ethylene glycol poisoning. Ethanol (sterile, for IV administration) or oral, nonsterile ethanol (when the IV formulation is not readily available) can also be given to effectively inhibit the metabolism of methanol or ethylene glycol and prevent the potentially devastating effects of the poisoning. Finally, in the specific case of methanol poisoning, the administration of folic acid in animal models has been shown to enhance the in vivo clearance of formate, the toxic metabolite of methanol (McMartin et al., 1977). Folate or folinic acid has been given to methanol-poisoned patients and is suggested to hasten formic acid metabolism, thereby accelerating its clearance (Noker et al., 1980). Many clinical toxicologists will include folic or folinic acid in their therapeutic regimen for methanol-poisoned patients. Poisoning with methanol or ethylene glycol is relatively common; several thousand cases per year are reported to the AAPCCs. A review of these cases has shown that even high exposures of either toxicant can be survivable, given early recognition of the poisoning and rapidly applied therapies (Kruse, 2012).

Valproic Acid

A 33-year-old male is brought to the ED after being found unresponsive by his brother at home with two empty prescription pill bottles of extended release valproic acid at his side. He was last seen 8 hours prior to being found unresponsive and was then in normal health. No further history is available. The patient's pharmacy confirmed that three monthly prescriptions, each containing 30, 250 mg extended release valproic acid tablets had been dispensed within the preceding 3 months. A call to the office of the prescribing physician did not reach anyone who could verify the prescription or provide past medical history for the patient.

The patient was unresponsive to verbal or tactile stimulation with vital signs: blood pressure 85/55 mm Hg, pulse 94/min, respiratory rate 20/min, and temperature 33.2°C. IV access was obtained and IV fluids were administered as rapidly as possible. Naloxone was administered without effect. The patient was placed on a cardiac monitor that showed sinus rhythm. The remainder of the physical examination showed the patient to be without obvious signs of trauma; the skin was cool and without track marks. The pupils were 2 mm and poorly reactive to light. Other significant findings included the examination of the abdomen, which showed diminished bowel sounds. The rectal examination was negative for occult blood. The neurological examination revealed coma without focal motor abnormalities and an absent gag reflex.

Initial clinical laboratory results showed mild metabolic acidosis with elevated serum lactate, an increased anion, slightly increased serum ammonia, normal glucose, liver function tests, and renal function tests. The chest and abdominal radiographs were normal. The 12-lead ECG showed a prolonged QT interval without arrhythmia. The patient was endotracheally intubated to protect his airway prior to gastric lavage that yielded some pill fragments only. The patient was placed on a ventilator to support his respiration. Activated charcoal (1.5 g/kg) was administered via the orogastric tube immediately following the lavage procedure. The blood pressure continued to remain low despite IV fluid administration. A STAT valproic acid serum measurement showed the concentration was 572 mcg/mL.

Blood pressure responded to low-dose vasopressors (IV dopamine) with continued IV fluid administration. A repeat serum valproic acid concentration was 890 mcg/mL at 2 hours post admission. Serial oral activated charcoal (every 4 hours) was initiated via the orogastric tube and hemodialysis was started 3 hours after admission. IV L-carnitine was given when a repeat serum ammonia concentration returned further elevated at 94 mg/dL. Subsequent measured plasma concentrations of vaproic acid gradually declined to <100 mcg/mL over the next 48 hours after one additional hemodialysis session was conducted. The patient regained consciousness 24 hours after admission and made a full recovery by hospital day 4. He was evaluated by the Psychiatry consult service, which accepted the patient in transfer to their inpatient service after he was medically cleared by the Toxicology service.

This case illustrates the significance of increasing plasma concentrations of the toxic substance despite gastric decontamination procedures. In this case, the most likely cause for this observation is the ingestion of an extended release formulation, which is pharmaceutically designed to slowly dissolve in the gastrointestinal tract and provide for ongoing sustained release of the active drug product as opposed to immediate release of the drug. Other drug substances can demonstrate the "slow release" profile without having been formulated in a special sustained release dosage form. Examples of these drug "bezoars" include salicylates and barbiturates as well as formulations of iron supplements. The presence of a drug bezoar or concretion can be dangerous because, without serial drug concentration measurements, the treating team could erroneously stratify a patient based on the initial measured plasma concentration and be unprepared for severe toxicity or prolonged toxicity time course not expected from the initial concentration alone.

The use of L-carnitine is an example of the application of basic biochemical knowledge to apply and effective therapeutic

intervention. In the case of valproic acid intoxication, the biochemical effects of valproate on fatty acid metabolism in liver mitochondria, specifically the depletion of L-carnitine, are thought to be causative for the hyperammonemia often observed in moderate and severe cases of valproic acid intoxication (Bohan et al., 2001; Lheureux and Hantson, 2009). Based on these findings and other experimental data, the FDA has approved the use of IV L-carnitine for the treatment of valproic acid poisoning in the setting of hepatotoxicity, hyperammonemia, large overdoses of valproate by history, or measured serum concentrations of valproic acid exceeding 450 mcg/mL.

Finally, the benefit of employing multiple methods simultaneously to enhance the elimination of a toxicant is also demonstrated in this case example. The total body clearance of valproic acid is known to be enhanced when serial oral activated charcoal is administered (Table 33-7). Hemoperfusion, or the more available method of hemodialysis has been shown to increase the total body clearance of valproic acid in overdose (Guillaume et al., 2004). This recommendation is based, in part, on the finding that the plasma protein binding of valproic acid is easily saturated, and in large overdoses, the free fraction (unbound) drug increases from 10% to more than 30%, making more of the drug available for effective removal during hemodialysis. Both modalities were likely beneficially employed in this potentially fatal valproic acid ingestion.

Salicylate

A 48-year-old female is brought to the ED by ambulance approximately 2 hours after a witnessed ingestion of approximately 35 aspirin tablets (each 325 mg) at home in an apparent suicide attempt. The patient complained of upper abdominal discomfort, nausea, and ringing in her ears (tinnitus) and reported one episode of spontaneous emesis at home before transport. The patient has a significant past medical history for major depression disorder, mild osteoarthritis, and hypothyroidism. Her current medications include the antidepressant agent sertraline, 50 mg per day and for hypothyroidism, levothyroxine 100 mcg per day. The patient has been a heavy cigarette smoker (1.5 packs per day) for at least 20 years. No other information about potential illicit substance or alcohol use is available.

Upon arrival in the ED, the vital signs were blood pressure 130/74 mm Hg, pulse 113/min, respiratory rate elevated at 28/min, and body temperature slightly elevated at 38.0°C. She was awake, but oriented only to person. The remainder of the physical examination was significant for slightly dilated pupils (4 mm with normal reactivity to light); heart and lung examinations revealed an increased heart rate and a normal lung exam; the abdominal examination revealed normal bowel sounds and slight epigastric tenderness; no organomegaly or masses were detected. The rectal examination was normal; the stool was without detectable gross or occult blood. Neuro examination was altered mental status, nonfocal with a diminished gag reflex.

Clinical laboratory analysis upon arrival in the ED found the following abnormalities: white blood cell count mildly elevated, serum potassium was slightly low at 3.1 mEq/L, arterial pH 7.15 and serum bicarbonate 11 mEq/L, consistent with a significant metabolic acidosis, anion gap calculation was 29 (normal < 12). At 2.5 hours after the reported ingestion time, salicylate was 36 mg/dL (therapeutic concentrations 10 to 30 mg/dL), acetaminophen was not detected, serum creatinine 1.9 mg/dL, the urine pH was 5.0, and the urine toxicology screen was negative. Chest and abdominal radiographs were normal.

In the ED the patient received normal saline IV and two bolus dosages of 50 mEq sodium bicarbonate to treat her acidosis. IV anti-emetics were given for nausea. At 6 hours post ingestion, the salicylate level was 51.4 mg/dL and no therapeutic action was taken. Five hours later (approximately 11 hours post ingestion) the patient became restless and displayed a tremor in her upper extremities and cramps in her legs. The vital signs at that time were blood pressure 150/105 mm Hg, pulse 144/min, respiratory rate elevated at 30/min, and body temperature slightly elevated at 38.5°C. A salicylate level at that time was 81 mg/dL. Thirteen hours after presentation the patient became significantly agitated, the heart rate increased to 224/min. An arterial blood gas demonstrated pH 7.25, pCO_2 12 mm Hg, and calculated serum bicarbonate 6.6 mEq/L, indicating that the significant metabolic acidosis has continued. Endotracheal intubation and mechanical ventilation were initiated; hemodialysis was not performed on this patient. Following a dose of IV labetalol, given in an attempt to control heart rate, the patient suffered a cardiac arrest from which she could not be resuscitated.

This unfortunate case demonstrates several potential pitfalls in the management of serious salicylate ingestion. First, the witnessed dose ingested was 35 tablets, each containing 325 mg, or more than 11 g of salicylate, which by risk stratification by estimated dose ingested should have alerted the ED staff that this ingestion was very likely to result in severe toxicity. The initial salicylate level was just above the upper limit of normal therapeutic values for salicylate. This lower than expected laboratory result may have steered the ED away from considering this to be a serious and potentially fatal ingestion and toward the obvious under-treatment that occurred. Gastric decontamination did not occur for unclear reasons. Perhaps the ED team assumed that the reported spontaneous emesis was sufficient to prevent significant absorption of the toxicant. Furthermore, the rising salicylate levels over time were apparently overlooked. This phenomenon is commonly seen with extended release formulations (not a factor in this case) or when there is the formation of a drug bezoar. Salicylates, in overdose, are well-known to form drug bezoars in the proximal gastrointestinal tract, which slowly dissolve over time, resulting in rising serum concentrations of salicylate over time. The result of formation of a drug bezoar is that, instead of rapid absorption and a relatively short apparent half-life, the ongoing absorption creates a prolonged and increasing systemic exposure profile for the drug being released from the bezoar. The end result is that the time course to peak toxic effects is delayed. Adequate dosage of activated charcoal upon arrival in the ED, and perhaps use of multi-dose activated charcoal, would likely have reduced the amount of salicylate reaching the systemic circulation. The administration of two bolus doses of sodium bicarbonate was certainly inadequate to overcome the systemic acidosis observed and achieve the needed alkalinizing of the urine to enhance salicylate elimination. The patient should have received a continuous IV sodium bicarbonate infusion with tracking of her urine output and pH with a target pH of 8 for the urine. Involvement of Nephrology consultants to initiate hemodialysis to facilitate salicylate elimination should have also taken place once rising plasma concentrations of salicylate, coincident with clinical deterioration were apparent. In summary, this case reinforces the importance of timely application of the basic, stepwise, procedures available to treat severe, poison-induced toxicity, and demonstrates the potential consequences of underestimating poisoning severity.

SUMMARY

The practice of Clinical Toxicology encompasses the expertise of the specialties of Medical Toxicology, Applied Toxicology, and Clinical Poison Information Specialists. Basic and clinical science continues to provide new information that allows for more effective diagnosis and treatment of the poisoned patient. The evolution of the poison control (information) center and regional poisoning

treatment centers has greatly aided advances in the field of Clinical Toxicology. The incorporation of evidence-based, outcome-driven practice recommendations has improved and standardized the evaluation of treatment modalities for poisonings. Application of a stepwise approach to the poisoned patient as described in this chapter remains an essential approach to the effective evaluation and treatment of the poisoned patient. This methodically performed, clinical diagnostic and therapeutic approach applied in a parallel fashion to the poisoned patient is essential, especially when the medical history is absent or likely unreliable. Skillful use of antidotes is an important component of the practice of Medical Toxicology. Continued research will increase the repertoire of effective treatments for poisoning and ultimately improve patient outcomes.

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REFERENCES

American Association of Poison Control Centers (AAPCC). National Poison Data System. 2017. Available at: http://www.aapcc.org/data-system/. Accessed March 31, 2017.

American College of Medical Toxicology. Guidance document: Management priorities in salicylate toxicity. *J Med Toxicol*. 2015;11(1):149–152.

Andersen AH. Experimental studies on the pharmacology of activated charcoal: adsorption power of charcoal in aqueous solutions. *Acta Pharmacol Toxicol (Copenh)*. 1946;2:69–78.

Andersen AH. Experimental studies on the pharmacology of activated charcoal: the effect of pH on the adsorption by charcoal from aqueous solutions. *Acta Pharmacol Toxicol (Copenh)*. 1947;3:199–218.

Andersen AH. Experimental studies on the pharmacology of activated charcoal. III. Adsorption from gastrointestinal contents. *Acta Pharmacol*. 1948;4:275–284.

Arena JM, Rourk MHJ, Sibrack CD. Acetaminophen: report of an unusual poisoning. *Pediatrics*. 1978;61:68–72.

Auerbach PS, Osterloh J, Braun O, et al. Efficacy of gastric emptying: gastric lavage versus emesis induced with ipecac. *Ann Emerg Med*. 1986;15(6):692–698.

Beerman R, Nunez D, Wetli C. Radiographic evaluation of the cocaine smuggler. *Gastrointest Radiol*. 1986;11:351–354.

Berg MJ, Berlinger WG, Goldberg MJ, et al. Acceleration of the body clearance of phenobarbital by oral activated charcoal. *N Engl J Med*. 1982;307:642–644.

Blizzard J, Michels J, Richardson W, et al. Cost-benefit analysis of a regional poison center. *Clin Toxicol*. 2008;46(5):450–456.

Boehnert MT, Lovejoy FH. Value of the QRS duration versus the serum drug level in predicting seizures and ventricular arrhythmias after an acute overdose of tricyclic antidepressants. *N Engl J Med*. 1985;313:474–479.

Bohan TP, Helton E, MacDonald I, et al. Effect of L-carnitine treatment for valproate-induced hepatotoxicity. *Neurology*. 2001;56:1405–1409.

Bosinski T, Bailie GR, Eisele G. Massive and extended rebound of serum lithium concentrations following hemodialysis in two chronic overdose cases. *Am J Emerg Med*. 1998;16:98–100.

Braue EH, Boardman CH, Hurst CG. Decontamination of chemical casualties. In: Tuorinsky SD, ed. *Medical Aspects of Chemical Warfare*. 2nd ed. Washington, DC: United States Department of the Army, Office of the Surgeon General at TMM Publications, Borden Institute; 2008:527.

Brown TR, Evans JE, Szabo GK, et al. Studies with stable isotopes II: phenobarbital pharmacokinetics during monotherapy. *J Clin Pharm*. 1985;25:51–58.

Burgess JL, Kirk M, Borron SW, Cisek J. Emergency department hazardous materials protocol for contaminated patients. *Ann Emerg Med*. 1999;34:205–212.

Cao D, Heard K, Foran M, Koyfman A. Intravenous lipid emulsion in the emergency department: a systematic review of recent literature. *J Emerg Med*. 2015;48(3):387–397.

Chyka PA. Multiple-dose activated charcoal enhancement of systematic drug clearance: summary of studies in animals and human volunteers. *J Toxicol Clin Toxicol*. 1995;33:399–405.

Chyka PA, Seger D, Krenzelok EP, Vale JA. Position statement: single dose activated charcoal. *Clin Toxicol*. 2005;43:61–87.

Clark R, Borirakchanyavat V, Davidson AR, et al. Hepatic damage and death from overdose of paracetamol. *Lancet*. 1973;1:66.

Cooney DO. *Activated Charcoal in Medical Applications*. New York: Dekker; 1995.

Cutler RE, Forland SC, Hammond PG, Evans JR. Extracorporeal removal of drugs and poisons by hemodialysis and hemoperfusion. *Annu Rev Pharmacol Toxicol*. 1987;27:169–191.

Dally SL, Garneir R, Bismuth C. Diagnosis of chlorinated hydrocarbon poisoning by x-ray examination. *Br J Indus Med*. 1987;44:424–425.

de Silva HA, Foneska M, Pathmeswaran A, et al. Multiple dose activated charcoal for treatment of yellow oleander poisoning: a single-blind, randomized, placebo controlled trial. *Lancet*. 2003;361:1935–1938.

Done AK. Salicylate intoxication, significance of measurements of salicylate in blood in cases of acute intoxication. *Pediatrics*. 1960;26:800–807.

Dugandzic RM, Tierney MG, Dickinson GE, Dolan MC, McKnight DR. Evaluation of the validity of the Done nomogram in the management of acute salicylate intoxication. *Ann Emerg Med*. 1989;18(11):1186.

Ellenhorn MJ. Alcohols and glycols. In: Ellenhorn MJ, ed. *Ellenhorn's Medical Toxicology, Diagnosis and Treatment of Poisoning*. 2nd ed. Philadelphia, PA: Williams & Wilkins; 1997a.

Ellenhorn MJ. *Ellenhorn's Medical Toxicology, Diagnosis and Treatment of Poisoning*. 2nd ed. Philadelphia, PA: Williams & Wilkins; 1997b.

Everson GW, Oudjhane K, Young LW, et al. Effectiveness of abdominal radiographs in visualizing chewable iron supplements following overdose. *Am J Emerg Med*. 1989;7:459–463.

Gaffney A. FDA grants approval to new drug under rarely used animal rule pathway. 2015. Available at: http://www.raps.org/Regulatory-Focus/News/2015/05/11/22135/FDA-Grants-Approval-to-New-Drug-Under-Rarely-Used-Animal-Rule-Pathway/. Accessed March 30, 2017.

Goodman JW, Goldfarb DS. The role of continuous renal replacement therapy in the treatment of poisoning. *Semin Dial*. 2006;19(5):402–407.

Goulbourne KB, Cisek J, et al. Small-bowel obstruction secondary to activated charcoal and adhesions. *Ann Emerg Med*. 1994;24:108–109.

Greensher J, Mofenson HC, Caraccio TR. Ascendency of the black bottle (activated charcoal). *Pediatrics*. 1987;80:949–950.

Guillaume CP, Stolk L, Dejagere TF, et al. Successful use of hemodialysis in acute valproic acid intoxication. *J Toxicol Clin Toxicol*. 2004;42(3):335–336.

Handy CA. Radiopacity of oral non-liquid medications. *Radiology*. 1971;98:522–533.

Heffner JE, Harley RA, Schabel SI. Pulmonary reactions from illicit substance abuse. *Clin Chest Med*. 1990;11:151–162.

Hoffman RS. In: Hoffman RS, Howland MA, Lewin NA, et al., eds. *Goldfrank's Toxicologic Emergencies*. 10th ed. New York: McGraw-Hill; 2015:Chap 3.

Holstege CP, Borek HA. Toxidromes. *Crit Care Clin*. 2012;28(4):479–498.

Holt LE, Holz PH. The black bottle: a consideration in the role of charcoal in the treatment on poisoning in children. *J Pediatr*. 1963;63:306–314.

Ilkhanipour K, Yealy K, Krenzelok EP, et al. The comparative efficacy of various multiple-dose activated charcoal regimens. *Am J Emerg Med*. 1992;10:298–300.

Institute of Medicine (IOM). *Forging a Poison Prevention and Control System*. Washington, DC: National Academy Press; 2004.

Jarcho S. Medical numismatic notes. VII: Mithridates IV. *Bull NY Cad Med*. 1972;48:1059–1064.

Jones JS, Lagasse J, Zimmerman G. Computed tomographic findings after acute carbon monoxide poisoning. *Am J Emerg Med*. 1994;12:448–451.

Juurlink DN. Activated charcoal for acute overdose: a reappraisal. *Br J Clin Pharmacol*. 2016;81(3):482–487.

King WD, Palmisano PA. Poison control centers: can their value be measured? *South Med J*. 1991;84:722–726.

Kramer RE, Lerner DG, Lin T, et al. Management of ingested foreign bodies in children: a clinical report of the NASPGHAN Endoscopy Committee. *J Pediatr Gastroenterol Nutr*. 2015;60(4):562.

Krenzelok EP, McGuigan M, Lheureuz P. Position statement: ipecac syrup. *J Toxicol Clin Toxicol*. 1997;35(7):699–709.

Krenzelok EP, McGuigan M, Lheureuz P; and the American Academy of Clinical Toxicology and European Association of Poisons Centres and Clinical Toxicologists. Position paper: ipecac syrup. *J Toxicol Clin Toxicol*. 2004;42(2):133–143.

Kruse JA. Methanol and ethylene glycol intoxication. *Crit Care Clin*. 2012; 28 (4): 661–711.

Levine M, Hoffman RS, Lavergne V, et al. Systematic review of the effect of intravenous lipid emulsion therapy for non-local anesthetics toxicity. *Clin Toxicol*. 2016;54(3):194–221.

Lewin Group. Final Report on the Value of the Poison Center System. 2012. Available at: http://www.aapcc.org/about/lewin-group-report/. Accessed March 31, 2017.

Lheureux PE, Hantson P. Carnitine in the treatment of valproic acid-induced toxicity. *Clin Toxicol (Phila)*. 2009;47(2):101–111.

Major RH. History of the stomach tube. *Ann Med Hist*. 1934;6:500–509.

Manoguerra AS, Temple AR. Observations on the current status of poison control centers in the United States. *Emerg Med Clin North Am*. 1984;2:185–197.

Martin JM, Arena JM. Lye poisoning and stricture of the esophagus: a report of 50 cases. *South Med J*. 1939;32:286–290.

Mayor A. *Greek Fire, Poison Arrows, and Scorpion Bombs: Biological and Chemical Warfare in the Ancient World*. Woodstock, NY: Overlook Press; 2003.

McCarron MM, Wood JD. The cocaine "body packer" syndrome. *JAMA*. 1983;250:1417–1420.

McElvaine MD, DeUngria EG, Matte TD, et al. Prevalence of radiographic evidence of paint chip ingestion among children with moderate to severe lead poisoning. St. Louis, Missouri, 1989 through 1990. *Pediatrics*. 1992;89:740–742.

McMartin KE, Martin-Amat G, Makar AT, et al. Methanol poisoning V: role of formate metabolism in the monkey. *J Pharmacol Exp Ther*. 1977;201:564–572.

Miller TR, Lestina DC. Costs of poisoning in the United States and savings from poison control centers: a benefit-cost analysis. *Ann Emerg Med*. 1997;29(2):239–245.

Mitchell JR, Jollow DJ, Potter WZ, et al. Acetaminophen induced hepatic necrosis. *J Pharm Exp Ther*. 1973;187:185–194.

Miura T, Mitomo M, Kawai R, Harada K. CT of the brain in acute carbon monoxide intoxication: characteristic features and prognosis. *Am J Neuroradiol*. 1985;6:739–742.

Mofenson HC, Caraccio TR, Greensher J, et al. Gastrointestinal dialysis with activated charcoal and cathartic in the treatment of adolescent intoxications. *Clin Pediatr*. 1985;24:678–684.

Mofenson HC, Greensher J. The nontoxic ingestion. *Pediatr Clin North Am*. 1970;17(3):583–590.

Mokrzycki MH, Kaplan AA. Therapeutic plasma exchange: complications and management. *Am J Kidney Dis*. 1994;23:817–827.

Mowry JB, Spyker DA, Brooks DE, Zimmerman A, Schauben JL. 2015 annual report of the American association of poison control centers' national poison data system (NPDS): 33rd annual report. *Clin Toxicol*. 2016;54(10):924–1109.

Nelson JC, Liu D, Olson KR. Radiopacity of modified release medications. *Vet Hum Toxicol*. 1993;35:317.

Noker PE, Eells JT, Tephly TR. Methanol toxicity: treatment with folic acid and 5-formyl tetrahydrofolic acid. *Alcohol Clin Exp Res*. 1980; 4:378–383.

O'Brien RP, McGeehan PA, Helmeczi AW, et al. Detectability of drug tablets and capsules by plain radiography. *Am J Emerg Med*. 1986; 4:302–312.

Ohning BL, Reed MD, Blumer Jl, et al. Continuous nasogastric administration of activated charcoal for the treatment of theophylline intoxication. *Ped Pharm*. 1986;5:241–245.

Orphan Drug Act. Relevant excerpts. In: Administration USFaD, ed. 21 CFR Part 316. Orphan Drugs: U.S. Government Publishing Office; 2013.

Osius TG. The historic art of poisoning. *Med Bull (Ann Arbor)*. 1957;23:111–116.

Park GD, Radomski L, Goldberg MJ, et al. Effects of size and frequency of oral doses of charcoal on theophylline clearance. *Clin Pharm Ther*. 1983;34:663–666.

Park GD, Spector R, Goldberg MJ, Johnson GF. Expanded role of charcoal therapy in the poisoned and overdosed patient. *Arch Intern Med*. 1986;146(5):969–973.

Peterson RG, Rumack BH. Treatment of acute acetaminophen poisoning with acetylcysteine. *JAMA*. 1977;237:2406–2407.

Plaitakis A, Duvoisin RC. Homer's moly identified as *Galanthus nivalis*: physiologic antidote to stramonium poisoning. *Clin Neuropharmacol*. 1983;6:1–5.

Pollack MM, Dunbar BS, Holbrook PR, Fields AI, et al. Aspiration of activated charcoal and gastric contents. *Ann Emerg Med*. 1981;10: 528–529.

Proudfoot AT, Krenzelok EP, Vale JA. Position paper on urine alkalinization. *J Toxicol Clin Toxicol*. 2004;42:1–26.

Proudfoot AT, Wright N. Acute paracetamol poisoning. *Br Med J*. 1970;2:557.

Reid DHS. Treatment of the poisoned child. *Arch Dis Child*. 1970;45: 428–433.

Rep. Terry L. H.R. 3527. Poison Center Network Act. In: America tCotUSo, ed. 2014; 113–177.

Rosenblatt MA, Abel M, Fischer GW, Itzkovich CJ, Eisenkraft JB. Successful use of a 20% lipid emulsion to resuscitate a patient after a presumed bupivacaine-related cardiac arrest. *Anesthesiology*. 2006;105(1):217–218.

Rumack BH, Matthew H. Acetaminophen poisoning and toxicity. *Pediatrics.* 1975;55:871.

Rumack BH, Peterson RC, Koch GG, et al. Acetaminophen overdose: 662 cases with evaluation of oral acetylcysteine treatment. *Arch Intern Med*. 1981;141:380–385.

Savitt DL, Hawkins HH, Roberts JR. The radiopacity of ingested medications. *Ann Emerg Med*. 1987;16:331–339.

Scherz RG, Robertson WO. The history of poison control centers in the United States. *Clin Toxicol*. 1978;12(3):291–296.

Smilkstein MJ, Knapp GL, Kulig KW, et al. Efficacy of oral *N*-acetylcysteine in the treatment of acetaminophen overdose. *N Engl J Med*. 1988;319:1557–1562.

Smithline N, Gardner KD. Gaps: anionic and osmolal. *JAMA*. 1976;236: 1594–1597.

Sporer KA, Firestone J. Clinical course of crack cocaine body stuffers. *Ann Emerg Med*. 1997;29:596–601.

Stern WZ, Spear PW, Jacobson HG. The roentgen findings in acute heroin intoxication. *Am J Roentgenol*. 1968;103:522–532.

Swartz RD, Millman RP, Billi JE, et al. Epidemic methanol poisoning: clinical and biochemical analysis of a recent episode. *Medicine*. 1981;60:373–382.

Tenebein M, Lheureuz P; and the American Academy of Clinical Toxicology and European Association of Poisons Centres and Clinical Toxicologists. Position paper: whole bowel irrigation. *J Toxicol Clin Toxicol*. 2004;42(6):843–854.

University of California San Francisco (UCSF). University of California San Francisco General Hospital Laboratory Lab Manual. 2017. Available at: http://labmed.ucsf.edu/sfghlab/test/StatServices.html. Accessed April 11, 2017.

Vale JA, Kulig K. Position paper: gastric lavage. *J Toxicol Clin Toxicol*. 2004;42(7):933–943.

Van de Graaff WB, Thompson WL, Sunshine I, et al. Adsorbent and cathartic inhibition of enteral drug absorption. *J Pharmacol Exp Ther*. 1982;221:656–663.

Walker JA, Schwartzbard A, Krauss EA, et al. The missing gap: a pitfall in the diagnosis of alcohol intoxication by osmometry. *Arch Intern Med*. 1986;146:1843–1844.

Wax PM. Analeptic use in clinical toxicology: a historical appraisal. *J. Toxicol Clin Toxicol.* 1997;35:203–209.

Weinberg GL, VadeBoncouer T, Ramaraju GA, et al. Pretreatment or resuscitation with a lipid infusion shifts the dose-response to bupivacaine-induced asystole in rats. *Anesthesiology*. 1998;88(4):1071–1075.

Weisman RS. Theophylline. In: Goldfrank, ed. *Goldfrank's Toxicologic Emergencies*. 6th ed. Stanford, CT: Appleton & Lange; 1998.

Wolkin AF, Martin CA, Law RK, Schier JG, Bronstein AC. Using poison center data for national public health surveillance for chemical and poison exposure and associated illness. *Ann Emerg Med*. 2012;59(1):56–61.

Woolf AD, Karnes DK, Kirrane BM. Preserving the United States' poison control system. *Clin Toxicol (Phila)*. 2011;49(4):284–286.

chapter 34

Occupational Toxicology

Peter S. Thorne

Introduction

Workplaces, Exposures, and Standards
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- Determinants of Dose
- Occupational Exposure Limits

Occupational Diseases
- Routes of Exposure
- Agents Associated With Diseases
- Occupational Respiratory Diseases
- Other Occupational Diseases

Toxicologic Evaluation of Occupational Agents
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  - *Establishing Causality*
  - *In Vitro Assays*
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- Animal Toxicology Testing for Establishing Acceptable Levels of Exposure
- Worker Health Surveillance
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Exposure Monitoring
- Environmental Monitoring for Exposure Assessment
- Biological Monitoring for Exposure Assessment

Conclusion

Acknowledgments

References

INTRODUCTION

Those who work full time over a working lifetime will spend about 94,000 hours in the workplace. If that workplace is dusty or laden with chemicals or allergens, it has the potential to induce disease. The higher the exposures, the greater the likelihood of disease. For centuries scholars have recognized that the work environment plays a significant role in the occurrence of adverse human health effects. Early writings by Ulrich Ellenbog (1435 to 1499), Agricola (1494 to 1555), and Paracelsus (1492 to 1541) revealed the toxic nature of exposures in mining, smelting, and metallurgy. A systematic treatise by Ramazzini (1633 to 1714) described the hazards as they applied to workers in over 50 common trades including miners, chemists, tinsmiths, tanners, pharmacists, grain sifters, stonecutters, sewage workers, and even corpse bearers. Legislation to protect worker health began in England with the Factory Act of 1833 which established a factory inspectorate and limited child laborers 9 to 13 years old to a 48-hour work week and 14- to 18-year olds to a 68-hour work week.

Today we continue to be concerned with occupational health and safety in a wide variety of work environments and child labor remains a concern worldwide. There are an estimated 151.6 million children between the ages of 5 and 17 engaged in child labor and half of them do hazardous work (ILO, 2017). Although occupational settings in developed countries are safer now than in the past, the levels of risk deemed acceptable have decreased while the recognition of the causal link of exposures to chronic diseases or diseases with long latencies has increased. As new hazards arise with the emergence of new technologies, we must be prepared to assess the risks and protect the health of workers. With increased globalization and rising prosperity in many middle-income countries, there exists a responsibility to extend health protections to workers in developing nations who too often bear the burden of high exposures to occupational toxicants.

Occupational toxicology is the application of the principles and methodology of toxicology toward understanding and managing chemical and biological hazards encountered at work. The objective of the occupational toxicologist is primary prevention, that is to prevent adverse health effects in workers that could arise from exposures in their work environment. Because nonoccupational exposures can act as confounders or can increase the susceptibility of individual workers, occupational toxicologists must evaluate the entire spectrum of exposures experienced by the work force under consideration. Occupational toxicology is a discipline that draws on industrial hygiene, epidemiology, occupational medicine, and regulatory toxicology. The occupational toxicologist must have an intimate knowledge of the work environment and be able to recognize and prioritize exposure hazards. Because the work environment can present exposures to complex mixtures, the occupational toxicologist must also recognize those that are particularly hazardous when occurring in combination.

It is often difficult to establish a causal link between a worker's illness and job. First, the clinical manifestations of occupationally induced diseases are often indistinguishable from those arising from nonoccupational causes. Second, there may be a protracted but biologically predictable latent interval between exposure and the expression of disease. Third, diseases of occupational origin may be multifactorial with other environmental factors or personal (e.g., genetic, epigenetic, or behavioral) risk factors contributing to the disease process. Nevertheless, studies have repeatedly shown that the dose of toxicant is a strong predictor of the likelihood, severity, and type of health effect.

WORKPLACES, EXPOSURES, AND STANDARDS

The Nature of the Work Force

The world's labor force totals 3.45 billion people (World Bank, 2017). This includes 803 million workers in China and 511 million workers in India. Approximately 31.3% of the global work force, some 1.1 billion people, work in agricultural production. Many of these workers are engaged in subsistence agriculture and fewer than half are waged workers (ILO, 2014). In contrast, the demographics and distribution of the work force in industrialized nations has undergone a progressive shift over the past three decades, moving away from jobs in heavy industry and agriculture toward jobs in the service sector and high-technology industries. In some instances, the manufacturing jobs have moved to less developed countries with less stringent worker health protection. There are currently 153 million people in the United States in the civilian, paid work force (seasonally adjusted data). The civilian labor force participation rate is 62.9% (USDL, 2017c), a figure that has grown markedly over the past 50 years owing to the increased entry of women into the labor force. The countries of the EU have an estimated 234 million workers (European Commission, 2017), and Japan has about 66 million (Japan Monthly Statistics, 2017). The 1.3 billion workers in the labor force of China and India includes many jobs in manufacturing, minerals extraction, agriculture, and waste processing (including electronic waste).

Fig. 34-1 shows the breakdown of employment in the United States. This illustrates that there are about 22.2 million workers (14.9%) engaged in manufacturing, construction, mining and logging, and agriculture—occupations that have the potential for significant exposure to chemical and biological agents. Furthermore, there are occupations in the service sector, such as automobile repair; other repair and maintenance; work in gasoline stations, pipeline transportation, truck and rail transportation; waste management and remediation services; and employment in botanical gardens that can also include exposures to hazardous chemicals. Service-producing occupations (including government jobs) account for the majority (85.1%) of U.S. jobs. On average, employees in the service sector work 6.4 fewer hours per week than in the goods-producing sector. Farm work employs an estimated 2.14 million workers 16 years of age and older, and the U.S. Bureau of Labor Statistics has documented an average of 40.4 hours worked per week. Work in agriculture is markedly different from most other occupations in four fundamental ways. First, 44% of those employed in agriculture are self-employed or unpaid family members. Second, the overwhelming majority of farm establishments (90%) are small family farms with fewer than 20 employees and annual gross cash farm income less than $350,000 (USDA, 2017). Third, although the annual national average hours worked per week is 40.4, many farmers, ranchers, and farm workers have periods (e.g., harvest time) when they work as many as 20 hours per day, 7 days per week. Fourth, according to a 1998, one-time U.S. government survey, there are in excess of 290,000 children who identify agricultural work as their major employment (GAO/HEHS, 1998). Department of Labor data indicate that, on average, 128,500 hired farm workers between the ages of 14 and 17 were working annually in crop production. The U.S. Fair Labor Standards Act establishes a 16-year minimum age for those occupations in agriculture that the Secretary of Labor finds and declares to be particularly hazardous. The presence of children in the work force has important ramifications for body burdens, disease latency, toxicokinetics, and biotransformation of toxicants.

Determinants of Dose

Dose is typically defined as the amount of toxicant that reaches a target tissue over a defined time span and is available for interactions with metabolic processes. In occupational environments, *exposure* is often used as a proxy for *dose*. The response to a chemical is dependent upon both host factors and dose. Fig. 34-2 illustrates the pathway from exposure to subclinical disease or to adverse health effect and suggests that there are important modifying factors: contemporaneous exposures, genetic and epigenetic susceptibility, age, gender, nutritional status, and behavioral factors. These modifying factors can influence whether a worker remains healthy, develops subclinical disease that is repaired, or progresses to frank illness. Workplace health protection and surveillance programs (shown in blue) can reduce exposures, disrupt the exposure-dose pathway, or identify internalized dose and early effects before irreparable

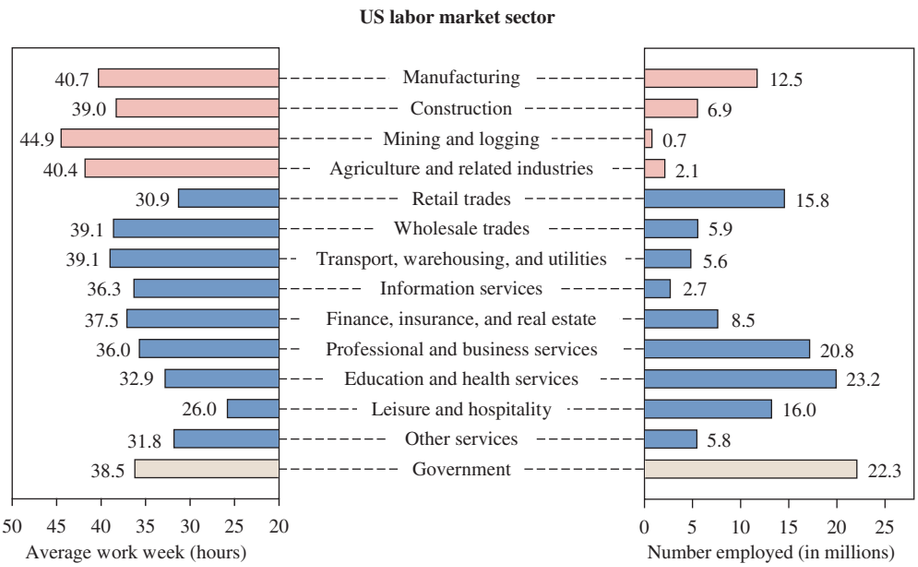

Figure 34-1. *Hours worked and number employed in the U.S. civilian labor market.* (From USDL [2017a, 2017b, 2017c].)

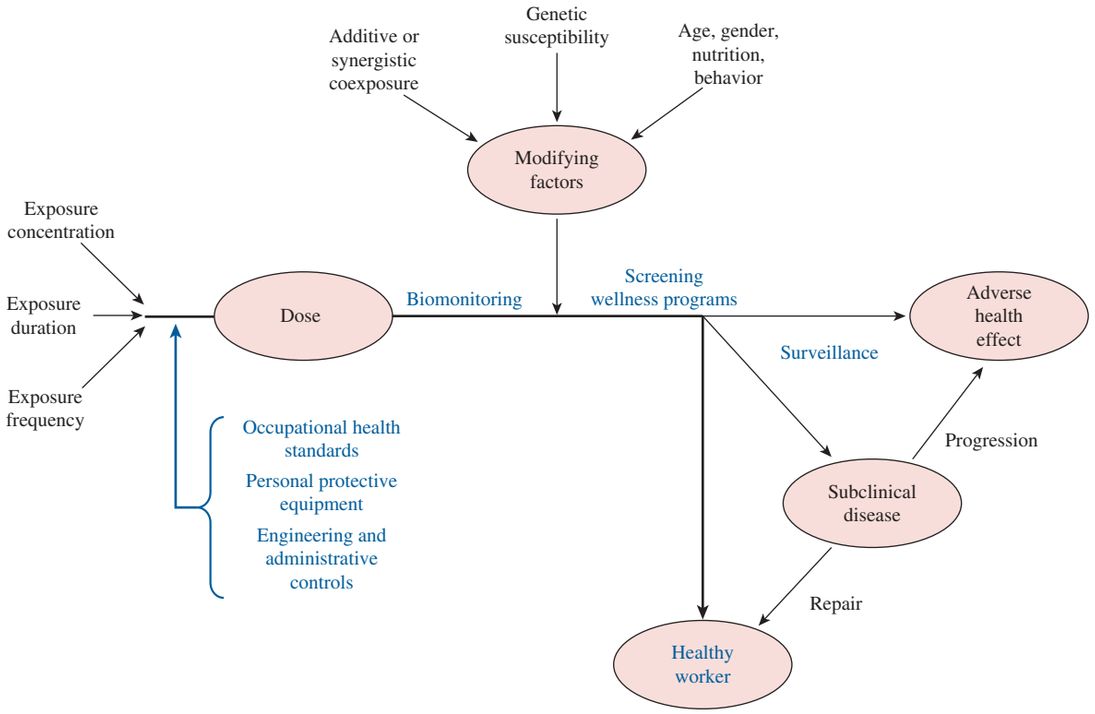

Figure 34-2. *Pathway from exposure to disease, showing modifying factors and opportunities for intervention.*

disease develops. These programs help to ensure a safe workplace and a healthier work force.

As illustrated in Fig. 34-2, dose is a function of exposure concentration, exposure duration, and exposure frequency. Individual and environmental characteristics can also affect dose. Exposure assessment is the process of quantifying the intensity, frequency, and duration of exposures and their determinants in order to better estimate dose. Table 34-1 indicates determinants of dose for exposure via the inhalation and dermal routes. For determining inhalation exposures, environmental conditions such as concentration, particle size distribution, and properties of the chemical are important. However, respiratory rate and breathing volume as well as other host factors contribute. Protection afforded by personal protective equipment (especially respirators) will reduce but not eliminate exposure. The degree of exposure reduction for a particular respirator (the workplace protection factor) varies with respirator design, fit, maintenance, manner of use, and environmental conditions from less than 5 to 10,000. A respirator with a workplace protection factor of 10, when used properly, will reduce particulate matter concentrations inside the mask to one-tenth that outside the mask. A commonly used two-strap fiber respirator for lowering particulate matter exposure, the N95, should reduce exposures by 95% but may fail to meet this criterion for submicron aerosols (Balazy et al., 2006).

Dermal exposures depend upon toxicant concentration; work conditions, including the degree and duration of wetness; and the ambient conditions at the work site (Table 34-1). Some determinants of dermal dosing relate to the physicochemical properties of the chemical as they affect the percutaneous absorption rate. These include solubility, temperature, pH, molecular size, and chemical characteristics of the vehicle. Host factors also influence dermal absorption and distribution. Important factors include the surface area of the skin that is exposed, the integrity of the skin, blood flow, and biotransformation. Because the stratum corneum, the outer layer of the epidermis, is the principal barrier to dermal uptake, the thickness of this layer in the exposed area has great significance. As an example, the absorption of hydrocortisone through the plantar foot arch is only 4% than through the back and 0.3% than through scrotal skin (Bason et al., 1991). A classic human in vivo study (Maibach et al., 1971) using the pesticide parathion demonstrated

Table 34-1

Determinants of Toxicant Dose

Inhalation exposure
Airborne concentration
Particle size distribution
Respiratory rate
Tidal volume
Other host factors
Duration of exposure
Chemical, physical, or biological properties of the hazardous agent
Effectiveness of personal protective devices
Dermal exposure
Concentration in air, droplets, or solutions
Degree and duration of wetness
Integrity of skin
Percutaneous absorption rate
Region of skin exposed
Surface area exposed
Preexisting skin disease
Temperature in the workplace
Vehicle for the toxicant
Presence of other chemicals on skin

a 12-fold range in percutaneous absorption between the anatomical site with the highest (scrotum) to that with the lowest (forearm) percent of dose absorbed. Trabaris et al. found 10-fold higher dermal fluxes of chloracetonitrite, a disinfection byproduct, for excised skin from the torso and dorsum as compared to the palm and scalp (Trabaris et al., 2012). The use of protective gloves and clothing or aprons and the application of barrier creams can greatly reduce exposure. For maximal protection, it is important that the glove be constructed of material tailored to the toxicant(s) of concern.

Occupational Exposure Limits

One of the roles of the occupational toxicologist is to contribute data to the process of establishing standards or determining the appropriateness of those standards. Workplace exposure limits exist for chemical, biological, and physical agents and are recommended as guidelines or promulgated as standards in order to promote worker health and safety. For chemical and biological agents, exposure limits are expressed as acceptable ambient concentration levels (occupational exposure limits [OELs]) or as concentrations of a toxicant, its metabolites, or a specific marker of its effects in biological systems (biological exposure indices).

OELs are established as standards by regulatory agencies or as guidelines by research groups or professional organizations. In the United States, the Occupational Safety and Health Administration (OSHA) under the Department of Labor promulgates legally enforceable standards known as permissible exposure limits (PELs). These standards are determined and supported by the best scientific evidence available and assure "to the extent feasible … that no employee will suffer material impairment of health or functional capacity" with regular exposure "for the period of his working life." OSHA has defined an 8-hour time-weighted average (TWA) PEL (U.S. Federal Register, 1992) as the "employee's average airborne exposure in any 8-hour work shift of a 40-hour work week which shall not be exceeded." The TWA PEL is established as the highest level of exposure to which an employee may be exposed without incurring the risk of adverse health effects (OSHA, 1995). Although approximately 500 PELs have been promulgated, there are not enough to regulate exposures to the vast number of compounds to which workers are exposed, and some of the existing PELs do not reflect current knowledge.

The National Institute for Occupational Safety and Health (NIOSH), under the Centers for Disease Control and Prevention, publishes recommended exposure limits (RELs) that are more frequently updated and are generally more stringent than PELs. NIOSH also performs research and disseminates information on workplace hazards and their prevention. Most developed countries have governmental inspectorate agencies analogous to OSHA that are responsible for establishing and enforcing OELs. In some countries, the insurance system also plays a significant role.

The European Commission has established legally enforceable binding occupational exposure limit values (BOELV) and biological limit values (BLV) for the protection of health and safety in the workplace. Socioeconomic and technical feasibility factors are also considered in setting these values. The OELs are established based on recommendations of the Scientific Committee on Occupational Exposure Limits (SCOEL), which evaluates published scientific data on hazardous compounds for regulation and provides assessment of exposure limits that "it believes will protect workers from chemical risks" (European Commission, 1995, 1998). Indicative Occupational Exposure Limit Values (IOELVs) are non-binding health-based OELs that do not consider technical or economic feasibility. A first list of IOELVs was established in 2000 followed by second (European Commission, 2006) and third lists in 2006 and 2009, respectively (European Commission, 2009). EU member countries may not promulgate OELs less stringent than these values.

The American Conference of Governmental Industrial Hygienists (ACGIH) is a non-profit scientific association that publishes OELs for chemicals and for physical agents. These take the form of threshold limit values (TLVs™) and biological exposure indices (BEIs™). They are frequently revisited and generally reflect current knowledge in occupational toxicology and industrial hygiene. They are developed as guidelines and are not enforceable standards; however, many industries adopt TLVs and BEIs as internal OELs. As stated by the ACGIH, "The TLVs and BEIs represent conditions under which ACGIH believes that nearly all workers may be repeatedly exposed without adverse health effects" (ACGIH, 2017). These guidelines are health based and do not include consideration of technical feasibility or economic effects of implementation.

Three types of TLVs are suggested, depending on the time scale of adverse effects inducible by the toxicants. The time-weighted average TLV (TLV-TWA) is an OEL for exposures averaged over an 8-hour day, 5-day work week regimen. These are generally applied to toxicants that exert their effects over long periods. The short-term exposure limit (TLV-STEL) is an OEL for a 15-minute measurement period. The TLV-STEL should not be exceeded in any 15-minute sampling window and there should be 60 minutes or more between exposures in this range. The ceiling limit (TLV-C) represents a concentration that should never be exceeded. These are usually applied to toxicants that cause acute effects (such as asphyxia or potent sensory irritation) and for which real-time monitoring devices are available. BEIs are guidance values for biological monitoring and represent "levels of determinants that are most likely to be observed in specimens collected from healthy workers who have been exposed to chemicals to the same extent as workers with inhalation exposure at the Threshold Limit Value" (ACGIH, 2016). BEIs are recommended for analysis of urine, blood, and exhaled air. While hair, fingernails, adipose tissue biopsies, and other specimens are used in research and forensic toxicology, there are no BEIs for these.

It is important to recognize that OELs do not correspond to exposure conditions devoid of health risk. The concept of acceptable exposure level must be understood as the level of exposure below which the probability of impairing the health of the exposed workers is acceptable. The process of deciding what is an acceptable risk to occupational or environmental hazards blends the scientific disciplines of exposure assessment and toxicology with often vexing policy issues. Historically, acceptable risk in a society is related to the general health of the population and to a host of factors that influence how risks are perceived. To determine that the risks from an occupational hazard are acceptable, it is necessary to characterize the hazard, identify the potential diseases or adverse outcomes, and establish the relationship between exposure intensity or dose and the adverse health effects. If biological markers of exposure or early reversible effects are identified, this can aid in the risk-assessment process.

OCCUPATIONAL DISEASES

Routes of Exposure

Diseases arising in occupational environments involve exposure primarily through inhalation, ingestion, or dermal absorption. In the vast majority of work environments, inhalation of toxicants is a primary concern. Inhalation exposures can occur with gases,

vapors, liquid aerosols, particulate aerosols, fumes, and mixtures of these. Dermal exposures are also important and can arise from airborne materials as well as liquids splashed onto the skin, immersion exposures, or from material handling.

Additional exposure hazards exist for infectious agents. Exposures leading to occupational infections may arise through inhalation or ingestion of microorganisms but can also arise from needle-sticks in health-care workers or through insect bites among farmers, natural resource workers, and others employed out of doors. Additionally, poisonings from toxic plants or venomous animals can occur through skin inoculation (e.g., zookeepers, horticulturists, or commercial divers).

Agents Associated With Diseases

There are a myriad of agents responsible for occupational diseases. While some act on a particular organ such as the liver or kidney, others can affect multiple organ systems. Table 34-2 presents a list of the major occupational diseases and examples of agents that cause them. This is not intended to be all-inclusive. Rather it is meant to highlight what are historically the most prevalent and widely recognized occupational diseases plus those that continue to be prevalent in the workplace. The toxicants listed are those for which there is a strong association with the disease or the most conclusive data to support causality. Examples are shown for cancer and for diseases of the lung and airways, heart, liver, kidney, skin, nervous system, immune system, and reproductive system. Several examples of occupational infectious diseases are also listed to highlight the fact that, in many work settings, infectious agents may constitute the major hazard and may coexist with chemical hazards. Concurrent chemical exposures may increase susceptibility to infectious agents. Most of the occupational diseases listed in Table 34-2 are associated with industrial chemicals. These are discussed in other chapters throughout this book.

Table 34-2

Examples of Occupational Diseases and the Toxicants That Cause Them

ORGAN SYSTEM OR DISEASE GROUP	DISEASE	CAUSATIVE AGENT
Lung and airways	Acute pulmonary edema, bronchiolitis obliterans	Nitrogen oxides, phosgene, diacetyl
	Allergic rhinitis	Pollens, fungal spores
	Asphyxiation	Carbon monoxide, hydrogen cyanide, inert gas dilution
	Asthma	Toluene diisocyanate, α-amylase, animal urine proteins
	Asthma-like syndrome	Swine barn environments, cotton dust, bioaerosols
	Bronchitis, pneumonitis	Arsenic, chlorine
	Chronic bronchitis	Cotton dust, grain dust, welding fumes
	Emphysema	Coal dust, cigarette smoke
	Fibrotic lung disease	Silica, asbestos
	Hypersensitivity pneumonitis	Thermophilic bacteria, avian proteins, pyrethrum, *Penicillium, Aspergillus*
	Metal fume fever	Zinc, copper, magnesium
	Mucous membrane irritation	Hydrogen chloride, swine barn environments
	Organic dust toxic syndrome	"Moldy" silage, endotoxin
	Reactive airways dysfunction syndrome	Ammonia, chlorine, acetic acid
	Upper respiratory tract inflammation	Endotoxin, peptidoglycan, glucans, viruses
Cancer	Acute myelogenous leukemia	Benzene, ethylene oxide
	Bladder cancer	Benzidine, 2-naphthylamine, 4-biphenylamine
	Gastrointestinal cancers	Asbestos
	Hepatic hemangiosarcoma	Vinyl chloride
	Hepatocellular carcinoma	Aflatoxin, hepatitis B virus
	Mesothelioma, lung carcinoma	Asbestos, arsenic, radon, bis-chloro methyl ether
	Skin cancer	Polycyclic aromatic hydrocarbons, ultraviolet irradiation
Skin	Allergic contact dermatitis	Natural rubber latex, isothiazolins, poison ivy, nickel
	Chemical burns	Sodium hydroxide, hydrogen fluoride
	Chloracne	TCDD†, polychlorinated biphenyls
	Irritant dermatitis	Sodium dodecyl sulfate
Nervous system	Cholinesterase inhibition	Organophosphate insecticides
	Neuronopathy	Methyl mercury
	Parkinsonism	Pesticides, polychlorinated biphenyls, trichloroethylene, carbon disulfide
	Peripheral neuropathy	*N*-hexane, trichloroethylene, acrylamide
Immune system	Autoimmune disease	Vinyl chloride, silica
	Hypersensitivity	See entries for allergic rhinitis, asthma, hypersensitivity pneumonitis, allergic contact dermatitis
	Immunosuppression	TCDD†, lead, mercury, pesticides
Renal disease	Indirect renal failure	Arsine, phosphine, trinitrophenol
	Nephropathy	Paraquat, 1,4-dichlorobenzene, mercuric chloride

(*continued*)

Table 34-2

(Continued)

ORGAN SYSTEM OR DISEASE GROUP	DISEASE	CAUSATIVE AGENT
Cardiovascular disease	Arrhythmias	Acetone, toluene, methylene chloride, trichloroethylene
	Atherosclerosis	Dinitrotoluene, carbon monoxide
	Coronary artery disease	Carbon disulfide
	Cor pulmonale	Beryllium
	Systemic hypotension	Nitroglycerine, ethylene glycol dinitrate
Liver disease	Fatty liver (steatosis)	Carbon tetrachloride, toluene
	Cirrhosis	Arsenic, trichloroethylene
	Hepatocellular death	Dimethylformamide, TCDD[†]
Reproductive system	Male	Chlordecone (Kepone), dibromochloropropane, hexane
	Female	Aniline, styrene
	Both sexes	Carbon disulfide, lead, vinyl chloride
Infectious diseases[*]	Arboviral encephalitides	Alphavirus, Bunyavirus, Flavivirus
	Aspergillosis	*Aspergillus niger, A. fumigatus, A. flavus*
	Cryptosporidiosis	*Cryptosporidium parvum*
	Hepatitis B	Hepatitis B virus
	Histoplasmosis	*Histoplasma capsulatum*
	Legionellosis	*Legionella pneumophila*
	Lyme disease	*Borrelia burgdorferi*
	Psittacosis	*Chlamydia psittaci*
	Tuberculosis	*Mycobacterium tuberculosis hominis*

[*]*For more on occupational infectious diseases, see Douwes et al. (2003).*
[†]*TCDD = 2,3,7,8-tetrachlorodibenzo-para-dioxin.*

Table 34-3 lists 39 agents that are known by the International Agency for Research on Cancer (IARC) to be carcinogens in humans (Group 1) and for which there has been or is currently extensive occupational exposure. This list includes agents such as asbestos, arsenic, benzene, vinyl chloride, and coal tars. IARC Group 1 refers to agents or mixtures that are known to be carcinogenic to humans based on sufficient epidemiologic evidence usually accompanied by limited or sufficient animal evidence.

Occupational Respiratory Diseases

Because inhalation generally represents the most significant route of exposure, many of the major occupational diseases affect the lung and airways. These diseases have been studied extensively and are largely responsible for the creation of the occupational regulatory framework. Deaths due to occupational lung diseases such as asbestosis, coal workers' pneumoconiosis, silicosis, byssinosis, and occupational asthma have been recognized for centuries and led to important legislation such as the U.S. Occupational Safety and Health Act (1970). These occupational lung diseases continue to have significant associations with morbidity and mortality worldwide but are diminishing steadily in the United States and Europe. Table 34-4 lists the U.S. age-adjusted death rate and annual deaths for 2014 and illustrates that while the death rates are fairly low, there are still about 1477 deaths per year attributable to asbestos, silica, coal dust, and other pneumoconiotic dusts; 2786 malignant mesothelioma deaths; and 141 deaths from hypersensitivity pneumonitis. However, fatalities are just the tip of the iceberg as some occupational lung diseases exhibit slow progression yet may still be debilitating. Every year in the United States, there are 20,000 hospital discharges related to cases of asbestosis and 188,000 coal workers receive federal Black Lung Benefits (NIOSH, 2017c). The U.S. Mine Safety and Health Administration has compiled data on inspector- and mine operator-collected samples in coal mining operations and found that 29.3% of these exceeded the PEL for respirable quartz and 7.5% were over the PEL for respirable coal dust.

Many of the diseases listed in Table 34-2 are known by other names that refer to a particular occupation or agent. One example is hypersensitivity pneumonitis, an allergic lung disease marked by interstitial lymphocytic pneumonitis and granulomatous lesions. Hypersensitivity pneumonitis is also known as extrinsic allergic alveolitis, farmer's lung disease, bagassosis (sugar cane), humidifier fever, Japanese summer house fever, pigeon breeder's lung, and maple bark stripper's lung, depending upon the occupational setting in which it arises. Although we often think of these as the same disease, it is important to recognize that the exposures and physiologic responses they induce are complex and may differ in the manifestation of the disease.

The U.S. Bureau of Labor Statistics tracks data for non-fatal occupational injuries and illnesses of which there were 3.3 million in 2009. The vast majority of these were injuries (e.g., falls, slips, strains, sprains, back injuries, and roadway incidents), but there were still over 187,900 occupational illnesses reported in 2015 (USDL, 2015). There were 17,200 reported respiratory conditions arising from exposure to toxic agents and dust diseases of the lung. The former category includes pneumonitis, pharyngitis, rhinitis, and acute lung congestion. Table 34-5 shows data for rates and cases in 2015 by industry division. Poisonings were rare whereas skin diseases occurred at rates as high as 4.3 per 10,000 workers per year. The manufacturing sector accounted for approximately 22% of all occupational illnesses. Industry codes with the highest respiratory condition incidence rates were aquaculture (94.0 per 10,000); oilseed and grain farming (33.7 per 10,000); coal mining (24.1 per 10,000); roasted nuts and peanut butter manufacturing (17.1 per 10,000); and copper, nickel, lead, and zinc mining (14.2 per 10,000). The rate for chemical manufacturing was much lower, 1.7 cases per 10,000 workers, down by 50% since 2000.

Table 34-3

Occupational Exposure Agents Classified by IARC as Group 1 Definite Human Carcinogens

AGENT	INDUSTRIES AND OCCUPATIONS WHERE SOME WORKERS MAY BE EXPOSED
Particulate matter	
Asbestos	Miners, abatement workers, construction workers, sheet metal workers, steam fitters, shipyard workers
Crystalline silica (quartz or cristobalite)	Stone and ceramics industry, foundries, construction, abrasives manufacturing
Erionite	Waste treatment workers, building materials manufacturing
Talc containing asbestiform fibers	Ceramics industry
Wood dust	Wood and wood-products industries, pulp and paper industry, wood working trades
Metals	
Arsenic and arsenic compounds	Miners, nonferrous metal smelting, arsenical pesticide manufacturers and applicators
Beryllium	Specialty metallurgy workers, avionics, electronics, nuclear industry
Cadmium and cadmium compounds	Cadmium smelting, battery production, dyes and pigment making, electroplating
Gallium arsenide	Microelectronics manufacturing
Hexavalent chromium compounds	Chromate production plants, dye and pigment making, welders, tanners
Nickel compounds*	Nickel smelting, welding
Organic chemicals	
Aflatoxins	Animal feed industry, grain handling and processing
4-Aminobiphenyl	Chemical industry, dyestuffs and pigment manufacturing
Benzene	Refineries, shoe industry, chemical, pharmaceutical and rubber industry, printing industry
Benzidine	Chemical industry, dyestuffs and pigment manufacturing
Benzo(*a*)pyrene	Coke oven emissions, coal tar pitch volatiles, diesel exhaust, ETS
Bis(chloromethyl) ether and chloromethyl ether (technical grade)	Chemical industry, laboratory reagent, plastic manufacturing
1,3-Butadiene	Chemical industry, petrochemical plants, styrene–butadiene rubber manufacturing
Coal tars and pitches	Coke production, coal gasification, refineries, foundries, road paving, hot tar roofing
Ethylene oxide	Chemical industry, dry vegetable fumigation, hospital sterilizing
Formaldehyde	Textiles, composite wood industry, chemical industry, medical laboratories
4,4′-Methylenebis(2-chloroaniline) [MOCA]	Epoxy resin manufacturing, polyurethane product fabrication
Mineral oils, untreated and mildly treated	Metal machining and honing, roll steel production, printing
2-Naphthylamine	Chemical industry, dyestuffs and pigment manufacturing
2,3,4,7,8-Pentachlorodibenzofuran	Hazardous waste processing, chlorophenoxy herbicide production and use, pulp and paper industry
3,4,5,3′,4′-Pentachlorobiphenyl‡ [PCB126]	Hazardous waste processing, waterway dredging, transformer handling, pulp and paper industry
Shale oils or shale-derived lubricants	Mining and processing, cotton textile industry
Soot	Chimney sweeps, heating and ventilation contractors, firefighters, metallurgical workers
2,3,7,8-Tetrachlorobibenzo-*para*-dioxin (TCDD)	Hazardous waste processing, chlorophenoxy herbicide production and use, pulp and paper industry
Vinyl chloride	Plastics industry, production of polyvinyl chloride products and copolymers
Other agents with occupational exposure	
Environmental tobacco smoke (ETS)	Restaurant, bar and entertainment industry; other smoke-exposed workers
Occupational exposures as a painter	Commercial painting
Leather dust	Garment industry, auto seat fabrication, saddle and tack manufacturing,
Magenta dye (rosaniline, pararosaniline)	Dye manufacture, textile dying, commercial art and printing
Mustard gas	Production, soldiers, some research laboratories
Exposures in the rubber industry	Work in rubber manufacturing industries
Strong inorganic acid mists containing sulfuric acid	Steel industry, petrochemical industry, fertilizer industry, pickling industry
Physical agents	
Ionizing radiation†	Radiology and nuclear medicine staff, nuclear workers, miners, hazardous waste workers
Solar radiation	Farmers, gardeners and landscapers, lifeguards, construction workers

**Certain combinations of nickel oxides and sulfides.*

†Includes x-rays, γ-rays, neutrons, radon gas, and α and β particle-emitting substances internally deposited.

‡PCBs as a class are listed by IARC as Group 1, carcinogenic to humans as of 2016.

SOURCES: From IARC (n.d., 2016), Siemiatycki et al. (2004), and Cogliano et al. (2011).

Table 34-4

U.S. Deaths and Age-Adjusted Death Rates Attributed to Selected Occupational Lung Diseases for U.S. Residents Age 15 and Over, 2014

DISEASE	NUMBER OF DEATHS†	DEATH RATE PER MILLION WORKING AGE PEOPLE
Asbestosis	1221	4.39
Coal workers pneumoconiosis	363	1.26
Silicosis	84	0.30
Byssinosis	2	0.01
Other pneumoconioses*	139	0.48
Malignant Mesothelioma	2786	9.89
Hypersensitivity pneumonitis	141	0.49

**This includes aluminosis, berylliosis, stannosis, siderosis, and fibrosis from bauxite, graphite fibers, wollastonite, cadmium, portland cement, emery, kaolin, antimony, and mica.*
†Includes underlying and contributing cause deaths.
SOURCE: From NIOSH (2017a).

Toxic gas injuries are often characterized by leakage of both fluid and osmotically active proteins from the vascular tissue into the interstitium and airways. Important determinants of the severity and location of injury are the concentration and water solubility of the toxic gas or vapor. Anhydrous ammonia, with its extremely high solubility, primarily damages the eyes, sinuses, and upper airways. The vapors combine with water in the tissue and form ammonium hydroxide, quickly producing liquefaction necrosis. Chemicals with lower solubility, such as nitrogen dioxide, act more on the distal airways and alveoli and take longer to induce tissue damage.

Occupational asthma may be defined as a "disease characterized by variable airflow limitation and/or airway hyperresponsiveness and/or inflammation due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace" (Bernstein et al., 2013). Workplace asthma is generally divided into allergic, non-allergic, and work-aggravated asthma. Data from the NIOSH Work-Related Occupational Lung Disease Surveillance System has shown that 22% of occupational asthma cases represented aggravation of pre-existing asthma by workplace exposures. The U.S. National Health Interview Survey has provided data on the prevalence of asthma based upon usual industry (Table 34-6). These data indicate that asthma prevalence is much higher for female than male workers and is highest in manufacturing of plastics and rubber; electrical equipment and appliances; chemicals; and transportation equipment. Among the service sectors asthma is prevalent among those engaged in health care and social assistance; education; and

Table 34-5

Nonfatal Occupational Illnesses* Requiring Days Away From Work, 2015 (Number of Cases and Rate Per 10,000 Full Time Workers)

	NUMBER	INCIDENCE RATES			
INDUSTRY DIVISION	TOTAL CASES	OVERALL	RESPIRATORY CONDITIONS**	POISONINGS	SKIN DISEASES OR DISORDERS†
Goods Producing	49,700	24.6	1.3	0.2	3.3
Manufacturing	40,800	32.9	1.3	0.2	3.4
Construction	5100	8.6	0.8	0.2	2.3
Agriculture, Forestry, Fishing, and Hunting‡	3100	31.2	3.9	1.6	10.0
Mining, Quarrying, Oil and Gas Extraction	700	7.5	2.1	–	–
Service Providing§	90,900	12.0	1.2	0.2	2.0
Education and Health Services	37,300	23.6	3.0	0.1	3.7
Trade, Transportation and Utilities	23,700	10.6	0.7	0.1	1.3
Leisure, Entertainment, and Hospitality	10,400	10.8	1.2	0.3	2.4
Finance, Insurance, and Real Estate	3,600	4.9	0.4	–	0.5
Professional and Business Services	11,800	7.8	0.8	0.2	2.1
Information	1,900	7.8	0.3	–	0.8
State and Local Government	47,400	32.0	3.4	0.5	4.3
Total number of reported cases	187,900		17,200	2500	28,300

**Illnesses excludes injuries. Not shown are the rates for hearing loss and illnesses.*
†These conditions are contact dermatitis, eczema, or rash caused by primary irritants and sensitizers or poisonous plants; oil acne; friction blisters, chrome ulcers; and inflammation.
‡Agricultural illnesses are grossly underreported because the data exclude farms with fewer than 11 employees.
§Includes auto repair, other repair, health care, hotel, personal, business, entertainment, educational, social, and engineering services.
***These conditions are pneumonitis; pharyngitis; rhinitis; acute congestions due to chemicals, dusts, gases, or fumes; and other dust diseases of the lung.*
SOURCE: From USDL (2015).

Table 34-6

U.S. Asthma Prevalence* by Current Industry and Sex for Selected Industries, Age 18 and Over (Annual Averages for 2004 to 2011)

INDUSTRY	ESTIMATED POPULATION	ASTHMA PREVALENCE (%)		
		TOTAL	FEMALE	MALE
Manufacturing				
Plastics and Rubber	613,195	7.7	11.8	5.9
Electrical Equipment, Appliances, and Components	435,146	6.8	13.4	3.5
Chemicals	1,077,030	6.2	10.1	4.2
Transportation Equipment	2,000,534	6.0	7.5	5.6
Miscellaneous Manufacturing	1,129,782	6.0	8.7	4.2
Textile Products	182,064	5.4	8.3	‡
Printing and Related Support Activities	661,067	4.8	7.7	3.5
Furniture and Related Products	569,204	4.6	5.3	4.3
Wood Products	496,446	4.2	7.2	3.4
Food	1,640,559	4.1	6.0	2.9
Retail and Services				
Gasoline Stations	446,793	8.7	13.8	‡
Health Care and Social Assistance	17,573,528	8.4	9.1	5.5
Furniture and Home Furnishings Stores	532,896	8.1	13.1	4.8
Education Services	13,160,030	7.8	8.8	5.8
Electronics and Appliance Stores	590,657	7.5	10.5	6.3
Personal Services†	1,997,487	6.7	8.1	3.1
Public Administration	6,944,968	6.4	8.3	4.9
Food and Beverage Stores	2,705,058	6.2	8.2	4.1
Merchant Wholesalers Durable Goods	2,048,004	6.0	7.9	5.4
Transportation	3,923,650	5.3	9.6	4.1
Construction	10,262,670	4.4	6.0	4.2
Agriculture (Animal and Crop)	1,614,617	4.1	5.0	3.9

**Current asthma defined as those who have ever been told by a health professional that they had asthma and currently still have asthma.*
†Barber shops, beauty salons, nail salons, laundry, funeral homes, and cemeteries.
‡Data suppressed due to standard error >50%.
SOURCE: From NIOSH (2015).

personal services. In retail, prevalence is highest among gasoline station workers and employees of furniture and home furnishings stores and businesses selling electronics and appliances.

There are a variety of industries in which there is increased risk of developing work-related asthma. In chemical-based industries, plastic and rubber polymer precursors, diisocyanates, reactive dyes, and acid anhydrides are recognized low-molecular-weight sensitizing compounds. Biocides and fungicides used in metal fabrication and machining, custodial services, lawn and turf growing, and agriculture are also chemicals associated with occupational asthma. A number of metals can induce sensitization and asthma, including chromium, cobalt, nickel, platinum, and zinc. Enzymes pose significant risks for occupational asthma (Heederik et al., 2002). Examples include α-amylase among bakery workers and subtilisin, a protease used in laundry detergents. The enzyme production industry has had to adopt strict environmental and process controls to reduce the incidence of occupational asthma in their production facilities.

Those working with animals or animal products are at increased risk of developing allergy (Elliott et al., 2005) and occupational asthma (Pacheco et al., 2003). Lab animal workers have concurrent exposure to animal allergens and bacterial endotoxin and being allergic modifies the relationship between endotoxin exposure and respiratory symptoms (Newton et al., 2017). Animal handlers, processors, and laboratory technicians who work with animals can become immunologically sensitized to urine or salivary proteins in many vertebrates; proteins in bat guano and bird droppings; animal dander; serum proteins in blood products; dust from horns, antlers, and tusks; or the shells of crustaceans. Very high rates of sensitization can occur in shellfish processors (Glass et al., 1998). Arthropods such as insect larvae, cockroaches, mites, or weevils are recognized inducers of work-related asthma. Plants and plant products (e.g., soy flour, spices, and coffee beans) can also cause asthma among workers. In a variety of occupations, exposure to fungi, especially of the genera *Aspergillus, Penicillium, Rhizopus, Mucor*, and *Paecilomyces*, is associated with allergic rhinitis and asthma. These are especially present in sawmills, woodchip handling, and composting facilities (Eduard et al., 1992; Duchaine et al., 2000). Apart from the contaminating microorganisms, certain woods themselves produce chemical sensitizing agents. Examples include western red cedar, redwood, and some tropical hardwoods.

Asthma emerged in the 1980s as a major occupational health concern among health-care workers. In order to reduce the risk of hepatitis B and other infectious diseases, health-care workers adopted the use of natural rubber latex gloves for barrier protection. Proteins from the latex of the rubber tree, *Hevea braziliensis*, led to immunologic sensitization. Thirteen of these high-molecular-weight

proteins have been characterized as allergens (Bernstein et al., 2003). People with latex allergy may also be sensitive to banana, avocado, chestnut, and kiwi due to cross-reactivity with the latex allergen fragment hevein (Radauer et al., 2011). Many other plants of less commercial value than *Hevea* produce a similar milky fluid when cut and have similar sensitizing properties. A shift to powder-free, nitrile, and vinyl examination gloves has reduced the incidence of latex allergy among health-care workers and patients.

Studies of asthma prevalence in occupational settings with exposure to low-molecular-weight chemicals have suggested prevalence rates of 5% to 10% for toluene diisocyanate (Baur, 1996; Becklake et al., 2006), 3.2% to 18% for anhydrides (Venables et al., 1985; Wernfors et al., 1986), 4% for plicatic acid from western red cedar (Chan-Yeung et al., 1984), and 54% in a platinum refinery (Venables et al., 1989). For high-molecular-weight allergens, prevalence among shellfish processors was estimated at 21% to 26% (Desjardins et al., 1995; Glass et al., 1998), 11% to 44% among lab animal workers (Cullinan et al., 1994; Fuortes et al., 1997; Hollander et al., 1997), and 5% to 7% among bakers exposed to wheat and α-amylase (Heederik et al., 2002).

Agricultural workers exposed to grain dust, cotton dust, or atmospheres in swine or poultry confinement barns may have reduced risk to developing allergy due to innate immune responses to inhaled endotoxin and microorganisms. However, they are at high risk for the development of an asthma-like syndrome. This syndrome is an acute nonallergic airway response characterized by self-limited inflammation with neutrophilic infiltrates and increased pro-inflammatory cytokines and chemokines (e.g., TNFα, IL-6, and IL-8), but it does not include persistent airway hyperreactivity, as in occupational asthma (Schenker et al., 1998). Asthma-like syndrome includes cough, mild dyspnea, fever, malaise, and cross-shift declines in lung function. Endotoxin in combination with other inflammatory bioaerosols is the likely etiologic agent (Douwes et al., 2003).

An emerging area of concern is adverse effects of respiratory exposures to manufactured nanomaterials. There are currently over 1800 consumer products that contain nanotechnology-based materials (Vance et al., 2015). Nanomaterials are engineered structures that have a primary size less than 100 nm in at least one dimension. Airborne nanoparticles generally form agglomerates in which groups of particles are held together by relatively weak forces (e.g., van der Waals). They may also aggregate forming clusters that are fused, sintered, or chemically bonded to one another (Pettibone et al., 2008). Manufactured nanomaterials find applications in electronics, construction materials including wall and floor coverings, cosmetics, drug delivery, medical imaging, food products, packaging materials, textiles, and sporting equipment, to name a few. Occupational exposures occur in the manufacture of the nanomaterials and in their use in fabricating materials and consumer products. Exposures can also occur when nanomaterials are cut or shaped and when product waste is discarded. Engineered nanomaterials may be carbon-based, metal-based, or biological in nature. Biological actions of nanoscaled materials may differ from the comparable bulk materials or micron-sized particles (NIOSH, 2013). Inhaled nanomaterials may induce pulmonary toxicity or they can cause adverse effects in other tissues through adsorption and transport, generation of toxic substances by their dissolution or degradation, or by crossing key physiological barriers, or cell and nuclear membranes (Kim et al., 2011). Mechanisms of nanotoxicity include oxidative stress, inflammation, cytotoxicity, genotoxicity, and developmental toxicity (Adamčaková-Dodd et al., 2014, 2015). Metal and metal-oxide nanoparticles absorb materials from the airway surface fluid and acquire protein coronas that can affect their pulmonary clearance (Konduru et al., 2017). In early 2011, NIOSH issued a new guideline on TiO_2, marking the first instance of two guidelines for the same compound based on particle size. In 2013, NIOSH issued a *Current Intelligence Bulletin* on carbon nanotubes and nanofibers that proposed an REL of 1 $\mu g/m^3$ elemental carbon as an 8-hour time-weighted average (TWA) concentration (NIOSH, 2013). Research programs and regulatory bodies in the United States and the EU are studying the toxicity of nanomaterials in commercial use and conducting human health risk assessments.

Other Occupational Diseases

Occupational diseases of the skin are common but less often fatal than those of the respiratory system. Irritant dermatitis and allergic contact dermatitis have the highest incidence of reported skin conditions. Table 34-5 shows data for rates of skin diseases or disorders in 2015 by industry division. These data indicate that agriculture, forestry, fishing, and hunting carry the highest risks with an incidence rate of 10 per 10,000 full-time workers. The incidence for the goods-producing sector overall is 3.3 per 10,000, higher than the rate for service sector workers. In the service sector, the education and health services division has the highest rate of skin diseases. This is due to the high rate of skin disorders among workers in nursing care facilities (10.4 per 10,000) and assisted living facilities (9.4 per 10,000).

Occupational toxicants may induce diseases in a variety of body sites distant from the lung or skin. These include tumors arising in the liver, bladder, gastrointestinal tract, or hematopoietic system and are attributable to a variety of chemical classes. Further discussion of other occupational diseases and the toxicants listed in Table 34-2 can be found in the relevant chapters in this book.

Nervous system damage can be central, peripheral, or both. It may be acute, as with some organophosphate exposures, or chronic, as with organomercury poisoning or acrylamide-induced neuropathy. Injury affecting the immune system may arise from the immunosuppressive effects of chemicals such as dioxins or toxic metals. Many occupational diseases of the immune system occur due to hypersensitivity leading to respiratory or dermal allergy or systemic hypersensitivity reactions. Autoimmune syndromes have been associated with occupational exposures to crystalline silica and vinyl chloride.

Occupational diseases of the cardiovascular system include atherosclerosis, a variety of arrhythmias, problems with coronary blood supply, systemic hypotension, and cor pulmonale (right ventricular hypertrophy usually due to pulmonary hypertension as with chronic obstructive pulmonary disease). Liver diseases such as carbon tetrachloride-induced fatty liver and hepatocellular death due to toxic concentrations of acetaminophen have classically been used to illustrate chemical mechanisms of cellular injury leading to organ failure. These are thoroughly discussed elsewhere in this book. Occupational diseases of the reproductive system can be sex- and organ-specific, but several toxicants—including carbon disulfide, lead, and vinyl chloride—may affect both sexes.

Exposures to infectious agents are a part of a variety of occupations (Thorne et al., 2015). Veterinarians, health-care workers, and biomedical researchers studying infectious agents have exposures that are largely known and infection control strategies can limit their risks. For others, such as farmers and foresters, specific risks may be less obvious. Zoonotic diseases such as Q-fever, rabies, leptospirosis, and brucellosis may affect abattoir workers, zookeepers, animal handlers, and veterinarians. Foresters, field biologists, and natural resource workers who spend time in wooded areas experience tick- and mosquito-borne illnesses at a higher frequency than that of the general population. These illnesses include the West Nile

Fever and other arboviral encephalitides, chikungunya, Dengue fever, Lyme disease, and ehrlichiosis. Occupational infections may arise as a result of work settings bringing people into close proximity with other people or animals, thus facilitating the transmission of microorganisms. Occupational infectious diseases attributable to the clustering of people affect workers in such facilities as day care centers, schools, health-care settings, correctional facilities, dormitories, military barracks, or shelters for the homeless. Industrial settings can place large numbers of workers in a shared space, leading to increased transmission of diseases. This is especially true for diseases with annual outbreaks, such as influenza. Exposures to chemicals may increase the susceptibility of workers to infection through irritation of mucosa or the pulmonary epithelium or through immunosuppression leading to impaired host defense.

Both industrial and nonindustrial occupational environments may pose occupational hazards due to the presence of chemical or biological agents. Reports of work environments with ineffective ventilation or decreased ventilation rates and increased utilization of synthetic building materials have demonstrated a rise in complaints associated with occupancy in buildings. In some cases, service sector workers in a problem building develop specific clinical conditions with recognized etiology. This is defined as building-related illness. In other cases, symptoms are nonspecific and disappear when the worker leaves the problem building. When this occurs with sufficient prevalence, it is termed *sick building syndrome*. This can arise from volatile and semivolatile chemicals released from office materials, building materials, floor coverings, furniture, cleaning products, and microorganisms. Office buildings and residential settings comprise a complex ecology consisting of people, molds, mites, volatile organic compounds of microbial and nonmicrobial origin, and sometimes plants, pets, cockroaches, and other vermin (Thorne and Heederik, 1999b). Molds, house dust mites, and animal proteins are potent human allergens that can lead to allergy and asthma. Exposure to chemicals and biomolecules such as endotoxin may enhance this process (Douwes et al., 2003). Workers in laboratory animal facilities exhibit a high prevalence of allergy to rodent urinary proteins. In some cases, the occupied space of a building may be clean and dry, but local amplification sites for molds may develop. These may arise in ventilation systems, utility closets, subfloors, or basements that serve as return air plenums, or in local sites of water damage. Such sites can become sources of microorganisms and aeroallergens of sufficient volume to generate significant bioaerosol exposures throughout the environment. Airborne viruses, bacteria, and fungi are responsible for a variety of building-related illnesses arising from organisms that are pathogenic to humans. Nonpathogenic microorganisms may induce symptoms or diseases through inflammatory processes, by stimulating innate or adaptive immune response, or by releasing noxious odors, allergenic compounds, or bioactive macromolecules. These may combine with industrial chemicals released into the air to create complex exposure environments.

TOXICOLOGIC EVALUATION OF OCCUPATIONAL AGENTS

Evaluation of Occupational Risks

In most instances of prolonged exposure to low levels of chemicals, there is a continuum between being healthy and being ill. The exposures may impart biochemical or functional changes that are without signs or symptoms, subclinical changes, or in cases of more significant toxicity, manifest as clinical disease. The health significance of the identified changes resulting from exposure to a particular agent must be assessed in order to determine which effects are adverse. Following this assessment, one must consider the interindividual variability or susceptibility factors that influence the risks. There is no single dose–effect relationship but a distribution of responses. Therefore, in order to recommend an acceptable exposure level to an industrial chemical, one must attempt to define the risk associated with adverse effects in the most sensitive populations exposed. It then remains to be determined what proportion of exposed subjects may still develop an adverse effect at the proposed acceptable exposure level. This acceptable risk level will vary according to a value judgment of the severity, permanence, and equity of the potential adverse effects and the characteristics of the most susceptible population. Clearly, inhibition of an enzyme without functional consequences will be viewed as more acceptable than a chronic non-fatal disease and in turn a more serious toxic effect, such as teratogenicity leading to a congenital malformation in the offspring of the exposed individual.

Establishing Causality In complex occupational environments, it may be difficult to establish a causal relationship between a toxic substance and a disease. For this reason a number of systematic approaches have been devised to help define causation. In 1890, Robert Koch proposed postulates for "proving" that a specific organism caused a specific disease. T. M. Rivers extended this approach to viruses in 1937. Sir Austin Bradford Hill suggested epidemiologic criteria for assessing causality in 1965 considering strength, specificity, consistency, temporality, exposure period, exposure gradients, and biological plausibility of the associations. These schema and modern weight-of-evidence determination criteria led to systematized approaches for establishing strength of associations. U.S. EPA criteria for establishing weight-of-evidence are illustrated in Fig. 34-3. Note that extensive study is required to establish a clear causal link between exposure to a toxicant and an adverse health outcome. Fig. 34-4 provides a matrix to evaluate the weight-of-evidence for a causal association between a

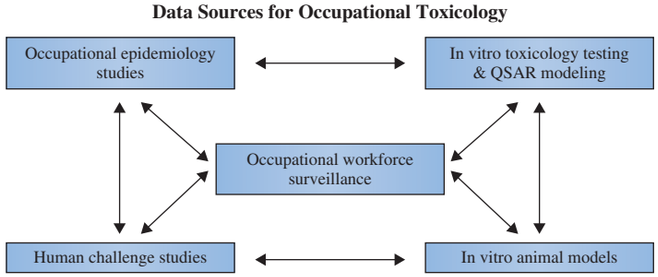

Figure 34-3. *Data sources for occupational toxicology decision making.*

	Assessment of exposure to specific agents	Consideration or control of confounders	Evidence of a dose – response relationship	Consistent results from different studies	Objective clinical data	Endpoints related to human pathology	Appropriate subjects or models
In vitro studies							
Animal studies							
Human challenge studies							
Case studies							
Epidemiology studies							

For each type of study listed in the first column weight the quality of data from existing studies based on the criteria listed in the column headings as follows:

0 No evidence or condition is not met
1 Equivocal evidence or condition is partially met
2 Some evidence or condition is mostly met
3 Clear evidence or condition is convincingly met
4 Squares shown in pink should be left blank

Figure 34-4. *Matrix for assessing the strength of an association between a toxicant and an occupational disease.*

toxicant and an occupational disease (Donham and Thorne, 1994). Evidence from well-conducted in vitro studies, animal toxicology studies, controlled human challenge studies, case reports, and epidemiologic investigations is evaluated with regard to data quality and clarity of evidence in support of the establishment of causality. This evaluation is guided by seven criteria. If a chemical were thoroughly studied in animals, humans, and in vitro studies and it produced clear and convincing evidence of an exposure–response relationship in controlled studies that used appropriate models and relevant endpoints, it would constitute compelling evidence of a causal relationship between that chemical and that disease. Fig. 34-3 reminds us that a consortium of study types contributes data used for the evaluation of occupational hazards. These are discussed below.

In Vitro Assays A number of useful in vitro assays have been developed over the past several decades in order to provide screening data and, in some cases, mechanistic insight without the need or expense of exposing animal or human subjects. While at this time there are few validated methods to determine complex toxicologic responses such as pulmonary hypersensitivity or peripheral neuropathy, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) lists recommended test method protocols for acute systemic toxicity, endocrine disruptors, eye corrosion and irritation, pyrogenicity, and skin sensitization (ICCVAM, 2017). Examples include the Direct Peptide Reactivity assay (OECD TG 442C) and the ARE-Nrf2 Luciferase (KeratinoSens) assay (OECD TG 442D) for predicting skin sensitizers. These methods were approved initially by the European Centre for the Validation of Alternative Methods (ECVAM) and were then accepted by the 32-nation Organization of Economic Cooperation and Development (OECD). Another example is the Bovine Corneal Opacity and Permeability (BCOP) test used to assess the potential for chemicals and consumer products to induce eye injuries (OECD TG 437). In addition, quantitative structure–activity relationships can help suggest potential toxicologic effects for an unstudied compound if structurally similar compounds have been evaluated.

Animal Toxicology Studies Animal toxicology studies serve an important function in terms of identifying adverse effects, providing mechanistic data, establishing dose–response relationships, and aiding the process of establishing standards. Because animal studies can be conducted before there is any human exposure, these studies play an important role in hazard identification and prevention of human disease. There are numerous animal models for occupational injury and illness; these are described throughout this textbook in the context of the affected organ system and the classes of toxicants. Generation of animal toxicology data to predict health effects in workers is a central function of experimental toxicologists.

Toxicologic investigations using animals often serve to establish a tentative acceptable exposure level. Other important information that may also be derived from these investigations concerns the relationships between the metabolic handling of the chemical and its interactions with target molecules (mechanism of action), identification of methods for biological monitoring of exposure and early health effects, and identification of preexisting pathologic states that may increase susceptibility to the chemical. However, animal testing can provide only an estimate of the toxicity of a chemical for humans. Animals do not always respond to a chemical exposure in the same way as humans. For instance, there are very significant species and strain differences in responsiveness to aryl hydrocarbon receptor agonists such as polychlorinated dibenzo-*p*-dioxins (Abnet et al., 1999; Boverhof et al., 2006). In some instances, interspecies differences in metabolism or mechanism of action cause certain chemicals to induce cancer in rodents but not in humans. One such example is kidney cancer, attributable to the accumulation of a rat-specific protein (α_{2u}-globulin) in proximal tubular cells, and produced in male rats chronically exposed to unleaded gasoline (Hard et al., 1993). There are a few compounds for which predictive animal models have not been found.

Human Challenge Studies Controlled human challenge studies, or clinical exposure studies, are a useful approach for verifying findings from animal toxicology studies in humans and for establishing whether biotransformation pathways in the animal models represent those in exposed humans. Human challenge studies with occupational toxicants are usually designed to answer very specific questions regarding rates of uptake, biotransformation pathways, the time course of metabolite excretion during and after exposure, evaluation of the threshold concentration for sensory responses (odor, irritation of the nasal mucosa, etc.), and acute effects of toxicant exposure on perception, vigilance, and lung, cardiac, or neurobehavioral function. Human challenge studies help to establish biomarkers of exposure. For reversible conditions, they can be useful for testing therapeutic options. They may also be useful for investigating bronchial hyperresponsiveness to inhaled agents. Extreme caution must be exercised to ensure the safety of research subjects and the scientific benefits must be balanced against the risks to participants (National Academies of Sciences, Engineering, and Medicine, 2017). Idiosyncratic responses can cause a subject to be exceptionally sensitive. For inhalation studies, equipment malfunction can result in overexposure, so real-time exposure monitoring is a necessity. In the past 25 years, there have been several serious injuries and fatalities associated with human challenge studies. These have been attributed to hypersensitivity reactions and generally used mock workplace simulations without rigorous control and monitoring of exposures, as is the current standard of practice. Thus, such studies should be undertaken only when the required data cannot be obtained through other means and under circumstances in which the risk for volunteers can reasonably be estimated as negligible.

Case Reports When new toxicants, new combinations of toxicants, or changes in process conditions occur in the workplace, a case or outbreak of cases can occur. These may be identified through workplace surveillance systems, OSHA 300 logs, or through workers associating their disease with workplace exposures. In some cases, the problem is identified quickly and resolved, while others take years to resolve. These are often published as case reports and may give rise to animal or epidemiologic studies. Cases of hypersensitivity pneumonitis among machinists exposed to metalworking fluids contaminated with mycobacteria is one example (Weiss et al., 2002; Thorne et al., 2006). Another example is diacetyl-induced bronchiolitis obliterans that was first recognized in workers manufacturing artificially flavored microwave popcorn (Kreiss et al., 2002). NIOSH has a Health Hazard Evaluation (HHE) program in place in which employees or their authorized representatives (i.e., union officials) or employers at a job site can request an investigation to evaluate a potentially hazardous situation (NIOSH, 2017b). This program issues HHE reports to disseminate information regarding the hazard. NIOSH also publishes NIOSH Alerts, Criteria Documents, Fatal Assessment and Control Evaluation Reports, and Current Intelligence Bulletins. It also publishes Joint Occupational Health Documents with similar entities in foreign governments. While useful for hazard identification and mitigation, case reports and HHE reports generally do not establish incidence or prevalence of diseases associated with an occupational hazard.

Epidemiology Studies Epidemiology studies help to unravel the associations between occupational diseases, exposures, and personal risk factors. Exposure may be characterized using a surrogate measure such as job classification or via questionnaire, or more directly through exposure monitoring or biomonitoring. Adverse effects may be expressed in terms of mortality, incidence, or prevalence of clinical disease, irreversible or reversible functional changes, or critical biological changes.

Several types of epidemiologic studies are used to gather data on the association of workplace exposures with human disease. Cross-sectional studies compare disease prevalence or health status between groups of workers classified according to job title, work site, or exposure status. Cohort studies compare exposed workers versus unexposed or less exposed workers either prospectively or retrospectively in order to associate the occurrence of disease with exposure. Because many occupational diseases have a long induction period or occur only rarely, prospective cohort studies may require a long time and need a large number of subjects to establish significant findings. Retrospective cohort studies can resolve the latency problem but require that relevant exposure data have been collected over time. In the absence of measured exposures, job titles, tasks routinely performed, and years of employment may allow categorical exposure classification (e.g., low, medium, and high). Exposure misclassification is frequently a problem in retrospective studies that reduces the likelihood of associating a disease with a particular exposure.

Case–control studies are useful for investigating rare diseases or diseases with long induction periods. As the name suggests, case–control studies compare workers with disease to workers without disease with regard to their past exposure intensity, frequency, and duration, plus other postulated risk factors. In some instances where the exposure–disease relationship is not understood, it may be difficult to identify an appropriate control group. Case–control studies are strongest when accompanied by a rich data set of measured exposures to the candidate causative agents spanning the relevant exposure period. If exposure history is assessed by questionnaire after workers have developed adverse health effects, there arises the potential for recall bias in which those with disease may recall past exposures differently than those who are free of disease. Error can also arise due to selection bias if those who agree to participate in the study are not representative of the population of interest.

Characteristics of observational epidemiology studies are listed in Table 34-7. Occupational epidemiology studies assess relationships between exposures and human health outcomes and, therefore, are particularly useful for risk assessment. Confounding may arise due to exposures of risk factors not associated with the work environment.

Because measures of effect may be subtle and may overlay a background level of incidence, results generally require sophisticated statistical comparisons between a group of exposed workers and a similar group of workers without the exposure of interest. Ideally, the group of unexposed workers should be matched on variables such as age, race, gender, socioeconomic status, and smoking habits. They should also undergo the same standardized clinical, biological, or physiologic evaluation at the same time as the exposed group. Comparison with the general population is ill-advised because an employed population is a highly selected group and may have a higher degree of physical fitness, called the healthy worker effect. Because occupational epidemiologic studies often last for several years, all methods of investigation—such as questionnaires, measurement instruments, and analytic techniques—must be validated and standardized before the start of the study. If results will be compared to prior or contemporaneous studies, the same questionnaires should be used. The number of subjects under study should be chosen based on a sample size calculation to be able to detect a difference between exposed and unexposed subjects (should there be a difference) and should take into account labor turnover and those declining participation in any aspects of the study. If exposures are high enough to induce an adverse effect, it is expected that

these studies may permit establishment of the relationship between integrated exposure (intensity × time) and frequency of abnormal results and, consequently, a redefinition of the OEL.

In most cases, occupational epidemiologic studies encompass the collection of samples from the subjects or data obtained through interaction with the subjects. Because this often includes identifiable private information, the confidentiality of the data must be protected. U.S., EU, and international laws require that subjects must always have the right to refuse participation and investigators must have written consent from a duly informed participant obtained without coercion.

Animal Toxicology Testing for Establishing Acceptable Levels of Exposure

It is evident that certainty as to the complete safety of a chemical can never be obtained, regardless of the extent of toxicologic investigations performed on animals. Nevertheless, animal studies provide valuable data from which to estimate the level of exposure at which the risk of health impairment is acceptable. Table 34-7 compares the information gained from animal studies to epidemiology studies. To the extent possible, animal studies should employ species for which the metabolic pathways and disease processes reflect those of humans. Guidelines and protocols for assessing experimentally the toxicologic hazards of chemicals have been formulated by various national and international agencies. These tests include local and systemic acute toxicity tests, tests of toxicity following repeated exposure, investigations of metabolism and mechanism of action, short-term tests for detecting potential mutagens and carcinogens, studies of effect on reproduction and of teratogenic activity, chronic studies to detect carcinogenesis and other long-term effects, interaction studies, tests for immunosuppression, and dermal and pulmonary hypersensitivity tests. The need for performing these testing protocols should be carefully evaluated for the inclusion of any occupational toxicant to which workers will be exposed. In selecting the studies most appropriate for safety evaluation, the toxicologist should be guided by an understanding of the following:

- Physicochemical properties of the chemical
- Potential for the generation of toxic derivatives when the chemical is submitted to heat, pH changes, and UV light
- Conditions of use and route of exposure
- Type of exposure (continuous, intermittent, or incidental)
- Degree of exposure

Toxicologic information already available on other chemicals with similar chemical structure and reactive chemical groups can suggest potential hazards and reactivity.

Conclusions drawn from any toxicologic investigation are useful only if the composition and physical state of the tested preparation is known. This would include the nature and concentration of impurities or degradation products, speciation of inorganic

Table 34-7

Comparison of Epidemiologic Studies and Experimental Exposure Studies

	OBSERVATIONAL EPIDEMIOLOGIC STUDIES	EXPERIMENTAL ANIMAL EXPOSURE STUDIES
Toxicant exposure	Reflects true exposure among population at risk	Controlled to represent major toxicant of interest
Character	Complex and variable in space and time	Usually one or two test compounds
	May include nonoccupational exposures to toxicant or related compounds	May not reflect complexity of human exposures
Frequency and duration	Work day, work week, and years in that job may be task specific	Acute, subacute, subchronic, chronic
Exposure route	Inhalation, ingestion, percutaneous, or a combination	Injection, inhalation, oral, or dermal; rarely a combination by design
Appropriateness of dose	Reflect the actual range of exposure	Often doses studied are far higher than human exposures
Assessment	Environmental sampling or measurement of biomarkers	Measurement of administered dose with or without measurement of biomarkers
	May be retrospective and based on employer records, group-based approaches, or questionnaires	Sampling of exposure chamber air for inhalation studies
Species considerations	Humans—cohorts or cases and controls	Laboratory animals, usually inbred strains of mice or rats
	Must protect the safety and confidentiality of subjects	Must ensure proper care and use of animals
Representativeness	May exist a selection bias such that the study population may not represent the occupational work force	Experimental animal species may not represent humans
Relevance to human health	Directly relevant if appropriate outcomes are studied	Relevant if species differences are known
		Of limited relevance if species or strain effects on absorption, distribution, metabolism, and disease are unknown
Analytical challenges	Selection bias, misclassification, and confounding in characterization of outcomes	Control of genetics, feeding, and housing between exposed and control groups
	Within- and between-subject variance may be high	Low variance in outcomes

compounds, characterization of physicochemical properties for inhaled materials, and characterization of the vehicle (the carrier, diluent, or excipient of the toxicant). Sensitive and specific methods of analysis of the chemical in solution, air, and biological material should also be available. The assessment of the toxicity of malathion illustrates this point. Malathion is an organophosphate insecticide that normally has relatively low human toxicity. This pesticide was responsible for an episode of mass poisoning among malaria workers in Pakistan because the specific product contained impurities (mainly isomalathion) capable of inhibiting tissue and plasma carboxyesterases (Aldridge et al., 1979) and inactivating acetylcholinesterase via formation of adducts (Doorn et al., 2003). The toxicity evaluation for malathion had not anticipated isomalathion coexposure. A recent study evaluated the pulmonary toxicity of inhaled aluminum nanowhiskers (Adamčaková-Dodd et al., 2012). The manufacturer of this material stated they were Al_2O_3 whiskers. Careful physicochemical analysis prior to toxicity evaluation using X-ray photoelectron spectroscopy and transmission electron microscopy revealed they were actually mixed phase aluminum hydroxy structures containing $Al(OH)_3$ and γ-AlOOH. Further, the whiskers were 100 to 400 nm in length, considerably shorter than the 2800 nm length claimed by the manufacturer.

The duration of tests necessary to establish an acceptable level for occupational exposure is primarily a function of the type of toxic action suspected. It is generally recognized that for systemically acting chemicals, subacute and short-term toxicity studies are usually insufficient for proposing OELs. Subacute and short-term toxicity tests are usually performed to find out whether the compound exhibits immunotoxic properties and cumulative characteristics. They also aid in the selection of the doses for long-term-exposure studies and the kind of tests that may be most informative when applied during long-term exposures. A number of studies have drawn attention to the fact that the reproductive system may also be the target organ of industrial chemicals (e.g., glycol ethers, styrene, lead, dibromochloropropane). Thus, studies designed to evaluate reproductive effects and teratogenicity should also be considered during routine toxicologic testing of occupational toxicants.

Information derived from exposure routes similar to those experienced by workers is clearly the most relevant. For airborne pollutants, inhalation exposure studies provide the basic data on which provisional OELs are based. Experimental methodology is much more complicated for inhalation studies than for oral administration experiments and requires more specialized equipment and expertise. For example, in the case of exposure to an aerosol, particle size distribution must be evaluated and the degree of retention in the respiratory tract of the animal species under study should be established. Ideally, particle size should be selected according to the deposition pattern of dry or liquid aerosols in the particular animal species used in order to represent human lung deposition with occupational exposures. Particle deposition and retention curves have been published for human, monkey, dog, rabbit, guinea pig, rat, and mouse (Schlesinger, 1985; Hsieh et al., 1999; Schroeter et al., 2013; Asgharian et al., 2014, 2016). It should also be kept in mind that the concentration of the material in the air and the duration of exposure do not give a direct estimate of the dose, because retained dose is also dependent on the minute volume and the proportion of inhaled particles retained. Measurement of pulmonary dust retention following exposure to a radiolabeled or fluorescently tagged test aerosol should be performed prior to conducting acute, subchronic, or chronic studies. This allows one to assess deposition and determine whether the selected levels of exposure may overwhelm pulmonary clearance mechanisms (ILSI, 2000; Oberdörster, 2002).

The choice of studies to perform and their routes of administration must be evaluated scientifically for each toxicant. Important considerations include its target sites and mechanism of action, metabolism, the nature of its adverse effects, and how workers are exposed to the toxicant. The morphologic, physiologic, and biological parameters that are usually evaluated, either at regular intervals in the course of the exposure period or at its termination, are described elsewhere in this book. Investigations that can make use of specific physiologic or biochemical tests, based on the knowledge of the principal target organ or function, produce highly valuable information and increase confidence in the OEL derived from them.

Worker Health Surveillance

The primary objective of worker health surveillance programs is to provide both periodic screening of general health and wellness plus health and exposure monitoring tailored to recognized hazards of the workplace. The monitoring of exposures to toxicants in the workplace may play an important role in detecting excessive exposures before the occurrence of significant biological disturbances and health impairment. A scheme for biological monitoring of exposure and of early biological effects is possible only when sufficient toxicologic information has been gathered from in vitro, animal, or human studies on the mechanism of action and the metabolism of xenobiotics to which workers are exposed. When a new chemical is being used on a large scale, the careful clinical surveillance of workers and monitoring of workplaces should be instituted in order to address three aims: (1) to identify overexposure or adverse effects on the health of the workers and quickly intervene, (2) to evaluate the validity of an existing or proposed OEL, and (3) to test the validity of a proposed method for biological monitoring.

Evaluation of the validity of the proposed OEL derived from animal experiments through workplace surveillance is the major aim because studies and observations on humans are the final basis for deciding whether an OEL set originally on the basis of animal toxicity testing is truly acceptable as one that will not produce excess health risks. This means that sensitive clinical, biochemical, physiologic, or behavioral tests for detecting an adverse effect of a toxicant should ideally be performed on the workers concurrent with exposure assessment. It is helpful if health surveillance programs can include the same biomarkers as used in prior animal or human exposure studies. Occupational toxicologists and occupational physicians cannot rely solely on the standard diagnostic tools used in clinical medicine, as they were established primarily to reveal advanced pathologic states and not to detect early adverse effects at a stage when they are still reversible. For example, the measurement of serum creatinine is still a widely used clinical test for assessing renal integrity; yet it is known that the glomerular filtration rate of the kidney must be reduced by more than 50% before serum creatinine rises significantly.

The main limitation of current OELs or BEIs is that some are based on limited experimental data or clinical studies in which only late effects have been investigated and correlated with past exposure. Furthermore, several BEIs are derived from the study of external–internal exposure relationships and not from relationships between internal dose and early adverse effects. The validity of an OEL is much stronger if it is based on the study of dose–response relationships in which the dose is expressed in terms of the cumulative target dose and the monitored effect reflects a critical biological event. However, for some chemicals and some adverse effects (e.g., induction of hypersensitivity and possibly genotoxic

effects), the frequency of peak exposure may be more important for health risk assessment than the integrated dose. For example, long-term low-level exposures to commercial enzymes rarely induce sensitization. However, a single exposure to a high concentration can produce hypersensitivity and occupational asthma.

In cases where a surveillance program was not instituted before the introduction of a new chemical, it is more difficult to establish the efficacy of the OEL. In this situation, evaluation depends on retrospective cohort studies or case–control studies on workers who have already sustained exposure. Evaluation of a "no observed adverse effect level" (NOAEL) is difficult because information on past exposures is often incomplete and frank effects are generally the focus of retrospective or case–control studies. Provided that a satisfactory assessment of past exposure is possible, cross-sectional studies that rely on preclinical signs of toxicity may, to a certain extent, overcome these difficulties. Whether or not clinical investigations are planned from the introduction of a new chemical or process, it is essential to keep standardized records of occupational histories and exposure. The need may arise for mortality or case history studies in order to answer an urgent question on a suspected risk.

Careful investigation of overexposures resulting from specific incidents such as containment breaches, chemical spills, or vessel or pipe ruptures can provide useful information. Although such observations are usually not helpful for determining the NOAEL in humans, they may indicate whether human symptomatology is similar to that found in animals and may suggest functional or biological tests that might prove useful for routine monitoring of exposed workers.

Linkage of Animal Studies and Epidemiologic Studies

In the field of occupational toxicology, perhaps more than in other areas of toxicology, cooperation between those conducting animal studies and studies of workers is helpful for examining risks associated with overexposure to chemicals and other toxicants. A few examples will serve to illustrate the complementarity of these disciplines.

Several occupational carcinogens have been identified clearly through combined epidemiologic and experimental approaches. For example, the carcinogenicity of vinyl chloride was first demonstrated in rats (Viola et al., 1971), and a few years later, epidemiologic studies confirmed the same carcinogenic risk for humans (Creech and Johnson, 1974; Monson et al., 1974). This observation stimulated several investigations on the metabolism of vinyl chloride in animals and on its mutagenic activity in in vitro systems. Identification of vinyl chloride metabolites led to the conclusion that there is microsomal oxidation leading to the formation of an epoxide derivative, which acts as a proximate carcinogen (ATSDR, 2006). This finding triggered further studies on the biotransformation of structurally related halogenated ethylenes, such as vinyl bromide, vinylidene chloride, 1,2-dichloroethene, trichloroethylene, and perchloroethylene. Comparison of their oncogenic activity in relation to their metabolism suggested that an interplay between the stability and reactivity in reaching the DNA target and reacting with it after being formed would determine their genotoxic risk. It is now recognized that vinyl chloride is metabolized to 2-chloroethylene oxide which rearranges to 2-chloroacetaldehyde and produces promutagenic etheno-DNA adducts including etheno-guanine, etheno-cytosine, and lesser amounts of etheno-adenine (Bolt, 2005; ATSDR, 2006; NTP, 2016).

1,3-Butadiene is a known human carcinogen with more than 75% of its use in the manufacture of synthetic rubber products. Experimental studies in rats and mice demonstrated carcinogenicity, with mice being particularly sensitive. Subsequent to these findings, 1,3-butadiene was shown to follow the same metabolic pathway in humans as in rats and mice, forming mutagenic and carcinogenic epoxides. That led to cohort and case–control studies establishing 1,3-butadiene as a human carcinogen (ATSDR, 2009; NTP, 2016). More recent studies have considered the relative importance of the metabolic pathway leading to the formation of the reactive metabolites, 3,4-epoxy-1-butene, 1,2:3,4-diepoxybutane, and 3,4-epoxy-1,2-diol that react with DNA and with nuclear proteins. Under the assumption of a genotoxic mechanism and cross-species comparisons of DNA binding, these data facilitate a more informed cancer risk assessment. In vitro studies showed that human lymphocytes were less sensitive to 1,2:3,4-diepoxybutane-induced chromosomal aberrations than mouse or rat cells (ATSDR, 2009). In vivo studies revealed a higher rate of formation of epoxides in mice than rats. Inhaled 1,3-butadiene produced N7-guanine DNA adducts in lung tissue due to 3,4-epoxy-1,2-diol (Koivisto and Peltonen, 2001) and 1,2:3,4-diepoxybutane (Goggin et al., 2009). These cross links were more prevalent in mice than rats and in females than males which agreed with the enhanced susceptibility to cancers of female mice. On this basis, rats were judged to be a more predictive species than mice for human risk assessment. This work illustrates that the measurement of DNA adducts is a sensitive method for monitoring 1,3-butadiene metabolism via the epoxide-forming pathway in workers.

In 1973, an outbreak of peripheral neuropathy occurred in workers exposed to the solvent methyl butyl ketone (2-hexanone, MBK) (McDonough, 1974; Allen et al., 1975). The same lesion was reproduced in animals (Mendell et al., 1974; Spencer et al., 1975). Biotransformation studies were then undertaken in rats and guinea pigs and some MBK metabolites (2,5-hexanedione, 5-hydroxy-2-hexanone) were also found to possess neurotoxic activity (Spencer and Schaumburg, 1975; DiVincenzo et al., 1977). Similar oxidation products are formed from *n*-hexane, the neurotoxicity of which is probably due to the same active metabolite as that produced from MBK. Because methyl ethyl ketone (MEK) cannot give rise to 2,5-hexanedione, it was suggested as a replacement solvent. Subsequent work demonstrated the potentiation of neurotoxic effects of *n*-hexane upon coexposure with MEK (Yu et al., 2002). Nevertheless, MEK remains a much less toxic solvent than MBK.

These examples demonstrate that studies of the metabolic handling of occupational toxicants in animals are instrumental in the characterization of reactive intermediates and may suggest unsuspected risks or indicate new methods of biological monitoring. Conversely, clinical observations of workers may stimulate studies of the metabolism or the mechanism of toxicity of a toxicant in animals, thereby revealing the health significance of a biological disturbance.

Arsenic is one of the very few compounds for which there are limited data of predictive value from animal studies to human health effects. It has been used as a medicine since the time of Hippocrates. Initially used to treat ulcers, arsenicals achieved notoriety as medicinals for a wide variety of ailments, and then, in the first half of the 20th century, for the treatment of syphilis and parasites. Many foods and beverages contaminated with arsenic have been associated with accidental and intentional poisonings. Inorganic pentavalent arsenic (arsenate) is readily absorbed across tissues and converted to the trivalent form (arsenite). This is then methylated to form monomethyl arsenic acid and dimethylarsenic acid (DMA) (ATSDR, 2007). These are primarily transported in the blood bound to sulfhydryl groups in proteins. The half-life in humans for arsenic compounds is 2 to 4 days and the major excretion is via the urine.

Inorganic arsenic was first noted as a human carcinogen by Hutchinson in 1887 (Hutchinson, 1887). Epidemiologic studies led to classification of arsenic by the IARC as a skin and lung carcinogen in 1980 (IARC, 1980). Since then, studies among occupationally exposed populations and populations with high arsenic in their drinking water have shown conclusively that arsenic causes human cancers of the skin, lung, bladder, kidney, liver, nasal tissue, and prostate. There is also evidence for arsenic-associated cutaneous effects, cardiovascular and cerebrovascular disease, diabetes mellitus, and adverse reproductive outcomes (EPA, 2000; WHO, 2001).

A large number of carefully executed cancer bioassays in mice, rats, beagles, and monkeys performed using sodium arsenate, sodium arsenite, lead arsenite, arsenic trioxide, and DMA were uniformly negative for cancer. A number of subsequent studies that tested for tumor-promotion activity following dosing with recognized tumor initiators also yielded negative results. The 2007 ATSDR Toxicological Profile for arsenic stated, "First and most importantly, no animal model exists for the health effect of greatest concern for human exposure: carcinogenicity in skin and other organs after oral exposure" (ATSDR, 2007). Studies since about 2000, using organic arsenicals, susceptible transgenic mice, high-dose in utero exposures, or administration of a co-carcinogen, have yielded lung adenomas and adenocarcinomas in mice and rats (Shen et al., 2003, 2007; Cui et al., 2006; Hughes, 2006). However, years of negative studies in standard animal carcinogenicity screens were problematic in the face of unquestionable oncogenic activity in humans.

The examples above demonstrate that the occupational toxicologist cannot rely solely on animal or epidemiologic studies. A combined approach is necessary in order to identify, elucidate, and prioritize risks and to develop interventions and techniques for worker health surveillance.

EXPOSURE MONITORING

Two important applications of occupational toxicologic investigations are compared below: environmental monitoring and biological monitoring. As described above under "Occupational Health Standards," both are important in worker health surveillance and are essential elements of toxicology studies with dosing via the inhalation or dermal routes.

Environmental Monitoring for Exposure Assessment

An important objective of experimental and clinical investigations in occupational toxicology is the proposal of safe levels of exposure. OELs must be reevaluated at regular intervals as new information on the toxicity of industrial chemicals develops. Adherence to OELs may not protect everyone and, therefore, cannot supplant close medical surveillance of workers. Various private and official institutions regularly review the toxicologic information on chemicals in order to propose or update permissible levels of exposure. These include governmental and professional organizations worldwide. A critical element of establishing OELs is the accurate assessment of exposure. Methodology for exposure assessment must be specifically tailored to the agent under study and the environment in which it appears. To assess airborne exposures for compliance purposes, personal samples taken in the breathing zone are generally used. In a few specific environments, area samples form the basis of an exposure standard (e.g., the OSHA standard for exposure to raw cotton dust specifies use of the vertical elutriator or an equivalent method). Occupational environmental surveys may employ area sampling to determine areas with higher or lower toxicant concentrations. However, concentrations determined from personal samples typically exceed area concentrations, depending on the work practices and environmental controls. For example, geometric mean concentrations of inhalable dust assessed from 159 personal samples in dairy barns were 1.78 mg/m^3, compared with 0.74 mg/m^3 for 252 area samples collected simultaneously in two locations in the same barns (Kullman et al., 1998). Thus, in this environment, area sampling alone would underestimate personal exposures by a factor of 2.4.

Repeated random sampling is theoretically the best approach to developing unbiased measures of exposure. However, this is rarely the approach that is taken. Variability in exposure, especially variability over time, is often large; therefore, a considerable number of repeated measurements are needed to obtain an accurate proxy of the true exposure. When the number of repeats is insufficient, the slope of the exposure–response relationship will be biased, usually leading to considerable underestimation of the relationship (Heederik and Attfield, 2000). Studies have demonstrated that approaches assessing exposures to groups rather than to individuals are more efficient in terms of measurement effort for obtaining a desired level of accuracy (Vermeulen and Kromhout, 2005). In a group-based approach, workers are grouped by job title, task performed, or through exposure modeling studies to elucidate determinants of exposure, and the group mean is used as the average exposure for each worker (Kromhout et al., 1996). Further statistical modeling of the exposure data can reduce problems of bias and large temporal and spatial variability (Preller et al., 1995; Tielemans et al., 1998). Whereas this approach is gaining acceptance among occupational epidemiologists for evaluating exposure–response data and assessing risks, it is not accepted for compliance monitoring.

Although one cannot assess dose directly through exposure monitoring, it does have several distinct advantages over biomonitoring. Exposure monitoring allows one to quantify workplace exposure by route through selective air monitoring in the breathing zone of the worker and dermal dosimetry using absorptive material affixed to the workers' skin or clothing. Biomonitoring generally does not provide route-specific exposure data. Environmental monitoring techniques are generally less expensive and less invasive than techniques involving the collection and analysis of biological samples such as blood or urine. Thus, a larger population of workers can be studied for the same amount of money. New personal sampling devices incorporate GPS, accelerometers, and smartphone technology to provide enhanced data on location and activity. Workers are accustomed to wearing personal samplers for exposure assessment and are generally quite willing to do so. However, they are often unwilling to give a blood or urine sample, fearing that the sample will be surreptitiously used for drug testing, DNA testing, or experimentation. Another benefit of air sampling in the workplace is that spatial, temporal, and work practice associations can be established and can suggest better interventions and engineering controls to reduce exposures. Finally, analytic interferences and variabilities are generally lower with environmental samples than with biological samples.

A fully validated sampling and analysis method requires specification of the sampling methods; sample duration, handling, and storage procedures; the analytic method and measurement technique; the range, precision, accuracy, bias, and limits of detection; quality assurance issues; and known interferences. It is also important to document intralaboratory and interlaboratory variability. Once a standard method is established, it must be closely followed in every detail in order to assure consistency of results.

The development of accurate and precise analytic methods for environmental assessment is an ongoing effort. NIOSH publishes the extensive *NIOSH Manual of Analytical Methods* (NIOSH NMAM, 2017) and these are widely used. The American Society for Testing Materials (ASTM) has also developed a rigorous system for the establishment of methods. It generally requires 5 or more years to establish a new ASTM method for exposure assessment. The International Organization for Standardization (ISO) is a global federation of national standards bodies with 162 member countries. The subcommittee on workplace atmospheres is administered by the American National Standards Institute (ANSI). ISO has completed harmonization on a number of air sampling methods—for example, the determination of the number concentration of airborne inorganic fibers by phase contrast optical microscopy. The American Industrial Hygiene Association (AIHA) and the ACGIH have published compilations with descriptions of analytic devices and methodology (Cohen and McCammon, 2001; Anna, 2010). Methods have also been developed for bioaerosol exposure assessment and these have been reviewed (Thorne and Heederik, 1999a; Heederik et al., 2003; Hess-Kosa, 2011).

Biological Monitoring for Exposure Assessment

Biomonitoring consists of the measurement of toxicants, their metabolites, or molecular signatures of effect in specimens from humans or animals, including urine, blood, feces, exhaled breath, hair, finger or toenails, bronchial lavage, breast milk, and adipose tissue. These may serve as biomarkers of exposure, biological effect, or susceptibility. New technologies are emerging that will allow measurement and monitoring of chemicals in the body and transmission of the data from indwelling biosensors. Biomonitoring data provide a measurement of exposure based upon internalized dose and, thus, account for all exposures by all routes for the assessed analyte.

Depending on the chemical and the analyzed biological material, the term *internalized dose* may have different meanings. The measured biomarker may reflect the amount of chemical absorbed shortly before sample collection, as with the concentration of a solvent in exhaled air or in a blood sample obtained during the work shift. It may reflect exposure during the preceding day, as with the measurement of a metabolite in blood or urine collected after the end of exposure. For toxicants with a long biological half-life, the measured parameter may reflect exposure accumulated over a period of weeks or months as with arsenic in toenails. *Internal dose* may refer to the amount of chemical stored in one or in several body compartments or in the whole body (*the body burden*).

When biological measurements are available to assess the internal dose, they offer important advantages over monitoring the air of the workplace or measuring deposition of chemicals onto dermal patches. The greatest advantage is that the biological measure of exposure is more directly related to the adverse health effects than environmental measurements because it reflects the amount of toxicant absorbed. Therefore, it may offer a better estimate of the risk than can be determined from ambient monitoring. Biological monitoring accounts for uptake by all exposure routes. Many industrial chemicals can enter the organism by absorption through the skin or the gastrointestinal tract as well as the lung. For example, some solvents (e.g., dimethylformamide) and many pesticide formulations exhibit substantial exposure via the dermal route. In these situations, exposures determined through monitoring airborne concentrations alone underestimate true exposure. Monitoring of an early biomarker of effect is useful when the subjects are exposed to a complex mixture and the agents responsible for the effect are unknown or several agents are working in synergy.

Several factors can influence uptake. Personal hygiene habits vary from one person to another, and there is some degree of individual variation in the absorption rate of a chemical through the lungs, skin, or gastrointestinal tract. Use of size-selective air sampling to determine the inhalable or respirable fraction can strengthen the exposure estimate. However, biological factors such as ventilatory parameters can affect the strength of such a correlation since increased workload can markedly increase the respiratory uptake of an airborne toxicant. Because of its ability to encompass and evaluate the overall exposure (whatever the route of entry), biological monitoring can also be used to test the overall efficacy of personal protective equipment such as respirators, gloves, barrier creams, or aprons. Another consideration with biological monitoring is the fact that nonoccupational exposures (through hobbies, residential exposures, dietary habits, smoking, second jobs) may also be expressed in the biological sample. The organism integrates the total external (environmental and occupational) exposure into one internal load. Whereas this is beneficial for worker health and safety, it may be confounding in epidemiologic studies or compliance monitoring. Thus, while biomonitoring is an important exposure measurement tool for health risk assessment, it generally does not allow one to relate sources and levels of exposure to adverse health effects unless the exposures are unique to the workplace (Albertini et al., 2006).

The value of biological monitoring is heightened when the relationships between external exposure, internal dose, and adverse effects are established. Normally, biological monitoring of exposure cannot be used for assessing exposure to substances that exhibit their toxic effects at the sites of first contact and are poorly absorbed. Examples include dermally corrosive compounds and primary lung irritants. In this situation, the only useful relationship is that between external exposure and the intensity of the local effects.

Relationships between air monitoring and biological monitoring may be modified by genetic or external factors that influence the fate of an occupational toxicant in vivo. Metabolic interactions can occur when workers are exposed simultaneously to chemicals that are biotransformed through identical pathways. Exposure to chemicals that modify the activity of the biotransformation enzymes (e.g., microsomal enzyme inducers or inhibitors) may also influence the fate of another compound. Furthermore, metabolic interferences may occur between occupational toxicants and alcohol, tobacco, food additives, prescription drugs, natural product remedies, or recreational drugs. Changes in any of several biological variables (weight, body mass index, pregnancy, diseases, immune status, etc.) may modify the metabolism of an occupational chemical. These factors have to be taken into consideration when the results of biomonitoring are interpreted. Whatever the parameter measured, whether it is the substance itself, its metabolite, or an early biomarker of effect, the test must be sufficiently sensitive and specific to provide meaningful data in the range of workplace exposures.

Some chemicals have a long biological half-life in various body compartments (e.g., lipophilic persistent organic pollutants) and the time of sampling may not be critical. For other chemicals, the time of sampling is critical because, following exposure, the compounds or their metabolites may be rapidly eliminated from the body. In these cases, the biological sample is usually collected during exposure, at the end of the exposure period, or sometimes just before the next work shift. When biological monitoring consists of the sampling and analysis of urine, it is usually performed on "spot" urine specimens or on the first morning void. It is a standard practice for most organic compounds to adjust the results for urine output by expressing the results per gram of creatinine in the urine.

Table 34-8

Control Approaches for Occupational Inhalation Hazards

Change the process to use or produce less hazardous compounds
Automate and enclose the process to isolate the compounds
Incorporate administrative and work practice controls to reduce duration or intensity of exposure
Install or upgrade local exhaust systems and dilution exhaust
Institute a comprehensive program for personal protective equipment use where necessary

Analyses performed on very dilute urine samples are not reliable. The WHO has specified acceptable limits for urine specimens of between 0.3 and 3.0 g/L creatinine or 1.010 to 1.030 specific gravity (ACGIH, 2016). When samples exhibit large interindividual variability or high "background" levels, the interpretation of a single measurement may be difficult. In such cases, it may be useful to analyze biological material collected before and after the exposure period and gauge exposure based upon the cross-shift change.

The majority of BEIs listed by the ACGIH refer to analysis of the parent compound or its phase I metabolite. Analytical advances over the past decade have yielded methods for detecting reactive intermediates of metabolism and macromolecular adducts that may induce mutations or cell cycle disruption. These methods are important for comparing human and animal toxicity data for risk assessment and may help to explain differential responses in animal models and susceptibility in human populations. These methods may also be useful for occupational health surveillance programs.

Environmental monitoring plays an important role in the evaluation and prevention of excessive exposure to toxicants in the workplace. However, the prevention of acute toxic effects on the respiratory tract, skin, or eye mucosa can only be achieved by keeping the concentration of the irritant substance below a toxic level or by eliminating the exposure. Local acute effects of chemicals do not lend themselves to a biological surveillance program. Likewise, biological monitoring is usually not indicated for detecting peak exposure to dangerous chemicals such as arsine (AsH_3), carbon monoxide, or prussic acid (HCN). Furthermore, identification of emission sources and the evaluation of the efficiency of engineering control measures are usually best performed by ambient air analysis. Table 34-8 lists the approaches most useful for controlling inhalation exposures in the workplace. These include process changes, engineering controls, and use of personal protective equipment. It should be emphasized that process changes and application of engineering controls are preferable to reliance on personal protective equipment.

In summary, environmental and biological monitoring should not be regarded as opposites but as complementary elements of an occupational health and safety program. They should be integrated as much as possible to ensure low levels of contaminants and optimal health for workers.

CONCLUSION

The working environment will always have the potential to overexpose workers to toxicants. Recognition of these risks should not wait until epidemiologic studies have uncovered hazardous levels. A combined experimental, clinical, and epidemiologic approach is most effective for evaluating and managing the potential risks. One can then promulgate scientifically based occupational health standards, apply effective workplace controls to ensure adherence to those standards, and institute worker health surveillance programs to identify unexpected effects in susceptible individuals.

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REFERENCES

Abnet CC, Tanguay RL, Heideman W, Peterson RE. Transactivation activity of human, zebrafish, and rainbow trout aryl hydrocarbon receptors expressed in COS-7 cells: greater insight into species differences in toxic potency of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners. *Toxicol Appl Pharmacol*. 1999;159(1):41–51.

ACGIH. *2016 TLVs® and BEIs®: Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices*. Cincinnati, OH: American Conference of Governmental Industrial Hygienists; 2016.

ACGIH. TLV™/BEI™ position statement. 2017. Available at: www.acgih.org/tlv-bei-guidelines/policies-procediures-presentations/tlv-bei-position-statement. Accessed October 22, 2017.

Adamčaková-Dodd A, Monick MM, Powers LS, Gibson-Corley KN, Thorne PS. Effects of perinatal inhalation exposure to copper nanoparticles on murine dams and offspring. *Part Fibre Toxicol*. 2015;12:30.

Adamčaková-Dodd A, Stebounova LV, Kim JS, et al. Toxicity assessment of zinc oxide nanoparticles using sub-acute and sub-chronic murine inhalation models. *Part Fibre Toxicol*. 2014;11:15.

Adamčaková-Dodd A, Stebounova LV, O'Shaughnessy PT, et al. Murine pulmonary responses after sub-acute exposure to aluminum oxide-based nanowhiskers. *Part Fibre Toxicol*. 2012;9:22.

Albertini R, Bird M, Doerrer N, et al. The use of biomonitoring data in exposure and human health risk assessments. *Environ Health Perspect*. 2006;114:1755–1762.

Aldridge WN, Miles JM, Mount DL, Verschoyle RD: The toxicological properties of impurities in malathion. *Arch Toxicol*. 1979;42:95–106.

Allen N, Mendell JR, Billmaier DJ, et al. Toxic polyneuropathy due to methyl *N*-butylketone. *Arch Neurol*. 1975;32:209–218.

Anna DH, ed. *The Occupational Environment: Its Evaluation, Control and Management*. 3rd ed. Vol 1 & 2. Fairfax, VA: American Industrial Hygiene Association; 2010.

Asgharian B, Price O, Kabilan S, et al. Development of a Zealand white rabbit deposition model to study inhalation anthrax. *Inhal Toxicol*. 2016;28(2):80–88.

Asgharian B, Price OT, Oldham M, et al. Computational modeling of nanoscale and microscale particle deposition, retention and dosimetry in the mouse respiratory tract. *Inhal Toxicol*. 2014;26(14):829–842.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Vinyl Chloride*. Atlanta, GA: Department of Health and Human Services; 2006. Available at: http://www.atsdr.cdc.gov/ToxProfiles/TP.asp?id=282&tid=51. Accessed October 20, 2011.

ATSDR (Agency for Toxic Substances and Disease Registry). *Toxicological Profile for Arsenic*. Atlanta, GA: Department of Health and Human Services; 2007. Available at: http://www.atsdr.cdc.gov/ToxProfiles/TP.asp?id=22&tid=3. Accessed October 31, 2017.

ATSDR (Agency for Toxic Substances and Disease Registry). *Draft Toxicological Profile for 1,3-Butadiene*. Atlanta, GA: Department of Health and Human Services; 2009. Available at: https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=459&tid=81. Accessed October 31, 2017.

Balazy A, Toivola M, Adhikari A, et al. Do N95 respirators provide 95% protection level against airborne viruses, and how adequate are surgical masks? *Am J Infection Control*. 2006;34:51–57.

Bason M, Lammintausta K, Maibach HI. Irritant dermatitis (irritation). In: Marzulli FN, Maibach HI, eds. *Dermatotoxicology*. 4th ed. New York: Hemisphere; 1991:223–252.

Baur X. Occupational asthma due to isocyanates. *Lung*. 1996;174:23–30.

Becklake MR, Malo JL, Chan-Yeung M. Epidemiological approaches in occupational asthma. In: Bernstein IL, et al. eds. *Asthma in the Workplace*. 3rd ed. New York: Taylor & Francis Group; 2006:37–77.

Bernstein DA, Biagini RE, Karnani R, et al. In vivo sensitization to purified *Hevea brasiliensis* proteins in health care workers sensitized to natural rubber latex. *J Allergy Clin Immunol*. 2003;111:610–616.

Bernstein IL, Bernstein DI, Chan-Yeung M, Malo JL. Definition and classification of asthma in the workplace. In: Malo JL, et al. eds. *Asthma in the Workplace*. 4th ed. New York: Taylor & Francis Group; 2013:1–5.

Bolt HM. Vinyl chloride: a classical industrial toxicant of new interest. *Crit Rev Toxicol*. 2005;35:301–323.

Boverhof DR, Burgoon LD, Tashiro C, et al. Comparative toxicogenomic analysis of the hepatotoxic effects of TCDD in Sprague Dawley rats and C57BL/6 mice. *Toxicol Sci*. 2006;94:398–416.

Chan-Yeung M, Vedal S, Kus J, et al. Symptoms, pulmonary function and bronchial hyperactivity in western red cedar workers compared with those in office workers. *Am Rev Respir Dis*. 1984;130:1038–1041.

Cogliano VJ, Baan R, Straif K, et al. Preventable exposures associated with human cancers. *J Natl Cancer Inst*. 2011;103:1827–1839.

Cohen BS, McCammon CS Jr. eds. *Air Sampling Instruments for Evaluation of Atmospheric Contaminants*. 9th ed. Cincinnati, OH: American Conference of Governmental Industrial Hygienists; 2001.

Creech JL, Johnson HM. Angiosarcoma of the liver in the manufacture of polyvinylchloride. *J Occup Med*. 1974;16:150–151.

Cui X, Wakai T, Shirai Y, Hatakeyama K, Hirano S. Chronic oral exposure to inorganic arsenate interferes with methylation status of p16INK4a and RASSF1A and induces lung cancer in A/J mice. *Toxicol Sci*. 2006; 91(2):372–381.

Cullinan P, Lowson D, Nieuwenhuijsen MJ, et al. Work related symptoms, sensitisation, and estimated exposure in workers not previously exposed to laboratory rats. *Occup Environ Med*. 1994;51:589–592.

Desjardins A, Malo JL, L'Archevçque J, et al. Occupational IgE-mediated sensitization and asthma due to clam and shrimp. *J Allergy Clin Immunol*. 1995;96:608–617.

DiVincenzo GD, Hamilton ML, Kaplan CJ, Dedinas J. Metabolic fate and disposition of ^{14}C-labeled methyl *n*-butyl ketone in the rat. *Toxicol Appl Pharmacol*. 1977;41:547–560.

Donham KJ, Thorne PS. Agents in organic dusts: criteria for a causal relationship. *Am J Ind Med*. 1994;25:33–39.

Doorn JA, Thompson CM, Christner RB, Richardson RJ. Stereoselective inactivation of *Torpedo californica* acetylcholinesterase by isomalathion: inhibitory reactions with (1R)- and (1S)-isomers proceed by different mechanisms. *Chem Res Toxicol*. 2003;16:958–965.

Douwes J, Thorne PS, Pearce N, Heederik D. Biological agents—recognition. In: Perkins JL, ed. *Modern Industrial Hygiene: Vol II. Biological Aspects*. Cincinnati, OH: American Conference of Governmental Industrial Hygienists; 2003:219–292.

Duchaine C, Meriaux A, Thorne PS, Cormier Y. Assessment of particulates and bioaerosols in Eastern Canadian sawmills. *Am Ind Hyg Assoc J*. 2000;61:727–732.

Eduard W, Sandven P, Levy F. Relationships between exposure to spores from *Rhizopus microsporus* and *Paecilomyces variotii* and serum IgG antibodies in wood trimmers. *Int Arch Allergy Immunol*. 1992;97:274–282.

Elliott L, Heederik D, Marshall S, Peden D, Loomis D. Incidence of allergy and allergy symptoms among workers exposed to laboratory animals. *Occup Environ Med*. 2005;62:766–771.

EPA. 40 CFR Parts 141 and 142, National primary drinking water regulations; arsenic and clarifications to compliance and new source contaminants monitoring; proposed rule. *Fed Reg*. 2000;65(121):38888–38983.

European Commission. European Commission Directive 95/320/EC. 1995. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31995D0320:EN:HTML. Accessed October 23, 2011.

European Commission. European Commission Directive 98/24/EC. 1998. Available at: http://osha.europa.eu/en/legislation/directives/exposure-to-chemical-agents-and-chemical-safety/osh-directives/75. Accessed October 22, 2011.

European Commission. EU Commission Directive 2006/15/EU. 2006. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:038:0036:0039:EN:PDF. Accessed October 22, 2017.

European Commission. EU Commission Directive 2009/161/EU. 2009. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:338:0087:0089:EN:PDF. Accessed October 22, 2017.

European Commission. *Employment and Social Developments in Europe*. 7th ed. 2017. Available at: http://ec.europa.eu/social/main.jsp?catId=738&langId=en&pubId=8030&furtherPubs=yes. Accessed October 22, 2017.

Fuortes LJ, Weih L, Pomrehn P, et al. Prospective epidemiologic evaluation of laboratory animal allergy among university employees. *Am J Ind Med*. 1997;32:665–669.

GAO/HEHS. *Child Labor in Agriculture—Changes Needed to Better Protect Health and Educational Opportunities*. GAO/HEHS-98-193. Washington, DC: U.S. Government Accountability Office; 1998:1–90.

Glass WI, Power P, Burt R, et al. Work-related respiratory symptoms and lung function in New Zealand mussel openers. *Am J Ind Med*. 1998; 34:163–168.

Goggin M, Swenberg JA, Walker VE, et al. Molecular dosimetry of 1,2,3,4-diepoxybutaneinduced DNA—DNA cross-links in B6C3F1 mice and F344 rats exposed to 1,3-butadiene by inhalation. *Cancer Res*. 2009;69(6):2479–2486.

Hard GC, Rodgers IS, Baetcke KP, et al. Hazard evaluation of chemicals that cause accumulation of α_2 *u*-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. *Environ Health Perspect*. 1993;99:313–349.

Heederik D, Attfield M. Characterization of dust exposure for the study of chronic occupational lung disease: a comparison of different exposure assessment strategies. *Am J Epidemiol*. 2000;151(10):982–990.

Heederik D, Thorne PS, Doekes G. Health-based occupational exposure limits for high molecular weight sensitizers: how long is the road we must travel? *Ann Occup Hyg*. 2002;46(5):439–446.

Heederik D, Thorne PS, Douwes J. Biological agents: monitoring and evaluation of bioaerosol exposure. In: Perkins JL, ed. *Modern Industrial Hygiene: Vol II. Biological Aspects*. Cincinnati, OH: American Conference of Governmental Industrial Hygienists; 2003:293–327.

Hess-Kosa K. *Indoor Air Quality: The Latest Sampling and Analytical Methods*. 2nd ed. Boca Ratan, FL: Taylor & Francis Group; 2011.

Hollander A, Heederik D, Doekes G. Respiratory allergy to rats: exposure–response relationships in laboratory animal workers. *Am J Respir Crit Care Med*. 1997;155:562–567.

Hsieh TH, Yu CP, Oberdorster G. Deposition and clearance models of Ni compounds in the mouse lung and comparisons with the rat models. *Aerosol Sci Technol*. 1999;31:358–372.

Hughes MF. Biomarkers of exposure: a case study with inorganic arsenic. *Environ Health Perspect*. 2006;114:1790–1796.

Hutchinson J. Arsenic cancer. *Br Med J*. 1887;2:1280–1281.

IARC. *Metals and Metallic Compounds. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. Vol 23. Lyon, France: International Agency for Research on Cancer; 1980.

IARC. *IARC Monograph 107: Polychlorinated Biphenyls and Polybrominated Biphenyls*. Lyon, France; International Agency for Research on Cancer; 2016. Available at: http://monographs.iarc.fr/ENG/Monographs/vol107/index.php. Accessed October 22, 2017.

IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans*. n.d. Available at: http://monographs.iarc.fr/ENG/Monographs/PDFs/index.php. Accessed October 22, 2017.

ICCVAM. 2017. Available at: https://ntp.niehs.nih.gov/pubhealth/evaluation/test-method-evaluations/protocols/index.html. Accessed October 29, 2017.

ILO. Key indicators of the labour market 2014. 2014. Available at: http://www.ilo.org/global/industries-and-sectors/agriculture-plantations-other-rural-sectors/lang--en/index.htm. Accessed October 22, 2017.

ILO. *Global Estimates of Child Labour: Results and Trends, 2012–2016*. Geneva: International Labour Office (ILO); 2017.

ILSI Risk Science Institute Workshop Participants. The relevance of the rat lung response to particle overload for human health risk assessment: a workshop consensus report. *Inhal Toxicol*. 2000;12:1–17.

Japan Monthly Statistics. Ministry of Internal Affairs and Communications, Statistics Bureau. August 2017. Available at: http://www.stat.go.jp/english/data/getujidb/index.htm#g. Accessed October 22, 2017.

Kim JS, Adamcakova-Dodd A, O'Shaughnessy PT, Grassian VH, Thorne PS. Effects of copper nanoparticle exposure on host defense in a murine pulmonary infection model. *Part Fibre Toxicol*. 2011;8(1):29.

Koivisto P, Peltonen K. N7-guanine adducts of the epoxy metabolites of 1,3-butadiene in mice lung. *Chem Biol Interact*. 2001;135-136:363–372.

Konduru NV, Molina RM, Swami A, et al. Protein corona: implications for nanoparticle interactions with pulmonary cells. *Part Fibre Toxicol*. 2017;14(1):42.

Kreiss K, Hubbs A, Kullman G. Correspondence: bronchiolitis in popcorn-factory workers. *N Engl J Med*. 2002;347:1981–1982.

Kromhout H, Tielemans E, Preller L, Heederik D. Estimates of individual dose from current measurements of exposure. *Occup Hyg*. 1996;3:23–39.

Kullman GJ, Thorne PS, Waldron PF, et al. Organic dust exposures from work in dairy barns. *Am Ind Hyg Assoc J*. 1998;59:403–413.

Maibach HI, Feldmann RJ, Milby TH, Serat WF. Regional variation in percutaneous penetration in man: pesticides. *Arch Environ Health*. 1971;23:208–211.

McDonough JR. Possible neuropathy from methyl-*n*-butyl ketone. *N Engl J Med*. 1974;290:695.

Mendell JR, Saida K, Ganasia MF, et al. Toxic polyneuropathy produced by methyl-*n*-butyl ketone. *Science*. 1974;185:787–789.

Monson RR, Peters JM, Johnson MN. Proportional mortality among vinyl-chloride workers. *Lancet*. 1974;2(7877):397–398.

National Academies of Sciences, Engineering, and Medicine. *Controlled Human Inhalation-Exposure Studies at EPA*. Washington, DC: The National Academies Press; 2017. doi:10.17226/24618.

Newton AN, Davis M, Koehler K, et al. Atopy as a modifier of the relationships between endotoxin exposure and symptoms among laboratory animal workers. *Ann Work Expo Health*. 2017;61(8):1024–1028.

NIOSH. Current Intelligence Bulletin 65: Occupational Exposure to Carbon Nanotubes and Nanofibers; April 2013, DHHS (NIOSH) Publication Number 2013-145.

NIOSH. Work-Related Lung Disease Surveillance System (eWoRLD). 2015-866 U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Respiratory Health Division, Morgantown, WV. 2015. Available at: https://wwwn.cdc.gov/eworld/Data/866. Accessed October 22, 2017.

NIOSH. Work-Related Lung Disease Surveillance System (eWoRLD). 2017-915 U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Respiratory Health Division, Morgantown, WV. 2017a. Available at: https://wwwn.cdc.gov/eworld/Set/Work-Related-Respiratory-Diseases/88. Accessed October 22, 2017.

NIOSH. Health Hazard Evaluations (HHEs). 2017b. Available at: https://www.cdc.gov/niosh/hhe. Accessed October 22, 2017.

NIOSH. Coal Workers' Health Surveillance Program. 2017c. Available at: https://www.cdc.gov/niosh/topics/cwhsp/default.html. Accessed November 4, 2017.

NIOSH NMAM. *NIOSH Manual of Analytical Methods*. 5th ed. 2017. Available at: https://www.cdc.gov/NIOSH/NMAM. Accessed October 31, 2017.

NTP (National Toxicology Program). *14th Report on Carcinogens*. Research Triangle Park, NC: National Toxicology Program, U.S. Department of Health and Human Services, 2016. Available at: https://ntp.niehs.nih.gov/pubhealth/roc/index-1.html. Accessed November 4, 2017.

Oberdörster G. Toxicokinetics and effects of fibrous and nonfibrous particles Regul. *Toxicol Pharmacol*. 2002;27:123–135.

OSHA. Standard Interpretations: 10/06/1995—8-hour total weight average (TWA) permissible exposure limit (PEL). 1995. Available at: http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=INTERPRETATIONS&p_id=24470.

Pacheco KA, McCammon C, Liu AH, et al. Airborne endotoxin predicts symptoms in non-mouse-sensitized technicians and research scientists exposed to laboratory mice. *Am J Respir Crit Care Med*. 2003;167:983–990.

Pettibone JM, Adamcakova-Dodd A, Thorne PS, et al. Inflammatory response of mice following inhalation exposure to iron and copper nanoparticles. *Nanotoxicology*. 2008;2(4):189–204.

Preller L, Kromhout H, Heederik D, Tielen MJM. Modeling long-term average exposure in occupational exposure-response analysis. *Scand J Work Environ Health*. 1995;21:504–512.

Radauer C, Adhami F, Fürtler I, et al. Latex-allergic patients sensitized to the major allergen hevein and hevein-like domains of class I chitinases show no increased frequency of latex-associated plant food allergy. *Mol Immunol*. 2011;48(4):600–609.

Schenker MB, Christiani D, Cormier Y, et al. Respiratory health hazards in agriculture. *Am J Respir Crit Care Med*. 1998;158(pt 2):S1–S76.

Schlesinger RB. Comparative deposition of inhaled aerosols in experimental animals and humans: a review. *J Toxicol Environ Health*. 1985;15:197–214.

Schroeter JD, Asgharian B, Price OT, McClellan GE. Computational fluid dynamics simulations of inhaled ano- and microparticle deposition in the rhesus monkey nasal passages. *Inhal Toxicol*. 2013;25(12):691–701.

Shen J, Liu J, Xie Y, Diwan BA, Waalkes MP. Fetal onset of aberrant gene expression relevant to pulmonary carcinogenesis in lung adenocarcinoma development induced by in utero arsenic exposure. *Toxicol Sci*. 2007;95(2):313–320.

Shen J, Wanibuchi H, Salem EI, et al. Liver tumorigenicity of trimethylarsine oxide in male Fischer 344 rats: association with oxidative DNA damage and enhanced cell proliferation. *Carcinogenesis*. 2003;24(11):1827–1835.

Siemiatycki J, Richardson L, Straif K, et al. Listing occupational carcinogens. *Environ Health Perspect*. 2004;112(15):1447–1459.

Spencer PS, Schaumburg HH. Experimental neuropathy produced by 2,5-hexanedione A major metabolite of the neurotoxic industrial solvent methyl-*n*-butyl ketone. *J Neurol Neurosurg Psychiatry*. 1975;38:771–775.

Spencer PS, Schaumburg HH, Raleigh RL, Terhaar CJ. Nervous system degeneration produced by the industrial solvent methyl-*n*-butyl ketone. *Arch Neurol*. 1975;32:219–222.

Thorne PA, Adamcakova-Dodd A, Kelley KM, et al. Metalworking fluid with mycobacteria and endotoxin induces hypersensitivity pneumonitis in mice. *Am J Respir Crit Care Med*. 2006;173:759–768.

Thorne PS, Duchaine C, BlaisLecours P. Airborne bacteria and endotoxin. In: Yates MV, Nakatsu C, Miller R, Pollai S, eds. *Manual of Environmental Microbiology*. 4th ed. Washington, DC: ASM Press; 2015:3.2.6.1–3.2.6.20.

Thorne PS, Heederik D. Assessment methods for bioaerosols. In: Salthammer T, ed. *Organic Indoor Air Pollutants—Occurrence, Measurement, Evaluation*. Weinheim, Germany: Wiley/VCH; 1999a:85–103.

Thorne PS, Heederik D. Indoor bioaerosols—sources and characteristics. In: Salthammer T, ed. *Organic Indoor Air Pollutants—Occurrence, Measurement, Evaluation*. Weinheim, Germany: Wiley/VCH; 1999b:275–288.

Tielemans E, Kupper LL, Kromhout H, et al. Individual-based and group-based occupational exposure assessment: some equations to evaluate different strategies. *Ann Occup Hyg*. 1998;42(2):115–119.

Trabaris M, Laskin JD, Weisel CP. Effects of temperature, surfactants and skin location on the dermal penetration of haloacetonitriles and chloral hydrate. *J Expo Sci Environ Epidemiol*. 2012;22(4):393-7.

USDA. United States Department of Agriculture. *Economic Research Service: Farming and Farm Income*. 2017. Available at: https://www.ers.usda.gov/data-products/ag-and-food-statistics-charting-the-essentials/farming-and-farm-income/. Accessed November 4, 2017.

USDL. Bureau of Labor Statistics, U.S. Department of Labor. 2015. Available at: https://www.bls.gov/iif/oshsum.htm. Accessed October 22, 2017.

USDL. American Time Use Survey, Bureau of Labor Statistics, U.S. Department of Labor. 2017a. Available at: http://www.bls.gov/bls/newsrels.htm#OEUS. Accessed October 18, 2017.

USDL. Employment Projections: Employment by Major Industry Sector, Bureau of Labor Statistics, U.S. Department of Labor. 2017b. Available at: https://data.bls.gov/cgi-bin/print.pl/emp/ep_table_201.htm. Accessed October 18, 2017.

USDL. The Employment Situation—August 2017, Bureau of Labor Statistics, U.S. Department of Labor. 2017c. Available at: http://www.bls.gov/bls/newsrels.htm#OEUS. Accessed October 18, 2017.

U.S. Federal Register. June 12, 1992;57(114):26539–26590.
Vance ME, Kuiken T, Vejerano EP, et al. Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory. *Beilstein J Nanotechnol*. 2015;6:1769–1780.
Venables KM, Dally MB, Nunn AJ, et al. Smoking and occupational allergy in workers in a platinum refinery. *Br Med J*. 1989;299:939–942.
Venables KM, Topping MD, Howe W, et al. Interaction of smoking and atopy in producing specific IgE antibody against a hapten protein conjugate. *Br Med J*. 1985;290:201–204.
Vermeulen R, Kromhout H. Historical limitations of determinant based exposure groupings in the rubber manufacturing industry. *Occup Environ Med*. 2005;62:793–799.
Viola PL, Bigotti A, Caputo A. Oncogenic response of rat skin, lungs, and bones to vinyl chloride. *Cancer Res*. 1971;31:516–522.
Weiss LPC, Lewis R, Rossmoore H, et al. Respiratory illness in workers exposed to metal working fluid contaminated with nontuberculous mycobacteria—Ohio, 2001. *Morb Mortal Wkly Rev*. 2002;51:349–352.
Wernfors M, Nielsen J, Schültz A, Skerfving S. Phthalic anhydride-induced occupational asthma. *Int Arch Allergy Appl Immunol*. 1986;79:77–82.
WHO. *Arsenic and Arsenic Compounds*. 2nd ed. Environmental Health Criteria 224. Geneva: World Health Organization; 2001. Available at: http://whqlibdoc/ehc/WHO_EHC_224.pdf. Accessed October 23, 2011.
World Bank. Labor force, total. 2017. Available at: https://data.worldbank.org/indicator/SL.TLF.TOTL.IN?view=chart. Accessed November 3, 2017.
Yu RC, Hattis D, Landaw EM, Froines JR. Toxicokinetic interaction of 2,5-hexanedione and methyl ethyl ketone. *Arch Toxicol*. 2002;75(11-12):643–652.

chapter 35

Regulatory Toxicology

Gary E. Marchant*

What Is Regulatory Toxicology?

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References

WHAT IS REGULATORY TOXICOLOGY?

Regulatory toxicology is a subfield of regulatory science, which addresses the intersection of science with regulation, namely how the science of toxicology is developed, evaluated, and applied in regulatory decision-making. Toxicology is one of the most common fields of scientific knowledge utilized in regulatory science, as regulatory agencies often are required to identify and quantify the health risks of the products and activities they seek to regulate. Regulatory toxicology, which must straddle traditional scientific methodologies with the public policy world of regulation, strains to adhere to the standards of good toxicological science while also providing relevant inputs to decision-making, which often asks questions that science cannot answer completely or with any certainty. It is the tension between these two different worlds of science and policy that raise many of the most important and challenging issues in regulatory toxicology, many of which are addressed in this chapter.

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Over the past 50 years, the field of regulatory toxicology has grown enormously as the intersection between regulation and toxicology has expanded dramatically. With the enactment of a series of environmental, health, and safety laws in the United States, Europe, and elsewhere in the 1970s and 1980s, regulatory agencies increasingly rely on toxicological science to identify potential hazards, prioritize chemicals and other potentially toxic substances, and provide the data used for assessing and managing risk. Regulators are not merely consumers of toxicological studies but also help shape toxicology science in important ways. Regulatory programs have provided a major impetus for improvements in toxicology methods, and they have stimulated a demand for toxicology studies that meet various regulatory requirements. Some programs, such as regulatory programs for licensing drugs, devices, food additives, and pesticides, explicitly demand toxicology studies as a condition for marketing products. Other statutory and regulatory requirements, such as the recent program requiring agencies to screen chemicals for their endocrine disruptor capability, or agencies to evaluate the safety of new nanotechnology applications, are a major driver for the development of new types of toxicological assays.

Regulatory agencies have exercised important influence over the design and conduct of toxicology studies. For example, the United States Environmental Protection Agency (EPA) is

empowered by the Toxic Substances Control Act (TSCA) to promulgate standards for different types of toxicology (and other scientific) investigations. Likewise, the U.S. Food and Drug Administration (FDA) has long issued guidelines for laboratory studies submitted in support of food additives and drugs. Both agencies have adopted requirements governing laboratory operations and practice, known as Good Laboratories Practice (GLP). Communication between government officials and laboratory scientists flows in both directions. Government testing standards are influenced strongly by the prevailing consensus among toxicologists, many of whom work in regulatory agencies. Similar standards have been established at the international level through the Organization for Economic Co-operation and Development (OECD).

THE SCIENCE/POLICY INTERFACE IN REGULATORY TOXICOLOGY

There are critical differences between the objectives and methods of science and regulatory policy. Science seeks to understand natural phenomena through objective, empirical, and neutral methodologies, and is cautious, incremental, and evidence-based. In contrast, government regulation seeks to affect human behavior and resolve human disputes; it is episodic, peremptory, and normative, pursuing goals such as human welfare, safety, efficiency, and fairness.

Science is open-ended and can continue to study a problem indefinitely without coming to any final resolution. As the National Research Council noted, "the scientific process of seeking the truth, by design and to its credit, has no natural endpoint" (NRC, 2009). In contrast, regulation usually does not have the luxury of waiting until the science has matured, but rather must come to decisions based on legislative, political, and societal deadlines. This creates two problems. First, finality is essential for regulations, so that regulated entities and other stakeholders have certainty about the applicable regulatory requirements, which often involve significant resources and lead time to achieve compliance. Consequently, regulations are adopted as final decisions, which can only be reconsidered at significant administrative burden. Science meanwhile continues to develop, resulting in regulations that often become increasingly outdated in relationship to constantly evolving scientific data and understanding.

The second problem created by the need to adopt regulations on a set timetable is often that the scientific inputs into that regulatory decision remain uncertain and underdeveloped. This leads to what Alvin Weinberg referred to as the "regulator's dilemma": "the regulator, by law, is expected to regulate even though science can hardly help him; this is the regulator's dilemma" (Weinberg, 1985). Regulatory agencies are therefore required to make numerous assumptions to fill the gaps in the available scientific data (NRC, 1983). This then has the inevitable effect of making agency determinations a mixture of science and policy, which can lead to confusion and obfuscation.

Scientists are often frustrated by the political and administrative requirements (often referred to colloquially as "red tape") associated with regulatory utilization of toxicological data. A regulatory proposal incorporating scientific evidence has to pass through many layers within a regulatory office, including the agency scientists, economists, program officers, office of the general counsel, political appointees, various centers, programs, and offices within the agency that are affected by the proposed decision, external advisory committees, and finally the agency head, subject at every stage to a bevy of internal control requirements and processes. When the agency has finally made its proposed decision, the proposal is often then scrutinized by the Office of Information and Regulatory Affairs (OIRA) in the White House, courts conducting judicial review, Congressional oversight committees, and of course external stakeholders and the media. At every step along this pathway, the scientific findings of the agency are subject to challenge, prodding, and second-guessing.

This elaborate regulatory process has a number of implications. First, regulatory agencies tend to attempt to buttress their regulatory decisions with extensive analytical support. As a comparative study of US and European regulation observed, "American regulators, being more politically exposed than their European counterparts, have a greater need to support their actions through formal analytical arguments. Their demand for expertise necessitates a continuing build-up of technical capabilities and the development of more sophisticated and detailed analytical methodologies. The structure of the American rule-making process subjects the analytical case for regulation to intense political scrutiny. Any weaknesses are quickly exploited, and the uncertainties and shortcomings of the relevant scientific base are readily exposed …" (Brickman et al., 1985). In response to these pressures, regulatory agencies will often compile extensive scientific assessments of the toxicological and other data supporting their regulatory decisions, sometimes thousands of pages in length.

Another consequence of the highly politicized and adversarial nature of regulatory decision-making is that regulatory agencies have an incentive to overstate the scientific basis of their decisions. Although scientific data are a critical input into sound regulatory decision-making, the actual decision of how much protection to provide and which requirements or limits to impose is inevitably a normative policy decision, even if informed by scientific information and knowledge. Yet, agencies receive greater deference from Congress, the White House, reviewing courts, and even the media and public if they justify their decision on scientific rather than policy or normative grounds. Accordingly, regulatory agencies are inclined to give disproportionate weight to scientific arguments and evidence in trying to justify their decisions, a tendency that has been labeled in the literature as the "science charade" (Wagner, 1995).

AN OVERVIEW OF REGULATORY APPROACHES

Different agencies and even different regulatory programs within the same agency use toxicological data in different ways. One important difference relates to the extent of premarket analysis or approval required before a new product may be commercialized. Some products require premarket approval from a regulatory agency (e.g., drugs and medical devices by FDA and pesticides by EPA), other products require only premarket notification to the agency (e.g., new chemical substances), whereas still other products require no premarket activity with a regulatory agency (e.g., foods, cosmetics) before they can be sold commercially. There are also differences in who has the burden of proof to demonstrate safety or hazard. For example, the manufacturer of a new food additive has the burden to demonstrate to the FDA the lack of hazard before humans may be exposed, while laws such as the Occupational Safety and Health Act (1970) put the burden on regulators to show that a substance is hazardous before exposures can be restricted. The approach chosen by Congress for a particular type of product or activity greatly influences an agency's ability to require comprehensive toxicological investigation prior to commercialization.

In determining what level of a substance to allow, regulatory agencies generally use one of three different approaches: (i)

acceptable risk, (ii) balancing, or (iii) feasibility. The determination of which of these three approaches is used for a particular hazard is generally determined by the applicable statutory framework.

Acceptable Risk

This approach determines the level of risk a substance presents, and then seeks to lower that risk (if necessary) to a level that is "acceptable." Because these risk-based programs only consider health evidence, toxicological data play a central role in these regulatory programs. In making the determination of acceptable risk, agencies have traditionally distinguished between carcinogens and noncarcinogens in their approach. For noncarcinogenic chemicals, regulators have generally embraced a standard safety assessment formula, built around the concept of acceptable daily intake (ADI) for the FDA or reference dose (RfD) or reference concentration (RfC) in the case of EPA. These levels are derived by applying a series of safety factors to the lowest "no observed adverse effect level" from animal experiments. Human exposures to a chemical that fall below the resulting ADI, RfD, or RfC are assumed to be "safe" or acceptable. This traditional approach to noncarcinogenic toxicants has not been considered appropriate for carcinogens. Regulators in the United States and many of their counterparts in other countries have operated on the premise that carcinogens as a class cannot be assumed to have "safe" or threshold doses. Furthermore, they have assumed that any chemical shown convincingly in animal studies to cause cancer should be considered a potential human carcinogen. Accordingly, for this group of compounds, regulators have generally assumed that no finite level of human exposure can be considered risk-free. As research has begun to illuminate the different mechanisms by which chemicals may cause cancer, however, regulatory agencies have cautiously accepted the possibility that "safe" thresholds may be established for at least some non-genotoxic carcinogens.

An example of an "acceptable" risk-regulatory program is the national ambient air quality program under the Clean Air Act (CAA). Under this program, EPA must set standards for criteria air pollutants such as particulate matter, ozone, and carbon monoxide that protect against "adverse effects" in the most susceptible subgroup with an adequate margin of safety. EPA may only consider risk data in setting these standards; no consideration of costs or feasibility is permitted (*Whitman v ATA*, 2001). A more stringent form of the acceptable risk approach is a "zero" or "negligible" risk requirement, epitomized by the famous Delaney clause, enacted in 1958 as part of the Food Additives Amendment. The amendment itself requires that any food additive be found "safe" before the FDA may approve its use (Food Additive Amendment, 1958). The Delaney clause stipulated that this finding may not be made for a food additive that has been shown to induce cancer in humans or in experimental animals. In 1996, Congress amended the provisions of the Food, Drug, and Cosmetics Act applicable to pesticide residues in food, revoking the Delaney proviso as it applies to food additives (but not color addictives), and adopting a standard that, though not expressed in these words, is understood to permit a tolerance for a carcinogenic pesticide if the estimated cancer risk is extremely small, on the order of one in one million (Food Quality Protection Act, 1996).

Balancing Approaches

Other regulatory programs require the agency to balance the health benefits of risk reduction against the costs of such reductions. These approaches known as cost-benefit or risk-benefit analysis generally require a quantified estimate of both the health risks and costs associated with various regulatory options, and the agency then chooses the regulatory option that offers the biggest estimated net benefit. Both toxicological and economic data play key roles in these statutory programs for calculating the benefits and costs of regulation, respectively. The original TSCA statute enacted in 1976 is an example of a statute utilizing a balancing approach, requiring EPA to set standards for toxic substances based on a quantified cost-benefit analysis (*Corrosion Proof Fittings v EPA*, 1991). The 2016 amendments to TSCA generally eliminate consideration of costs and other non-risk factors in determining unreasonable exposures, thereby converting TSCA from a cost-benefit to an acceptable risk statute. Pharmaceutical drugs approved by the FDA continue to use a risk-benefit approach, whereby safety is determined by considering whether the health benefits of the drug exceed the health risks associated with the drug.

Feasibility/Best Available Technology

The third major approach requires the agency to reduce exposures to the lowest feasible level, or to require companies to install the best available technology. This approach does not explicitly rely on any health data, and this skirting of the complexities and uncertainties of toxicological evidence is seen by some as a major advantage of this approach. The consequence of ignoring health impacts, though, is that the resulting standards may overprotect or underprotect health. An example of such a feasibility-based approach is the Clean Water Act's effluent standards, which requires dischargers of pollutants to install the best available technology determined to be feasible for that industrial category. A program using a hybrid feasibility/acceptable risk approach is the Occupational Safety and Health Act, which directs the Occupational Safety and Health Administration (OSHA) to set workplace standards for hazardous exposures to the level "which most adequately assures, *to the extent feasible* … that no employee will suffer material impairment of health or functional capacity" (Occupational Safety and Health Act, 1970). This language has been interpreted by OSHA and the courts as requiring the agency to set occupational health standards at the lowest exposure level that is technologically and economically feasible, to the extent necessary to reduce a "significant" risk (*American Textile Manufacturers Institute v Donovan*, 1982).

SPECIFIC REGULATORY PROGRAMS UTILIZING TOXICOLOGICAL DATA

At the federal level, four agencies are chiefly responsible for regulating human exposure to toxic materials: the FDA, EPA, OSHA, and the Consumer Product Safety Commission (CPSC). Together they administer over two-dozen statutes whose primary goal is the protection of health. The statutes administered by these four agencies utilize different regulatory approaches and require divergent safety benchmarks. This diversity has several explanations. The statutes were enacted in different eras. They originated with different political constituencies and remain under their influence. Perhaps most significant, statutory standards often reflect differences in the technical capacity to control different types of exposures, and they embody different Congressional judgments about the economic implications of limiting exposures.

Food and Drug Administration (FDA)

The oldest of the major health regulation laws, the FFD&C Act, was originally enacted in 1906 and, as amended over time, now covers food for humans and animals, human and veterinary drugs, biologics, medical devices, cosmetics, and tobacco products.

Food The initial Food and Drug Act enacted in 1906 prohibited "adulterated" foods that contain "any poisonous or deleterious substance, which may render it injurious to health." This provision, which remains in the statute to date [FFD&C Act § 402(a)], does not require premarket approval of foods and puts the burden of proof on FDA to demonstrate that a food meets the definition of "adulterated."

Congress has since amended the act several times to strengthen the FDA's ability to ensure the safety of foods (FFD&C Act, 1938). The most important of these amendments was the 1958 Food Additives Amendments that require the safety of food additives to be demonstrated prior to marketing (Food Additive Amendments, 1958). The manufacturer of a food additive must submit a petition demonstrating that the substance is "reasonably certain to be safe"; no inquiry into the benefits of an additive is undertaken or authorized (Cooper, 1978). The food additive regulations require that the quantity of the substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritive, or other technical effect in food. Two categories of nonnaturally occurring food ingredients were exempted from this food additive requirement: (a) substances generally recognized as safe among experts qualified by scientific training and experience to evaluate safety; and (b) substances that either FDA or the US Department of Agriculture (USDA) had sanctioned for use in food prior to 1958 (so-called "prior sanction" substances).

Separate regulatory standards have been developed for several classes of indirect food constituents. For example, a food-contact substance requires approval as a food additive if, when used as intended, it "may reasonably be expected to become a component of food." In 1997 omnibus legislation that addressed most of FDA's regulatory programs, Congress created a new premarket notification system for food-contact materials that permits the agency to delay introduction if it questions a material's safety, but does not require affirmative agency approval (FDA Modernization Act, 1997).

In 2011, Congress enacted the FDA Food Safety Modernization Act (FSMA, 2011), which seeks to strengthen food safety by shifting the focus of federal regulators from responding to contamination to preventing unsafe foods. The legislation mandates the FDA to establish comprehensive, science-based preventive controls across the food supply, which includes a requirement for food facilities to adopt and implement written preventive control plans, a mandate that FDA establish science-based, minimum standards for the safe production and harvesting of fruits and vegetables, and new authority to prevent intentional contamination of food. The statute also gives FDA enhanced enforcement and inspection authority, authorizes FDA to conduct mandatory recalls of unsafe foods, and new powers to ensure that imported products meet U.S. standards and are safe for U.S. consumers.

Human Drugs and Biologicals Preclinical studies in animals play an important role in the FDA's evaluation of human drugs and biologicals. The current law requires premarket approval, for both safety and efficacy, of all "new" drugs, a category that embraces virtually all prescription drug ingredients introduced since 1938 (Hutt et al., 2007). Premarket approval of drugs for commercial use primarily relies on randomized controlled trials in human subjects to establish safety and efficacy. However, animal and in vitro studies are the primary source of information about a substance's biological effects before human trials are begun, and their results influence not only the decision whether to expose human subjects but also the design of clinical protocols.

The FDA reviews the clinical evidence before authorizing human clinical trials through review of an Investigational New Drug (IND) application. The IND must include (i) preclinical data to permit an assessment as to whether the product is reasonably safe for initial testing in humans, including animal pharmacology and toxicology studies (with the core battery of studies including the assessment of effects on cardiovascular, central nervous, and respiratory systems, as well as acute toxicity, with other types of studies conducted on a case-by-case basis as warranted), in vitro and in silico data, and any previous experience with the drug in humans (often foreign use); (ii) manufacturing information to ensure that the company can adequately produce and supply consistent batches of the drug, including data on the composition, manufacturer, stability, and controls used for manufacturing the drug; and (iii) clinical protocols and investigator information, including detailed protocols for proposed clinical studies, information on the qualifications of clinical investigators, and commitments to obtain informed consent from the research subjects and to obtain review of the study by an institutional review board (IRB).

Once an IND has been approved, the drug sponsor may proceed with human clinical studies consistent with the IND. The drug testing traditionally proceeds in three sequential phases. Phase 1 involves 20 to 100 healthy volunteers or people with the disease/condition and lasts several months, with the objective of evaluating drug safety and dosage. Phase 2 trials involve up to several hundred patients with the disease/condition, and last several months to 2 years, with the purpose of evaluating efficacy and side effects. Finally, at least two Phase 3 trials are required, which involve 300 to 3000 subjects who have the disease or condition, and these trials take 1 to 4 years. In some cases, such as when the FDA grants accelerated approval to a therapeutic, the agency can require a post-marketing study known as a Phase 4 study, to monitor the safety, efficacy, and side effects of the drug in clinical use.

Another category of drugs regulated by FDA is over-the-counter (OTC) drugs, which do not require a prescription. An OTC drug can receive regulatory authorization in two ways. First, the sponsor of an FDA-approved prescription drug can petition the FDA to change the status of the drug to OTC, a process referred to as an "Rx-to-OTC switch," if certain requirements regarding safety, efficacy, risk-benefit ratio, and consumer understanding of labeling can be demonstrated. The second method is the FDA "monograph" program, which consists of criteria for various categories of OTC drugs (e.g., antacid, cough/cold, dandruff shampoo, and sunscreens). A new product that meets the Monograph criteria for the relevant category of OTC drug can be marketed without FDA pre-approval.

Medical Devices In 1976, Congress overhauled the FFD&C Act's requirements for medical devices, providing the FDA major new authority to regulate their testing, marketing, and use. The elaborate new scheme contemplates three tiers of control, the most restrictive of which (class III) is premarket approval similar to that required for new drugs. To obtain FDA premarket approval of a class III device, the sponsor must demonstrate safety and efficacy. The bulk of the data supporting such applications will be derived from clinical studies but also will include toxicology studies of any constituents likely to be absorbed by the patient.

Cosmetics The statutory provisions governing cosmetics do not require premarket approval of any ingredient, or demand that manufacturers test their products for safety, though many manufacturers routinely do so. The basic safety standard for cosmetics is similar to that for food ingredients: no product may be marketed if it contains "a poisonous or deleterious substance, which may render it injurious to health" [FFD&C Act § 601(a)]. The case law establishes that this language, too, bars distribution of a product

that poses any significant risk of more than transitory harm when used as intended, but it places on the FDA the burden of proving a violation (Hutt et al., 2007). The FDA has brought few cases under this standard, in part because acute toxic reactions to cosmetics are readily detected and immediately result in abandonment of the offending ingredient. The FDA encourages cosmetic firms to participate in its Voluntary Cosmetic Registration Program (VCRP), which is an online registration system that cosmetic manufacturers, distributors, and packers can file information on their products that are currently being marketed to consumers in the United States and register their manufacturing and/or packaging facility locations in the VCRP database. The FDA also relies on the Cosmetic Ingredient Review (CIR), a private expert assessment body established in 1976 and operated by the Personal Care Products Council. The CIR appoints an expert panel that conducts safety assessments of cosmetics ingredients that are subject to public review and comment and then published as monographs.

Environmental Protection Agency (EPA)

The EPA is responsible for administering most of the nation's environmental laws, which include protection of both human health and the environment. A comprehensive review of the EPA's numerous programs is not possible here; the following summary focuses on those EPA activities in which toxicology evidence plays a central role: the agency-wide Integrated Risk Information System (IRIS) program, pesticide regulation, regulation of industrial chemicals, regulation of drinking water supplies, hazardous waste control, and regulation of toxic pollutants of water and of air.

IRIS Program EPA's IRIS provides an agency-wide scientific assessment program to evaluate the carcinogenic and noncarcinogenic human health risks of various chemicals. EPA prepares a Toxicological Review for a chemical, which goes through a series of internal and external reviews before being finalized, which is then used to derive an RfD and/or inhalation RfC for noncarcinogenic effects, and a weight-of-evidence determination as well as oral and inhalation unit risks for carcinogenic effects. IRIS values are used to support a variety of agency regulatory programs, including many of those described below. EPA has established IRIS values for over 500 chemical substances. In response to a critical National Research Council study of EPA's IRIS assessment of formaldehyde (NRC, 2011), the EPA has restructured its IRIS process to adopt systematic review methods and information management tools for study selection and analysis, and "data integration" approaches to develop overall hazard findings. In addition, IRIS assessments now derive organ-specific reference values (in addition to overall RfD and RfC values) when health effects data are available. The future of the IRIS program is in question, as both the Trump Administration and Congress are calling for major changes and possible funding cuts to the program.

Pesticides Pesticides are subject to two types of regulation: (i) registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA, 1972), which is required for a pesticide to be marketed in the United States; and (ii) tolerance (under the FFD&C Act), which sets the allowable level of pesticide residue in food for human consumption. Pesticide residues on raw agricultural commodities for years were initially regulated by the EPA under a 1954 amendment to the FFD&C Act, which allowed the agency to set tolerance levels based on weighing the potential adverse health effects of residues against the benefits of pesticide uses. If the concentration of a pesticide in a processed food exceeded the established tolerance, or the pesticide was a carcinogen, the Delaney clause prohibited its approval for that use (*Les v Reilly*, 1992). This framework was revised by Congress in 1996 by the Food Quality Protection Act (FQPA) to exempt pesticide residues from the operation of the Delaney clause but also restricted the consideration of pesticide benefits in setting tolerances.

The FQPA integrated the standard for registration and tolerance of a pesticide to require a pesticide to be shown to be safe, a standard defined as "reasonable assurance of no harm" (Food Quality Protection Act, 1996). The 1996 amendments also introduced some additional novel elements to the tolerance process. First, it required EPA to determine the safe level of a pesticide residue in a cumulative context, considering "all anticipated dietary exposures and all other exposures for which there is reliable information" [21 USC § 346a(b)(2)(A)(ii)]. This includes other nonoccupational sources of exposure, including food, drinking water, and residential exposures [21 USC § 346a(b)(2)(D)(vi)]. EPA must also base its safety determination on a consideration of exposures to other pesticides that share the same mechanism of toxicity [21 USC § 346a(b)(2)(C)(i)(III)].

Second, the 1996 FQPA required EPA to give special consideration to the health of infants and children, by ensuring "that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue …" [21 USC § 346a(b)(2)(C)(ii)(I)]. To implement this requirement, the statute directs EPA to "use an additional 10-fold margin of safety in assessing the risks to infants and children to take into account potential for pre- and postnatal toxicity and the completeness of the toxicology and exposure databases." EPA can deviate from the 10-fold margin of safety "only if, based on reliable data, the resulting margin would be safe for infants and children" [21 USC § 346a(b)(2)(C)(ii)(I)]. EPA's failure to provide a legally sufficient rationale for not applying the presumptive 10-fold extra safety factor for children has been found to be arbitrary and capricious by reviewing courts (*Northwest Coalition for Alternatives to Pesticides v EPA*, 2008; *NRDC v EPA*, 2011).

Third, EPA must develop a program to test for endocrine disrupting effects of pesticides. There has been a flurry of research over the past couple of decades addressing the identification and mechanisms of toxicity from endocrine-disrupting chemicals (Marty et al., 2011). The FQPA required EPA to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine effect as the Administrator may designate" [21 USC § 346a(p)(1)]. Although EPA was required to establish this program by 1999, it did not meet this deadline.

EPA has been moving forward nevertheless and has created an Endocrine Disruptor Screening Program, which involves a two-tier screening and testing process: In Tier 1 testing, EPA seeks to identify chemicals that have the potential to interact with the endocrine system. Tier 1 consists of 11 different assays, which are being applied to 67 pesticide ingredients on EPA's initial list of chemicals to be tested, which was released in 2009. EPA completed this first round of Tier 1 testing in September 2015 with results for 52 chemicals (the other 15 chemicals on the original list were dropped because they are no longer in use). Of these 52 chemicals, 34 were found to have no significant endocrine disruptor activity, while the other 18 were found to have some activity and were selected for Tier 2 testing. EPA finalized in 2013 a second list of 109 additional chemicals to be tested in Tier 1 (of which 2 were subsequently removed from the list in 2014). In Tier 2, EPA will determine the endocrine-related effects caused by each chemical that indicated endocrine disruption activity in Tier 1, and obtain information

about effects at various doses in order to enable risk assessment. The agency finalized three Tier 2 testing guidelines in August 2015 (EPA, 2015a). Also in 2015, EPA published a proposal to allow companies to use high-throughput assays and computational toxicology as an alternative screening method for endocrine disruptors (EPA, 2015b).

After the FQPA of 1996, the pesticide registration process now includes a broader review of the human health impacts from commercial use of the pesticides on applicators and other individuals, as well as effects on the environment. The manufacturer of a pesticide must produce a variety of data from tests done according to EPA guidelines. EPA's test guidelines, most recently revised in 2013, now encompass guidelines for several hundred types of tests recommended for pesticides (40 CFR 158 and 161). These tests evaluate whether a pesticide has the potential to cause adverse effects on humans, wildlife, fish, and plants, as well as possible contamination of surface water or ground water from leaching, runoff, and spray drift. The potential human risks that are evaluated include both acute and chronic toxicity. The EPA then conditions the use of the pesticide on various protections to ensure that the pesticide is used safely, which are summarized on the pesticide label.

The EPA has been engaged in a comprehensive review of previously registered pesticides and "reregistration" of those that meet contemporary standards for marketing for approximately the past three decades. Under this program, many older pesticides have been subjected to comprehensive toxicology testing, including carcinogenicity testing, for the first time, and the results have required modification of the terms of approved use and, in some instances, cancellation for several pesticides. The FQPA required EPA to review the registration ("reregistration") and tolerance of older pesticides. In addition, EPA initiated a new program in 2006 called "registration review" in which it intends to re-evaluate all pesticides every 15 years. Finally, EPA may initiate a "Pesticide Special Review" process whenever it discovers that the use of a registered pesticide may result in unreasonable adverse effects on people or the environment.

Industrial Chemicals The Toxic Substances Control Act (TSCA) was promulgated in 1976 to regulate all chemical substances manufactured or processed in or imported into the United States, except for substances already regulated under other laws (e.g., pesticides, tobacco products, firearms, foods, drugs, and cosmetics). *A chemical substance* is defined broadly as "any organic or inorganic substance of a particular molecular identity." Forty years after the initial adoption of TSCA in 1976, in 2016 the Frank R. Lautenberg Chemical Safety for the 21st-Century Act was signed into law, the first major amendments to the TSCA statute (TSCA, 2016).

As amended, TSCA gives the EPA three main powers. First, the agency is empowered to restrict or even ban the manufacturing, processing, distribution, use, or disposal of a chemical substance when there is a reasonable basis to conclude any such activity poses an "unreasonable risk of injury to health or environment." Prior to the 2016 amendments, the agency was instructed to balance the costs of restricting the substance against the health benefits of the proposed regulation and to choose "the least burdensome requirement" [TSCA § 6]. This trade-off approach to regulation proved a major challenge to the EPA and the agency exercised its regulatory authority under the original section 6 against only a handful of substances, and its most significant regulations, such as its attempted ban on asbestos products, have been struck down by the courts for failing to do an adequate cost-benefit analysis (*Corrosion Proof Fittings v EPA*, 1991). The statute itself imposes restrictions on two types of chemicals: mercury and polychlorinated biphenyls (PCBs).

The 2016 amendments to TSCA seek to rejuvenate section 6. The amended statute deletes the "the least burdensome requirement" provision, and requires EPA to undertake a prioritization process that involves screening existing chemicals based on factors such as hazard, exposure potential, persistence, and bioaccumulation, but without considering cost. Any chemical that is found to "may present an unreasonable risk" based on this screening analysis must be categorized as high priority and is subject to a risk evaluation. The risk evaluation is intended to determine whether a chemical presents an unreasonable risk, without considering costs or other non-risk factors. EPA must then promulgate a risk management standard for any chemical that is found to present an unreasonable risk in the risk evaluation.

The second major authority under TSCA is that if the EPA suspects that a chemical *may* pose an unreasonable risk but lacks sufficient data to take action, TSCA empowers the agency to require testing to develop the necessary data. Similarly, it may order testing if the chemical will be produced in substantial quantities that may enter the environment and/or result in significant human exposure whose effects cannot be predicted on the basis of existing data. In either case, the EPA must consider the "relative costs of the various test protocols and methodologies" and the "reasonably foreseeable availability of the facilities and personnel" needed to perform the tests [TSCA § 4]. The 2016 revisions to the TSCA testing authority require EPA to use tiered testing and screening approaches, and to reduce and replace vertebrate animal testing when this can be scientifically justified.

Finally, to enable the EPA to evaluate chemicals before humans are exposed, TSCA requires the manufacturer of a new chemical substance to notify the agency 90 days prior to production or distribution [TSCA § 5(a)(1)]. The manufacturer's or distributor's notice (called a pre-manufacturing notice or PMN) must include (i) chemical identification, including the Chemical Abstract (CA) name (when available), the chemical formula, and the molecular structure of the substance, and any synonyms, trade names, or generic names for the chemical; (ii) information on the physical properties of the substance; (iii) amounts and identities of any impurities in the chemical substance; (iv) any byproducts that result from the manufacture, processing, use, and disposal of the new chemical substance; (v) production volume for the first 12 months of production and estimate of the maximum production volume for any consecutive 12-month period during the first 3 years of manufacture; (vi) intended use of the substance including the degree of containment; (vii) any information on manufacturing, processing, and use operations involving the new chemical substance or products containing the new substance; (viii) any hazard warning statement, label, material safety data sheet (MSDS), or safety data sheet (SDS) available for the substance; (ix) anticipated environmental release and disposal information; and (x) (optionally), information on pollution prevention and recycling activities involving the new substance. In addition, the manufacturer must submit any hazard, toxicology, or safety data or studies it has on the substance.

Prior to the 2016 amendments, if EPA did not take action within 90 days, the manufacturer may proceed with commercialization of the chemical substance. Under the 2016 amendments, EPA *must* review all new chemicals and significant new uses (SNUs) and must make one of three findings: (1) that the new chemical or SNU presents an unreasonable risk of injury to health or the environment, in which case the EPA must regulate those risks; (2) that there is insufficient information to make a reasoned evaluation of the chemical or SNU, in which case the agency must obtain additional information from the manufacturer; or (3) that the chemical or SNU is not likely to present an unreasonable risk.

In contrast, the European Union (EU) is implementing a comprehensive regulatory program for chemical substances, known as the Registration, Evaluation, Authorization, and Restriction of Chemical substances (REACH) program. REACH puts the responsibility for ensuring the safety of chemicals on the companies that produce, import, and use chemicals. The program applies to both new and existing chemicals, and is phased-in over an 11-year period based primarily on the production volume of individual chemicals. REACH employs a tiered-testing approach, with the amount of initial testing determined by production volume. A technical dossier is required for all registered chemicals; however, only chemicals with a production volume of 10 or more tons per year require a chemical safety assessment documented in a chemical safety report. REACH allows the use of alternative testing strategies to evaluate specific endpoints in lieu of experimental testing; however, whole animal testing requirements exist for each level based on production volume.

Hazardous Wastes The two primary statutes regulating hazardous wastes in the United States are the Resource Conservation and Recovery Act (RCRA, 1976) and the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA, 1980). With some exceptions, the primary purpose of CERCLA is the cleanup of contamination from pre-existing hazardous waste sites, whereas RCRA regulates the generation, transport, and disposal of new hazardous wastes. Both RCRA and CERCLA have their own extensive lists of substances and waste streams of toxic materials that are defined as hazardous wastes. In addition, under RCRA, wastes that meet any of four "characteristics" are also treated as hazardous wastes. The four characteristics are ignitability, corrosivity, reactivity, and toxicity, and EPA has identified standardized protocols for determining whether a particular waste stream meets one or more of these characteristics. Under RCRA, EPA has developed standards for generators, transporters, and those who treat, store, or dispose hazardous wastes that are required to "protect human health and the environment." The EPA's regulations applicable to generators and transporters establish a manifest system that is designed to create a paper trail for every shipment of waste, from generator to final destination, to ensure proper handling and accountability. The agency has the broadest authority over persons who own or operate hazardous waste treatment, storage, or disposal facilities. Pursuant to the RCRA, it has issued regulations prescribing methods for treating, storing, and disposing of wastes; governing the location, design, and construction of facilities; mandating contingency plans to minimize negative impacts from such facilities; setting qualifications for ownership, training, and financial responsibility; and requiring permits for all such facilities (RCRA, 1976).

CERCLA, often referred to as Superfund, authorizes the cleanup of hazardous waste sites and provides for liability of persons responsible for releases of hazardous waste at these sites. EPA can conduct cleanup of hazardous waste sites itself, and then seek recovery for the costs from potentially responsible parties (PRPs), or it can direct one or more PRPs to conduct cleanup, who may then seek contribution from other PRPs for their share of the cleanup. Under CERCLA, EPA has created the National Priorities List (NPL), which is a list of hazardous waste sites that are deemed the most hazardous sites and eligible for use of federal funds for long-term cleanup, and the National Contingency Plan (NCP), which provides the guidelines and procedures needed to respond to releases and threatened releases of hazardous substances, pollutants, or contaminants. For sites on the NPL, EPA conducts a Remedial Investigation and Feasibility Study to support the risk management process, which includes a site-specific human health and ecological risk assessment.

Toxic Water Pollutants The EPA has had responsibility for regulating toxic water pollutants since 1972. As originally enacted, Section 307 of the Federal Water Pollution Control Act required the EPA to adopt standards for toxic water pollutants that provided an "*ample margin of safety*"—a difficult criterion to meet for most toxic pollutants and arguably impossible for any known to be carcinogenic. Given EPA's difficulties in complying with this statutory directive, Congress amended the statute in 1977 to focus standards on economic cost and technologic feasibility. In 1987, Congress amended the statute again to toughen standards for toxic pollutants. Under the prior law, the EPA had developed health-based "water quality criteria" for 126 compounds it had identified as toxic. These criteria essentially described *desirable* maximum contamination levels, which, because the EPA's discharge limits were technology-based, generally were substantially lower than the levels actually achieved. The 1987 amendments gave what had been advisory criteria real bite by requiring that states incorporate them in their own mandatory standards for water quality and impose additional effluent limits on operations discharging into below-standard waterways (Heineck, 1989).

Drinking Water The 1974 Safe Drinking Water Act (SDWA) was enacted to ensure that public water supply systems "meet minimum national standards for the protection of public health." Under the SDWA, the EPA is required to regulate any contaminants "which may have an adverse effect on human health." Over time and several Congressional amendments, this system has evolved into a two-tier system in which EPA adopts for each contaminant a maximum contaminant level goal (MCLG) that represents the optimal level to be achieved to ensure safety and the usually less stringent, feasibility-based maximum contaminant level (MCL). Only the MCL is legally enforceable. As part of the 1996 Safe Drinking Water Amendments, Congress directed EPA to use the "best available science" in setting MCLGs and MCLs.

Toxic Air Pollutants The original 1970 version of the Clean Air Act (CAA) provision for regulation of hazardous air pollutants (section 112) required the agency to set emission standards for individual toxic pollutants that protect public health with "an ample margin of safety." The implication of this language—that standards were to provide absolute safety without regard to the costs of emissions control—generated intense debate from the beginning and contributed to the EPA's glacial pace of implementation. The EPA finally attempted to escape the strict language of section 112 when it issued a standard for vinyl chloride in 1986 in which the agency claimed it could consider costs and declined to adopt a standard dictated solely by safety. A court ruled that the EPA had improperly considered costs, but it did make clear that in determining what emissions level was safe, even for a carcinogen, the EPA was not obligated to eliminate exposure but rather was to "decide what risks are acceptable in the world in which we live" (*NRDC v EPA*, 1987).

Amendments to the CAA in 1990 responded to the difficulties presented by the strict health-based approach of old section 112. The statute now provides a list of 188 hazardous air pollutants, which EPA may modify by adding or deleting items. The EPA must establish national emissions standards for sources that emit any of the listed pollutants with a two-tier system of regulation. The EPA must first issue standards that are technology-based, designed to require the "maximum achievable control technology" (MACT) [CAA § 112(d)(2)]. If the MACT controls are insufficient to protect human health with an "ample margin of safety," the EPA must issue residual risk standards [CAA § 112(f)]. The 1990 Amendments essentially define "ample margin of safety" for carcinogens by requiring the EPA to establish added residual risk limits for any

pollutant that poses a lifetime excess cancer risk of greater than one in one million. However, as subsequently clarified in a judicial decision, while EPA must promulgate residual risk standards if the MACT standards do not reduce risks to one in one million, the residual risk standards are not required to reduce the risks to one in one million, but rather to 100 in one million (*NRDC v EPA*, 2008).

Occupational Safety and Health Administration (OSHA)

The 1970 Occupational Safety and Health Act requires employers to provide employees with safe working conditions and empowers OSHA to prescribe mandatory occupational safety and health standards (OSHA, 1970). OSHA's most controversial actions involved its attempts to set occupational exposure limits for hazardous chemicals.

The Act specifies that in regulating hazardous occupational exposures, OSHA shall adopt the standard "which most adequately assures, to the extent feasible, on the basis of the best available evidence, that no employee will suffer material impairment of health or physical capacity" [OSHA § 6 (b)(5)]. Judicial challenges to OSHA standards have clarified OSHA's responsibilities. In a famous case, the U.S. Supreme Court overturned OSHA's benzene standard because the agency had not shown that prevailing worker exposure levels posed a "significant" health risk (*Industrial Union Department, AFL-CIO, v American Petroleum Institute*, 1980). This prerequisite proved a major obstacle when OSHA attempted to establish standards for 428 air contaminants in a single proceeding in 1989. Although it found that OSHA's generic approach to regulation was permissible in theory, a court vacated the standards because OSHA failed to show that each individual contaminant posed a "significant risk" at current levels (*American Federation of Labor v OSHA*, 1992). However, the Supreme Court earlier upheld OSHA's cotton dust standard, rejecting arguments that the agency was obligated to balance the costs of individual standards against the benefits of reducing hazardous workplace exposures (*American Textile Manufacturers Institute v Donovan*, 1982). The end result of these judicial challenges is that OSHA may adopt standards that reduce a "significant" risk to workers down to levels that are technologically and economically feasible, but cannot base its standards on a cost-benefit analysis.

OSHA promulgates substance-specific occupational exposure limits known as Permissible Exposure Limits (PELs), where compliance is measured with an 8-hour time-weighted average (TWA). For a few hazardous exposures (e.g., 1,3-butadiene, benzene, and ethylene oxide) OSHA has promulgated short-term exposure limits (STELs), which sets an average exposure limit for any 15-minute average that cannot be exceeded.

Another important standard promulgated and enforced by OSHA is the hazard communication standard (HCS). The HCS requires chemical manufacturers and importers to evaluate the hazards of the chemicals they produce or import, and prepare labels and material safety data sheets (MSDSs) to convey the hazard information to their downstream customers; and requires employers with hazardous chemicals in their workplaces to provide labels and MSDSs for their exposed workers, and train them to handle the chemicals appropriately. As of 2016, the HCS has been harmonized with the United Nations' (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS). OSHA has provided detailed criteria in Appendix A to §1910.1200—Health Hazard Criteria for determining whether a substance is hazardous under the HCS. For example, any substance listed in the National Toxicology Program (NTP) "Report on Carcinogens" (latest edition) or the International Agency for Research on Cancer (IARC) "Monographs on the Evaluation of Carcinogenic Risks to Humans" will be considered a carcinogen or possible carcinogen under the HCS.

In addition to the HCS and substance-specific exposure limits, OSHA also enforces more generic standards such as the general duty clause, the particles otherwise not regulated (total dust) standard, and the respiratory protection standard. Unlike EPA, OSHA does not have authority to require chemical safety testing.

Other organizations also issue occupational exposure limits, but unlike OSHA, these standards are guidance and not legally enforceable. For example, the American Conference of Governmental Industrial Hygienists (ACGIH) publishes recommended but nonenforceable exposure standards known as threshold limit values (TLVs) which can be for an 8-hour time-weighted average exposure (TLV-TWA) or a 15-minute short-term time-weighted average (TLV-STEL). The TLV is a level that a worker can be exposed to day after day for a working lifetime without adverse effects. In addition, ACGIH also publishes threshold limit value–Ceiling limit (TLV-C) which is an absolute exposure limit that should not be exceeded at any time. The National Institute of Occupational Safety and Health (NIOSH) also publishes some Recommended Exposure Limits or RELs (although as of January 2017, NIOSH recommends a new value for carcinogens known as a Risk Management Limit for Carcinogens, or RML-CA).

Consumer Product Safety Commission (CPSC)

Of the four agencies discussed here, the CPSC has played the least important role in federal efforts to control toxic chemicals to date. The commission was created in 1972 by the Consumer Product Safety Act (CPSA, 1972) with authority to regulate products that pose an unreasonable risk of injury or illness to consumers. The commission is empowered to promulgate safety standards "to prevent or reduce an unreasonable risk of injury" associated with a consumer product. If no feasible standard "would adequately protect the public from the unreasonable risk of injury" posed by a consumer product, the commission may ban the product [CPSA § 8]. In assessing the need for a standard or ban, the agency must balance the likelihood that a product will cause harm, and the severity of harm it will likely cause, against the effects of reducing the risk on the product's utility, cost, and availability to consumers.

The CPSC also administers the older Federal Hazardous Substances Act (FHSA, 1976). The FHSA authorizes the CPSC to regulate, primarily through prescribed label warnings, products that are toxic, corrosive, combustible, or radioactive or that generate pressure. The FHSA is unusual among federal health laws because it contains detailed criteria for determining toxicity. It defines "highly toxic" in terms of a substance's acute effects in specified tests in rodents; substances capable of producing chronic effects thus fall within the "toxic" category. The FHSA contains another unique provision [FHSA § 2(h)(2)] specifically addressing the probative weight of animal and human data on acute toxicity: "If the [commission] finds that available data on human experience with any substance indicates results different from those obtained on animals in the above-named dosages or concentrations, the human data shall take precedence."

The CPSC has prescribed labeling for products containing numerous substances that are acutely toxic. It has also acted to ban from consumer products several substances that pose a cancer risk, including asbestos, vinyl chloride as a propellant, benzene, tris(hydroxymethyl)aminomethane, and formaldehyde (Merrill, 1981).

In 2008, Congress adopted the Consumer Product Safety Improvement Act (2008), which significantly strengthened the CPSC's enforcement authority, especially for children's products, in response to a number of high-profile recalls of children's toys. The Act imposes bans on products containing lead or phthalates that exceed stringent levels, and requires third-party testing of certain children's products.

INSTITUTIONAL OVERSIGHT AND CONTROLS

As the previous subsection describes, regulatory agencies are required to evaluate and apply toxicological and other scientific data in a variety of statutory contexts. In addition to the statutory language, a number of internal and external institutional control mechanisms and processes greatly influence the agencies' treatment of toxicological data.

Advisory Committees

Regulatory agencies frequently rely on scientific advisory committees composed of external scientific experts to help guide their scientific determinations, including toxicological evaluations. For example, the EPA has a Scientific Advisory Board (SAB), established by Congress in 1978, to advise the agency on the quality and relevance of the scientific and technical information being used in regulatory programs as well as to review the agency's research programs. The SAB's work is done by a relatively large number of subcommittees and panels that focus on special subjects. The EPA also has two other independent scientific advisory committees associated with specific regulatory programs—the Clean Air Science Advisory Committee for air pollution, and the Science Advisory Panel for pesticides. These committees consist of external scientists who are appointed to serve on the committees for specified terms. Similarly, the FDA has over 50 external advisory committees that provide advice on the agency's significant product approvals and other scientific issues.

Regulatory agencies are not required to automatically follow the advice of their external advisory committees, but frequently do. Failure to follow the independent advice of their own advisory committee tends to call into question the soundness of the agency's scientific findings, whereas advisory committee endorsement of the agency's position can provide important scientific credibility and political cover to the agency.

Peer Review

Beyond peer review by agency advisory committees, regulatory agencies frequently utilize external peer review of scientific reports and assessments. In December 2004, the Office of Management and Budget (OMB) formalized the requirement for peer review by issuing its Final Information Quality Bulletin for Peer Review (OMB, 2004). This Peer Review Bulletin requires federal regulatory agencies to ensure that all "influential scientific information" they disseminate is peer-reviewed. Influential scientific information is defined as "scientific information the agency reasonably can determine will have or does have a clear and substantial impact on important public policies or private sector decisions." The Bulletin specifically identifies "scientific assessments" as one type of information subject to the peer review requirement, which are defined as "an evaluation of a body of scientific or technical knowledge, which typically synthesizes multiple factual inputs, data, models, assumptions, and/or applies best professional judgment to bridge uncertainties in the available information." These assessments include, but are not limited to, state-of-science reports; technology assessments; weight-of-evidence analyses; meta-analyses; health, safety, or ecological risk assessments; toxicological characterizations of substances; integrated assessment models; hazard determinations; or exposure assessments.

The Peer Review Bulletin directs agencies to choose a peer review mechanism that is appropriate and commensurate with how the information will be used, giving due consideration to the novelty and complexity of the science involved, the relevance of the information to regulatory decision-making, the extent of prior peer reviews, and the expected benefits and costs of additional review. The Peer Review Bulletin requires the most rigorous peer review requirements for highly influential scientific assessments.

Risk Assessment Guidance/Guidelines

Regulatory agencies often adopt guidelines to promote consistency and predictability in their treatment of toxicological data and the conduct of risk assessment. For example, EPA has adopted risk assessment guidelines for carcinogenicity, mutagenicity, developmental toxicity, chemical mixtures, exposure analysis, reproductive toxicity, neurotoxicity, and ecotoxicity. The genesis of EPA's risk assessment guidelines was a series of "cancer principles" prepared largely at the instigation of EPA lawyers in the mid-1970s (Albert, 1994). The agency lawyers were frustrated by the delays in regulating pesticides as a result of scientific disagreement about the mechanisms and causes of human cancer. In an attempt to circumvent such scientific disputes, the lawyers developed a series of 17 "cancer principles" with the assistance of EPA scientists during hearings on the pesticides heptachlor and chlordane. The 17 principles were intended to be nonrebuttable guidelines that would govern and simplify carcinogenicity determinations.

Shortly thereafter, EPA began to develop a more comprehensive set of guidelines for evaluating carcinogens. This led to EPA publishing its "interim" guidelines for carcinogenic risk assessment in 1976, which were subsequently updated and finalized in 1986 (EPA, 1986). The 1986 guidelines primarily consisted of a series of "default options" that were intended as presumptive principles that would apply in the absence of sufficient data to establish an alternative assumption. Some of the most important default options included in EPA's 1986 carcinogen guidelines are: (i) laboratory animals are assumed to be a valid surrogate for humans in assessing risk; thus, positive cancer results in animal bioassays are taken as evidence of human carcinogenicity; (ii) humans are assumed to be as sensitive as the most sensitive animal species, strain, or sex evaluated in an appropriately designed animal bioassay; (iii) benign tumors are assumed to be as significant as malignant tumors; (iv) the dose–response of humans to potential carcinogens is assumed to be linear all the way to the zero exposure levels with no threshold; and (v) a given intake of a substance is assumed to have the same effect regardless of the rate or route of intake (EPA, 1986). Most of the default options selected by EPA were deliberately chosen to be "conservative," in that they are intended to estimate the plausible upper-bound of actual risk (NRC, 1994).

EPA's risk assessment guidelines not only promoted consistency in risk assessment approach and procedure across the broad array of EPA regulatory programs, which use risk assessment, but also furthered the objective of efficiency, by sparing the agency the need to revisit the same controversial issues in each successive rulemaking proceeding (Wiltse and Dellarco, 1996). The courts were generally supportive of the predictability and consistency provided by the agency's risk assessment guidelines, giving greater deference

to EPA risk assessments that comported with the guidelines. As one court stated, "EPA's specific enunciation of its underlying analytical principles, derived from its experience in the area, yields meaningful notice and dialogue, enhances the administrative process, and furthers reasoned agency decision making" (*Environmental Defense Fund, Inc, v Environmental Protection Agency*, 1976).

Notwithstanding these benefits of risk assessment guidelines, EPA's implementation of its guidelines was criticized in a 1994 NRC report that found that EPA applied the default principles too rigidly and had failed to articulate clear criteria for departing from the defaults (NRC, 1994). EPA itself acknowledged that "[i]n practice, the agency's assessments routinely have employed defaults and, until recently, only occasionally departed from them" (EPA, 1996). EPA published draft revisions to its carcinogen guidelines in 1996 (EPA, 1996) that were finalized in 2005 (EPA, 2005), which take a more flexible, data-based approach to default options. As explained by EPA, the revised guidelines "are intended to be both explicit and more flexible than in the past concerning the basis for making departures from defaults, recognizing that expert judgment and peer review are essential elements of the process" (EPA, 1996). The revised guidelines incorporate "a weight-of-the-evidence approach that considers all relevant data in reaching conclusions about the potential human carcinogenicity of an agent" (EPA, 1996). In 2014, EPA published "Framework for Human Health Risk Assessment to Inform Decision Making," which adopts a framework for risk-based decision-making that maximizes the utility of risk assessment (EPA, 2014).

Judicial Review

Agency regulations are often subject to legal challenges by stakeholders who disagree with the agency's decision. Under the Administrative Procedure Act, such judicial review tends to be deferential to the agency's decision, overturning the decision only if it is "arbitrary and capricious" or lacking "substantial evidence." Nevertheless, a significant proportion of major agency regulations are overturned in whole or in part, or sent back to the agency for further explanation. Scientific determinations, such as toxicological findings, are generally given the highest level of deference under a decision by the U.S. Supreme Court, which held that "[w]hen examining this kind of scientific determination, as opposed to simple findings of fact, a reviewing court must generally be at its most deferential" (*Baltimore Gas & Elec v NRDC*, 1983).

Even with this deference to scientific determinations, there have been many important judicial decisions impacting the way regulatory agencies address toxicological information. One prominent example was a challenge to the EPA's decision to retain an MCLG of zero under the Safe Drinking Water Act for chloroform in the face of evidence, accepted by its own scientific advisory committee, that the mechanism by which chloroform produces tumors in rodents is threshold-limited. The reviewing court held that given its acceptance of the evidence of a threshold, the EPA was required by the SDWA's "best available" evidence provision to base its decision on that evidence (*Chlorine Chemistry Council v EPA*, 2000). This ruling marks the first time that a court has directed a federal regulatory agency to recognize a "safe" finite level of exposure for an animal carcinogen. Another example of proactive judicial oversight is two recent decisions where the court second-guessed EPA's determination that there was sufficient toxicological evidence to justify departing from the presumptive 10-fold extra safety factor to protect children in pesticide regulatory decisions (*Northwest Coalition for Alternatives to Pesticides v EPA*, 2008; *NRDC v EPA*, 2011).

Congressional Oversight

Congress has ultimate oversight of regulatory agencies, both by delegating the authority to regulate in the form of statutes, and by controlling the purse strings for the agencies. Regulatory statutes determine many of the fundamental choices for regulatory programs by specifying what factors may be considered in adopting regulatory standards and the objectives of the standards in terms of the level of safety to be attained. Statutes differ greatly on these questions, with some mandating standards being based on technological feasibility, whereas others specifying health-based regulation. The statutes can provide additional guidance that can influence the agency's consideration of toxicological evidence, such as the 1996 amendments to the Safe Drinking Water Act, which direct EPA to use "the best available, peer-reviewed science and supporting studies conducted in accordance with sound and objective scientific practices" (Safe Drinking Water Act, 1996).

Congress also controls regulatory programs through its budgetary authority. By controlling how much money is directed to specific activities or programs, Congress can directly influence agency priorities and emphasis. If Congress is not pleased with an agency's actions, it can adopt appropriation riders prohibiting the agency from spending money on controversial topics. In the past, for example, Congress has prevented EPA from working on climate change or radiofrequency issues by imposing annual riders for a time period.

In addition to these primary legislative controls, Congress has a number of other ways it can influence agency decision-making. Congress can pressure agencies toward a preferred policy outcome through oversight hearings, letters to agency officials, and press releases. Congress has also adopted some oversight statutes to further control agency rulemaking. The Congressional Review Act requires federal regulatory agencies to file final rules with Congress before the rules can become effective. If the regulation meets the definition of a major rule ($100-million impact on the economy), Congress has 60 days to introduce a resolution of disapproval that, if adopted by both the House and Senate, can nullify the agency's rule, unless vetoed by the President. If a disapproval resolution is enacted, the rule may not take effect and the agency may issue no substantially similar rule without subsequent statutory authorization. The Congressional Review Act was only used once prior to 2017 to disapprove an agency regulation (OSHA ergonomic standard in 2001), the U.S. Congress has used this authority to disapprove more than ten agency rules in the first half of 2017.

Congress also enacted section 515 of the Treasury and General Government Appropriations Act of 2001, known as the Information Quality Act (IQA), which required the Office of Management and Budget (OMB) to promulgate guidance to agencies ensuring the quality, objectivity, utility, and integrity of information (including statistical information) disseminated by federal agencies (IQA, 2001). Federal agencies were also required by the IQA to publish their own agency-specific guidelines no later than 1 year after OMB's guidelines, and also provide a mechanism for stakeholders to file petitions challenging agency actions that allegedly fail to comply with the data quality objectives. Private parties have challenged a number of scientific determinations by federal agencies under this provision, but the courts have determined that the agency decisions on these petitions are not judicially reviewable (*Salt Institute v Leavitt*, 2006).

Executive Oversight

The Executive branch also exerts overview of regulatory decisions, particularly through the Office of Information and Regulatory Affairs (OIRA) within the White House OMB. OIRA reviews all

draft significant regulations proposed by regulatory agencies, and although its review most often focuses on the economic aspects of the proposed rule, it also reviews the agency's scientific assumptions and findings. Although OIRA cannot itself revise the proposed regulation, it can hold up its publication until it has reached agreement with the agency on acceptable revisions.

The Executive Branch has other mechanisms for oversight and influence of regulatory science determinations by agencies. The White House under various Presidents has put out a series of Executive Orders providing regulatory guidance that agencies must follow to the extent consistent with the governing regulatory statute. For example, Executive Order 13563 issued by President Obama in January 2011 requires that federal regulations "must be based on the best available science. It must allow for public participation and an open exchange of ideas. It must promote predictability and reduce uncertainty. It must identify and use the best, most innovative, and least burdensome tools for achieving regulatory ends. It must take into account benefits and costs, both quantitative and qualitative" (EO 13563, 2011).

The White House also issued a memorandum on Scientific Integrity in 2009 that required each regulatory agency to develop its own scientific integrity policy that implemented several principles of scientific integrity, including: "(a) The selection and retention of candidates for science and technology positions in the executive branch should be based on the candidate's knowledge, credentials, experience, and integrity; (b) Each agency should have appropriate rules and procedures to ensure the integrity of the scientific process within the agency; (c) When scientific or technological information is considered in policy decisions, the information should be subject to well-established scientific processes, including peer review where appropriate, and each agency should appropriately and accurately reflect that information in complying with and applying relevant statutory standards …" (White House, 2009).

REGULATORY INFLUENCES ON TOXICOLOGY

This chapter has focused thus far on how toxicological data are used in federal regulatory programs. Yet, the relationship between regulation and toxicology is reciprocal, in that regulatory programs have also shaped and influenced the practice of toxicology. Modern toxicology has developed, in substantial part, in response to the information needs of contemporary regulation. But government regulation impinges on the discipline of toxicology in more direct ways as well. Regulatory agencies often prescribe the specific objectives and design of studies that are conducted to satisfy regulatory requirements. In addition, pressure to protect animals used in research has produced laws and regulations that govern toxicologists themselves.

Testing Guidelines

An agency's influence over the conduct of toxicology studies depends on its regulatory responsibilities. An agency such as the FDA or the EPA, which must confirm the safety of new substances before marketing, can dictate the kinds of tests that manufacturers must conduct to gain approval. By contrast, an agency or program that has no premarket approval function has less leverage.

Agencies such as the FDA and EPA that have been authorized to require premarket approval for certain categories of products have generally specified the types of tests they require before they will consider the safety of a compound (e.g., tests for acute toxicity, subchronic effects, and chronic effects). Within each of these categories, an agency might set out more detailed requirements, essentially enumerating its "base set" data demands. In addition, the agencies usually adopt guidelines that describe preferred or acceptable methods for executing particular tests, such as a bioassay for carcinogenesis (FDA, 2000; EPA, 2011a). In addition, the EPA has established guidelines for several of the tests that it may mandate by rule or consent agreement for individual chemicals under TSCA § 4 (EPA, 2011a). Multinational bodies such as the Organization for Economic Cooperation and Development have sought to secure multilateral adherence to standardized test guidelines and minimum testing requirements for new chemicals (Page, 1982).

Both the FDA and the EPA have adopted another set of requirements that specify laboratory procedures for conducting tests required or submitted for regulatory consideration. These GLP regulations prescribe essential but often mundane features of sound laboratory science, such as animal husbandry standards and record-keeping practices (EPA, 2011b, 2011c; FDA, 2011a). The GLP regulations including requirements for (i) organization and personnel responsibilities (e.g., study director should be "a scientist or other professional of appropriate education, training, and experience, or combination thereof"); (ii) a quality assurance program; (iii) facilities; (iv) equipment, reagents, and materials; (v) test systems; (vi) test and reference items; (vii) standard operating procedures; (viii) performance of study; (ix) reporting of results; and (x) archival storage of records and reports. All of these requirements are intended to contribute to sound regulatory decision-making by ensuring the quality and integrity of toxicology data submitted to support agency decisions.

Food and Drug Administration The FDA exercises premarketing approval authority over several classes of compounds, of which the most important, for present purposes, are new human drugs and direct additives to food.

Toxicology Testing Requirements for Human Drugs In 1962, Congress expressly authorized the FDA to exempt investigational drugs from the premarket approval requirement so that they could be shipped for use in clinical testing, subject to conditions the agency believed appropriate to protect human subjects [FFD&C Act § 505(i)]. One condition that the FDA established was that an investigational drug first must have been evaluated in preclinical studies, which are then submitted to FDA as an Investigational New Drug (IND) application. This requirement appears in current regulations that amplify, in the text and in referenced guidelines, the types of tests that are to be performed and the design they should follow (FDA, 2011b). A drug's sponsor is encouraged to consult agency personnel to get a precise understanding of what sorts of toxicology studies the agency expects prior to submitting the IND. Preclinical studies of substances that are candidates for use as human drugs must also meet the standards set by the FDA's GLP regulations (FDA, 2011a). These regulations apply to all laboratories—university, independent, and manufacturer-owned—in which such studies are conducted. The work of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) highlights the trend toward international agreement on test methods. Since its founding in 1990, the ICH—comprising the pharmaceutical regulatory agencies and industries from the European Union, Japan, and the United States—has issued over 50 harmonized guidelines on various aspects of pharmaceutical regulatory approval, including numerous guidelines on toxicology testing methods for human drugs.

Testing Requirements for Food Additives The Food Additives Amendment and the Color Additive Amendments require premarket approval of new additives to human food. Both laws

assume that laboratory studies in animals will provide the principal data for assessing safety. Thus, a petitioner must submit "full reports of investigations made with respect to the safety for use of such additives, including full information as to the methods and control used" [FFD&C Act § 409(c)]. The FDA has adopted regulations specifying the types of data that an applicant must submit (FDA, 2011c).

The FDA's regulations contain only general statements about the need for and features of toxicology studies. For many years, the agency maintained an advice-giving system in which it prescribed the type and design of tests to be performed. In 1982, the FDA first codified this "common law" in *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food,* known thereafter as the "Red Book." The Red Book, revised in 2000, describes the types of tests the FDA believes necessary to evaluate an additive's safety (FDA, 2000). The agency's requirements, which are in the form of guidelines rather than regulations, are calibrated to the purposes for which the additive will be used, to estimated levels of human exposure, and to the results of sequential studies. Tests of food color and additives must also comply with the FDA's GLP regulations (FDA, 2011a).

Environmental Protection Agency The EPA's premarket approval authority over pesticides places it, like the FDA, in a position to dictate the design and conduct of studies on such compounds. The 1976 TSCA gave the EPA authority to mandate testing of other chemicals in use or scheduled for introduction and to specify, by regulation, test standards.

Toxicology Requirements for Pesticides The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requires the submission of toxicology studies, as well as other types of investigations, to support the EPA's evaluation of a pesticide [FIFRA § 136(b)]. The statute also requires the EPA to publish guidelines specifying the kinds of information that will be required to support the registration.

The agency has issued regulations outlining the procedures for submission of registration petitions and their basic content (40 CFR part 158 and 161). The Office of Chemical Safety and Pollution Prevention (formerly the Office of Prevention, Pesticides, and Toxic Substances) has issued Harmonized Test Guidelines for all toxicology testing done under both FIFRA (pesticides) and TSCA (chemicals) (EPA, 2011a). For example, the Health Effects Test Guidelines (Series 870) include guidelines for approximately 50 different specific assays for acute toxicity, subchronic toxicity, chronic toxicity, genetic toxicity, neurotoxicity, and other toxicity testing. EPA has attempted to harmonize these guidelines whenever possible with applicable Organization for Economic Cooperation and Development (OECD) guidelines. Animal studies of pesticides must also comply with EPA's own GLP regulations, which were inspired by the same investigations that led the FDA to promulgate its standards for testing laboratories and impose similar requirements.

Testing of Industrial Chemicals The primary means by which the EPA may mandate health effects testing of new or existing industrial chemicals is Section 4(a) of the TSCA. Prior to the 2016 amendments to the TSCA (TSCA, 2016), the agency was required to promulgate a regulation to require testing of a specific compound, but the 2016 amendments provide more flexibility and allow EPA to also require testing by administrative order. This obligation to order testing is triggered by an administrative finding that a chemical presents a potential risk (based on the suspicion of toxicity) or that humans or the environment will be exposed to substantial quantities. The statute creates an Interagency Testing Committee (ITC) with members from EPA, CPSC, FDA, Department of the Interior (DOI), OSHA, Council on Environmental Quality (CEQ), National Institute for Occupational Safety and Health (NIOSH), National Institute of Environmental Health Sciences (NIEHS), National Cancer Institute (NCI), National Science Foundation (NSF), Agency for Toxic Substances and Disease Registry (ATSDR), National Library of Medicine (NLM), USDA, and the Department of Commerce to recommend a list of chemicals that should be tested and in what order of priority. Once the ITC has recommended a chemical substance for testing, the EPA is required by statute to either initiate testing or publish its reasons for not doing so within 12 months, although given the administrative burdens of such action the agency has frequently failed to meet this deadline.

Validation of Alternative Test Methods

Regulatory agencies have also been instrumental in encouraging the development of new safety test methods. Federal agency validation of new toxicological test methods was formalized and harmonized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Authorization Act of 2000, which permanently established the ICCVAM. Some 15 federal agencies participate in ICCVAM. Congress directed that one of the purposes of ICCVAM was to "ensure that new and revised test methods are validated to meet the needs of Federal agencies" [Pub L 106-545, §3(b)(4)]. In that regard, ICCVAM is directed to review and evaluate new or revised or alternative test methods and to provide test recommendations to federal agencies. Each federal agency administering a program that "requires or recommends acute or chronic toxicological testing" is required to adopt an alternative test method recommended by ICCVAM unless the agency determines that the alternative test method would not be consistent with its statutory goals.

The ICCVAM statute also requires that each federal agency "that requires or recommends acute or chronic toxicological testing shall ensure that any new or revised acute or chronic toxicity test method, including animal test methods and alternatives, is determined to be valid for its proposed use prior to requiring, recommending, or encouraging the application of such test method" [Pub L 106-545, §4(c)]. ICCVAM has established detailed criteria for the validation and regulatory acceptance of toxicological test methods (NIEHS, 1997). ICCVAM defines validation as "the process by which the reliability and relevance of a test method are evaluated for the purpose of supporting a specific use." The ICCVAM criteria stipulate that test methods that have been validated according to the ICCVAM criteria are not automatically acceptable for regulatory agencies. The ICCVAM criteria thus specify a second set of criteria for regulatory acceptance, which address the existence of standard operating procedures for the test method, its cost-effectiveness, its utility for agency risk assessment, its potential for harmonization with similar testing requirements by other entities, and its potential for reducing the use of animals. The ICCVAM statute preserves the individual agency's final authority to accept or reject a specific test method based on its statutory mandate and regulatory mission.

The ICCVAM process has now been used to validate a significant number of alternative test methods, including for acute oral toxicity, dermal irritation, acute inhalation toxicity, endocrine disruptors, biologics testing, genetic toxicity, ecotoxicity, ocular irritation, allergic contact dermatitis, and reproductive and developmental toxicity. The ICCVAM Authorization Act also directs federal agencies to reduce, refine, and/or replace the use of animals in testing where feasible.

Computational Toxicology

Another area where regulatory demands and programs are pushing the development of new toxicology tests is the field of computational toxicology. Computational toxicology uses high-powered

computers to discern patterns and findings from large data sets derived from quantitative structure–activity relationships, in vitro assays, and various 'omic technologies (e.g., toxicogenomics, proteomics, and metabolomics). The regulatory driver for computational toxicology is the need to evaluate much larger numbers of chemicals much quicker and at lower costs, and perhaps eventually even with more robust and relevant results, than traditional animal studies. The shift toward computational toxicology was boosted by a 2007 Natural Research Council report entitled *Toxicity Testing in the 21st Century: A Vision and a Strategy*, which endorsed greater reliance on computational methods (NRC, 2007). EPA has launched an ambitious program to implement computational methods into its regulatory programs, which is called the ToxCast program. The ToxCast program has so far screened over 1800 chemicals using over 700 high-throughput assays that cover a range of high-level cell responses and approximately 300 signaling pathways.

In 2008, EPA and the National Institutes of Health (NIH) signed a memorandum of agreement to work together to develop these new toxicological methods for regulatory applications (Collins et al., 2008), which grew into an inter-agency collaboration called Toxicology in the 21st century (Tox21). This federal collaboration among EPA, NIH, including National Center for Advancing Translational Sciences and the National Toxicology Program at the National Institute of Environmental Health Sciences, and the Food and Drug Administration, has the objective of developing better toxicity assessment methods to quickly and efficiently test whether certain chemical compounds have the potential to disrupt processes in the human body that may lead to negative health effects. Using a high-throughput robotic screening system, the program is screening 10,000 environmental chemicals (called the Tox21 10K library) for their potential to disrupt biological pathways that may result in toxicity.

Animal Welfare Requirements

Researchers who conduct studies funded by federal agencies must comply with the Animal Welfare Act (AWA), and some are also subject to restrictions imposed by the Public Health Service (PHS). Recipients of grants from the Department of Education, the Department of Agriculture, or the EPA are subject only to the AWA. Those funded by the Department of Energy or by the PHS (which includes all research agencies within the Department of Health and Human Services, including the NIH) must also comply with PHS policies. Restrictions on animal use also appear in the GLP regulations adopted by the FDA and the EPA (Reagan, 1986).

Animal Welfare Act The AWA was initially adopted in 1966 and has been subsequently amended several times (1970, 1976, 1985, 1990, 2002, 2007, and 2008). It is administered by the Animal and Plant Health Inspection Service (APHIS), a part of the USDA. The AWA, which protects only warm-blooded animals and excludes birds, rats, and mice bred for research, requires all covered research facilities to register with APHIS and agree to comply with applicable AWA standards for humane treatment of animals. Each facility must file an annual report signed by a responsible official that shows that "professionally acceptable standards governing the care, treatment, and use of animals" were followed for the year in question (Animal Welfare Act, 1988).

Pursuant to the AWA, APHIS has established specific requirements for the humane handling, care, and transportation of dogs and cats, guinea pigs and hamsters, rabbits, nonhuman primates, marine mammals, and other warm-blooded animals. The regulations governing facilities address living space, heating, lighting, ventilation, and drainage. The health and husbandry provisions address feeding, watering, sanitation, veterinary care, grouping of animals, and the number and qualifications of caretakers (APHIS, 2011).

The AWA requires each research facility to establish an Institutional Animal Care and Use Committee (IACUC), composed of three or more members, one of whom must be a veterinarian and one of whom must represent community interests and who may not be affiliated with the institution. In 1989, the APHIS expanded the responsibilities of the IACUC and increased the minimum size to five members, and must include a veterinarian, an animal research scientist, a nonscientist, and an individual not affiliated with the institution. At least one member of the IACUC must now review and approve the animal care and use components of all proposed research activities. Prerequisites to approval include the avoidance or minimization of discomfort, distress, and pain; the use of pain-relieving drugs where appropriate; the consideration of pain-free alternatives; and euthanization when an animal would otherwise experience severe or chronic pain or distress that cannot be relieved.

The IACUC is also responsible for conducting semiannual inspections of the facility itself and of the program for humane care and use of animals. Committee reports are filed with the APHIS and with any federal agency funding the research.

Public Health Service Policy The PHS Policy on Humane Care and Use of Laboratory Animals applies to research funded by the NIH and other federal agencies that uses any vertebrate animals, and thus has a broader reach than the AWA. The PHS policy requires each facility to submit an annual report, called an "Assurance," which is evaluated by the NIH Office of Laboratory Animal Welfare (OLAW) to determine the sufficiency of animal care.

The PHS policy imposes two primary obligations on researchers as a condition for receiving federal funding: each institution must adopt an Institutional Program for Animal Care and Use, and it must establish an IACUC. The IACUC must review all applications for research funding and review the institution's programs to ensure compliance with NIH standards. The Health Research Extension Act of 1985 requires that PHS-funded institutions provide training on methods to reduce animal suffering similar to that mandated by the AWA for its personnel. It also requires that researchers' grant applications justify any proposed use of animals (NRC, 1988).

Research facilities subject to either the AWA or PHS may wish to consult a National Academy of Sciences' report that details suggestions for developing institutional compliance programs (NRC, 1991). Scientists working with no federal funding who expect their research to be submitted to the FDA or the EPA are not subject to the AWA or PHS policies, but they must comply with the animal protection provisions of those agencies' GLP regulations. These regulations prescribe adequate living conditions, detail requirements for veterinary treatment, and impose specific record-keeping requirements (EPA, 2011b, 2011c; FDA, 2011a).

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REFERENCES

Cases

American Federation of Labor v OSHA, 965 F2d 962 (11th Cir 1992).
American Textile Manufacturers Institute v Donovan, 452 US 490 (1982).
Baltimore Gas & Elec v NRDC, 462 US 87 (1983).

Chemical Manufacturers Association v EPA, 899 F2d 344 (5th Cir 1990).
Chlorine Chemistry Council v EPA, 206 F3d 1286 (DC Cir 2000).
Corrosion Proof Fittings v Environmental Protection Agency, 947 F2d 1201 (5th Cir 1991).
Environmental Defense Fund, Inc, v Environmental Protection Agency, 548 F2d 998 (DC Cir 1976).
Industrial Union Department, AFL-CIO, v American Petroleum Institute, 448 US 607 (1980).
Les v Reilly, 968 F2d 985 (9th Cir 1992).
Natural Resources Defense Council, Inc., v United States Environmental Protection Agency, 595 F Supp 1255 (SD NY 1984).
Natural Resources Defense Council, Inc., v United States Environmental Protection Agency, 824 F2d 1146 (DC Cir 1987).
Natural Resources Defense Council, Inc., v United States Environmental Protection Agency, 529 F3d 1077 (DC Cir 2008).
Natural Resources Defense Council, Inc., v United States Environmental Protection Agency, 658 F3d 200 (2nd Cir 2011).
Northwest Coalition for Alternatives to Pesticides v EPA, 544 F3d 1043 (9th Cir 2008).
Salt Institute v Leavitt, 440 F3d 156 (4th Cir 2006).
Whitman v American Trucking Associations, Inc, 531 US 457 (2001).

Secondary Sources

Albert RE. Carcinogen risk assessment in the U.S. Environmental Protection Agency. *Crit Rev Toxicol*. 1994;24:75–85.
Brickman R, Jasanoff S, Ilgen T. *Controlling Chemicals: The Politics of Regulation in Europe and the United States*. Ithaca, NY: Cornell University Press; 1985.
Collins FS, Gray GM, Bucher JM. Transforming environmental health protection. *Science*. 2008;319:906–907.
Cooper R. The role of regulatory agencies in risk-benefit decision-making. *Food Drug Cosmet L J*. 1978;33:755–757.
GAO. *EPA Implementation of Selected Aspects of the Toxic Substances Control Act*. Washington, DC: U.S. General Accounting Office; 1982.
Heineck D. New clean water act toxics control initiatives. *Nat Resour Environ*. 1989;4(1):10–12, 49–50.
Hutt PB, Merrill R, Grossman LA. *Food and Drug Law: Cases and Materials*. 3rd ed. Mineola, NY: Foundation Press; 2007.
Marty MS, Carney EW, Rowlands JC. Endocrine disruption: historical perspectives and its impact on the future of toxicology testing. *Toxicol Sci*. 2011;120(S1):S93–S108.
Merrill R. CPSC regulation of cancer risks in consumer products: 1972–81. *Va L Rev*. 1981;67:1261.
National Research Council. *Risk Assessment in the Federal Government: Managing the Process*. Washington, DC: National Academy; 1983.
National Research Council. *Use of Laboratory Animals in Biomedical and Behavioral Research*. Washington, DC: National Academy; 1988.
National Research Council. *A Guide for Developing Institutional Programs*. Washington, DC: National Academy; 1991.
National Research Council. *Science and Judgment in Risk Assessment*. Washington, DC: National Academy; 1994.
National Research Council. *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Washington, DC: National Academy; 2007.
National Research Council. *Science and Decisions*. Washington, DC: National Academy; 2009.
National Research Council. *Review of the Environmental Protection Agency's Draft IRIS Assessment of Formaldehyde*. Washington, DC: National Academy; 2011.
Page NP. Testing for health and environmental effects: The OECD guidelines. *Toxic Substances J*. 1982;4:135.
Reagan K. Federal regulation of testing with laboratory animals: future directions. *Pace Environ L Rev*. 1986;3:165.
Wagner W. The science charade in toxic risk regulation. *Columbia L Rev*. 1995;95:1613–1723.
Weinberg A. Science and its limits: the regulator's dilemma. *Issues Sci Tech*. 1985;Fall:59–72.
Wiltse J, Dellarco VL.U.S. Environmental Protection Agency guidelines for carcinogen risk assessment: past and future. *Mutat Res*. 1996;365:3–15.

Statutory and Regulatory Materials

Animal Welfare Act, 7 USC § 2131 et seq (1988).
APHIS, Animal Welfare, 9 CFR Parts 2 & 3 (2011).
Comprehensive Environmental Response, Compensation and Liability Act, 42 USC § 9601 et seq (1980).
Consumer Product Safety Act, 15 USC § 2051 et seq (1972).
Consumer Product Safety Improvement Act, 15 USC § 2051 et seq (2008).
EPA. Guidelines for carcinogen risk assessment. *Fed Reg*. 1986;51(185):33992–34003.
EPA. Proposed guidelines for carcinogenic risk assessment. *Fed Reg*. 1996;61(79):17960–18011.
EPA. Notice of availability of the document entitled "Guidelines for carcinogen risk assessment". *Fed Reg*. 2005;70(66):17766–17817.
EPA. Harmonized test guidelines. Available at: http://www.epa.gov/ocspp/pubs/frs/home/guidelin.htm (2011a).
EPA. Good Laboratory Practice Standards (FIFRA), 40 CFR Part 160 (2011b).
EPA. Good Laboratory Practice Standards (TSCA), 40 CFR Part 792 (2011c).
EPA. Framework for Human Health Risk Assessment to Inform Decision Making. EPA-100/R-14/001 April 2014.
EPA. Final test guidelines; endocrine disruptor screening program test guidelines (Series 890); Three Tier 2 non-mammalian tests; notice of availability. *Fed Reg*. 2015a;80(164):5158–51560.
EPA. Use of high throughput assays and computational tools; endocrine disruptor screening program; notice of availability and opportunity for comment. *Fed Reg*. 2015b;80(118):35350–35355.
Executive Order 13563. Improving regulation and regulatory review, 76. *Fed Reg*. 2011;3821–3823.
FDA Food Safety Modernization Act (FSMA), Pub L No 111–353 (2011).
FDA Modernization Act, Pub L No. 105–115 (1997).
FDA. *Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook 2000*. Washington, DC: U.S. Food and Drug Administration; 2000 (periodically updated).
FDA. Good Laboratory Practice for Nonclinical Laboratory Studies, 21 CFR. Part 58 (2011a).
FDA. Investigational New Drug Application, 21 CFR Part 312 (2011b).
FDA. Food Additive Petitions, 21 CFR Part 171 (2011c).
Federal Food, Drug, and Cosmetic Act, 21 USC § 321 et seq (1938).
Federal Hazardous Substances Act, 15 USC § 1261 et seq (1976).
Federal Insecticide, Fungicide, and Rodenticide Act, 7 USC § 135 et seq (1972).
Federal Water Pollution Control Act Amendments of 1972, 33 USC § 307.
Food Additive Amendments to the Federal Food, Drug, and Cosmetic Act, 21 USC § 348 et seq (1958).
Food Quality Protection Act, Pub L No. 104–170 (1996).
ICCVAM Authorization Act of 2000, Pub L No. 106–545, 106th Congress.
Information Quality Act, Pub L No. 106–554, 31 USC § 3516 note (2001).
NIEHS. *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods*. NIH Publication No. 97–3981. Research Triangle Park, NC: NIEHS; 1997.
Occupational Safety and Health Act, 29 USC § 651 et seq (1970).
OMB. *Memorandum for the Heads of Departments and Agencies*, M-05-03 (2004). Available at: http://www.whitehouse.gov/omb/memoranda/fy2005/m05-03.pdf.
Resource Conservation and Recovery Act, 42 USCA § 6901 et seq (1976).
Safe Drinking Water Act, 42 USC §§ 300f to 300j-9 (1974, amended 1996).
Toxic Substances Control Act (2016), 15 USC § 2601 et seq.
White House. Memorandum for the Heads of Executive Departments and Agencies. Subject: Scientific Integrity (March 9, 2009).

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